Environmental Science, Engineering and Technology

Ecotoxicology in Latin America

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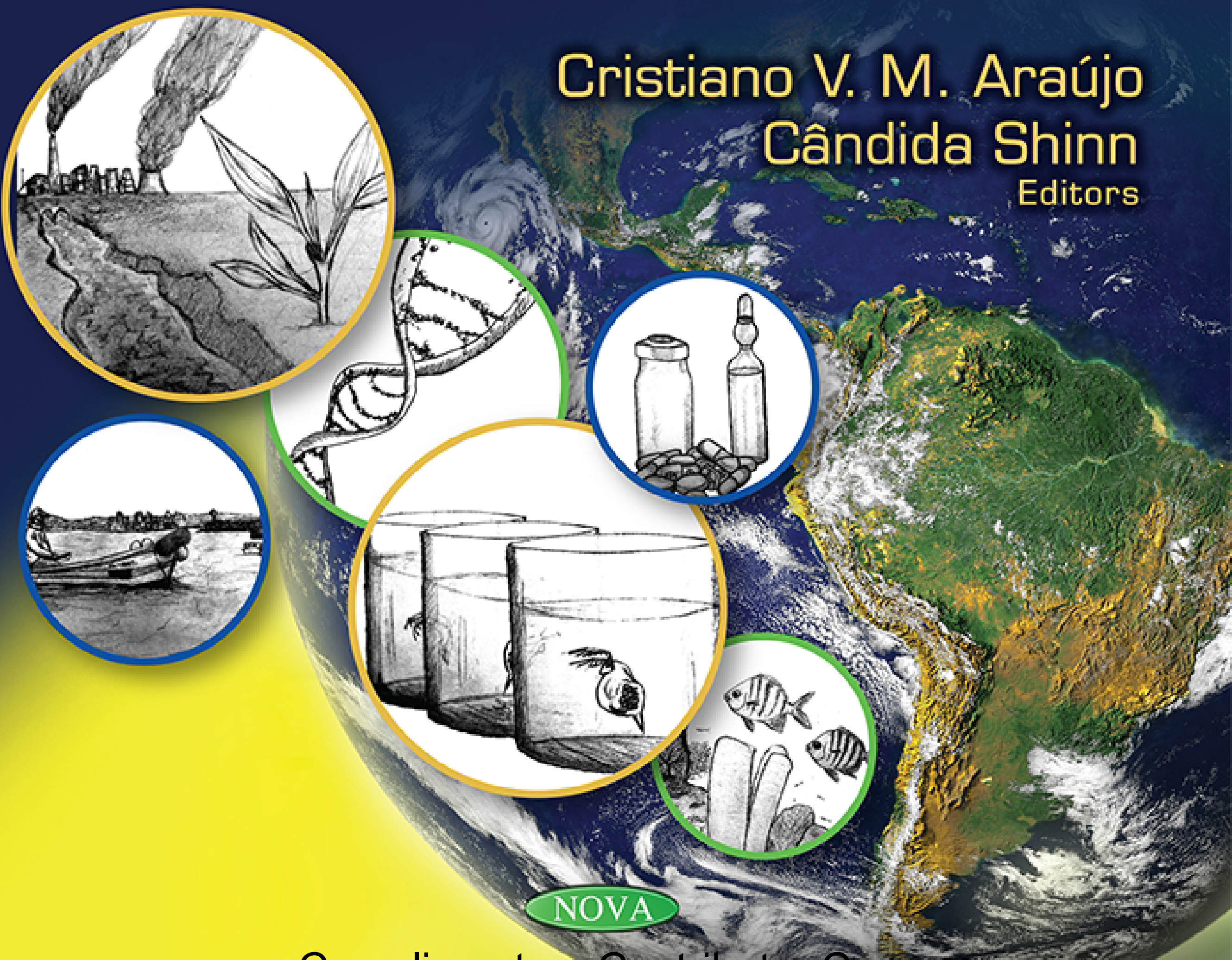

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ECOTOXICOLOGY IN LATIN AMERICA

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ABOUT THE BOOK

Studies on the interactions between biotic and abiotic components of ecosystems, structure and functioning of habitats as well as recovery, rehabilitation and recolonization of the environments cannot neglect the presence of contaminants and their effects at the individual, population, community and ecosystem levels. Since in Latin America many countries are in increasing development, and considering that what we called development assumes, to some extent, contamination and some loss of the environmental quality, it is a serious error to consider ecotoxicology a luxury for environmental risk assessment, because the price to pay for this development has been severe environmental disturbances.

Considering that in Latin America there are many vulnerable areas concentrating one of the most diverse regions in the world, one can question what is the level of disturbance in these areas? What are the types of studies carried out in those countries? What is going on in the field of ecotoxicology in Latin America?

Ecotoxicology in Latin America was proposed, therefore, to offer a collection of studies in ecotoxicology and environmental risk performed in different countries from Latin America. Researchers from several Latin American countries were invited to submit a chapter focusing on any of the following topics: (i) Contaminant entrance, transportation, distribution and fate; (ii) Environmental risk in freshwater ecosystems; (iii) Ecological risk in coastal zones; (iv) Biomonitoring programs: water, sediment and air; (v) Physiological effects and biomarkers; (vi) Soil ecotoxicology; (vii) Bioaccumulation and human risk; (viii) Toxicity of emerging contaminants; and (ix) Frontiers in Ecotoxicology. These issues tried to cover the most important subjects and applications of ecotoxicology, including classical and novel subjects.

The original idea to develop a book like this arose after our experience with different groups from Latin America that work in the area of ecotoxicology and environmental risk assessment, particularly during our researches in Ecuador in 2014. It became evident to us the notorious and increasing urban and industrial development of Latin American countries and the impact caused by anthropogenic activities that greatly threaten the biodiversity and environmental quality of many ecosystems. Although some studies and monitoring programs have been developed in those countries, the results obtained and decisions taken by different groups are poorly known because groups fail to publish their studies or do it exclusively at the local or regional scale, such that they are often difficult to access. In fact, even among specialized researchers in ecotoxicology working in Latin America, the transfer of knowledge is incipient and countries treating similar environmental problems do not know how the other is acting. Moreover, despite the geographic particularity that lead to differences between countries in landscape, biodiversity, functioning, biotic and abiotic dynamics, and physical and chemical properties of habitats, that could justify the lack of interest to publish their studies at a wider scale, problems tend to be very similar and many decisions and management plans could be applied transversally in different countries. Obviously, the publication of many studies at the local or regional scale is not incompatible with a simultaneous international projection. Therefore, we were motivated by the idea that for a successful monitoring and management for preservation of disturbed ecosystems it is crucial to optimize efforts, share experiences and transfer knowledge among different groups working in areas related to ecotoxicology, as well as among them and other internationally recognized groups. With this book, it is possible to provide a diverse and relevant collection of the studies performed in important subareas of ecotoxicology and to expose the main work area of some research groups within which experiences could be shared.

As the main authors of this book are from Latin America, an obvious question that arises is "why was the book edited in English?" Since this book is envisioned to have an international projection not only within Latin American countries, it is a great opportunity to increase the networks not only among Latin American researchers working in similar themes, but also with teams from other regions. The idea of this book is, therefore, to favor the connection among groups to quickly improve the development of methods and their application in ecotoxicological and environmental risk studies in Latin America.

We believe that the book will be useful to important sectors of environmental sciences (and related areas - agriculture, biology, chemistry, ecology, environmental engineering, hydrology, limnology, oceanography, soil sciences, public health and others)- and to the target public such as students (under-graduate students, and Master and PhD degrees), researchers acting in environmental studies and decision makers (politicians and environmental organizations), given that it covers themes related to contamination of water (freshwater and coastal environments), sediment, soil, air and human beings while taking into account effects produced by different sectors - industries, agriculture, urban discharges and natural phenomena.

We gathered 34 chapters authored by 111 researchers from 12 Latin American countries (Argentina, Brazil, Chile, Colombia, Costa Rica, Cuba, Ecuador, Mexico, Panama, Peru, Uruguay, and Venezuela) and from 6 non-Latin American countries (Austria, Belgium, Italy, Portugal, Spain, and USA). Although these data may seem to indicate a considerable number of participants for a book, taking into account the geographical dimension of Latin America, uncountable research groups were left out and it was not possible to consider many studies/projects/researches. Regardless of that, we hope that *Ecotoxicology in Latin America* helps to considerably increase the consolidation of networks and knowledge transfer to the target public, and that ecotoxicological and environmental risk assessment studies can be improved.

As Editors it has been an incredibly enriching experience to collaborate with scientists of a very diverse research field in an equally diverse region regarding ecosystems, climate, culture and history. The entire creative process of the book was achieved using virtual tools for the uncountable online meetings and exchange of hundreds of emails. This is clear proof that fruitful collaborations are nowadays possible regardless of the geographical distance, so long as there is respect, strong intention and clear communication.

FOREWORD

Ecotoxicology investigates the fate of contaminants in the biosphere and their effects on constituents of the biosphere, the biota. Environmental pollution is a problem worldwide, not restricted to any specific region of the earth. Why then is a book devoted to "Ecotoxicology in Latin America" needed?

Latin America is the region with the highest biodiversity on Earth. It holds highly productive marine zones and a major fraction of available freshwater resources. Latin American ecosystems range from tropical, temperate and desert regions to alpine and polar zones. At the same time, these ecosystems are under increasing pressure of anthropogenic threats. Progressive urbanization and overall population growth go along with increasing habitat degradation, waste water production and the concurrent usage of fresh- and groundwater resources. The strong agricultural expansion which has taken place over recent decades results in massive deforestation and depends on intensive use of pesticides. In parallel, industrial activities have strongly developed, including industries with high environmental impact such as oil and gas drilling or mining activities. These few examples may be sufficient to highlight that Latin American ecosystems are under stress of man-made activities and that there is an urgent need to assess the ecological consequences of environmental pollution in Latin America.

Historically, many pollution-related environmental problems have first become evident during industrialization of the countries in Northern America and Europe. As a consequence, ecotoxicological methods and concepts for environmental surveillance and ecological risk assessment have been designed for the purposes and under the perspectives of these regions. This raises the question to what extent these methods and concepts can be transferred to Latin America, and whether their "applicability domain" is universal enough to address the specific ecotoxicological problems of this continent and its ecosystems. For instance, the difference in the toxicant sensitivity and vulnerability between tropical and temperate species and ecosystems has interested scientists for a long time, but currently we have no conclusive answer to this question. Still, simply because of the relative scarcity of ecotoxicological data for Latin American species, risk assessments are largely performed with data determined for North American and European species. To overcome this biogeographic bias, ecotoxicological research in Latin America has to grow rapidly in order to learn about the commonalities as well as the differences in the approaches needed to protect and manage the integrity of Latin American ecoystems.

This book provides an excellent overview of current ecotoxicological research devoted to the specific problems and needs of Latin America. The book assembles case studies of typical pollution problems in Latin America, and it informs on current progress in the monitoring, risk assessment and management of such problems. At the same time, the book serves as a platform to bring together scientists from the various Latin American countries, thereby stimulating the collaboration and exchange that is essential to further advance the study field. As someone from outside Latin America, I found the progress and vitality of ecotoxicological research in Latin America, as highlighted by the contents of this book, to be most impressive. I am convinced that it will become a milestone in the development of ecotoxicology in Latin America.

Helmut Segner (Centre for Fish and Wildlife Health, Universität Bern, Switzerland).

Since the ‘80s, research in Ecotoxicology in Latin America has slowly but steadily grown, involving professionals from several countries. More recently a burst in the number and diversity of activities was observed due to periodical meetings of different scientific societies and the publication of the extended abstracts as grey literature in regional books. These meetings helped to increase the frequency of the interactions among researchers from this geographic area. For example, in 2012, a selected number of studies presented in the Lima (Perú) and Cumana (Venezuela) biennial SETAC-LA (Society of Ecotoxicology and Environmental Chemistry, Latina America branch) meetings were published for the first time, as a special section of the journal Environmental Toxicology and Chemistry. This section aimed to promote the publication of environmental studies conducted in Latin America as white literature, allowing readers from all over the world to know about current investigations in Latin America.

In this context, this book will help to show the current status of research regarding Ecotoxicology in Latin America presenting 34 chapters by authors from 12 Latin American countries: Argentina, Brazil, Chile, Colombia, Costa Rica, Cuba, Ecuador, Mexico, Panama, Peru, Uruguay and Venezuela; and from 6 non-Latin American countries: Austria, Belgium, Italy, Portugal, Spain and USA.

The chapters deal with different ecotoxicological issues: bioaccumulation, biomarkers and bioindicators, toxicity tests organisms and laboratory intercallibration, and ecological and human health risks assessments. The environmental stressors included are: heavy metals, hydrocarbons, pesticides, nanomaterials, endocrine disruptors, pharmaceutical and personal care products, dredging sediments and sewage effluents. These assessments were conducted in relation with soil, sediments, fresh and marine waters and air. The following human activities are also covered: mining, agriculture, wastewater treatment and restoration and conservation.

We think this book represents a screenshot of research in Ecotoxicology in Latin America and nevertheless there are still challenges ahead; we hope that it will be a new starting point for further substantial advances.

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Chapter 1

WHAT RISKS DO THE CONTAMINANTS REALLY REPRESENT? A STANDPOINT ON EFFECTS FROM ORGANISMS TO ECOSYSTEMS/LANDSCAPES BASED ON NON-FORCED AQUATIC EXPOSURE SCENARIOS

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ABSTRACT

The present essay has as main goal to discuss the risks of the contaminants when their effects are assessed under non-forced aquatic exposure scenarios. Endpoints in ecotoxicology have initially focused on mortality, but promptly evolved to more sensitive and relevant sub-lethal responses, although forced exposures to contamination have continued to be almost exclusive. Recently, a complementary approach using non-forced exposures, thus allowing organisms to actively avoid contaminants, has been fostered. This perspective provides a novel paradigm for ecotoxicological studies by shifting the focus from individuals to higher levels of biological organisation, even under mild contamination scenarios in which noxious (physiological) effects at the individual level

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are not reached. Additionally, the concept of habitat selection/preference due to the simultaneous presence of contaminants with other attractive or repulsive stimuli is integrated and here discussed in light of a multi-stressor approach. This essay therefore discusses the above-mentioned concepts and how non-forced exposures can increase the ecological relevance in environmental risk assessment schemes and resulting environmental decisions.

Keywords: avoidance, environmental risk assessment, non-forced exposure

ENDPOINTS BEYOND SURVIVAL

For many centuries, contamination has been a theme linked exclusively to human health. This is the reason why toxicology is a more developed and robust science than ecotoxicology[1]. Only in the last century the word “health” has been extended to environmental questions (Maltby and Calow, 1989; Newman and Unger, 2002). Physical, chemical and biological conditions became determinant factors to classify an environment as fit for life or not. Moreover, recent ecological studies have realized that community structure, ecosystem processes and temporal and spatial patterns become difficult to be understood if the presence of contaminants is not considered (Cairns, Jr., 1992; Chapman, 2002). Therefore, concepts, methods and skills from ecology, chemistry, biology, toxicology, and many other environmental and life sciences have been integrated to compose the field of ecotoxicology.

In general terms, the first steps for a more integrated environmental assessment of aquatic ecosystems were focused on chemical analysis. Many chemical indicators were (and still are) used to define the environmental quality by monitoring contaminant concentrations comparatively to reference or acceptable values and by verifying the presence of xenobiotic compounds. Besides traditional and simple but very relevant measurements such as pH, conductivity, redox potential, temperature, organic matter, dissolved oxygen, nutrient levels, oxygen chemical demand, and oxygen biochemical demand, other more toxicologically-related parameters (e.g., concentration of metals, hydrocarbons, agrochemicals, nanoparticles, pharmaceuticals, etc.) have been added to environmental risk assessment schemes. However, it is widely recognized that the risk of a contaminant is not exclusively related to its presence, but mainly to its bioavailable concentration. As organisms respond to and recover from contamination in a species-specific manner, a chemical compound can be risky for one given species and, at the same concentration, pose no effect to many others. This vision regarding vulnerability was initially based on survival: how lethal are the contaminants? Thousands of studies during the 60’s to the beginning of 80’s were mainly centred onthe lethal effects of contaminants; finding the most sensitive species was the greatest challenge (Maltby and Calow, 1989). Researchers then realized that a healthy ecosystem could not be defined based on whether organisms are dead or alive. Being alive is a primary condition for living beings, and, ideally, no level of mortality should be acceptable (Maltby and Calow, 1989). Besides, sub-lethal effects occur at lower and more environmentally realistic contaminant concentrations than lethal effects. From this point onwards, a new paradigm based on sub-lethal responses pervaded ecotoxicological studies.

[1]No difference regarding environmental toxicology has been considered.

In this new (sub-lethal effect) context, more sensitive and ecologically relevant responses started to be considered: biochemical, histological, morphological, physiological, behavioural, genetic, etc. The vast majority of the studies monitoring such responses, including those using survival as endpoint, have been almost exclusively performed under forced exposure scenarios, in which organisms are exposed to contamination in a confined environment (e.g., aquaria, plates, flasks, tubes). A concentration-response relationship is then derived and potential effects on organisms can be predicted. Even in *in situ* studies, organisms are very often caged, representing also a forced exposure. Such forced approaches lead experts to consider that adverse effects at the population level due to contamination are a consequence of a cascade of alterations that go from the biochemical level, to cellular, physiological, and finally whole population effects (Newman and Unger, 2002; Walker et al., 2001; Figure 1). However, in natural environments, a forced exposure scenario is not always the case. Mobile organisms that have a chemical sensorial system may be able to move towards less polluted areas avoiding contact with contaminants (avoidance[2] response; Figure 1). Even organisms that present a limited displacement ability may evade from disturbed areas by drifting (Beketov and Liess, 2008; Berghahn et al., 2012). Forced exposure could then represent a limited approach.

CHANGING THE EXPOSURE PARADIGM: RESPONSES IN NON-FORCED SYSTEMS

One of the first attempts to verify if organisms actively avoid contaminated areas to escape from toxic effects was performed with the fish *Pygosteus pungitius* by simulating a contamination gradient in a tube containing clean water and a contaminant (alcohol, chloroform, copper sulphate, formalin, mercuric chloride, or zinc sulphate) in the extremities (Jones, 1947). Since then, different systems have been employed: bi-compartmented (Folmar, 1976; Svecevičius, 1999), steep-gradient, laminar flow chambers (Hartwell et al., 1989), avoidance/preference chambers (Smith and Bailey, 1990) and fluvarium systems (Richardson et al., 2001). Many other studies have shown the ability of fish to detect and actively avoid a large range of contaminants (Sprague, 1968; Gunn and Noakes, 1986; Scherer and McNicol, 1998; Svecevičius, 2001). Based on the assumption that contamination in aquatic habitats disperses, forming a gradient which is increasingly diluted with increasing distance from the contamination source (except when an abrupt discharge forms a contamination plume), a multi-compartmented system was recently proposed (Lopes et al., 2004; Moreira-Santos et al., 2008). This system simulates a linear contamination gradient through which organisms can move freely with access to sequential zones containing different contamination levels: the exposure is facultative and non-forced, provided the organisms are mobile and have the ability to detect the contaminant. This simulation of a contamination gradient allows for an estimation of what could occur in the environment when contaminants are discharged. Therefore, the focus shifts from individual (i.e., noxious physiological effects) to ecosystem (e.g., pond, stream, estuary, beach) and landscape (e.g., river basin, coastal ocean) levels.

[2]Although in some studies spatial avoidance has been indirectly defined as changes in the patterns of specific movements (swimming velocity, direction, area of use), in the present study spatial avoidance is exclusively considered as the spatial displacement to escape disturbed areas.

Using the non-forced multi-compartmented exposure system, Lopes et al. (2004) assessed the avoidance response of the cladoceran *Daphnia longispina* exposed to a copper gradient. In that study, a continuous flow was used to maintain the contamination gradient. An optimization of this system was later on used by Moreira-Santos et al. (2008), who exposed the fish *Danio rerio* to a gradient of copper and acid mine drainage effluent, and by Rosa et al. (2008), who exposed *D. magna* to a gradient of pulp mill effluents. Rosa et al. (2012) modified that system to a static condition with no flow, such that a simpler and more practical avoidance assay system began to be used (Figure 2). Those authors tested the avoidance magnitude of *D. magna* when exposed to an atrazine gradient. Recently, several studies have been performed using this non-forced multi-compartmented static system: copepods exposed to a polycyclic aromatic hydrocarbon mixture (Araújo et al., 2014a), amphibians exposed to copper (Araújo et al., 2014b), to the biopesticide abamectin (Vasconcelos et al., 2016) and to the fungicide pyrimethanil (Araújo et al., 2014c), freshwater fish exposed to pyrimethanil (Araújo et al., 2014d), and seawater fish and shrimp exposed to copper (Araújo et al., 2016a). In all these studies, avoidance showed to be a sensitive endpoint with the potential to be used in ecotoxicological studies and risk assessment schemes.

Non-forced exposures are specially relevant and realistic in the presence of a gradient of contamination and in the case of non-sessile organisms. For instance, lethal responses are to be expected when discharges at very high concentrations are suddenly disposed off in the environment and organisms have no time to avoid them. Hare and Shooner (1995) observed that the abundance of *Chironomus* (*salinurius* gp.) sp. larvae was negatively correlated with the sediment Cd gradient. This abundance pattern could be related to avoidance behaviour. However, when larvae of this chironomid species were tested for avoidance to field control and Cd-spiked sediments, avoidance was not evidenced. The lower abundance of *Chironomus* sp. at high Cd concentrations in the field might thus be attributed to the direct toxic effects of Cd and not to spatial avoidance. Therefore, this new paradigm does not intend to replace the forced exposure, but to complement it.

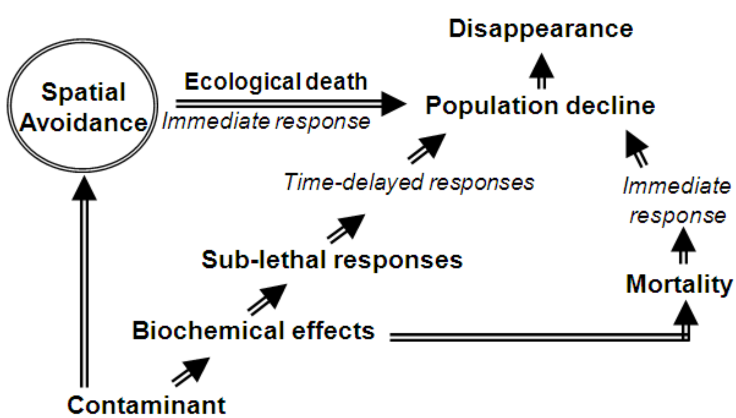

Figure 1. Cascade of time-delayed effects expected from the exposure to a contaminant: from biochemical alterations to sub-lethal responses, to the decline of the population and its disappearance in the long term. Immediate mortality is observed in parallel to this cascade effect, at higher concentrations and with the same consequences, but within a shorter time frame. Spatial avoidance can also occur regardless of the cascade effect, immediately producing similar ecological consequences: disappearance of the population. Modified from Walker et al. (2001).

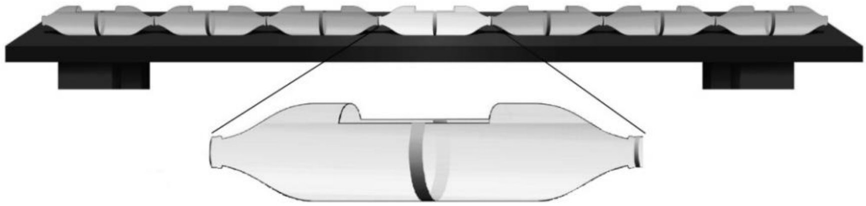

Figure 2. Schematic representation of a non-forced multi-compartmented static exposure system used in avoidance experiments (Araújo et al., 2014a).

CHANGING THE EXPOSURE-EFFECTS PARADIGM: EFFECTS ON ORGANISMS VS. EFFECTS ON ECOSYSTEMS/LANDSCAPES

The idea behind "what risks do the contaminants really represent" is to reflect on how we perform ecotoxicological studies, what they really indicate and, the most important question: can non-forced exposure approaches improve our understanding about the effects of contaminants on the environment? Although initially one could think that the present essay tries to convey the idea that contamination is not a problem, the idea is completely the opposite. Given that an extensive number of studies have shown many organisms are able to escape contaminated habitats, one could suppose that toxic effects become irrelevant since organisms are not passively exposed to the contaminants. However, a change at higher levels of biological organisation in biodiversity, functioning and temporal patterns is expected due to organism's dispersion caused by the presence of contaminants. This complementary approach, in which neither the uptake of contaminants nor individual noxious effects are mandatory, suggests that contaminants can be less dangerous at the organism level than what is often expected. However, the ability to avoid contamination creates, at community and ecosystem levels, an effect similar to mortality, as organisms partially or totally disappear (De Lange et al., 2006; Rosa et al., 2012).

A novel concept called *Population Immediate Decline* (PID) including the "ecological death" caused by the population disappearance was proposed by Rosa et al. (2012). The PID concept indicates that a local population can be immediately reduced not only due to mortality, but also due to spatial avoidance (ecological death), mainly when the contaminant concentration is not lethal or does not even cause moribundity. A simple integration of the immediate mortality and the immediate avoidance can more accurately predict the population decline exposed to a contaminant. For instance, in a study with *D. magna* exposed to atrazine, it was shown that at levels at which lethal effects are minimal (5%, for example), the predicted local PID was of approximately 50% due to spatial avoidance (Rosa et al., 2012). The same authors also observed that, at the 48 h-LC50, the predicted PID was more than 90%. In a study with the copepod *Boeckella occidentalis intermedia*, it was observed that the concentration of polycyclic aromatic hydrocarbons that triggered an avoidance of 30% caused 25% mortality among non-avoiders; therefore, the PID was of 55% (Araújo et al., 2014a). Similarly, in a study with tadpoles of the amphibians *Leptodactylus latrans, Lithobates catesbeianus* and *Pelophylax perezi* exposed to copper, Araújo et al. (2014b) showed that, at copper concentrations lower than 200µg L^{-1}, the PID was exclusively dictated by avoidance; above this concentration, organisms began first to lose the ability to avoid (moribundity) and then, due to the continuous exposure, mortality started to occur. In a recent study with marine organisms, the fish *Rachycentron canadum* and the shrimp *Litopenaeus vannamei* avoided copper contamination at concentrations below those causing lethal effects (Araújo et al., 2016a).

When considering all the above-mentioned results, it is clear that extrapolations of immediate toxic effects based on mortality and forced exposure could lead to a severe underestimation of ecological risks. The proportion of the population expected to disappear at the short term has to consider both avoiders and dead organisms. Under a long term and sub-lethal exposure, population decline will also integrate reduction in feeding rate, delayed growth, impaired reproduction, susceptibility to predation, loss of competitiveness, etc. Avoidance should therefore be also considered in chronic exposure scenarios. Although the integration of avoidance in long term experiments with other sub-lethal endpoints has not yet been performed, avoidance data on *L. vannamei* exposed to copper obtained by Araújo et al. (2016a), when compared with other endpoints published in the literature, strongly suggests that it is more sensitive than predation rate (Wong et al., 1993), immune response (Yeh et al., 2004), weight gain (Chen and Li, 2001), and osmoregulatory capacity and hyperplasia of gill tissue (Soegianto et al., 2013) in the same species. It is thus possible to hypothesize that spatial avoidance of this marine shrimp might be triggered as a mechanism to escape those damages, since concentrations considered to produce a significant effect are, in general, time delayed and higher than the concentration triggering avoidance.

When measured in a non-forced exposure system, avoidance represents an important change in the exposure paradigm. Moreover, it usually is a quick (short term) and sensitive (low concentrations) response when compared with lethal (observed in the short term and at high concentrations) and sub-lethal (observed in the long term and at low concentrations) responses (Figure 3). Although organisms continue to be the key focus in exposure studies, the final target/receptor of the ecological consequences is the ecosystem, as no toxic effect at the individual level is (or at least should be) expected. Long term forced and non-forced exposure studies with observation of sub-lethal responses and avoidance should be simultaneously carried out to improve our understanding regarding the effects of contaminants on population decline due to sub-lethal effects and organism evasion.

A Multi-Stressor Approach: Avoidance of Contaminants vs. Attractive and Other Repulsive Stimuli

Undoubtedly, avoidance can, to some extent, give indications about species spatial distribution and biodiversity in contaminated ecosystems as well as in neighbouring uncontaminated areas. According to Hansen et al. (1999), the abundance and distribution of the fish *Oncorhynchus mykiis* in areas with metal contamination and acidity could be partially explained by the escape of organisms from those areas. Similarly, the distribution of amphipods and chironomids has been inversely associated to the sediment toxicity levels that indicated a contaminant-driven spatial arrangement (Swartz et al., 1982; Hare and Shooner, 1995). However, it is important to consider that the decision to livein a habitat does not exclusively depend on the presence of the contamination, even if concentrations are recognizably risky. Factors such as food availability, presence of predators and conspecifics, and habitat complexity may also play an important role. If, on the one hand, the presence of contaminants represents a repulsive stimulus, on the other hand many other attractive factors can influence the decision of the organisms to stay or move towards other areas regardless of the contamination levels. Even in habitats in which contamination occurs at concentrations expected to produce deleterious effects on organisms, high biodiversity and complex biological interactions have been observed. Cardoso et al. (2013) observed that density, biomass and growth productivity of the snail *Peringia ulvae* along a mercury gradient in a shallow coastal lagoon (Ria de Aveiro, Portugal) reached higher values in the area with intermediate contamination. This apparent preference of *P. ulvae* to Hg-contaminated areas might be influenced by many factors such as age, resource availability, presence of refuges, and other biotic or abiotic factors.

Preliminary observations by Araújo and collaborators (Araújo et al., 2016b) have shown that the habitat selection process by tilapia fry tends to be influenced by contamination levels. However, in the presence of two conflicting stimulus (contamination and food) organisms were compelled to intermittently move towards contaminated areas where the food availability was higher. This decision was taken regardless of the potential toxic effects caused by contaminants. The tilapia fry were more intensely stimulated by the attractiveness of the food than by the repulsion of the contamination. A similar behaviour was observed in rainbow trout (*Salmo gairdneri*) exposed to copper and food in an attraction-avoidance experiment using a two-channel system (Pedder and Maly, 1986). This plasticity of the avoidance response indicates that organisms may make a decision based on the identification of the most repulsive and attractive stimuli (Harper et al., 2009).

Avoidance experiments, in non-forced exposure systems, should also be conducted under a multi-stressor approach to improve the understanding of the ecological risk posed by contaminants. Although the forced exposure approach is important to determine concentration-response relationships, and fundamental to immobile organisms, the non-forced exposure including repulsive and attractive stimuli could provide ecologically relevant data to predict the effects of contaminants on ecosystems and their functional and temporal dynamics.

FINAL CONSIDERATIONS

The non-forced exposure system does not focus on the health of individuals, but on the ecological consequences at higher levels of biological organisation due to the effects of habitat disturbance on the spatial distribution patterns of organisms. If organism's spatial distribution is driven by contaminants, a serious restriction of potentially habitable areas can occur as avoidance modifies the migratory routes and processes of (re)colonization (Gray, 1990; Smith and Bailey, 1990). "Chemical barriers" due to contamination result in habitat fragmentation and prevent the flux of individuals and, thus, alleles among populations, making the latter more susceptible to other perturbations, namely inbreeding and mutational load in small-sized populations (Ribeiro and Lopes, 2013). This approach implies considering that contaminants can be environmental disturbers, even if no effect is observed at the individual level. This new paradigm drives us to take a closer look at the role of contaminants at the wider and more complex ecosystem/landscape level.

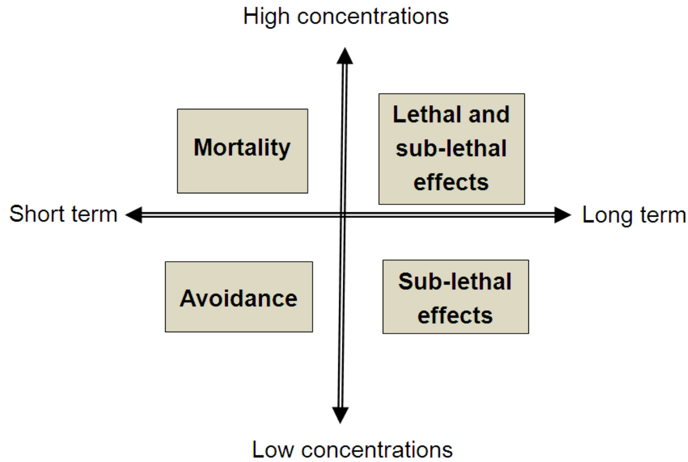

Figure 3. Theoretical representation of lethal, sub-lethal and avoidance responses in relation to the exposure time and sensitivity (concentration). Mortality is expected to occur at the short term and at high concentrations; sub-lethal noxious effects are expected at the long term (at least longer than lethality) and at low concentrations; finally, spatial avoidance is expected to be immediate and to occur at low concentrations.

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Chapter 2

BIOMARKERS IN NATIVE CENTRAL AMERICAN SPECIES

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ABSTRACT

In an ecotoxicological framework, biomarkers are considered a source of information that helps to establish exposure-effects relationships. Data at the biochemical or physiological level can help to elucidate the mechanisms by which a pollutant can cause negative effect on an organism. Knowing those mechanisms is key in the identification of signals of effects or exposure to a given compound. In general, ecotoxicological information is scarce for tropical species compared to standard temperate counterparts. The application of biomarkers has provided an alternative method for ecotoxicology research which can be adapted to local or standard species as well. In the tropics, where higher biodiversity is found, it is very important to develop research in the field of biomarkers in order to assess the sensitivity of species to the pollutants that diverse human activities are releasing into the environment. This chapter summarizes the efforts carried out during the past two decades to characterize the sensitivity and physiological responses of several native Central American species exposed to pesticides. At the same time, the biomarker approach has been applied in the field as a means to assess early warning signals of pollution in ecosystems.

Keywords: Costa Rica, pesticides, ecotoxicology, biomarkers, native species

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INTRODUCTION

Biomarkers have become a widely used tool in ecotoxicology for the evaluation of early effects or early warning signs of pollution on organisms. The use of biomarker techniques has been increasing since the 1990's when this concept was adapted to an ecotoxicology context (Amiard-Triquet et al., 2012).

During almost three decades of intensive application, this low-biological-level endpoint, related to exposure and/or effects of pollutants to organisms, as defined by Peakall (1994), has gained weight in ecotoxicology. The inclusion of biomarkers in regulatory frameworks for protection of aquatic resources is being currently considered as an improvement in developed countries (Handy et al., 2003; Sanchez and Porcher 2009; Sanchez et al., 2012; Capela et al., 2016).

In Costa Rica, studies in this field have been carried out during two decades, progressing with the implementation of analytical methods for the evaluation of different biomarkers throughout the years. All work developed so far, has been based on the use of local species as assay organisms for the characterization of their sensibility to relevant pollutants, but also for the assessment of environmental samples and *in situ* evaluations (Broman and Hedene, 1996; Navarro et al., 2014; Echeverría-Sáenzet al., 2016 *in press*).

A battery of assays has been developed with the implementation of methods for the evaluation of cholinesterase (ChE), glutathione S-transferase (GST) and catalase (CAT) activities, and lipid peroxidation (LPO) and vitellogenin (VTG) levels (Mena et al., 2014a; Lelieveld, 2004). Collaborative work has also allowed the application of molecular techniques for phase I biotransformation (cytochrome P450) and endocrine disruption (vitellogenin) biomarkers at a gene expression level (Navarro et al., 2014).

The use of biomarkers contributes to enhance the specificity and complexity of information produced in ecotoxicology by characterizing and measuring the effects of pollutants in lower levels of biological organization. For tropical environments where this kind of information is scarce, the use of biomarkers should be a resource to improve the protection of such fragile and rich ecosystems. In this chapter we will summarize the outcomes of biomarker responses (exposure and effects) that have been observed in organisms exposed in the laboratory to specific toxicants or to environmental samples collected in polluted sites, and also the responses of organisms that have been exposed *in situ* at sites studied for pollution in Costa Rica.

METHODS

Biomarkers have been evaluated in several native freshwater species which include fish (*Astyanax aeneus* and *Bryconamericus scleroparius,* Characidae*; Parachromis dovii,* Cichlidae*; Atractosteus tropicus*, Lepisosteidae; *Poecilia gillii,* Poecilidae), bivalve molluscs *(Anodontites luteola,* Mycetopodidae), freshwater shrimp (*Macrobrachium digueti,* Palaemonidae), amphibians (*Agalychnis callidryas, Smilisca baudinii* and *Isthmohyla pseudopuma*, Hylidae) and crocodiles (*Crocodilus acutus,* Crocodylidae).

The first approach of these investigations is the characterization (in the laboratory) of biochemical or physiological responses triggered by specific pollutants or environmental samples by toxicity tests coupled with measurement of biomarkers in organisms exposed to sub-lethal concentrations of toxicants.

A second approach has been the evaluation of biomarkers in free-living organisms, mostly in fish, but also ChE activity in plasma samples from sloths (*Bradypus variegatus* and *Choloepus hoffmanni*), and vitellogenin, as a biomarker for endocrine disruption, has been evaluated in fish (*Oreochromis aureus*, *Rhamdia guatemalensis*, *Herotilapia multispinosa*, *Dormitator latifrons*, *Archocentrus nigrofasciatus*, *A. aeneus*, *P. dovii* and *P. gillii*) and the crocodile (C. *acutus)*.

The third approach undertaken is the *in situ* exposure of caged fish. This method has allowed the evaluation of both survival and biomarkers, keeping uniformity in the assay organisms by using laboratory reared animals and measuring the responses under realistic environmental conditions.

Biochemical tests to measure the activity of the enzymes ChE, GST, CAT and LPO have been established in the Laboratory of Ecotoxicological Studies (ECOTOX) at the Universidad Nacional and are applied routinely. An immunological method for VTG determination has been established in the laboratory and collaborative work with European counterparts has also allowed the application of molecular biology and proteomic approaches (Tedengren et al., 2000; Navarro et al., 2014).

Biochemical Methods

ChE activity is measured with the method described by Ellman et al. (1961), based on the metabolism of the synthetic substrate acetylthiocholine iodide by tissue endogenous enzyme; the method was adapted to microplate by Guilhermino et al. (1996). GST activity is determined as described by Habig et al. (1974), measuring the conjugation of reduced glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB). Lipid peroxidation is evaluated by the thiobarbituric reactive species (TBARS) assay, as described by Oakes and Van Der Kraak (2003). CAT activity is measured according to Aebi et al. (1974) by the consumption of H_2O_2. In all the protocols we use, biomarkers are normalized to protein content in sample homogenates which is determined by the method of Bradford (1976) using γ-globulin as standard.

Proteomic, Molecular and Immunological Methods

Differential expression of CYP1A and VTG genes in tissues has been evaluated with quantitative real-time polymerase chain reaction (qRT-PCR) technique, using β-actin as a housekeeping gene (Navarro et al., 2014). Differential protein expression in tissues of animals exposed to pesticides has been measured with two-dimentional polyacrylamide gel electrophoresis (2D-PAGE) (Tedengren et al., 2000). Presence of VTG in plasma of fish and crocodiles has been measured with an indirect enzyme-linked immunosorbent assay (ELISA) (Lelieveld, 2004).

Table 1. Biomarker responses observed in native Central American species exposed to pollutants or polluted sites in Costa Rica

Taxonomic group	Species	Measured biomaker(s)	Main findings	Reference
Fish	*Parachromis dovii** (L-F)	ChE	Method implementation and application in tissues (brain, muscle and blood) of native species.	Broman and Hedene, 1996
Fish and bivalves	*P. dovii* (L) *Anodontites luteola* (L)	Physiology, Protein expression patterns (2DGE)	Increased respiration in mussels exposed to a mixture of pesticides. Suppression of 15 proteins and induction of 12 in mussels exposed to a mixture of pesticides.	Tedengren et al., 2000
Fish	*P. dovii* (L)	ChE	Comparison of the ChE inhibition caused by terbufos (organophosphate) in three tissues (brain, muscle and blood). Brain showed the major response and lower variability.	Abicht and Pfennig, 2003
Fish	Several species (F)	VTG	Method implementation, optimization and application in native species. High levels of VTG in male fish captured near rice and banana plantations.	Lelieveld, 2004
Fish	*P. dovii* (L) *Astyanax aeneus* (F)	ChE	ChE inhibition by ethoprophos in brain and muscle of *P. dovii* exposed to ethoprophos ChE inhibition in *A. aeneus* collected at 2 sites downstream from banana plantations	Sánchez, 2005
Fish	*A. aeneus* (F) and *Poecilia gillii* (F)	ChE	Muscle ChE activity in both species correlated inversely with fish standard length. Lower levels of ChE activity in muscle of *P. gillii* correlated positively with presence of ectoparasites on fish.	Pfennig, 2006
Fish	*Atractosteus tropicus* (L)	ChE	Dose-response of ChE inhibition after exposure to ethoprophos. EC_{50} on ChE inhibition.	Mena et al., 2012
Fish	*P. gillii*(F) and *Bryconamericus scleroparius* (F)	ChE, GST, LPO	ChE inhibition in brain and muscle of *P. gillii* caged at three sites influenced by pineapple plantations. ChE inhibition in *B. scleroparius* muscle caged at one site influenced by pineapple plantations. GST induction in *P. gillii* at four sites near pineapple plantations and LPO increase at three sites. LPO increase in *B. scleroparuis* caged at one site influenced by pineapple plantations.	Echeverría-Sáenz et al., 2012
Bivalves	*A. luteola* (L)	ChE, GST, LPO, Behavior	Sediment avoidance, weight loss and increased GST and ChE in mussels exposed to sediment from a swine farm.	Arias-Andrés et al., 2014
Fish	*P. dovii* (L)	ChE	Dose-response of brain and muscle ChE inhibition vs ethoprophos and chlorpyrifos. ChE inhibition by exposure to water samples from three sites downstream from banana plantations.	Diepens et al., 2014
Fish	*A. aeneus* (L-F)	ChE	Dose-response of brain and muscle ChE inhibition vs ethoprophos. Muscle ChE inhibition in muscle of fish collected at a site that receives effluent from banana plantations.	Mena et al., 2014b
Fish	*P. dovii* (F) and *P. gillii* (F)	ChE, GST, LPO and CAT	Increase of GST, CAT and LPO in *P. dovii* caged at one site associated with rice plantations. ChE inhibition in *P. gillii* caged at a site with presence of organophosphate pesticides. GST, LPO and CAT variations in *P. gillii* caged at sites associated with rice and sugarcane plantations.	Mena et al., 2014a

* In the original paper, the species was called *Cichlasoma dovii*, the name of the genus changed afterwards. L=Laboratory study; F=Field study.

Behavior and Metabolism

Evaluation of avoidance has been carried out in mussels exposed to contaminated sediments (Arias-Andrés et al., 2014) and respiration has been measured in mussels and fish exposed to a mixture of pesticides (Tedengren et al., 2000).

RESULTS AND DISCUSSION

Assessment of the toxicity of pollutants and the evaluation of their effects on aquatic ecosystems of Costa Rica have been approached from different angles: from the detection and quantification of residues in environmental matrices, to the evaluation of effects at different biological organization levels (See chapter 11).

Studies with biomarkers in Costa Rica have supplied broad evidence regarding sub-individual responses (in the lower biological organization level) and the sensitivity of tested species. Moreover, effects in the field have been evaluated with a diverse group of native species of different taxonomic groups (Table 1).

Since agriculture is one of the most important economic activities in Costa Rica and crops are cultivated with high pesticide usages, these compounds are identified as one of the most relevant groups of environmental pollutants. Presence of pesticide residues in the environment is constant in agricultural areas and high sensitivity of some native species to this group of pollutants has already been described (see Table 2 in chapter 11). Regarding biomarkers, all of the native species tested so far have shown significant responses when exposed to sub-lethal concentrations of relevant pollutants. This suggests that many of them are candidates for being used as sentinel species in the region where they are naturally distributed.

Fish is the group of organisms on which more studies have been done. Three species (*A. aeneus*, *P. dovii* and *P. gillii*) have shown to be the most suitable to work with. A common characteristic that makes these species appropriate is their broad distribution in the region and, in the case of Costa Rica, their presence in both Pacific and Caribbean versants: *A. aeneu*s ranges from Mexico to Panama, *P. dovii* from Honduras to Costa Rica and *P. gillii* from Guatemala to Colombia (Bussing, 2002). Besides their distribution, *A. aeneus* and *P. gillii* also have the advantage of numerous populations that can be occasionally sampled without significantly reducing them. When there is the necessity of having fish raised in controlled conditions, *P. dovii* and *P. gillii* can be easily reproduced in the laboratory to obtain clean and well characterized experimental organisms. Finally, when running *in situ* caging experiments or laboratory tests, it is important to maintain minimal stress and aggression among organisms during exposures. In this regard, behavior of the fish is an important issue and *P. gillii* has shown to be the least aggressive among the species tested.

Regarding other taxonomic groups, more work should be done in order to suggest suitable sentinel species, although bivalves (*A. luteola*), decapodes (*M. diguetti*) and amphibians (*S. baudinii*) have shown positive results regarding sensitivity to pesticides and biomarker responses.

A robust battery of biomarkers, integrating different physiological mechanisms related to effects or responses to pollutants is highly recommended; and the link of sub-individual responses measured at higher levels of biological organization should be also pursued (Galloway et al., 2004; Moore et al., 2004). The interpretation of several biomarkers measured in the field can be difficult and even confusing. In our field studies, significant responses of biomarkers have been obtained, but not always in direct correlation with the detected pesticide residues (Fournier et al., 2016 *submitted*; Echeverría-Sáenz et al., 2016 *in press*). In order to overcome this situation, integrative analysis have been developed that help merge as many responses one can measure in one index (Broeg and Lehtonen, 2006). For our data, application of the integrated biomarkers response (IBR) (Beliaeff and Burgeot, 2002; Devin et al., 2014) has helped to elucidate the relationship between biomarkers and gross pollution of sites. For instance, in a study where no correlation could be established between individual biomarker responses and pesticide residues in individual samples, IBR responses were consistently significant at the overall most polluted sites (Echeverría-Sáenz et al., 2016 *in press*).

There is a continuous innovation and adaptation in the techniques applied for the evaluation of biomarkers. This evolution enables researchers to improve the specificity and refinement of the information in this field, but also requires constant updating in order to keep up with the advances in biochemistry and molecular biology.

So far, data generated with the application of biomarkers shows the suitability of these techniques to characterize the sensitivity of some native species to pesticides and chemical pollution.

Many ecotoxicologists have reservations about the value of biomarkers and their application in the field, mostly because in many cases the responses measured might be influenced by natural variations and environmental parameters (Forbes et al., 2006; Guilhermino, 2006). Also, there might have been some frequent misuse or misinterpretation of this information that is known to represent a low ecological relevance level in an ecotoxicological framework (Forbes et al., 2006). Nevertheless, in the end, biomarkers should be used and interpreted as they are intended (early warning signals) and if applied correctly (Adams et al., 2001; Handy et al., 2003; Guilhermino, 2006), they can be a valuable tool in the protection of ecosystems.

CONCLUSION

The inclusion of biomarkers in a set of ecotoxicological tests has improved the capacity of researchers to detect and characterize effects of pollutants (pesticides) in a group of Central American native species.

From all the studies that have been executed over the years in Costa Rica, the clearer results have been obtained from the laboratory experiments, where toxicant-effect causality has been demonstrated and several new potential test species have been recognized. This is highly valuable information for the tropics. However, more research is needed to elucidate the different mechanisms and interactions that take place in field studies, where natural and anthropogenic stressors get mixed.

The use of integrative methods such as IBR helps to process and interpret the results of a vast battery of biomarkers. This tool has been useful in order to compare responses between sites and finding the overall, most intense response to the toxicants that are present in the field.

This summary of information regarding sensitivity at a physiological level should be used in order to protect biodiversity, by setting lower limits to the allowed levels of pollutants in the environment that should not compromise organisms even at a sub-individual level.

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Chapter 3

AQUATIC ECOTOXICOLOGY: NATIVE FRESHWATER GASTROPODS FROM ARGENTINA

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ABSTRACT

The increasing worldwide contamination of freshwater systems with a wide range of chemical compounds is one of the main environmental problems that humanity faces nowadays. These contaminants, such as pesticides, disturb natural ecosystems and can have a great impact on the species that inhabit them. Traditionally, environmental monitoring programs were based on detecting and determining the concentration of contaminants. However, they did not necessarily reflect the actual impact on the environment. Lately, programs have included bioassays and sensitive biomarkers as effective tools to obtain an integrated overview of aquatic ecotoxicology. Our research group uses bioassays to study whether environmental concentrations of toxic compounds used in Argentina can produce effects on native gastropods. Our research lines involve the native freshwater snails *Biomphalaria straminea*, widely distributed in northeastern Argentina, and *Chilina gibbosa*, endemic to freshwaters of the Argentine Patagonia. The first studies we carried out were focused on the organophosphate azinphos-methyl, which

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has been detected in freshwater courses at concentrations higher than those recommended for aquatic life protection. We found different sublethal effects after exposures to environmental concentrations in both species. *B. straminea* is resistant to an acute exposure but shows toxic effects after subchronic exposures, which has driven further studies on reproduction and offspring. *C. gibbosa,* however, is highly sensitive to azinphos-methyl, which has led us to consider it as a sentinel species. Our research allows us to contribute with a wide range of biomarker information, propose new sentinel species and provide data to reassess current guidelines for aquatic life protection.

Keywords: pesticides, azinphos-methyl, freshwater gastropods, native species, biomarkers

PESTICIDES

A great number of pesticides are used around the world in gardening, public health and agriculture to provide protection from pests. Most of them are generally sold as formulations that consist of an active ingredient, which is responsible for the mechanism by which the pesticide kills the target pest, plus other compounds that act as solvents, carriers or adjuvants.

Pesticides can easily enter the soil, surface and ground water through direct application, terrestrial runoff, rainwater, floods and soil leaching, among others. Pesticide, water and soil properties together with weather conditions determine the destination and persistence of the compound in the environment. The use of pesticides in agriculture has led to an increasing concern about the potential implications for human and environmental health and many strategies have been developed to evaluate them. A traditional strategy is to determine the presence and/or concentration of an array of compounds of interest in the environment (US EPA, 2001; Blasco and Picó, 2009; Lam, 2009). However, this approach is limited because it is impossible to identify every potential toxic compound and it does not reflect how the effect to organisms is modified by bioavailability, bioaccumulation, continuous and successive exposures, among other factors. The use of bioassays and sensitive biomarkers arose from the need to develop new tools to assess the impact of hazardous compounds in the environment (Wolska et al., 2007; Lam, 2009; Smital and Ahel, 2015; de Castro-Català et al., 2016). Consequently, the use of both chemical and biological evaluations provides a more integrated overview of water quality in environmental monitoring programs (Zhou et al., 2008; Blasco and Picó, 2009; Guasch et al., 2012; Burgess et al., 2013).

Organophosphorus Insecticides: Azinphos-Methyl

Organophosphorus compounds comprise a large and diverse family of organic chemicals. They are esters, amides or thiol derivatives of phosphoric, phosphonic and phosphinic acids. Most organophosphorus insecticides are derived from phosphoric acid and present a phosphorus atom with a P=O or P=S group. Their main mechanism of action involves the inhibition of the enzyme acetylcholinesterase, responsible for the hydrolysis of the neurotransmitter acetylcholine. They can also alter the function of other proteins and enzymes due to their highly reactive nature (Chambers and Levi, 1992; Terry, 2012).

Organophosphorus pesticides have acquired importance for pest control due to their low to moderate persistence in the environment (Chambers and Levi, 1992) in relation to other chemicals used; nevertheless, many of them have been banned or restricted in several countries because of their high acute toxicity to non-target organisms (Chambers and Levi, 1992; Terry, 2012).

Azinphos-methyl is an organophosphate (phosphoric acid ester) insecticide-acaricide that contains a phosphorothioate group (P=S). Its half-life in water is 26 days at 30 C and pH 7, and its water solubility is 28 mg L^{-1} at 20 ºC (US EPA, 2001). It is commonly applied for agricultural pest control in the Upper Valley of Río Negro and Río Neuquén in North Patagonia, Argentina. It is applied between October and March mainly to control *Cydia pomonella*, an important agricultural pest in fruit production (Soleño et al., 2008).

This pesticide has been reported as the most frequently detected organophosphate pesticide in surface and ground water of the Upper Valley region (Loewy et al., 2006, 2011; Tosi et al., 2009). The recommended value of this insecticide for aquatic life protection according to Argentine legislation is ≤0.02 μg L^{-1} (Subsecretaría de Recursos Hídricos de la Nación, 2003). Nevertheless, Loewy et al. (1999) detected a maximum concentration of 79.30 µg L^{-1} (from October 1995 to September 1997), and Loewy et al., (2011) a maximum concentration of 22.48 µg L^{-1} (from September 2008 to October 2009). It has also been registered in samples from the Luján river, located in east-central Argentina, at concentrations below 0.634 µg L^{-1}, the detection limit of the analytical method used for its determination (Castañé et al., 2015) and in Concordia, Entre Ríos, where it is used for pest control in blueberry crops (Acosta, 2014).

BIOMARKERS

The concept of biological markers or biomarkers has been defined in many ways (Amiard-Triquet et al., 2013). According to The National Research Council (NRC, 1987), a biomarker in environmental health research is any biochemical, physiological, histological or behavioral response in an organism that indicates the presence of xenobiotics. In a more generalized way, Depledge and Fossi (1994) re-defined the term biomarker as “a biochemical, cellular, physiological or behavioral change which can be measured in body tissues or fluids or at the level of the whole organism that reveals the exposure to/or the effects of one or more chemical pollutants.”

Biomarkers can be classified as biomarkers of exposure, effect or susceptibility (NRC, 1987; Amiard-Triquet et al., 2013). Biomarkers of exposure indicate that the organism has been exposed to pollutants, through the measurement of the contaminant itself, its metabolites, or the interaction product with a target molecule (NRC, 1987). A biomarker of effect is any quantifiable change in an individual after exposure to a contaminant such as biochemical, physiological, histological or other alterations (NRC, 1987). Biomarkers of susceptibility, which can be inherited or acquired, indicate that an organism is particularly sensitive to the effect of a toxic compound (van der Oost et al., 2003; Manini et al., 2007).

In ecotoxicology, bioassays can be defined as biological systems that allow the study of the impact of chemical compounds, mainly from anthropogenic activity, on ecosystems. Exposure to pollutants triggers a cascade of biological responses and multiple toxic effects in organisms. The use of a wide battery of biomarkers can provide a suitable evaluation of the toxic responses in organisms and an early warning tool to prevent a significant deterioration of the biota or of entire ecosystems, due to the presence of contaminants (Cajaraville et al., 2000; Amiard-Triquet et al., 2013).

An ideal biomarker for environmental monitoring should be easy to measure, specific, of fast induction and slow recovery to pre-exposure levels (Grandjean et al., 1994; Wu et al., 2005). Few biomarkers meet all the requirements but they are useful nonetheless because they provide complementary and relevant information about potential risks of pollutants for organisms (Amiard-Triquet et al., 2013).

FRESHWATER GASTROPODS

Working with Native Species

Aquatic invertebrate species have been amply recommended and used as bioindicators of water and sediment toxicity. For example, the oligochaete *Lumbriculus variegatus* has been widely adopted as a standard organism mainly for the study of toxicokinetic processes of organic substances and heavy metals (Phipps et al., 1993; ASTM, 1995; US EPA, 2000; Liebig et al., 2005). However, the use of native invertebrate species is not always taken into account in ecotoxicological research as a criterion to select model organisms (Freitas and Rocha, 2014).

Conducting studies and biomonitoring programs with native species has been suggested by US EPA (1976) and recommended by several authors (Buikema et al., 1982; Krull and Barros, 2010; Gagneten et al., 2012; Freitas and Rocha, 2014). The results of such integrated studies could be considered more ecologically relevant because native species are likely to be key organisms within the food webs and the ecosystems they are part of (Krull and Barros, 2010, Baird et al., 2007). Thus, working with native invertebrate species could be more pertinent for extrapolation to natural scenarios.

Also, there are studies that have shown that some native species are less tolerant to exposure to toxics than exogenous species and have highlighted this as an important factor in the success of invasive species (Piola and Johnston, 2009; Lenz et al., 2011; Oliveira et al., 2015; Varó et al., 2015). Consequently, using non-native species in bioassays could lead to misguided decision-making if, for instance, they are used to determine aquatic guide levels for different contaminants (Freitas and Rocha, 2014).

Biomphalaria straminea

The freshwater snail *B. straminea* (Figure 1a,b) (Dunker, 1848) belongs to the Planorbidae family, a large and diverse family of aquatic pulmonate gastropods distributed worldwide (Dillon, 2000). In Argentina, specimens of *B. straminea* were recorded in Salta, Formosa, Chaco, Misiones, Corrientes, Entre Ríos, Córdoba, Buenos Aires and Río Negro provinces (Rumi et al., 2008).

B. straminea gastropods have the same type of radula as scraper herbivore organisms but they are unspecialized feeders (Yipp, 1983; Vera-Ardila and Linares, 2005). This species is able to survive within a wide variety of habitats, being hard water, warm temperatures and eutrophic habitats optimal for its development (Yipp, 1983). Organisms are also able to withstand periods of drought as their metabolic rates can be reduced to basal levels. Olivier and Barbosa (1956) found that a population of snails studied in the laboratory was able to survive for up to 380 days in mud which was left to dry naturally.

They are simultaneous hermaphrodites, reproducing by both self-fertilization and cross-fertilization, which allows them to easily reproduce and colonize new habitats. Organisms lay several eggs inside gelatinous capsules (Figure 1b) which they adhere to hard substrates such as plants, rocks, shells of other snails or other surfaces; they can store these egg masses inside the shell for some days before oviposition. Embryos develop inside the egg and hatch out as juveniles without going through larval stages (Yipp, 1983).

B. straminea is a species that can be easily handled and maintained in laboratory cultures. The snails do not require stringent conditions to survive and reproduce and, under favorable conditions, they have great reproductive potential, rapid growth and early maturation (Yipp, 1983).

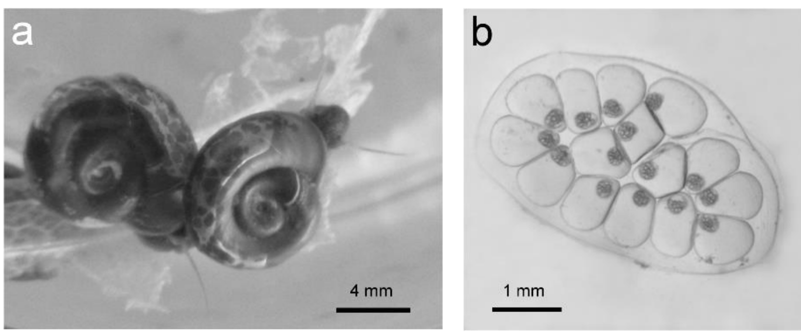

Figure 1. Photographs of *Biomphalaria straminea* adults (a) and egg mass (b).

B. straminea Bioassays

There are almost no reports in the literature regarding toxicology studies performed with *B. straminea*, except for a study conducted in our laboratory by Bianco et al., (2014). These authors characterized *B. straminea* cholinesterases (ChEs) and carboxylesterases (CEs), and evaluated their sensitivity to a 48 h exposure to azinphos-methyl through no observed effect concentration (NOEC) and 50% inhibition concentration (IC_{50}) estimations. They also evaluated subchronic effects after exposure to environmental concentrations of this pesticide.

In order to measure CE activity, they used both p-nitrophenyl acetate (p-NPA) and p-nitrophenyl butyrate (p-NPB) substrates, since CE activity and sensitivity to pesticides depend on the substrate used (Laguerre et al., 2009; Kristoff et al., 2012). They found that CE activity of total soft tissue resulted more sensitive to organophosphates when using p-NPA and p-NPB compared to other substrates (Kristoff et al., 2012; Cacciatore et al., 2013).

After an acute exposure (48 h) to azinphos-methyl at different concentrations (50, 100, 250, 500, 2500, 5000, 6500 and 10000 µg L^{-1}), Bianco et al. (2014) found that CE and ChE activities, when using p-NPA, were not inhibited even at the highest concentration used (Table 1). Conversely, CEs measured with p-NPB were inhibited at 500 µg L^{-1} and above, being the values of NOEC and IC_{50} 250 µg L^{-1} and 2200±750 µg L^{-1}, respectively (Table 1). Lethality or evident neurotoxic signs were not observed in the acute assay.

A different picture was observed when evaluating the response of the organisms after 21 days of exposure to environmental concentrations of azinphos-methyl (20 and 200 µg L^{-1}). A decrease in survival, protein content and CE activity (measured with p-NPB and p-NPA) was registered. In accordance with the acute exposure results, ChEs were not inhibited. Contrastingly, CE activity using both substrates was inhibited. With p-NPB as substrate it was reduced by 90% with the highest concentration of the pesticide and, with p-NPA as substrate, it was inhibited by almost 50% with both concentrations.

Recent subchronic studies (14 days), carried out in our laboratory at environmental concentrations of azinphos-methyl, showed no significant alterations in several reproductive parameters when we exposed *B. straminea* adults and egg masses until hatching (Cossi et al., 2015a). However, preliminary results showed effects in some oxidative stress parameters in adults exposed to the pesticide.

Chilina gibbosa

C. gibbosa (Figure 2) (Sowerby 1841) is a freshwater gastropod from the Chilinidae (Pulmonate) family, endemic to South America and especially abundant in southern Chile and Argentina (Bosnia et al., 1990; Rumi et al., 2008; Fuentealba et al., 2010; Gutiérrez-Gregoric et al., 2010).

C. gibbosa can be readily found along the Limay, Neuquén and Negro rivers, as well as in lakes and reservoirs of the Río Negro and Neuquén provinces (Bosnia et al., 1990; Valdovinos, 2006; Rumi et al., 2008). The species' most suitable environment is clear, well oxygenated waters, associated with macrophytes. It feeds on periphyton and benthic diatoms from the riverbed or from the macrophytes and is thus benefited by areas with high primary productivity (Bosnia et al., 1990).

According to Bosnia et al. (1990), *C. gibbosa* is a semelparous species with an annual life-cycle, with largest population growth in summer and almost ceasing in winter. Preliminary histological results indicate that it is hermaphrodite, but the exact reproductive strategy has not yet been described. Fertilized eggs are laid within capsules (Figure 2b), which we have observed can host from a few to more than 200 eggs at a time. A single juvenile hatches from each egg.

These snails have several characteristics that make them potential model organisms for ecotoxicology bioassays. On the one hand, adults are easy to collect and handle, as they can usually be found in shallow waters, in an aggregated dispersion pattern (Bosnia et al., 1990). On the other hand, their limited mobility and reduced ability to excrete pollutants may result in several negative effects at low environmental concentrations of toxicants (Oehlmann and Schulte-Oehlmann, 2003), as well as ensuring an effective exposure to any pollutant present in the environment. Furthermore, they have been described as a relevant part of the benthic fauna due to their high productivity and turnover rate, which make them an important food source for different species (Bosnia et al., 1990; Ferriz, 1993).

Nevertheless, we have found that *C. gibbosa* requires special conditions for surviving and breeding in the laboratory, such as controlled temperature and day-night cycles and permanently aerated and clean water, which represent a challenge in order to keep laboratory cultures. This is made harder by the fact that little has been studied and reported on *C. gibbosa*.

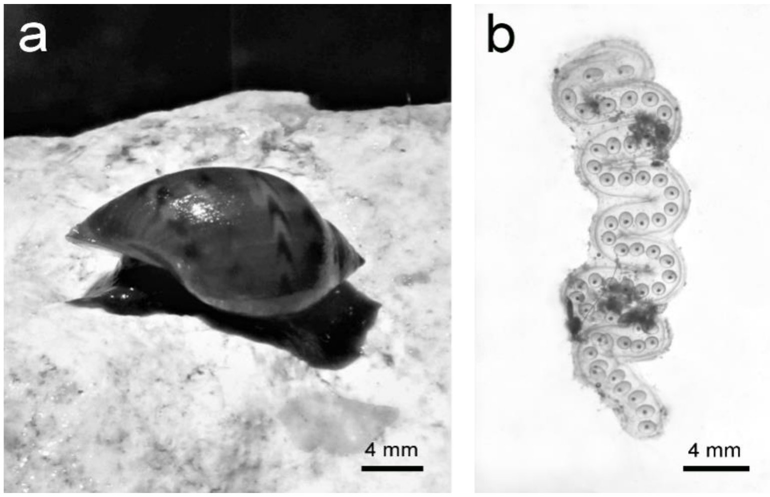

Figure 2. Photographs of *Chilina gibbosa* adult (a) and egg mass (b).

***C. gibbosa* Bioassays**

The first toxicological studies using *C. gibbosa* as model organisms were carried out in our group by Bianco et al. (2013). ChE and CE activity were measured on whole organism soft tissue after a 48 h exposure to a range of azinphos-methyl concentrations (0, 0.001, 0.005, 0.01, 0.1, 5, 100, 500, 1000 and 1500 µg L^{-1}). ChE activity was strongly inhibited, becoming significant even at low concentrations such as 0.005 µg L^{-1}. NOEC for ChEs was 0.001 µg L^{-1} and IC_{50} 0.02 ± 0.01 µg L^{-1} (Table 1). CEs proved to be less sensitive than ChEs, being significantly inhibited only at concentrations higher than 1000 µg L^{-1} (Table 1). CE activity using p-NPB was in turn slightly more sensitive (NOEC 500 µg L^{-1} and IC_{50} 1300 ± 100 µg L^{-1}) than using p-NPA (NOEC 1000 µg L^{-1} and IC_{50} 1290±40 µg L^{-1}) (Table 1).

Even though lethality was not significant, a conspicuous neurotoxic sign was observed in animals exposed to 0.01 µg L^{-1} or higher concentrations of azinphos-methyl (Figure 3a,b). The entire head-foot region of these snails protruded outside of the shell (Figure 3b), they lacked adherence and spontaneous movement and hardly responded to mechanical stimuli.

Bianco et al., (2013) also evaluated antioxidant defenses of animals exposed to two environmentally relevant concentrations of azinphos-methyl. At 0.02 and 20 µg L^{-1}, azinphos-methyl causes approximately 50 and 85% of inhibition of ChEs, respectively, but has no effect on CE activity. An exposure to 0.02 µg L^{-1} of azinphos-methyl caused an increase in glutathione (GSH) levels and catalase (CAT) activity. An exposure to the highest concentration only caused an increase in GSH levels. No effect was found on superoxide dismutase (SOD) and glutathione S-transferase (GST).

Based on these results, Cossi et al., (2015b) carried out an acute exposure (48 h) of *C. gibbosa* to 20 µg L^{-1} of azinphos-methyl and then monitored their recovery for 21 days after transferring the organisms to pesticide-free water. They found that while ChE and CE activity remained constant in control organisms during the bioassay period, in exposed animals ChEs were inhibited by 85% after 48 h and remained inhibited up to day 21, when a slight recovery in activity was registered. CE activity in exposed animals also remained constant and uninhibited during the experiment. Neurotoxicity was observed after 48 h, with most exposed snails showing weak or lack of adherence to the vessels and almost all of them exhibiting a protruded head-foot region and an absence of spontaneous movement. Most snails recovered adherence capacity during the 21 days in water free of pesticide, and by the end of the bioassay none of the snails showed any sign of head-foot protrusion.

Table 1. No observed effect concentration (NOEC) and 50% inhibition concentration (IC_{50}) of azinphos-methyl in the total soft tissue of *Biomphalaria straminea* and *Chilina gibbosa* after a 48 h exposure. ChE: cholinesterase; CE: carboxylesterase; AcSCh: acetylthiocholine iodide; p-NPA: p-nitrophenyl acetate; p-NPB: p-nitrophenyl butyrate

Species	Neurotoxic signs	Enzyme (substrate)	NOEC⁺ (µg L^{-1})	IC_{50}⁺⁺ (µg L^{-1})	Reference
B. straminea	No	ChE (AcSCh)	>10000*	>10000*	Bianco et al., 2014
		CE (p-NPA)	>10000*	>10000*	
		CE (p-NPB)	250	2200 ± 750	
C. gibbosa	Yes	ChE (AcSCh)	0.001	0.02 ± 0.01	Bianco et al., 2013
		CE (p-NPA)	1000	1290 ± 40	
		CE (p-NPB)	500	1300 ± 100	

⁺ Experimentally estimated as the maximum concentration causing no significant effect.

⁺⁺Calculated using the 4-parameter logistic model using OriginPro 7.5. Model equation: $y=A_2 + (A_1-A_2) / (1+(x/x_0)^p)$; y enzyme activity (expressed as % of control); x inhibitor concentration; A_1 and A_2 upper and lower bound for function values y; x_0 (relative IC_{50}) concentration corresponding to a response midway between A_1 and A_2.

* Not reached at the studied concentrations (50-10000 µg L^{-1}).

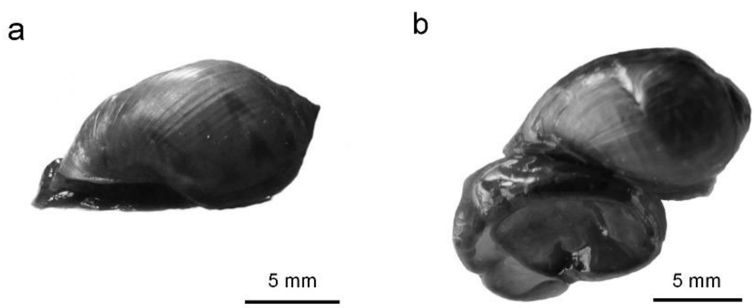

Figure 3. Organisms of *Chilina gibbosa*, control (a) and exposed to 20 µg L^{-1} of azinphos-methyl for 48 h (b).

Boburg et al., (2015) exposed *C. gibbosa* snails subchronically during 21 days to 20 µg L^{-1} of azinphos-methyl. By day 21 most of the snails had lost adherence to the walls and all presented a protruded head-foot region. At 48 h, ChE activity was greatly inhibited and remained inhibited during the rest of the bioassay. CE activity measured using p-NPB and p-NPA was only inhibited after 14 days of exposure. Preliminary histological studies have not shown any differences between control and treated animals.

In current studies we are focusing on questions that arose from the first bioassays. On the one hand, we intend to find other biomarkers that could be relevant when using an endemic native species such as *C. gibbosa*. For instance, we have recently found that an acute 48 h exposure to 20 µg L^{-1} of azinphos-methyl affects the immune system of these snails (Castro et al., 2015). On the other hand, we are exposing *C. gibbosa* to other contaminants, such as the carbamate carbaryl or river water polluted by a natural petroleum spill, in order to determine whether the conspicuous neurotoxic sign is specific to anticholinesterase insecticides and can be used as a simple and effective biomarker.

CONCLUSION

Our research group has evaluated the effects of the organophosphate pesticide azinphos-methyl, a relevant agrochemical used in Argentina and found in its watercourses (Loewy et al., 1999, 2011; Castañé et al., 2015) at concentrations higher than those recommended for the protection of aquatic wildlife, on two different species of native freshwater snails. *B. straminea* and *C. gibbosa*, our model organisms, have different natural habitats, life cycles and lifestyles. Thus, they provide an interesting possibility for comparing toxic effects between organisms. *B. straminea*, found throughout South America and widely distributed in northeastern Argentina, is a species with a great reproductive potential, rapid growth and an early maturation (Yipp, 1983), that can be easily handled and maintained in laboratory cultures. On the contrary, *C. gibbosa*, endemic to freshwater habitats of the Argentine Patagonia, is a semelparous species with an annual life-cycle (Bosnia et al., 1990) that requires special conditions for surviving and breeding in the laboratory.

Our results show clear differences in the responses to azinphos-methyl between *B. straminea* and *C. gibbosa*. Whilst *B. straminea* does not show acute toxic effects at environmental concentrations, resulting particularly resistant to azinphos-methyl, *C. gibbosa* is a highly sensitive species that exhibits a conspicuous physical neurotoxic sign even after an acute exposure to the pesticide (Bianco et al., 2013, 2014). In *B. straminea*, however, toxic effects were observed at environmental concentrations after subchronic exposures, which further demonstrated the need to evaluate effects at different exposure times (Bianco et al., 2014). These dissimilar responses have led to individual branching questions. On the one hand, could *C. gibbosa* be a suitable sentinel species to indicate the presence of azinphos-methyl and/or organophosphate pesticides? On the other hand, given that *B. straminea* appears to be more tolerant to azinphos-methyl, could there be long-term effects on the organisms and their offspring?

Our findings also reinforce the notion that different species within a same trophic level should be taken into account for biomonitoring programs, especially considering that even species belonging to the same family can respond differently to the same toxicant (Kristoff et al., 2006; Bianco et al., 2014). Working with bioassays and native species implies that our results could provide relevant information to be used for re-assessing guideline maximum levels of water contaminants for aquatic life protection and new tools to evaluate aquatic toxicity risk.

Further research will allow us to continue contributing with biochemical, histological, behavioral and physiological information about native gastropods; propose early warning biomarkers; provide evidence for the potential use of these species as sentinels; and develop a baseline to perform ecotoxicological assays with water sampled from the field.

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Chapter 4

ENERGY PHYSIOLOGY AS BIOMARKER IN ASSESSING ENVIRONMENTAL POLLUTION

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ABSTRACT

An ecosystem's health is more and more difficult to assess due to the variety and amount of xenobiotic substances. Physiological energy measurements are therefore used together with chemical analysis of pollutants in the body's tissues to detect and quantify the effects of pollution. The Scope for Growth (SFG) is the most common measurement because it integrates basic physiological responses such as feeding, digestion, respiration and excretion. This is the case of *Choromytilus chorus,* a native mussel from the Chilean coast, where it was observed that an increase in the concentration of PAHs (polycyclic aromatic hydrocarbons) or Ochs (organochlorine pesticides) in tissue produced a negative effect on their physiological processes. When the concentrations of these pollutants were low in the tissue, the population responses were better. This chapter describes the SFG in a gradient of pollution along the coast in spring and summer, finding the high pollution site with a negative SFG (-4.56 J/h/g in spring and -3.45 J/h/g in summer). The energy balance in *C. chorus* in the site with less pollution was positive with values of 6.2 J/h/g in spring and 25.7 J/h/g in summer. In general, the physiological responses showed a significant negative correlation with organochlorine pesticides (r=-0.87) and PAHs (r=-0.89).

Keywords: scope for growth, biomarker, aquatic pollution, bivalve, *Choromytilus chorus*

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INTRODUCTION

The assessment of environmental pollution using physiological biomarkers has been carried out with success for the easiness to collect the data and its ecological relevance (Bayne, 1985; Barton et al., 2002; Chambers et al., 2002). The physiological energetic responses not only provide information about fundamental processes of acquisition, spending and available energy for growth and reproduction, but also reflect some of the main mechanisms of toxicity (Smaal and Widdows, 1994). For evaluating the energy balance, physiological processes such as Scope for Growth (SFG), the Oxygen/Nitrogen Ratio and Condition Index are used. The SFG is the most applied measurement since it integrates basic physiological responses (feeding, digestion, respiration and excretion), and because available total body energy may be affected by altering one or more of these processes (Widdows, 1985).

The assessment of SFG in the organism together with chemical analysis in its tissue allows for the prediction of possible negative impacts at different levels of biological organization, from the cell to the ecosystem. In addition, this biomarker has high sensitivity to environmental changes, accuracy, simplicity and the physiological measurements are of short duration (Bayne et al., 1979; Peck et al., 1987; Widdows and Johnson, 1988; Widdows and Donkin, 1991; Widdows and Salked, 1992; Quian, 2011).

The SFG has been used to assess contamination with heavy metals such as cadmium (Poulsen et al., 1982) and lead (Widdows et al., 1990), organochlorines (Toro et al., 2003; Shuhong et al., 2005), aromatic hydrocarbons (Toro et al., 2003; Halldórsson et al., 2005), tin compounds (Widdows et al., 1990; Halldórsson et al., 2005) and other highly toxic compounds such as polychlorinated biphenyls (Widdows et al., 1990). The main marine organisms used for the evaluation of pollution are bivalves such as *Mytilus edulis* (Poulsen et al., 1982; Halldórsson et al., 2005), *M. galloprovinciales* (Widdows et al., 1997; Albentosa et al., 2012) and *Anadara granosa* (Din andAhamad, 1995) as well as the marine gastropods *Thais lima* (Stickle et al., 1984) and *Haliotis fulgens* (Farías et al., 2003). The main goal of this chapter is to show that Scope for Growth is a useful biomarker for assessing aquatic pollution and it has great relevance at an ecological level.

METHODS

Study Area

Specimens of the mussel *Choromytilus chorus* were collected at three locations on the south-central coast of Chile (Figure 1). Two samplings were carried out at each location: one in spring 1998 and another in summer 1999. Site A (highly polluted) is in San Vicente Bay (36°44´S; 73°09´W), a location surrounded by fish processing, steel, and petrochemical industries. Site B (medium pollution) is in Corral Bay (39°52´S; 73°25´W), which presents a fishing port and small human populations along its shore. Site C (low pollution) is in Yaldad Bay (43°08´S; 73°44´W), an isolated location where the main activity was mussel culture (*M.chilensis*).

Sampling

C. chorus specimens were collected from natural banks in San Vicente and Corral Bays, while individuals from Yaldad Bay were obtained from cultures suspended at 4-8 m depth. At each station, adult specimens ~120 mm in length were collected for chemical analysis and individuals ~70 mm long for physiological measurements.

Chemical Analyses

Tissue Preparation

For each sampling site, between 15 and 20 specimens were pooled as a single sample to minimize natural variance among individuals (n=four analytical replicates). Standard operation procedures for sample handling and preparation followed guidelines of FAO (1983). Subsamples of 10 g of pooled material were prepared for analyses of PAHs (polycyclic aromatic hydrocarbons) and Ochs (organochlorines).

Standards and Solvents

The analytical methodology was tested on Standard Reference Material (SRM 2974) containing 14 PAHs and seven OChs at known concentrations in mussel tissue (*M. edulis*), obtained from the National Institute of Standards and Technology (NIST).

Analysis of Subsamples

The extraction, clean up and analyses of Ochs in the tissue of *C. chorus* were carried out as described by Knickmeyer and Steinhart (1988) and FAO (1983). The UNEP (1992) methodology was followed for extraction, purification, and analyses of PAHs. Detection of the OChs was performed with a GC-ECD Perkin-Elmer 9000 with a^{63}Ni electron capture detector. PAHs were identified and quantified by using a HP 6980 (Hewlett Packard) gas chromatograph equipped with HP 5973A mass selective detector. The Estimated Detection Limit (EDL) of the analytical method was 5 µg/kg dry tissue for 10 g sample.

Physiological Measurements

Acclimation

Before beginning physiological measurements the mussels were cleaned of epibionts and maintained for 12-48 h as recommended by Widdows (1993). Test specimens were maintained at standard laboratory conditions, at a salinity of 30 and temperature of 12 ºC for spring experiments and 15°C for those performed in the summer.

Since many of the physiological processes are dependent on the body size of the individual, the size effect was removed by standardization of the physiological rates to per-gram dry weight values, using the following equation:

$$\mathrm{Y}s = \left(\frac{Ws}{We}\right)^{b} \text{ x Ye}$$

Where *Ys* is the physiological rate for a normalized weight, *Ws* the normalized weight, *We* the observed weight, *Ye* the measured physiological rate and *b* the weight exponent (regression potential; Navarro, 1988).

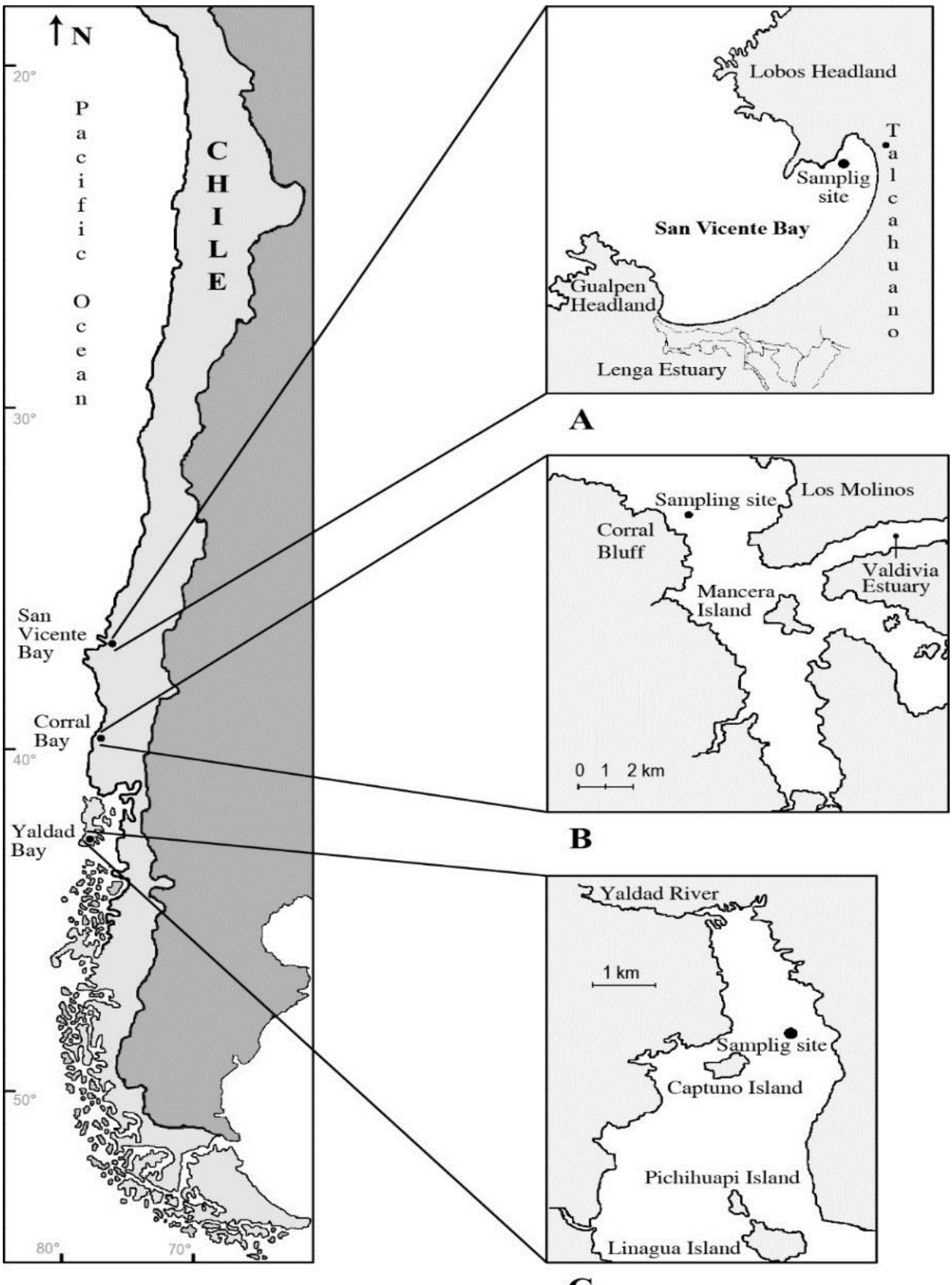

Figure 1. *Choromytilus chorus* sampling sites: San Vicente Bay (A); Corral Bay (B); Yaldad Bay (C).

Clearance Rate (CR)

The feeding rate in bivalves is usually measured in terms of clearance rate, and it is evaluated using a static system, following the methodology described by Bayne et al. (1985). Fifteen aquariums of 6 L received a diet of 20×10^6 cell/L of laboratory-cultured *Isochrysis galbana*. The clearance rate for each individual was calculated using the following equation:

$$CR = \frac{V(lnC_1 - lnC_2)}{t}$$

where, *CR* is the clearance rate, *V* the volume of water, and C_1 and C_2 are the *I. Galbana* concentrations at the beginning (1) and end (2) of the test interval t.

Absorption Efficiency (AE)

Faecal material produced during the measurements of particle clearance was filtered onto filters and processed by the same methods as for the diet samples. The ratio of diet ash free dry weight (AFDW) of the faeces was used to determine the absorption efficiency using the method of Conover (1966):

$$AE = \frac{(F - E)}{(1 - E)\ x\ F}$$

where*F* is the ash free dry weight to dry weight ratio for the food and *E* the ash free dry weight to dry weight ratio for the faeces.

Absorption Rate (AR)

AR represents the amount of ingested material absorbed per unit of time, and is calculated as a product of the absorption efficiency and the organic ingestion rate (OIR; Bayne et al., 1985):

AR (mg/h)=*AE* x *OIR*

Oxygen Consumption (OC)

OC was measured by placing each mussel into a sealed 800 mL respirometer chamber containing oxygen-saturated seawater. Oxygen in the water was measured by the Winkler method (Strinckland and Parsons, 1972). Oxygen consumption was calculated using the formula:

$$OC\ (mLO_2/h) = \frac{[(mLO_2/Lcontrol - mLO_2/Lchamber) \times Vc]}{t}$$

Where mL O_2 per L control is the O_2 concentration in the chamber without mussels; ml O_2 per L chamber is the O_2concentration in the chamber containing a mussel; *Vc* the volume of the chamber, and *t* the incubation time (h). Oxygen consumptions were expressed as energetic values using the oxycaloric conversion value (1 mL O_2=19.9 J) given by Elliot and Davinson (1975).

Scope for Growth (SFG)

After completion of all the physiological measurements, the soft tissues of each mussel were removed and dried to constant weight at 60°C for 48 h, and used to convert the physiological rates to per-gram dry body weight values. To calculate the SFG, all the physiological values were converted to energetic equivalents (J/h per g) following Bayne et al. (1985):

$$SFG = A - R$$

where *SFG* is the scope for growth, *A* the absorbed energy and *R* the respired energy.

Statistical Analyses

Data on OChs could not be submitted to statistical analyses because many of the compounds occurred in trace amounts which affected their detectability, producing very high variations in tissue subsamples. Conversely, PAH compounds were found in relatively high concentrations in the *C. chorus* tissues, allowing for statistical comparisons between samples. Since the data did not fit criteria for applying analysis of variance tests, the non-parametric Mann-Whitney test was used to compare PAH contents of tissues of mussels from the three sampling sites for the two sampling seasons. This test was also used to compare physiological responses of the mussels from the different populations in the two seasons, since the data did not show homoscedasticity of variances, nor could be normalized by transformation. Correlation between different physiological responses and SFG of *C. chorus* and the concentrations of the organic pollutants (PAHs and OChs) present in their tissues was determined using a Spearman multiple correlation matrix (Sokal and Rohlf, 1979).

Table 1.Total organochlorines (OChs) and polycyclic aromatic hydrocarbons (PAHs) accumulated in tissue of *Choromytilus chorus* from the south/central coast of Chile

Sampling site	OChs (Σμg/kg dry wt)		PAHs (Σμg/kg dry wt)	
	Spring	Summer	Spring	Summer
San Vicente	29.5	29.5	3380.0	2993.0
Corral	7.5	6.0	82.0	215.0
Yaldad	17.0	0.5	347.0	49.0

RESULTS

Chemical Analyses

C. chorus individuals from San Vicente, Corral, and Yaldad Bays contained low levels of OChs in their tissues. The total concentrations of OChs were always higher in tissues of specimens from San Vicente Bay population both in spring and summer when compared with populations from Corral and Yaldad. The values for PAHs were much higher than for OChs, following the same tendency (Table 1).

Organochlorines

The OChs results obtained from the samples were classified according to the toxicity groups established by the US Environmental Protection Agency (EXTOXNET, 2000). The San Vicente mussel population contained the highest levels of the substances in both seasons measured (Figure 2), and the highest concentrations were of moderate toxicity (Class II), i.e., such as chlordane (α and β), heptachlor, heptachlor epoxide, DDT (and metabolites) and hexachlorocyclohexane isomers. Others chlorinated compounds highly toxic such as aldrin, dieldrin, endrin, endrin aldehyde, endosulfan and endosulfan sulfate were also recorded at relatively high concentrations, particularly in individuals from Bay of San Vicente during the spring and summer, while in the sites of Corral and Yaldad they were below the detection limit.

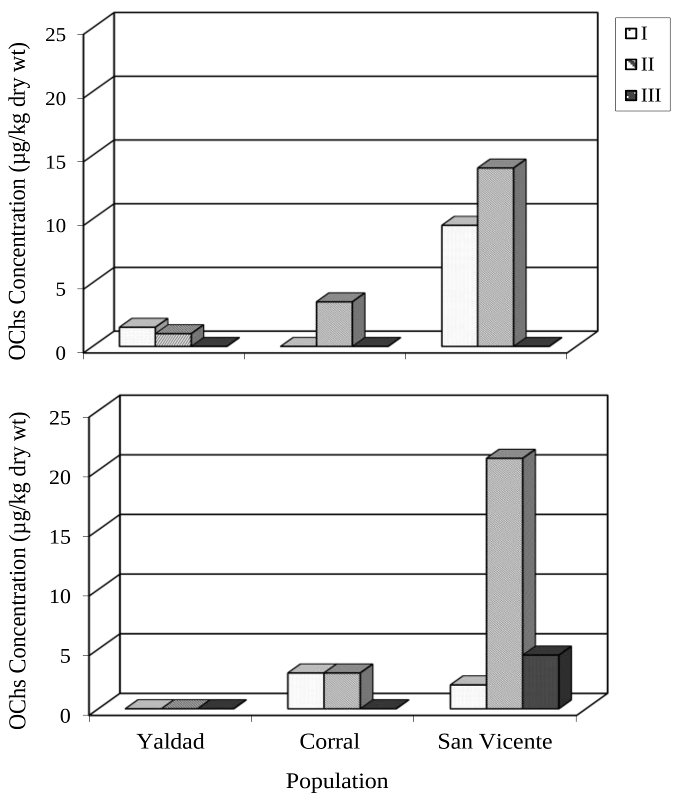

Figure 2. Concentrations of organochlorine (OChs) compounds in tissues of *Choromytilus chorus* grouped according to toxicity level established by the US Environmental Protection Agency (EXTOXNET, 2000). Upper: spring; lower: summer. I: high toxicity; II: moderate toxicity; III: non-toxic.

Polycyclic Aromatic Hydrocarbons

In the scan for 16 PAH, 13 were found and quantified by the GC-MS methods. Total levels of PAH were always higher than total OCh levels in the mussel tissues, in an order of magnitude of 10-35 times in individuals Corral Bay, in 20-100 in Yaldad, and 100 -130 in San Vicente Bay. The population from San Vicente demonstrated the highest total concentration of PAHs both in spring and summer (Table 1). Mussel population from San Vicente bay contained higher levels of potential carcinogens (RAIS, 2000) than those of the other two locations sampled (Figure 3).

The PAHs were grouped according to their aromatic rings. It was found that in the Bay of San Vicente the highest percentage corresponded to hydrocarbons with 4rings (52%).While the PAHs with 2-3 rings showed the highest percentage in the bays of Yaldad and Corral (73 and 79%, respectively; Table 2).

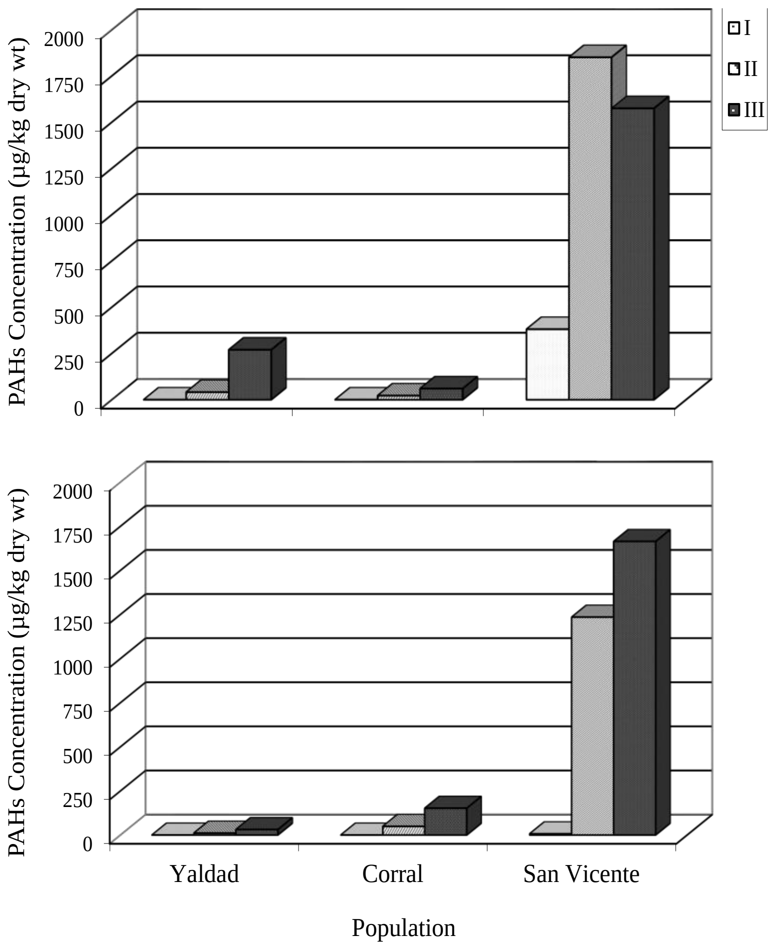

Figure 3. Concentrations of polycyclic aromatic hydrocarbons (PAHs) in tissues of *Choromytilus chorus* grouped according to carcinogenic potential (RAIS, 2000). Upper: spring; lower: summer. I: carcinogenic; II: probably carcinogenic; III: non-carcinogenic (n=4).

Physiological Measurements

During spring, *C. chorus* from San Vicente Bay had significantly lower CR (0.67 ± 0.32 L/h per g) than those from Yaldad (1.41 ± 0.38 L/h per g; $p<0.0005$). In summer, individuals from San Vicente bay only showed significant differences with those of Yaldad bay (CR 2.25 ± 0.21), obtaining an average value of 0.97 ± 0.42L/h per g, corresponding to 43% of the CR found for *C. chorus* from Yaldad (Figure 4).

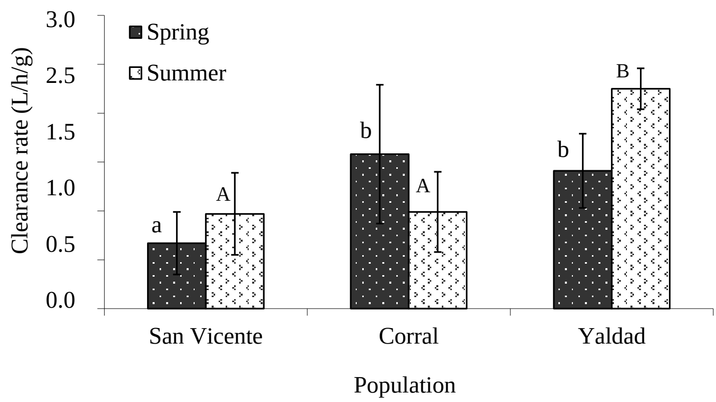

Figure 4. Clearance rate in *Choromytilus chorus* exposed to organochlorines and polycyclic aromatic hydrocarbons from Chilean coast. Values represent the mean ± standard deviation (vertical lines; hereafter SD); letters indicate statistical differences between sites during spring (lowercase) and summer (uppercase).

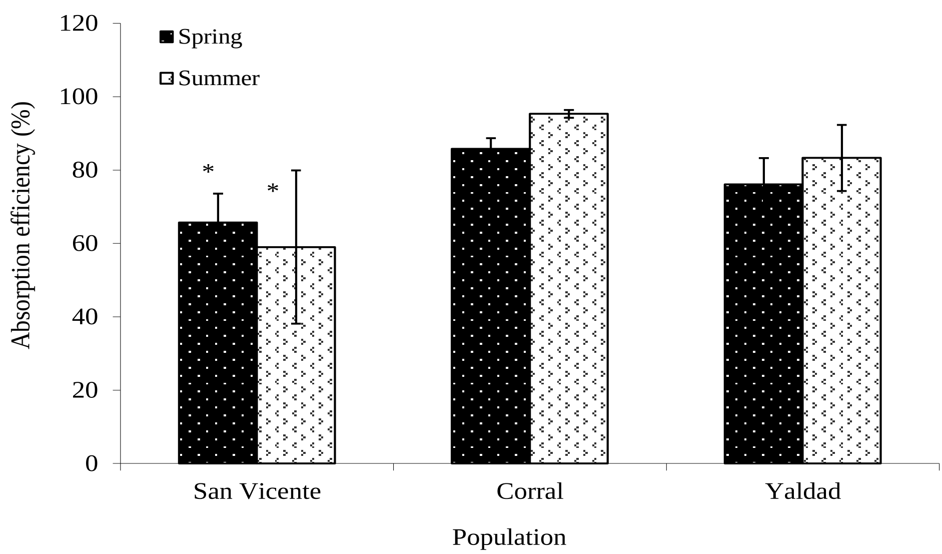

Figure 5. Absorption efficiency in *Choromytilus chorus* exposed to organochlorines and polycyclic aromatic hydrocarbons from Chilean coast. Values represent the mean ± one SD; * indicates no significant differences in relation to other sites.

Table 2.Concentration of polycyclic aromatic hydrocarbons (PAHs) in tissue of *Choromytilu chorus* from the south/central coast of Chile, according to aromaticity. The number between () represents the percentage of PAH with this number of rings. DL=detection limit (5 µg/kg)

Number of rings	Concentration (µg/kg dry wt)					
	San Vicente		Corral		Yaldad	
	Spring	Summer	Spring	Summer	Spring	Summer
2-3	695 (18)	1339 (46)	55 (67)	147 (73)	245 (79)	25 (60)
4	1325 (35)	1513 (52)	27 (33)	54 (27)	66 (21)	17 (40)
5-6	1787 (47)	54 (2)	<DL	<DL	<DL	<DL

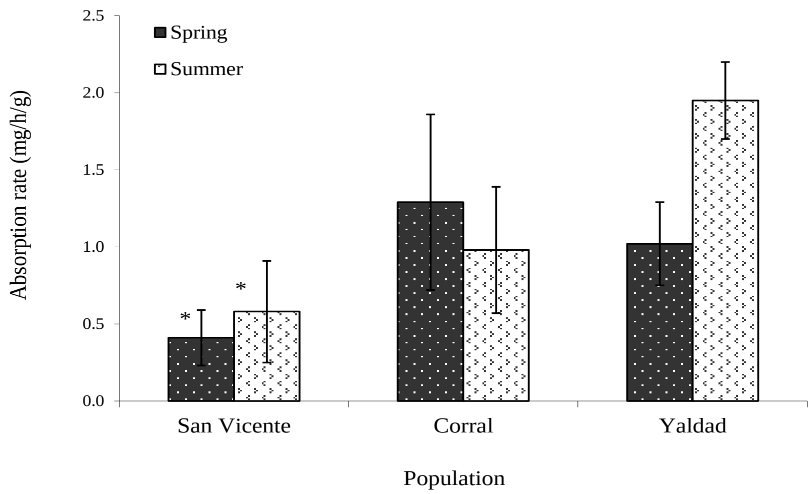

Figure 6. Absorption rate in *Choromytilus chorus* exposed to organochlorines and polycyclic aromatic hydrocarbons from Chilean coast. Values represent the mean ± one SD; * indicates no significant differences between seasons.

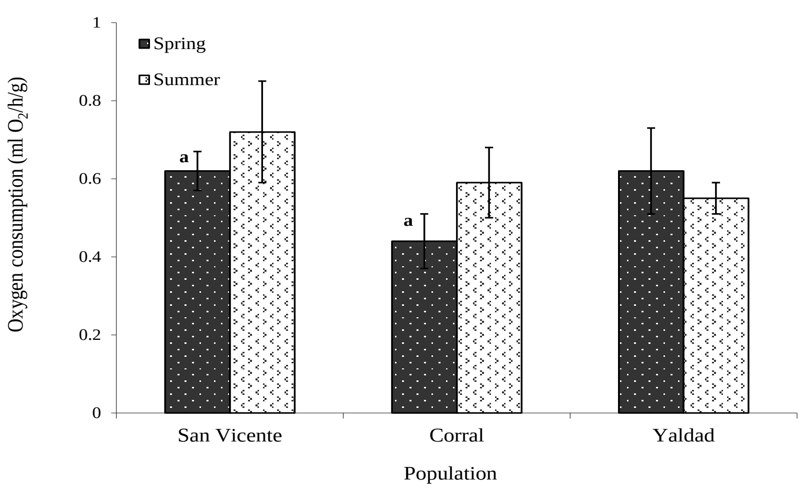

Figure 7. Oxygen consumption in *Choromytilus chorus* exposed to organochlorines and polycyclic aromatic hydrocarbons from Chilean coast. Values represent the mean ± one SD; the lowercase *a* indicates statistical differences between seasons.

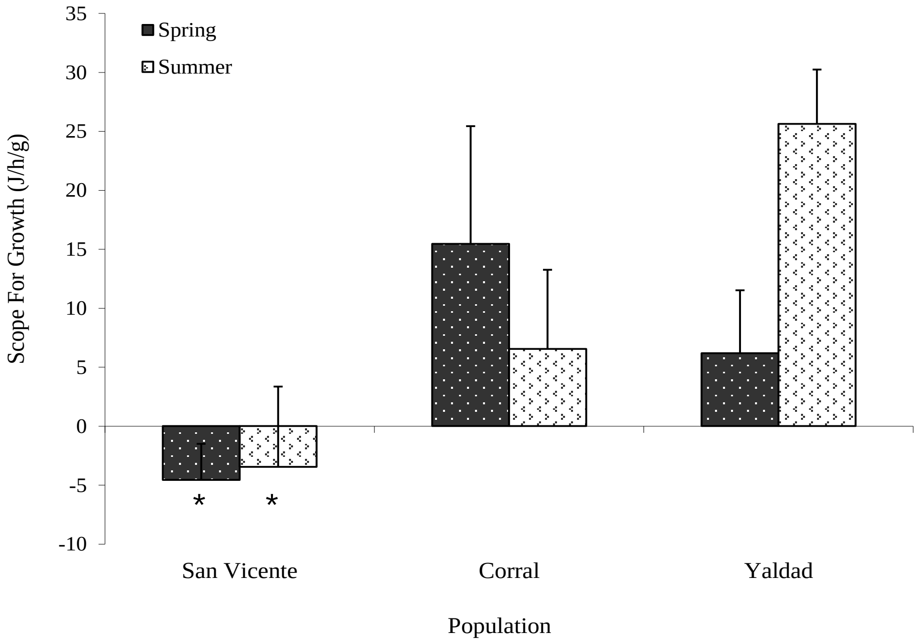

Figure 8. Scope for growth in *Choromytilus chorus* exposed to organochlorines and polycyclic aromatic hydrocarbons from Chilean coast. Values represent the mean ± one SD; * indicates no significant differences between seasons.

The absorption efficiencies measured in individuals from Corral and Yaldad Bays were significantly higher ($p<0.005$) than those measured for the mussels from San Vicente Bay, where the values never exceeded 70% during both seasons (Figure 5).

The AR showed the same tendency as the CR, since it represents an integrated response to the CR, ingestion rate, and absorption efficiency (Figure 6).

The oxygen consumption rates varied between populations and between seasons. Although there were significant differences ($p<0.01$) between spring and summer for all three populations, there were no differences in rates between the populations of San Vicente and Yaldad in spring, and between Corral and Yaldad in summer (Figure 7).

The SFG of individuals from Corral bay (15.5 and 6.6 J/h per g) and Yaldad bay (6.2 and 25.6 J/h per g) were positive in both spring and summer, in contrast to individuals from San Vicente bay which showed negative values in both seasons (-4.6 and -3.5 J/h per g). Significant differences were found ($p<0.01$) among populations and between seasons except for individuals from San Vicente which showed similar values in both spring and summer (Figure 8).

The Spearman multiple correlation matrix showed that the physiological responses related to energy balance have a significant correlation with the presence of organic pollutants in the tissues ($p<0.001$). In the three populations studied, a negative effect on the physiological parameters was observed with an increase in PAHs and OChs concentrations in the mussel tissues. The absorption rate showed the greatest correlation to organic pollutants: $r=-0.86$ for OChs and $r=-0.91$ for the PAHs. This was followed by the CR: $r=-0.71$ for the OChs and $r=-0.82$ for the PAHs. The AE showed a lesser correlation ($r=-0.66$) with PAHs while the OChs it was -0.75. Oxygen consumption was the only physiological response with a positive and significant ($p<0.001$) correlation with the pollutant concentrations: 0.42 for the

OChs and 0.51 for the PAHs. The index of SFG showed a significant negative correlation to tissue pollutant levels: r=-0.87 for the OChs and r=-0.89 for the PAHs (Figure 9).

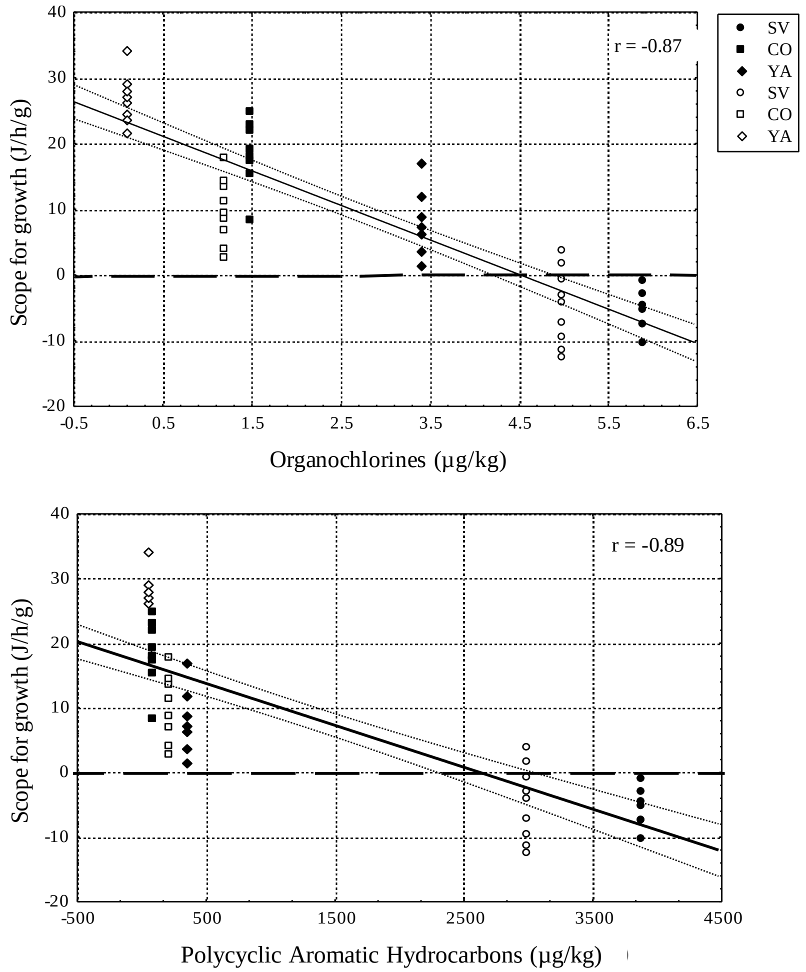

Figure 9. Correlation between the Scope for Growth and concentration of organochlorines and polycyclic aromatic hydrocarbons in *Choromytilus chorus* populations from San Vicente (SV), Corral (CO) and Yaldad (YA) in spring (dark symbols) and summer (clear symbols).

DISCUSSION

Chemical Analyses

Organochlorines

The low concentrations of OChs in tissues of *C. chorus* may be the result of the banning of most of these compounds over the last 20 years in Chile. Residues detected in the mussel may have originated from resuspended sediments as these (lipophilic) compounds may adsorb to sedimentary organic material, and remain in bottom sediments for many years (EPA, 1982; Balls and Campbell, 1999).

None of the compounds detected occurred above action levels established by the Food and Drug administration (USA-FDA, 1992). Compared with values for OChs burden in *M. edulis* obtained from the highly polluted Baltic Sea (Jensen et al., 1969; Lee et al., 1996), the present values for *C. chorus* are low, even those of San Vicente which were the highest among the values from the present study. Present values obtained from Corral and Yaldad are indeed at insignificant levels, as would be expected of non-industrialized regions.

Polycyclic Aromatic Hydrocarbons

The greater concentrations of PAHs when compared with the OChs is due to the multiple sources of the former (pyrogenic, diagenetic, petrogenic) which liberate these compounds into the environment (CEPAL, 1990; EHC, 1996, 1998). Thus the elevated concentrations of PAHs in the specimens from San Vicente bay during both spring and summer, when compared with individuals from Corral and Yaldad Bays, are probably related to the industrialization at San Vicente. This bay is subject to extensive pollution in the form of petroleum and pyrogenic wastes (Mudge and Seguel, 1999), while the anthropogenic influence in the other bays is low, particularly at Yaldad, shown by the low degree of these pollutants in the respective *C. chorus* tissues.

Values of PAHs for *C. chorus* populations were within the ranges observed in other bivalve species such as *M. galloprovincialis* (Shchekaturina et al., 1995), *M.edulis* (Baumard et al., 1999) and *Crassostrea virginica* (Noreña et al., 1999). The elevated concentrations of PAH from San Vicente samples in both seasons explain the higher uptake of oil pollution (Mudge and Seguel, 1999) by mussels when compared with individuals from Corral and Yaldad. According to the criteria presented by the preceding authors and Axelman et al. (1999), mussels from the San Vicente population are highly polluted with PAH, while those from Corral and Yaldad would be considered unpolluted. Populations of *C. chorus* from Corral and Yaldad had a qualitative pattern of distribution of hydrocarbons similar to those in other species such as *Arca zebra* (Widdows et al., 1990) and *Dreissenia polymorpha* (Gossiaux, 1998) where there was a tendency to accumulate PAH of low molecular weight (di and triaromatics), principally anthracene and phenanthrene. In contrast, mussels from San Vicente accumulated a higher molecular weight (3-4 ring) hydrocarbon burden, including indeno(1,2,3-*cd*)pyrene, dibenzo(a,h)anthracene, and benzo(*ghi*)perylene. These hydrocarbons may have been absorbed from resuspended benthic sediments, since the mussel bed was located in a zone experiencing a high degree of turbulence.

Physiological Responses

The CR measured in *C. chorus* was significantly ($p<0.001$) affected by the presence of OChs and PAHs in their tissues, as is reflected by high correlation coefficients linking the physiological responses with concentrations of the studied pollutants. Various ecotoxicological studies have shown that the CR is the component of the energy budget the most affected by toxic compounds (Howell et al., 1984; Axiak and George, 1987a; Bourdelin, 1996). These pollutants may produce a narcotizing effect on the ctenidial cilia or cause morphological alterations in branquial filaments and severe disturbances of ciliated epithelial cells (Bayne et al., 1982; Axiak and George, 1987b; Auffret, 1988). For example, concentrations of 100 mg/g dry tissue of 1-3 ring aromatic hydrocarbons may produce a 50%

reduction in CR of *M. edulis* when the octanol-water partition coefficient (K_{ow}) is less than five (Donkin et al., 1989; Widdows and Salkeld, 1992). The former suggests the decline in CR in *C. chorus* may be due in part to the presence of tri- and tetra-aromatic PAHs which were detected at high concentrations in the mussels (Table 2).

The significant reduction in the AE, as experimentally observed in mussels from San Vicente bay when compared with the same species from Corral and Yaldad bays during two seasons of the year, indicated deficiencies in the functioning of the digestive systems in mussels from the polluted bay. These results are comparable to results obtained in other studies on the efficiency of absorption rate in other bivalve species, where this rate declined in the presence of organic chemical compounds as aromatic hydrocarbons (Widdows et al., 1982, 1987). In mussels the exposition to aromatic hydrocarbons induces pathologic changes in digestive and basophilic cells, unstablizing the lysosomal membrane and thus making it more permeable to hydrolytic enzymes toward the cytoplasm, which accelerates protein hydrolysis and causes cellular atrophy (Lowe et al., 1981; Moore and Lowe, 1985; Lowe, 1988; Moore, 1988; Lowe and Clarke, 1989).

The high value for oxygen consumption measured in *C. chorus*, primarily in individuals from San Vicente Bay, may be related to the greater expenditure of metabolic energy produced by significant concentrations of pollutants in tissues of this bivalve species. Increased consumption of oxygen in bivalves has been observed in response to low levels of petroleum hydrocarbons (Gillfillan et al., 1976). Some authors suggest that the hydrocarbons may cause an uncoupling of oxidative phosphorylation, increasing the metabolic rate (Bayne et al., 1982), while others maintain that the increase in the rate of oxygen consumption is due to enzymatic activity related to the degradation of the petroleum components (Stekoll et al., 1980).

The negative SFG demonstrated by mussels from San Vicente Bay in spring (-4.6 J/h per g) and summer (-3.5 J/h per g) indicates severe stress, given that these individuals are consuming bodily reserves for survival. The reduction of the SFG to negative values is a result of the low clearance rate observed for individuals from San Vicente bay which were unable to obtain enough energy to fulfill their metabolic requirements. Exposure of marine invertebrates to pollutants, principally PAHs and other organic pollutants, often produces a loss of fitness of the organism, observable as reduction in SFG or reproductive capacity, or increase in mortality (Bayne et al., 1982; Widdows, 1985). Ultimately this may affect population dynamics by interfering with material and energy flows (Kooijman, 1998). The SFG in *C. chorus* was reduced in proportion to the degree of pollution to which they were exposed (Figure 9) and agrees with the results of Widdows et al. (1997) who obtained results for maximum SFG in *M. edulis* of 20-25 J/h per g in low-pollution environments, 10-16 J/h per g in coastal regions with urban influence, and -5 J/h per g in industrialized, highly polluted urbanized environments. The results obtained on the physiological responses of *C. chorus*, in addition to the chemical analyses of their tissues, allow to infer that the populations at Corral and Yaldad are still able to counteract the negative effects of pollutants present in their environments, since they demonstrate a positive energy budget. It may, therefore, be supposed that these populations are capable of normal growth and reproduction, and are thus capable of maintaining the survival of the species within the ecosystem. San Vicente bay is one of the most polluted sites of Chile, characterized by a strong hydrocarbon pollution (Mudge and Seguel, 1999). Hence, individuals of this population showing a negative SFG are under a constant stress, being more susceptible to diseases, predation and parasites. If these conditions persist in the bay, the population could disappear in a long term.

CONCLUSION

The mussel *C. chorus* presented levels of PAHs and OChs that reflected the concentrations found in their environments. These organisms would therefore be good bioindicators of local pollution.

The combination of physiological measurements of energy balance and chemical analyses of pollutants in tissues of *C. chorus* is a useful tool for the evaluation of environmental pollution, given the sensitivity and ecological relevance. Since the responses measured are at the organism level, they have the advantage of being readily interpreted as beneficial or deleterious. Furthermore, they may be predictive of long-term consequences for the growth and survival, not only of the individuals but also of populations, with individual measurements predicting pollution effects at the ecosystem level.

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Chapter 5

LABORATORY AND FIELD BASED STUDIES PROVIDE INSIGHTS OF COPPER TOLERANCE MECHANISMS IN BROWN SEAWEEDS AND BIOTECHNOLOGY TOOLS FOR ENVIRONMENTAL DIAGNOSIS

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ABSTRACT

The model brown alga *Ectocarpus siliculosus*, a cosmopolitan small seaweed, is distributed in the subtidal and intertidal ecosystem, and has been identified in different impacted ecosystems, including copper-polluted locations. In this chapter we describe different laboratory- and field-based investigation aiming to identify defence mechanisms behind intra-specific tolerance in *E. siliculosus* toward copper (Cu) pollution. The *E. siliculosus* strain Es524, isolated from a Cu-polluted location in northern Chile, showed the highest defences compared to other four strains when exposed to up to 2.4 µM copper for 10 d. The response manifested in less intracellular copper, higher induction and polymerization of metal-chelating phytochelatins, increased syntheses of antioxidants glutathione (GSH) and ascorbate (ASC), and higher expression and activities of antioxidant enzymes such as ascorbate peroxidase (APX), catalase (CAT), and superoxide dismutase (SOD); all the latter were responsible, at least in part, for the low signs of oxidative damage and physiological impairment observed in this strain. Laboratory experiments help identifying specific metabolic responses, but do not represent what happens in the natural, more complex, environment. Thus, we designed a novel transplantation technique for field experiments with *E. siliculosus*. The algae was enclosed in a dialysis tubing device and deployed in Cu-polluted and pristine locations. Antioxidant responses of Es524 in the field were similar to those recorded in laboratory experiments, supporting that the recorded defences were mediated by copper excess.

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Moreover, the method was successful for environmental diagnosis and can be applied globally in temperate coastal ecosystems.

Keywords: copper stress, tolerance mechanisms, inter-population tolerance, brown algae, biomonitoring

INTRODUCTION

There is growing concern regarding the increasing anthropogenic impacts on marine ecosystems, among which metal pollution is one of the most important (Brown and Depledge, 1998; Sáez et al., 2012a). In Chile, coastal areas of the north have been the most impacted by metal contamination, mainly copper (Cu); mining activities of Cu are mostly located in this area due to the presence of important reservoirs in the Atacama Desert (Ramirez et al., 2005). Copper pollution in Chile is derived mainly from the disposal of extracting and refinery residues, as in the bay of Chañaral city, where miners have historically discharged metal tailings in coastal ecosystems, negatively impacting their biodiversity (Correa et al., 2000). Records show that copper concentrations in coastal sediments of Chañaral exceed 900 µg g^{-1} (De Gregori et al., 1996; Andrade et al., 2006); in contrast, in locations near less impacted cities such as Coquimbo, sediment concentrations of copper have been observed to be around 15 µg g^{-1} (De Gregori et al., 1996).

Copper is an essential metal, involved in many metabolic functions of living organisms such as acting as a cofactor of the enzymes superoxide dismutase (SOD) and cytochrome c oxidase (Leary et al., 2004), and as component of plastocyanin, which takes part in electron transport in chloroplasts of photosynthetic organisms (Tapken et al., 2012). However, beyond certain threshold concentrations, copper can become highly toxic and affect the metabolism. For example, excess copper can induce over-production of reactive oxygen species (ROS) such as superoxide anions (O_2^-) from hydrogen peroxide (H_2O_2) in Fenton reaction, and through the disruption of electron transport chains in mitochondria and in chloroplasts of photosynthetic organisms (Gómez et al., 2015; Sáez et al., 2015a). It is important to highlight that although ROS play an important role in signal transduction for the induction of gene expression, their over-accumulation can produce oxidative stress and damage to biomolecules, disrupting the metabolism, and eventually affecting physiological processes such as growth and reproduction (Roncarati et al., 2015; Sáez et al., 2015a; Gómez et al., 2016; Laporte et al. 2016; Moenne et al., 2016).

Brown macroalgae are the main primary producers and the base of trophic networks in coastal rocky shores in temperate environments, providing food, shelter and habitat for a variety of organisms; thus, their diversity and abundance control the complexity of entire coastal ecosystems (Sáez et al., 2012a; Ortega et al., 2014). It is because of the latter that environmental impacts on brown seaweeds will undoubtedly have consequences at higher levels of biological organization, such as at population and community levels, compromising the availability of species with high ecological and economic importance (Sáez et al., 2012a; Ortega et al., 2014). Although brown macroalgae share certain metabolic and ecological characteristics with other photosynthetic organisms such as green and red macroalgae, and even plants, they are phylogenetically distantly related (Charrier et al., 2008). In spite of the economic and ecological importance of brown seaweeds, little is known about their biology at low levels of biological organization when compared with other photoautotrophs; therefore, further research is required to explain the biology of such a relevant and distinctive group of marine organisms.

The bioavailability of Cu is an important factor mediating development and growth in photosynthetic organisms and brown algae. For instance, Cu is involved in enzyme activation and electron transport in photosynthesis (Falkowski and Raven, 2007); however, beyond certain levels, Cu can represent a risk for the survival of brown seaweeds. For example, it has been found that photo-inhibition can be caused by the substitution of magnesium (Mg^{2+}) by copper (Cu^{+2}) in the chlorophyll molecule, incapacitating it to perform photosynthesis (Kuepper et al., 2002). Besides the latter, the main negative effects of Cu on brown macroalgae are associated with the induction of an over-production of ROS and oxidative stress (Sáez et al., 2015a; Moenne et al. 2016). Tolerance to Cu in brown seaweeds depends on mechanisms that involve both metal-exclusion and intracellular antioxidant and defence responses. Exclusion mechanisms can be related to different factors, such as the permeability of the cellular membrane, adsorption to the cell wall and/or epybionts (i.e., epiphytic bacteria), and the exudation of organic substances (e.g., polysaccharides, phenolic compounds) (Gledhill et al., 1999; Sáez et al., 2015a). On the other hand, metal-complexing compounds such as phytochelatins (PCs), oligomers of glutathione (GSH) synthesized by the enzyme phytochelatin synthase (PCS), and transcriptionally produced metal-binding proteins such as metallothioneins (MET) (Roncarati et al., 2015), are also important intracellular defence mechanisms. Reactive oxygen defences are based on the syntheses of antioxidants molecules such as glutathione (GSH), ascorbate (ASC), and phenolic compounds, and in the activation of antioxidant enzymes like ascorbate peroxidase (APX), glutathione peroxidise (GPX), catalase (CAT), glutathione reductase (GR) and SOD, to prevent (or at least reduce) oxidative damage (Ritter et al., 2014; Roncarati et al., 2015; Sáez et al., 2015a,b).

While it is widely recognised that metal-pollution induces stress-mediated changes at population, community, and ecosystem levels, these higher level changes are typically too complex and far removed from the causative events to be used in developing tools for the early detection and prediction of the consequences of anthropogenic activity. The impacts of environmental conditions on organism fitness can be studied by detecting stress signals at the cellular to molecular level, and linking these to higher level impacts. These stress signals can act as early warning 'biomarkers' of reduced individual health and fitness. Development of appropriate biomarkers requires an understanding of the cellular and physiological processes associated with cell injury, molecular damage and impairment of protective systems as well as how they are manifested as reductions in reproductive output, competitive ability and survival of individuals (Brown and Depledge, 1998). This understanding could be gained using a combination of laboratory-based investigations and field studies (biomonitoring) (Sáez et al., 2015a). Although there is extensive literature demonstrating the effects of environmental stressors in living organisms, most of the mechanistic and stress metabolism observations have been conducted in simple models of single exposure under laboratory conditions. While they can provide valuable information on specific metabolic responses against stress, it does not necessarily represent the mechanisms by which organisms respond in natural, more complex, environments. In contrast, the majority of field environmental assessments display a descriptive approach and lack of mechanistic evidence permitting an effective identification of the action of a specific stressor (Sáez et al., 2015c).

Metal biomonitoring using brown seaweeds has been conducted for over 60 years, mostly because of their sessile nature, relatively high metal-tolerance, and accumulation capacity (Sáez et al., 2012b). Most studies have been conducted with a 'passive biomonitoring' approach, where local populations are sampled, and different parameters such as accumulation and metabolism changes (biomarkers) are measured (Pawlik-Skowronska et al., 2007; Sáez et al., 2012b); however, this method could lead to uncertainties and false environmental diagnosis due to the known intra-specific responses to metal stress observed in brown seaweed species (Ritter et al., 2010; Roncarati et al., 2015; Sáez et al., 2015a). On the other hand, 'active biomonitoring' has been proposed as a better alternative: species are cultivated in the laboratory and then transplanted to locations of interest, after which bioaccumulation and/or biomarkers are measured (Brown et al., 2012; Sáez et al., 2012b). Advantages of active biomonitoring lie on aspects such as: known populations can be used and thus avoiding errors raised by intra-specific responses; similar age individuals can be assessed; transplants can be exposed for the same time-periods in polluted and control sites; and the assessment can be conducted even if the biomonitoring species is absent in the locations of interest (Brown et al., 2012; Sáez et al., 2012b). Although scarce, active biomonitoring using brown seaweeds have been mostly conducted with Fucales or Kelps (e.g., Serisawa et al., 2002; Hédouin et al., 2008); however, transplantation efforts with these organisms are time-consuming and logistically demanding due to their slow growth, morphological complexity, and large size. It is clear now that active biomonitoring efforts could be improved by using broadly distributed, small, fast growing, and less-complex species such as *Ectocarpus siliculosus*.

E. siliculosus is a small filamentous brown alga that belongs to the order Ectocarpales, closely related to more physiologically complex brown seaweeds such as Laminariales and Fucales (Charrier et al., 2008). *E. siliculosus* is distributed worldwide in temperate regions, especially in marine and low salinity habitats; however, it has also been found in freshwater (West and Kraft, 1996) and hyper-saline (Geissler, 1983) ecosystems, which makes *E. siliculosus* an environmentally versatile species. *E. siliculosus* was chosen as a model organism for the study of brown algae, and the genome was recently published (Cock et al., 2010). There are records of *E. siliculosus* in Cu-polluted locations, such as in Chañaral in Chile, and Restronguet Creek in England (Sáez et al., 2015a). Even though it has been found that *E. siliculosus* can be highly adaptable to fluctuating environmental conditions, in terms of Cu-tolerance, an important degree of intra-specific variation has been observed. For instance, Hall (1980) found that a an *E. siliculosus* population collected from a Cu-polluted location in Salcombe, England, had higher growth rates than a population sampled from a pristine site when exposed to 1.6 µM Cu for 14 d. Similarly, Ritter et al. (2010) observed that the *E. siliculosus* strain Es32, from a pristine site in Peru, presented 70% cell death when grown under 0.8 µM Cu for 10 d; on the other hand, the Es524 strain, isolated from the Cu-polluted Chañaral, in Chile, presented a similar percentage of cell death only when exposed to 4 µM Cu.

The aim of this chapter is to present results of a series of laboratory and field studies conducted with the brown alga *E. siliculosus* to assess molecular, biochemical and physiological intra-specific responses to Cu excess, which describe tolerance mechanisms and, furthermore, provide information suitable for the development of biotechnology tools for environmental diagnosis.

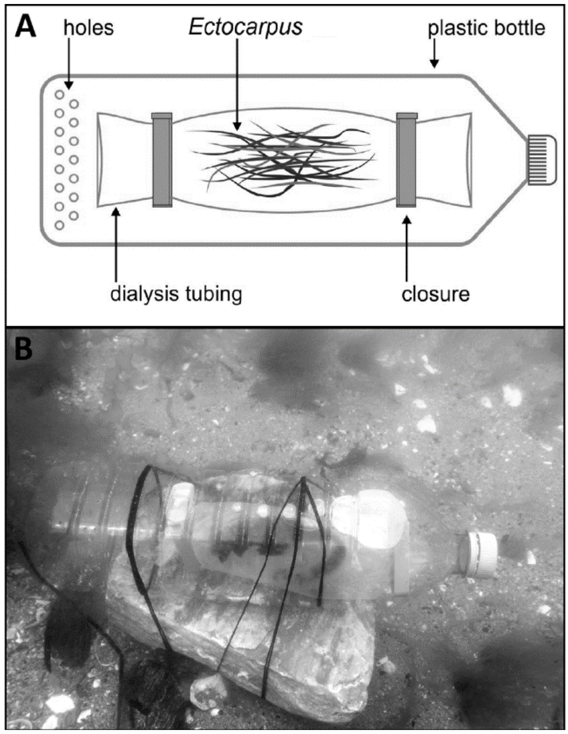
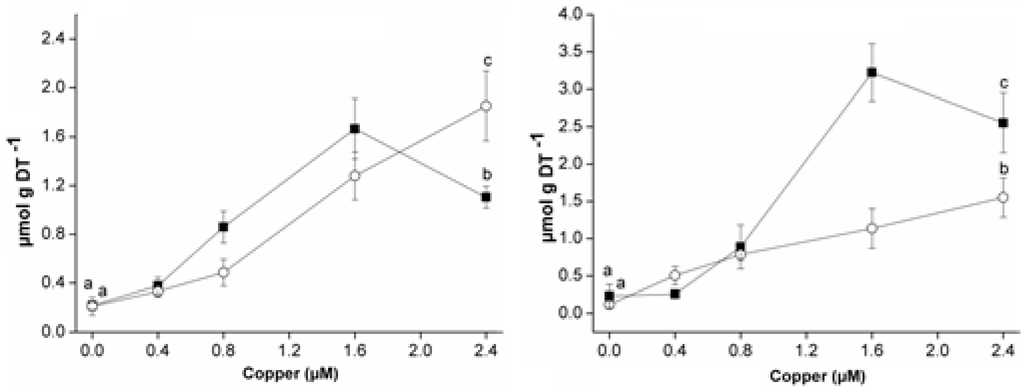


Figure 1. Designed transplantation device for active-biomonitoring using *Ectocarpus siliculosus*. (A) Model of the transplantation device, and (B) prototype of the transplantation device in the field. Modified from Sáez et al. (2015c).

METHODS

The investigations were conducted on five different *E. siliculosus* strains, isolated from locations with different degrees of Cu contamination. The strains were: LIA (Culture Collection of Algae and Protozoa (CCAP) accession number 1310/339), sampled at Lon Liath, Scotland, a pristine location; Es524 (CCAP 1310/333), isolated from a Cu-polluted site in Chañaral, northern Chile; REP (CCAP 1310/338), sampled at a metal (including Cu)-polluted location at Restronguet Creek, south-west England; and Es147 (CCAP 1310/340), isolated from site with a recent history of low Cu-pollution in Caleta Coloso, northern Chile.

To assess for responses to Cu excess in controlled environmental conditions, the *E. siliculosus* strains Es524, LIA and REP were exposed in triplicates for 10 d to a range of Cu concentrations of 0 (control), 0.4, 0.8, 1.6 and 2.4 µM; details of culture conditions in Sáez et al. (2015a). Different parameters were measured: relative growth rates (RGR), Cu-exclusion mechanisms (extracellular and intracellular accumulation), expression of enzymes involved in GSH and PCs syntheses, and concentration and levels of PCs polymerization (see Greco et al., 2014; Roncarati et al., 2015); oxidative stress and damage responses, levels of antioxidants GSH, glutathione disulphide (GSSG), ASC, dehydorascorbate (DHA) and phenolic compounds, and activity and expression of ASC, CAT, SOD and GR (see Greco et al., 2014; Sáez et al., 2015a,b).



A B

Figure 2. Concentrations per g dry weight (DT) of intracellular (A), and extracellular (B) Cu in *Ectocarpus siliculosus* strains Es524 (black squares), sampled from a Cu-polluted location, and LIA (empty circles), from a pristine site, after Cu exposure for 10 d. Error bars were calculated from three independent replicates ± SD. Different letters represent significant differences at 95% confidence interval. Modified from Roncarati et al. (2015).

To investigate responses to Cu (and other metals)-excess in semi-controlled environmental conditions, 6 g fresh weight (FW) *E. siliculosus* strains Es524 and Es147 were transplanted in triplicates to polluted (Ventanas site) and non-polluted (Quintay site) locations in central Chile for 10 d, using a novel active-biomonitoring method based on a designed dialysis tubing device (see Figure 1). Parameters assessed were: oxidative stress and damage, concentration of antioxidants GSH, ASC and phenolic compounds, and activities of antioxidant enzymes (see Sáez et al., 2015c).

Significant differences were calculated by one-way analysis of variance (one-way ANOVA) and *post-hoc* Tukey Test, previous assessment of the requirements of normality and homogeneity of variance (Zar, 1999).

RESULTS

Cu exposure laboratory experiments evidenced that physiological parameters are intra-specific dependent. When exposing the strains Es524 and LIA to increasing Cu concentrations, although both strains showed a decrease in relative growth with increasing Cu exposure, above 0.4 µM the growth levels were always higher in Es524 (from a Cu polluted site) than in LIA (from a pristine site) (Roncarati et al., 2015).

When comparing Cu exclusion mechanisms, the results evidenced that under high Cu exposure extracellular and intracellular Cu accumulation were higher and lower, respectively, in Es524 than LIA (Figure 2). Related to intracellular accumulation, we observed higher levels and degree of polymerization of PCs in Es524 compared to LIA; indeed, Es524 displayed PCs with 2 and 3 GSH oligomers (PC2-PC3), whereas LIA had only PC2 under Cu excess (Figure 3).

Regarding antioxidant defences, laboratory experiments showed that ASC, DHA, GSH and GSSG were higher at greater Cu exposure in Es524 in relation to the strains LIA and REP (Figure 4). Moreover, phenolic compounds increased similarly in Es524 and REP upon Cu exposure, but in a greater manner than in LIA (see Sáez et al., 2015a). Continuing the trend, the activities of the antioxidant enzymes SOD and CAT were higher in Es524 than in LIA and REP under increasing Cu exposure (Figure 5A and B). In contrast, the pattern of increase of APX activity was similar between the *E. siliculosus* strains assessed (Figure 5C).

After transplanting *E. siliculosus* strains Es524 (from a highly Cu-polluted site) and Es147 (from a low Cu polluted site) for 10 d to Ventanas (polluted) and Quintay (non-polluted), metal accumulation was shown to be similarly higher for both stains at the polluted location in relation to the pristine site. Results on oxidative stress and damage were, as in the laboratory experiments, different between strains but in agreement with metal pollution degrees. On the other hand, levels of GSH, GSSG, ASC and DHA were always higher in Ventanas than in Quintay for both strains, although those levels were usually higher for Es524 than Es147 (Figure 6). Furthermore, in terms of the activities of antioxidant enzymes, while the activities of all SOD, APX, CAT and GR increased in Ventanas in relation to Quintay for the strain Es524, this pattern was only observed for SOD in Es147 (Figure 7). Indeed, no significant changes were shown in the activities of APX, CAT and GR in Es147 between transplantation sites (Figure 7).

DISCUSSION

Cu exposure laboratory experiments on *E. siliculosus* have provided valuable information on tolerance mechanisms that allow this species to thrive in Cu-polluted environments. In addition, it has been demonstrated that intra-specific tolerance to Cu excess is likely to be based on inherited features developed in populations after a long history of being subjected to different degrees of pollution, varying in strength upon the intensity of the exposure. In this respect, it is clear that Cu-tolerance mechanisms in *E. siliculosus* and other brown algae are essentially based on exclusion mechanisms, syntheses of metal-chelators and antioxidant defences.

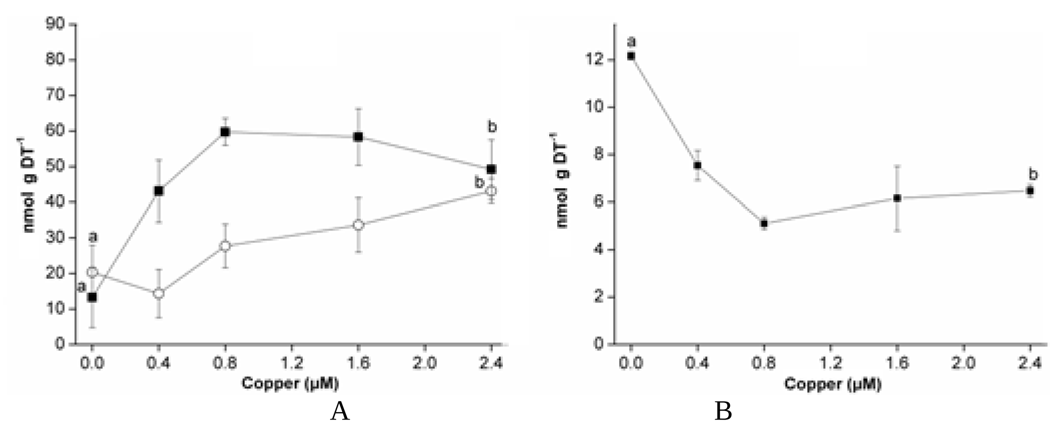


Figure 3. Levels per dry weight (DT) of phytochelatins 2 (PC2; A), and 3 (PC3; B) in *Ectocarpus siliculosus* strains Es524 (black squares), sampled from a Cu-polluted location, and LIA (empty circles), from a pristine site, after Cu exposure for 10 d. Error bars were calculated from three independent replicates ± SD. Different letters represent significant differences at 95% confidence interval. Modified from Roncarati et al. (2015).

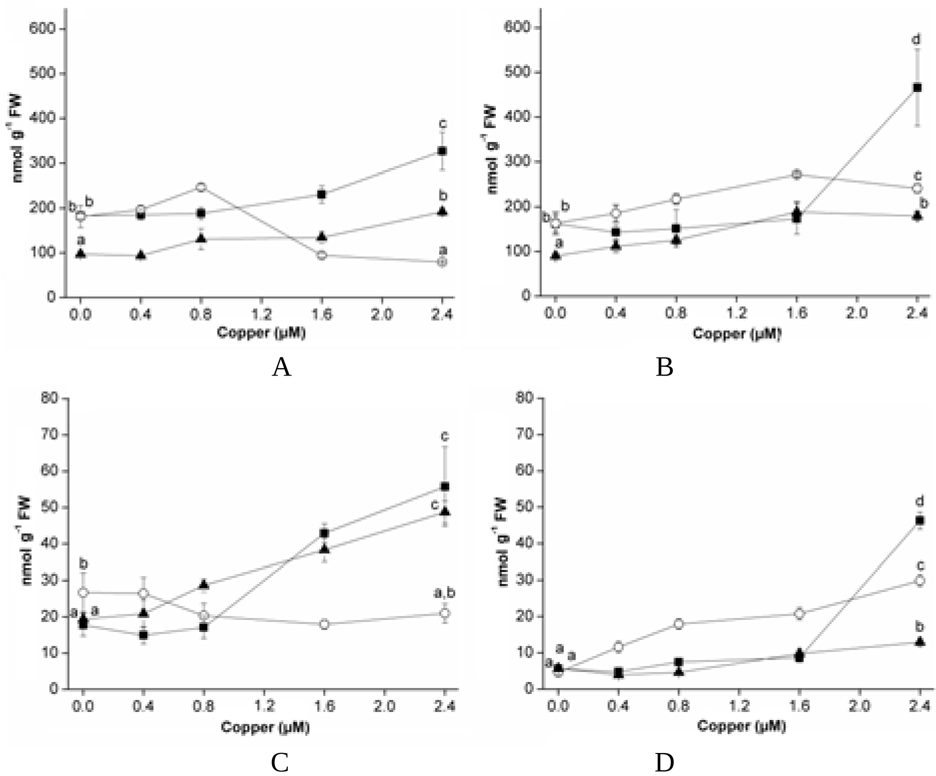
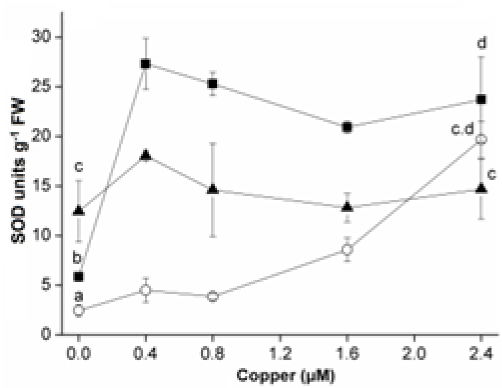


Figure 4. Concentrations per fresh weight (FW) of reduced glutathione (GSH) (A), glutathione disulphide (GSSG) (B), reduced ascorbate (ASC) (C), and dehydroascorbate (DHA) (D) in *Ectocarpus siliculosus* strains Es524 (black squares), sampled from a Cu-polluted location, REP (black triangles), isolated from a metal (including Cu)-polluted site, and LIA (empty circles), from a pristine site, after Cu exposure for 10 d. Error bars were calculated from three independent replicates ± SD. Different letters represent significant differences at 95% confidence interval. Modified from Sáez et al. (2015a).

Physiological observations have disclosed that although tolerance to Cu is concentration-dependent and can vary between strains/populations of *E. siliculosus*, all organisms were subject to stress, which was evident upon a decrease in RGRs (Roncarati et al., 2015), but RGRs were always higher in Es524, the *E. siliculosus* Cu-tolerant strain. Results on lipid peroxidation, production of hydrogen peroxide, and content of photosynthetic pigments disclosed that Es524 is less affected than other strains assessed in terms of ROS production and subsequent oxidative damage under Cu excess; the latter would explain, at least in part, why there is less physiological impairment in Es524 when compared with other strains (Roncarati et al., 2015; Sáez et al., 2015a).

Another important aspect of these investigations was that, for the first time, PCs were described as important mechanisms to counteract Cu stress in brown algae, presenting inter-population differences in levels of production and polymerization. It was identified that the *E. siliculosus* strain Es524 was capable of synthesizing PC2 and PC3 under Cu excess, whereas LIA (isolated from a pristine site) was able to produce only PC2 and in lower quantity than Es524. This is an interesting feature of Es524 since it is well known that the longer the PC the greater the capacity to chelate metals, as more binding sites are available (see Roncarati et al., 2015). It was also observed that genes encoding for γ-GCS, GS and PCS, responsible for

GSH-PCs syntheses, were more up-regulated in Es524 in relation to the strain LIA, which also suggests that enzymatically-mediated production of GSH dimers and then PCs is a transcriptionally regulated process during exposure to high Cu levels in this species (Roncarati et al., 2015).



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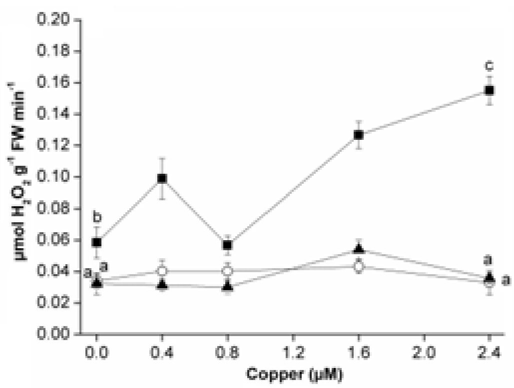

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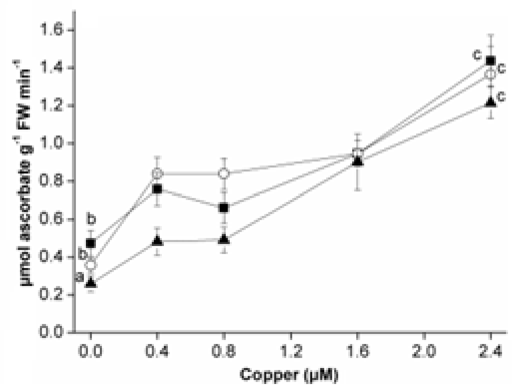

C

Figure 5. Activities per fresh weight (FW) of the antioxidant enzymes superoxide dismutase (SOD) (A), (B) catalase (CAT) (B), and ascorbate peroxidase (APX) (C) in *Ectocarpus siliculosus* strains Es524 (black squares), sampled from a Cu-polluted location, REP (black triangles), isolated from a metal (including Cu)-polluted site, and LIA (empty circles), from a pristine site, after Cu exposure for 10 d. Error bars were calculated from three independent replicates ± SD. Different letters represent significant differences at 95% confidence interval. Modified from Sáez et al. (2015a).

In relation to the reactive oxygen metabolism of *E. siliculosus* under Cu excess, intra-specific differences were also observed. Es524 showed greater production of antioxidants GSH and ASC, and their respective oxidized forms GSSG and DHA, and higher levels of phenolic compounds (Sáez et al., 2015a). The fact that reduced and oxidised forms of glutathione and ascorbate were higher than in other strains suggests that syntheses and recycling within the Halliwell-Asada cycle are more efficient mechanisms in Es524 (Sáez et al., 2015a). Indeed, for example, we could observe that the expression of the genes coding for γ-GCS and GS was higher in Es524 than in LIA under high Cu exposure (Roncarati et al., 2015). In addition, we observed that the activities of the antioxidant enzymes SOD and CAT were higher in the Es524 strain than in LIA and REP under Cu excess. Interestingly, APX activity increased similarly for all strains in parallel with Cu exposure, which suggests that APX activity is a constitutive feature among *E. siliculosus* populations to withstand Cu excess (Sáez et al., 2015a). The information is conclusive and indicates that antioxidant defences are one of the most important mechanisms to control Cu-mediated ROS excess in *E. siliculosus* and responsible, at least in part, for intra-specific Cu tolerance (Sáez et al., 2015a). The latter is in agreement with the fact that the lowest levels of lipid peroxidation and ROS were found in the Es524 strain, phenomena accomplished by displaying stronger antioxidant defences and thus higher syntheses of antioxidant compounds and increased activities of antioxidant enzymes.

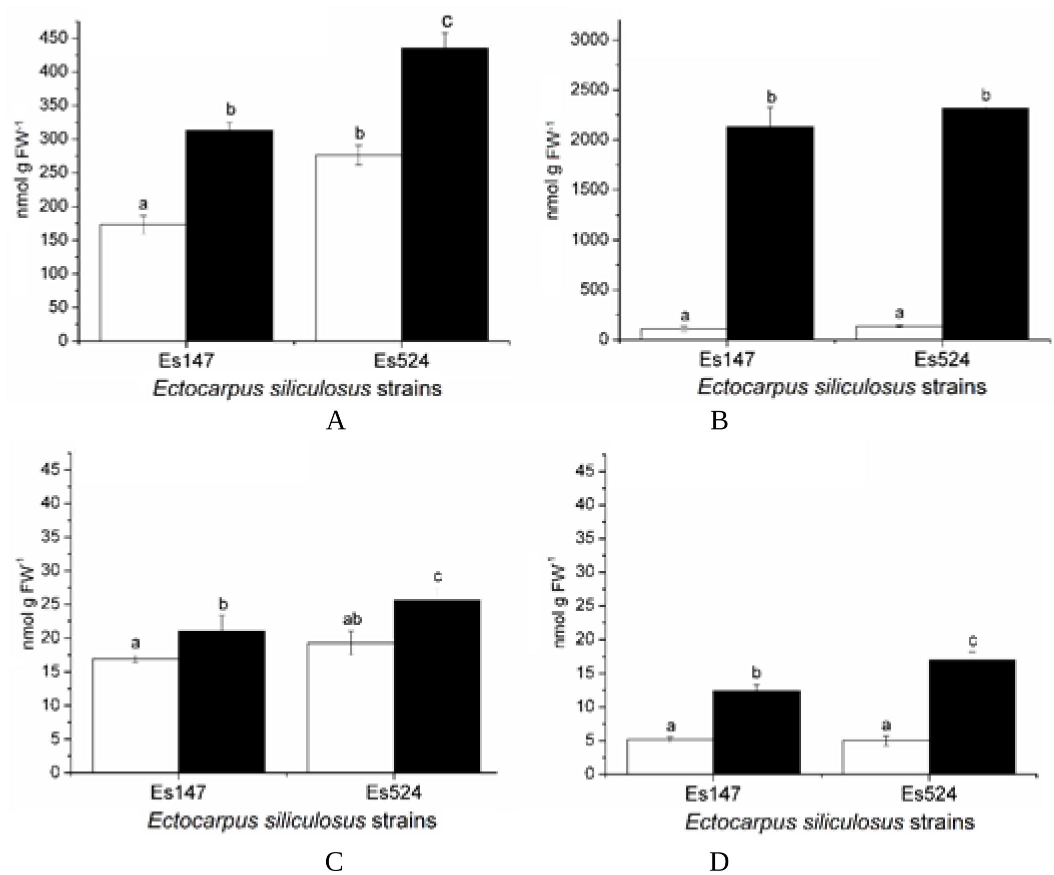

Figure 6. Concentrations of reduced glutathione (GSH) (A), glutathione disulphide (GSSG) (B), reduced ascorbate (ASC) (C), and dehydroascorbate (DHA) (D) in *Ectocarpus siliculosus* strains Es524, sampled from a Cu-polluted location, and Es147, from a site with recent history of Cu inputs, after transplantation experiments for 10 d to the the pristine Quintay (empty bars) and the metal-polluted Ventanas (black bars), in central Chile. Error bars were calculated from three independent replicates ± SD. Different letters represent significant differences at 95% confidence interval. Modified from Sáez et al. (2015c).

Further steps on the study of metabolic responses of *E. siliculosus* under Cu excess in the laboratory focus on the transcriptome and investigate the molecular basis of tolerance mechanisms as a whole. We have recently conducted laboratory experiments on *E. siliculosus* strain Es524 exposed to 2.4 μM Cu for 10 d and assessed the transcriptome using Next

Generation Sequencing (NGS) Technologies (RNA-seq). Preliminary results showed that the transcriptome of the Es524 strain cultured in control conditions expresses 48 different genes encoding for heat shock proteins (HSPs) of type HSP33, HSP40, HSP70, and HSP90. In contrast, when exposed to 2.4 μM Cu we observed the expression of 65 HSPs of the kind HSP20, HSP40, HSP70, and HSP90 (unpublished data). Our results are in agreement with findings by Ritter et al. (2014), which observed up-regulation of HSPs of the classes 70, 40, and 20 in the Es32 strain under 3.9 μM Cu. The authors suggested that the syntheses of HSPs intended to counteract protein denaturalization due to Cu-mediated oxidative stress. Regarding the expression of antioxidant enzymes, in Es524 under control conditions, we identified two genes encoding for APX, two CATs, and seven SODs, while in the strain exposed to Cu we found two APX, four CATs, and eight SODs (unpublished data). Observing results on Es524 under control conditions, it appears that HSPs and antioxidant enzymes are induced, and that these are part of 'baseline defence-mechanisms' for maintaining cellular homeostasis. Despite the latter, the general trend seems to be that more HSPs and antioxidant enzymes are getting expressed under Cu excess.

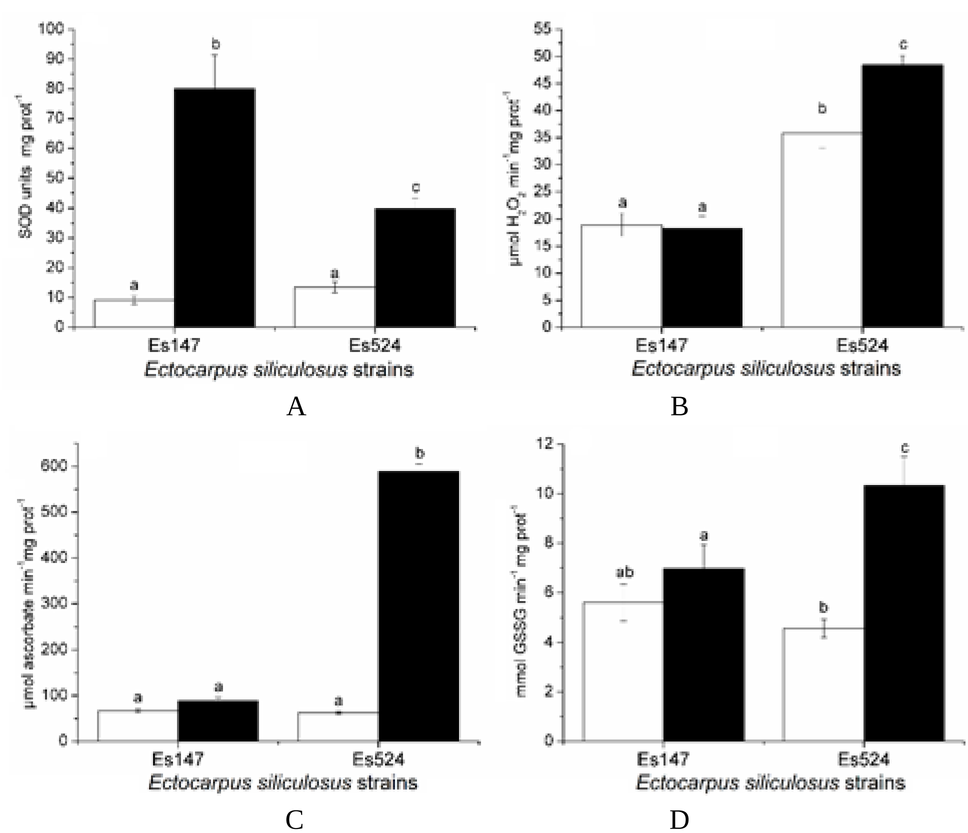

Figure 7. Activities of the antioxidant enzymes superoxide dismutase (SOD) (A), catalase (CAT) (B), ascorbate peroxidase (APX) (C), and glutathione reductase (GR) (D) in *Ectocarpus siliculosus* strains Es524, sampled from a Cu-polluted location, and Es147, from a site with recent history of Cu inputs, after transplantation experiments for 10 d to the pristine Quintay (empty bars) and the metal-polluted Ventanas (black bars), in central Chile. Error bars were calculated from three independent replicates ± SD. Different letters represent significant differences at 95% confidence interval. Modified from Sáez et al. (2015c).

The novel active-biomonitoring method designed to assess metal pollution in coastal environments using *E. siliculosus* transplants was successful for its biomonitoring purpose and in agreement with the findings on the reactive oxygen metabolism from laboratory Cu-exposure experiments. Even though both Es524 and Es147 strains showed higher levels of metal accumulation at Ventanas (impacted area) than at Quintay (non-impacted location), antioxidant responses were different between them. Es524 displayed higher defences manifested in greater levels of GSH and ASC than Es147, although phenolic compounds were higher in Es147. Moreover, the activities of antioxidant enzymes SOD, APX, CAT and GR were higher in Ventanas than Quintay for Es524, whereas the latter only occurred for SOD in Es147. The data were also concordant regarding oxidative responses, which showed lower lipid peroxidation and ROS levels in Es524 in relation to Es147 (Sáez et al., 2015c). In relation to its biomonitoring purpose, the best biomarker candidates proposed were those that had similar inter-strain behaviour, in other to avoid false environmental diagnoses. These were metal accumulation, production of antoxidants GSH, ASC, and phenolic compounds, and the activity of SOD (Sáez et al., 2015c). As the active-biomonitoring results were similar to those recorded under controlled Cu-exposure experiments (Sáez et al., 2015a), the information suggests that the oxidative responses observed in situ were indeed influenced by metal excess (Sáez et al., 2015c). This novel active biomonitoring method showed to be effective in representing metal bioavailability in the environment. Moreover, in agreement with other investigations (Sáez et al., 2012a,b), the transplantation method confirmed that Ventanas remains as a highly polluted location (Sáez et al., 2015c).

CONCLUSION

In this chapter we have described different laboratory- and field-based investigations on the model brown algae *E. siliculosus*, which were conducted in order to identify intra-specific tolerance mechanisms that allow this species to thrive in Cu polluted environments. Even though it was observed that defences to Cu excess in *E. siliculosus* are mainly based on cellular exclusion mechanisms, syntheses of metal chelators and in the antioxidant metabolism, these were shown to vary depending on the exposure history of the strains that were subject to investigation. Indeed, Es524 (isolated from a Cu-polluted location) displayed enhanced defences that allowed it to withstand Cu excess more efficiently than other strains, which was manifested as less physiological and metabolic impairment. Moreover, the novel transplantation method designed for metal assessment in the environment using *E. siliculosus* was successful for diagnosis, can be applied globally in temperate ecosystems, is cost-effective, and logistically straightforward.

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Chapter 6

ENDOCRINE GENE EXPRESSION IN AQUATIC ANIMALS AS INDICATOR OF THE PRESENCE OF XENOBIOTIC COMPOUNDS

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ABSTRACT

This chapter provides an overview on how the application of gene profiling can be used in different aquatic organisms as biomarkers of endocrine disruption in the presence of xenobiotic compounds. The application of gene expression qualification provides the means to identify complex pathways and strategies that an organism applies in response to an environmental stressor. In this study we used four estrogenic genes: Vitellogenin I and II (Vtg I and Vtg II), estrogen receptor α (Eα) and estrogen receptor ß (Eß). For the validation of a gene expression as a biomarker of exposure to a xenobiotic compound we performed various experiments with different animal species: 15 d exposure to17ß-estradiol using male mosquito fish (*Gambussia yucatana*); 48 h exposure of the Japanese pond turtle (*Mauremys japonica*) to petroleum; and 48 h exposure of males of black mollies (*Poecilia sphenops*) to petrogenic and pyrogenic hydrocarbons. A different gene expression was observed in relation to the compound, species, time and concentration; therefore, these genes can be used as biomarkers regarding the presence of xenobiotic compounds in aquatic animals. For the application of these biomarkers to environmental samples, we performed the analysis with the Atlantic angel shark (*Squatina dumeril*); the results indicate that the gene expression can be used as biomarker of the presence of xenobiotic compounds in the natural environment and of the consequent damage in wild organisms.

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Keywords: endocrine gene expression, estrogenic disruptor, toxicity; biomarkers

INTRODUCTION

An endocrine-disrupting compound (EDC) has been defined by the U.S. Environmental Protection Agency (EPA) as "an agent that interferes with the synthesis, secretion, transport, binding, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior" (Kavlock et al., 1996). This means that endocrine disruptors are chemicals, or chemical mixtures that interfere with normal hormone function.

The endocrine disruptors are known to mainly alter the hormonal and homeostatic systems of living system. These systems are of primary importance since they are involved in the regulation of several significant processes such as metabolism, sexual development, insulin production and utilization, growth, stress response, gender behavior, reproduction and even in fetal development (Kabir et al., 2015).

Different studies demostrate that endocrine disruptors are present in the air, water and even in the soil (Kabir et al., 2015). The evidences indicate that endocrine disruptors are responsible for different wildlife crisis. However, wildlife is not exposed to individual contaminants, but to a complex mixture of EDCs. The exposure route of wildlife to EDCs is also very critical, because many of those compounds do not persist in the environment and organism. On the other hand, a large number of chemicals persist in the food and habitat of wildlife. Although it largely depends on the properties and persistent nature of EDCs, wildlife is normally exposed to them via air, water, food, soil and sediment, and even via skin absorption (Kidd et al., 2012). The EDCs are degraded in the environment, for example by sunlight, bacterial activity and chemical processes, while others persist in the environment for different periods of time. Some EDCs are found to be highly soluble in water and might be present in water from the level of parts per trillion to parts per billion.

The EDCs such as organochlorines and pesticides, as well as plasticizers, pharmaceutics, and natural hormones can interact with various receptors, such as the estrogen receptor (ER), vitellogenin (VTG), androgen receptor, and aryl hydrocarbon receptor (Iguchi et al., 2002). Clarifying the molecular basis of the EDCs and the endogenous estrogens on developing organisms is essential, if we are to understand the linkages between exposure levels, timing of exposure, genes responsive to these chemicals, and adverse effects (Iguchi et al., 2007). Various modes of action of chemicals and non-traditional targets of the EDCs have been summarized (Tarrant, 2005). Understanding the effects of the EDCs on various species at the level of molecular biology is greatly needed to help explain observations at the cellular and organism levels that typically are obtained with traditional toxicological approaches.

The sub-discipline combining the fields of genomics and toxicology is defined as "Toxicogenomics" (Inoue and Pennie, 2002), and has become an important field in ecotoxicology. "Ecotoxicogenomics" is the application of toxicogenomics to organisms that are representative of ecosystems and is used to study the hazardous effects of chemicals on ecosystems as well as individuals (Miracle and Ankley, 2005). Although the availability of genomic information regarding non-model organisms is still limited, the application of toxicogenomics to a variety of organisms could be a powerful tool for evaluating the effects of chemicals on ecosystems (Watanabe and Iguchi, 2006).

Profiling of transcripts, proteins, and metabolites can help discriminate classes of the EDCs and toxicants and be helpful in understanding modes of action. Through systematic efforts to generate mechanistic information, diagnostic and predictive assessments of the risk of the EDCs and other toxic chemicals will be established in model species for ecological risk assessment. Toxicogenomics and ecotoxicogenomics also provide important information on the basic biology of animals (Iguchi et al., 2007).

DNA microarray methodology has been applied to obtain genome-wide analysis of gene expression stimulated by hormones and/or chemicals (Watanabe and Iguchi, 2003). An understanding of the patterns of expression of estrogen-responsive genes is essential to comprehend the mechanisms of action of estrogenic chemicals on non-target organs (Iguchi et al., 2007).

For the validation of the gene expression as a biomarker of the xenobiotic compounds we performed various experiments with different animal species and applied these biomarkers to environmental samples in wildlife.

METHODS

First Experiment: Expression of Estrogenic Response Genes in Male Mosquito Fish (*Gambusia yucatana*)

In this study we evaluated the expression of genes involved in vitellogenin production by analyzing mRNA in adult male mosquito fish (*G. yucatana*) exposed to different concentrations of 17ß-estradiol.

The mosquito fish were collected from a small and non-contaminated pond in a Ria Creek in Campeche, Mexico, and were maintained and cultivated in an artificial pond for three months. The organisms were fed *ad libitum*, three times a day with a commercial fish food (TretaMin, Tetra Holding, USA). The fish were acclimated for 1 week in 20 L glass aquarium containing 18 L of aerated and dechlorinated water according to Rendón von Osten et al., (2005). The stocking rate for the *in vivo* study was 3 fish/aquarium. The fish were not fed 24 h prior to the experiments and the food was provided during the test before the medium was renewed.

A total of 15 male fish were exposed to 17ß-estradiol at nominal concentrations of 0.5, 1, 10, and 15 µg L^{-1} with three replicates per treatment. The test solutions were prepared by dissolving the appropriate amount of 17ß-estradiol stock solutions in ASTM hard water. Two controls were included in the experimental design with male fish, one with ASTM hard water and with ethanol (solvent control) and the other control was performed with female fish without treatment. The bioassays were carried out for 14 days in a semi-static test design according to chronic exposure criteria by Orlando et al., (2002). After the experiments, all the organisms were sampled for analysis of the gene expression by Q-PCR method.

The total RNA was isolated from liver and gonad tissue of male fish according to the kit instructions (Ultra Clean Tissue and Cells RNA Isolation, Mobio INC.). The RNA was diluted at 1 mg ml^{-1} for RT-PCR (Reverse Transcriptase-Polimerase Chain Reaction) and stored at -80ºC. The RT-PCR was performed with the total RNA extracted from the mosquito fish livers and gonads. The RT reaction mixture contained 5 µg of total RNA, 1 µl of oligo (dT) primer and diethyl pyrocarbonate (DEPC)-treated water. The reaction mixture was heated to 70ºC for 10 min and quickly chilled on ice. After cooling, 4 µl of reaction buffer containing 25 mM $MgCl_2$, 2 µl of deoxynucleotide triphosphate (dNTPs; 10 mM each), 1 µl of RNase inhibitor, and 1 µl of ReverTraAce (BIORAD) was added to a total volume of 20 µl, and the reaction mixture was incubated for 60 min at 42 ºC. The reaction mixture was then heated to 90ºC for 5 min to stop the RT.

The PCR reactions contained 2 µl of the RT reaction mixture as the cDNA template, 5 µl of 10x PCR buffer, 1 µl of Taq polymerase (5 U µl^{-1}, BIORAD), 3 µl of 25 mM $MgCl_2$, 1 µl of dNTPs (10 mM each) and 1.5 µl of sense and antisense primers. The primer pair sequences for the quantification of the genes involves in vitellogenin production are shown in Table 1. The total volume of the reaction mixture was 50 µl. The PCR conditions were as follows: initial denaturation at 94ºC for 2 min, 45 cycles of denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s, and extension at 72ºC for 1 min, and final extension at 72ºC for 10 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide (15 µl of a 10 mg ml^{-1} ethidium bromide solution per 100 ml of water). The band densities of amplified products were calculated using Quantity One Software (BIORAD).

Second Experiment: Expression of Estrogenic Response Genes in Japanese Pond Turtle (*Mauremys japonica*)

In this study we evaluated the expression of genes involved in the vitellogenin production by analyzing the mRNA in adult male Japanese pond turtle (*Mauremys japonica*).

The Japanese pond turtles were acquired in a commercial aquarium and were maintained and cultivated three weeks in 20 L glass aquarium containing 3 cm of depth of dechlorinated water. The organisms were fed *ad libitum*, three times a day with a commercial reptile food (Reptile sticks, Wardley INC). The stocking rate for the *in vivo* study was 1 turtle/aquarium. The turtles were not fed during the experiment.

A total of 6 turtles were exposed to 1 mg L^{-1} of a sample of a hydrocarbon leaked from the Usumacinta oil ring as source of petrogenic hydrocarbon; and 3 turtles were kept as controls. All the turtles were stocked in ASTM hard water. The bioassays were carried out for 36 h in a static test design according to chronic exposure criteria by Orlando et al., (2002). Every 12 h, two turtles (except those from the control, which were sacrificed at 36 h) were sacrificed to remove the gonads.

The total RNA was isolated from the gonad tissue of all the tested turtles according to the kit instructions (GeneJET RNA Purification, Thermo Fisher Scientific). The RNA was diluted at about 1 mg ml^{-1} for RT-PCR. The RT-PCR was performed with the total RNA extracted from the turtle gonads. The RT reaction was performed according to the kit instructions (TaqMan Reverse Transcription Reagent, Thermo Fisher Scientific).

For the quantification of the gene expression, a Q-PCR was performed in a StepONE RT-PCR equipment (Applied Biosystem) using a Maxima SYBRGreen/ROX QPCR Master Mix (Thermo Fisher Scientific). The primer pair sequences are shown in Table 1. The PCR conditions were as follows: initial denaturation at 94ºC for 2 min, 45 cycles of denaturation at

94ºC for 30s, annealing 60ºC for 30 s, and extension at 72ºC for 1 min. Quantification was performed by using the ß-actin gene as reference and applying the$2^{\Delta\Delta C_T}$method (Kenneth et al., 2001).

Third Experiment: Expression of Estrogenic Response Genes in Black Mollies (*Poecilia sphenops*)

In this study we evaluated the expression of the genes involved in the vitellogenin production by analyzing the mRNA in adult male black mollies (*Poecilia sphenops*).

The black mollies were acquired in a commercial aquarium and maintained and cultivated for three weeks in 20 L glass aquarium containing 18 L of aerated and dechlorinated water according to Rendón von Osten et al., (2005). The organisms were fed *ad libitum*, three times a day with a commercial fish food (TretaMin, by Tetra Holding, USA). The stocking rate for the *in vivo* study was 3 fish/aquarium. Fish were not fed during the experiment.

A total of 12 fish were exposed to 1 mg L^{-1} of burned grass as a source of pyrogenic hydrocarbon; another group of 12 fish was exposed to 1 mg L^{-1} of a sample of a hydrocarbon leaked from the Usumacinta oil ring as a source of petrogenic hydrocarbon; and 12 fish were kept as controls. All the fish were stocked in ASTM hard water. The bioassays were carried out for 36 h in a static test design according to chronic exposure criteria by Orlando et al., (2002). Every 12 h, four fish from each treatment were sacrificed (except those from the control, which were sacrificed at 36 h) to remove the gonads.

Table 1. Sequences of primer pairs used in the RT-PCR study (Ishibashi et al., 2008)

Gene name	Primer sequences
ERα (AB033491)	5´-GTCAGTCGGGTTACTTGGCC-3´
	5´-CATCACCTTGTCCCAACCTG-3´
ERß (AB070901)	5´-GTGGACTCAACTTTCGGC-3´
	5´CACGTCGCAGCAGGATCTT-3´
VTG I (AB064320)	5´-TGGAAAGGCTGATGGGGAAG-3´
	5´-AACTGCAGGCATGGTGAGCC-3´
VTG II (AB074891)	5´-GTCYYCAGGAGGTCTTCTTC-3´
	5´-GGTAGACAATGGTATCCGAC-3´
ß-Actin (S74868)	5´-AGACCACCTACAGCATC-3´
	5´-TCTCCTTCTGCATTCTGTCT-3´

The total RNA was isolated from the gonad tissue of tested fish according to the kit instructions (GeneJET RNA Purification, Thermo Fisher Scientific). The RNA was diluted at about 1 mg ml^{-1} for RT-PCR. The RT-PCR was performed with total the RNA extracted from black mollies gonads. The RT reaction was performed according to the kit instructions (TaqMan Reverse Transcription Reagent, Thermo Fisher Scientific).

For the quantification of the gene expression a Q-PCR was performed in a StepONE RT-PCR equipment (Applied Biosystem) using a Maxima SYBRGreen/ROX QPCR Master Mix

(Thermo Fisher Scientific). The primer pair sequences are shown above in Table 1. The PCR conditions were as follows: initial denaturation at 94ºC for 2 min, 45 cycles of denaturation at 94ºC for 30s, annealing at 60ºC for 30 s, and extension at 72ºC for 1 min. The quantification was performed by using the ß-actin gene as reference and applying the $2^{\Delta\Delta C_T}$ method (Kenneth et al., 2001).

Fourth Experiment: Presence of Expression of Estrogenic Genes in Wild Atlantic Angel Shark (*Squatina dumeril*)

In this study we evaluated the the expression of genes involved in vitellogenin production by analyzing the mRNA in adult Atlantic angel shark (*Squatina dumeril*) for the application of these biomarkers in wildlife.

The samples were taken from the catch of fisherman from San Francisco de Campeche city, Campeche, México. Ten grams of the liver were sampled from 4 sharks (3 males and 1 female) and the samples were transported on ice to the laboratory and frozen at -80ºC until further analysis.

The total RNA was isolated from the shark liver tissue according to the kit instructions (Gene Jet RNA Purification Kit, Thermo Fisher Scientific). The RNA was diluted at about 1 mg ml^{-1} for RT-PCR. The RT-PCR was performed with the total RNA extracted from shark gonads. The RT reaction was performed according to the kit instructions (TaqMan Reverse Transcription Reagent, Thermo Fisher Scientific).

For the quantification of the gene expression a Q-PCR was performed in a Step One RT-PCR equipment (Applied Byosystem) using a Maxima SYBRGreen/ROX QPCR Master Mix (Thermi Fisher Scientific). The Primer cycles of denaturation were at 94ºC for 30 s, annealing 60ºC for 30s, and extension at 72ºC for 1 min. Quantification was performed by using the ß-actin gene as reference and applying the $2^{\Delta\Delta C_T}$ method (Kenneth et al., 2001).

RESULTS

First Experiment

The results of the expression of the estrogen receptor transcripts in the liver and gonads are presented in the Figure 1. The expression of the gene in the liver was greater than in the gonads for all of the samples, with the exception of the negative control where it was greater in the gonads. The females exhibited the highest expression in the liver, significantly different from the other samples. However, in the gonads there were no differences relative to the negative control and the concentration of 10 µg L^{-1} 17ß-estradiol; this concentration caused the highest gene expression in both types of tissues when compared to the other tested concentrations.

The strongest expression of the vitellogenin gene was observed in the treatment with 10 µg L^{-1} 17ß-estradiol, which was significantly different from the other treatments and the controls (Figure 2). The females had the greatest expression of this gene with the exception of the treatment with 1 µg L^{-1} 17ß-estradiol (both types of tissue) and the treatment with 0.55 µg L^{-1} 17ß-estradiol for liver.

Second Experiment

The expression of the transcripts showed a linear increment over time, with significantly higher expression of the VTG I than the females at 24 and 36 h (Figure 3). In the case of the VTG II the increment is linear but lower than the females (Figure 3). For The estrogen α and ß the increment was linear and presented higher values than the females only at 36 h (Figure 4).

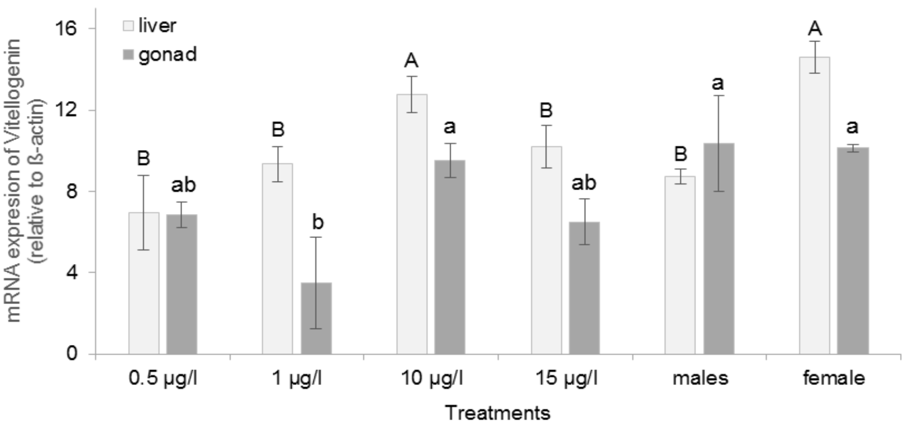

Figure 1. Mean and standard deviation of the relative intensity of vitellogenin receptor transcript in the liver and gonads of mosquito fish exposed to 17ß-estradiol. Values with the same superscript are not statistically different (p > 0.05).

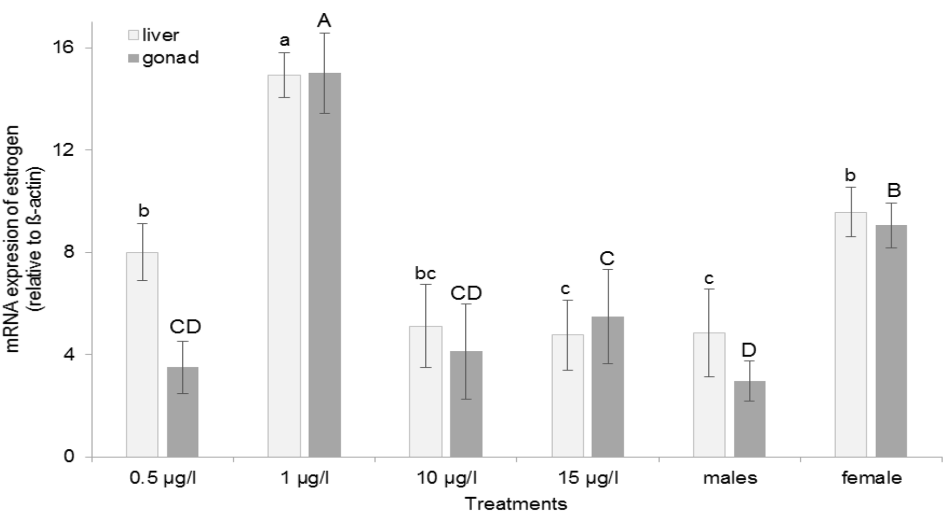

Figure 2. Mean and standard deviation of the relative intensity of estrogen receptor transcript in the liver and gonads of mosquito fish exposed to 17ß-estradiol. Values with the same superscript are not statistically different (p > 0.05).

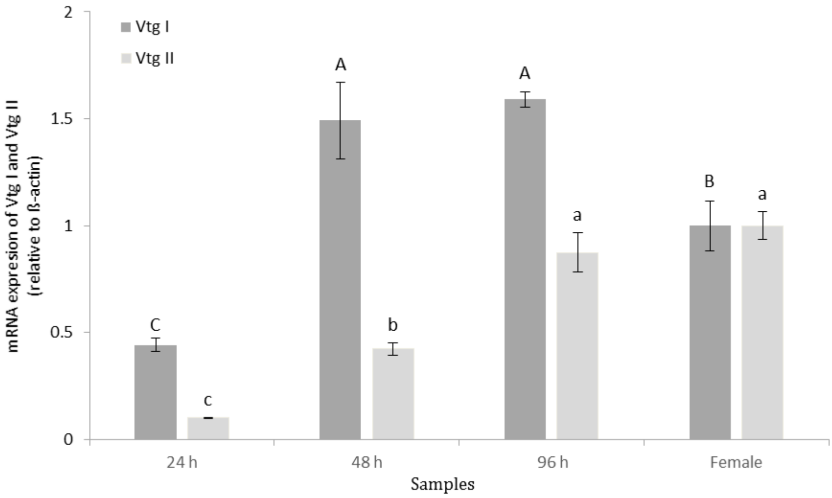

Figure 3. Mean and standard deviation of the relative intensity of Vitellogenin I (Vtg I) and Vitellogenin II (Vtg II) receptor transcript in the gonads of Japanese pond turtle exposed to petrogenic hydrocarbon for 24, 48 and 96 h. Values with the same superscript are not statistically different ($p > 0.05$).

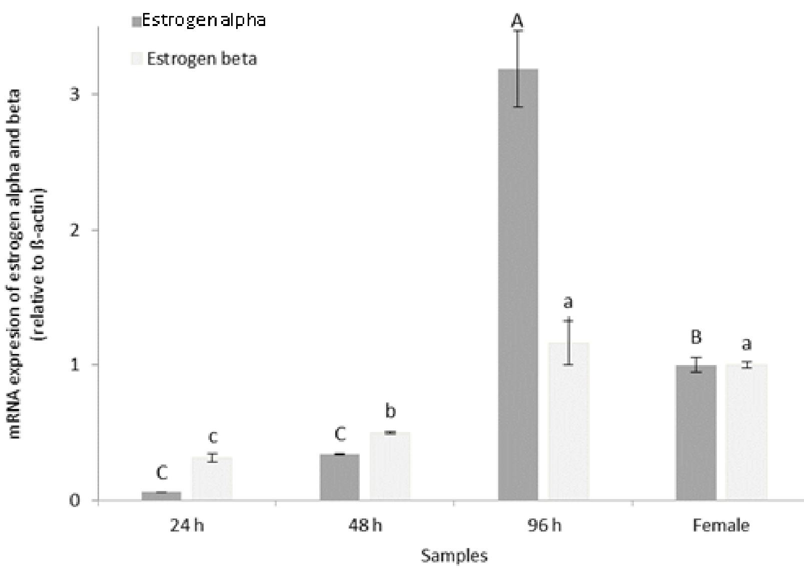

Figure 4. Mean and standard deviation of the relative intensity of estrogen alpha and estrogen beta receptor transcript in the gonads of Japanese pond turtle exposed to petrogenic hydrocarbon for 24, 48 and 96 h. Values with the same superscript are not statistically different ($p > 0.05$).

Third Experiment

The expression of the VTG I gene in black molly in response to petrogenic hydrocarbon is shown in Figure 5. At 24 h the VTG I expression was maximum, decreasing after 36 h. The maximum expression of the VTG II was observed at 36 h of exposure to the pyrogenic hydrocarbon; on the other hand, with petrogenic hydrocarbon the maximum expression was at 12 h with a linear decrease over time (Figure 6).

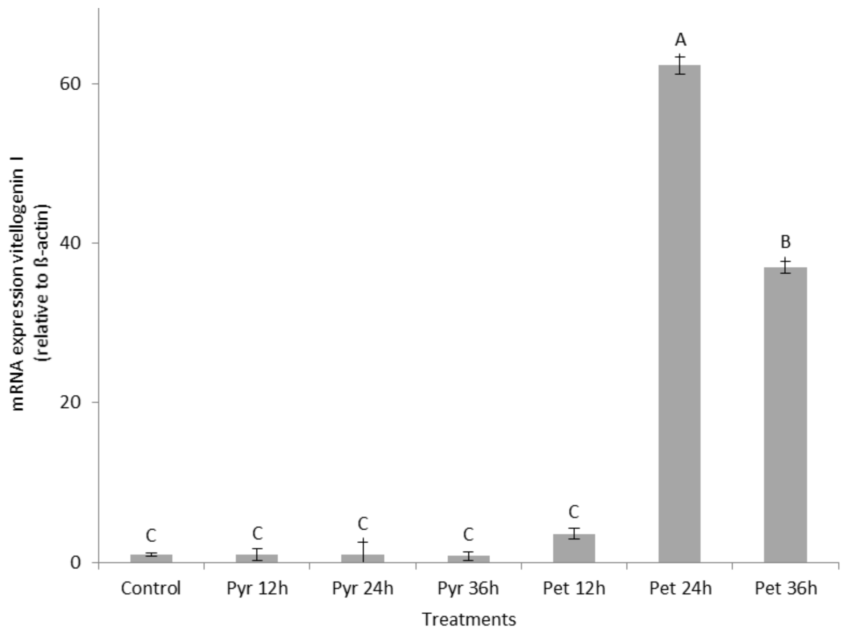

Figure 5. Mean and standard deviation of the relative intensity of vitellogenin I receptor transcript in the gonads of black mollies fish exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon for 12, 24 and 36 h. Values with the same superscript are not statistically different ($p > 0.05$).

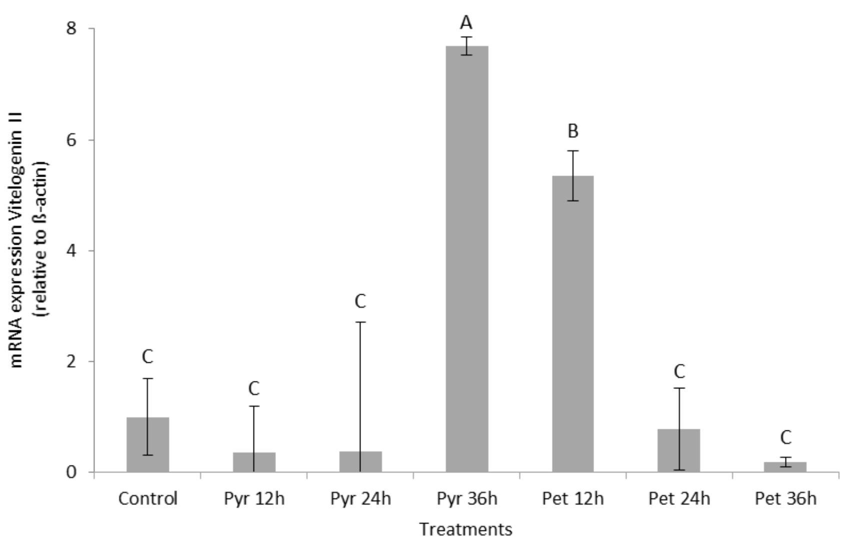

Figure 6. Mean and standard deviation of the relative intensity of vitellogenin II receptor transcript in the gonads of black mollies fish exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon. Values with the same superscript are not statistically different ($p > 0.05$).

For the estrogen receptor α gene, an increase over time with maximum values at 36 h in both hydrocarbon sources was observed; the highest expression was observed at 36 h exposure to hydrocarbon source (Figure 7). Only after 24 h an increase was observed in the expression of estrogen receptor ß gene in organisms exposed to petrogenic hydrocarbons, without expression in other samples (Figure 8).

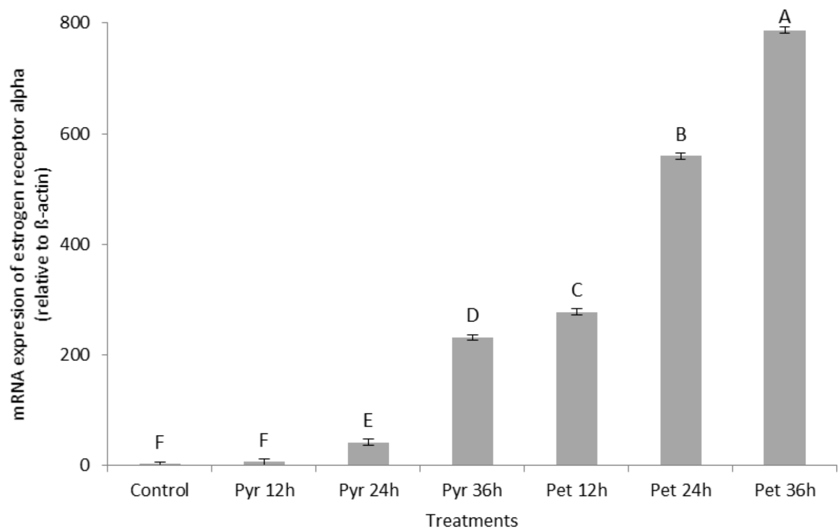

Figure 7. Mean and standard deviation of the relative intensity of estrogen receptor alpha transcript in the gonads of black mollies fish exposed to petrogenic and pyrogenic hydrocarbon. Values with the same superscript are not statistically different ($p > 0.05$).

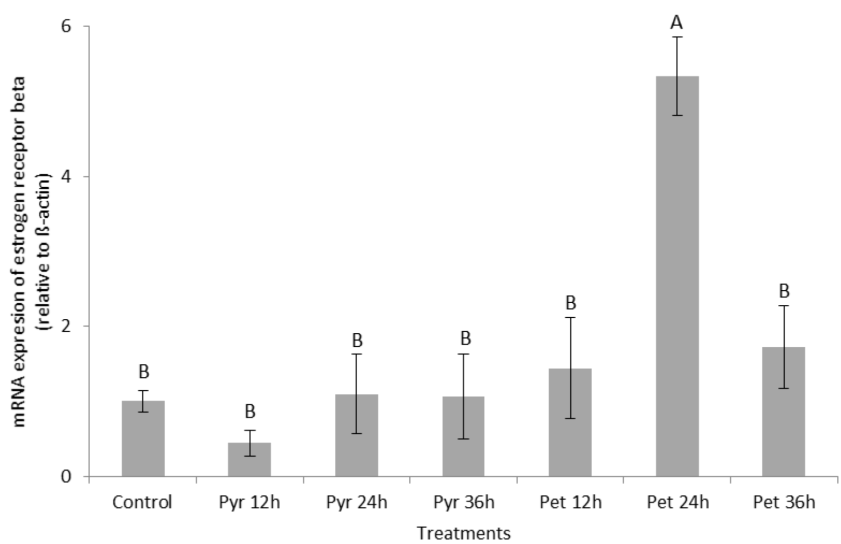

Figure 8. Mean and standard deviation of the relative intensity of estrogen receptor beta transcript in the gonads of black mollies fish exposed to Petrogenic (Pet) and Pyrogenic (Pyr) hydrocarbon. Values with the same superscript are not statistically different ($p > 0.05$).

Fourth Experiment

In the Atlantic angel shark male #2 presented the highest expression in the VTG I and estrogen receptor α. No expression of the other genes (VTG II and estrogen receptor ß) was observed in the males.

DISCUSSION

The steroid hormones that circulate in the plasma of organisms are essential to the reproduction and sexual differentiation. The estrogenic hormones increasingly discarded in the environment are contaminants and detecting them requires efficient, sensitive methods, such as the use of molecular biomarkers. The species used as indicators in the first study was the mosquito fish (*G. yucatana*), a poecilid that has been used to evaluate toxicity biomarkers such as acetylcholinesterase (AChE) and glutathione S-transferase (GST) (Rendón von Osten et al., 2005), along with several response parameters such as vitellogenin concentrations, sperm count and gonodopodial indices. Exposure to 17ß-estradiol stimulated the induction of vitellogenin and the expression of the respective genes, allowing us to conclude that this compound causes changes in the endocrine system of the fish similar to those reported by Lori et al. (2008) for Nile tilapia (*Oreochromis niloticus*) exposed to 17ß-estradiol concentrations of 5 µg L^1. In the study of Jin et al. (2008) the vitellogenin genes were expressed at 17ß-estradiol concentrations of 10 to 1000 ng L^{-1} in the zebra fish (*Danio rerio*) liver, with significant differences starting at 50 ng L^{-1} and the greatest expression at 1000 ng L^{-1}. In the present study the concentration of 15000 ng L^{-1} for the male mosquito fish was comparable to the expression observed for the females. The family of estrogen receptors can be induced by xenoestrogens and produce different responses: phosphorylation of signals regulated by kinases, abnormal cell growth and the increased expression of the genes (Albino et al., 2009). Hawkins et al., (2000) mentioned that the estrogen receptors participate in the regulation of vitellogenesis and have been isolated in teleost fish; these receptors have been proposed to be sensitive to variations in estrogen levels. The present study revealed that the estrogen receptor α was expressed to differing degrees at all of the concentrations of 17ß-estradiol used, which is in agreement with the results of Yamaguchi et al., (2005); they observed gene expression at concentrations between 10 and 10 000 ng L^{-1}. In contrast, Choi et al., (2007) studied the effects of the induction by 17ß-estradiol on estrogen receptors α and ß in male olive flounder (*Paralichthys olivaceus*), and observed that gene expression was dose dependent. A decrease in the intensity of the gene expression was observed when the fish were exposed to a concentration of 1 000 ng L^{-1} of 17ß-estradiol. In this regard, Palstra et al., (2010) report that when the organisms make an effort and are under stress there is a decrease in the levels of estrogen gene expression as a consequence of directing energy to their muscles as a strategy to allow them to flee quickly and/or prevent muscular atrophy.

The induction of the Vtg in oviparous species is used as a standard biomarker of exposure to estrogenic compounds (Cheek et al., 2001; Irwing et al., 2001; Rey et al., 2006). Many environmental contaminants are considered to be xenoestrogens since they show an estrogenic response in different *in vivo* and/or *in vitro* assays (Nakada et al., 2004). Little information exists concerning hormonal profiles in turtles. The synthesis of estrogens in organs other than ovaries (adrenal and hepatic tissue) has been observed in various reptilian species, including the turtles (Zaccaroni et al., 2010). Therefore, small amounts of the estrogens was produced by these organs might trigger VTG synthesis (Pieau and Dorizzi, 2004). Short-term production of the estrogens as a result of pre-activation or preparation of the gonads rather than ovarian differentiation could also occur. The possible effect of the endocrine disrupters (organochlorine compounds, heavy metals, tributyltin) on the VTG synthesis should also be considered. In this study we observed an activation of the VTG gene over time during exposure to petrogenic hydrocarbon; these results confirm that the turtles could be used as bioindicators.

The hepatic VTG is a sensitive biomarker for the estrogenic effect of hydrocarbons. The VTG is synthesized in response to stimulation by estrogenic chemicals that bind to the ERs to activate the VTG transcription. When the estrogens enter into the liver, they bind to the ERs, forming ER complexes that then bind to the estrogen response elements of the target genes to regulate their expression (Watanabe et al., 2009). A recent *in vitro* study with primary cultured zebrafish hepatocytes suggested that the hydrocarbons have a common estrogenic effect but may interact differently with the ER isoforms, perhaps contributing directly to differential the ER and the VTG gene transcription (Maradonna et al., 2013). Several studies have reported the induction of the VTG synthesis or transcription in the fish or in cultured male fish hepatocytes upon exposure to hydrocarbons (Carnevali et al., 2010; Uren-Webster et al., 2010; Maradonna et al., 2013). Although the induction of the VTG could depend on species sensitivity, exposure time, and concentrations, these studies demonstrate the estrogenic activity of hydrocarbons (Wang et al., 2013). In our study the expression of the vitellogenin-related genes in the black molly fish was observed only when exposure was to petrogenic hydrocarbon and not to pyrogenic hydrocarbon, probably due to the nature of the compounds.

There are no studies that demonstrate the effect of pollutants on the expression of the genes of endocrine disruption in sharks. These results show an important potential use of the biomarkers in different aquatic species of commercial interest, such as the shark, to determine the effects of pollutants. The high levels of expression in one of the tested organisms indicate that it had contact with some kind of pollutant. This can be confirmed by reproductive age, eating habits and proximity to the coast where the organism was captured. These findings are being confirmed by the stomach contents and concentrations of pollutants in the organisms.

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Chapter 7

SENSITIVITY OF TROPICAL CLADOCERANS AND CHIRONOMIDS TO TOXICANTS AND THEIR POTENTIAL FOR ROUTINE USE IN TOXICITY TESTS

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ABSTRACT

We conducted toxicity tests with three reference toxicants, following NBR-12713 procedures (2009), with an aim to establish *Daphnia laevis* and *Ceriodaphnia cornuta* as routine toxicity test species by the Brazilian Association of Technical Procedures. To evaluate the sensitivity of these native cladocerans, standardized species were also tested, namely *D. similis* and *C. dubia* from temperate regions as well as the neotropical *C. silvestrii*. EC50 mean values for *D. laevis* were 0.025 mg L^{-1} for potassium dichromate, 1.06 g L^{-1} for sodium chloride and 5.07 mg L^{-1} for sodium dodecyl sulfate. Mean values of EC50s for *D. similis* corresponded to 1.63 g L^{-1} (sodium chloride) and 5.64 mg L^{-1} (sodium dodecyl sulfate). *Ceriodaphnia* spp. EC50 mean values for sodium chloride and sodium dodecyl sulfate were, respectively: 1.04 g L^{-1} and 3.27 mg L^{-1} (*C. cornuta*); 1.24 g L^{-1} and 4.42 mg L^{-1} (*C. silvestrii*); 1.43 g L^{-1} and 4.12 mg L^{-1} (*C. dubia*). EC50 values for potassium dichromate and sodium chloride showed higher sensitivity of *D. laevis* than *D. similis*, already used in Brazil. *C. cornuta* was also more sensitive to sodium chloride than *C. dubia* and *C. silvestrii.* These results, beside high fecundity and easy laboratory maintenance, support their use in toxicity tests. Regarding sediment toxicity, *Chironomus xanthus* 96h-LC50 for instars II, III and IV exposed to potassium chloride were 3.99, 4.50

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and 4.91, and 4.64 g L^{-1}, respectively. The results obtained for this tropical chironomid showed a similar sensitivity to their temperate zone counterparts.

Keywords: tropical aquatic invertebrates, sensitivity tests, potential test species

INTRODUCTION

An important aspect in ecotoxicological studies is the choice of test species. In order to provide precise and reproducible results, a good background knowledge on the biology of the test species is required as well as sensitivity to a wide range of toxicants; wide geographic distribution and representativeness of its ecosystem; easy and cost-effective laboratory culturing; small size; short life cycle; high reproduction rates and genetic stability (Rand, 1995; Santos, 2011).

Most toxicity test procedures used in Latin America are based on protocols developed by regulatory agencies of temperate climate countries (e.g., USEPA in United States, Environment Canada in Canada, and OECD in Europe) and utilize species such as *Daphnia magna, D. similis, Ceriodaphnia dubia* and *Hyalella azteca* as test organism (e.g., Sotero-Barbosa et al., 2007; Rodgher and Espíndola, 2008; Botelho et al., 2013; Maranho et al., 2014). However, such protocols are inadequate to evaluate the effects of toxicants in tropical and sub-tropical countries (Daam and Van Den Brink, 2010). They demand physical and chemical adjustments since their specifications differ from those of tropical environments and are not necessarily appropriate to their optimum conditions. As an example, *D. magna*, cultured in hard water, cannot precisely evaluate impacts on Brazilian environments, mostly represented by soft waters (Baptista et al., 2000).

Furthermore, the use of exotic species, confined to the laboratory, should be avoided whenever possible, since their escape to the wild poses a risk of them becoming invasive and hazardous to native species (Santos et al., 2007; Fracácio et al., 2009; Braidotti, 2014; Freitas and Rocha, 2014). Nevertheless, the knowledge of the sensitivity of tropical and subtropical species is relatively small compared to temperate regions. Therefore, studies with tropical species from different habits and origins remain necessary in order to establish an adequate database for ecotoxicological studies (Kwok et al., 2007; Daam and Van den Brink, 2010).

On the other hand, ecotoxicological studies conducted in tropical aquatic environments have shown the potential of native species as test organisms. Trenfield et al. (2012) tested *Hydra viridissima* and *Moinodaphnia macleayi* to evaluate aluminum toxicity in two Australian aquatic systems, both demonstrating consistent results related to sensitivity. The same was found for Brazilian water bodies nearby industries, where the native *Pseudosida ramosa* showed higher sensitivity to metals Cr and Cd than *D. magna*, besides similar sensitivity to *D. similis* (Freitas and Rocha, 2011a).

Other tropical species, including *C. rigaudi, M. macleayi* and *Diaphanosoma brachyurum* have also shown sensitivity to salinity (Mohammed and Agard, 2007). The potential of *C. rigaudi* for ecotoxicological studies was tested in Central America (Trinidad), including life cycle data, salinity tolerance and sensitivity to toxicants. Results contrasted to those of *D. magna* indicated that the use of *C. rigaudi* is recommended (Mohammed, 2009).

Moreover, *C. silvestrii*, a Brazilian daphnid (ABNT, 2010) has been used for evaluating the quality of water (Takenaka et al., 2006; Kuhl et al., 2010) and for eutrophic sediment remediation purposes (Janke et al., 2011). It has also demonstrated to be sensitive to pesticides (Moreira et al., 2014; Casali-Pereira et al., 2015) and metals (Rodgher and Espíndola, 2008; Santos et al., 2008).

Although sediment evaluation is still not a basic requirement of Brazilian environmental agencies, ecotoxicological studies have been carried out, most of which utilized non-native species and based on international protocols, including bacteria (*Vibrio fisheri*) and invertebrates such as amphipods *(H. azteca*), cladocerans (*D. magna*; *D. similis*; *C. dubia*) and oligochaetes (*Branchiura sowerbi*; *Tubifex tubifex*).

According to Böhrer-Morel et al. (2005), *D. laevis, C. silvestrii and Chironomus xanthus* are the autochthonous species whose biology has been studied and which have been frequently used as test organisms in Brazil. These authors have pointed out that despite this, routine actions still necessary in many laboratory tests, include sensitivity evaluations and establishment of control-charts.

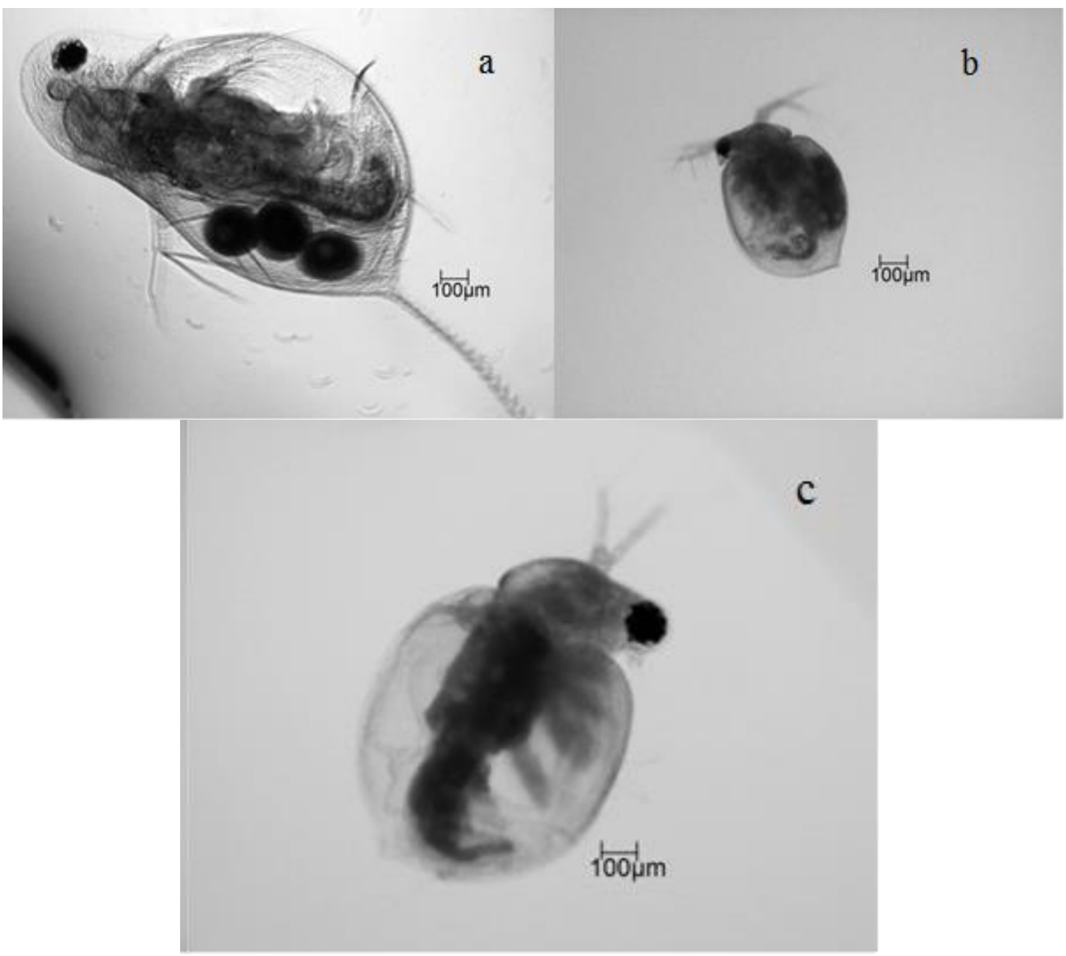

Figure 1. *Daphnia laevis* (a), *Ceriodaphnia cornuta* (b) and *C. silvestrii* (c).

In this context, the present chapter investigated the potential of tropical species as test organisms, aiming at their routine use in water and sediment toxicity tests using standardized toxicity-test protocols from the Brazilian Association of Technical Procedures (ABNT). Two cladoceran species were considered: *D. laevis*, which is one of the three *Daphnia* species found in Brazil (Matsumura-Tundisi, 1984; Rocha and Matsumura-Tundisi, 1990), endemic to the American continent; and *C. cornuta*, another daphnid widely distributed in tropical and subtropical regions. Both species are among the most abundant in tropical freshwaters (Do Hong et al., 2004; Brandão et al., 2012).

Regarding sediment toxicity studies, the tropical endemic benthic macroinvertebrate *C. xanthus* (Diptera, Chironomidae) has high survival rates, short life cycle and easy culturing in laboratory. These features make it adequate as test organism for sediment toxicity tests (Almeida, 2002; Fonseca and Rocha, 2004).

METHODS

Zooplankton Culturing and Sensitivity Tests

Tropical cladocerans considered in the present chapter (Figure 1) were cultured in natural non-reconstituted water with neutral pH, conductivity of 130 to 150 µS cm^{-1} and hardness of 40 to 44 mg L^{-1} $CaCO_3$. The temperate species (*D. similis* and *C. dubia*) were included for comparison. The cultures were fed 10^6 cells per mL of *Raphidocelis subcapitata* and a compound of fermented fish ration (TetraMin/Tetra) and yeast (*Saccharomyces cerevisiae*/Fleischmann) every two days.

Sensitivity tests (24h-EC50* and 48h-EC50) were conducted with the following reference toxicants: potassium dichromate ($K_2Cr_2O_7$*), sodium chloride (NaCl) and sodium dodecyl sulfate ($C_{12}H_{25}NaO_4S$). The methodology followed ABNT procedures (2009). In each treatment 5 to 10 organisms were added to 10-20 mL of tested solution in 3 to 4 replicates. Concentrations of toxicants for *Daphnia* spp. varied from 0.55 to 2.3 g L^{-1} (NaCl); 1.5 to 7.0 mg L^{-1} ($C_{12}H_{25}NaO_4S$) and 0.01 to 0.05 mg L^{-1} ($K_2Cr_2O_7$). In the case of *Ceriodaphnia* spp., their concentrations ranged from 0.6 to 1.9 g L^{-1} (NaCl) and 1.0 to 7.0 mg L^{-1} ($C_{12}H_{25}NaO_4S$). Controls consisted of laboratory culturing water.

Chironomus xanthus Culturing and Sensitivity Tests

The tropical chironomid considered was cultured in natural non reconstituted water with neutral pH, conductivity of 130 to 150 µS cm^{-1} and hardness of 40 to 44 mg L^{-1} $CaCO_3$. Culturing was initiated by transferring ca. 100 larvae to plastic trays. The trays contained a 3 cm thick layer of sterilized sand and 4 L of maintenance water. Larvae were fed 10^5 cells per mL of *R. subcapitata* on the first day plus 0.04 mg mL^{-1} volatile suspended solids/day fish ration throughout the development of the four larval instars. The system was maintained under continuous aeration at 24 ± 1°C and photoperiod of 12 h. The trays also consisted of a screened wooden support covered by tulle (±100 µm) for adult retention (Fonseca and Rocha, 2004).

C. xanthus larvae sensitivity to potassium chloride (KCl) was tested following USEPA (2000) procedures. Five KCl concentrations were used with individual addition of 10 organisms (from instars I to IV) to 20 ml of test solution. Controls used laboratory culturing water. 96h-LC50 values were estimated by Trimmed Spearman-Karber Program based on Hamilton et al. (1977). Statistical analysis applied to all sensitivity tests included T-test, one-way ANOVA and Tukey test, using PAST software package (version 2.17c) for data analysis (Hammer et al., 2001).

RESULTS

Cladoceran Sensitivity Tests

24h-EC50 mean (n=15) value for *D. laevis* was 0.025 ± 0.009 mg L^{-1} for potassium dichromate. 48h-EC50 mean values for *D. laevis* using sodium chloride and sodium dodecyl sulfate (SDS) were 1.06 ± 0.18 g L^{-1} (n=11) and 5.07 ± 0.77 mg L^{-1} (n=6), respectively (Table 1). These results showed the higher sensitivity of *D. laevis* to potassium dichromate (t=-11.043; p=0.000) and sodium chloride (t=-7.192; p=0.000) compared to *D. similis,* although similar sensitivity to sodium dodecyl sulfate (t=-1.028; p=0.323). Coefficients of variation ranged from 15 to 35%, mostly below 30%, expressing adequate results for test validation (Environment Canada, 1990). Sensitivity control charts for *D. laevis* were also included (Figure 2).

For *Ceriodaphnia* spp., 48h-EC50 mean values using sodium chloride corresponded to 1.04 ± 0.20 g L^{-1} for *C. cornuta* (n=13), 1.24 ± 0.27 g L^{-1} for *C. silvestrii* (n=23) and 1.43 ± 0.23 g L^{-1} for *C. dubia* (n=10). 48h-EC50 mean values using sodium dodecyl sulfate were 3.27 ± 0.61 mg L^{-1} for *C. cornuta* (n=10), 4.42 ± 1.46 mg L^{-1} for *C. silvestrii* (n=9) and 4.12 ± 0.97 mg L^{-1} for *C. dubia* (n=6). Coefficients of variation ranged from 16 to 33%, in general within ranges expected (0 to 30%; Environment Canada, 1990). Sensitivity control charts for *C. cornuta* were also included to complement the results (Figure 3).

Table 1. Mean values of 24 and 48h-EC50 for *Daphnia laevis* and *D. similis* using sodium chloride (g L^{-1}), sodium dodecyl sulfate (mg L^{-1}) and potassium dichromate (mg L^{-1}) as reference substances

Values	Sodium chloride		Sodium dodecyl sulfate		Potassium dichromate	
	D. laevis	*D. similis*	*D. laevis*	*D. similis*	*D. laevis**	*D. similis***
Average	1.06	1.63	5.07	5.64	0.025	0.138
Standard deviation	0.18	0.23	0.77	1.20	0.009	0.027
Coefficient of variation	17%	14%	15%	21%	35%	20%
Sensitivity range	0.70-1.42	1.16-2.09	3.53-6.61	3.25-8.04	0.007-0.043	0.084-0.192

* 24h-EC50; ** Source: Zagatto (1988).

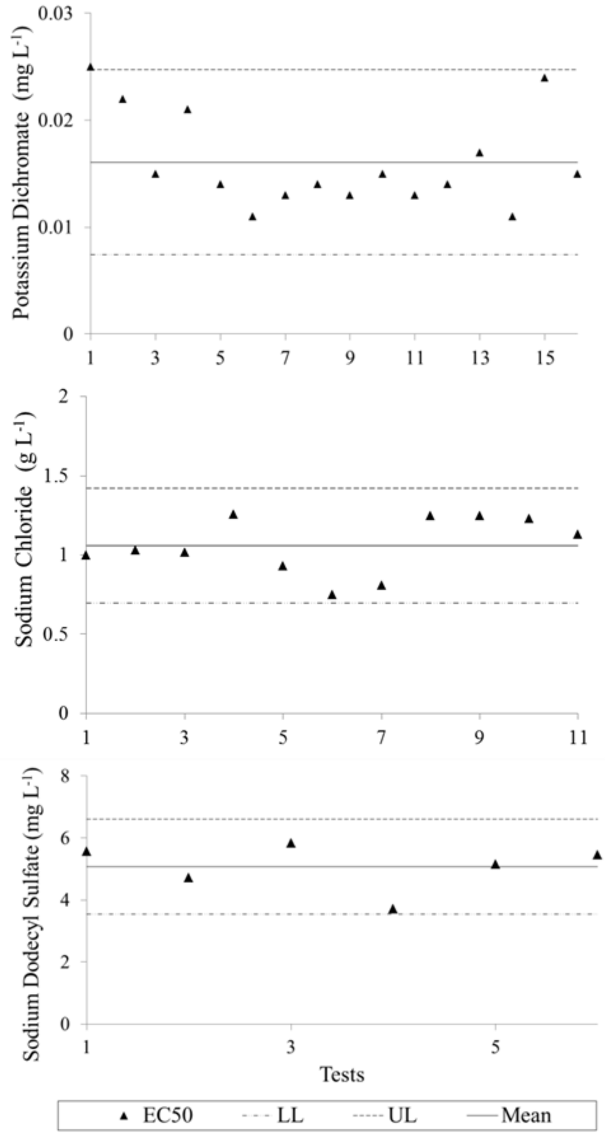

Figure 2. Mean values of 48h-EC50 ± 2 standard deviations (Sensitivity control charts) for *Daphnia laevis* exposed to potassium dichromate, sodium chloride and sodium dodecyl sulfate (LL=Lower Limit; UL=Upper Limit).

Table 2. Mean values of 48h-EC50 for *Ceriodaphnia cornuta, C. silvestrii* and *C. dubia* using sodium chloride (g L^{-1}) and sodium dodecyl sulfate (mg L^{-1}) as reference substances

Values	Sodium chloride			Sodium dodecyl sulfate		
	C. cornuta	*C. silvestrii*	*C. dubia*	*C. cornuta*	*C. silvestrii*	*C. dubia*
Average	1.04	1.24	1.43	3.27	4.42	4.12
Standard deviation	0.20	0.27	0.23	0.61	1.46	0.97
Coefficient of variation	19%	22%	16%	19%	33%	23%
Sensitivity range	0.64-1.45	0.70-1.79	0.98-1.89	2.05-4.49	1.50-7.33	2.19-6.06

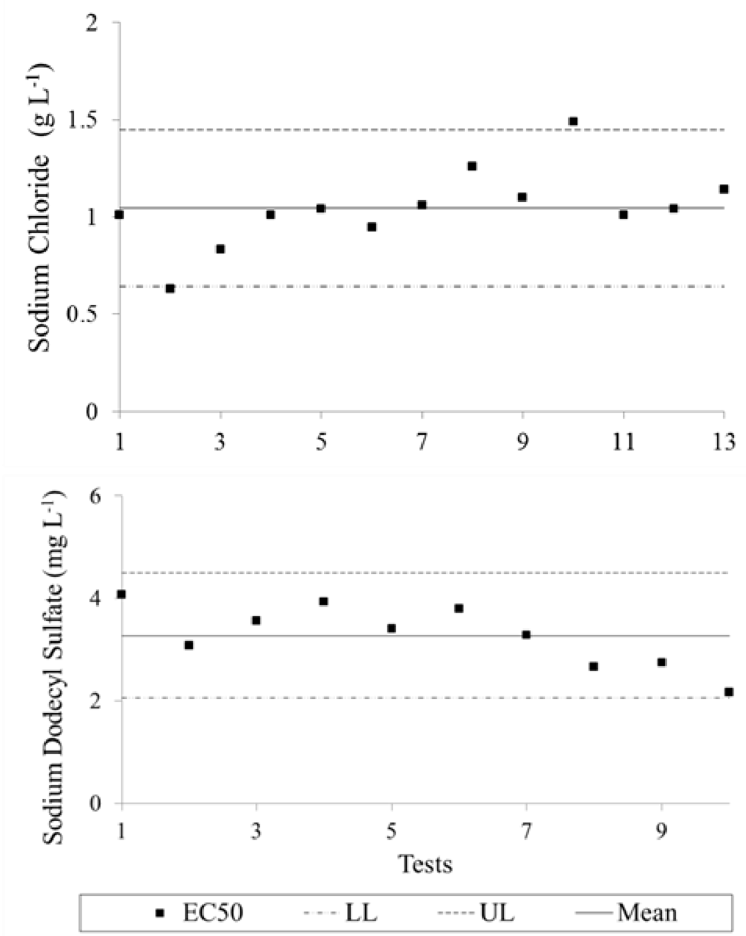

Figure 3. Mean values of 48h-EC50 ± 2 standard deviations (sensitivity control charts) for *Ceriodaphnia cornuta* exposed to sodium chloride and sodium dodecyl sulfate (LL=Lower Limit; UL=Upper Limit).

In considering sodium chloride as reference substance, *C. cornuta* showed higher sensitivity than *C. silvestrii* and *C. dubia* (F=7.214; p=0.002). No differences among these congeners were found in the case of SDS (F=2.924; p=0.074).

Sediment Sensitivity Tests Using Benthic Organisms

Mean values of 96h-LC50 for instars II (n=10), III (n=13) and IV (n=12) of *C. xanthus* (Table 3) corresponded to 3.99 ± 0.63 g L^{-1}, 4.50 ± 0.94 g L^{-1} and 4.64 ± 0.68 g L^{-1}, respectively, showing no differences between sensitivity of instars (F=2.08; p=0.14). The long term results for instar III larvae compared with other tests conducted with *C. xanthus* larvae of the same instar aiming at an interlaboratorial calibration, also showed no difference in their sensitivity (t=1.230; p=0.230). Sensitivity control charts for potassium chloride could provide more information on the usual instar adopted for toxicity tests with these chironomids (Figure 4).

Table 3. Mean values of 96h-LC50 for *Chironomus xanthus* (instar III) and *C. xanthus* (instars II*, III* and IV*), using potassium chloride (g L^{-1}) as reference substance. Data were obtained in 2015-2016 and 1996-1997 (*)

Values	Instar III	Instar II*	Instar III*	Instar IV*
Average	4.91	3.99	4.50	4.64
Standard Deviation	0.80	0.63	0.94	0.68
Coefficient of Variation	16%	16%	21%	15%

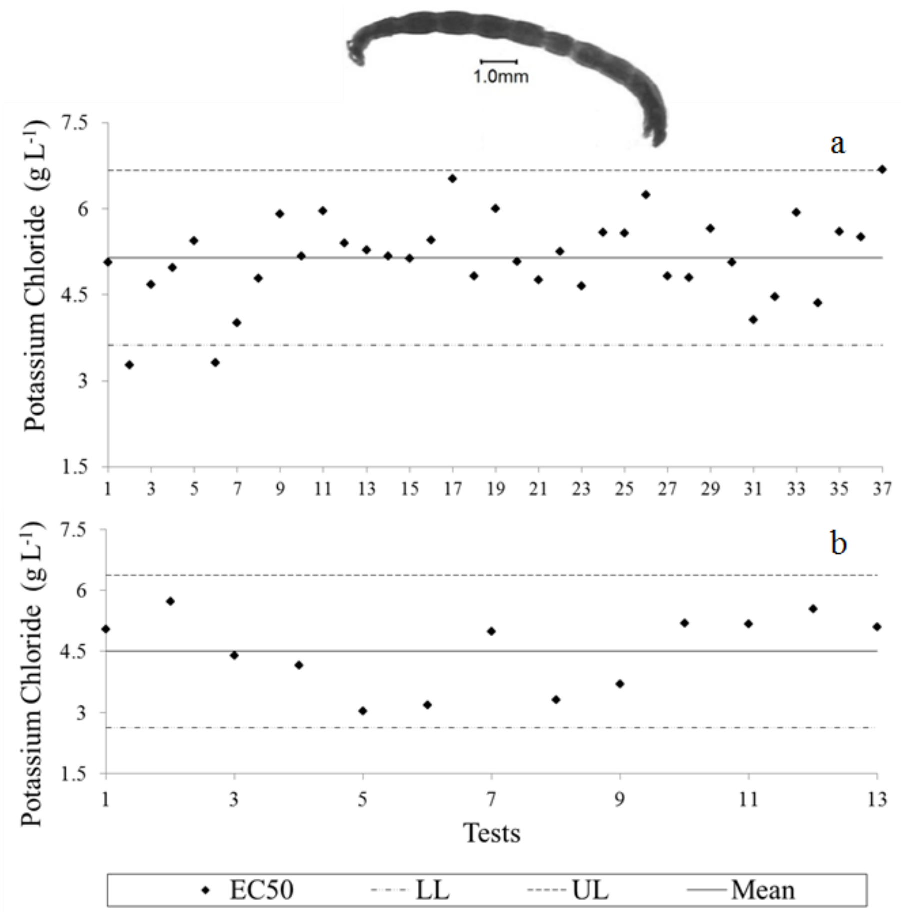

Figure 4. Mean values of 48h-EC50 ± 2 standard deviations (Sensitivity control chart) for *Chironomus xanthus*, instars III 2014-2015 (a) and 1996-1997 (b) exposed to potassium chloride as reference substance (LL=Lower Limit; UL=Upper Limit).

Discussion

Tropical Cladocerans as Test Species

There is a general feeling that at higher temperatures and increased runoff from torrential rainfall, toxicity effects in the tropics can substantially differ from those in temperate regions, in which pollutants toxicity, process rates, compound transformations and fate of residues are now well understood (Daam and Van den Brink, 2010). Not only are the processes not directly applicable, but the responses of temperate region selected test organisms might not show the same sensitivity or be totally appropriate to show the effects of toxic pollutants whether in laboratory or in complex natural tropical conditions (Baptista et al., 2000; Lopes et al., 2007; Tables 1 and 2).

An intensification of ecotoxicological studies is required in developing tropical and subtropical countries in order to cope with the exponential loss of environmental quality (Soares and Callow, 1993).

Among tropical freshwater zooplankton, a number of species have already been tested and recommended for ecotoxicological studies, such as *M. macleayi* (Semaan et al., 2001; Orchard et al., 2002), *P. ramosa* (Freitas and Rocha, 2014), *D. laevis* (Rietzler and Viegas, 2002; Böhrer-Morel et al., 2005; Braidotti, 2014; Sales et al., 2016) and *C. cornuta* (Lopes et al., 2011; Ribeiro, 2011). However, the most frequently used species has been *C. silvestrii*, for both acute and chronic toxicity tests following standardized procedures recommended by the Brazilian methodology standardization agency (ABNT, 2009, 2010).

In the present chapter, *D. laevis* showed higher sensitivity than *D. similis* for sodium chloride and sodium dodecyl sulfate (Table 1). When testing similar substances for *D. ambigua* (another native species to South America), Harmon et al. (2003) found 48h-EC50 values of 2.00 g L^{-1} for sodium chloride and of 44.0 mg L^{-1} for sodium laurel sulfate as reference substances. A comparison of these values to our data also shows higher sensitivity of *D. laevis*. In addition, EC50 mean values for potassium dichromate also showed higher sensitivity of *D. laevis* than *D. similis* (Zagatto, 1988), a temperate species commonly utilized in ecotoxicological studies in Brazil (Zoratto, 2007; Araújo et al., 2010; Sales et al., 2016), thus supporting the idea that the use of *D. laevis* instead of *D. similis* in ecotoxicological studies should be adopted.

D. magna, the temperate species most widely used as test organism (Ratte et al., 2003; Wong et al., 2009), has lower sensitivity than other tropical cladocerans to many pesticides (Lopes et al., 2007) when assessing its performance as a laboratory surrogate. The authors found no lethal effects of deltamethrin to this species and sublethal effects were only observed at the highest concentrations, being less sensitive to the feeding depression endpoint compared to the tropical and subtropical cladoceran *D. brachyurum.*

Freitas and Rocha (2011a) have also observed higher sensitivity of *P. ramosa* than *D. magna* when testing NaCl, KCl, Cr, Cd, SDS and atrazine as reference substances. According to Moreira et al. (2014), *Macrothrix flabelligera* and *C. silvestrii* were also more sensitive to atrazine than *D. magna* (12.37 ± 2.6 mg $L^{–1}$, 14.30 ± 1.55 mg $L^{–1}$ and 50.41 ± 2.64 mg $L^{–1}$, respectively).

In considering other daphnids, *C. cornuta*, a native tropical species, was more sensitive to sodium chloride when compared with *C. silvestrii*, another native species, and *C. dubia*, their temperate counterpart (Table 2). *C. cornuta* was also more sensitive to sodium dodecyl sulfate (3.39 ± 0.50 mg L^{-1}) than *P. ramosa* (10.30 ± 1.60 mg L^{-1}) (Freitas and Rocha, 2011b). In previous studies, *C. rigaudi* contrasted to two other tropical cladocerans, *D. brachyurum* and *M. macleayi*, presented higher sensitivity to Cu, K, Cd, Cr and triton X100 (Mohammed and Agard, 2006; Mohammed, 2009).

Among invertebrates, cladocerans are an important tool in ecotoxicological studies. Wogram and Liess (2001), when comparing the sensitivity of different invertebrate groups, including arthropods and non-arthropods, concluded that Cladocera was one of the groups most sensitive to organic compounds and was the most sensitive to metallic compounds. Their study was based on *D. magna* which, in fact, is equally or less sensitive than other daphnids, as shown in the present chapter, or than copepods (Wong et al., 2009).

Tropical Benthic Macroinvertebrates as Test Species

Regarding sediment ecotoxicological studies, most species adopted in monitoring and risk assessment are temperate species. Little has been done to provide protocols using native test organisms for tropical freshwaters (Marchese and Brinkhurst, 1996; Ducrot et al., 2010; Lobo and Alves, 2011).

Among studies carried out in Brazil, Almeida (2007) has evaluated the potential of *Branchiura sowerbyi* (Oligochaeta, Tubificidae) as test species and could verify a 96h-LC50 mean value to potassium chloride (0.364 g L^{-1}) in a low sensitivity range (0.177-0.551 g L^{-1}). High sensitivity of *B. sowerbyi* was observed by Dhara et al. (2015) when testing Cd. However, this species showed slower development and reproduction than *C. xanthus*.

C. xanthus, an endemic chironomid species to the tropics, has been subject of investigation in many studies carried out in Brazilian aquatic systems (Tonissi et al., 2004; Santos et al., 2007; Araújo, 2008; Sales, 2009; Janke et al., 2011; Silva, 2013; Alves and Rietzler, 2015; Sueitt et al., 2015), presenting feasible features in terms of culturing (Fonseca and Rocha, 2004) and data reproducibility (Almeida, 2002; Dornfeld et al., 2006; Table 3 of the present chapter). The genus *Chironomus* is also well represented in the neotropics (Correia, 2004).

Studies performed to determine sensitivity ranges for *C. xanthus* found lower 96h-EC50 values than in temperate congeners (*C. crassiforceps*, *C. riparius*, *C. decorus* and *C. tentans*) for Cu (0.27 mg L^{-1}, Masutti, 2004; 0.30 mg L^{-1}, Dornfeld et al., 2006). Almeida (2007) also verified higher sensitivity of *C. xanthus* to pentachlorophenol (96h-LC50 of 0.111 mg L^{-1}; sensitivity range of 0.082-0.141 mg L^{-1}) compared with the values from the literature for *Callibaetis okianos*, Neuroptera (1.78 mg L^{-1}; 1.27-2.48 mg L^{-1}) and *Monhystera disjuncta*, Nematoda (2.10 mg L^{-1}; 0.50-7.0 mg L^{-1}). Considering potassium chloride as toxicant, our results (Table 3) show similar sensitivity of this tropical species compared with chironomids *C. riparius* and *C. tentans* from temperate regions (USEPA, 1994; Burton Jr et al., 1996). Thus, *C. xanthus* is at least as sensitive as the non-native species.

Stoughton et al. (2008) conducted toxicity tests using insecticides (imidacloprid) to compare the sensitivity of *C. tentans* and *H. azteca*, finding much lower EC50 values for the chironomids (5.75 μg L^{-1} versus 65.43 μg L^{-1}). *H. meinerti*, a tropical amphipod, has not yet been sufficiently tested to compare it to *H. azteca*, an exotic species still often used in toxicity tests in tropical laboratories (Silvério et al., 2005; Favaro et al., 2014).

Sublethal effects have also been important endpoints in ecotoxicological studies related to the genus *Chironomus*, including morphological deformities from environmental exposition (Bonani, 2010) and genotoxic and bioaccumulative effects of cyanobacteria toxins (Santiago, 2012).

CONCLUSION

Our results for *D. laevis* and *C. cornuta,* besides high fecundity, easy maintenance in the laboratory and data reproducibility of both species, support their use as preferential test species in water and sediment toxicity tests, in order to construct a strong data base for toxicity evaluation and risk assessments in tropical conditions.

C. xanthus can also be a useful test species, being representative of the benthic community and appropriate to evaluate sediment toxicity. This species should be more widely used, together with tropical cladocerans and other tropical macroinvertebrates, for carrying out much needed ecotoxicological studies in Brazil.

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Chapter 8

ANTHROPOGENIC IMPACT MODIFIES THE BLOOD ANTIOXIDANT STATUS IN *PYGOSCELIS ADELIAE* AND *PYGOSCELIS PAPUA* AT HOPE BAY, ANTARCTIC PENINSULA

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ABSTRACT

Antioxidant blood status was measured in *Pygoscelis adeliae* (Adélie penguin) and *Pygoscelis papua* (Gentoo penguin) populations coming from areas with high and low anthropogenic impact at Hope Bay, Antarctic Peninsula. Superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPx) enzyme activities, as well as the levels of reduced glutathione (GSH), uric acid (UA), protein oxidation (PO) and lipid peroxidation (LPO) were measured. The SOD and CAT activities showed significant differences for areas and species. Adélie and Gentoo penguins from the high impact area exhibited an increase in GST activity. Only Gentoo penguins, that inhabit the high impact area, registered an increase in GPx activity. The GSH levels did not show significant differences, suggesting that these penguins would not use GSH *per se* as primary hydrosoluble antioxidant response. However, UA levels presented differences among the analyzed factors. The oxidative damage levels registered in erythrocytes (PO_e and LPO_e) showed similar patterns for both species. Adélie penguins from the high impact area and Gentoo penguins from the low impact area had the highest levels of oxidative damage. In view of the present results, we conclude that penguin populations located in the high impact area are affected by human activities. These disturbances were evident on the upset of the antioxidant defences and/or the oxidative

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damage. Accordingly, the analyzed parameters used in the present study proved to be reliable biomarkers to reflect anthropogenic impact on penguin populations.

Keywords: Antarctica, *Pygoscelis adeliae*, *Pygoscelis papua*, blood antioxidant status

INTRODUCTION

Expeditions to and settlement in Antarctica began in the early 19th century, with seal and whale hunting in order to obtain their oil (Bargagli, 2008; Tin et al., 2008; Xin, 2009). At the beginning of the 20th century, the explorers to Antarctica built wooden huts and brought large quantities of supplies and equipment to support their geographical and scientific studies for several years. When the expeditions ended and relief ships arrived, a rapid exodus frequently allowed only essential items to be taken north. The huts and thousands of items were left behind. Fuel depots with unused containers of petroleum products, asbestos materials, and diverse chemicals were also left inside the huts (Blanchette et al., 2004). Since then, the Antarctic continent has been occupied and visited uninterruptedly (Bargagli, 2008), and nowadays constructions such as tents, shelters, stations and trails can be found everywhere (Tejedo et al., 2011).

Despite anthropogenic pollutants (such as DDT, among others) being found in Antarctica (Bargagli, 2008; Aronson et al., 2011), it is possible to focus on local impacts which include: oil spills and paintings; heavy metals and metalloids coming from waste disposal sites; materials used in constructions; and chemicals used in effluent treatment plants (Negri et al., 2006; Corsolini, 2008; Tin et al., 2008; Aronson et al., 2011).

Esperanza station is located at Hope Bay (Antarctic Peninsula) and its activity is on-going throughout the entire year. Electric supply is provided by a diesel generator that works continuously during the year. Even though gasoil containers have protection to avoid occasionally spills, it is possible to find oil slicks in the surroundings of the station. Besides this type of pollution, the disposal of incinerated waste, which has not been yet fully retracted, and the historical trash can be found spread around the station. Moreover, light aircraft (Twin-Otter) and helicopters, used for personal and cargo transportation, fly over the areas causing another type of perturbation: noise. Such activities are increased during the summer season supporting the scientific and touristic activities, coinciding with the reproductive season of different Antarctic species (Acero et al., 1996; Carlini et al., 2007).

Several studies have registered the effect caused by human activities on different bird species. These may include the decrease in populations, behavioural changes and the alteration of physiological responses (Harris, 2005; Tin et al., 2008; Ninnes et al., 2010). The anthropogenic activity could also modify these responses increasing the production of reactive oxygen species (ROS) which exert an unbalanced state called oxidative stress (Halliwell and Gutteridge, 2007). It has been seen that the exposure to heavy metals (Koivula and Eeva, 2010), hydrocarbons (Perez et al., 2010) and even anthropogenic activities such as nocturnal working and ecotourism (Burger and Godchfel, 2007; Navarra and Nelson, 2007) produce ROS, increasing oxidative damage (Stohs and Bagchi, 1995; Bagchi et al., 2012). As a consequence, the antioxidant responses are increased (Berglund et al., 2007). As was noted by Costantini (2008), the oxidative damage level is closely related to animal fitness, meaning that high oxidative stress levels may compromise survival and reproduction. Moreover, animal population health may be affected by oxidative stress which has significant effects on fitness components (Beaulieu et al., 2013).

Many organisms are used to evaluate environmental pollution. They are recognized as sentinels and include species such as molluscs, crustaceans, fish and birds (Dee Boersma, 2008). Marine birds usually used as sentinels are: gulls, albatroes, skuas, petrels and penguins. Besides having sentinel characteristics (Costantini, 2008; Dee Boersma, 2008), penguins are adapted to swimming and diving, so they can also reflect ocean productivity variation (Furness and Camphuysen, 1997; Burger and Gochfeld, 2004).

The aim of the present study was to assess the physiological state of *Pygoscelis adeliae* (Adélie penguin) and *Pygoscelis papua* (Gentoo penguin) populations that were nesting in areas with high and low human impact at Hope Bay, using biomarkers of oxidative damage and antioxidant responses.

METHODS

Samples

P. adeliae and *P. papua* penguin populations breed sympatrically during the summer season at Hope Bay, Antarctic Peninsula. The colonies are located near the Esperanza Argentinean Antarctic station. The nests are placed in areas which have been identified by Acero et al. (1996) as high and low impact areas. The high impact area is located close to (sometimes inside) the station, and the low impact area is far from the station and its associated activities.

Sampling was carried out in the colonies of both penguin species at the different areas, during the summer 2012/2013. Blood samples of 3 ml were taken from 15 adults with heparinized syringes and sterile 23G x 1” needles.

Biomarkers

In the laboratory, samples were centrifuged (1500 rpm x 10 min) in order to separate erythrocytes and plasma. A small portion of erythrocytes was separated for determining reduced glutathione (GSH) levels. The remaining erythrocyte fraction was diluted with potassium phosphate buffer (100 mM, pH 7.4; dilution ratio sample/buffer 1:50) for superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities, and lipid peroxidation (LPO_e) and protein oxidation (POe) levels determination. Diluted plasma (potassium phosphate buffer 100 mM, pH 7.4; dilution ratio sample/buffer 1:10) was used for measuring oxidative damage (LPO_p and PO_p) and uric acid (UA).

In the diluted erythrocyte fraction, SOD, CAT, GST and GPx activities were determined using spectrophotometric methods described by Misra and Fridovich (1972), Aebi (1984), Habig et al. (1974) and St Clair and Chow (1996), respectively. Specific enzyme activity was calculated considering the total protein content. One SOD unit is the amount of enzyme necessary to inhibit 50% of the rate of autocatalytic adrenochrome formation measured at 480 nm. One CAT unit is the amount of enzyme necessary to degrade 1 µmol of H_2O_2, measured at 240 nm. One GST unit represents the amount of enzyme required to conjugate 1 µmol of 1-chloro-2.4-dinitrobenzene, determined at 340 nm. One GPx unit is expressed as the amount of enzyme necessary to oxidize 1 pmol of NADPH/mg of protein per minute at 340 nm.

The GSH level was determined by the method of Moron et al. (1979). After deproteinization with trichloroacetic acid (TCA 50%), free endogenous GSH was determined using 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) 0.5 mM. The absorbance was read at 412 nm. GSH was used as standard to calculate nmol/ml hemolysate.

The PO level was evaluated according to Reznick and Packer (1994), with minor modifications (Ansaldo et al., 2007), by detecting the formation of protein hydrazones as a result of the reaction of dinitrophenyl hydrazine (DNPH) with protein carbonyls. After the protein hydrazone formation, they were precipitated using TCA 30% (Fagan et al., 1999), and then washed 3 times with ethanol:ethyl acetate (1:1). After the final wash, the protein was solubilised in 1 ml of urea (6 M in 20 mM potassium phosphate, pH 2.5). To speed up the solubilisation process, the samples were incubated in a 37 ºC water bath for 60 min. The final solution was centrifuged to remove any insoluble material. The carbonyl content was calculated from the absorbance measurement at 375 nm.

The LPO level was measured according to Buege and Aust (1978), by the formation of thiobarbituric acid reactive substances (TBARS). Fresh hemolysates were added to the reaction mixture (trichloroacetic acid 15% (w/v), 2-thiobarbituric acid 0.375% (w/v), and butylhydroxytoluene 0.147 mM) in a ratio of 1:5 (v/v). The mixture was vigorously shaken, maintained in boiling water for 60 min, and immediately cooled at 5 °C for 5 min (Ohkawa et al., 1979). It was then centrifuged at 5000 x g for 10 min, and the supernatant was measured spectrophotometrically at 535 nm.

The UA level was quantified using the uricase enzyme method. Uric acid is converted by uricase into allantoin, hydrogen peroxide (H_2O_2) and CO_2. The H_2O_2 initiates the coupling of 4-aminophenazone to 3,5-dichloro-2-hydroxybenzenesulfonic acid to form a chromogen, red quinoneimine, which is measured at 505 nm and is proportional to the amount of H_2O_2 generated from the UA (Fossati et al.,1980). Units are expressed as mg/dl hemolysate.

The total protein quantity was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistics

Data were analyzed with a general linear model using as factors the type of area (high and low impacted) and species (Gentoo and Adélie). The best model was obtained by Akaike and Schwartz IC comparison (Infostat, 2012). When the interaction was not significant, the main effects were evaluated and plotted independently on various graphs. On the other hand, when the interaction is significant, the impact of one factor depends on the level of the other factor, so they are plotted all together on a graph. Interaction effects were thus evaluated given that the analysis of the main effect results uncompleted or misleading. When results were significant, post-hoc comparisons were performed using the Di Rienzo, Guzman, and Casanoves test (universally referred to as the DGC test; Di Rienzo et al., 2002).

RESULTS

The SOD and CAT activities did not show significant interaction between the analyzed factors (areas and species), therefore the main effects of each factor are plotted (Figure 1). The SOD activity was higher in Adélie than Gentoo penguins ($p<0.0001$; Figure 1-I). Besides, penguins from the low impact area showed higher SOD activity than those located in the high impact area ($p<0.001$; Figure 1-II). The Gentoo penguins had increased values of CAT activity ($p<0.0001$; Figure 1-III) but, independently of their species, penguins from the low impact area showed more CAT activity than those from the high impact area ($p<0.001$; Figure 1-IV).

The GST and GPx activities were related to the interaction of the studied factors, type of area and species ($p<0.05$ and $p<0.01$, respectively; Figure 2). Both species from the high impact area presented increased GST activity in comparison with those from the low impact area. However, Gentoo had higher enzyme activity than Adélie penguins (Figure 2-I). Moreover, the Gentoo penguins located in the high impact area registered a 25% higher GPx activity than those from the low impact area and the Adélie penguins from both areas (Figure 2-II).

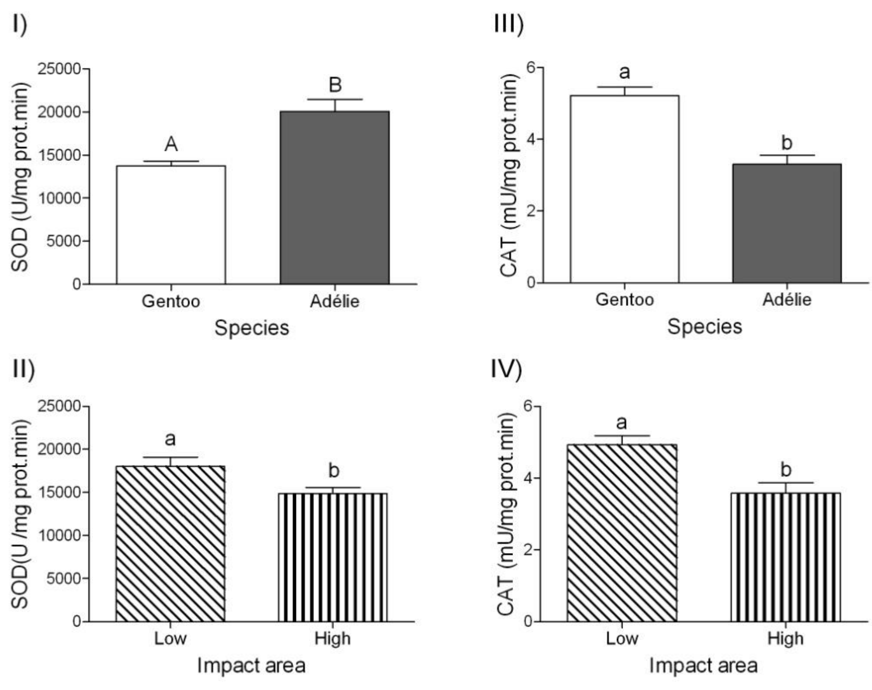

Figure 1. The SOD (I, II) and CAT (III, IV) activities in penguin erythrocyte fractions. Values are expressed as means ± standard error, n=15 animals. Different letters mean significant differences (a,b: $p<0.001$).

The measured hydrosoluble antioxidants (Figure 3) showed no significant differences for GSH levels in penguins of both species and from both areas (Figure 3-I), while UA levels depended on the interaction among the analyzed factors ($p<0.05$). The Gentoo from both studied areas had the highest UA levels, while Adélie penguins from the high impact area had 50% more UA levels than those from the low impact area (Figure 3-II).

The erythrocyte protein oxidation (PO_e) and lipid peroxidation (LPO_e) levels showed the interaction of the studied factors ($p<0.01$ and $p<0.05$, respectively; Figure 4), indicating that PO_e and LPO_e levels depend on the nesting area. Adélie from the high impact area had the highest protein damage levels (16.67±0.89 carbonyl nmol/mg protein; Figure 4-I). The PO_e levels for both species belonging to the low impact area were similar (Gentoo: 13.82±1.11 carbonyl nmol/mg protein; Adélie: 12.30±1.57 carbonyl nmol/mg protein). Gentoo penguins that inhabit the high impact area presented the highest LPO_e levels (16.87±0.86 TBARS mmol/ml hemolysate; Figure 4-II) contrasting with values from the low impact area and with the Adélie penguins. Considering only the Adélie penguins, those from the high impact area registered higher LPO_e levels (9% more than individuals from the low impact area).

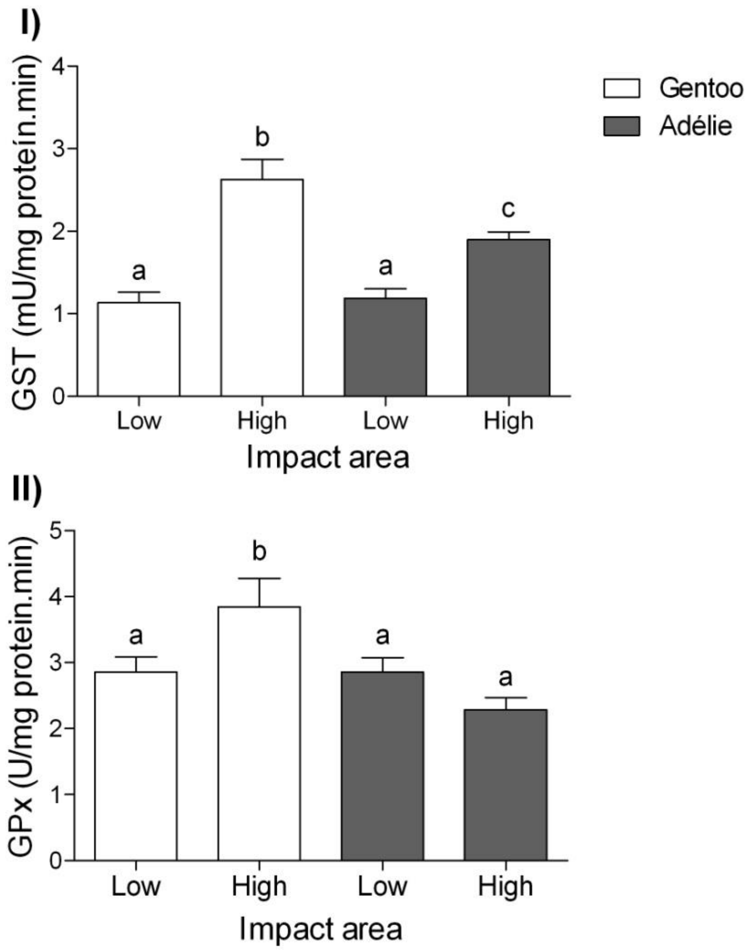

Figure 2. I) GST and II) GPx enzyme activities in penguin erythrocytes from both study areas. Values are expressed as means ± standard error, n=15 animals. Different letters mean significant differences ($p<0.05$).

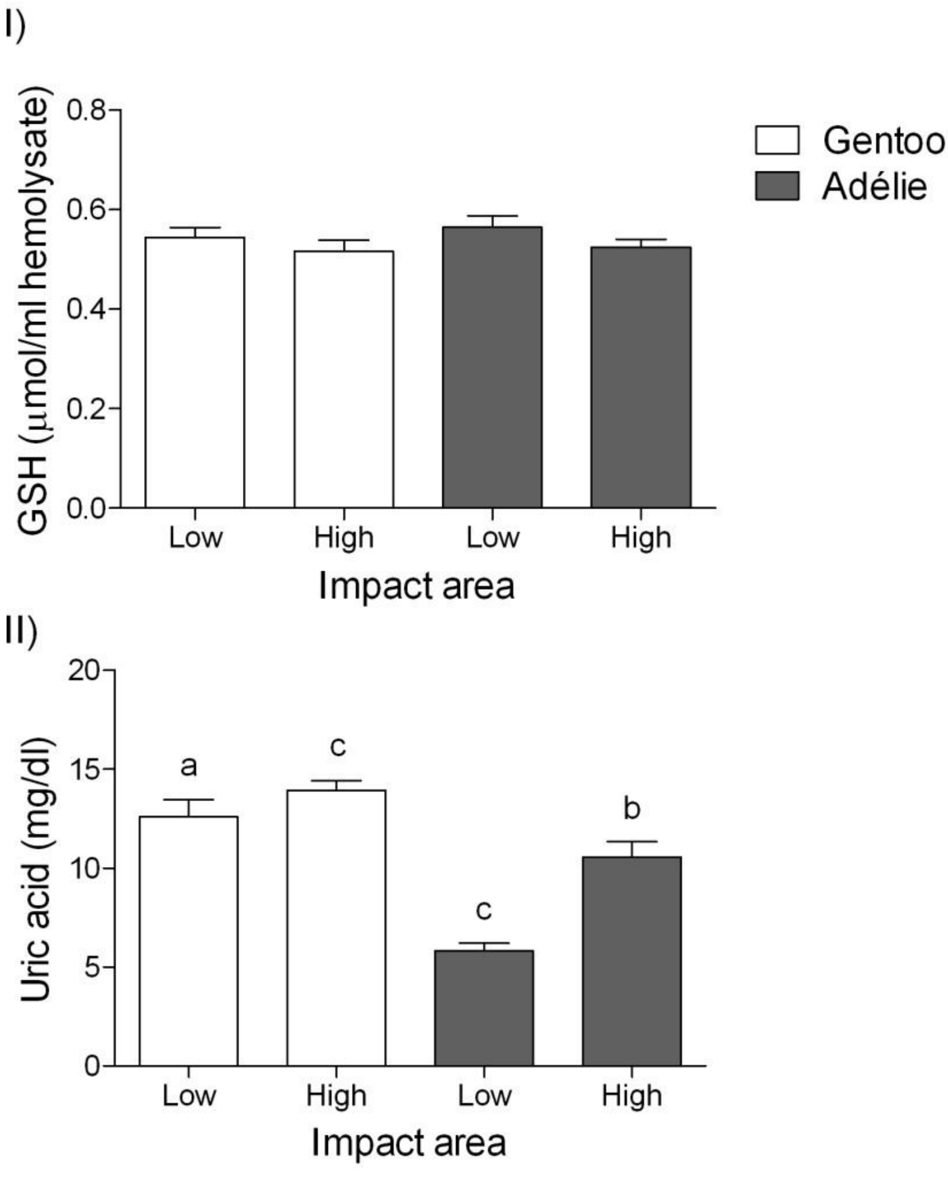

Figure 3. I) GSH and II) uric acid levels in penguin erythrocyte and plasma fractions, respectively. Values are expressed as means ± standard error, n=15 animals. Different letters mean significant differences ($p<0.05$).

Registered plasma oxidative damage levels were about 50% lower than those of the erythrocytes (Figure 4-III, IV). The Gentoo showed PO_p levels significantly higher ($p<0.01$) than the Adélie penguins, particularly those Gentoo inhabiting the low impact area presented the highest levels (7.78±0.62 carbonyl nmol/mg protein). Adélie penguins from the high impact area were more affected (≈54%) than those located in the low impact area (Figure 4-III). The LPO_p levels did not show significant differences between both areas and species (Figure 4-IV).

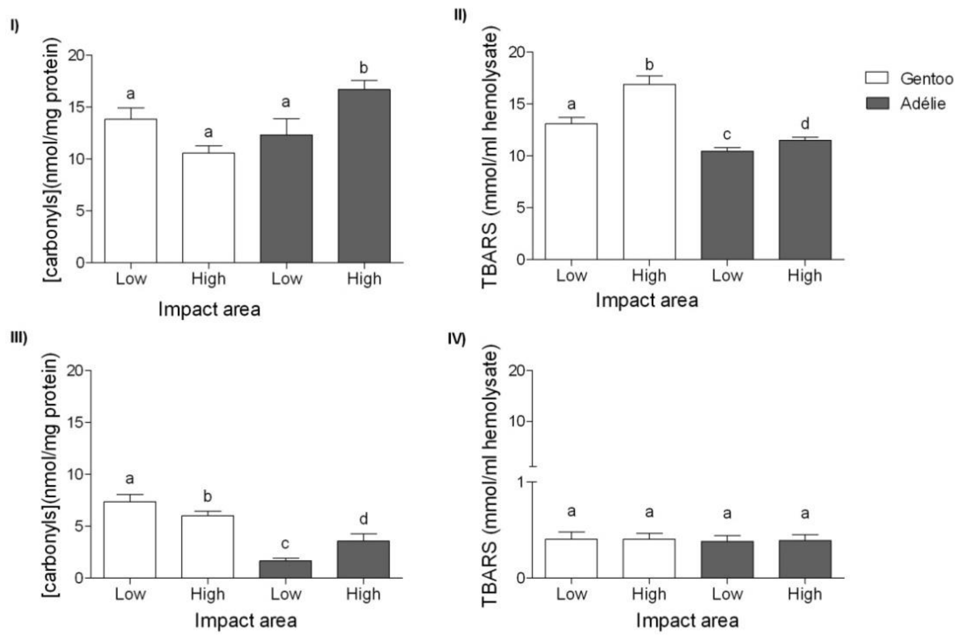

Figure 4. I) PO_e, II) LPO_e, III) PO_p and IV) LPO_p levels of Gentoo and Adélie penguins. Values are expressed as means ± standard error, n=15 animals. Different letters mean significant differences ($p<0.05$).

DISCUSSION

In the light of the present results, it is possible to assess the physiological status of *P. adeliae* (Adélie) and *P. papua* (Gentoo) populations that are nesting in areas near and distant to Esperanza Argentinian Station at Hope Bay, Antarctic Peninsula. There is a clear difference in antioxidant enzyme activities and oxidative damage biomarkers either between penguin species or the studied areas. It may be argued that penguins from the high impact area would be more affected by the station activities. These results agree with those reported by other authors who notice negative effects in many bird species due to human activity (De Villiers et al., 2006; Burger and Goshfield, 2007; Tin et al., 2008; Isaksson et al., 2009; Perez et al., 2010).

The SOD and CAT activities varied with the species or with the areas in which birds nest. Adélie showed increased SOD activity and Gentoo showed increased CAT activity and, in both cases, enzyme activities were increased in penguins which belonged to the low impact area. These results are not those to be expected for the disturbed areas: high ROS production followed by altered enzyme activities. The enzyme activity declines may be correlated with some inhibitory effect owing to heavy metals such as cadmium (Cd) and lead (Pb) present in the environment, which were detected by Guerra et al. (2011) in the vicinity of Esperanza station soil. Furthermore, Stohs and Bagchi (1995) evidenced that some heavy metals such as Cd, can inhibit SOD and CAT activities. Thus, it would be assumed that the low activity in SOD and CAT from the high impact area could be due to the inhibitory effect of Cd present in the environment.

Herrera-Dueñas et al. (2013) worked with sparrows which came from polluted areas (industrial zones) and unpolluted areas of Madrid and surroundings. In their work they compared the SOD and CAT activities, the GSH/GSSG relation, the lipid oxidative damage, the total antioxidant capacity and the hemoglobin concentrations. Those results only showed significant alterations for both the total antioxidant capacity and the hemoglobin concentration, being lowest in urban areas. The rest of the analyzed parameters did not show any differences between the compared areas. In the present work, differences in SOD and CAT activities were registered in both analyzed areas. These changes may indicate that human activities affect penguin oxidant/antioxidant blood parameters.

The GSH is a hydrosoluble antioxidant with low molecular weight, wide tissue distribution and considered a key molecule in the antioxidant process because of its reduction capacity (Manduzio et al., 2005; Halliwell and Gutteridge, 2007). No differences were registered in GSH levels of penguins for either species or area. Thus, GSH possibly would not be used as an antioxidant *per se* by these penguin species. In the detoxification process, GSH works as substrate and cofactor for GST and GPx enzymes, respectively. The GST activity was higher, as expected, in penguins from the high impact area. The GST and GPx showed similar response patterns for the Gentoo penguins, while the Adélie penguins only had differences for the GST enzyme activity.

Plasma UA is a hydrosoluble molecule with antioxidant properties. It is also the main form of nitrogen excretion in avian species, which is produced from hypoxanthine and xanthine by xanthine oxidase and xanthine dehydrogenase enzyme catalysis (Halliwell and Gutteridge, 2007; Costantini, 2008). High correlation between UA levels and total antioxidant capacity was found in 526 individuals of 92 bird species (Koivula and Eeva, 2010). Uric acid also displayed a strong and positive correlation with antioxidant capacity in studies with chickens and greenfinches (Cohen et al., 2007; Hõrak et al., 2007). Hõrak et al. (2007) found that there is a positive correlation between UA and LPO in greenfinches, which might be due to the production of UA in response to increased LPO. In the present study, we also found a correlation between UA and LPOe (Pearson: 0.37, $p<0.05$) for Adélie penguins but not for Gentoo penguins. In the same way, the significant differences observed in UA levels between Adélie penguins suggest that these penguins could be using the UA as its main circulating antioxidant.

For erythrocyte and plasma fractions, protein oxidation (PO_e and PO_p, respectively) showed higher levels of damage in Gentoo located in the low human impact area when compared with those located in the high impact area. On the contrary, the Adélie penguins which inhabit the high impact area showed augmented levels of both PO_e and PO_p in comparison to those from the low impact area. The LPO_e was increased in the penguins that inhabited the area of high impact; being the Gentoo penguins the ones with the highest LPO_e levels. These results differ to those registered by Herrera-Dueñas et al. (2013), because we found that LPOe levels showed differences between the sampled areas.

The LPOp levels did not show significant differences between the studied species. These results agree with Perez et al. (2010), whose study was carried on in breeding adults of the yellow-legged gull (*Larus michahellis*) stressed by the addition of fuel to diet. They did not register a significant increase in the LPOp levels from the exposed birds. One possible explanation given for this fact is that the LPO_p damage was partially prevented by antioxidants such as vitamin E, which protects polyunsaturated fatty acids from the oxidative attack.

In the Antarctic Peninsula, Beaulieu et al. (2013) reported that Adélie penguins had lower antioxidant capacity (an index for the hydro-soluble antioxidant levels) and increased levels of oxidative damage compared to Gentoo penguins. In contrast, the present results showed no differences in GSH levels and oxidative damage was observed for both species at high and low impact areas. Only GSH and UA were analyzed in the present study while the total antioxidant capacity, as measured by Beaulieu et al. (2013), involves ascorbic acid, vitamin E and bilirubin, in addition to the above mentioned antioxidants. The Adélie penguins showed lower levels of damage than Gentoo in either proteins or lipids for the oxidative damage.

Anthropogenic activity tends to alter the oxidation status in animals (Navarra and Nelson, 2007); in addition, urbanization and its associated pollution are the main activities that affect the environment (Baydas et al., 2001; Cruz et al., 2003; Beaulieu and Costantini, 2014). The Esperanza station generates many disturbances that may affect the penguin colonies.

In agreement with the present observations, Barbosa et al. (2013) registered stronger alterations in Gentoo penguins from the high tourist traffic area than those from the low traffic area. They observed lower immunoglobulin levels, erythrocyte malformations, and augmented levels of Pb and Ni in the most transited area. Moreover, Higham and Luck (2007), in their book Marine Wildlife and Tourism Management, collected many studies on the responses of different penguin species regarding tourist transit into the colonies. In most cases, the registered responses lead to heart rate increase and release of corticosterone. These reports reaffirm that human activities produce negative effects on animals.

In the review done by Carney and Sydeman (1999), a total of 17 studies examined the effects of scientific activity, tourism, and aircraft operations on physiology, behaviour and reproductive success of penguins. Due to their apparent indifference to the presence of humans, penguins were thought to be relatively immune to human disturbance. It is noticeable that Adélie penguins from protected areas far from disturbances were more susceptible to minor contacts than those populations where human contact was common. This means that the more regular the disturbance, the smaller the response. The present study showed that, although changes in penguin behaviour were not observed, any disturbance (overflying aircraft and helicopters) might generate oxidative stress by increasing the ROS generation.

As seen in the present study, Gentoo penguins from the high impact area have higher GST and GPx antioxidant enzyme activities and lower PO_e and PO_p levels than those Gentoo penguins which inhabit the low impact area. One might then assume that this increment of enzyme activities would counteract the oxidative damage, which was reflected in its low levels. On the contrary, Gentoo penguins from the low impact area had low enzyme activity, such that levels of oxidative damage would not be offset. Thus, for any small disturbance, as expressed by Carney and Sydeman (1999) in their review, oxidative damage of these animals will be higher, as reflected in the present results. Both species of penguins from the low impact area present PO and LPO "baseline" levels. Unlike what might be happening with Gentoo penguins from the high impact area, the antioxidant defences registered in Adélie penguins did not reach the balance of the natural (basal) damage, producing an even greater damage in response to anthropogenic activity.

CONCLUSION

Adélie penguins from the high impact area presented higher GST activity and UA levels than those from the low impact area. Besides, those penguins registered high LPO_e, PO_e and PO_p levels. On the other hand, Gentoo penguins from the high impact area presented higher GST and GPx activities and high LPO_e levels.

Penguins are being affected by human activity, which has altered the analyzed biomarkers such as the blood oxidative damage and the antioxidant responses. These biomarkers have proved to be useful and effective tools in reflecting antioxidant responses and the damage to the exposed penguins. Thus, this work gives the first report on the state of Hope Bay penguin colonies as well as baseline values of oxidative stress related parameters.

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Chapter 9

METALLOTHIONEINS AS BIOMARKERS OF CONTAMINATION BY METALS IN BIVALVES OF EASTERN VENEZUELA

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ABSTRACT

Metallothioneins (MTs) are proteins with molecular mass of 6-7 KDa, a high content of cysteine and lack of aromatic amino acids. They have the ability to bind more metals than any other protein. For this reason, they have been considered as biomarkers in the presence of zinc, copper, cadmium and mercury. This chapter presents studies on the evaluation of MTs in bivalve molluscs under confined conditions and in natural environments, particularly in eastern Venezuela where the greatest diversity and abundance of these organisms are found. Although *Perna viridis* is an introduced species in Venezuela, it is the most widely used bivalve in ecotoxicological studies in the region. Other bivalves have also been evaluated, such as *Arca zebra, Anadara floridana, Atrina seminuda, Tivela mactroides, Donax denticulatus* and *Lima scabra*. The laboratory bioassays have shown that, in general, metals such as cadmium and mercury induce the expression of MTs. However, studies in natural environments have determined that MTs have a seasonal variation associated with the reproductive cycles of bivalves. The latter, in turn, are influenced by the seasonal variation of physical, chemical and biological parameters in the region. These results highlight the physiological role of MTs in the biological processes of bivalves. It is necessary to elucidate the mechanisms of physiological processes and toxicity of metals, particularly MT isoforms that seem to play a key role in toxicity and life cycles of molluscs.

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Keywords: metallothionein, bivalve, heavy metal, pollution

INTRODUCTION

Metallothioneins (MTs) were isolated for the first time from the cortex of the horse kidney as a protein that binds Zn (zinc) and Cd (cadmium). Its name is attributed to the high content of metal and sulfhydryl groups, lack of aromatic amino acids and a molar mass in the range 6-7 kDa (Margoshes and Vallee, 1957; Kägi and Vallee, 1960).

MTs are present in all life forms, from bacteria to mammals (Capdevilla and Atrian, 2011). Their cysteine content can vary between 25 and 30%; they are heat stable proteins (Klaassen et al., 2009) and lack stable tertiary structure, determined by the bound metal ions. Among marine invertebrates, bivalve molluscs are the most studied organisms. A greater study of MTs in these invertebrates is enabled by the association between the toxic metals and MTs, once they were isolated and linked to Cd (Kägi and Vallée, 1960). Accordingly, these proteins have been linked to the detoxification of metals.

Induction mechanisms of MTs have been established from the information available from higher vertebrates and similar mechanisms seem to be present in all organisms. The expression of MTs can be induced by a wide variety of physiological agents, environmental stressors and transition metals (Juárez-Rebollar and Méndez-Armenta, 2014), and is controlled at the level of transcription. MT gene promoter sites present metal response elements (MRE) that are present in multiple copies in the promoter regions of all MTs induced by metals (Culotta and Hammer, 1989).

A protein called MT transcription factor (MTF-1) acts as a mediator to start the MT gene expression. This factor requires a high concentration of Zn for binding to DNA (Westin and Schaffner, 1988). Studies in mammals (Heuchel et al., 1994) point out that MTF-1 is a ubiquitous protein that contains six domains of Zn and some domains of trans activation, rich in the amino acids proline and serine/threonine, which are required for the inducible translation by metal. The active form of MTF-1 is initially inhibited by an inhibitor sensitive to Zn, called metal transcription inhibitor (MTI). In the presence of Zn and Zn-ions, the complexes MTF-1 and MTI dissociate. This condition allows that MTF-1 interacts with the MRE in the MT developer site to activate transcription. The binding of the newly synthesized MT to Zn, promotes the formation of the MTF-1/MTI complex (Palmiter, 1998). At the same time, the transcription of MTs via MTF-1/MRE is controlled by multiple cascades of transduction signals that affect the phosphorylation of MTF-1 (Adams et al., 2002).

MTs in bivalve molluscs have been widely described in their structure and function, particularly as a response to contamination by metals in coastal marine ecosystems, and their evaluation has been incorporated into pollution biomonitoring programs (Phillips, 1979; Phillips and Rainbow, 1993) However, it has not always been possible to determine associations between metal levels and the expression of the protein in natural environments (Monserrat et al., 2007; Machreki-Ajmi et al., 2008), since previous exposures of organisms to any type of metal inductor of MTs modulate the expression of the protein in new exposures (Lemus et al., 2014a). Most studies performed under laboratory conditions show a dose-response relationship (Baudrimont et al., 1997; Geret and Cosson, 2002). However, it has been pointed out that induction mechanisms of these molecules are modulated by biotic factors such as developmental stage, sex, reproductive condition, growth rate, and also by some abiotic factors such as temperature, salinity, dissolved oxygen, light intensity, among others (Ladhar-Chaabouni et al., 2012; Zapata-Vívenes et al., 2014).

Studies in marine ecotoxicology in Northeastern Venezuela began to increase in the 1980s when research started on the effect of metals to fish (Lemus et al., 1989; Márquez et al., 2008) and polychaetes (Marcano et al., 1996, 1997), and on levels of metals in bivalves (Jaffé et al., 1998; Lista et al., 2006). Evaluation of metals in bivalves was initiated in order to evaluate the human health safety of these organisms due to the large consumption by the population in general.

Bivalves have been the most widely studied marine invertebrates in the area of biomonitoring of heavy metals and ecotoxicology. They are ideal organisms in studies of toxicity and biomonitoring (Phillips, 1979; Phillips and Rainbow, 1993; Rainbow, 2002), given that they: (a) accumulate high levels of pollutants; (b) are sedentary or able to make short migrations; (c) can present large abundances; (d) have wide distribution (cosmopolitan); (e) are long-lived; (f) are easy to sample; (g) are easy to transport, handle and keep under conditions of confinement; (h) have a good relationship dose-response; (i) are available throughout the year; (j) have simple feeding habits, usually by filtration; (k) support a wide range of climatic and environmental conditions; (l) are taxonomically well described; and (m) are well documented regarding species life history and biology. Other important conditions, also identified by researchers, are: (a) the patterns of accumulation in the target organism should also be reflected in some other species in the study area; (b) the studied organism should preferably be of economic importance; (c) collection or purchase of organisms must be low cost; and (d) samples should be transportable internationally without legal impediments (Rainbow, 1995; Zhou et al., 2008; Páez-Osuna and Osuna-Martínez, 2011).

The eastern region of Venezuela is characterized for having the greatest landings of fishery products of the country, and studies in the field of ecotoxicology, particularly with marine bivalves, have been performed to evaluate their safety for human consumption and to assess their physiological responses to metal incorporation. The bivalve species most widely used for these studies have been *Perna viridis* (Linneaus 1758), *Arca zebra* (Swainson, 1833), *Lima scabra* (Born, 1778), *Anadara floridana* (Conrad, 1869), *Donax denticulatus* (Linnaeus, 1758) and *Atrina seminude* (Lamark, 1869).

A. zebra is the most commercialized bivalve species in Venezuela, due to its large natural banks and landings; in second place is the mussel *P. perna*, which is being displaced by *P. viridis*, an invasive species to the Venezuelan coasts with origin in the Indopacific Ocean. Other species of commercial importance, but with much lower natural density, are *A. floridana*, *A. seminuda* and *Tivela mactroides* (Born, 1778). On the other hand, *L. scabra* is a representative of coralline environments and *D. denticulatus* of intertidal sandy beaches. Ecotoxicological studies have been conducted on all of these species under controlled conditions as well as in their natural environments.

This chapter presents results from studies on MTs in bivalve molluscs of the eastern region of Venezuela. The use of bivalves in the presence of metals in laboratory bioassays and in natural environments is evaluated and finally two cases are shown on the seasonal variation of MTs in *P. viridis* and *A. zebra*.

ASSAYS UNDER LABORATORY CONDITIONS

Toxicological assays under controlled conditions have allowed determining the relationship between an inductor (heavy metal) and a specific response from the organism. The responses can range from alterations in the behaviour, to physiological or biochemical responses. Most bioassays have established a relationship between the biomarker and the inductor agent (Ma et al., 2008; Li et al., 2015).

Although laboratory bioassays are widely used today, they have little predictive power in natural environments, due to their low level of environmental realism (Farris and Van Hassel, 2006). In ecotoxicological studies conducted under controlled conditions with organisms from the Caribbean Sea, specifically from the eastern region of Venezuela, *P. viridis* has been the most widely used (Nusetti et al., 2010; Lemus et al., 2012a; Zapata-Vívenes et al., 2012, 2014; Lemus et al., 2014a,b). However, *L. scabra* (Lemus et al., 2012b), *D. denticulats* (Antón et al., 2008), *Pinctada imbricata* (Nusetti et al., 2004) and *T. mactroides* (Acosta and Lodeiros, 2009) have also been considered.

The green mussel, *P. viridis,* is the mytillid most widely studied as biomonitor, after *Mytilus edulis,* attributed to its wide distribution and ease in colonizing environments. *P. viridis* is a native species to the Indo-Pacific, which was detected for the first time in Trinidad in 1990 and has spread to the eastern region of Venezuela, being detected in the Gulf of Paria and Northern Sucre state, in Margarita Island and the Gulf of Cariaco (Rylander et al., 1996). Due to its tropical character and its high fertility, this species has easily established along the coasts of Venezuela and much of the Caribbean Sea.

Studies carried out with *P. viridis* under confinement conditions at different stages of development show that the species has a great capacity for acclimatization to long periods of time. Thus, it is easily manipulated for laboratory tests and shows physiological and biochemical responses, such as growth and respiration rates, oxidative stress, glutathione, lipid peroxidation, and MTs, in the presence of heavy metals (Narváez et al., 2005; Lemus et al., 2012a; Acosta et al., 2013; Lemus et al., 2014a; Zapata-Vívenes et al., 2014).

The modulation of MT expression is determined by a great variety of external factors. One of them is the pre-exposure of the organism to toxic agents, particularly to heavy metals (Lemus et al., 2014a). In the case of juveniles of *P. viridis* exposed to sublethal doses of Cd and Cu (copper)/Cd under controlled conditions, MT levels increased with exposure time to Cd, originating a dose-response behavior in the presence of the metal (Figure 1).

The induction of MTs in organisms pre-exposed to sublethal doses of Cu can also be modulated. In these organisms, the incorporation of Cd shows no relationship with the expression of MTs, suggesting that the expression of the MTs by pre-exposure to Cu raises the ability of the organisms to increase the concentration of this protein in the presence of Cd (Figure 2). This response, under extremely controlled conditions of pH, temperature, salinity, feeding and photoperiod, does not occur in natural environments. It has thus been proposed that studies conducted under natural environmental conditions require more complex analyses, involving factors and variables that may be affecting the biomarker. Many of the variations shown by MTs in natural environments have been interpreted using multivariate models (Lemus et al., 2014a).

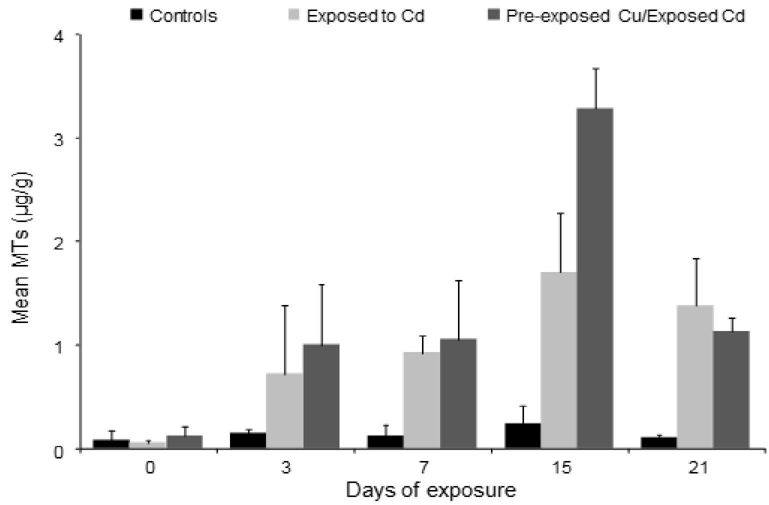

Figure 1. Mean metallothionein (MTs) levels in *Perna viridis* exposed to Cd and pre-exposed to Cu and later to Cd (Lemus et al., 2014a). Vertical lines are one standard deviation.

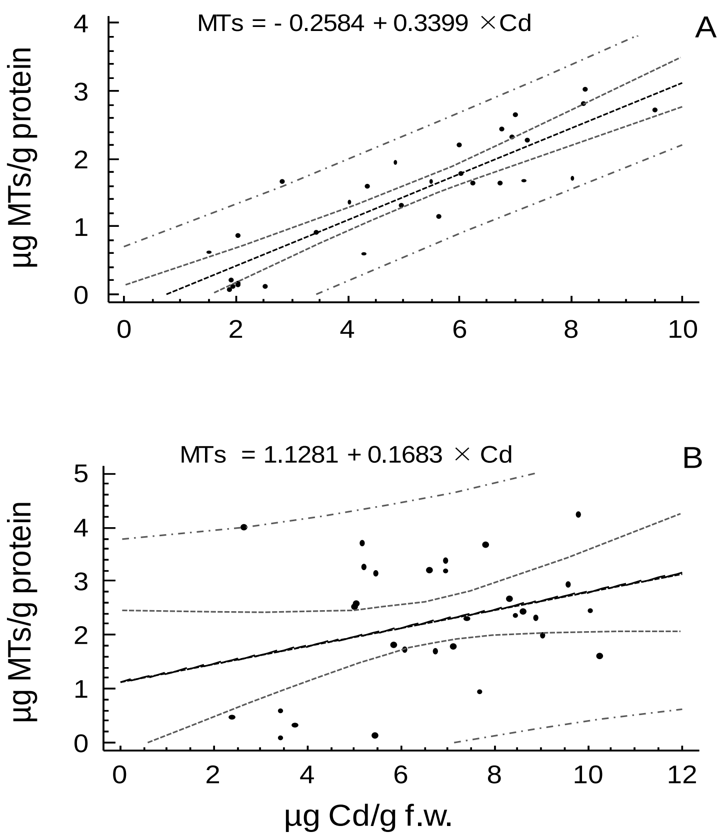

Figure 2. Linear relationship between the content of metallothioneins (MTs) and levels of Cd in *Perna viridis* during 21 days of exposure. A. Exposed to Cd (r=0.88; Fs=102.73, p<0.001); B. Preexposed to Cu and exposed to Cd (r=0.323; Fs=0.323, p=0.0815). Dotted line: confidence interval 95%; dashed line: prediction interval 95%. After Lemus et al., 2014a.

Furthermore, it has been suggested that the levels of MTs could mediate the resistance of bivalves to anoxic conditions, since it has been shown that the antioxidant system of the digestive gland of *P. viridis* is not affected by the acute exposure to Cd in the short term; this is possibly associated to the high concentration of MTs recorded, which in turn could explain the resistance to anoxic conditions of the organisms (Zapata-Vívenes et al., 2014).

D. denticulatus is a representative of the benthos from high energy sandy beaches and has been used with great success under controlled conditions in toxicological assays, showing good adaptation to confined conditions (maintains similar growth rates to that of wild individuals). Individuals exposed to Cd show a significant bioaccumulation of this metal, depending on the length of exposure, and a significant decrease in growth (RNA/DNA ratio). This is possibly related to the energy expenditure involved in the processes of incorporation, metabolization and excretion of the metal, compromising the energy for growth (Antón et al., 2008).

L. scabra, commonly called fire clam, is a bivalve which inhabits coralline environments in very clear waters. It is widely distributed throughout the Caribbean Sea, from the coast of North Carolina in the United States to the north-eastern coast of Brazil, in a depth range between 1 and 40 m (Lodeiros and Himmelman, 1999). The species is a good representative to assess disruption of this type of environments, although its maintenance under confined conditions is more limited than other species evaluated. Under confined conditions and exposed to Cd, MT expression is induced after 21 days of exposure.

METALLOTHIONEINS IN NATURAL ENVIRONMENTS

More recent studies have characterized MTs as a family of multifunctional proteins related to many routes of intermediary metabolism, given the implications in the homeostasis of essential metals (Zapata-Vívenes and Nusetti, 2007; Takahashi, 2012). This is achieved by controlling the levels of free metals that are available for the proteins associated to cellular processes: they act as a mechanism of cell protection against toxic metals by binding them through mercapto links and preventing their linking to other molecules, and they protect against oxidative stress caused by toxic metals (Lemus et al., 2016).

Studies on the function of MTs in bivalves have not advanced at the same speed as those in vertebrates. By January 2015, more than 9800 nucleotide sequences had been recorded in the database of nucleotides of the NCBI as MTs, only 155 of which have been identified in 38 species of bivalves (Lemus et al., 2016). Metallothioneins have shown a large number of isoforms in these invertebrates, with very different functions that can be induced or modulated by temperature, anoxia, stage of development and essential or non-essential metals (Lemus et al., 2016). Researchers point out that since the lifecycles of these sessile, or with limited mobility, organisms, are directly associated with the physical, chemical and biological variables of the ecosystems where they develop, their metabolism is subject to multiple variations and adaptive mechanisms.

Of the species of bivalves studied in eastern Venezuela, only for *P. viridis* are the MT primary structure and isoforms known. Leung et al. (2014) characterized two types of MT isoforms in the digestive gland of the green-lipped mussel *P. viridis*, using Cd and hydrogen peroxide as inducers of MTs. The two isoforms differed in their deduced protein sequences, with 73 amino acids for MT10-I and 72 for MT10-II (a novel type), but both contained a high percentage (27.4 to 29.2%) of cysteine. The MT proteins were present in multiple isoform spots. The MT10-I responded promptly to Cd but had a lagged induction to H_2O_2 treatment, while tm10-II was exclusively induced by the Cd treatment.

Studies so far shows that the assessment of concentrations of MTs as biomarkers of exposure to metals is a field that still needs much development, because the isoforms have different inductors (Isani et al., 2000; Lemoine et al., 2000; Ivanina et al., 2015) and this mechanism is possibly fundamental to maintain the homeostatic functions of the organism and to respond to the presence of metals or other types of stressors (Lemus et al., 2016). Further knowledge of MTs isoforms is thus essential to elucidate the function of MTs in bivalves.

The presence of isoforms in bivalves could explain the reason why many studies evaluating MTs in this group of organisms in their natural environments are so controversial. Some of them demonstrate associations with their life cycle, while others identify the presence of metals, or both aspects, as determinants of the expression of MTs.

Perna viridis

Analyses of MTs in *P. viridis* from eastern Venezuela show seasonal variations, with higher concentrations between February and March and lower ones between September and December (Figure 3), coinciding with the periods of high and low phytoplankton productivity in the area, respectively. On the other hand, immature individuals have higher concentrations of MTs in comparison to mature and spawned ones (Figure 4), which leads to a negative relationship between MTs and Condition Index. Results show that MTs of *P. viridis* are influenced by physical and chemical factors of the marine environment as well as by the physiological and reproductive condition of individuals (Lemus et al., 2013).

Studies performed so far show that there is bioaccumulation of metals associated to the reproductive annual cycles, and that this fact is a response to annual variation of environmental factors in the coastal marine ecosystems. The latter, in turn, determines metabolic variations and modulates the reproductive mechanisms of bivalves (Martínez-Castro and Vázquez, 2012).

Arca zebra

The Turkey wing, *A. zebra*, is the largest bivalve in northeastern Venezuela. It represents the second category in terms of volume of fishing landings for the entire artisanal fishery of Sucre state, after the sardine, being the fundamental support of several fishing communities that rely almost exclusively on this activity (Jiménez, 1999; Prieto et al., 2001). This species, which belongs to the Arcidae family, is distributed from the coast of the Gulf of Mexico and South Florida to the North of Brazil, but is in Venezuela where it forms banks of commercial importance (Prieto et al., 2001). In Venezuela this bivalve has been successfully used as a biomonitor of heavy metals (Acagua, 2008; Lanza et al., 2011).

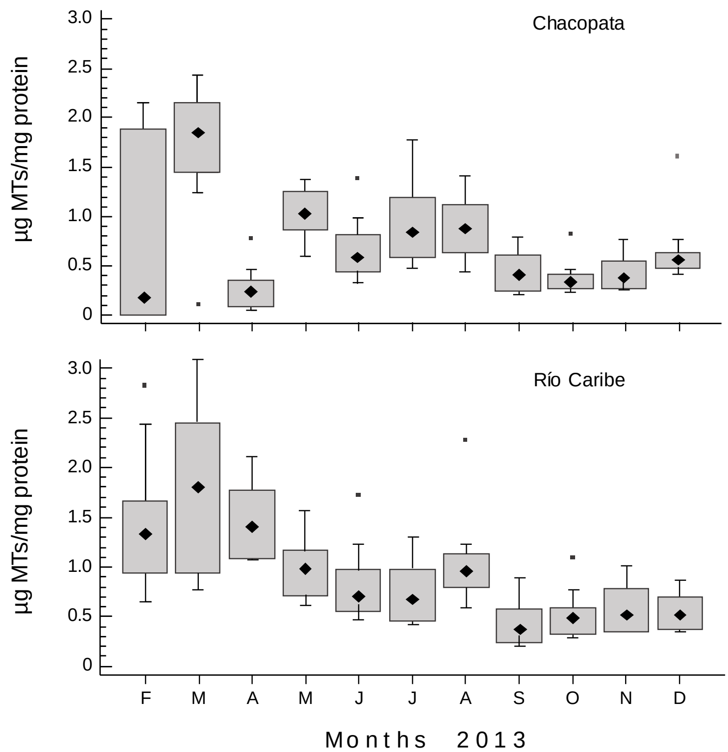

Figure 3. Monthly variation of metallothioneins (MTs) in *Perna viridis* collected in 2013 from two coastal locations of Sucre state, Venezuela: Chacopata and Rio Caribe (Lemus et al., 2013). Diamonds denote medians, boxes enclose the 25th and 75th percentiles, and error bars represent the 10th and 90th percentiles.

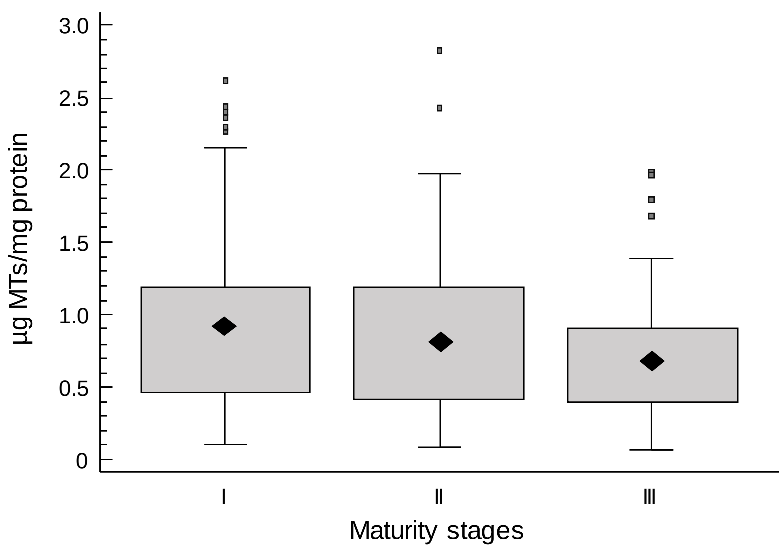

Figure 4. Metallothioneins (MTs) at different developmental stages of the gonad of *Perna viridis*, from the coastal zone of Sucre state, Venezuela (Lemus et al., 2013). Diamonds denote medians, boxes enclose the 25th and 75th percentiles, and error bars represent the 10th and 90th percentiles.

he concentrations of MTs in *A. zebra* also showed seasonal variations associated to the Condition Index of the organisms, registering the highest values during August, a period in which the Orinoco River increases the discharge into the coastal zone. In this species, there was not a significant correlation between the concentration of toxic metals (Pb and Cd) and the level of MTs, but it was significant with the levels of Cu (personal communication).

These results show that the levels of MTs in bivalves of eastern Venezuela are affected by environmental and physiologic factors that should be taken into account when determining the sources inducing MTs in the organisms. In this regard, the variations of physical and chemical parameters as well as the life cycle stages of the bivalves should be evaluated in order to determine the intervals of annual fluctuation of this protein. That is why knowledge about the physiological variations of MTs during the life cycle of bivalves is essential to consider MTs as a biomarker of metal contamination.

Atrina seminuda

The pen shell, *A. seminuda* (Lamarck, 1819), is an endobenthic bivalve that usually uses a strong byssus secreted by the animal to adhere to rocks and gravel substrate in areas of high energy. It can be found along the Atlantic coast of North America, from North Carolina to Texas and the Caribbean Sea, down to the shores of Argentina (Lodeiros and Himmelman, 1999; Macsotay and Campos, 2001). Within the Venezuelan Caribbean Sea region, it has been found on the coasts of the states of Vargas, Miranda, Anzoátegui, Sucre and Nueva Esparta (Ramos and Robaina, 1994). This bivalve shows reproductive activity during most of the year with several intense reproductive periods (June-July and September-November and February) as well as a year-round gametogenesis, a behavior of asynchronous type (Freites et al., 2010).

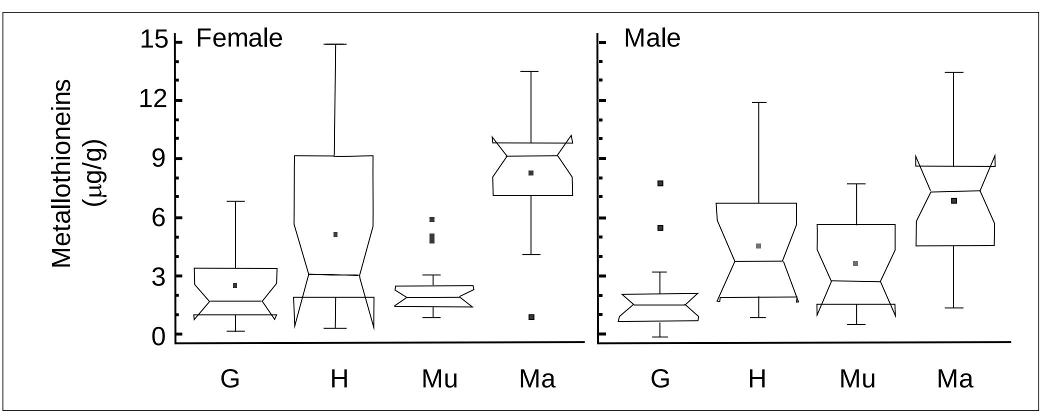

Figure 5. Levels of metallothioneins (MTs) in somatic tissues of *Atrina seminuda*. G: gonads; H: hepatopancreas; Mu: muscle; Ma: mantle.

In *A. seminuda* (sampled along the north eastern coast of Venezuela), the levels of MTs tend to be higher in the parenchymatous tissue, particularly in hepatopancreas. However, this species also presented a high concentration of MTs in the mantle (Figure 5). This is possibly associated with the highest levels of Cd that were detected in this tissue. It is known that Cd has a greater affinity for mercapto groups of the MTs than Zn, and thus has a great ability to displace it. At the same time, this free Zn is a strong inducer of the synthesis of MTs and could thus explain the high levels of MTs in the mantle. On the other hand, it has been shown that in many bivalves Cd can be removed via synthesis of the shell that takes place in the mantle.

Nevertheless, when MTs are evaluated in the whole organism a significant relationship is found between the level of MTs and the gonadosomatic index (Figure 6).

Anadara floridana

Comparing the levels of MTs obtained in *A. floridana* with those determined in other bivalve species, hepatopancreas and gills are usually seen to carry higher levels than other tissues (Amiard et al., 2006; Barrera-Escorcia and Wong-Chang, 2010). However, in *A. floridana*, the highest concentrations of MTs were found in muscle, which suggests that this tissue has a greater synthesis capacity of this protein, and that it is probably involved in specific processes of this species to regulate bioessential metals or the metabolization and clearance of toxic metals.

This species of bivalve has the distinction of presenting a very large muscle with great pigmentation and also with extreme force to close the shells, unlike many bivalves. Even though all studies performed on toxicity of metals in bivalves have considered the hepatopancreas as the target organ and have used this tissue to evaluate enzymes, biomarkers and physiological and biochemical responses, it is possible that there are species-specific responses which still have not been discussed.

Donax denticulatus

This species is distributed in the West Central Atlantic Ocean from the Caribbean Sea to Brazil, inhabiting the intertidal zone in the sweep of the swell area of many sandy beaches with high energy. Populations of this bivalve in the Caribbean Sea vary annually in density, size and color of the shell. The factors that influence this variation are mainly the size and arrangement of the sand grains, the slope of the beach, the degree of exposure to the waves and the organic content of the sand.

Analyzing the levels of MTs in males and females of *D. denticulatus* during different times of the year, a significant increase of the concentration of MTs was found during the month of January (Figure 7), when phytoplankton levels are the highest in the eastern region of Venezuela (Velásquez-Martínez and Lemus, 2011). This pattern shows once again that the MTs are modulated by the availability of food.

In the bivalve species studied in eastern Venezuela, it has been determined that there are seasonal variations in the incorporation of Cd, Cu, Hg, Zn and Pb, which are mainly associated to the variation of the upwelling periods that characterize the zone. These variations are regulated by the upwelling promoted by trade winds and by the influence of the Orinoco River discharge, which is associated to the precipitation regime. Both processes expand or contract in an alternating way under an annual cycle, dominated by upwelling during the first months of the year until June, while the river influence prevails approximately from June to December and generally carries a heavy load of suspended matter (Varela et al., 2003). These two aspects modulate both the incorporation of metals of natural and anthropic origin. In particular, the Orinoco River discharges considerable amounts of metals to the coastal zone, especially Hg and Cd, as waste or sub products of the mining and metallurgical industries established along this river.

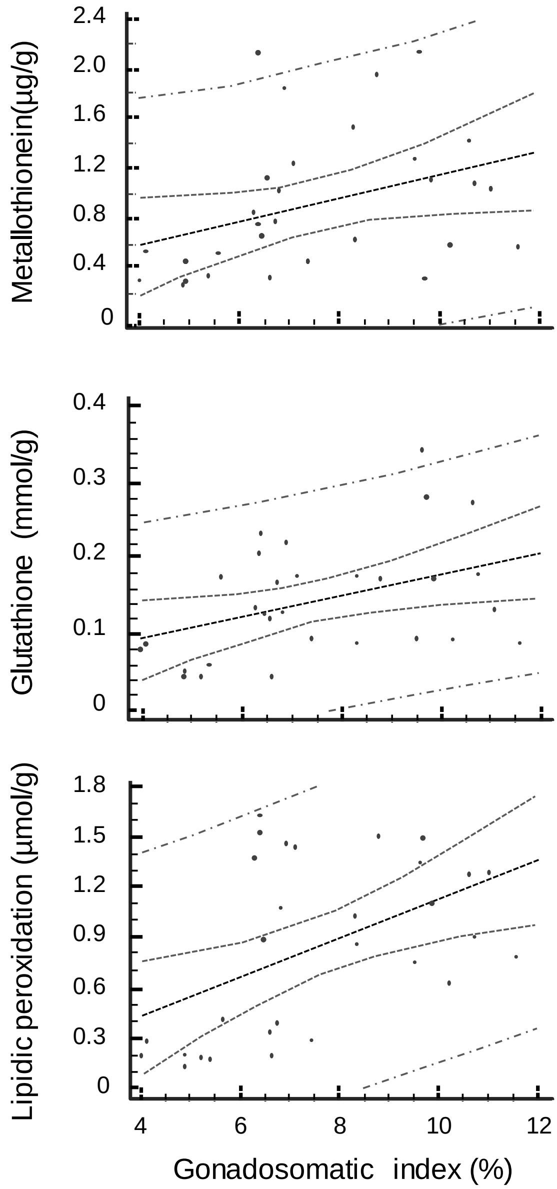

Figure 6. Relationship between levels of metallothionein (MT), glutathione or lipidic peroxidation, and gonadosomatic index, in *Atrina seminude*. Solid line: regression line; Dotted line: confidence interval 95%; Dashed line: prediction interval 95%.

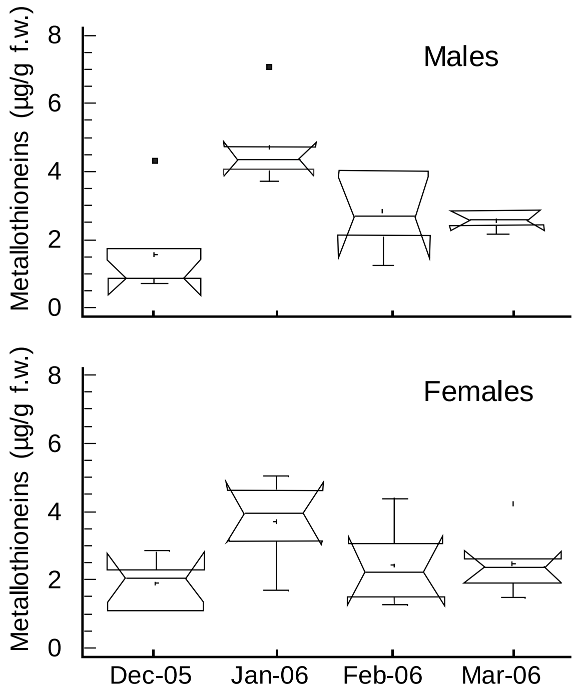

Figure 7. Concentration of metallothioneins (MTs) in *Donax denticulatus* collected over four months (from December 2005 to March 2006) at Playa Brava, Araya Peninsula, Sucre state, Venezuela. f.w., fresh weight (Velásquez-Martínez and Lemus, 2011).

CONCLUSION

MTs participate in several metabolic routes and there is limited knowledge about them in marine organisms, particularly in invertebrates. It is known that environmental factors and the stage of development of the organisms are determinants in the modulation of this protein. The previous life history of the organism also introduces a modulation to the expression of these molecules. In the case of tropical bivalves from eastern Venezuela, MTs are modulated by the reproductive cycles, which in turn are affected by physical, chemical and biological factors. Studies should be geared towards knowledge of isoforms in the different bivalves and the role played by each of them. It is essential to establish methodologies of easy reproducibility to evaluate isoforms routinely.

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Chapter 10

Ecotoxicological Studies of Freshwater Ecosystems in Latin America: Diagnosis, Perspectives, and Proposals

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Abstract

Latin America and the Caribbean are the regions with the greatest biological diversity on earth, and endemism in these regions is very high. Regarding freshwater ecosystems and water availability, Latin America and the Caribbean hold more than 30% of earth's available freshwater resources, but this water is very unequally distributed and at risk. Most ecotoxicological studies have been performed in countries and ecosystems in temperate zones, neglecting the tropics. In tropical environments most research has focused on water quality and aquatic toxicology, with regulations varying from country to country. Therefore, the present chapter aims at presenting the current status of ecotoxicological studies regarding the major risks to which Latin American freshwater resources are subjected, which can be included in six categories: 1) municipal discharges; 2) mining activities; 3) industrial/agricultural contamination; 4) depletion and

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contamination of aquifers/glaciers; 5) petroleum industry contamination; and 6) cyanobacteria toxins. In addition, information related to these threats is presented as evidence of the adverse effects caused to freshwater ecosystems: a) loss of biodiversity; b) eutrophication/bacterial-parasite contamination; c) bioaccumulation, bioconcentration and biomagnification; d) aquifer contamination and depletion; e) freshwater salinization. Despite all efforts of the scientific community, information is lacking regarding fate and effects of contaminants on freshwaters in Latin America. Thus, it is still necessary to coordinate the participation of several associations (national and/or international) and authorities at different hierarchical levels to achieve regulatory consensus regarding pollutants and their effects on Latin American freshwater ecosystems.

Keywords: eutrophication, pesticides, metal toxicity, aquatic toxicology, environmental toxicology

INTRODUCTION

Latin America and the Caribbean are the regions with the greatest biological diversity on earth, holding almost 50% of the world's tropical forests, 33% of mammals, 35% of reptiles, 41% of birds and 50% of amphibian species, and endemism in these regions is very high (UNEP, 2010). Regarding freshwater ecosystems and water availability, Latin America and the Caribbean hold more than 30% of earth's available freshwater resources (UNEP, 2010), but this water is very unequally distributed and in risk due to: a) increasing pollution, b) degradation of watersheds, and c) the depletion and unsustainable use of aquifers, which are all mainly due to anthropogenic activities. Ecotoxicology has focused on countries and ecosystems in temperate zones, with little research carried out in tropical environments. Techniques and procedures developed for temperate environments are often applied in tropical regions, even though physical and chemical environmental parameters in the tropics can be very different. The regulatory environment also varies among countries (Lacher and Goldstein, 1997). With this background, in the present review we focused on one main question: What are the major risks to which Latin American freshwater resources are subjected? The answer presented in this chapter is enclosed in six categories: 1) municipal discharges causing eutrophication; 2) mining activities; 3) industrial/ agricultural contamination; 4) depletion/contamination of aquifers/glaciers; 5) petrochemical contamination; and 6) cyanobacteria and cyanobacterial toxins.

MUNICIPAL DISCHARGES CAUSING EUTROPHICATION

Still by large the most common threat to freshwater ecosystems in Latin America is domestic and agricultural discharges that cause eutrophication. In Latin America there are many illegal and incidental discharges of unknown contaminants. Latin America has 578 million inhabitants (8% of the World population), and provides water supply for 93% of its population but sanitation for only 79% of its population (120 million lack sanitation) (Noyola, 2013). Wastewater treatment only covers around 20% of total water (Noyola, 2013). Many countries in Central and South America are highly dependent on transboundary waters because the origin of most of their water resources is not within their territory (IDB, 1998).

Heads of Environmental Protection Agencies of Latin American countries have expressed their commitment, through the use of existing transboundary agreements, to the sustainable use of water resources; there are many potential sources of conflict, but also opportunities for cooperation (Garcia, 1999). One of these cooperations is the case of the risk assessment of the Paraguay River Basin (The Nature Conservancy, WWF-Brazil, 2012) which included four countries (Argentina, Bolivia, Brazil and Paraguay); the risk assessment report concluded that the central portion of the basin, i.e., the Pantanal and the Dry Chaco, displayed low ecological risk, but that the floodplain presented a proportionately high risk.

MINING ACTIVITIES

Rising prices and expanding global demand have detonated an explosive growth in gold mining. Latin American and the Caribbean, mainly in Venezuela, Guyana, Suriname, French Guiana, Colombia, and Brazil, have experienced some of the highest growth rates in production since the year 2000 (Hammond et al., 2013). Mining is a legacy of our Spanish/Portuguese/English/French/Dutch ancestors in their quest for gold, silver and other precious metals. The largest mining industry globally is Canadian, with the majority of its investment in Latin America and the Caribbean: this industry increased up to 35% since 1990 to 2014 (Gordon and Webber, 2008). The gold rush in South America was triggered in 1980 with the discovery of a large gold mine in Sierra Pelada, Amazon Region; from there the fever spread to Venezuela, Guyana, French Guiana and Suriname (Mol et al., 2001). In the 1990s and 2000s, the largest gold exploration in the world was in Latin America, where 12 of the 25 largest mining investments took place in Peru, Chile and Argentina (Urkidi, 2010). From the 1980´s, Brazil was the largest producer of gold in South America followed by Colombia, Venezuela, Peru and Bolivia (Malm, 1998; Nevado et al., 2010).

Mining liquid waste or suspension tailings and deposits of discarded rock and leaks are a source of metals. When these deposits are in contact with sulfur and oxygen, they result in Acid Mine Drainage (AMD), with high levels of dissolved metals (for example, pyrite (FeS_2) associated to coal is exposed to air, and is oxidized to H_2SO_4 by bacterial action producing AMD). When these leached or filtered particles reach rivers, a wide dispersion of the metals in solution, and as particles occurs (Salomons, 1995). Most of the new gold mines are open pits and use cyanide that is released in the melting. Among the major impacts are the high consumption of water and the acidification of the water drained into the tailings (Urkidi, 2010). However, the ionic form of a metal is usually the most toxic, and when associated with suspended matter or dissolved ligands its toxicity decreases (Salomons, 1995).

Mercury

In Mexico and South America, mercury has been widely used since colonization for recovery of gold and silver, where it is directly released to aquatic systems as mining disposal (Malm et al., 1995a; Malm, 1998). Brazil is the first producer of gold in South America, of which 90% of mining sites are small-scale or “garimpos” (Nevado et al., 2010). The largest “garimpo” is located in the basin of the River Tapajós (Brazil) and releases a large amount of mercury into the Amazon River (Malm et al., 1995a; Nevado et al., 2010). The second largest gold mine in South America is the Pascua-Lama project at the borders of Chile and Argentina and the Andes mountains, which began in 2009 (Urkidi, 2010). Mercury pollution is recognized as one of the main environmental problems of tropical South America (Mol et al., 2001).

Mercury is used for the separation of fine particles of gold through the amalgamation in an open pit at high temperatures (350-600 °C). The resulting waste is then sent to tailing dams creating concentrated mercury. This process is repeated to improve the extraction (Salomons, 1995). In addition, the use of cyanide for gold recovery results in the formation of highly soluble mercury cyanide complexes, contributing to their mobilization (Nevado et al., 2010). Metallic mercury is volatile and when oxidized and methylated it becomes its more toxic organic form, methyl mercury, which is biomagnified in the food web eventually reaching human beings (Malm et al., 1995a; Nevado et al., 2010). High mercury concentrations in air from urban, rural or occupational exposures such as burning amalgam reflect the volume of discharge to the atmosphere due to gold mining activities. In addition, the Amazonian aquatic ecosystems environmental conditions favor high rates of mercury methylation (Salomons, 1995; Malm, 1998). Although Malm et al. (1995a) reported that Hg found in sediments in the Madeira River of Brazil have values below the global average (0.3 µg/g), high levels of methyl mercury have been found in carnivorous and piscivorous fishes of the Tapajós river (Akagi et al., 1995; Malm et al., 1995a,b), and in the Madeira river basin, showing levels of 0.7 µg/g which are higher than the 0.5 µg/g maximum established by the Brazilian legislation (Malm et al., 1995a,b). High levels of mercury have been found even in human hair along the Tapajós and Madeira rivers, attributed to the consumption of fish (Akagi et al., 1995). Some authors argue that the Hg is transported over long distances in the inorganic form, associated with suspended particles or organic matter. They have therefore suggested to avoid the ingestion of carnivorous fish such as piranha (*Serrasalmus* spp.), peacock bass (*Cichla ocelaris*), piraíba (*Brachiplatystoma filamentosum*), gilded catfish (*B. flavicans*), apapá (*Pellona castelnaeana*), spotted surubim (*Pseudoplatystoma fasciatum*), and tiger catfish (*Pseudoplatystoma* sp.) (Malm et al., 1995a).

The safety limits for ingestion of mercury are based on the concentration of this metal in muscle of consumed products (0.5 µg Hg/g, wet weight) (Nevado et al., 2010). In the Brokopondo reservoir, Suriname, there have been reported exceeding concentrations of mercury within several fish species, in which the highest concentrations (3.13 and 4.26 µg/g) were found in the piranha *S. rhombeus* (Mol et al., 2001). Neurotoxic effects have been observed in adults with levels of mercury in hair below 50 µg/g, due to chronic exposure of communities living close to the Amazon River (Passos and Mergler, 2008; Nevado et al., 2010). In the Tapajós River basin gold mining is the principal economic activity since the 70´s, representing a serious risk to environment and human health (Nevado et al., 2010). Malm et al. (1995b) suggest forbidding of the use of mercury, followed by a better understanding of the mining practice and development of alternative clean techniques.

Arsenic

Arsenic is released to aquatic systems, soil and atmosphere by the mining industry and metallurgy. Its residues can be dispersed by wind or water after its provision, and may be associated with AMD (Razo et al., 2004). Gold and copper mining in Brazil have contributed to increasing levels of arsenic for hundreds of years. High levels of arsenic in drinking water sources in many countries in Latin America such as Argentina, Bolivia, Brazil, Chile, Colombia, Cuba, Ecuador, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Peru, and Uruguay have been detected (McClintock et al., 2012). Bundschuh et al. (2012) mentioned that the main sources of arsenic in Latin America including for drinking water are geogenic and anthropogenic, such as mining. In addition, Salomons (1995) mentions that the erosion of active and inactive mining areas or direct discharge of tailings or waste rock introduce metals to the aquatic environment; even in pristine rivers more metals in the inert fraction have been reported than in polluted rivers.

Razo et al. (2004) reported the presence of arsenic (265 μg/L) in rainwater storage dams 105 km from the mining site of Villa de la Paz-Matehuala, S.L.P., Mexico. In Zimapan (Hidalgo, Mexico), the concentration of arsenic in groundwater is greater than Mexican drinking water standards, due to the contribution of tailings which can potentially lixiviate into not so deep aquifers (Ortega-Larrocea et al., 2010). The Pilcomayo basin (Argentina, Bolivia and Paraguay) presents an average of 50 μg/L of arsenic, while the secondary channel used for irrigation has a concentration of 200 μg/L. In addition, the arsenic concentration in fish is related to its concentration in water and is attributed to the mining activities of Sn, Ag, Zn, and Pb (Bundschuh et al., 2012).

Other Metals and Radionuclides

The Chilean economy depends heavily on the export of Cu, constituting 55.7% of national exports in 2007. In addition, Chile exported 47.5% of the total amount of Cu sold worldwide (Urkidi, 2010). The mining of Ni and other mining activities in the Bay of Levisa (Cuba) has generated high concentrations of metals in sediments such as Ni, Fe, Co and Mn (Gonzalez and Ramírez, 1995). The mining and milling of ores to produce uranium yields large amounts of waste, including AMD, waste rock, tailings, and radionuclides, transported by air, as the ^{222}Rn and its derivatives of short life. Acid mining and drainage waste rock are the main sources of ^{226}Ra, ^{238}U, Al and Fe (Fernandes et al., 1995).

INDUSTRIAL/AGRICULTURAL CONTAMINATION

The contamination of aquatic ecosystems by industry and agriculture in Latin America is complex and difficult to eradicate. Many categories of waste are produced: nitrogen, pesticides, solvents, hydrocarbons, personal care products, analgesics, antibiotics, hormones, chemicals and agro-industrial waste. Unfortunately all these compounds are difficult to remove in wastewater treatment plants (Hillstrom and Hillstrom, 2004). Köhler and Triebskorn (2010) stated that Latin American ecosystems are contaminated with 120 different pesticides with half a million tons applied without control, and that these pesticides produce critical adverse effects on aquatic biota.

Latin America generates 40% of the total world production of soybean in a total of 15,000 km^2 of land which was burned and cleared for crops. This has caused increasing levels of nitrogen in the atmosphere together with the pesticides that were used. It is estimated that levels of nitrogen in the water will duplicate by 2050 (Austin et al., 2013). The implications of contamination by nitrogen in aquatic ecosystems are catastrophic, causing: 1) acidification of rivers and lakes with low or reduced alkalinity; 2) eutrophication of freshwater and marine ecosystems (with the additional problem of algal toxicity); and 3) direct toxicity of nitrogenous compounds to aquatic organisms (Camargo and Alonso, 2007). The main products produced in Latin America and the pesticides that are used or associated are presented in Table 1.

Countries such as Argentina, Bolivia, Brazil, Paraguay and Uruguay use large amounts of herbicides like glyphosate because they use genetically modified soybean with resistance to glyphosate (Fishbein, 2012). In Mexico, Colombia, Ecuador, and Argentina, especially in flower-producing regions, several pesticides are used, such as: DDT, endosulfan, carbofuran, methomyl, dimethoate, aldrin, endrin, dieldrin, isodrin, and heptachlor epoxide (Benitez-Diaz and Miranda-Contreras, 2013). In countries such as Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua and Panama, the most used pesticides are: paraquat, mancozeb, terbufos, methamidophos, methyl, carbofuran, methyl bromide parathion, CCA, and aldicarb (Wesseling et al., 2003). As a result, pollution from pesticides in Latin America is evident and the probability increases as the demand for agricultural products increases.

In Mexico it is estimated that 20,000 tons per year of pesticides are used, and in the 2000's the Mexican states where the most pesticides were used were: Sinaloa, Veracruz, Jalisco, Nayarit, Sonora, Baja California, Michoacán, Colima, Tabasco, State of Mexico, Puebla, and Oaxaca (González-Arias et al., 2012).

Finally, several ecotoxicological studies in Latin America indicate the occurrence of high concentrations of pesticides in heavily populated and industrialized areas (Table 1).

Table 1. Ecotoxicology studies conducted with organic compounds in water (w), sediments (s) and organisms (o) in different countries of Latin America. The values are in mg/L for water and in µg/g for sediments and organisms. Abbreviations: mxv: maximum values, avv: average values, dw: dry weight, ww: wet weight

Country	Site	Concentration	Reference
Bolivia	a) Chanériver b) Del Palo river c) Grande river d) Piraí river e) Well	w: fluroxypyrmethylhepthyl ester a) 9.11, b) 0.025, c) 0.57, d) 0.26, e) 0.032. mxv	Vargas et al. (2005)
Brazil	Santa Catarina Rio Grande do Sul Paranagua Estuarine System	w: Azoxystrobin 0.65 µg/L s: Benzo(*a*)pyrene 1.58. mxv, dw s: PCB 0.0066. mxv	Stolberg et al. (2015) García et al. (2010) Combi et al. (2013)
Costa Rica	Grande de Tárcoles river	o: caudal scutes of American crocodiles, p,p´-DDE 0.34, p,p´-DDT 254.8, Dieldrin 0.008, Endrin 0.22, Methoxychlor 0.53. avv	Rainwater et al. (2007)
Mexico	Champotón river	s: Lindane 70.12. mxv	González et al. (2014)

Additional problems regarding agriculture contamination to aquatic ecosystems are the intensive use of fertilizers on crops. Fertilizers are applied to remediate the deficiencies of primary and secondary nutrients and less frequently of micro-nutrients (Fageria et al., 2002). Other contaminants associated with agriculture and recently created by nanotechnology materials such as new pesticides, which include several formulations that can consist of metal nanoparticles, mixtures of metal nanoparticles and pesticides (e.g., imidacloprid or deltamethrin), nanoemulsions (e.g., permethrin, garlic essential oil, or β-cypermethrin, among others), nanodispersion (e.g., novaluron and triclosan), among others, could represent an additional environmental problem to aquatic ecosystems (Kha, 2015).

The industrial problem coming up is the high demand for hydrocarbons and consequently contamination of aquatic ecosystems. The strong demand for oil will come from transport since emerging countries are experiencing an expansion of the automobile industry, which could reach 1,700 million vehicles before 2035 (Gokulakrishnan and Ganeshkumar, 2015). By then, oil demand will have increased to about 100 million barrels per day (Shell International, 2008). The intensification of oil extraction increases the environmental risk, for example: in April 2010 in the Gulf of Mexico 4.9 million barrels of crude oil leaked from the Macondo well, to which one of the first responses was to apply more than 1 million gallons of the oil dispersants Corexit 9500® and Corexit 9521A8®; however, when Corexit 9500® and oil are mixed, the toxicity to *Brachionus manjavacas* increases up to 52-fold (Rico-Martinez et al., 2013).

Finally, contamination by endocrine disruptors, pharmaceuticals, and care products is of concern, and the increase of discharges of these contaminants is complex. For example, tourism is a sector of great importance and dynamism that generates large profits to many Latin American countries. Large hotel settlements on coastal zones or waterfronts cause degradation and pollution of the coastal zone. Additionally, the population pressure and the development of coastal tourism and infrastructure have significantly affected coastal and marine ecosystems. Coral reefs have unfortunately been affected: in the Caribbean, 61% of coral reefs are threatened by the pressure from tourism and pollution (Miguel and Tavares, 2015).

In general, contamination from agriculture and industry in Latin America will be devastating if it continues with this progress. It is necessary to implement efficient and continuous monitoring mechanisms to obtain ecological risk of all of these pollutants in Latin American aquatic ecosystems.

DEPLETION AND CONTAMINATION OF AQUIFERS/GLACIERS

The expansion of mining, industrialization, and other anthropogenic activities has led to metal contamination in many aquatic ecosystems of the Neotropics (Figure 1). Analysis of scutes from Morelet's crocodiles (*Crocodylus moreletii*) from Belize and American crocodiles (*C. acutus*) from Costa Rica revealed the presence of mercury, cadmium, copper, lead, and zinc (Rainwater et al., 2007) (Table 2).

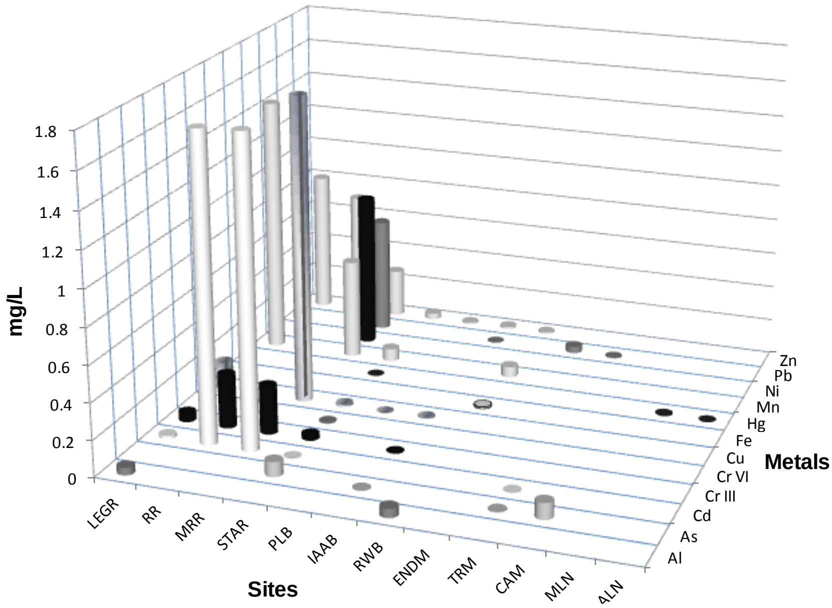

Figure 1. Determination of metals in water of different countries of Latin America. Abreviations: LEGR-Luján, Escobar and Garín Rivers, Argentina; RR-Reconquista River, Argentina; MRR-Matanza-Riachuelo River, Argentina; STAR-System Tres Arroyos River, Argentina; PLB-Patos Lagoon, Brazil; IAAB-Iguaçú-Sarapuí System, Brazil; RWB-Refinery wastewater, Brazil; ENDM-El Niagara Dam, Mexico; TRM-Tula River, Mexico; CAM-Calera aquifer, Mexico; MLN-Managua Lake Nicaragua; ALN-Apoyo Lake, Nicaragua. Sources: Magdaleno et al. (2001), Silva and Santa (2005), McCrary et al. (2006), Salibián (2006), Rubio-Franchini et al. (2008), Peluso et al. (2011), Pinhão et al. (2011), Fonseca and Wallner-Kersanach (2013), Daflon et al. (2015), Rubio-Franchini et al. (2016).

Table 2. Ecotoxicology studies conducted with metals in sediments and organisms in different countries of Latin America. The values are in µg/g for sediments and organisms. Abbreviations: mxv: maximum values, avv: average values, dw: dry weight, ww: wet weight

Country	Site	Concentration		Reference
		Sediments	Organisms	
Argentina	Beagle Channel	Cu 18.48, Fe 6.0, Hg 0.41, Pb 27.65, Zn 62.35. avv, dw		Amin et al. (1996)
	Bahía Blanca		Females of *N. granulate*, Ni 13.61, Pb 18.23, Zn 35.61. Eggs of *Neohelice granulate*, Ni 6.13, Pb 2.47, Zn 54.07. mxv, dw	Simonetti et al. (2012)
Belize	Sapote Lagoon		Morelet's crocodile eggs, Hg 0.23. mxv, ww	Rainwater et al. (2002)
	Hondo river		Caudal scutes of Morelet's crocodile. Cd 0.085, Hg 0.0561. mxv, ww	Buenfil-Rojas et al. (2015)
	Gold Button Lagoon (GBL) and the New River Watershed (NRW)		Caudal scutes of Morelet's crocodile. GBL, Cu 346.0, Hg 98.7 NRW, Cd 70.7, Cu 451.8, Hg 72.7, Pb 109.7. avv, ww	Rainwater et al. (2007)

Country	Site	Concentration		Reference
		Sediments	Organisms	
Brazil	a) Puruzinho lake b) Negro and Tapajós rivers c) Ribeirão das Lajes dam d) Hydropower Plant Reservoir Samuel		plankton, Hg a) 337.0 b) 452.0 and 264.0 c) 100.0 d) 309.0 mxv	Nascimento et al. (2007) Pacheco-Peleja (2002) Palermo (2002) Nascimiento (2006)
	a) Negro River b) Santarém and Tapajós river c) Madeira river		*Hoplias malabaricus*, Hg a) 0.35 b) 0.133 c) 0.38. avv	Belger and Forsberg (2006) Uryu et al. (2001) Boischio and Henshel (2000)
Costa Rica	Río Grande de Tárcoles		Caudal scutes of Morelet's crocodile, Cd 0.337, Cu 0.125, Hg 0.093, Pb 0.49, Zn 4.14. avv, ww	Rainwater et al. (2007)
Colombia	Magdalena river		Fish tissues, Hg 0.41 mxv	Alvarez et al. (2012)
Cuba	Sagua la Grande River		*Clarias gariepinus*, Hg 0.16. mxv, ww	De La Rosa et al. (2009)
	Guacanayabo Gulf		*Crassostrea rhizophorae*, Cu 53,000, Fe 278,000, Pb 130, Zn 219,000 mxv, ww	Díaz Rizo et al. (2010)
Guyana	a) Konashen river and creek sediments b) Iwokrama river sediments	Hg a) 0.30 b) 0.29 mxv, wd		Howard et al. (2011)
Mexico	SE Gulf of California		*Crassostrea corteziensis*, As 11.04 mxv, wd	Bergés-Tiznado et al. (2013)
	a) Chetumal Bay b) Coastal region of Campeche		Bone tissue of Antillean manatees, a) Cd 4.9, Cr 10.7, Cu 4.7, Pb 17.7, Ni 46.5, Zn 129.5, b) Cd 4.6, Cr 11.2, Cu 6.3, Pb 16.2, Ni 11.8, Zn 140.7 mxv, ww	Romero-Calderón et al. (2015)
	El Niagara dam	Pb 20,870. mxv	Zooplankton, Pb 1.22 mxv	Rubio-Franchini et al. (2008)
	Tula River	As 490, Cd 490, Pb 8. mxv	Muscle of *Oreochromis niloticus*, As 0.0149, Cd 0.06, Pb 0.83. mxv	Rubio-Franchini et al. (2016)
Nicaragua	a) Managua lake, b) Apoyo lake		Fish, Hg a) 0.35, b) 0.19. mxv	McCrary et al. (2006)
Uruguay	Montevideo Harbour	Ag 2.3, Cd 1.6, Cr 253, Cu 135, Hg 1.3, Ni 34, Pb 128, Zn 491. mxv		Muniz et al. (2004)
Virgin Islands	River Gut	Cd 2,400, Cu 70,000, Ni 32,000 Zn 870, 000. mxv, dw		Ross and DeLorenzo (1997)

Another way to evaluate water quality is via Toxic Units (TU), frequently used in ecotoxicology. It represents the ratio between the concentration of a component in a mixture and its toxicological acute (TUa; e.g., LC50) or chronic (TUc; e.g., long term NOEC) endpoint. The U.S. Environmental Protection Agency (2004) has recommended a maximal value of 0.3 TUa and 1.0 TUc. Herkovits et al. (1996) performed a chronic and acute toxicity test using *Bufo arenarum* embryos to evaluate the water quality in The Reconquitsa River in Argentina, obtaining: 10 TUa and 20 TUc. Herkovits et al. (2002) evaluated by means of the AMPHITOX test acute and chronic exposure in rivers and streams from the Metropolitan area of Buenos Aires, obtaining a maximal value of 1000 TUc (leach from landfills and solid industrial wastes) and a minimal value of 1.4 TUa (surface water samples).

In Brazil, De Paiva et al. (2014) sampled effluents of a large steel industry, finding a maximum value of 3030 TUa. In Aguascalientes (Mexico) Torres-Guzman et al. (2010) sampled influents and effluents of the most important wastewater treatment plants, obtaining between 0.1 and 8.3 TUa. Santos-Medrano et al. (2007) determined the toxicity levels in the San Pedro River in Aguascalientes which were found ranging from 0.36 to 9.91 TUa.

The role of high mountains as ‘cold condensers’ was hypothesized by Calamari et al. (1991) and confirmed by many authors (Galassi et al., 1997; Blais et al., 1998; McConnell et al., 1998). High mountains may represent condensation sites for Persistent Organic Pollutants (POP). Mountain glaciers may be used as ‘natural archives’ for studying historical trends of pollution. Blais et al. (2001a,b) showed that glaciers are important contributors of POPs to freshwater systems: melting glaciers supply 50 to 97% of the organochlorine (OC) inputs to the water system downstream. The prevalence of inputs of OC in the dissolved phase and the low organic content of glacial-fed waters determines a high bioavailability and toxic risk of these compounds to aquatic biota. Quiroz et al. (2009) found, in snow samples in the Aconcagua Mountains (Argentina), polychlorinated biphenyls (PCB) at levels ranging from 0.020 to 0.190 ng/L. These results point out the need of investigating the role of mountains in the trapping of POPs and the associated risks, including climate change.

PETROLEUM SPILLS/PETROCHEMICAL INDUSTRY CONTAMINATION IN FRESHWATER AND COASTAL LAGOONS

With globalization, emerging pollutants are a threat in the region arising from, industry and mining activities. Surfactants and in particular linear alkylbenzene sulfonates (LAS) are widely employed in detergent formulations (Penteado et al., 2006).

Although polycyclic aromatic hydrocarbons (PAH) are widespread contaminants, little information exists relative to most Latin American countries. Available information on PAH in sediment from Argentina and Uruguay is presented in Figures 2 and 3, respectively. In the case of Cuba and Guatemala some PAH have been also detected (Figure 4).

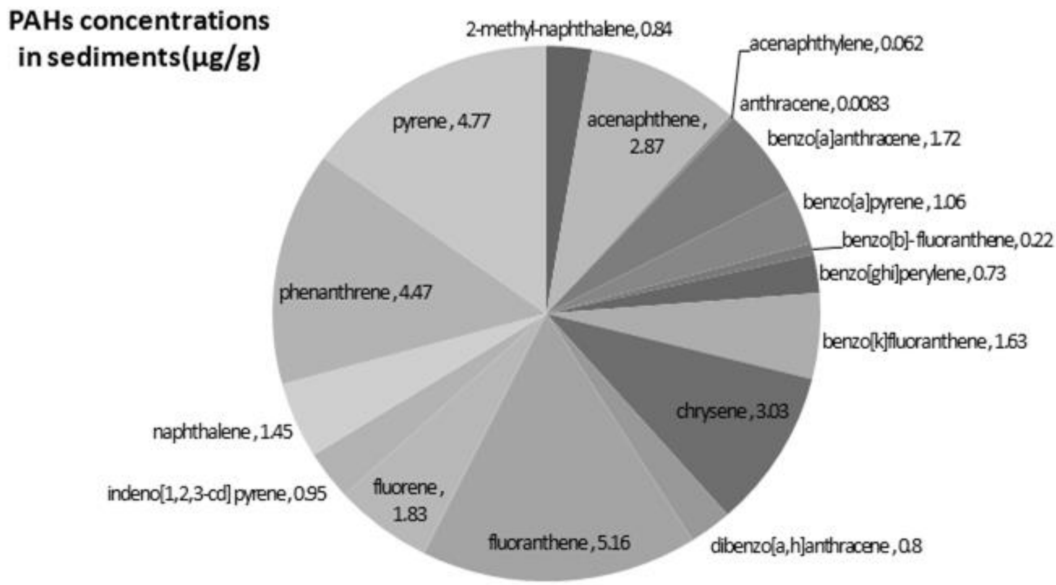

Figure 2. Concentrations of PAH in sediments of Bahía Blanca, Argentina. Source: Oliva et al. (2015).

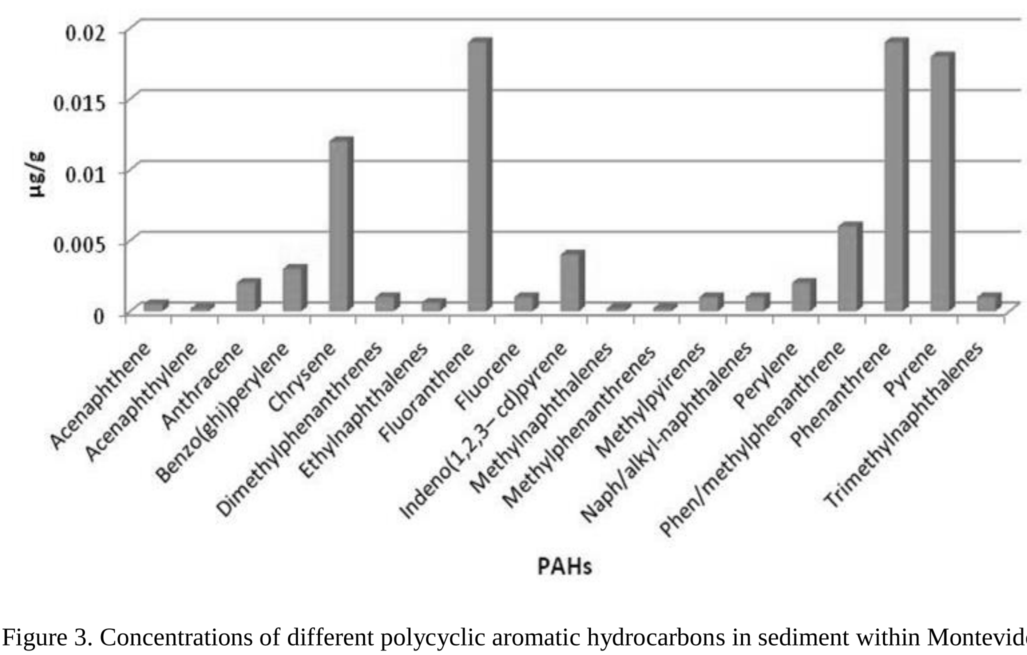

Figure 3. Concentrations of different polycyclic aromatic hydrocarbons in sediment within Montevideo Harbour, Uruguay. Source: Muniz et al. (2004).

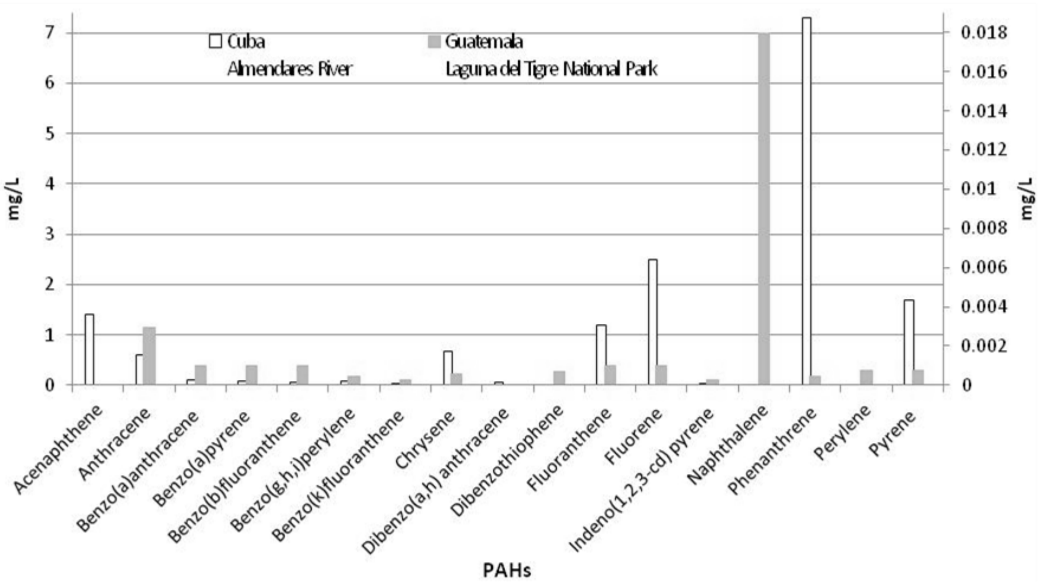

Figure 4. Occurrence of polycyclic aromatic hydrocarbons in Cuba and Guatemala. The left axis corresponds to Cuba and the right axis to Guatemala. Source: Theodorakis et al. (2012) and Santana et al. (2015).

Shale Gas

The existence of natural gas in shale has for long been known but technology could not develop those resources at economically viable costs. Specifically for shale gas, hydraulic fracturing techniques (fracking) have been developed in which a mixture of water, chemicals, and sand is pumped into the well to crack open the rock and release the natural gas into the well (Helms, 2008).

The environmental risks associated with shale gas exploration and production are:

a) Competition for water affects drinking water, wildlife habitat, recreation, agriculture and industry.
b) Methane leakage into groundwater.
c) Pollution from frackwater disposal on the surface.
d) Induced earthquakes from frackwater injection into disposal wells.
e) Environmental footprint of industrialized landscapes as new wells are constantly being drilled.

In Latin America, Argentina is attracting the most attention. Preliminary estimates indicate that only 20% of the prime Vaca Muerta shale basin has liquids (Wilkinson, 2011). Regarding environmental issues, Argentina starts out with an advantage as the shale gas reserves are largely in the sparsely populated regions of Patagonia and Neuquen. Nevertheless, there is a growing NGO (non-governmental organization) movement that is focusing on fracking issues. The growing opposition has linked environmental issues to those of indigenous rights to create significant obstacles to shale gas development (MercoPress, 2012).

Mexico has the second largest technically recoverable shale gas deposits in Latin America and the third largest in the world. Mexico's national oil company (NOC) Pemex drilled its first well in the Burgos region across the border from Texas, but it cost almost five times that of those drilled on the Texas side, and came up dry. Pemex produced shale gas for the first time in March 2011 in Coahuila state (CSUR - Canadian Society for Unconventional Resources, 2012). The main environmental obstacle for development of shale gas in Mexico is scarcity of water and the potential contamination of aquifers due to fracking to obtain shale gas (Mares, 2012).

Cyanobacteria and Cyanobacterial Toxins

Cyanobacteria blooms are related to high concentrations of nutrients, a process called eutrophication that is accelerated by human activities such as modern agricultural practices, fossil fuel extraction and combustion, use of phosphate-containing cleaning agents, etc. (Serediak et al., 2014). Moreover, due to bloom formation some cyanobacteria genera can reduce water quality by depleting dissolved oxygen, altering physicochemical and organoleptic properties, and releasing toxic metabolites (Sivonen and Jones, 1999).

In Latin America, several studies have been conducted to determine cyanobacteria species distribution and abundance, their ecological role, the effects on the aquatic biota, and the effects on human population, which in certain occasions have been intoxicated by cyanobacterial metabolites. Despite the importance of cyanobacteria in freshwater systems, little information is available regarding bloom formation and persistence in countries from Central and South America, where limnological studies mainly present algal lists and in which some bloom-forming cyanobacteria are also included (González, et al. 2004; Fontúrbel-Rada, 2005). Nonetheless, media and international organizations have documented the occurrence of blooms in the aforementioned countries (MARN, 2012; Julajuj, 2015; La Prensa, 2015). Table 3 presents some of the most representative cyanobacterial events in Latin America.

Some cyanobacteria strains can produce very toxic secondary metabolites, including toxins such as microcystins, anatoxins, lyngbyatoxin, cylindrospermopsin, microviridins, among others. The most studied cyanotoxins are microcystins (MC), which have more than 70 chemical variants and the general structure of cyclo(-d-Ala-l-X-erythro-b-methyl-d-isoAspl-Y-Adda-d-isoGlu-N-ethyldehydro-Ala) (De Figueiredo et al., 2004). Their toxicity was first described in mice liver. Nowadays there are reports of the potential deleterious effects on several organs, affecting both phytoplankton and zooplankton, fish, and even humans due to recreational or accidental exposure, such as that reported in Brazil in which people died because of the use of MC-contaminated hemodialysis water (Jochimsen et al., 1998; Carmichael et al., 2001; Azevedo et al., 2002).

Determination of MC has been performed by instrumental or immunological methods in several countries in Latin America. According to Dörr et al. (2010), there are no official data or references related to MC occurrence in Colombia, French Guyana, Guyana, Paraguay, Peru and Venezuela. However, these authors presented a list of MC analogs found in environmental samples from Argentina, Brazil, Chile, and Uruguay. Additionally, Mexico counts with few reports regarding MC detection in environmental samples and ecotoxicological studies assessing the toxic potential of colonial and filamentous cyanobacteria (Ramírez-García et al., 2004; Arzate-Cárdenas et al., 2010; Sánchez-Chávez et al., 2011; Pineda-Mendoza et al., 2012; Tomasini-Ortiz et al., 2012).

Table 3. Cyanobacteria found in Latin American freshwater bodies between 1988 and 2012

Country	Dominant cyanobacteria	Effects	Reference
Argentina	*Anabaena* *Microcystis*	Bloom formation Fish mortality Cyanotoxin production	Dörr et al., 2010
Brazil	*Anabaena* *Cylindrospermopsis* *Microcystis* *Planktothrix* *Pseudanabaena*	Bloom formation Fish mortality Human intoxication and death Cyanotoxin production	Azevedo et al., 1994; Beyruth, 2000; Bittencourt-Oliveira et al., 2001; Magalhães et al., 2001; Costa et al., 2006; Dos Anjos et al., 2006
Chile	*Cylindrospermopsis* *Microcystis* *Nostoc* *Oscillatoria* *Planktothrix*	Cyanotoxin production	Campos et al., 1999, 2005; Neumann et al., 2000
Colombia	*Anabaena* *Microcystis* *Nostoc* *Radiocystis*	Bloom formation Cyanotoxin production	Manceraand Vidal, 1994; Rivera-González and Gómez-Gómez, 2010; Abella and Martínez, 2012
Mexico	*Arthrospira* *Anabaena* *Cylindrospermopsis* *Microcystis* *Synechocystis*	Bloom formation Microcystins production	Oliva-Martínez et al., 2008; Arzate-Cárdenas et al., 2010, Vasconcelos et al., 2010; Pineda-Mendoza et al., 2012
Uruguay	*Anabaena* *Microcystis* *Nodularia*	Bloom formation Cyanotoxin production	Pérez et al., 1999; Kruk and De Leon, 2002

Cylindrospermosin (CYL) is a very toxic cyanobacterial metabolite that was named after *Cylindrospermopsis raciborskii*, although other species can also perform its biosynthesis. It was first described as a hepatotoxic compound, but it has been proven to be cytotoxic, dermatotoxic, or genotoxic. CYL is frequently found in tropical water bodies and also in temperate zones. In Australia it generated the most famous human intoxication due to consumption of CYL-contaminated drinking water. CYL detection is not a routine analysis for water quality in Latin America, although it has been found in Brazilian drinking water supplies. This should be a warning call to improve and increase monitoring and studying of the causes and effects of cyanotoxins (Bouvy et al., 1999; Bittencourt-Oliveira et al., 2014).

Despite the progress in the description of potential toxic species, limnological studies and research of the effect of MC on zooplankton, there is not enough information in Latin America related to other toxins (e.g., anatoxins, lyngbyatoxin, microviridins, nodularins, etc.)

that are also important because of their mode of action. Moreover, cyanotoxins have also been found in treatment plants where they could not be removed by "conventional" processes. Instead, tertiary treatments that include advanced oxidation processes have been tested for their potential use to purify cyanotoxin-contaminated water (Sharma et al., 2012).

Nevertheless, water treatment and research focused on cyanobacterial-blooms effects should be extended in order to study and implement methods for their prevention. Human activities have been the main causes that exacerbate the eutrophication process not only in temperate regions but also in tropical and subtropical areas, where climate easily promotes the abundance and proliferation of cyanobacterial species. Thus, a synergy between prevention and remediation should have the desired effect on reducing cyanobacterial related events and guaranteeing water quality and safety.

CONCLUSION

All the threats that we have presented here have resulted in severe adverse effects on our freshwater resources. Many of these adverse effects can be categorized as follows:

- *Loss of biodiversity in freshwater ecosystems:* Almost one quarter of the world's inland water fish species are found in Latin America and the Caribbean (UNEP, 2010). However, human use of freshwater resources and water pollution have adversely affected catches (UNEP, 2010). Many aquatic invertebrate species might have already been lost due to contamination.
- *Eutrophication/Bacterial-Parasite Contamination:* Many problems related to human health issues are related to simple water sewage issues. A part of the population still has no access to sewage/treatment plants or drinking water systems. Illegal discharges worsen the problem.
- *Bioaccumulation, bioconcentration, biomagnification:* These processes affect many aquatic ecosystems and cause serious human health concerns.
- *Aquifer contamination and depletion occurs as a consequence of severe exploitation of underground water:* Arsenic and fluor are found at higher concentrations in water as result of deeper drilling to obtain water from aquifers. Drinking water and wastewater treatment costs are rising.
- *Freshwater salinization:* Construction in coastal areas sometimes causes freshwater salinization, affecting estuarine and riverine populations. Mangroves are especially sensitive because they act as detoxification centers in rivers, coastal lagoons, and estuaries.

In order to face all these adverse effects we need a better integration in the subcontinent. Despite the existence of several ecotoxicological agencies in Latin America, the lack of integration is clear. Although there is an entity called SETAC – Latin America, this society is clearly a South American branch of the Society for Environmental Toxicology and Chemistry (SETAC); the representation from North America (Mexico) and the Caribbean is very poor. Regional or national chapters are also present: Asociación Mesoamericana de Ecotoxicología y Química Ambiental (AMEQA) is a Mexican chapter of SETAC. There is also a SETAC

Argentina and an ECOTOX Brazil. A real integration of ecotoxicological societies is missing in Latin America perhaps because they face the same problems that the national chapters experience: a) lack of governmental support; b) low number of associates; c) economical penuries; d) lack of interest or conflicts among associates. Many countries have governmental agencies that deal with environmental issues at the Municipal, State and Federal level. In some cases, like La Plata River Basin, there is interaction among countries and international agencies to deal with freshwater resources (Natale, 2005).

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Chapter 11

FRESHWATER ECOTOXICOLOGY IN COSTA RICA

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ABSTRACT

Synthetic pesticides were introduced in Costa Rica at the beginning of the last century, and even though the cultivated area in the country has remained relatively stable over the last 20 years, pesticide usage has increased substantially. Currently, Costa Rica is the largest importer of pesticides in Central America (24.8 kg active ingredient (a.i.)/ha/yr) and one of the biggest importers per capita in the world. Intensive use of pesticides coupled with the climatic and topographic characteristics of the country have triggered continuous aquatic pollution, biota mortalities, changes in communities' structure, and biochemical and physiological effects. Since before 1990 Central American Institute for Studies on Toxic Substances (IRET) researchers have used ecotoxicological tools such as bioassays and biomarkers to study pesticide pollution and exposure effects. Both standard test organisms and native species, found at different levels of biological organization, have been used. Results of those studies are presented in this chapter, highlighting the vulnerability of Costa Rica's freshwaters. Wiser management of pesticides is necessary to protect the integrity of this tropical ecosystem.

Keywords: Costa Rica, freshwater ecosystems, pesticides, ecotoxicology

INTRODUCTION

Costa Rica, a Central American country, is divided by a middle mountainous chain, which results in a hydrographic system with two versants, one towards the Pacific and one towards the Caribbean (Trejos, 1991). The Pacific side has dry (December to April) and rainy

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(May to November) seasons, while the Caribbean versant lacks clear seasonality, with rain (1500–>7000 P/mm) throughout the whole year (Figure 1).

The climatic and topographic characteristics of Costa Rica are suitable for agriculture, and the country´s economy relies on export of products such as coffee, pineapple, and bananas. This business requires continuous production, and therefore pesticides were introduced at the beginning of the 1900s (Hilje et al., 1987).

The import of pesticides has increased greatly and keeps increasing, even though the cultivated area has remained relatively stable over the last 20 years (de la Cruz et al., 2014a). Costa Rica is the largest importer of pesticides in Central America at 24.8 kg active ingredient (a.i.)/ha/yr (Bravo et al., 2015) and one of the largest importers per capita in the world, resulting in environmental problems in aquatic ecosystems.

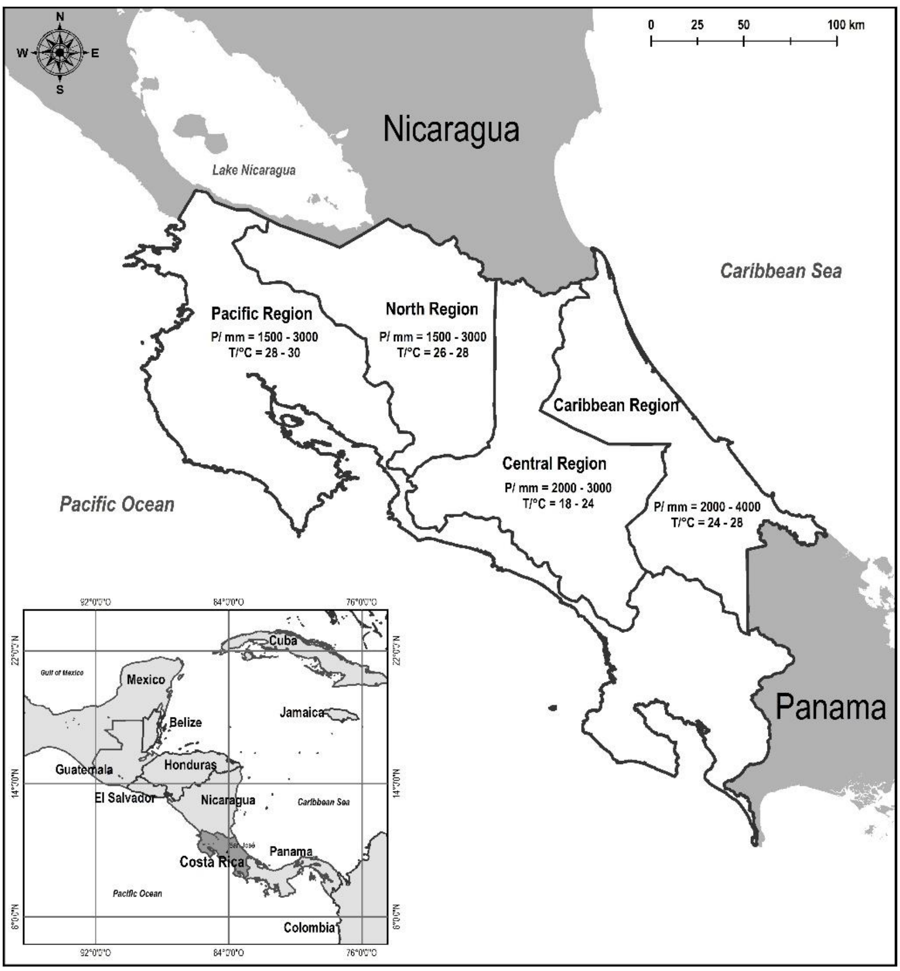

Figure 1. The four regions of Costa Rica where ecotoxicological studies have been conducted, with respective average annual temperature (T/ºC) and precipitation (P/mm).
Geannina Moraga López, IRET-UNA, Costa Rica. 2016. Administrative Division of GADM database of Global Administrative Areas, 2016. Climatological Atlas, National Meteorological Institute of Costa Rica, 2010.

Table 1. Methods used for freshwater ecotoxicological studies at IRET

Type of study	Methods
Biomarkers	Cholinesterase activity (ChE), lipid peroxidation (LPO), glutathione S-transferase (GST), catalase activity (CAT), vitellogenin (VTG), and cytochrome P450 (CYP1A) on organisms exposed to sublethal concentrations of pesticides (Mena et al., 2014a,b). (See chapter 2, "Biomarkers in Native Central American Species," of this book.)
Clam *Anodontites luteola*	Mortality, avoidance, weight loss and biomarker analysis after 96 h exposure to either oxitetracycline (OTC) or environmental sediment samples (Arias-Andrés et al., 2014a).
Water flea *Daphnia magna*	Acute toxicity (48 h) (OECD, 2004). Chronic test (21 d) (EPS, 1990); *in situ* toxicity for survival and 24 h feeding rate (de la Cruz et al., 2012).
Green algae *Pseudokirchneriella subcapitata*	Growth inhibition (72 h) (EPS, 2007a).
Macrophyte *Lemna minor*	Growth inhibition (7 d) (EPS, 2007b).
Onion *Allium cepa*	Root growth test (72 h) (Fiskesjö, 1993).
Hydra attenuata and *Hydra viridissima*	Morphological changes (96 h), microplate-based bioassay (Trottier et al., 1997).
Nematode *Panagrellus redivivus*	96 h assay: Survival, growth, maturation, and total fitness values of nematodes (Samoiloff, 1990).
Lettuce seed *Lactuca sativa*	120 h assay: Seed germination inhibition, root and stem growth inhibition (Dutka, 1989).
Muta-ChromoPlate test kit (fluctuation kit)	5 d test: Numbers of positive wells compared to those containing only sterile water and bacterial test strain (*Salmonella typhimurium*) (Rao and Lifshitz, 1995).
Hyalella azteca	10 d standard sediments test (EPS, 2013).
Microalgae indicators	Chlorophyll concentration in water (Eaton et al., 1995); phytoplankton abundance and diversity (Throndsen, 1978); microphytobenthos abundance and diversity (CHEbro, 2005).
Macroinvertebrate community (MC) structure	Abundance, richness, diversity, colonization of artificial substrates (Castillo et al., 2006) and BMWP-CR (MINAE-S, 2007)
Other biological assessment	Riparian vegetation and river habitat evaluated applying indexes (Acosta et al., 2009) and fish mortalities recorded.
Ecological risk assessment (ERA)	Tier I and tier II risk index (RI) based on predicted environmental concentrations/predicted no effect concentration (Posthuma et al., 2002; ECJRC, 2003).

BMWP-CR, Biological Monitoring Working Party index modified for Costa Rica.

Before 1995, most studies involving agrochemicals on freshwater ecosystems of Central America focused only on pesticide presence, with no further analysis on ecotoxicological responses (Castillo et al., 1997). With creation of the Central American Institute for Studies on Toxic Substances (IRET) of the Universidad Nacional there have been assessments of the agricultural import and use of pesticides (Bravo et al., 2011, 2015), levels of human and environmental exposure (CGR, 2005, 2013; Castillo et al., 2012), and environmental effects (Castillo et al., 2006; Ugalde, 2007; Castillo et al., 2012; Echeverría-Sáenz et al., 2012a; de la Cruz et al., 2014b) and risks for human and wildlife populations of tropical ecosystems (Arias-Andrés, 2011; de la Cruz et al., 2014a,b; Echeverría-Sáenz et al., 2015b). Created in

1994, the Laboratory for Ecotoxicological Studies (ECOTOX) of IRET was the first laboratory of its kind in Central America, driving and supporting all the aforementioned ecotoxicological research.

In this chapter the most relevant results of the investigations conducted by IRET are presented in five sections, four representing regions of the country—Central, North, Caribbean, and Pacific (Figure 1)—and a fifth section on studies with native species. Results address the most important ecotoxicological effects, with relevant conclusions from ecotoxicological studies in Costa Rica.

METHODS

IRET takes a multidisciplinary approach to its research, combining biology, ecology, toxicology, chemistry, agriculture, technology, and remote sensing including land use mapping. Each investigation diagnoses pesticide use in the crops of the study area (Bravo et al., 2011). Water and sediment sampling follows the standard protocols described by the U.S. Environmental Protection agency (USEPA, 2013).

Samples were taken randomly and were not associated with pesticide applications, so pesticide concentration peaks that may occur after applications and rain events were not always reflected in the results. Pesticide residue analyses in water, sediments, and tissues were performed at IRET in the Laboratory of Pesticide Residue Analysis (LAREP) according to the multi-residue method for pesticide analysis in water samples, using solid phase extraction and gas chromatography, according to SW846 3546 and ASTM 6010 procedures (Castillo et al., 1998, 2000a; Diepens et al., 2014). All the information was related to land use so that empirical relationships can be developed to predict levels of pesticide patterns in other areas. Predictive models can also be developed for management use.

The effects of toxic substances on aquatic biota were assessed using an established battery of techniques including organisms from different trophic levels at ECOTOX. All tests were based on available standards for environmental assessments (Table 1).

RESULTS AND DISCUSSION

Central Region

The Central Valley (CV) is the most urbanized, populated (60% of the 4.5 million total population), and economically active region of Costa Rica. Surface and groundwater of the CV are receptors of many pollutants from agricultural, domestic, and industrial activities (Astorga and Coto, 1996; CGR, 2005; Fournier et al., 2010; CGR, 2013; Reynolds, 2013).

Astorga et al. (1997) conducted research of water quality along the basin of the Grande de Tárcoles River. The study included chemical analysis of industrial and domestic effluents, river water and sediments, benthic MC composition, and toxkit tests with *Thamnocephalus platyurus*, *Brachionus calyciflorus,* and *Selenastrum capricornutum* (currently known as *Pseudokirchneriella subcapitata*). Taxa richness was higher during the rainy season, and decreased during the dry period. According to toxicity tests, water quality decreased from upstream to the middle and downstream, where there is greater influence of industrial, urban, and agricultural activities.

Mo (2001) assessed the impact caused by pesticides used in flower and leather leaf fern production to surface and groundwater, and the effects on *Ceriodaphania dubia* survival and reproduction. Acute toxicity was found in one-third of surface water samples, in which the detected pesticide residues were chlorothalonil, propiconazole, vinclozolin, prochloraz, diazinon, dimethoate, endosulfan, pirimicarb, malathion, and pirimiphos-methyl.

Fournier et al. (2010) conducted a study to determine agrochemical contamination of water, soil, and vegetables in an horticulture watershed located in Plantón-Pacayas, in Cartago Province. Chlorpyrifos was detected in 55% of the water samples ($\bar{X}$ 0.07 μg/L), pentachloroaniline in 48% ($\bar{X}$ 0.03 μg/L), flutolanil in 40% ($\bar{X}$ 0.66 μg/L), clorotalonil in 36% ($\bar{X}$ 0.70 μg/L), and quintozene in 24% ($\bar{X}$ 0.12 μg/L). Soil samples also were positive to pesticide presence: pentachloroaniline was detected in 90% of the samples ($\bar{X}$ 0.35 mg/kg), hexachlorobenzene in 76% ($\bar{X}$ 0.06 mg/kg), chlorpyrifos in 73% ($\bar{X}$ 0.15 mg/kg), clorotalonil in 72% ($\bar{X}$ 0.24 mg/kg), DDE-pp in 59% ($\bar{X}$ 0.09 mg/kg), quintozene in 48% ($\bar{X}$ 0.17 mg/kg), flutolanil in 41% ($\bar{X}$ 0.89 mg/kg), prothiofos in 36% ($\bar{X}$ 0.30 mg/kg), DDT-pp in 35% ($\bar{X}$ 0.14 mg/kg), and pentachlorobenzene in 24% ($\bar{X}$ 0.02 mg/kg). Finally, concentrations (mg/kg) found in vegetables that exceed national and international standards were clorotalonil (0.06), permethrin (0.15), pentachloroaniline (0.08), hexachlorobenzene (0.04), pentachlorobenzene (0.04), flutolanil (0.27), and quintozene (0.04).

MC was assessed during the third year at Pacayas (upstream, middle, and downstream) and at Plantón (upstream and middle). The BMWP-CR index was applied; water quality was poor, polluted, or heavily polluted at all sampled points. The authors concluded that the water quality is not suitable for biological communities and ecosystem function.

North Region

The north zone of Costa Rica is characterized by flat lowlands dedicated mainly to livestock and agriculture (orange, tubers, pineapple, and rice) and a very rainy climate. Higher irregular lands on the mountain slopes are dedicated to horticulture and milk farming.

During the 1990s the presence and effect were studied of pesticides used in agricultural areas on the San Juan River (SJR) ecosystems. Chlorothalonil, chlorpyriphos, and cadusaphos were detected in SJR effluent. However, surface water presented no acute toxicity to *D. magna*, *T. platyurus*, *B. calyciflorus*, and *H. attenuata;* nor were effects on MC diversity observed (Astorga, 1997).

In 2011 an evaluation was started on the Frío River basin (Fournier et al., 2016). The diagnosis of pesticide use revealed that 58 active ingredients are applied to the main crops in the area, including herbicides, fungicides, insecticides, nematicides and/or acaricides, and compounds with antimicrobial activity. Standard tests are used to determine toxicity of a single compound, but with 58 different chemicals, the synergistic response cannot be discarded. The Frío, Sabogal, Thiales, Mónico, and Samen Rivers and the Caño Negro wetland were sampled for presence of pesticides, acute and chronic toxicity to cladocerans, biomarker alterations in fish exposed *in situ*, and effects in the MC. Ametryn, diuron, bromacil, cypermetryn, diazinon, and ethoprophos were detected in water samples from the different rivers. No acute effects were observed on the zooplankter *Simocephalus semiserrulatus*, and effects on reproduction were obtained in samples from sites where no pesticides were detected. MC diversity was similar among sites, and the BMWP-CR index classified most of the sites (60%) as moderately polluted aquatic environments. Biomarkers measured in fish (fingerlings of *Parachromis dovii*) demonstrated cholinesterase inhibition at two of the four evaluated sites (Mónico and Thiales) and GST induction at one of them (Mónico); these sites had presence of pesticides.

Recently, a study was started in the higher-altitude Zarcero horticulture area (Ramírez et al., 2015). Evaluation of pesticide use has identified at least 65 a.i. (among fungicides, insecticides, and herbicides), detecting 21 of them in river and underground water samples. Acute effects have been observed on primary producers: *L. sativa* root growth inhibition in water samples from four sites; inhibition of *P. subcapitata* growth in a sample taken downstream from a potato field treated with paraquat; and growth stimulation in samples taken from sites with high phosphorus content. Acute effects (20% mortality) on *D. magna* have been observed in results obtained after exposure to a river water sample with diazinon residues. The studied sites were classified as moderately polluted, polluted, and very polluted by the BMWP-CR index.

Caribbean Region

This region has been subjected to agricultural pressure since the late 1800s, when banana cultivation first started. Today other products, such as rice, ornamental plants, papaya, cassava, cacao, and especially pineapple are also grown in this region. Pesticide consumption has reached 73 kg a.i./ha/yr in banana plantations, followed by pineapple (43 kg a.i./ha/yr), plantain (25 kg a.i./ha/yr), ornamental plants (≈17 kg a.i./ha/yr), and papaya (13 kg a.i./ha/yr) (Echeverría-Sáenz et al., 2015b).

Pesticide usage combined with high precipitation (3500 P/mm/yr) and very few soil conservation practices enhance the transport of agrochemicals into surface waters. These aquatic ecosystems receive pesticides almost every day of the year due to the lack of a marked dry season, year-round cultivation, and consequent continuous agrochemical application.

The most used pesticides in the Caribbean region include mancozeb, chlorothalonil, conazoles (fungicides), ethoprophos, diazinon, carbaryl, terbufos (insecticides/nematicides), glyphosate, diuron, 2,4-D, bromacil, and ametryn (herbicides). The toxicity of these substances to aquatic organisms is known to be very high (de la Cruz et al., 2012), and some of these are regularly detected in water, with the aggravation of being reported in mixtures of more than five a.i. in most samples (Castillo 2000a, 2006; Echeverría-Sáenz et al., 2012b, 2015a,b, 2016). This scenario poses high risk to aquatic ecosystems in this region and, as noted above, most laboratory tests involve single chemicals instead of mixtures. Therefore, acute and chronic ecotoxicology tests have been performed to assess the toxicity of field water samples in a battery of organisms from primary producers to invertebrates and fish.

Acute tests have not provided good information on effects in the majority of cases, since pesticide concentrations in the field are usually below the lowest lethal concentration for 50% of exposed individuals (LC50). Nevertheless in some cases, acute toxicity was detected. Echeverría-Sáenz et al. (2012b) found 80% and 60% immobility of *D. magna* (48 h) when exposed to water from the Santa Clara and Limbo Rivers, respectively, which contained (ug/L) diazinon (0.5–1.0), diuron (0.6–2.0), carbaryl (0.7–7.0), ametryn (0.4–9.0), hexazinone (1.5), bromacil (1.0), and ethoprophos (0.4). The same water samples caused 45% and 26% stem growth inhibition and 35% and 24% root growth inhibition of *L. sativa*, respectively. In another study area, Echeverría-Saénz et al. (2016) found 60% immobility of *D. magna* (48 h) when exposed to water from the Caño Azul River (Madre de Dios River watershed). In the latter site 21 a.i. were detected throughout the study, with highest concentrations (µg/L) reported for diuron (0.17–3.01), carbaryl (0.98–2.22), azoxystrobin (0.23–2.10), ethoprophos (0.05–1.56), and diazinon (0.02–0.63).

Chronic tests have helped us understand the effects of field pesticide concentrations on aquatic biota. Suárez et al. (2008) reported 50% reduction in reproduction of *D. ambigua* (15 d bioassay) exposed to water from a banana drainage canal with detected ethoprophos (0.2–4.0 µg/L), difenoconazole (0.1–3.0 µg/L), and chlorpyrifos (traces to 0.03 µg/L). Echeverría-Sáenz et al. (2015b), in a 21-day *D. magna* bioassay, registered 100% mortality (0% reproduction) when exposing the organisms to three water samples from the Jiménez and Parismina Rivers. Other samples from the Parismina River watershed showed <50% reproduction compared to a negative control. The reported diminished reproduction of *D. magna* was directly related to pesticide concentrations in the water. A total of 23 pesticide residues were detected in the study area, but the three samples with the highest toxicity consistently contained (µg/L) chlorpyriphos (0.05–0.08), diazinon (0.12–0.30), ethoprophos (0.04–0.36), propiconazole (0.10–0.24), metalaxil (0.1–0.2), ametryn (0.20–0.55), bromacil (0.8–1.6), and diuron (0.4–1.0). Other pesticides detected in those water samples included carbaryl, buprofezin, difenoconazole, epoxiconazole, pyrimethanil, azoxystrobin, and hexazinone in concentrations <0.85 µg/L.

Neurotoxic biomarkers used to indicate the impact of pesticides that affect the nervous system—such as ChE—have been included in recent studies. Mena et al. (2014a) conducted 96-hour laboratory experiments with the fish *Astyanax aeneus* with solutions of 0–1 mg/L ethoprophos, resulting in ChE inhibition and detection of this substance in fish muscle after exposure. However, *in situ* experiments (caged *A. aeneus*, 48 h) did not render consistent results, as ChE activities did not vary between sites along a pesticide gradient. Echeverría-Sáenz et al. (2012b) found ChE inhibition in muscle and brain and increased stress enzyme (GST) in liver of *Poecilia gilli* exposed *in situ* along agricultural sites of the Jiménez River in the same water samples that generated acute toxicity in *D. magna*. Echeverría-Saénz et al. (2016) applied a biomarker battery (GST, LPO, CAT, and ChE) (Table 1) and found intensified responses in highly polluted sites of the Madre de Dios River watershed. More details are given in "Biomarkers in Native Central American Species," chapter 2 of this book.

Ugalde (2007) analyzed differences in the abundance and diversity of phytoplankton between two sites on the Limbo River (influenced by pineapple plantations) and a reference stream. The results indicated increased abundance of microalgae in the most impacted site, where pesticide residues (µg/L) of bromacil (0.3–2.3), ametryn (0.1–0.3), diuron (0.06–0.30), chlorothalonil (0.1), triadimefon (0.1–0.2), diazinon (0.07–0.40), and ethoprophos (0.2) were detected, also with higher nitrates. Other results included absence of sensitive species that were present at the reference sites. Species known to be indicators of pollution were more abundant in the Limbo River.

Castillo et al. (2006), Suárez et al. (2008), and Echeverría-Sáenz et al. (2012b, 2015a) collected MC samples in several impacted and reference sites throughout the Caribbean; in all of them, a clear difference in MC structure between reference and impacted agricultural sites was noticed. Richness, biodiversity, and percentage of sensitive organisms as well as biotic indices for organic pollution (BMWP-CR) diminished in agricultural streams. The MC was also affected by riparian habitat degradation and stream/river channel characteristics. This community of organisms provided valuable information on effects and sensitivity to pesticides, in some cases even more than that obtained by laboratory toxicity tests.

Degradation rate (DR) of organic matter was measured by Suárez et al. (2008) as an ecological functioning endpoint. They compared DR in rivers inside the La Selva biological station (LSBS) and drainage canals of a banana plantation. Leaf DR was highest in the third-order streams where higher diversity of macroinvertebrates was also found. Smaller-order streams inside the LSBS had lower DR than third-order banana drainage waterways. Increased water velocity and temperature could account for enhanced mechanical and microbial degradation in that ecosystem.

Overall, more than 30 pesticide residues and two metabolites have been detected in water samples of the Caribbean region, in mixtures of herbicides, insecticides (the majority of them being organophosphates), and fungicides. Field concentrations of individual substances rarely exceed 2 µg/L; however the sum of all the a.i. detected in a single water sample has reached more than 10 µg/L. Aquatic biota and ecosystems of this region are in high risk.

Pacific Region

Seven of the major crops of the Pacific Versant (PV) cover 71%–98% of the area cultivated in this region (SEPSA, 2012, 2014). Coffee, palm oil, and some pineapple are produced in the central and southern PV, sugar cane and melon in the central and northern regions, and rice and pastures towards the north and the south. Among these crops, melon has the highest pesticide use (60.5–258.0 kg a.i./ha/yr), followed by pineapple (24.5–73.0 kg a.i./ha/yr), rice (9.5–18.9 kg a.i./ha/yr), and sugar cane (15.05 kg a.i./ha/yr). All other crops have pesticide usages below 7 kg a.i./ha/yr (CGR, 2005; MAG, 2007, 2011a,b,c; CNP, 2010; Bravo et al., 2013; Chaves and Chavarría, 2013; Echeverría-Saénz et al., 2015a; INEC, 2015).

Most commonly used in these crops are herbicides 2,4-D, glyphosate, paraquat, terbutryn, ametryn, bromacil, and diuron; insecticides permethrin, diazinon, and dimethoate; and fungicides mancozeb, fosetyl, and epoxiconazole (CGR, 2005; MAG, 2007, 2011a,b,c; CNP, 2010; Bravo et al., 2013; Chaves and Chavarría, 2013). The toxicity of these substances to aquatic biota is well known (Solomon et al., 2001; Berenzen et al., 2005; Campagna et al., 2006; Liu et al., 2013; Marin-Morales et al., 2013; de la Cruz et al., 2014a; Lewis et al., 2016).

Ecotoxicological studies on freshwater systems of the PV have been mostly focused on exposure and effects and less on the overall risk to the aquatic ecosystem (Castillo et al., 2012; CGR, 2013; de la Cruz et al., 2014a,b). Before the year 2000, pollution studies were primarily on organochlorine (OC) residues and their metabolites. Hidalgo (1986) described the relationship between OC levels (specifically *p,p´*-DDE) and egg shell thickness of waterfowl inhabiting the lower reaches of the Tempisque River basin (TRB). High concentrations (4.16 mg/kg fw) of OC (including heptachlor epoxide, HCB, *p,p´*-DDT, *p,p´*-DDE, and endrin) and of p,p´-DDE (3.19 mg/kg fw) were found in eggs of wood stork *Mycteria americana*. The eggs with higher concentrations of DDE had cracks. Fyfe et al. (1990) found high levels of DDE in eggs of *Nyctiocorax nyctiocorax* (4.59 mg/kg fw) and *Hirundo rustica* (3.35 mg/kg fw) and low levels (0.03–1.84 mg/kg fw) in other water birds such as *Calidris mauri and Tringa semipalmata*, prey of the peregrine falcon. Amphibians and turtles of the Santa Rosa National Park (where pesticides are not applied) showed low levels but high frequency of OC pesticide contamination (Klemens et al., 2003). Maximum concentrations (ng/g fw) of OC reported for amphibians and turtles were *p,p´*-DDE (55 and 125), delta-BHC (40 and 11), heptachlor (32 and 17), and dieldrin (3.7 and 4.6). Higher OC levels were also reported in mayflies' larval tissue (*Euthyplocia hecuba*) from agriculture-impacted forests of the upper and middle TRB compared with non-impacted forests (Standley and Sweeney, 1995). OC pesticide (ng/g ww) found in the mayfly were aHCH (<6–250), gHCH (<6–250), heptachlor epoxide (38), alfa-endosulfan (51), aldrin (54), dieldrin (100), beta-endosulfan (150), endosulfan sulfate (2001), DDE (67), DDT (<6–250), aldrin (54), dieldrin (100), endrin (<12–500), and endrin aldehide (150).

Most of the ecological effects of pesticides in aquatic ecosystems of the PV have been in the Arenal Tempisque Irrigation District (ATID) of the TRB, which drains into several protected areas, among them Palo Verde National Park (PVNP), which protects valuable wetlands. Nearly 28,000 ha of rice, sugar cane, and pastures, among other crops, are irrigated through a series of channels (de la Cruz et al., 2014a). Twenty-eight a.i. have been reported in surface waters of this region. Maximum concentration (µg/L) of the most frequent herbicides are ametryn (3.0), buthaclor (13.3), bentazon (4.39), 2.4-D (1.02), diuron (8.4), hexazinon (4.6), and terbutryn (6.5); insecticides carbofuran (0.107), cypermethrin (12.4), dimethoate (8.0), endosulfan (4.0), and triazophos (6.7); and fungicides epoxiconazole (1.7), propiconazole (2.5), tebuconazole (1.2), organophosphate insecticide-nematicide, and terbufos (7.2) (CGR, 2005, 2013; Arias et al., 2014c; de la Cruz et al., 2014a).

Martínez (1998), Rizo-Patrón (2003), Fournier et al. (2006), and Echeverría-Sáenz et al. (2012a) studied the MC of water ecosystems before and after planting of rice and sugar cane plantations in the ATID. The biodiversity and percentage of pollution-sensitive taxa (e.g., Trichoptera and Ephemeroptera) diminished in streams with agricultural influence, as did the BMWP-CR index. In contrast, the abundance of organisms resistant to poor water quality organisms such as snails increased. The most common pesticides in those surface waters were ametryn, cypermethrin, diazinon, dimethoate, diuron, endosulfan, epoxiconazole, methamidophos, propiconazole, terbutryn, and triazophos.

Laboratory studies reported 16% mortality in *D. magna* (48 h), but no growth difference in *P. subcapitata* (72 h) exposed to water from aquatic ecosystems influenced by rice and sugar cane production in the northern sector of the PVNP. Water samples contained low levels of dimethoate (toxic to *D. magna*) and high nutrient content (Fournier et al., 2006). Up to 55% mortality was obtained in laboratory tests with *D. magna* (48 h) exposed to water from a contaminated Tempisque River site, which drains rice and sugarcane crops, and 3% mortality from the Barbudal reference site (B). More than six a.i. have been reported in the

CVT. The same water samples from CVT and B sites show stem growth inhibition of *L. sativa* (120 h) of 3% and 8%, respectively, and slight root growth promotion in both sites. Survival percentage and feeding rate (intake of *P. subcapitata* cells per hour) of *D. magna* from *in situ* exposure experiments were also higher in B than in the CVT. Risk estimations (tier 1) of the pesticides in the aquatic ecosystem of the PV were high for almost all the a.i. detected, and were especially high for cypermethrin, endosulfan, triazophos, diuron, propiconazole, dimethoate, and terbutryn (de la Cruz et al., 2012).

Pesticide pollution and integrated responses of biochemical markers in transplanted fish (*P. dovii* and *P. gillii*) were used to monitor environmental hazards in the PVNP. High GST and CAT enzyme activities and elevated levels of LPO in *P. dovii* were observed at the Cabuyo (C) site impacted by rice fields. Mena et al. (2014b) associate these effects with HCB and triazole fungicides in the water. Fish transplanted across pesticide-contaminated sites near the PVNP presented significant changes regarding hepatic Cyp1A: *P. dovii* Cyp1A levels were enhanced in B and C during the rainy season, whereas in *P. gillii* fish, Cyp1A levels responded seasonally. VTG induction in field experiments was not observed in either of the two species (Navarro et al., 2014).

In the ATID, antibiotics are used in pig farms, aquaculture, and rice farming (de la Cruz et al., 2014b). Water from sediments impacted by pig farming affected burrowing behavior in *A. luteola* and caused higher mortality in *D. magna* (48 h), in comparison to aquaculture and rice farming. Integrated biomarker responses (ChE, GST, and LPO) in *A. luteola* tissue classified the pig and rice farms as the most stressful for exposed organisms. Sulfamethazine and oxytetracycline (OCT) were detected in effluents of pig farms and OCT in fish farms (Arias-Andrés et al., 2014b). A laboratory test of several days with tetracycline (TET) and 4-epitetracycline (ETC) showed functional and structural alteration in sediment microbial communities from an ATID tilapia farm. When community-level physiological profiles and phospholipid fatty acid profiles of the exposed microbial communities were contrasted, both compounds caused opposing metabolite responses. At concentrations >50 mg/kg, TET had a tendency to inhibit respiration, whereas ETC showed the opposite effect. From fatty acid analyses it was shown that the sediment analyzed was predominantly colonized by gram-negative bacteria. The authors concluded that TET and ETC antibiotics impacted the sediment microbial community in a unique way, with TET effects stronger. Derivative studies are important in antibiotics ecotoxicology (Granados-Chinchilla et al., 2013). Bacterial profiles in sediment collected from a protected wetland and from pig, rice, and fish farms exposed to various concentrations of OTC indicated basal levels of OTC tolerance in the protected wetland and increased levels in proportion to the intensity of antibiotic exposure: agriculture followed by aquaculture and swine farming (Arias-Andrés et al., 2014a).

The pesticides most frequently reported in the waters of the Volcan River in the south Pacific area of Costa Rica were >75% bromacil, >10% diazinon and permethrin, and <1% chlorpyrifos and fenthion. Laboratory studies reported 50%–70% mortality in *D. magna* (48 h), and 41%–48% root growth inhibition of *L. sativa* (120 h) exposed to water samples collected from the river and streams under the influence of pineapple crops. Pineapple production does not have a control system to prevent runoff and soil loss, favoring agrochemical presence in the surrounding water bodies (Castillo and Ruepert, 2001).

Table 2. Toxicity of relevant pesticides used in Costa Rican agriculture in native species of different taxonomic groups

Taxonomic group	Test species	Pesticide	Native vs. standard species sensitivity*	Lethal or effective concentration for 50% of exposed individuals	Source
Crustacea	*Simocephalus semiserratus*	Ethoprophos	≈ *C. dubia* > *D. magna* (10x)	LC50-48 h	Not published
		Diazinon	< *C. dubia* > *D. magna*	LC50-48 h	
	Daphnia ambigua	Ethoprophos	≈ *C. dubia* > *D. magna* (10x)	LC50-48 h	1,2
		Diazinon	≈ *C. dubia* > *D. magna* (10x)	LC50-48 h	3
		Carbofuran	> *C. dubia* (20x), *D. magna* (30x)	LC50-48 h	
		Chlorpyrifos	≈ *C. dubia* > *D. magna* (2x)	LC50-48 h	2,3
	Macrobrachium diguetti	Ethoprophos	< *Peneaus* (saltwater) > decapoda (Uca)	LC50-96 h	3
		Diazinon	≈ *Peneaus* < *Paratya* (10x)	LC50-96 h	
		Carbofuran	> *M. kistnensis* (6x)	LC50-96 h	
Fish	*Parachromis dovii*	Ethoprophos	> *Lepomis macrochirus, Cyprinus carpio, Oncorhynchus mykiss, Carassius auratus*	LC50-96 h	2
		Carbofuran	< *Danio rerio* (2x) > *Salmo trutta, O. mykiss, L. macrochirus, Pimephales promelas, C. carpio*	LC50-96 h	3
		Chlorpyrifos	< *L. macrochirus* (20x), *O. mykiss* (13x) ≈ *P. promelas, C. carpio*	LC50-96 h	2
	Astyanax aeneus	Ethoprophos	< *P. dovii* (2x), *L. macrochirus* > *C. carpio, A. tropicus, O. mykiss, C. auratus*	LC50-96 h	4
	Atractosteus tropicus		< *P. dovii, L. macrochirus, A. aeneus, C. carpio* > *O. mykiss, C. auratus*	LC50-96 h	4
Amphibians	*Agalychnis callidryas*	Chlorothalonil	>*Bufo bufo japonicas* (6x), *Rana limnocharis* (10x)	LC50-96 h	5
	Isthmohyla pseudopuma		>*Bufo bufo japonicas* (6x), *Rana limnocharis* (10x)	LC50-96 h	
	Smilisca baudinii		>*B. bufo japonicas* (6x), *R. limnocharis* (10x)	LC50-96 h	
Aquatic plants	*Lemna sp.*	Diuron	≈*L. perpusilla*, *L. minor*, *L. polyrhiza*	EC50-7 d	3

[1]Arias-Andrés et al., 2014b. [2]Diepens et al., 2014. [3]Arias-Andrés et al., 2016. [4]Mena et al., 2012. [5]Méndez et al., 2016.

*Comparison with species within same taxonomic group, same toxicological endpoint, and similar assay conditions. For comparison, ECOTOX-EPA database (USEPA, 2015) and PAN pesticide database (PAN, 2015) were consulted.

Pesticide Toxicity with Native Species

Since the first studies of water quality with standard (temperate) species in Costa Rica (Castillo et al., 2000b), it became evident that inclusion of relevant native organisms is ideal for environmental impact assessment in the tropics. Therefore, further investigation on native species has been conducted at ECOTOX (Table 2) to check their suitability as test species.

Conclusions

Agriculture is the second most important sector of the Costa Rican economy, and the use of pesticides has been identified as a major problem for our ecosystems. At IRET, researchers have been concerned about the high use of toxic substances in agriculture and have been working on it for several years, trying to determine the impact of these substances on ecosystems. Considering the current context of global climate change, emergent pollutants and elucidation of the complexity with which pollution affects ecosystems, ecotoxicologists should keep adapting and improving the research field in order to characterize and protect ecosystem functions and services.

It should be a goal in our countries to increase the sociopolitical impact of results obtained from ecotoxicological studies for creation of new public policies, aimed for better environmental protection and management of aquatic ecosystems. The current situation of agrochemical water contamination in Costa Rica makes it imperative to establish national freshwater quality monitoring to rigorously assess the use and emissions of pesticides, environmental health, and the effect of the constant agrochemical exposure of biota.

Implementation of good agricultural and soil conservation practices, integrated pest management, development of alternatives to pesticide usage, and enhancement of clean technologies in agriculture are key issues of reduction of pesticide loads in freshwaters.

Tourism being a key economic sector in Costa Rica, it is vitally important to ensure that natural ecosystems are maintained in order to protect this essential industry. In addition, the health of the workers and the people using the rivers for drinking water and irrigation of their crops must be protected from the harmful effects of pesticides.

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Chapter 12

ENVIRONMENTAL RISK IN A COASTAL ZONE OF RIO DE JANEIRO STATE (BRAZIL) DUE TO DREDGING ACTIVITIES

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ABSTRACT

This chapter presents an evaluation of the effects caused by sediments resuspension events on the biogeochemical behaviour of cadmium (Cd), using, as case study, samples from a highly polluted coastal system (Sepetiba bay, Brazil). These effects were evaluated on different depths of three sediment cores and on 12 surface sediments samples collected with increasing distances from the major Cd source. Physical-chemical characteristics, Fe and Mn concentrations, grain size composition and organic carbon contents were also evaluated to characterize potential factors affecting Cd mobility. Cadmium was nearly 100% in the reactive phase (HCl-soluble) and showed extremely high concentrations (up to 14.1 mg Kg^{-1}), with a clear decreasing gradient from the main source of contamination towards the bay. The differences in potential bioavailability after resuspension were low for the sediment cores, but surface sediments presented up to 30% more Cd in the reactive phase after resuspension. The changes on Cd concentrations observed after resuspension may be associated to the dynamics of Fe and Mn compounds.

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It is important to highlight that Cd concentrations are extremely high at this area. So even a small change in its relative bioavailability can imply an increase on the potential risks to local biota.

Keywords: bioavailability, trace metals, Sepetiba bay, resuspension

INTRODUCTION

Contamination of coastal aquatic systems is a worldwide problem and the evaluation of the risks associated to contaminant release for humans and aquatic biota is of utmost importance. This assessment should include biological, chemical and toxicological indicators in order to elucidate the complexity of contaminants dynamics within aquatic systems, considering the water column and sedimentary compartments. Sediment quality assessments are therefore recognized as critical steps for estimating the risks associated with man-made pollution in aquatic systems (Castillo et al., 2013), since these environments can be a sink for most pollutants, including heavy metals. On the other hand, sediments are also considered as potential sources, in case of resuspension events (Duarte and Caçador, 2012).

The sediment resuspension can occur naturally due to bioturbation, cold front entry and strong winds, or artificially due to anthropogenic activities such as dredging and watercraft navigation (Turner and Millward, 2002). Dredging activities are commonly used as a required operation to navigation channels maintenance and to remove contaminated sediments of aquatic ecosystems as a remediation action (Cappuyns et al., 2006). However, it causes sediments resuspension to water column, which implies in metal mobilization, making these elements potentially bioavailable in the aquatic system. These changes may lead to higher exposure and it could enhance the risks of biological uptake of contaminants (Morse, 1994; Machado et al., 2011).

In situ and *ex situ* studies have been developed to evaluate the mobilization of metals. In relation to sediment resuspension *ex situ* evaluations, laboratory tests are used in order to contribute to the assessment of ecological risks linked to resuspension events, simulating the effects of dredging in different time intervals of a pre-defined mechanical agitation. For instance, Machado et al. (2011) observed that the copper (Cu) content in a potentially bioavailable geochemical phase (extracted in HCl 1 M) increased by more than 10 times after the resuspension of sediments from a tropical coastal system (Guanabara Bay, SE Brazil).

The dredging effects on contaminants mobility and their availability to biological uptake are poorly known, especially in tropical ecosystems. Moreover, trace elements, that typically undergo high incorporation into pyrite, are susceptible to changes in their geochemical fractionation in case of sediment resuspension in oxidizing water. Pyrite can become a source of these elements to the water column if oxidized (Morse, 1994; Saulnier and Mucci, 2000). However, potential changes in the bioavailability of trace metals that generally do not present elevated pyritization, such as cadmium (Cd) and zinc (Zn) (Huerta-Diaz and Morse, 1992), can be also a concern in strongly contaminated areas. This is the case of heavily-polluted sites affected by metallurgical activities, as observed in Sepetiba Bay, SE Brazil (Monte et al., 2015). It is a tropical coastal system influenced by combined effects of severe Cd and Zn contamination (mainly due to past activities of a Zn smelting plant), urbanization and harboring activities (Barcellos et al., 1991; Machado et al., 2008; Ribeiro et al., 2013). Cd and

Zn emissions from this major industrial source were estimated as 24 and 3,660 t yr^{-1}, respectively, until the metallurgical plant was closed in 1997 (Molisani et al., 2004). These anthropogenic pollutants were mainly transferred to the bay by a tidal creek (Saco do Engenho Inlet) that received the drainage from a large refuse pile of the metallurgical plant (Molisani et al., 2004).

Therefore, the comprehension of the behaviour of contaminants associated to dredged sediments is essential for an adequate management of dredging areas and monitoring of sites used for the final disposal of dredged materials (Cappuyns et al., 2006). Given this scenario, the inclusion of *ex situ* experiments of sediment resuspension is important to predict risks associated to resuspension and deposition of contaminated materials in coastal and oceanic waters. This chapter addresses these issues, presenting a study on Sepetiba Bay sediments resuspension. Samples from surface sediments and sediment cores from a heavily-polluted area were used to evaluate possible changes in the biogeochemical behaviour of Cd, and the potential risks to local biota are discussed.

METHODS

Sampling

The study area is highly impacted by Cd and Zn contamination, located between Saco do Engenho Creek (SEC) and the Itaguaí Harbor in the northern region of Sepetiba Bay, Rio de Janeiro Brazil. In July 2012, surface sediment samples (0–10 cm depth) were collected using a van Veen grab at 12 sampling stations (Figure 1). Four transects (~350m apart) with 3 sampling stations per transect (~350m apart) were delimited to observe possible gradients of Cd concentration with increasing distances from the main point source of Cd. Station 1 is the closest sampling station to the old point source of Cd - a Zn smelting plant (Mercantil Ingá Company). Even after a program of environmental recuperation in response to decades of contamination due to mining wastes, high Cd concentrations are observed in this area (Barbosa et al., 2015).

Physical-chemical characteristics were measured *in situ* using an YSI probe. Sub-superficial water presented salinity of 32.8, pH of 7.9, dissolved oxygen of 4.6 mg L^{-1}, dissolved oxygen saturation of 65%, electrical conductivity (EC) of 48.5 mS cm^{-1} and temperature of 23.4ºC. Water was sampled only at station 12 at approximately 15 cm depth, using acid-cleaned glass bottles (total of 6 L) and it was used only for the sediment resuspension experiment, being analysed before (t0) and after resuspension. Station 12 was chosen due to its higher salinity and dissolved oxygen - conditions commonly observed at disposal areas of dredged material in Brazil, simulating what would happen if these 12 sediment samples were resuspended under these different physical-chemical conditions.

Three sediment cores (C1, C2 and C3) were obtained using acrylic tubes (10 cm diameter, 50 cm length). They were collected along a transect starting at SEC and going 1 km East into Sepetiba bay (Figure 1), with increasing distances from the major Cd source to the bay. C1 was collected in a tidal flat containing a mangrove ecosystem affected by anthropogenic sources of cadmium, while C2 and C3 were sampled at subtidal sites on the margins of the Cação River, responsible for the fresh water input on Saco do Engenho Creek and also with lower influence of the Cd point source. All cores were sectioned at 5cm intervals. C1 had 50 cm depth, C2 had 40 cm and C3 had 45 cm. Samples were stored in plastic bags and frozen until analysis. Only the sub-samples used for the resuspension experiment were not frozen, since the resuspension experiment was performed as soon as the cores arrived at the laboratory, on the same day of the sampling campaign.

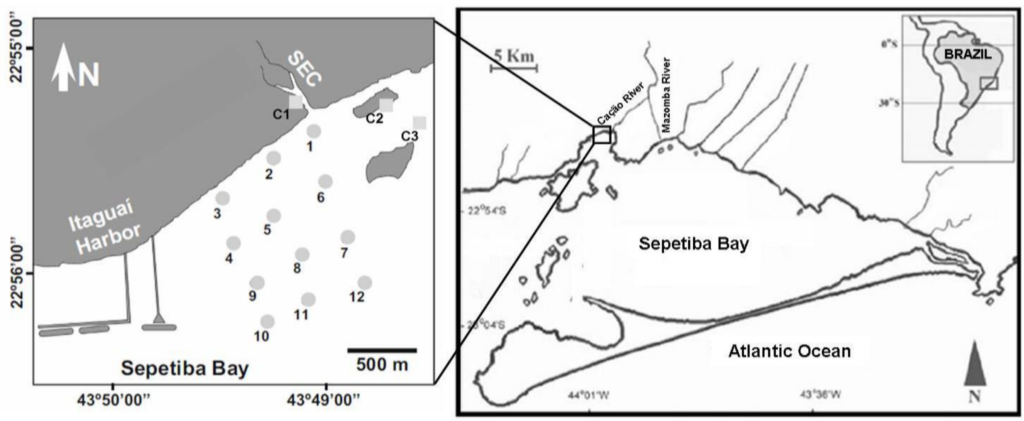

Figure 1. Sampling stations in Sepetiba Bay, Rio de Janeiro, SE Brazil. SEC, Saco do Engenho Creek. Adapted from Monte et al. (2015) and Santos et al. (2007).

Resuspension Experiment

Resuspension experiments were carried out at room temperature (25ºC). The experiments compared short (1h) and long (24h) time periods of resuspension. Wet sediment subsamples (7g) were transferred to 125-mL Erlenmeyer flasks and were shaken in 100mL of unfiltered estuarine water in contact with atmosphere. The sediment:water proportion was based on the study of Morse (1994), adapted by Machado et al. (2011). EC and pH were measured after each time interval of resuspension. Unfiltered estuarine water was also agitated during each time interval to be used as analytical blanks. The experiments were made in duplicates.

The choice of these time intervals was based on previous studies, which demonstrated that in the first hour (t1) most bioavailibility changes occur (Machado et al., 2011). The second interval (t2: 24h) has been also previously adopted (Morse, 1994), and was used to evaluate the stability of the contaminant response, simulating longer resuspension events. Additionally, the initial condition of all sediment samples and of the water used for the test was evaluated (t_0). After the resuspension experiments, the sediment was centrifuged (3,000 rpm/5 min), dried (<40ºC) and homogenized to posterior analysis.

Metal Determination

The reactive Cd phase (adsorbed and/or associated to carbonates, monosulphides and iron and manganese oxides) was extracted by 16h agitation in 1mol L^{-1} HCl solution, which is a usual approach for metal bioavailability evaluation (Huerta-Diaz and Morse, 1992; Morse, 1994; Hatje et al., 2009; Machado et al., 2011; Birch and Hogg, 2011; Monte et al., 2015). HCl-extracted subsamples were washed with deionized water and centrifuged three times to remove HCl residues prior to a second-step extraction of strongly bound metal fractions (Machado et al., 2011). Fe and Mn were also determined, with the purpose of assessing possible relations of Fe and Mn oxides formation with Cd bioavailability. The differences observed for analytical replicates (duplicate) for the reactive phase were 6.7% for Cd, 4.4% for Fe and 6.1% for Mn.

The method adopted for the extraction of strongly-bound metals (applied only for sediment cores) was a microwave assisted digestion with concentrated HNO_3 (USEPA method 3051A; USEPA, 1994). Cd, Mn and Fe concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES). The detection limits were: 0.01 mg Kg^{-1} for Cd, 3.00 mg Kg^{-1} for Fe and 0.02 mg Kg^{-1} for Mn. The differences observed for analytical replicates for the strongly bound phase were: 11.6% for Cd, 9.9% for Fe and 8.5% for Mn.

Sediment Characterization

Besides Fe and Mn determination, sediment grain size was characterized using a particle size analyser CILAS 1064. Total organic carbon (TOC) contents in sediments (after carbonate removal by acidification) and total carbon concentration in water, before and after the resuspension experiment, were determined using a Shimadzu TOC analyser. Possible associations between target variables were evaluated using correlation analysis (Spearman Test) with a significance level of $p < 0.05$.

Bioavailability Change Index (BCI)

The relative change in the percentage of trace metals in HCl-extractable fractions was calculated to evaluate possible differences in bioavailability after sediment profiles resuspension, hereafter referred as bioavailability change index (BCI). It is expressed by the formula (Monte et al., 2015): $BCI=(\%HCl_{AR} - \%HCl_{BR})/\%HCl_{BR} \times 100$, where $\%HCl_{AR}$ is the percentage in the HCl-extractable fraction after resuspension and $\%HCl_{BR}$ is the percentage in the HCl-extractable fraction before resuspension.

The percentage in the HCl-extractable fraction (%HCl) of Cd used for BCI calculation was obtained by the following formula: $HCl=([Cd_{RP}]/([Cd_{RP}]+ [Cd_{SBP}])) \times 100$, where $[Cd_{RP}]$ is the Cd concentration in the reactive phase and $[Cd_{SBP}]$ is the Cd concentration in the strongly bound phase (EPA 3051a method).

As we did not evaluate the strongly bound phase of superficial sediments, a simple calculation of reactive Cd losses and gains as the percentage related to t_0 (*in natura* samples, before resuspension) was done, as explained in the following equation: $(([Cd_{AR}] - [Cd_{BR}])/[Cd_{BR}])*100$, where $[Cd_{AR}]$ is the Cd concentration on reactive phase after resuspension and $[Cd_{BR}]$ is the Cd concentration on reactive phase before resuspension. The same equation was applied for Fe and Mn.

Contamination Factors (CF) and Environmental Guidelines

Contamination factors (CF) is the ratio between the metal concentration in the present (superficial sediment) and the background concentration, usually obtained from sediment cores (Hakanson 1980). Then, an average background value obtained from ^{210}Pb-dated sediment cores sampled in Sepetiba bay, reported by Gomes et al. (2009) was used (0.27 mg Kg^{-1} for Cd) to calculate CF. Moreover, the results were compared to Brazilian legislation (CONAMA Resolution 454/2012), which establishes guidelines and procedures for the management of dredged materials of fresh and estuarine waters.

CONAMA 454/2012 establishes two threshold levels for metals. The first threshold (Level 1) represents a limit for the low probability of any adverse effect occurrence on biota. The second threshold (Level 2) represents the limit for a high probability of adverse effect occurrence on biota. For estuarine waters, the Cd concentrations established as Levels 1 and 2 are 1.2 mg Kg^{-1} and 7.2 mg Kg^{-1}, respectively. According to this resolution, if the sediment samples show concentrations above level 2, ecotoxicity tests should be performed before dredging.

RESULTS

Surface Sediments

The surface sediment samples were classified as very coarse silt (stations 1, 8 and 12), coarse silt (stations 2, 3, 4 and 11), medium silt (stations 5, 6 and 7), very coarse silty fine sand (station 9) and very fine sandy very coarse silt (station 10). The average for TOC in sediment from Saco do Engenho Creek was 2.4 ± 1.2% (Table 1). The concentrations in the reactive phase ranged from 0.9 to 14.1 mg Kg^{-1} (stations 9 and 1, respectively) for Cd, from 1,870 to 9,871 mg Kg^{-1} (stations 9 and 12, respectively) for Fe and from 28.5 to 151.9 mg Kg^{-1} (stations 9 and 1, respectively) for Mn (Figure 2). Reactive Cd concentrations are correlated with reactive Fe (Spearman; r=0.68; $p < 0.05$; n=12) and Mn (Spearman; r=0.82; $p < 0.05$; n=12). Besides, Cd concentrations are correlated to TOC (Spearman; r=0.83; $p < 0.05$; n=12) and percentage of silt (Spearman; r=0.72; $p < 0.05$; n=12).

These are very high Cd concentrations, particularly in the reactive phase, reaching an average concentration one order of magnitude higher than the background concentration (CF=13.6 ± 11.4; n=12). The CF reached 41.5 at station 1. Even for the most distant station from the contamination hotspot, the contamination factors were above 5.7, what indicates high Cd contamination (Figure 3). These concentrations are almost 12 times higher than level 1, established by the Brazilian legislation guideline and 2 times higher than level 2, considering the most contaminated station (station 1).

For an initial discussion about the effects of resuspension on a bioavailable phase of Cd, we will discuss some modifications in physical-chemical parameters and TOC contents (Table 1) and possible losses and gains after 1h and 24h only on Cd reactive phase (Table 2). The pH decreased almost 3% after 1 h of resuspension and 5.7% after 24 h (Mann-Whitney U test; $p < 0.001$). EC presented an important increase after the first hour (42%), as also observed after 24h (43.8%), and the most significant changes were observed in transect 4 (sampling stations 10, 11 and 12) (Mann-Whitney U; $p < 0.001$). Also, conductivity was significantly correlated to Cd concentrations after 1h of agitation (Spearman; $r=-0.61$; $p < 0.05$).

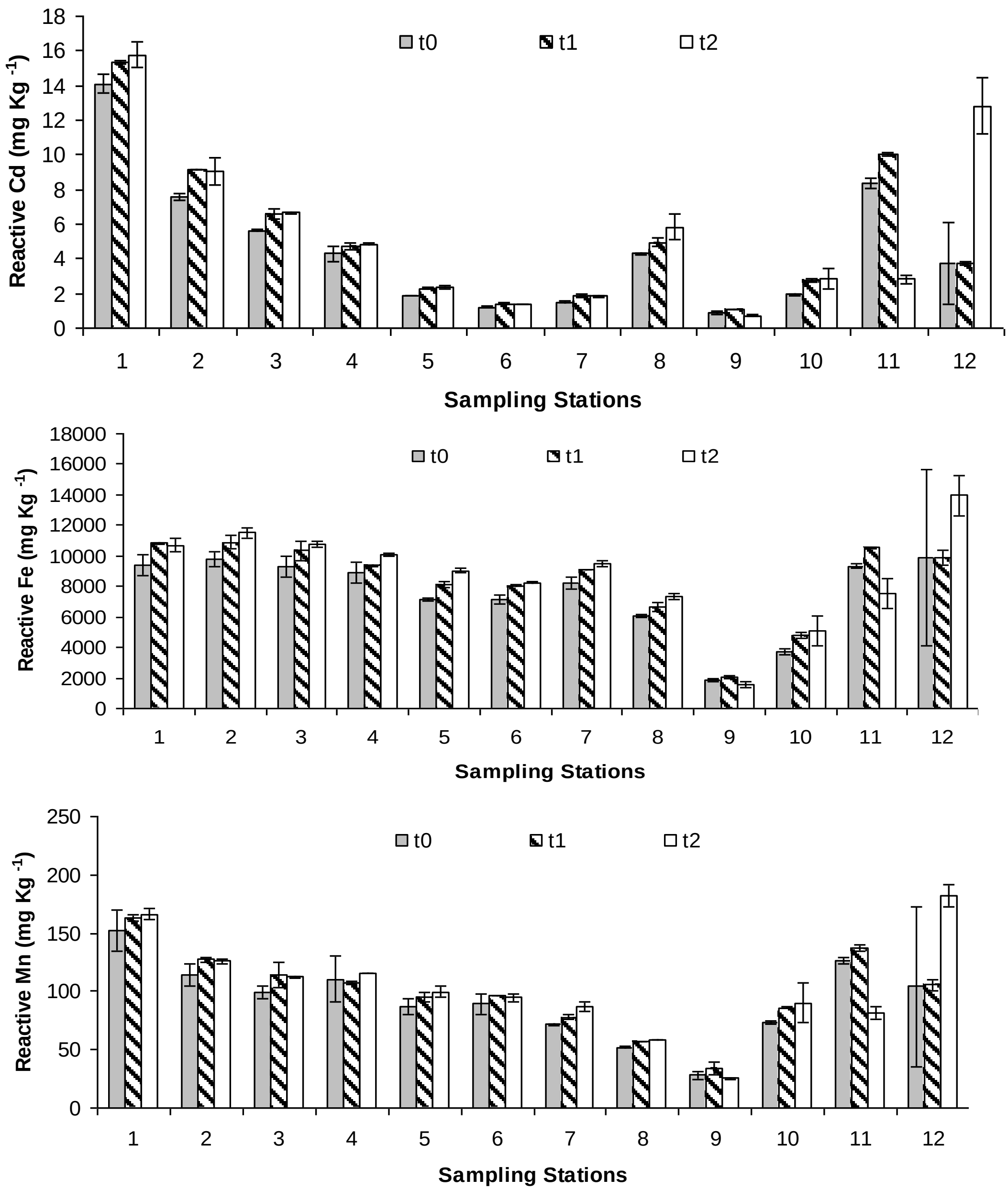

Figure 2. Reactive Cd, Fe and Mn concentrations in 12 surface sediment samples from Sepetiba bay-RJ, Brazil. t0: concentrations in sediments before resuspension; t1: concentrations in sediments after 1h of resuspension; t2: concentrations in sediments after 24h of resuspension.

Table 1. Average (± standard deviation) pH, electrical conductivity (EC) and total carbon (TC) in water; and, total organic carbon (TOC) in sediments before (t_0) and after 1h (t_1) and 24h (t_2) of resuspension in laboratory of 12 surface sediment samples from Sepetiba bay-RJ, Brazil

Stations	pH (water)			EC (water; mS cm^{-1})			TC (water; mg L^{-1})			TOC (sediment; %)		
	t_0	t_1	t_2	t_0	t_1	t_2	t_0	t_1	t_2	t_0	t_1	t_2
1	-	7.7 ± 0.0	7.5 ± 0.0	-	68.5 ± 0.9	70.3 ± 0.5	-	10.6	5.0	4.2 ± 0.1	3.9 ± 0.1	4.6 ± 0.1
2	-	7.8 ± 0.0	7.5 ± 0.0	-	68.9 ± 0.3	69.7 ± 0.5	-	9.1	5.8	3.6 ± 0.0	3.7 ± 0.1	4.2 ± 0.0
3	-	7.8 ± 0.0	7.5 ± 0.0	-	69.0 ± 0.3	68.5 ± 0.4	-	10.0	4.6	3.3 ± 0.1	3.5 ± 0.1	3.9 ± 0.0
4	-	7.6 ± 0.0	7.4 ± 0.0	-	68.6 ± 0.6	68.4 ± 0.2	-	9.4	2.4	2.7 ± 0.1	2.7 ± 0.0	2.9
5	-	7.6 ± 0.0	7.4 ± 0.0	-	69.2 ± 0.0	68.8 ± 0.7	-	11.0	5.6	2.5 ± 0.0	2.5 ± 0.0	2.9 ± 0.2
6	-	7.6 ± 0.0	7.4 ± 0.0	-	69.4 ± 0.4	70.2 ± 0.3	-	9.3	3.8	2.5 ± 0.0	2.5 ± 0.0	2.9 ± 0.1
7	-	7.6 ± 0.0	7.4 ± 0.0	-	69.2 ± 0.6	70.5 ± 0.0	-	4.5	4.5	2.4 ± 0.1	2.5 ± 0.1	2.8 ± 0.0
8	-	7.9 ± 0.0	7.5 ± 0.0	-	69.3 ± 0.3	69.3 ± 1.2	-	10.8	10.8	1.4 ± 0.0	1.6 ± 0.1	2.2 ± 0.1
9	-	7.7 ± 0.0	7.7 ± 0.0	-	69.1 ± 0.2	69.6 ± 0.3	-	9.4	6.6	0.2 ± 0.1	0.2 ± 0.0	0.1
10	-	7.7 ± 0.0	7.7 ± 0.0	-	69.0 ± 0.6	71.3 ± 0.6	-	10.1	6.8	0.4 ± 0.0	0.3 ± 0.4	0.8 ± 0.4
11	-	7.5 ± 0.0	7.4 ± 0.0	-	68.6 ± 1.7	70.5 ± 0.6	-	7.3	2.4	3.0 ± 0.0	3.1 ± 0.0	3.1
12	7.9	7.8 ± 0.0	7.5 ± 0.0	48.5	69.6 ± 0.1	70.1 ± 2.1	7.6	7.2	4.0	2.5 ± 0.1	2.6 ± 0.2	2.7 ± 0.1

Regarding TC in water, an increase of almost 20% after the first hour was observed, followed by a significant reduction (more than 30%) after 24h (Mann-Whitney U test; $p < 0.05$). An opposite trend was observed for TOC concentrations in sediments, with no significant loss after the first hour (0.24%) and a relatively accentuated increase (16.3%) after 24h, but not significantly different from t_0.

Cd concentrations in the reactive phase increased after the first hour of resuspension (ranging from 8.68%, at station 1, to 40.66%, at station 10), while Fe and Mn concentrations also increased but in lower proportions (Table 2). After 24h of agitation, there was an abrupt increase of Cd concentrations at station 12, not observed at the first hour. Strong correlations between Cd and both Fe and Mn were observed after agitation (Spearman; r=0.8; $p < 0.05$). This may be associated to Fe and Mn changes after 24h of resuspension (they became slightly more bioavailable after resuspension), suggesting a relation with the Cd dynamic.

Table 2. The percentage of gains (+) and losses (-, in italic) of Cd, Fe and Mn on reactive geochemical phase after 1 and 24h of resuspension of surface sediments from Sepetiba bay, RJ, Brazil

Stations	Campaign 2011 (Summer) (Monte et al., 2015)						Campaign 2012 (Winter)					
	1h			24h			1h			24h		
	Cd (%)	Fe (%)	Mn (%)	Cd (%)	Fe (%)	Mn (%)	Cd (%)	Fe (%)	Mn (%)	Cd (%)	Fe (%)	Mn (%)
1	6.53	6.17	3.70	*-6.34*	*-3.08*	*-7.55*	8.68	15.04	7.35	11.65	13.73	9.29
2	24.22	13.99	30.17	7.66	3.43	19.70	20.71	11.49	11.27	19.66	17.85	10.5
3	*-9.20*	0.38	3.97	*-11.53*	*-11.73*	*-9.45*	16.6	11.09	15.67	18.15	15.23	13.34
4	*-21.80*	*-0.82*	*-0.66*	*-22.10*	*-0.86*	*-3.91*	10.3	4.99	*-3.09*	12.58	13.33	4.12
5	11.65	0.59	5.22	0.58	*-8.18*	*-8.41*	21.04	13.48	8.57	23.82	25.94	14.49
6	60.01	44.78	38.46	*-3.55*	1.12	*-0.71*	14.8	12.84	8.34	11.73	15.74	5.77
7	15.20	25.27	23.22	*-1.38*	*-4.37*	5.48	22.47	10.97	8.91	20.15	15.72	21.73
8	6.95	3.49	6.30	30.49	36.22	41.73	15.71	9.69	9.54	36.22	21.24	12.09
9	16.73	19.51	12.08	12.80	12.76	10.50	23.74	9.72	19.16	*-18.69*	*-18.45*	*-9.72*
10	7.40	29.69	17.01	4.09	20.97	14.63	40.66	27.99	17.61	47.17	35.56	22.85
11	9.01	20.89	10.72	*-1.87*	5.94	0.57	20.23	13.38	8.46	*-66.31*	*-19.06*	*-35.52*
12	16.31	23.95	6.92	8.38	11.42	0.56	*-0.89*	0.05	1.23	239.15	41.44	74.48

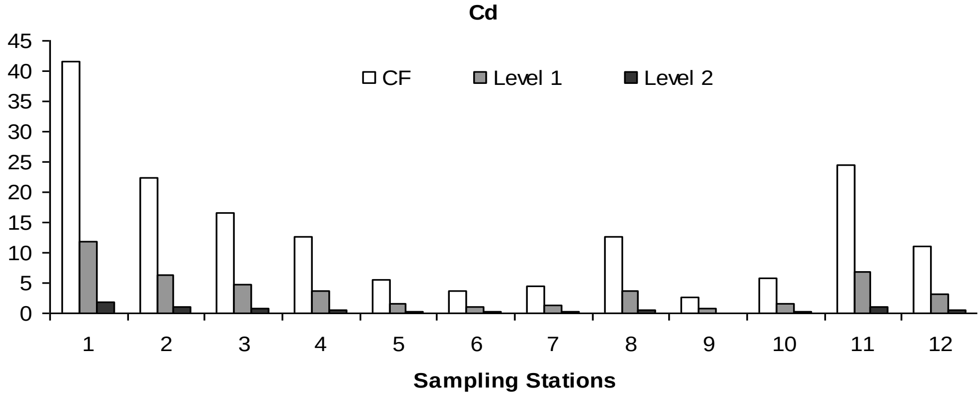

Figure 3. Contamination factors (CF) of Cd and ratios between measured Cd concentrations and Brazilian guideline values (CONAMA 454/2012 legislation: levels 1 and 2) of 12 surface sediment samples from Sepetiba bay-RJ, Brazil.

Sediment Cores

The C1 sediment core was mainly composed of fine particles (above 90%), with occasional sand influences from 15 to 35cm of depth (maximum of 85.3% of sand). TOC (maximum 9.33% at 0-5 cm and minimum of 0.3% at 30-35 cm) and metals (Cd, Fe and Mn) showed higher concentrations in the top and in the bottom sections of the sediment profile (Figure 4). In intermediate layers, where more sand contents generally occurred, metal concentrations were lower. The bioavailable percentages of Cd were very high - above 90% in the exchangeable geochemical phase. Positive correlations of Cd in the exchangeable phase with TOC (Spearman; r=0.73; $p < 0.05$; n=10), silt (Spearman; r=0.67; $p < 0.05$; n=10), Fe (Spearman; r=0.88; $p < 0.05$; n=10) and Mn (Spearman; r=0.90; $p < 0.05$; n=10) were found. In the strongly bound phase, these correlations with Cd were also observed with TOC (Spearman; r=0.77; $p < 0.05$; n=10), silt (Spearman; r=0.79; $p < 0.05$; n=10), Fe (Spearman; r=0.85; $p < 0.05$; n=10) and Mn (Spearman; r=0.87; $p < 0.05$; n=10), which suggests the influence of grain size on Cd sorption.

The C2 sediment core showed predominance of silt (above 80%), with low variability along the sediment profile (minimum of silt: 75.8% at 15cm; maximum: 95.5% at the top). The TOC content ranged from 1.81% (at 35cm) to 5.67% (at 10 cm). Unlike C1, the metals concentrations in C2 did not follow grain size oscillations (Figure 4). The highest Cd concentration (81.2 mg Kg^{-1}) occurred at 25cm, decreasing towards the top where the lowest concentration was observed (14.0 mg Kg^{-1}). The percentages of the exchangeable phases of Cd and Mn were close to 100%, while Fe was mainly in strongly-bound phases (<50% in the exchangeable phase). Strongly-bounded Cd was only correlated to Fe concentrations in this geochemical phase (Spearman; r=0.71; $p < 0.05$; n=8). Manganese showed significant correlation with Fe (Spearman; r=0.74; $p < 0.05$; n=8).

The C3 sediment core also showed a predominance of silt particles (above 70%), with two peaks of sand contents at 10 cm (73.8%) and 40 cm (83.9%). TOC contents, as observed for C1 and C2, increased from the base (0.62%) to the top (3.1%) of the profile. C3 showed higher metal concentrations at the base (Figure 4). The reactive phase of Cd was also close to

100%. The reactive Cd and Fe were significantly correlated (Spearman; r=0.82; $p < 0.05$; n=9) and, unlike for C1, the reactive phase of Cd was positively correlated to sand percentages (Spearman; r=0.97; $p < 0.05$; n=9). Cd in the strongly bound phase was only correlated to the percentage of sand (Spearman; r=0.93; $p < 0.05$; n=9).

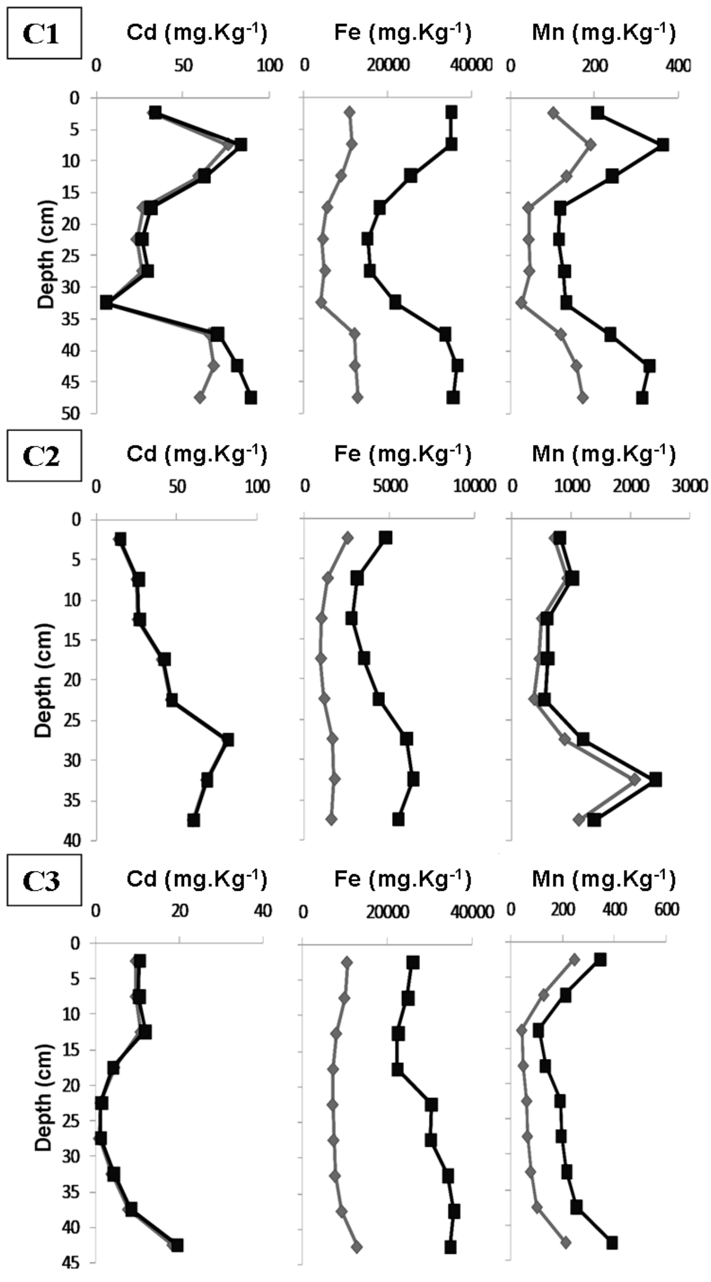

Figure 4. Reactive (losangle in gray) and strongly bound (rectangles in black) metal geochemical phases for the sediment cores C1, C2 and C3.

Table 3. Average (Ave), minimum (Min) and maximum (Max) contamination factors (CF) of Cd and ratios between measured Cd concentrations and Brazilian guideline values (CONAMA 454/2012 legislation: levels 1 and 2), considering the sediment cores samples

	C1			C2			C3		
Cd	Ave	Min	Max	Ave	Min	Max	Ave	Min	Max
CF	142.1	18.1	262.4	130.7	44.2	240.0	25.1	3.6	57.4
CONAMA Level 1	40.3	5.1	74.4	37.0	12.5	68.0	7.1	1.0	16.3
CONAMA Level 2	6.7	0.9	12.4	6.2	2.1	11.3	1.2	0.2	2.7

Background Cd concentration: 0.34 mg Kg^{-1} (Gomes et al., 2009); Cd guideline values from CONAMA 454/12 legislation: Level 1=1.2 mg Kg^{-1} and Level 2=7.2 mg Kg^{-1}.

Contamination Factors

The CF (Table 3) calculated using the maximum Cd concentration found in core C1 was up to 262 times above the background value for this area. Considering the Brazilian legislation guidelines, Cd concentrations were 74 times above level 1 and 12 times higher than level 2. For the C2 core, these contamination factors are even higher, corresponding to 240 times above background, corresponding to 68 and 11 times higher than levels 1 and 2, respectively. On the other hand, core C3 contamination factors were lower, with Cd concentrations up to 57 times above the background, corresponding to 16 and 2 times higher than levels 1 and 2, respectively. These results clearly show a concentration gradient of Cd at the study area, considering the distance from the point source.

Resuspension Experiments

A general trend of pH increasing in the first hour and decreasing at 24h of resuspension was observed (Table 4). Only for the C1 sediment core the pH decreased after the first hour. Conductivity increased at both time intervals. For TOC in sediment and TC in water, there was an apparent inverse trend for the C1 and C3 sediment cores, while it was not so clear for C2. At 0-5 cm depth and 15-20 cm depth of C1 the increase of TC in water was of 42.9% and 11.7%, respectively, after 1h of resuspension. Within 30-35 cm depth, TC decreased 35.1%. After 24h, all the tested layers showed a decrease of TC in relation to t_0 values, in average by 53.7% (maximum: 71%). For C2 and C3, this decrease was also observed, being more pronounced after the first hour (58.3%) for core C2, and after 24 h (50.9%) for core C3.

Considering the results obtained to metals determination in reactive and strongly bound phases, it was calculated the percentage in the HCl-extractable fraction (%HCl) for each metal (Table 4). The percentages of Cd and Fe in the reactive phase did not present noticeable changes after resuspension (Table 4). In the case of Cd, it was almost 100% present in the reactive phase even before resuspension, implying no changes in its geochemical partitioning.

Table 4. Average pH, conductivity, total carbon (TC) in water and total organic carbon (TOC), cadmium (Cd), iron (Fe) and manganese (Mn) percentages in the reactive phase in sediments of the three layers of sediment cores from Sepetiba Bay-RJ (Brazil), before (t_0) and after (t_1=1 h and t_2=24 h) resuspension in local water under oxidizing conditions. Samples with percentages of reactive phase more than 10% higher than t_0 are in bold and italic

Parameters		C1			C2			C3		
	Time	0-5 cm	15-20 cm	30-35 cm	0-5 cm	15-20 cm	30-35 cm	0-5 cm	15-20 cm	30-35 cm
pH	t_0	7.95	7.95	7.95	7.95	7.95	7.95	7.95	7.95	7.95
	t_1	7.84 ± 0.01	7.88 ± 0.02	7.92 ± 0.00	7.86 ± 0.01	8.17 ± 0.01	8.21 ± 0.01	7.85 ± 0.03	8.21 ± 0.01	8.27 ± 0.05
	t_2	7.70 ± 0.01	7.82 ± 0.03	7.96 ± 0.00	7.94 ± 0.01	7.47 ± 0.01	7.64 ± 0.02	7.93 ± 0.00	7.78 ± 0.01	7.82 ± 0.02
Conductivity (mS cm^{-1})	t_0	48.5	48.5	48.5	48.5	48.5	48.5	48.5	48.5	48.5
	t_1	75.2 ± 0.6	74.9 ± 0.1	74.3 ± 0.8	75.8 ± 0.2	74.6 ± 0.8	75.2 ± 0.1	76.1 ± 0.1	75.9 ± 0.0	75.1 ± 0.6
	t_2	78.5 ± 0.5	79.1 ± 0.5	78.8 ± 0.7	79.0 ± 0.3	78.6 ± 0.1	78.8 ± 0.2	78.6 ± 0.1	78.1 ± 0.9	78.5 ± 0.1
TC (mg L^{-1})	t_0	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6	7.6
	t_1	10.9	8.5	4.9	1.7	4.5	3.3	4.6	6	3.4
	t_2	2.2	5.7	2.7	3.5	2.6	4.2	3.9	3	4.2
TOC (sediment; %)	t_0	9.3 ± 0.2	2.7 ± 0.2	0.3 ± 0.0	4.5 ± 0.2	5.2 ± 0.2	2.4 ± 0.1	3.1 ± 0.2	2.1 ± 0.1	2.1 ± 0.1
	t_1	9.3 ± 0.5	1.9 ± 0.1	0.3 ± 0.0	4.6 ± 0.3	4.9 ± 0.2	2.5 ± 0.3	2.8 ± 0.0	2.0 ± 0.1	2.1 ± 0.1
	t_2	9.8 ± 1.1	1.9 ± 0.2	0.3 ± 0.1	4.4 ± 0.0	4.1 ± 0.3	3.5 ± 0.4	2.8 ± 0.3	2.3 ± 0.0	2.1 ± 0.1
Cd (sediment; %)	t_0	95.4	84.6	95.1	93.4	96.7	99.0	91.9	100	87.3
	t_1	98.1	93.8	99.9	97.0	96.4	99.0	98.8	99.2	***98.7***
	t_2	98.1	***95.2***	99.9	97.0	98.7	99.0	98.7	98.8	***98.9***
Fe (sediment; %)	t_0	31.2	30.7	18.6	52.8	27.1	27.6	40.6	32.4	22.5
	t_1	37.5	32.7	16.7	45.6	29.0	29.6	45.4	26.9	21.6
	t_2	34.9	33.2	15.7	***40.4***	23.7	29.5	42.7	29.2	23.9
Mn (sediment; %)	t_0	49.9	36.7	20.2	88.3	77.1	86.1	71.2	37.6	36.5
	t_1	***77.9***	***65.7***	***37.6***	93.6	77.6	83.4	***92.1***	***58.0***	***58.8***
	t_2	***73.8***	***66.5***	***36.3***	92.0	***64.5***	83.4	***91.2***	***60.3***	***61.8***

Applying the bioavailability change index (BCI), it was possible to observe important changes on bioavailability of Cd on the basis of C3. Changes on Fe were not observed, however the bioavailability of Mn increased (by over 20%) for most layers from cores C1 and C3. This trend was not observed in C2, where the results for the index were below 5% (Table 5).

Table 5. Bioavailability changes index (BCI, %) for three layers (0-5 cm; 15-20 cm; 30-35 cm) of three distinct sediment cores (C1, C2 and C3) from Sepetiba Bay-RJ (Brazil), after resuspension experiments (t_1=1 h and t_2=24 h). Samples with BCI above 10 or below -10 are in bold and italic

Sediment Cores	Time	Depth (cm)	Cd	Fe	Mn
C1	t_1	0-5	2.6	6.3	***28.0***
		15-20	9.2	2.1	***29.0***
		30-35	4.9	-1.9	***17.4***
	t_2	0-5	2.6	3.7	***23.9***
		15-20	***10.6***	2.5	***29.8***
		30-35	4.9	-2.9	***16.1***
C2	t_1	0-5	3.6	-7.2	5.2
		15-20	-0.3	1.9	0.5
		30-35	0.0	2.0	-2.7
	t_2	0-5	3.6	***-12.4***	3.7
		15-20	1.9	-3.4	***-12.5***
		30-35	0.0	1.9	-2.6
C3	t_1	0-5	6.9	4.8	***20.9***
		15-20	-0.8	-5.5	***20.4***
		30-35	***11.4***	-0.9	***22.3***
	t_2	0-5	6.9	2.0	***20.0***
		15-20	-1.2	-3.2	***22.7***
		30-35	***11.6***	1.4	***25.3***

DISCUSSION

The transects of surface sediments showed a clear trend of Cd distribution, being station 1 the most contaminated, as demonstrated by the contamination factor. The correlations between Cd concentrations and Fe and Mn, besides the correlation with silt, may indicate the origin linked to mining residuals or even the process of adsorption to small grain size particles, being accumulated in sediments and stabilized in oxides and hydroxides of Fe and Mn. Additionally, the relation found between Cd and total organic carbon in sediments indicates possible complexation of this element by organic matter. However, the carbon contents are not so high, as well the sulfides contents, which could maintain Cd linked to sediments (Rodrigues, 2013).

The observed Cd concentrations are considered extremely high and essentially correspond to the weakly bound fraction present in these sediment samples. Even studying only the reactive phase, these concentrations are almost 12 times higher than the Brazilian legislation guideline, which established its thresholds based on a strongly-bound extraction. Reminding that this area includes a port activity that demands periodical dredging, this environmental scenario is critical in relation to Cd pollution, since after resuspension, this contaminated material suffered considerable changes in Cd bioavailability (Monte et al., 2015).

Cd concentrations in the reactive phase increased after the first hour of resuspension and, in comparison with a previous publication discussing the data from sampling carried out in the summer of 2011 (Monte et al., 2015) (Table 2), the remobilization was more effective in the present study (sampling during winter of 2012). Possible explanations for this observation include: (i) lower pH at this second campaign; (ii) lower carbon contents, in comparison to campaign of 2011, which may result in less metal-organic matter complexes; (iii) higher control of the Fe and Mn dynamics on the Cd mobilization, also indicated by the strong correlations found between these metals, before and after resuspension.

Regarding the sediment cores, the C1 core was collected in a mangrove area, which is rich in organic carbon. The accumulation of most trace metals is favoured under reducing conditions of sediments, while Mn has a different behaviour, since it does not form stable sulphide, showing higher mobility under reducing conditions (Gueiros et al., 2003). All correlations described for C2 suggest an important function of Fe compounds (such as oxides and sulphides) in Cd sorption. Analogous to C2, C3 showed higher Cd concentrations in the core base, possibly due to the similar influence of Cação River (both cores are located in the margins of this river). Also it was observed an enrichment in Mn concentrations at the top, which may be expected due to Mn remobilization within pore waters, migration by diffusion to the surface and reprecipitation under the oxidizing top layers (Gueiros et al., 2003). In contrast, Cd presents higher mobility under oxidizing conditions and lower mobility in reducing environments, which is closely linked to its chemical affinity to sulphides (Levinson, 1980; Gobeil et al., 1997).

Rees et al. (1998) observed higher Cd concentrations in the base of sediment cores taken near the sampling point of C1 than those found in the present chapter, reflecting the tendency of recent reduction of anthropogenic sources of cadmium to the Sepetiba bay. Although this decreasing tendency is observed in more recent layers of sediment profiles, the contamination scenario is very clear for the northern region of Sepetiba bay, and there are more than 100 publications in the Web of Science related to its contamination by Cd, Zn and Hg (Gomes et al., 2009). Wasserman (2005), in his report concerning dredging activities in the bay, indicated a persistency of Cd contamination and its distribution throughout the bay, far from the original source, especially in northeast area.

Despite all the published works, this contamination remains a challenge to environmental managers, especially considering discussions regarding metal bioavailability and ecotoxicology. The high Cd concentration, especially in reactive phases, could be capable of inducing negative effects on local biota. Higher toxicity responses in surface sediments from Engenho Creek were found using acute toxicity tests with the amphipod *Tiburonella viscana* (Rodrigues, 2013) and with two shrimp species *Penaeus schimitti* and *Penaeus paulensis* (Moraes et al., 2000).

Although the sediment cores of this study were exposed to oxidizing waters during the resuspension experiment, Cd had low bioavailability changes (BCI $< 10\%$ in most samples). It is important to highlight that almost all Cd was already in reactive phases. Lacerda et al. (1987) described similarly that most metals transported by the suspended particulate matter at Sepetiba bay were in the reactive phase, using an HCl 0.1 M extraction to evaluate bioavailability (10 times weaker than the extraction applied in this chapter). According to the authors, Cd was completely in the reactive phase (100%) in suspended matter and in surface sediment, evidencing that its transport and accumulation in bottom sediments was essentially in an HCl 0.1 M-extractable form along the bay.

These high percentages of Cd in the reactive phase reflect the predominant anthropogenic contributions (especially the industrial wastes), which appears to occur as oxidized Cd input, besides the influence of activities that cause sediment oxidation, such as dredging, shipping, among others, being released in a reactive form. Additionally, when Cd is associated to oxides and hydroxides forming a stable solid compound or it is co-precipitated with hydrated iron oxides (even associated to carbonates), there is a lower risk of mobilization, in case of resuspension in oxidizing waters (Azevedo and Chasin, 2003).

Maddock et al. (2007) performed resuspension experiments with sediments from Sepetiba and Guanabara bays (SE Brazil) and found significant differences of dissolved metals only for samples from Guanabara Bay (in agreement with our results), where metals were initially bound to sulphides. The authors also found no difference of the release of metals to water after resuspension of Sepetiba bay sediments, which are not bound to sulphides. Fathollahzadeh et al. (2015) evaluated the effects of dredging at a port area in Sweden and also observed a low Cd remobilization after resuspension in laboratory.

In some cases, after resuspension, Fe and Mn showed negative BCI values, indicating a reduction of these elements in the reactive phase (i.e., they were solubilised and/or incorporated in strongly bound phases). Caetano et al. (2003) also found a decrease in Mn and Fe concentrations in HCl 6 M after 4 h of resuspension of sediments from the Tejo Estuary, Portugal.

Other authors evaluated in laboratory the effects of resuspension to metals mobility. Simpson et al. (1998) observed that anoxic sediments, when resuspended, released Cd and other metals to water, due to the oxidation of sulphides. According to Calmano et al. (1988), the sorption of metals to sediments depends not only on ionic exchanges, but also on complexation reactions, which enable stability at bonding processes on the surface of sediments. The authors observed, just as the former, during resuspension experiments with seawater, Cd remobilization to solution and bonding preference of metals such as Cd and Cu to organic matter (Forstner et al., 1989), where the organic phase could be considered an entry for these elements into the trophic chain.

Evaluating the role of organic matter on metal mobility, Cantwell et al. (2008) developed an experiment using artificial sediment, adding different TOC concentrations (0, 2, 1, 3, 5, 8 and 10%). The authors observed that Cd was released to the water column in the samples with lower TOC concentrations. Thus, the organic matter has particular relevance in the retention of dissolved metals, making them less available to the solution (Di Toro et al., 2005; Rickards, 2012; Rodrigues, 2013).

It is important to note that even a small relative change in Cd bioavailability could be of concern when the absolute concentrations are high. For instance, using a 10% threshold for the change in Cd bioavailability in the presented study area, this change corresponds to an absolute concentration of nearly 3 mg Kg^{-1} being transferred to a reactive phase in the case of core C1 (0-5 cm depth). This amount is already above the CONAMA Level 1 guideline and may lead to increased Cd toxicity to local biota. Lopes-Rosa (2011) observed that the metabolic response of the bacterial community of superficial sediments from the Engenho Creek was negatively affected after the resuspension, implying higher toxicity.

CONCLUSION

Resuspension of both surface (0-10 cm) and deeper (0-35 cm) sediment profiles altered significantly the physical-chemical conditions of water and the TOC contents of sediments, which influenced on Cd behaviour. The surface sediments had a higher Cd mobility due to stronger changes in physical-chemical parameters, which increased the Cd bioavailability, apparently linked to Fe and Mn oxides. The resuspension experiments allowed the evaluation of Cd behaviour in the case of a dredging activity in Sepetiba Bay (e.g., for remediation purposes) and demonstrated strong concerns associated to this activity, evidencing that ecotoxicological studies in association to resuspension experiments are necessary for dimensioning environmental risks.

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Chapter 13

ASSESSMENT OF THE RISK GENERATED BY NATURAL AND ANTHROPIC AGENTS ON PERUVIAN COASTAL MARINE ENVIRONMENTS

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ABSTRACT

The Peruvian coast is one of the most productive in the world due to the current system and the rich upwelling of nutrients. Therefore, it sustains the development of large and diverse populations, especially within the pelagic ecosystem. Different processes and events caused by natural and anthropic activities occur along that coast, calling for scientific research and its diffusion to provide a basis for decisions concerning this national resource. Natural environmental phenomena, such as El Niño, La Niña and tsunamis (124 over the last six centuries), have caused disasters involving millions of people and significant economic losses. The displacement of river sediment also threatens the use of water resources, although drought is possibly the phenomenon that causes most damage but the extent is difficult to quantify. It is estimated that the economic impact of this event is greater than the flooding. Among the anthropogenic risks, there are: threats posed by the exploitation of oil and natural gas from the continental shelf and from the installations sited in bays; erosion along the coastline that destroys piers and houses; and discharge of domestic and industrial wastewater. Furthermore, as the regulations for the management of solid waste are not implemented in many cities, solid waste is also a serious environmental problem. This problem should be overcome by improving prevention systems to mitigate the impacts of natural disasters and avoid the risks of anthropic need, via appropriate planning, monitoring and control of socioeconomic activities.

Keywords: coastal zone, natural catastrophes, anthropic pressures, environmental risk

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INTRODUCTION

A strategic environmental planning based on the analysis of the vulnerability of the coastal marine area, as well as knowledge of the risks that may be presented by different factors, allows for proper management of coastal resources for the economic and social development of any part of the world.

Peruvian coastal waters are known for being one of the most productive fishing areas in the world, due to the system of currents and upwelling phenomenon, which bring water rich in nutrients that sustain the development of an abundant and diverse biological community. The anchovy is the main pelagic resource that represents 90% of the total catch and which is used mostly for fishmeal and oil production (IMARPE, 2014). The coastal zone presents specific risks produced by natural agents like El Niño, which due to lack of prevention causes serious environmental damage and economic losses. During the El Niño of 1982-1983, listed as the strongest of the century (Jordán, 1985), there were losses of 3,283 million dollars equivalent to 11.6% of the annual gross domestic product (GDP) of 1983 (Vargas, 2009). During El Niño 1997 - 1998, there was a lack of an environmental assessment for the main peak of the global event. It was thus not possible to accurately describe its effects on the various resources of the Peruvian marine ecosystem, which decreased catches of the main pelagic species, mainly Peruvian anchovy (Sánchez et al., 2000), causing the collapse of the fishery and a 3.4 million dollar loss. Moreover, this event had a great impact on the Peruvian economy as a result of disasters caused by heavy rainfall, droughts and floods; losses are estimated at 1,000 million dollars, of which 800 correspond to the north of Peru due to floods and 200 due to drought in the southern highlands (Lavado-Casimiro and Espinoza, 2014).

The city of Trujillo, in the La Libertad Region, also suffered from the lack of water resources, leading to the setting up of the CRIS USAID project (MINAM, 2014). The 1997-98 El Niño caused an estimated 3,000 million dollars damage, equivalent to 6.2% of annual GDP 1998 (Vargas, 2009).

Another major problem is the erosion of the coastline, a process that is occurring worldwide in 70% of sandy beaches (Bird, 1985), as a result of the intensification of ocean storms (Zhang et al., 2004), fuelled by the rise of sea levels caused by the melting of the polar regions. However, coastal erosion is also caused by human activities such as ventures in ports and which may be manifested even at a great distance from the point of origin.

This chapter provides a review of the main risks to which the marine coastal environment of Peru is exposed, whether they are naturally occurring or the result of anthropic activities. For this the most recent publications were consulted regarding recurring natural events affecting coastal and marine ecosystems and where human activities generate risk situations.

DATA COMPILATION

Documentation and technical regulations of various public and private sectors involved in the assessment and risk management, as well as publications of international institutions and non-governmental organizations were reviewed for the preparation of this chapter.

The first step was to determine natural events or human activities that threaten coastal marine ecosystems by the high degree of vulnerability. As a second step, each of the risks was described with examples that occurred recently, reflecting the current situation of the national contingency when facing these facts.

STUDY AREA

The marine coastal area is an environmentally sensitive and economically valuable area with a rich biodiversity and benefits such as a reliable source of food and a range of opportunities for the production of renewable energy.

The Peruvian sea is under pressure from industrial activities, transport, trade, energy processing, tourism, recreation, whilst also playing an important role in our cultural heritage. The important role of the services and goods produced by export aquaculture, artisanal fisheries and the extraction of benthic resources should also be recognized as the environmental threats they represent.

The coastal zone of Peru, according to the Brack's (2000) ecosystemic classification, is shaped by the Tropical Sea, the Pacific Ocean's Tropical Forest, the Dry Equatorial Forest, the Pacific Desert and the Cold Sea. The first three ecoregions mentioned are present on the north Peruvian coast. The Tropical Sea is characterized by its warm waters and the presence of equatorial and tropical currents. The latter come from the Central American Pacific Ocean, which is characterized by the existence of mangrove forests along its coast, continuing from the Colombian Pacific Ocean down to the northern coasts of Peru.

The Dry Equatorial Forest is also present in the northern area, where 7 different types of habitats, shaped by tidelands (irrigation channels and creeks), have been identified; a great extension is occupied by prawn farms, which were the most serious threats to the area in the 70s. This ecoregion includes, besides Tumbes, the coast of Piura and Lambayeque, with the presence of dune landscapes where forests of carob-trees and ceibos predominate. In this ecoregion are located the most southern mangrove forests of the Southeast Pacific, threatened by the waters of the river Piura that contain untreated waste water discharges from Piura city and the populations settled on its banks.

The most extensive and rich ecosystem is the Cold Sea, because the Peruvian Current or Humboldt Current that runs from the south to Piura is an area of great primary productivity. It is the basis of a food web of marine biodiversity, which provides a resource of great socioeconomic importance for the country based mainly on the capture of anchovies.

NATURAL ENVIRONMENTAL RISKS

Hydrometeorological Events El Niño/La Niña

The overall natural climate cycle known as El Niño-Southern Oscillation (ENSO) has two phases: a warm one known as El Niño, and a cold phase known as La Niña, called this way because it presents opposite conditions to El Niño (CENEPRED, 2013).

El Niño and La Niña are an obvious example of the natural oscillations occurring in the region and are an essential part of a much vaster and more complex system of climatic fluctuations. La Niña is characterized by the presence of long-lasting cold temperatures, unlike El Niño which is characterized by unusually warm ocean temperatures in the equatorial Pacific Ocean (CENEPRED, 2013). The warm phase causes local flooding due to heavy rains and during which warm fronts come together on a general scale for months creating intense rainfall that saturates the soil on a local scale.

Flooding causes many disasters all over the world, affecting millions of people with significant economic loss and has been increasing in recent years. In 2010, about 178 million people were affected by floods (Table 1). The regions most affected are urban areas and include roads and irrigation infrastructures which suffer due to poor maintenance and construction type. Associated with this phenomenon are the mudslides and landslides that occur at the headwaters of rivers and streams because of the pore pressure caused by water infiltration in a loosely cohesive, friable soil, creating large deforested areas in the northern and central coastal regions of Peru.

The "El Niño" events of 1982-83 and 1997-98 affected many cities in northern Peru. Sullana, Piura Region, was one of the cities most affected. Not only infrastructures were affected, but also agriculture and industries suffered significantly. It is estimated that the country lost approximately 3,283 million dollars in the 1982-83 event and 3,500 million dollars in the 1997-98 El Niño (Vargas, 2009).

A project called "*Aliados ante inundaciones: Gestión de riesgos y resiliencia ante inundaciones de Piura y Lima*" has been established as a tool for risk management of the floods caused by such events in Piura and Lima in order to reduce vulnerability to flooding in the basins of Piura and the Rimac (Figure 1). This works by promoting a greater integration of the various entities for flood management, a more scientific approach to construction and other institutional approaches.

Another key point is the operation of the emergency systems and the Early Warning System, which, in the case of El Niño, is sometimes able to detect the phenomenon in advance. This measure helps to reduce losses by the implementation of emergency measures prior to the event, as occurred in 1997-1998. The Civil Defense System issues a "stay on alert" warning upon the occurrence of external geodynamic phenomena and promotes the implementation of contingency plans (MINAM, 2014).

Table 1. Positive and negative impacts of El Niño in Peru

Positive impacts	Negative impacts
Emergence of other pelagic species.	Accelerated glacial retreat.
Increased rainfall and air temperature favoring the development of rice farming on the coast.	Loss of farmland.
Heavy rains in a strong El Niño event favor the natural regeneration of dry forests on the north coast.	Silting of reservoirs.
The emergence of temporary pastures on the north coast is important for livestock.	Land salinization.
Excessive rainfall favors restocking groundwater reserves.	Destruction of productive infrastructures (irrigation channels, intakes, dams, etc.).
Higher sea temperatures during the fall and winter favor a decrease in the intensity of frost in the central and north highlands.	Destruction of roads and collapsing of bridges.
	Death or migration of some plant and animal species.
	High probability of occurrence of forest fires due to high temperatures.
	High temperatures impact livestock (low production of meat and milk).
	The destruction of basic sanitation infrastructures.
	Displacement of schools of anchovy to deeper zones, which cannot be compensated by the presence of a new species.

Source: SENAMHI (2014).

Figure 1. Heavy rain causing landslides and floods in Piura highlands in January of 1998. (Photo: A. Indacochea).

While the cold sea conditions during La Niña favors anchovy, it also causes a wider dispersion of this species away from the coast, which does not benefit the fishermen as they must thus travel further than usual to capture the resource (Table 1). Furthermore, a decline in landings of other fish species such as hake, mackerel, horse mackerel and sardine and other fishery resources such as squid, octopus, scallops, etc. (CENEPRED, 2013) has been observed.

Tsunamis

Over the past six centuries, 124 tsunamis have been registered in Peru and the most disastrous were caused by earthquakes. In 1586, an 8.6 Mw magnitude earthquake occurred off the coast of Lima, causing waves up to 26 meters high which completely destroyed La Punta (Callao) and entered 10 km inland. The tsunami spread and flooded all the beaches from Tacna to Piura. In 1604 there was an earthquake with a magnitude of 7.8 Mw causing 16-meter high waves, causing widespread destruction mainly in the ports of Pisco, Camana, and Arica (Carpio and Talavera, 2002). In 1687 a tsunami resulting from an 8.2 Mw magnitude earthquake that occurred south of Lima, seriously affected the ports and towns of Chimbote, Callao, Lima, Cañete, Chincha, Pisco and Camana. Nearly 500 people died, 300 of them in Callao (Carpio and Talavera, 2002). There was an earthquake west of Lima with an 8.6 Mw magnitude in 1746 that generated waves of up to 24 m in height, killing between 5000-7000 people, mainly the inhabitants of Callao, Chorrillos and La Herradura (Carpio and Talavera, 2002). In 1868, an earthquake of magnitude 8.8 Ms in the town of Arica in Chile had several aftershocks that resulted in tsunamis with waves up to 18 m high, destroying much of the Peruvian and Chilean coast and killing 300 people in Arica and 30 in Arequipa (Carpio and Talavera, 2002). In 1960, an earthquake that occurred off the coast of Piura with a magnitude of 6.8 Mw produced 9 m high waves, causing severe damage to the ports of Eten and Pimentel and Caletas in Santa Rosa and San Jose (Carpio and Talavera, 2002). Finally, in 2001, an earthquake of 8.4 Mw magnitude, with its epicenter in the city of Ocoña in Arequipa, created a tsunami that also affected the populations of Ocoña, Camana Quilca and Matarani, causing the destruction of houses in Camana and penetrating more than 1 km inland (Carpio and Talavera, 2002).

Sediment Transport in Rivers

The mountain basins of the Peruvian coast suffer intense physical-chemical soil degradation. They are also the main source of water for 65% of the Peruvian population (Morera, 2014). The main and largest water projects in Peru are located in this region. For these reasons the use of such water resources (by the population and for hydroelectricity, irrigation, etc.) is at risk due to high loads of river sediment, which affect aquatic ecosystems, reduce the lifetime of waterworks and even contribute to social conflicts due to the decreasing availability of good quality water (Morera, 2014).

A further problem is that, naturally, due to the characteristics of the Andes that have a polymetallic formation (Soler and Lara, 1983; Tumialan, 2004), many rivers carry clay sediments with a load of metallic waste.

In this regard, it is important to consider the variability of the contributions of the sediments brought by the 53 Peruvian coastal rivers, especially in times of flooding as a result of alternating wet and dry periods throughout the year. The increased use of water due to population growth and farming must be considered, as well as the fact that sediment transported from agricultural land or mining areas is also associated with carrying traces of heavy metals (Sánchez et al., 2010).

Drought

The variation in water resources makes difficult to quantify the millions in losses that often occur during a period that may last several years. Also, the associated intangible losses are many and very diverse (ANA, 2010). It is likely that drought is the phenomenon that causes most damage, but it is difficult to be certain due to such a challenging quantification. However, it is estimated that the economic impact of drought is greater, in general, to flooding, even though the latter is of greater intensity and higher losses are registered (ANA, 2010). In 2011, drought events affected areas of the coast such as: Lambayeque, Piura, La Libertad, Lima, Moquegua and Tacna. The consequences of this drought were the loss of crops, livestock mortality and the proliferation of pests and diseases. This mainly affects small farmers and urban populations, and the production of electricity (ANA, 2012).

The Risk Management Plan and adaptation to climate change in agriculture, PLANGRACC-A (2012) (FAO support) has identified five areas on the coast as being of low risk of drought, including Lima; 12 regions of medium risk, including, Ancash, La Libertad, Piura and Tumbes; and 3 regions of high risk: Ica, Lambayeque and Tacna (ANA, 2012).

Regarding the use of water, the trend is growing as sectors requiring water are increasing, as shown in the growth of the population and GDP of the Piura region (from 1997 to 2010 economic activity increased by 70%). The latter is thanks to an increase in economic output in the unregulated basin: intensive agriculture, fisheries, industries associated with the fishing industry and associated with the ports (ANA, 2012). It should be noted that the valleys of Zaña, Chancay, La Leche, Motupe and Olmos that supply the major export crops in the region, have below average discharges and the reservoir that supplies Tinajone's 15 irrigation committees is only working at 30% of its storage capacity. In the Piura River, the biggest use of water is for irrigation, which consumes 80% of the water in the lower reaches of the Piura basin (ANA, 2012).

Soil Salinization

There are two major sources of soil salinization. One is a consequence of drought that favors seawater intrusion in coastal areas, causing the salinization of groundwater and wells as well as degrading soils. This saturations affect agricultural production, mostly in the case of the lower basins of the Piura and Chira rivers, and generates health problems in the local population (ANA, 2012).

Another cause of soil salinization on the coast is the use of irrigation in the upper reaches of the valley. Ramírez et al. (2007) showed that there is a slight soil degradation in the lower basin of the province of Santa, Ancash Region, namely in the agricultural area of Samanco Valley, due to the effect of salinity and poor drainage (Figure 2), leaving the land unfit for agriculture. However, the positive influence of the Special Irrigation Project (CHINECAS) has led to soils being recovered in the agricultural sector and has changed land use by creating conditions for permanent crops or grazing.

Figure 2. Photo illustrates agricultural land affected by salinization caused by the lack of drainage of the irrigation system in Samanco, Nepeña Valley, Ancash Region (Ramirez et al., 2007).

ANTHROPOGENIC ENVIRONMENTAL RISKS

Exploration and Exploitation of Oil and Natural Gas on the Continental Shelf

Licenses have been authorized along the Peruvian coast for this activity, and although they employ technologies used quite successfully in other countries, there still remains a risk of accidents, with varying degrees of threats to marine and coastal ecosystems. There are 18 sedimentary basins with hydrocarbon potential in Peru, seven of which are located in the coastal marine area (Perupetro, 2015). The hydrocarbon extraction activity takes place mainly along the coastal strip and on the continental shelf. There is also a significant flow of tankers carrying petroleum products from refineries in La Pampilla and Conchan to fuel storage tanks located along the coast, for distribution to cities or places with intense industrial, fishery or port activity, water sports, etc. The third refinery on the Talara coast is supplied through an oil pipeline in the Peruvian jungle.

The northwest coast of Peru is the oldest area of oil exploitation in the country with the province of Talara including the oil centers of La Brea, Pariñas, Lobitos, El Alto, Talara and Los Organos. This area constitutes a high-risk zone for oil spills. These and other marine coastal areas where there are refineries and fuel storage tanks are monitored periodically (Figure 3).

An example of these risks is the burning of the BAP Supe oil tanker on January 30th of 2008 off the coast of Zorritos, Tumbes, creating an oil spill (Sánchez et al., 2010). There was another spill of oily waste in the sea at Zorritos that occurred on January 20th, 2016 from a tanker at the La Cruz pier. A total of 550 L was spilled and the Contingency Plan of the Navy of Puerto Zorritos was immediately activated.

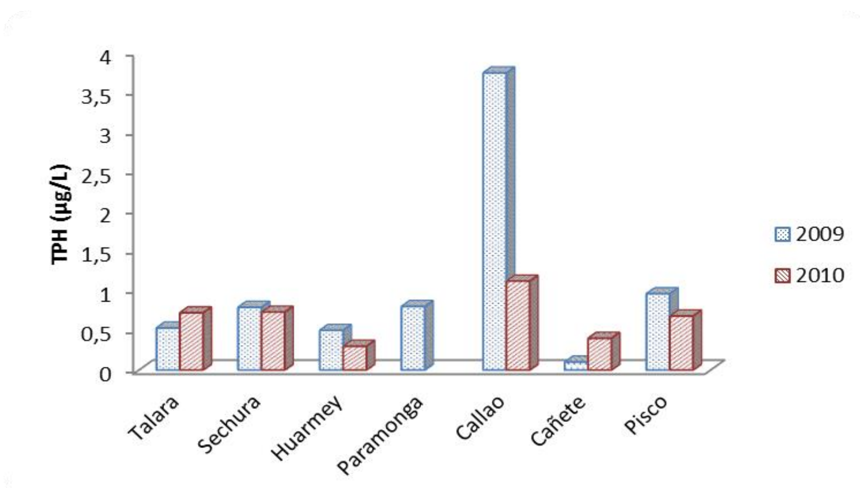

Figure 3. Total petroleum hydrocarbon (TPH) concentrations in the seawater and coastal areas of Peru (Sánchez et al., 2010).

Gas exploitation takes place primarily in the jungle of Peru. Gaseous products travel through pipelines to the coast where fractionation plants are located in Pisco and Cañete, with a processing capacity of 128 thousand barrels per day and a storage unit for natural gas liquids (NGL). The other liquefied natural gas is the Pampa Melchorita, Cañete, Lima Region, with an estimated annual production of 4.4 million tons. In each of these plants port terminals have been built for export of natural gas. This activity can be a risk for the development of tourism and recreation, mainly in Paracas Bay in Pisco, and in the case of Pampa Melchorita, Cañete, and the impact would affect artisanal fisheries.

The Erosion of the Coastline

The wind, waves or tidal currents carry away soil stripped from the cliffs, such that coastal inclines may suffer a considerable loss of material. This is what is occurring on the northern coast of Peru, with the inherent risk of part of the beaches or towns located within the coastal strip disappearing. However, in most cases it is the result of human activity, such as: construction, building docks, marinas, piers, breakwaters and embankments or even just with the intention of retaining sediment particles. Such erosion has changed the size of sandy beaches, sometimes transforming them into narrow pebble beaches and causing the destruction of coastal embankments.

In the Bay of Paita a port terminal is located, and 10 km north is the beach of Colan, where houses have been built in the intertidal beach area near the mouth of the Chira River (Figure 4). The study by Rondón (2011) with data covering 61 years (1946-2007) shows a clear trend towards sedimentation of a large area in Colan since 1992 to 1999, reflecting the 1997-1998 El Niño event.

Figure 4. Colan Beach, Bay of Paita, Piura Region, showing strong sedimentation caused by natural events such as El Niño 1997-98 (Photo: A. Indacochea).

The sea erosion on the coast of Trujillo extends from the Port of Salaverry to the beach of Buenos Aires (Guerrero-Padilla et al., 2013). It has been caused by the construction of a 1 km long breakwater at the Port of Salaverry, with the objective of preventing the natural movement of sediment from the south to the north. That breakwater (Figure 5) was not an effective solution for the port terminal and has destroyed the main resorts of Trujillo. It is estimated that 60 million m^3 of sand have accumulated in the Port of Salaverry as a result. According to Veneros et al. (2012), the Trujillo beaches have been negatively impacted, primarily by a combination of factors such as: destruction of the sandy beaches, retreat of the coastline, destruction of the ecosystem of wetlands and dunes that have triggered similar erosion processes, and sedimentation.

It is estimated that between 1976 and 1997, the average erosion of the beaches of Las Delicias and Buenos Aires (Figures 6 and 7) was 3.2 m/year, with a maximum erosion of 7.6 m/year (160 m erosion in 21 years of Las Delicias beach) and a sedimentation rate of 5-10 m/year at the Las Delicias beach during the 1995 (Veneros et al., 2012).

Figure 5. Satellite view of Salaverry Terminal Port, Peru, in January 2015, with about 600 meters of sand accumulation. Strong erosion is evident from the Las Delicias beach (Image from Google Earth).

Figure 6. Heavy erosion on the beach in Buenos Aires, Trujillo. Photo taken in August 2010. (Bocanegra, 2014).

Figure 7. Protection infrastructure with rocks to prevent erosion and destruction of the houses on the beach in Buenos Aires, Trujillo. Photo taken in September 2015 (Photo: F. Benites).

Discharge of Domestic and Industrial Wastewater

On the Peruvian coast, wastewater resulting from the combination of water residences, offices, commercial buildings and institutions, together with waste from industries and agricultural activities. The dumping of domestic sewage along the Peruvian coast is done after some treatment. In Lima and Callao, 20 m^3/s are generated per drain. One of the primary treatments, performed in the Wastewater Treatment Plant Taboada (PTAR Taboada), started to operate in 2013, with a treatment capacity of $14 m^3/s$ that serves 27 districts, equivalent to 75% of the sewage produced in the northern and central districts of Lima and Callao. This treatment plant has a submarine emitter, which is 3 m in diameter and 3.8 km offshore from Oquendo, Callao Bay.

In Lima, 40% of the wastewater of southern zone is treated by 4 PTARs: San Juan de Miraflores, Villa El Salvador, San Bartolo (the largest but only works for 50% of its capacity) and La Chira. These four PTARs comprise the San Bartolo project that aims to use the wastewater treated to irrigate desert lands, cultivated fields, parks and berms (the Pan American South Highway).

In the city of Huacho, located in Huaura province, 150 km from Lima with a population of 55,442 inhabitants (INEI, 2007), the municipality's wastewater is discharged directly into the sea without any treatment. The Huaura River, polluted by domestic and industrial wastewater from the upper basin, discharges into Caleta Carquín, which is also part of Huacho (Sánchez et al., 2010). The Municipal Water and Sewage Company of Huacho (EMAPA-Huacho) is in charge of two continuous domestic water discharges: one is downriver from Huaura, with an average discharge flow of 3.2 m^3/s, and the other is near Puerto Huacho with an average discharge flow of 2.4 m^3/h. However, the situation is considerably better at Pisco, which is a coastal city 260 km from Lima and has a population of 125,879 inhabitants. The wastewater from Pisco and San Andrés is treated at two anaerobic lagoons, two facultative lagoons and two maturation ponds, using biological treatment without any chemical agents. The treated water from the Municipal Water and Sewage Company (EMAPISCO) that is responsible for the collection, transport, storage, treatment and disposal of wastewater, is discharged into the Pisco River; however, tourism and recreational beaches located south are not at risk of contamination (Sánchez et al., 2010).

Solid Waste

Waste such as paper bags, candy wrappers, etc. produced by the inhabitants of urban and rural areas, end up in the sea after being transported by wind, rain water and even rivers, becoming marine litter. The bulkier group is comprised of domestic waste mixed with organic waste. This solid waste impacts the coastline by becoming marine debris that is carried far out to sea by runoffs from the land or in river water.

Between 17,000 to 18,000 tons of waste (Figure 8) are produced in Peru, daily. Lima and Callao alone produce around 9,000 tons each day and only 25% is treated (MINAM, 2009). This environmental problem is mainly dealt with by local governments that try to comply with the General Law of Solid Waste (Law 27314 year 2000) and its regulations (DS 057-2004/PCM) in order to solve the poor management of solid waste. This Act establishes the Integrated Environmental Management Plans for Solid Waste (PIGARS) to be implemented by the municipalities, but by the year 2008 only 25% of local governments had developed their PIGARS, mainly comprised of coastal municipalities.

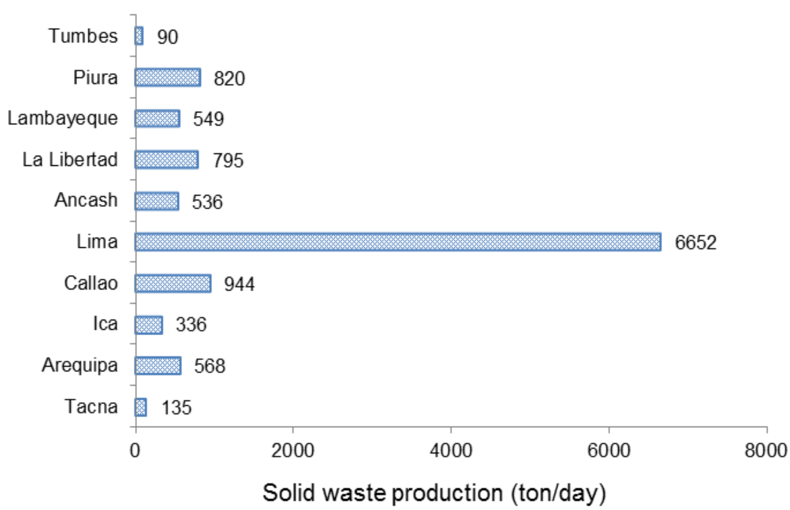

Figure 8. Production of solid waste in tons per day in Coastal Regions of Peru during 2008 (MINAM, 2009).

Peru requires approximately 100 landfills to store the waste that people produce. However, there are only ten such waste storage facilities in the country, which indicates a considerable deficit. This problem leads to the majority of municipalities to use solid waste dumps, many of which are located in areas close to population centers. Lima is located near five of the ten landfills in the country: Huaycoloro, located in the province of Huarochirí; Portillo Grande in Lurin; Ancón; Modelo of Callao in Ventanilla and the Zapallal in Carabayllo (MINAM, 2014).

Meanwhile, in Lima there are 14 districts with selective collection services operated by solid waste recyclers such as: downtown Lima, Barranco, Chorrillos, San Juan de Miraflores, Villa Maria del Triunfo, Villa El Salvador, Comas, La Victoria, Pachacamac, Puente Piedra, Punta Hermosa, San Juan De Lurigancho and San Martin de Porres; 9 cities have this service in the Piura Region.

The presence of marine debris at sea poses risks to marine life and has serious effects on ecosystems due to changes in behavior and life cycles, increased amounts of disease, including the presence of viruses, bacteria, protozoa among other causative agents of death affecting species of fish, turtles and marine mammals.

CONCLUSION

Peruvian coastal marine ecosystems have been impacted by natural (El Niño, tsunamis, droughts, loads) and anthropogenic (salinization, erosion, solid waste, domestic an industrial wastewater) factors that threat their environmental quality, reduce the biodiversity and represent a risk for human health.

Since the ENSO events are recurring phenomena an economic allocation should be considered in the annual budget of the nation, within the Program for Disaster Prevention, which deals with the most sensitive and vulnerable critical points in coastal environments, mainly focused on infrastructure and capacity building.

The Peruvian coast has often been hit by tsunamis. Despite advances in international early warning systems, Peru does not have a warning system that efficiently covers the entire coastline.

Erosion on the Peruvian coast calls for studies to identify the causative agents, using time series on factors such as ocean currents, waves, climatic variations and human actions which have altered the geomorphology of a given coastal area.

The problem of salinization of agricultural land can be avoided with the use of technical irrigation and improved drainage systems, in addition to a proper selection of the type of crop.

Reports from the monitoring conducted by Sánchez et al. (2010) have determined an increase in pollution by domestic and industrial wastewater in some bays, such as: Végueta, Carquín, Huacho and Chancay with the inevitable risk of food safety and the populations' health due to severe contamination. It is important to increase the installation of wastewater treatment plants in coastal cities, which allow reuse of treated water or discharge to water bodies by technically designed emitters, avoiding sensitive areas such as those used for aquaculture and artisanal fisheries.

There is an urgent need to reverse the deficits in infrastructures, human resources and funding in the short term. It is also necessary to increase the number of landfills, encourage recycling, promote environmental education, and develop proposals for funding and strengthening cooperation and technical assistance for local governments in order to facilitate a better management of solid waste.

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Chapter 14

ASSESSING THE ECOLOGICAL EFFECTS OF CONTAMINANTS IN ESTUARIES IN BRAZIL: GAPS AND FUTURE DIRECTIONS

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ABSTRACT

This chapter reviews published papers from the last 15 years that estimate the effects of contamination in Brazilian estuaries. The goals were to identify (i) the main classes of contaminants studied in estuarine systems in Brazil; (ii) the most used lines of evidence (LOEs) to estimate the effects of contaminants; (iii) the most common experimental designs and statistical analysis; and (iv) the major gaps in the field and suggest future directions. A total of 130 papers were reviewed. Metals were the most measured contaminant (34.6%) followed by organic contaminants (13.1%). Most papers (56.9%) only used one line of evidence (LOE) and the most common LOE were bioaccumulation (40.8%) followed by media chemistry (39.2%) and biomarkers analysis (33.8%). Most papers (59.6%) did not report the salinity range of the studied system, had three or less spatial replicates (56.4%), and did not include temporal replication (48.1%) or reference sites (52.6%) in their design. The most used analyses were ANOVA (44.6%) and correlation (26%). Some suggestions for future studies are: (i) consider the salinity range of the studied sites when planning the experimental designs; (ii) include appropriate reference areas; (iii) clearly state the question and the null hypothesis to be tested, (iv) evaluate the possible effect of covariates when performing bioaccumulation and biomarker studies; and (v) include more than one LOE in the design if possible.

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Enhancing the collaboration among scientists in different states and regions of Brazil may reduce the gap in our knowledge of the effects of contaminants in many estuaries.

Keywords: ecotoxicology, estuary, pollution, experimental design

INTRODUCTION

Estuaries are among the most degraded coastal ecosystems on the planet (Agardy et al., 2005). The human impact on these systems has led to the depletion of populations and extinction of estuarine species in different parts of the world (Lotze, 2006). As a consequence, many of the goods and services provided by these highly productive environments, such as viable fisheries and provision of nursery habitats, are threatened (Worm et al., 2006).

Estuarine systems are subject to a wide variety of human activities as they are sites of port, industrial, urban, aquaculture, and recreational development. As a consequence, they are exposed to many different point and non-point sources of contaminants such as oil spills, untreated sewage discharge, urban and agriculture run-offs, and air pollution deposition (Kennish, 2002). They often serve as sinks for different types of contaminants that accumulate in the organisms and sediments, and cause adverse effects to the biota (Chapman and Wang, 2001; Oliveira et al., 2016). Estuarine pollution is a major concern in tropical emerging countries due to the rapid change in land use, agriculture expansion, and economic and population growth (Kennish, 2002). Estuaries will continue to degrade under increased demands unless proper management is applied to maintain their integrity and function (Rapport et al., 1998). For this reason, robust environmental impact studies are necessary to evaluate the status of estuaries and improve conservation efforts.

In Brazil, estuaries are the most abundant coastal environment, covering an area of 6.696.787 ha, that is 43.7% of the available coastal habitat (MMA, 2010). Due to the country’s long coast line (approximately 7,400 km), estuaries can be found in a variety of climates such as humid and semi-humid climates in the south and southeast and semi-arid regions in the north Atlantic coast (Souza and Knoppers, 2003). The climate also influences the presence and distribution of mangroves (Schaeffer-Novelli et al., 1990) and salt marshes. Most of the population, ports and heavy industries are located in Brazilian estuaries and bays, and as a result, pollution is heavily concentrated in these areas (Diegues, 1999).

This chapter reviews the most commonly used approaches to estimate the effects of contamination in Brazilian estuarine systems for the last 15 years. Our goals are to identify (i) the main classes of contaminants studied in estuarine systems in Brazil; (ii) the most used lines of evidence (LOEs) to estimate the effects of contaminants; (iii) the most common experimental designs including sampling size, spatial and temporal replication; and (iv) the major gaps in the field and suggest future directions to researchers in Brazil.

METHODS

A literature survey was conducted using the *ISI Web of Science* search platform for the keywords estuar* followed by contaminant*, pollut*, ecotoxicol*, ortoxicol*. Only papers conducted in Brazil and authored by Brazilian authors between 2000 and 2015 were included in the analysis. The studies considered in this chapter must have been conducted in an estuarine system and aimed at identifying possible effects of contaminants in the environment. Studies conducted in mangroves were included. In this sense, papers that conducted bioassays with estuarine organisms to estimate the effect concentration of a selected contaminant, or only measure the concentration of a chemical in the environment were excluded. However, papers that only measured the concentration of contaminants in estuarine organisms were included because of the possible metabolic, energetic and long term costs associated with bioaccumulation and thus considered as an effect *per se*. Papers that evaluated a species potential as a biomonitoring tool were also included.

All papers that met these criteria were subsequently reviewed and summarized in a database according to the following groups: (i) general information, such as the name of the system(s), region(s) and state(s) where the study was conducted and what LOEs were used; (ii) statistical analysis and experimental design (e.g., spatial and temporal replication, sample size, number of reference areas and statistical methods); (iii) measured contaminants, (e.g., type of chemical and environmental media); (iv) species and levels of organization (e.g., species or group of species analyzed); and (v) bioassays (e.g., species and endpoints used in the bioassays).

Whenever some of this information was unclear or not presented in the text, tables or figures of a paper, it was classified as “not reported.” In order to estimate how many papers were published in one geographic area, studies conducted in different rivers within the same bay or estuarine complex systems were combined. For instance, studies that were conducted in the Subaé, Jaguaripe and Paraguaçu estuaries within Todos os Santos Bay, BA, were counted and grouped under the same bay. Studies that evaluated sites across more than one estuarine system, including reference areas, were separated by estuarine system in the analysis, unless the reference area was only used in bioassays. If the design was unbalanced, sample size was estimated as the mean sample size per treatment. If the sample size was only reported as the minimum and maximum number of samples (e.g., higher than 5 and lower than 10) it was considered as not reported, as the mean sample size cannot be precisely estimated.

The salinity range of the sampling stations reported in each study was recorded in order to estimate the most frequently studied estuarine salinity regions in Brazil. Categories were defined as oligohaline (0.5-5), mesohaline (5-18), polyhaline (18-30) and euhaline (30-40) following the Venice System (Anon, 1959). In cases where the study was conducted in more than one system and the salinity range was reported for both systems, they were both included in the analysis. The number of reference sites was analyzed separately from the spatial replicates within each system.

Six broad categories of LOEs were considered: (i) chemistry, in which contaminants are measured and estimated in samples of water, sediments, and/or porewater; (ii) bioaccumulation, in which the concentration of contaminants are measured in tissues of organisms; (iii) biomarkers, including a wide range of biomarkers such as enzymatic activities and histopathological lesions; (iv) community and/or population structure, in which measurements are taken from *in situ* samples of natural populations and/or communities; (v) bioassays, controlled laboratory experiments in which organisms are exposed to an environmental media to assess the possible effects of contaminants in predetermined endpoints; and (vi) manipulative field experiments. Due to its broad definition, biomarkers were further classified into biochemical biomarkers (e.g., enzymatic activities and metallothionein), hematological parameters, histopathological lesions, imposex, and genotoxiceffects (e.g., DNA damage and micronucleus) to quantify which are the most commonly used in Brazil.

The contaminants measured by each study were classified in four broad classes: (i) organic contaminants, such as polycyclic aromatic hydrocarbons (PAHs), aliphatic hydrocarbons (HA), benzene, polychlorinated biphenyls (PCBs) and pesticides; (ii) metals and metalloids (such as As); (iii) nutrients, and (iv) biological, pathogens such as *Escherichia coli* or fecal steroids such as coprostanol and coprostanone. Each measured element and/or compound was recorded to evaluate the most studied among each class of contaminants. Contaminants measured in the chemistry and bioaccumulation LOEs were pooled together in order to estimate what are the contaminants of greater concern to ecotoxicologists studying Brazilian estuaries.

RESULTS

Out of the 549 papers initially sampled by the literature search, only 130 papers met the criteria for inclusion. The number of papers generally increased over time with a peak in 2013 with 24 papers (Figure 1). The 130 papers were published in 49 different journals, with eight journals accounting for 50.8% of the total (Figure 2). Marine Pollution Bulletin was the journal with the highest number of publications (17) and with the highest impact factor (2.991) among the eight journals.

The studies were conducted in 49 different estuaries along the Brazilian coast with a total of 190 samples in these systems. Santos/São Vicente Bay (São Paulo state - SP) was the most studied system with the highest number of papers, followed by Paranaguá Bay (Paraná state - PR) and Guanabara Bay (Rio de Janeiro state- RJ), with 30, 21 and 15 papers respectively (Figure 3). In the Northeast region, Todos os Santos Bay (Bahia state - BA) was the most studied system with seven papers, followed by Goiana Estuary (Pernambuco state -PE), Itamaracá (PE) and São Marcos Bay (Maranhão state - MA) with four publications each. Guajará Bay (Pará state - PA) was the only studied system in the North region with 4 papers. Most studies were conducted in the Southeast region with 58 papers (44.6%). Both Northeast and South regions had 31 papers each (23.8% each). Six papers (4.6%) were conducted in more than one region.

More than half of the studies (84 papers) did not report the salinity range of the sampling stations (Figure 4). Of those papers that reported the salinity range, 22 studies were conducted in only one salinity zone (15.6%) and 24 over two zones (17%). Only four studies (2.8%) were performed across three zones and seven (5%) along all four salinity zones.

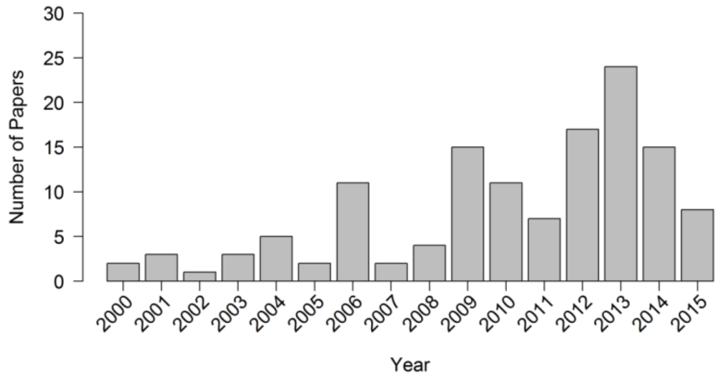

Figure 1. Number of published papers per year.

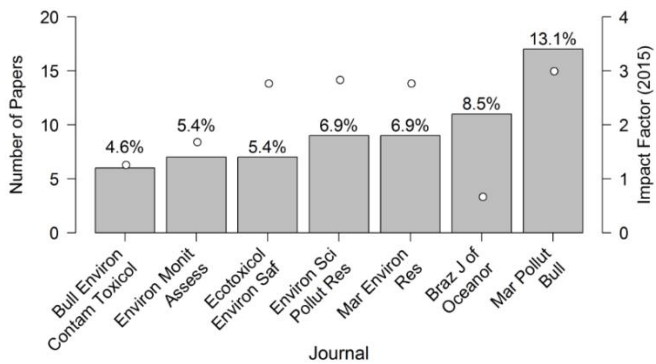

Figure 2. Number of papers published in the most frequent scientific journals. The impact factors (2015) are indicated by white circles.

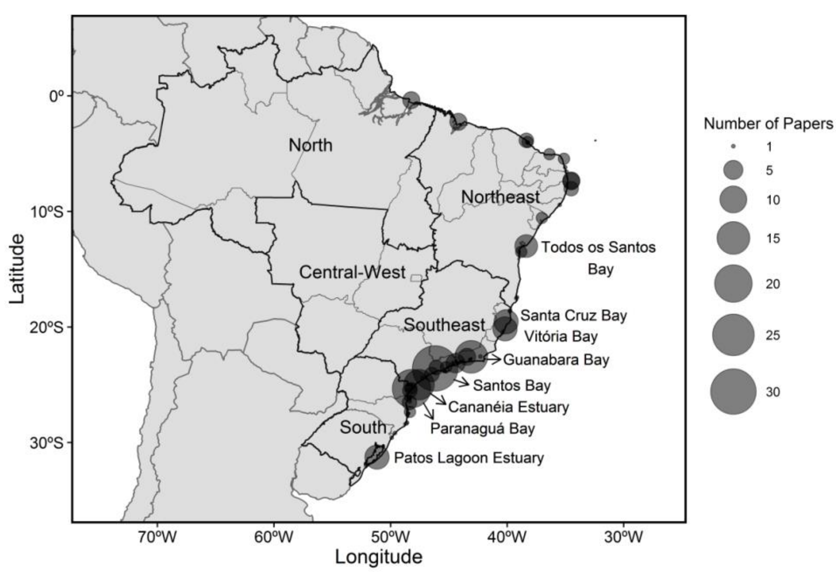

Figure 3. Spatial distribution of the papers conducted in Brazilian estuarine systems. The names of the systems with more than seven publications are shown.

Figure 4. Number of papers conducted per salinity zone.

Regarding spatial replication along the estuaries, 38 studies (27.1%) did not include spatial replication in their design (Figure 5). More than half of the systems (57.2%) had three or less spatial replicates and 15 systems (11.4%) had ten or more replicates. Only ten studies (7.1%) used hierarchical nested designs.

Almost half of the studies had no temporal replication in their designs (48.1%) and 93.2% had four or less replicates over time (Figure 6). More than half of the studies (52.6%) did not include any reference site in their design and 33.6% of the studies included only one reference site (Figure 7). Only 5.2% of the studies had 2 or more reference sites and 5.9% used nested designs.

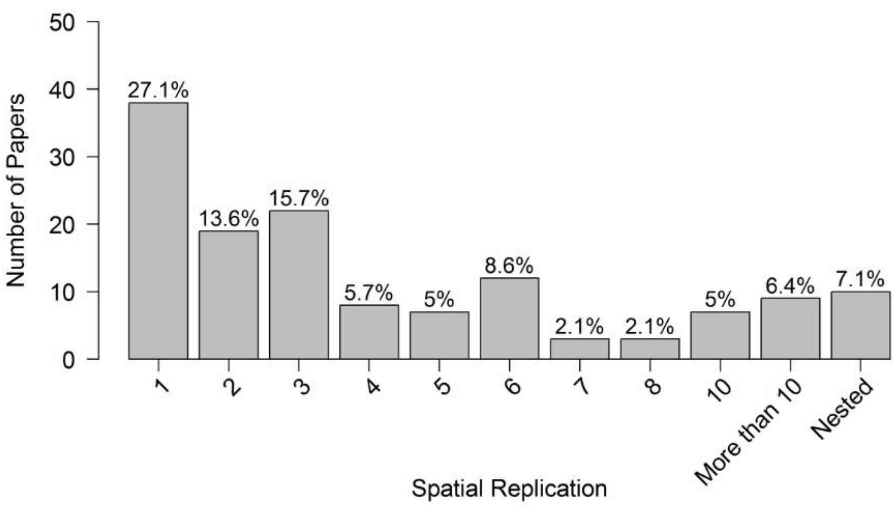

Figure 5. Number of papers for each of the number of spatial replicates.

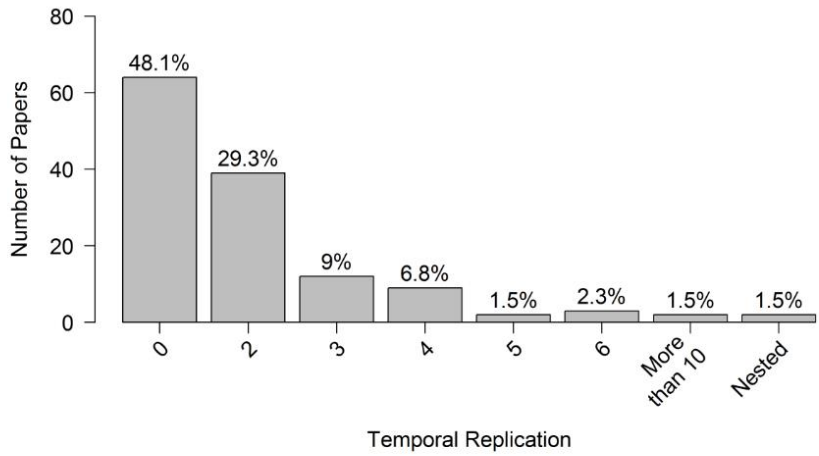

Figure 6. Number of papers for each of the number of temporal replicates.

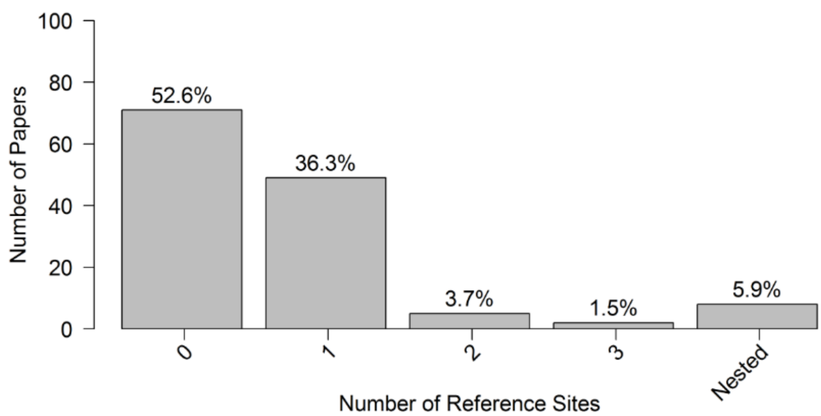

Figure 7. Number of papers for each of the number of reference sites.

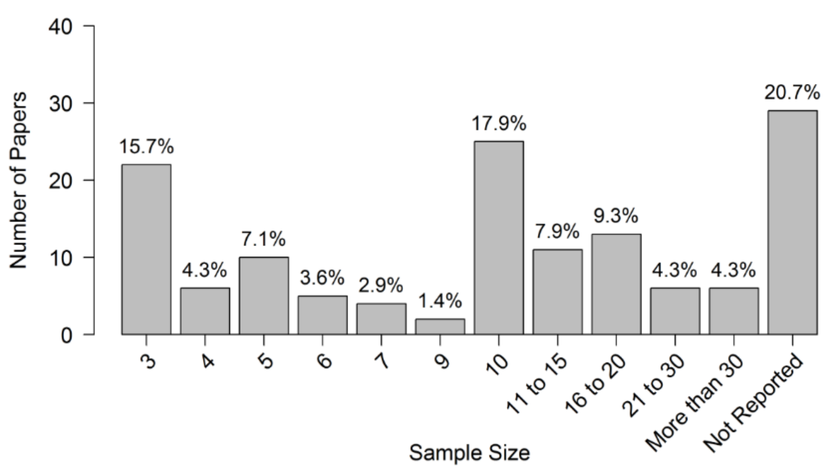

Figure 8. Number of papers for each of the sample sizes.

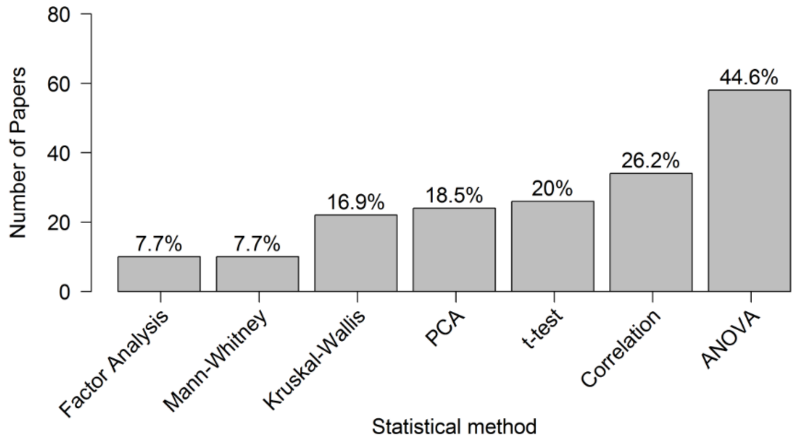

Figure 9. Number of papers for each of the most common statistical methods.

Forty-nine of the sampled estuarine systems (35%) had 9 or fewer replicates per treatment with 22 papers (15.7%) having three replicates. More than half of the papers (43.7%) had 10 or more replicates per treatment in their design and in 29 papers (20.7%) the number of replicates was unclear or not reported (Figure 8).

Most papers used only univariate statistical methods (59.2% or 77 papers) to analyze their data, while 10 papers (7.7%) used only multivariate methods and 41 papers (31.5%) used both univariate and multivariate techniques. Two papers (1.5%) were descriptive and did not use any formal analysis. No papers performed *a priori* power analysis or used Bayesian methods. Among the most used statistical methods were ANOVA (58 papers; 44.6% of all analyzed papers), correlation (34 papers; 26.2%), or a t-test (26 papers; 20%) (Figure 9).

Non-parametric Spearman's rank correlation and parametric Pearson's correlation were combined because some papers did not specify which method was used, while the distinction was clear for the parametric ANOVA and non-parametric Kruskal-Wallis tests. The number of factors used in the ANOVA was not always clear and thus one, two and three-way ANOVA were pooled together as well. Analysis of covariance (ANCOVA) was used in only six papers (4.6%). Among the multivariate techniques, Principal Component Analysis (PCA) was the most used with 24 papers (18.3%), followed by Factor Analysis (FA; 10 papers; 7.6%). Non-metric multidimensional scaling (nMDS), Cluster and BIOENV analyses were used in seven papers each (5.3%), and Canonical Correspondence Analysis (CCA) in six papers (4.6%).

Regarding the number of LOEs, 74 papers (56.9%) used only one line of evidence, 46 used two (35.4%), nine papers used three (6.9%) and only one paper used five LOEs (0.77%). The most common LOE was bioaccumulation with 53 papers (40.8%), followed by chemistry analysis with 51 papers (39.2%) and biomarkers with 44 papers (33.8%) (Figure 10). Community structure, bioassays, and manipulative experiments were less common with 27 (20.8%), 22 (18.9%) and 3papers (2.3%), respectively.

Among the papers that used biomarkers as a line of evidence, 73.3% considered more than one class of biomarkers. Biochemical biomarkers were the most commonly used, being recorded in32 papers (41% of all biomarkers), of which 33% (24 papers) evaluated enzymatic activities. Genotoxic biomarkers were measured in 16 papers (22.2%), followed by histopatological (18.1%; 13 papers).

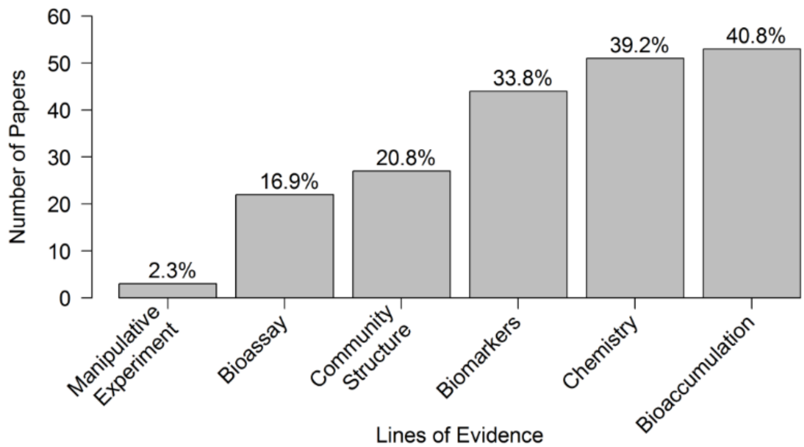

Figure 10. Number of papers for each line of evidence.

Among the classes of contaminants, metals were measured in 45 papers (34.6%), followed by organic contaminants with 17 papers (13.1%), and 9 papers measured both metals and organic contaminants (6.9%) (Figure 11). Studies that only measured nutrients (4.6%) or biological contaminants (3.1%) were less frequent. Fifty-five papers (26.9%) did not measure any contaminant in the study.

Among the classes of organic compounds, PAHs were the most common, being measured in 19 papers (14.5%), followed by PCBs with 11 papers (8.4%), organotins (e.g., TBT, DBT, MBT) with 5 papers (3.8%) and aliphatic hydrocarbons with 4 papers (3%). PBDE and organochlorides were measured in three papers each (2.3%). The most measured metals were Cu (50 papers; 38%), Zn (47 papers; 35.9%), Pb (41 papers; 31.3%), Cd (37 papers; 29.2%), Ni and Cr (33 papers; 29.2% each). Mercury (Hg) was measured in 29 papers (22.1%), methylmercury (MeHg) in 5 (3.8%) and the metalloid As in 12 papers (9.2%).

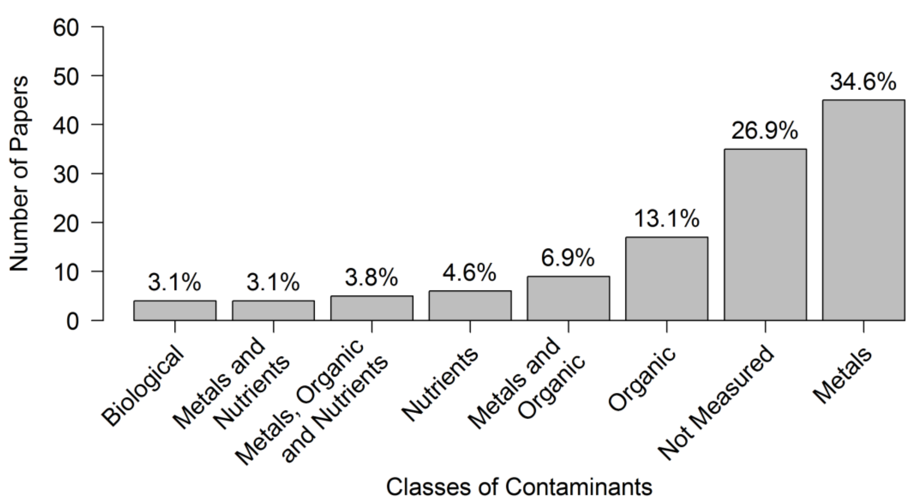

Figure 11. Number of papers for each of the measured contaminants.

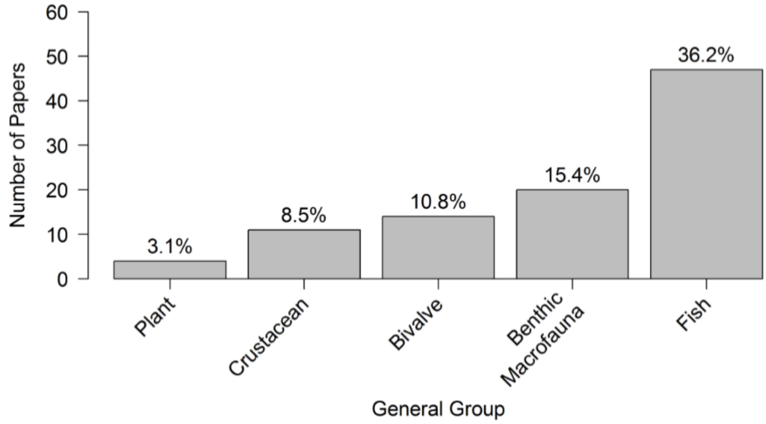

Figure 12. Number of papers for each of the general groups of studied organisms.

Regarding the media in which the contaminants were measured, 39 papers measured contaminant concentration only in organisms (30%), 28 papers measured it only in sediments (21.5%) and 5 papers only in water samples (3.8%). Fourteen papers (10.7%) measured the concentration of contaminants in two different environmental media and 6 papers (4.6%) measured it in three or more.

Fish were the most studied organisms (47 papers; 36.2%), followed by benthic macrofauna (20 papers; 15.4%), bivalves (14 papers; 10.8%) and crustacean species (11 papers; 8.5%) (Figure 12). Only 4 papers (3.1%) studied the effects of contaminants in mangrove plants, and no work was conducted in salt marshes. Only 2 papers (1.5%) studied mammals (dolphins) and no paper studied birds.

Most studies were conducted with the fish species *Cathorops spixii* (12.3%), *Micropogonias furnieri* (8.5%), *Genidens genidens* (5.4%), *Mugil lisa* (4.6%), *Sciades herzbergii* (3.1%) and *Centropomus parallelus* (3.1%) (Figure 13). Among the bivalve species, *Crassostrea rhiziphorae* (6.2%) and *Perna perna* (3.8%) were the most frequently studied.

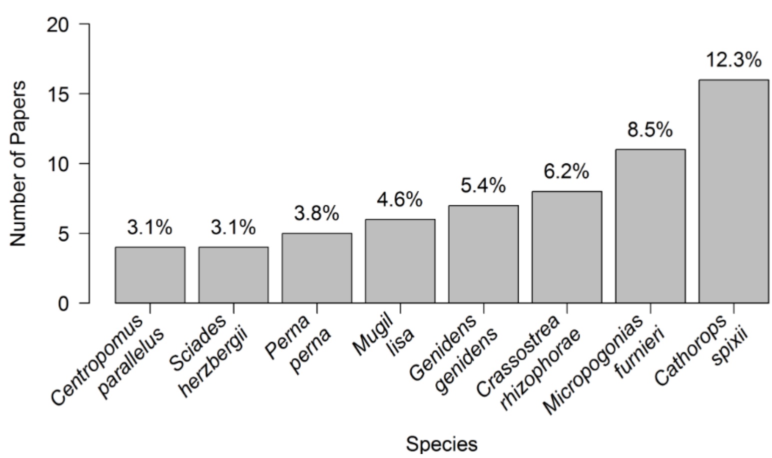

Figure 13. Number of papers for each of the most frequently studied species.

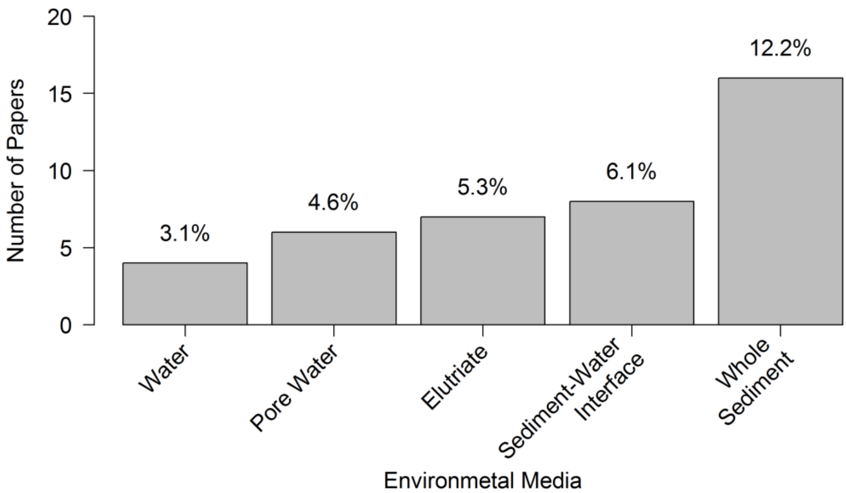

Figure 14. Number of papers for each media used in the bioassays.

Regarding the bioassays, 12 papers (9.2%) conducted bioassays with only one species, 8 papers (6.1%) used two species, and 2papers (1.5%) used three species. The most common species used in bioassays were the amphipod *Tiburonella viscana* with mortality as the measured endpoint (11 papers; 8.4%), the sea urchin *Lytechinus variegates* with abnormal larval development as the endpoint (6 papers; 4.6%), the copepod *Nitokra* sp. with reproduction as the endpoint (3 papers; 2.3%).

Thirteen papers (9.9%) conducted bioassays with only one environmental media, 2 papers (1.5%) with two media and 7 papers (5.3%) with three or four media. The most frequent media were whole sediments with 16 papers (12.2%). Due to the relative high frequency of studies that used sediment-water interface (6.1%) we included it here as another environmental media (Figure 14).

DISCUSSION

Although most studies were conducted in the Southeast region of Brazil, only 1.3% of the estuarine area of the country is in this region (MMA, 2010). The least-studied North and Northeast estuarine systems make up 77.8% and 19% of all estuarine areas, respectively (MMA, 2010). In fact, most papers in different areas published by Brazilian authors are from institutions in the Southeast (74.5%), followed by the South (19%), Northeast (12.2%) and North (2.7%) (Faria et al., 2011). As discussed by Leta et al. (2006), Brazilian scientific and technological capacity is concentrated in a relative small number of institutions, mainly in the more wealthy states. Many estuarine systems in the south and southeast regions are heavily industrialized and have a long history of contamination (e.g., Torres et al., 2015). However, the low number of papers in the north and northeast region does not mean that both regions are not impacted by pollutants. For instance, PAHs in most sediment samples of the Guajará Bay (PA) in the North region were found at concentrations above threshold effect levels (TEL) and in the same range of the Guanabara Bay (RJ) (Sodré et al., 2014). The northeast region also had an intense use of organochlorine pesticides before its ban in Brazil (Oliveira et al., 2016). In fact, the highest concentrations of total DDTs in mussels along the Brazilian coast were found in the northeast region (Sericano et al., 1995).

Salinity Zones

Most papers (60%) did not report the salinity range of the sampling stations (Figure 4), thus estimates of what are the most studied salinity zones in Brazil might be biased. The salinity was also usually reported based on single measurements during the time of sampling, therefore the classification of the salinity zones are likely to be inaccurate. However, because 46.7% of the papers had three or fewer spatial replicates (Figure 5), it is reasonable to conclude that most studies are conducted in just a portion of the studied estuary.

Salinity is well established as a major factor influencing the distribution of estuarine species (e.g., Sanderset al., 1965). It also affects the osmorregulation of organisms (McLusky and Hagerman, 1987), and the bioavailability and uptake of metals in the environment (Chapman and Wang, 2001). Likely for these reasons, enzymatic activities such as AChE and biomarkers of exposure such as MT can also be strongly influenced by salinity (Pfeifer et al., 2005; Monserrat et al., 2007). Salinity can also play an important role on phenotypic plasticity of functional traits of the mangrove *Avicennia schaueriana* (Arrivabene et al., 2014). Besides, because the salinities of environmental samples are adjusted to standard values in bioassays, toxicity could also potentially be over or underestimated among sampling sites (Chapman and Wang, 2001). In this sense, salinity can potentially influence different LOEs used to estimate the effects of contaminants in estuarine systems.

In many cases, the salinity gradient might also overlap the pollution gradient (e.g., Fitzpatrick et al., 1997) and attention is required when interpreting the obtained results. For instance, the number of different groups of species tends to naturally decline from the euhaline to the oligohaline salinity zone in both temperate (Dauvin, 2008) and tropical systems (Barros et al., 2014). One could conclude that the number of species decreases with distance from a pollutant source or that the number of species in a contaminated site in an oligohaline zone is lower than in a reference site located downstream. In such a case, the distinction between natural and anthropogenic stress is often unclear. This is known as the estuarine quality paradox (Elliot and Quintino, 2007).

As discussed earlier, not only the number of species is affected by estuarine gradients, but other LOEs might be subjected to the natural stressful conditions of the estuarine system. Researchers have suggested that it is important to include multiple LOEs in a study to account for the heterogeneity and variability of estuarine systems (e.g., Chapman and Wang, 2001; Dauvin, 2008). Multiple reference areas might also be necessary to account for the spatial heterogeneity along the salinity gradient. Thus, in most cases, the salinity gradient should not be dismissed when designing the study and it should be reported. Effort should also be made to collect a comprehensive salinity dataset of the studied system(s) in order to have a more accurate estimate of the temporal and spatial fluctuation of salinity. In addition, other gradients (e.g., temperature, pH, organic carbon, grain size) should be measured as they can also influence the fate and effects of contaminants in estuaries. Most papers measured at least one of these variables, thus they were not discussed in this chapter.

Spatial and Temporal Replication

Given that studies of estuaries are subject to many confounding variables, experimental designs aiming at evaluating the effect of contaminants need to take into account spatial gradients and comparisons to reference sites. As discussed by Green (1993), natural environmental heterogeneity can coincide with control versus impact locations, and spatial replication of both contaminated and reference locations are important. In this chapter, 27.1% of the papers had only one spatial replicate (Figure 5) and 52.6% of the papers (Figure 6) had no reference sites in their design. Some bioaccumulation studies just evaluated if the concentrations of contaminants in organisms where within the maximum limits for human consumption, and in this case reference sites may be not necessary. However, if the goal is to evaluate if the concentrations are due to a potential source of contaminants, reference sites may be important.

Many studies (48.1%) also did not include temporal replication in their designs, and of those that did, most looked at differences among seasons within a year (Figure 6). The main concern with this approach is that seasons are not replicated among years and the only inference that can be made is about the effect of season in that specific year. Thus, results of such a design need to be carefully interpreted. There are good reasons for why season can influence the effects of contaminants in estuaries, such as increase in contaminants runoff, freshwater input, changes in salinity and redox potential of sediments (Chapman and Wang, 2001). How seasons affect these variables can vary among years.

For instance, Costa et al. (2009) evaluated the concentrations of Hg in tissues of the fish *Trichiurus lepturus* in the Goaiana Estuary (PE) using a nested design with months nested within seasons (dry in 2005, rainy in 2006 and dry in 2006). The data were analyzed using two-way ANOVA considering a possible interaction of months and season instead of months nested within season. The authors did not reject the null hypothesis of no difference in the mean of Hg in fish tissues among months. The null hypothesis of no difference between the dry season in 2005 and rainy season in 2006 was rejected. However, it was not rejected in the rainy season in 2006 and the dry season in 2006. In fact, both dry seasons were considered significantly different from each other. The authors concluded that rainfall might be the main factor explaining the variation in Hg concentrations in fish. However, if only two seasons were sampled, the conclusion would depend on the year of sampling.

Including replication among years can be prohibitively costly in many cases; however, long term studies are necessary to evaluate seasonal effects. Nested designs can also help identify the source of variation in different temporal scales. If funding is limited and replication in the long term is infeasible, increasing sample size and including reference areas in the design can be a better allocation of resources. Due to the importance of reference areas in environmental impact studies, Brazilian scientists should increase efforts of mapping reference areas at a regional and national scale.

Sample Size

Sample size is also an extremely important element of the experimental design as higher number of samples can increase statistical power. Statistical power is the probability of rejecting the null hypothesis H_0 when it is false, in favour of an alternative hypothesis H_A

(i.e., 1- β (Type-II error rate)). This means that an alternative hypothesis must be specified *a priori* (i.e., the effect size must be defined) in order to estimate the power of the test (Green, 1993)[1]. In this sense, without *a priori* defining the hypothesized effect size and β, no conclusions can be made about $H_A$if the *p* value is lower than the selected α. For this reason, power not only depends on the sample size, but also on the Type-I error (α) and the population effect size (Cohen, 1992). Power curves can be used to help estimate the number of samples necessary to achieve the desirable β to detect a hypothesized effect size given an α. Nowadays, power analysis can be easily implemented in many statistical packages such as the R environment (R Core Team, 2015).

As is generally the case in the ecotoxicology literature, of the studies included in this chapter none had conducted power analysis[2]. All papers that used frequentist inferential statistics assumed an α of 0.05 and in only one case α was adjusted to 0.01. Some papers stated that the adopted α was 0.05 or 0.01 which is a fundamentally flawed, but common, practice. As discussed above, the selected α should be defined according to the number of replicates, β and the effect size. There are many cases in the field of ecotoxicology in which the consequences of a Type-II error are much more costly than that of a Type-I error, and the opposite can also be true. As discussed by Gigerenzer (2014), the level of significance should not be fixed by convention and used mechanically for either Fisher's and Neyman-Pearson framework. Even though descriptive and frequentist statistics were the only used approach, other prominent methods are available such as Bayesian statistics and confidence intervals (Hobbs and Hilborn, 2006).

In this chapter, the number of papers with sample size from three to around nine seems to decrease and tends then to increase for ten or more replicates (Figure 8). This bimodal distribution can be explained by the distinct designs and sampling methods used by different LOEs. The lower number of replicates was more common in papers that used community structure and bioassays as LOE, while the higher number of replicates was associated with biomarkers and bioaccumulation analyses. The number of replicates per treatment was often unclear, especially for studies conducted with fish, where usually only the total number of caught fish is reported. In many cases, the number of organisms and the degrees of freedom were also not reported.

Lines of Evidence

Many researchers suggest that multiple LOEs should be used when evaluating the effects of contaminants in estuaries, especially for sediment quality assessment (e.g., Batley et al., 2002, Torres et al., 2015). Most studies reviewed in this chapter generated only one (57.3%) or two (35.1%) LOEs. Among the reasons to use multiple LOEs is that no single line can reliably predict the effect of the others and there are uncertainties associated with each LOE (Batley et al., 2002). For instance, Torres et al. (2015) might conclude based on the AVS-SEM (Acid Volatile Sulfide/Simultaneously Extracted Metals) approach that metals are biologically unavailable in sediments of the Santos Estuary (SP); however, high

[1]Note that the concept of power and β are only applied in a *Neyman–Pearson decision theory* logic because, in *Fisher'snull hypothesis testing* framework, noalternative hypothesis is specified (Gigerenzer, 2004).

[2]Because many bioassays are standardized, it is assumed that power analyses were conducted during the development of the protocols (ASTM, 1996; however see Crane and Newman, 2000).

concentrations of some metals were observed in soft tissues of both transplanted and native oysters. Krull et al. (2014) also found a lack of agreement between sediment bioassays, metal contamination and the benthic macrofauna assemblage in the Jaguaripe Estuary (BA). Even though it is highly advisable to use more than one LOE, the total number and particular LOEs to be used will depend mostly on the question, logistics and funding (Chapman and Hollert, 2006).

Bioaccumulation was the most commonly used LOE (Figure 10) and bioaccumulation studies were conducted mainly with fish and bivalves (Figures 12 and 13). Even though most of these studies measured total length and weight, ANCOVAs were only used in six papers. For instance, Silva et al. (2003) found a size effect using log dry weight and some metal concentrations (e.g., Cu, Ni) in soft tissues of *C. rhizophorae* in the Curimatau Estuary (Rio Grande do Norte state - RN). Some elemental concentrations can also decrease with animal size, such as Cr and Ni in *C. spixii* specimens of the Paranaguá Bay (PR) (Angeli et al., 2014). In such cases, using ANOVAs without accounting for size or weight effects (i.e., without adjusting the mean factor level for the effect of the covariate) can lead to biased and incorrect results. In cases where the covariate is not significant, the statistical model can be reduced to a single factor ANOVA[3]. Most studies conducted correlation analysis between species length and the bioconcentration of contaminants, or t-tests to estimate the differences in mean of total length among sites or seasons; however, this is insufficient as the covariate was not formally included in the final linear model.

Length and size of the organism might also be an important covariate when analyzing biomarkers. For instance, Flammarion et al. (2002) found that if length of the fish *Leuciscus cephalus* were not included as a covariate, ChE activity would indicate that in eight sites out of 17, the null hypothesis of no difference between reference sites and contaminated sites would be rejected. However, after adjusting for organism size effect, the null hypothesis was rejected in only two, notably polluted, sites. The effects of size as a covariate on fish biomarkers were also demonstrated in other enzymatic activities such as glutathione S-transferase (GST) (Siscar et al., 2015) and ethoxyresorufin-O-deethylase (EROD) (Wunderlich et al., 2015). Plotting the data before running the analyses is recommended for investigating possible effects of covariates on the response(s).

Another common approach is to measure multiple biomarkers in different tissues from the same organisms and perform multiple statistical tests such as ANOVAs for each response variable. Most papers (73.3%) that used biomarkers as a LOE measured more than one class of biomarkers. As Krull et al. (2013) pointed out, these measures are not independent from each otheras they might be correlated and multivariate analysis might be a better option to overcome this issue. For instance, Arrivabene et al. (2014) used Linear Discriminant Analysis and Factor Analysis to analyze anatomical and morphological parameters of mangrove leaves and physico-chemical parameters among sites. Gusso-Choueri et al. (2015) also used Factor Analysis to analyze metal and organic bioaccumulation and eight biomarkers in the fish *C. spixii*.

[3] The general ANCOVA statistical model can be written as: $y_{ij} = \mu + \alpha_i + \beta(x_{ij} - \bar{x}) + \varepsilon_{ij}$; where y_{ij} is the response of the ith level of the factor in the jth replicate, μ is the overall mean or the intercept, α_i is the effect of the ith treatment of the factor, β is the effect of the covariate, $\bar{x}$ is the mean value of the covariate and x_{ij} is the covariate value of the ith level of the factor and the jth replicate, and ε_{ij} is the random unexplained error with mean of 0 and variance of σ_ε^2. If the covariate does not have an effect on the response variable (i.e., β=0) the statistical model is reduced to a single factor ANOVA.

Most multivariate techniques were used to analyze biological communities or multiple LOEs (e.g., chemistry, community structure and bioassays). Similar to a review of multivariate methods in ecology (James and McCulloch, 1990) the most commonly used ordination method was PCA (18.3%). Exploratory Factor Analysis (7.6%) was the second most used ordination technique and was more common among studies that conducted sediment quality analysis (7 out of 10 papers). The high relative frequency of Factor Analysis (among sediment quality analysis studies) was not observed in general ecology (James and McCulloch, 1990) and in benthic ecology papers (Carvalho et al., 2015). As discussed by James and McCulloch (1990) both PCA and Factor Analysis are exploratory analyses which may help understanding the patterns in the data and formulating testable hypothesis.

Bioassays and manipulative experiments were less common approaches (Figure 10). This is probably because of the low number of developed standard bioassays with native estuarine species in Brazil (Krull and Barros, 2012). For instance, the native sea urchin *L. variegates* was the second most used species in estuaries. Regarding manipulative experiments, two papers transplanted oysters from uncontaminated to possibly contaminated areas (Maranho et al., 2012, Torres et al., 2015) and one paper performed a reciprocal sediment transplant among contaminated and reference sites to look at the colonization of benthic species (Gern and Lana, 2013).

Classes of Contaminants

Among the classes of contaminants, organics were studied much less often than metals (Figure 11). However, this is probably not related to the fact that organic contaminants are not a major concern in estuarine systems in Brazil. For instance, high concentrations of PAHs were found in oyster tissues and sediments of the Santos Estuary (SP) and might be contributing to the negative effects on the benthic community (Torres et al., 2015). The concentrations of benzene, total phenols, TBT, and PCB in sediments and water of the São Marcos's Bay (MA) were above the limits set by Brazilian legislation and possibly influencing the enzymatic activity of the fish *S. herzbergii* (Carvalho-Neta et al., 2012). The biomagnification factor of PBDEs in dolphin liver in relation to scabbardfish and croaker of the Paraíba do Sul Estuary (RJ) were similar to the ones estimated for teleost fishes and bottlenose dolphins in Florida (Quinete et al., 2011) even though PBDEs were never produced in Brazil (Lavandier et al., 2013).

In fact, the lower number of papers that analyzed organic contaminants can possibly be related to the lack of facilities to conduct such analysis and associated higher costs. Dachs and Méjanelle (2010) argue that in the last decade, organic pollutants were not being studied around the world due to limitations in the analytical methods available and also the time and money required for analyses. In Brazil, the first paper that measured the concentrations of TBT in sediments was dated 2003 (Godoi, et al., 2003) even though methods had been available for more than ten years in other parts of the world (e.g., Langston et al., 1987). Concentrations of organochlorine pesticides (OCPs) were measured for the first time in sediments of the Jaguaribe Estuary (Ceará state - CE) in 2016, even though this is a region of historical high use of OCPs (Oliveira et al., 2016). In this study, the concentrations of γ-HCH and heptachlor were judged to present high risk and DDTs presented moderate risk when compared to sediment guidelines. Studies that evaluated the effect of metals in bioassays

(Nilin et al., 2013) and enzymatic activities of crabs (Davanso, 2013) of an estuary in the same state (CE) concluded that other contaminants such as pesticides might also be affecting the biota. However, there is insufficient data available regarding pesticide contamination in this region.

Historically, metal analysis in estuarine sediments in Brazil can be traced from the early eighties (e.g., Pfeiffer et al., 1980), and by the end of that decade metal contamination in sediments was already recorded in different regions of the Brazilian coast (Pfeiffer et al., 1988). By that time, concentrations of PAHs, PCBs, and DDT were first being measured in sediments of the coast of Rio de Janeiro (Japenga et al., 1988) and in mussels of Todos os Santos Bay (BA) (Tavares et al., 1988). Currently, there is considerable evidence that many Brazilian estuaries are affected by metal contamination (e.g., Cesar et al., 2006; Hatje et al., 2006; Souza et al., 2015) indicating that metals are a major class of contaminants in estuaries. However, organic pollution is still growing in Brazil. As suggested by Oliveira et al. (2016), ecotoxicology studies evaluating the effect of OCPs are urgent in mangrove and estuarine systems of Brazil. One way of overcoming this issue is improving the network connectivity among scientists from different institutions, states and regions so that the lack of facilities and funding are not a major barrier driving the assessment of contaminants in estuarine systems in Brazil.

CONCLUSION

Brazil has made great strides in addressing the impact of contaminants on estuarine ecosystems. This chapter identified the most common patterns of publications in the field and made some recommendations for future studies, as follows: (i) because salinity is one of main factors affecting the distribution of organisms and the bioavailabily of contaminants in estuaries, salinity, and other variables, need to be taken into account when selecting the sampling sites (including the reference areas) and planning other elements of the design; (ii) appropriate reference areas need to be included more in designs; (iii) in the absence of sufficient temporal replication to address the study question, resources should be allocated to other important elements of the design, such as increasing the sample size, and inclusion of reference areas or other LOEs; (iv) conducting power analysis is necessary to select *a priori* the appropriate α, β, sample size, and effect size; (v) the covariates effect needs to be considered more when performing bioaccumulation studies; and (vi) include different LOEs in the design when possible. Clearly stating the question and the hypotheses to be tested is extremely important to help making decisions about the temporal and spatial scales of the study, allocation of resources and selection of the LOEs. Currently, most studies have focused on metal contamination and provided evidences that metal contamination is a major concern in Brazilian estuaries. On the other hand, organic contaminants are much less studied in Brazil even though there are many different sources and types of contaminants that could potentially be harmful to estuarine ecosystems.

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Chapter 15

AVOIDANCE AND RECOLONIZATION RESPONSES OF THE GASTROPOD *OLIVELLA SEMISTRIATA* EXPOSED TO COASTAL SEDIMENTS

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ABSTRACT

The spatial distribution of the snail *Olivella semistriata* along the Ecuadorian coast near the city of Manta seems to be influenced by urban discharges. This observation leads us to hypothesize that contamination might determine inhabitable areas for the snails. Therefore, the ability of the snails to detect the local contamination and react by moving away from contaminated sediment has been assessed. In addition, the ability of the snails to recolonize contaminated sediment under recovery was also studied. Six sediment samples (El Murciélago beach – reference point, El Puerto, La Poza, Río Burro, Los Esteros, and Río Muerto) were taken and tested in two different assays: avoidance and recolonization. Assays were performed in a non-forced exposure system in which a contamination gradient was formed by mixing the test sample and the reference sediment. Avoidance was more intense in the samples from Río Burro and Río Muerto. In the

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recolonization assays, the reference sample was always preferred regarding all test samples. As there is no physical barrier to prevent the displacement of the organisms among the studied areas, the absence (visual field observation) of snails in the sediment from Rio Burro and Rio Muerto is suggested to be due to the ability of the organisms to avoid these areas. The present data indicate that contaminants can affect the spatial distribution of the snails by triggering avoidance or even preventing the colonization of contaminated areas, acting as a chemical barrier that might isolate populations.

Keywords: habitat selection, sediment, spatial distribution, toxicity

INTRODUCTION

The ability of gastropods to sense and react to environmental stimuli has been widely documented, being differences in sediment grain size and availability and nutritional quality of the food some of the stimuli recognized by these organisms (Barnes and Greenwood, 1978; Forbes and Lopez, 1986, Marklevitz et al., 2008a, Araújo et al., 2015). Regarding gastropod ability to sense contamination levels, laboratory studies have attested that they can use different mechanisms such as retraction inside their shell (Hellou et al., 2009; Araújo et al., 2012), floating, burying, crawling (Hampel et al., 2009; Campana et al., 2013), or grouping (Leung et al., 2004), to reduce their contact with contaminated sediments. The gastropods *Ilyanassa obsoleta* (Marklevitz et al., 2008a,b; Hellou et al., 2009) and *Peringia ulvae* (= *Hydrobia ulvae*) (Araújo et al., 2012) have already been shown to be capable of avoiding contaminated sediments also by moving towards less stressful areas. In a field study a change in the population density of the snail *P. ulvae* was observed along an eutrophication gradient (Lillebø et al., 1999), which could be partially explained by their avoidance behaviour to escape from the contamination. More recently, an experimental study showed that the gastropod *P. ulvae* was able to detect patches of sediment with different contamination levels (Araújo et al., 2016). According to these results, in a contamination scenario in which contaminated and non-contaminated sediment patches are heterogeneously distributed, local gastropod populations could rearrange their spatial distributions by avoiding contamination. For organisms able to detect and avoid contaminants, such behaviour might have serious implication in their habitat selection and spatial distribution pattern (De Lange et al., 2010; Hellou, 2011). In this sense, contaminants may play a decisive role in the habitat structure even if no noxious physiological effect at the individual level is triggered (Ares, 2003; Hellou et al., 2008).

Contamination can not only trigger the avoidance response of the organisms which are able move to less stressful areas, but also prevent (re)colonization processes (Hellou, 2011; De Lange et al., 2013; Moe et al., 2013). In areas where a contamination input is stopped and is subsequently followed by a decrease in contamination levels, it is expected that populations gradually recolonize them, as of the moment contaminant concentrations are no longer repulsive. Therefore, by using avoidance and recolonization experiments it is possible, on one hand, to predict how repulsive a habitat is and, on the other hand, to assess the potential of this area to be recolonized. The use of both avoidance and recolonization approaches incorporates the concepts of population dynamics and disturbance ecology in ecotoxicological studies by considering organism displacement as a response to contamination, largely contributing to understand at which temporal and spatial scales populations are expected to recover from disturbance (Worm and Duffy, 2003; Hellou et al., 2008; Schmitt-Jansen et al., 2008).

As deposit-feeding organisms inhabiting sediment, gastropods are suitable key organisms to be used in sediment environmental quality assessment schemes, and due to their responsiveness to avoid contamination, they can provide valuable information about how habitable an area can be. The present study aimed at assessing the quality of coastal sediments from the urban coast of Manta (Ecuador) by using as endpoint the ability of the native gastropod *Olivella semistriata* to avoid contamination. Additionally, a novel approach using experiments of recolonization was employed in order to assess the potential of sediment to be recolonized by gastropod populations and, in the case of the occurrence of avoidance, to predict at which contamination levels recolonization could be expected.

METHODS

Test Organisms

Individuals of the snail *O. semistriata* were collected from the El Murciélago beach (Manta, Ecuador, [80° 44' 0.18" W and 0° 56' 24.25" S: El Murciélago beach]) during low tide. Organisms were selected according to size (mean [n=10] 17±2 mm length) and maintained in a 20 L aquarium with 5 L of 0.7 µm-filtered natural seawater (salinity between 26-30, pH of 7.8, alkalinity of 162 mg $CaCO_3$ L^{-1}, 0.25 mg L^{-1} of ammonium, and 0.15 mg L^{-1} of nitrite) and a 3 cm layer of El Murciélago beach sediment. The culture was kept at 25°C with a photoperiod of 12/12 h (light/dark) and renewed daily. As the main food source to gastropods is microphytobenthos, additional food besides natural sediment from El Murciélago beach was not provided.

Sediment Samples

Sediment samples were taken from the Ecuadorian coast near the city of Manta at six different points (Figure 1): El Murciélago beach, El Puerto (80° 43' 15.36" W and 0° 56' 47.07" S), La Poza (80º 43' 9.08" W and 0° 56' 54.21" S), Río Burro (80° 42' 55.66" W and 0º 56' 57.56" S), Los Esteros (80° 42' 13.43" W and 0° 56' 56.90" S) and Río Muerto (80° 41' 49.42" W and 0° 56' 52.90" S). El Murciélago beach was the sampling point from where the organisms for assays were collected and thus was considered as the reference point. Superficial sediment (top 2 cm) was sampled by hand during low tide. Samples were kept at 4°C until the start of the experiments. Total metal concentrations (Al, Cd, Cu, Fe, Hg, Mn, Ni, and Pb [inductively coupled plasma mass spectroscopy – ICP-MS, Thermo, ICAP 7000]), total organic carbon and organic matter contents [titration; Walkley and Black, 1934], levels of NO_3, PO_4, and SO_4 [spectrophotometer, Hach, DR2800]), and granulometry (wet sieving) were analysed in the sediment samples (Table 1).

A visual survey of the presence of *O. semistriata* in all the studied areas revealed that this species is found in El Puerto, La Poza and Los Esteros, besides El Murciélago beach where test organisms were collected from. Along the sampled area no organisms were found in Río Burro and Río Muerto, areas where two important discharges of domestic and industrial effluents occur.

Avoidance Assays

Before the assays, each tested sediment was mixed with reference sediment (El Murciélago beach) to obtain four different contamination levels: 25, 50, 75 and 100% (proportions of contaminated sediment). The mixture proportions were based on wet volume. Sample mixtures were placed on rectangular trays (27.5 cm x 6 cm x 4 cm), which were divided into five sections (5.5 x 6 cm). In each section, each of the four different proportions of the sediment plus the reference sediment (0% of contaminated sediment) were disposed forming a gradient of the test sediment as described in Araújo et al. (2012) such that the reference and 100% tested sediment were at opposite extremities of the tray (Figure 2). A control tray with only reference sediment was also prepared to test for preference/avoidance response of organisms to any section of the tray in particular. Trays were then filled with 20 mL of seawater used in the culture of the organisms, enough to keep the sediment sufficiently wet but without the possibility of migration of the organisms by floating. Once the trays were prepared, five organisms were introduced in each section, totalling 25 organisms per tray. Each tray was assayed in triplicate. After 30 min and 6 and 24 h, the distribution of the organisms in the sections was checked by counting. Assays were performed at 25°C and in darkness.

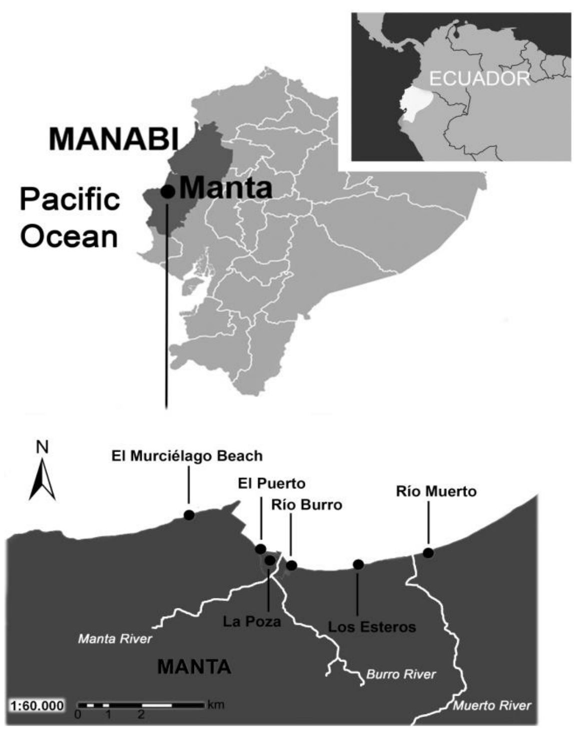

Figure 1. Sediment sampling points on the Ecuadorian coast near the city of Manta.

Table 1. Physical-chemical characterization of the sediment samples collected from study sites along the coastal area of Manta city, Ecuador

Parameters	Samples					
	El Murciélago	El Puerto	La Poza	Río Burro	Los Esteros	Río Muerto
Al (mg kg^{-1})	5550	5747	5774	4535	5959	3752
Cd (mg kg^{-1})	0.67	0.53	0.65	0.48	0.53	0.45
Cu (mg kg^{-1})	9.4	18.8	33.9	10.7	13.8	7.7
Fe (mg kg^{-1})	11015	8954	10245	7571	8668	6908
Hg (mg kg^{-1})	<0.3	<0.3	1.3	0.4	<0.3	0.3
Mn (mg kg^{-1})	163.3	82.2	81.0	86.0	85.5	105.3
Ni (mg kg^{-1})	14.5	13.6	15.6	11.1	12.7	9.1
Pb (mg kg^{-1})	2.1	1.8	2.9	1.2	1.4	1.2
Zn (mg kg^{-1})	39.3	48.9	65.2	33.4	40.2	27.1
TOC (mg kg^{-1})	1400	500	5500	3500	2000	1800
OM (%)	0.25	0.08	0.95	0.60	0.36	0.31
NO_3 (mg kg^{-1})	<0.420	<0.420	<0.420	<0.420	<0.420	<0.420
PO_4 (mg kg^{-1})	1.6	2.8	48.5	2.5	4.8	3.9
SO_4 (mg kg^{-1})	490	430	640	230	500	330
Particle size (%)(< 75 µm)	2.2	17.9	34.6	5.3	14.1	6.5
(75 – 425 µm)	97.8	82.1	65.4	94.7	85.9	93.5

Total metal concentrations are in dry weight. TOC: total organic carbon; OM: organic matter.

Recolonization Assays

For recolonization assays, the same sediment gradient was used and the sediment dilutions were distributed to form a toxicity gradient as previously described. In these assays, all 25 organisms were introduced in the section containing the reference sediment (Figure 2). As for the avoidance assays, recolonization assays solely with reference sediment were performed to verify whether the snails were able to uniformly colonize the entire tray along its five sections. Assays were also performed in triplicate and under the same environmental conditions as described above. The distribution of the organisms was similarly recorded after 30 min and 6 and 24 h.

Statistical Analysis

Avoidance assays with reference sediment were used to test absence of preference/ avoidance to any section of the tray. This assumption, considered as criterion for the validation of the results, was tested by using Yates' chi^2 test as described by Moreira-Santos et al. (2008) with the number of expected organisms (considering a homogeneous distribution along the five sections) and observed organisms. This statistical test was also applied to the avoidance assays with potentially contaminated sediment samples, so that calculation of avoidance percentage was only performed when the uniformity of the organisms' distribution was statistically rejected. Avoidance was calculated at the three exposure times (30 min and 6 and 24 h) from the number of expected organisms (N_E). For each exposure time, N_E was calculated considering a homogenous distribution of the observed organisms, which assumes no preference for any sediment concentration lower than that of the respective section. For instance, for the most contaminated section (#5; 100% contaminated sediment; Figure 2), the number of expected organisms was equal to the total number of organisms observed within sections #1 to #5, divided by the corresponding total number of sections (i.e., 5). For the adjacent section (#4; 75% contaminated sediment), N_E was calculated considering the number of organisms observed within sections #1 to #4, divided by the corresponding number of sections (i.e., 4). For section #2 (less contaminated section excluding the reference sediment; 25% contaminated sediment), N_E was equal to the total number of organisms observed in the first (reference sediment) and second sections, divided by 2 (the corresponding number of sections). The number of avoiders was determined for each sediment section by calculating N_E minus the number of observed organisms (N_O) in that section: Avoiders=N_E - N_O. Finally, avoidance (in %) was calculated using the formula: Avoiders/N_E×100. For the calculation of the recolonization (in %): Recolonizers=N_O in each section (except the one with the reference sediment) and Recolonization=Recolonizers/N_E×100, which leads to the same result as Recolonization=100–Avoidance. Mean avoidance and recolonization and their respective standard errors were calculated with the three replicates. The concentration of sediment causing the avoidance and recolonization of 50% of the population after the total exposure time of 24 h (AC_{50} and RC_{50}, respectively), and corresponding 95% confidence intervals, was determined using the software PriProbit 1.63 (Sakuma, 1998). Finally, to better infer the relationship between avoidance and recolonization responses under similar contamination levels, the expected recolonization at 24 h was calculated directly from the data of the respective avoidance assays in order to compare it with the observed recolonization obtained in the recolonization assays. This 24 h expected recolonization was determined using the formula: 24 h expected recolonization=100% - avoidance.

RESULTS

Reference Sediment

Results from the avoidance assay with solely reference sediment (control) aimed at verifying the uniformity of the final distribution of the organisms along the tray in the absence of contamination. The number of observed organisms in each section at any observation time (30 min, 6 and 24h) was not statistically different from the number of initially deployed organisms (Yates' chi^2 tests; $p>0.05$), confirming that there was no preference/avoidance of the snails for any section in particular.

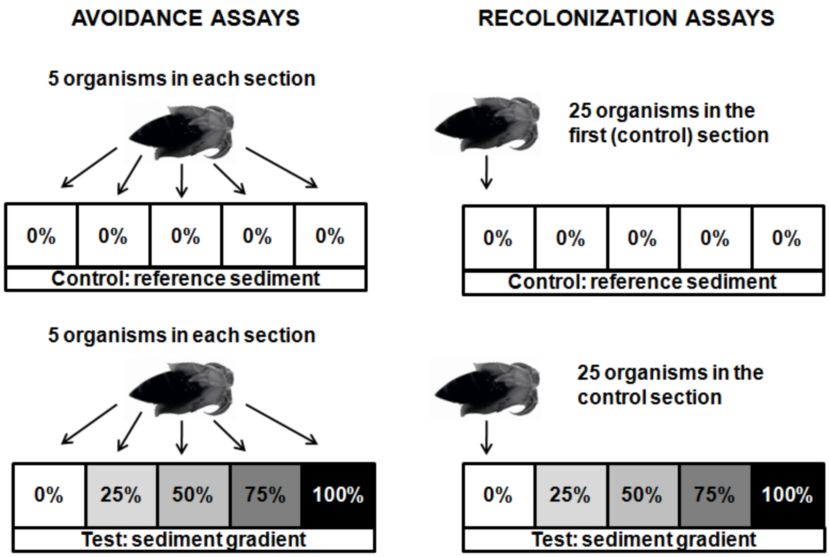

Figure 2. Experimental setup of the avoidance and recolonization experiments. The order of the sections (numbered from #1 to #5) referred to in the text goes from left to right: section #1 is at the left extremity and section #5 is at the right extremity.

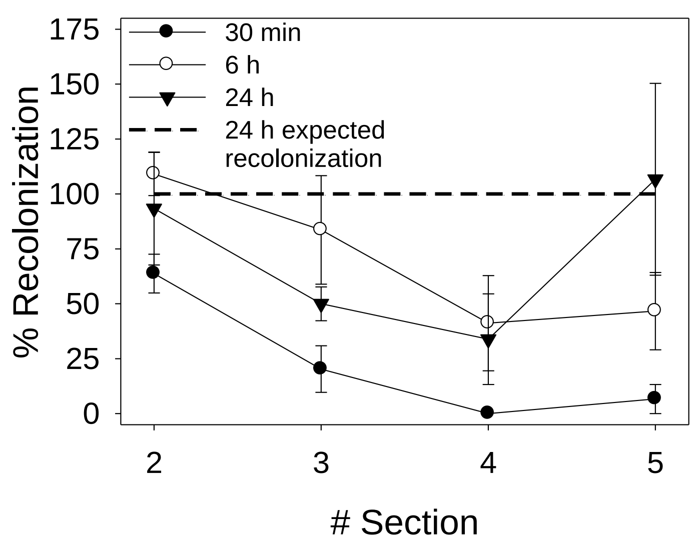

Figure 3. Recolonization percentage of the snail *Olivella semistriata* for each section expected to be colonized (as all organisms were initially placed in section #1) in the recolonization assays with reference sediment only.

As for the recolonization assays with reference sediment, the distribution of snails along the entire tray was influenced by exposure time (Figure 3). After 30 min, organisms mainly colonized sections #2 and 3, the two closer to the section where they were introduced (#1). The farthest section was colonized by 50% of the expected number of organisms after 6 h, but after 24 h the colonization percentage increased to 100%. It was also observed that after 24 h the variability of the recolonization response increased with the distance covered from the reference sediment. The observed recolonization was similar to the 24 h expected recolonization for sections #2 and #5, but lower for intermediate sections #3 and #4.

El Puerto

No avoidance was observed for any concentration of the sediment from El Puerto (graph suppressed). Although recolonization increased with exposure time, it was less than 20% for the two most contaminated sections. Thus, the 24 h expected recolonization was in general overestimated except for the concentration of 25% (Figure 4).

La Poza

Avoidance was observed during the whole assay, but it was not concentration-dependent. The most intense avoidance was observed for 25% of sediment with values around 60%, although only after 6 h of exposure. For the other concentrations, avoidance was lower than 25%. Recolonization assays showed that only the concentration of 25% was colonized. The 24 h expected recolonization was similar to the observed recolonization percentage for the concentration of 25% at 6 h; however, for this same concentration it was underestimated after 24 h exposure. For all the other concentrations it was overestimated throughout the entire exposure period (Figure 5).

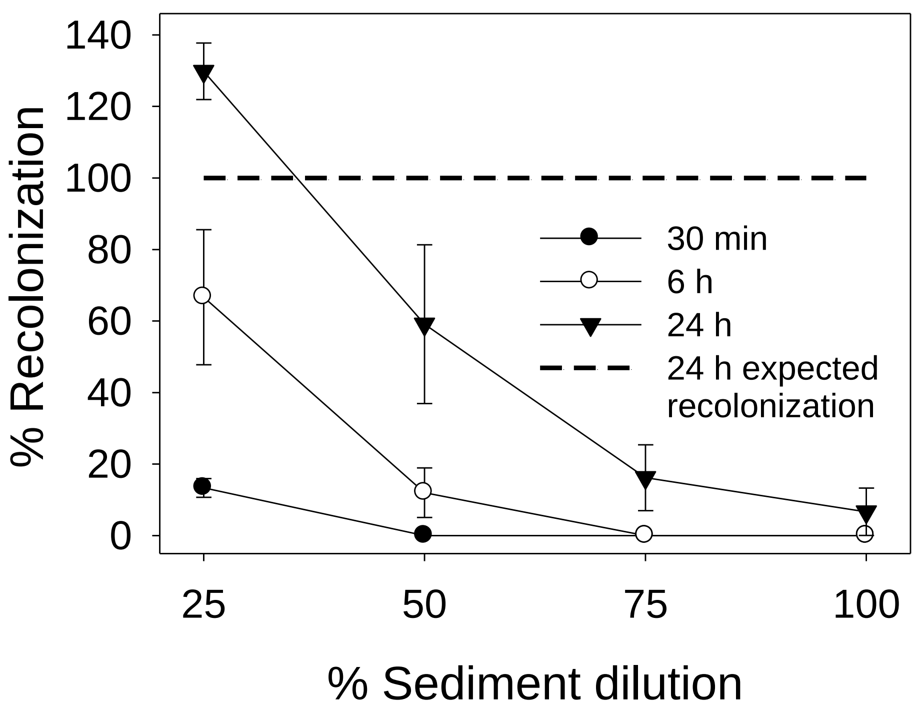

Figure 4. Recolonization percentage of the snail *Olivella semistriata* exposed to different dilutions of the sediment from El Puerto.

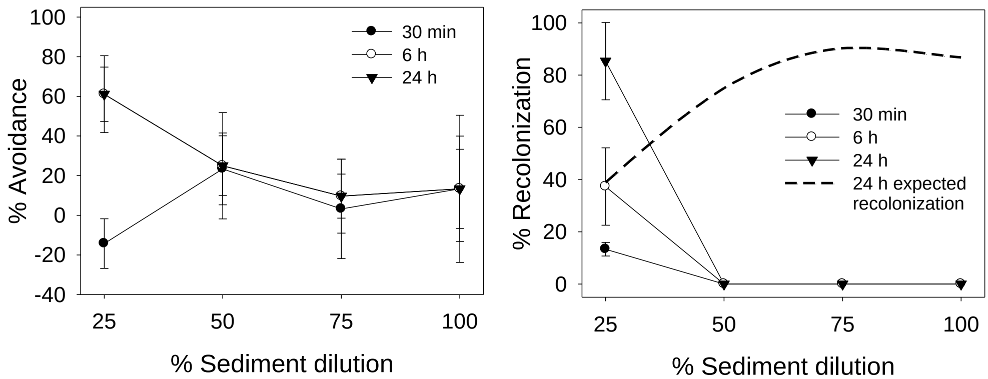

Figure 5. Avoidance (left) and recolonization (right) percentages of the snail *Olivella semistriata* exposed to different dilutions of the sediment from La Poza.

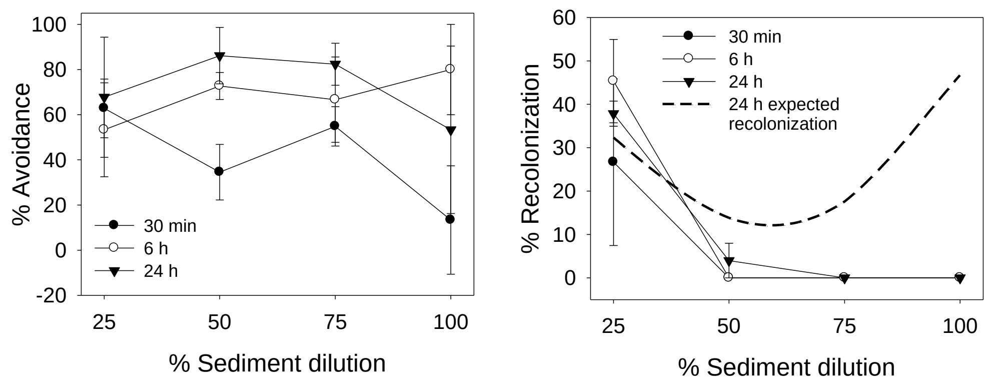

Figure 6. Avoidance (left) and recolonization (right) percentages of the snail *Olivella semistriata* exposed to different dilutions of the sediment from Río Burro.

Río Burro

Snails avoided the sediment sample from Río Burro by more than 60% after 6 h of exposure. Yet, for the most concentrated sediment, avoidance was slightly reduced compared to other concentrations. With regard to recolonization, only the concentration of 25% was recolonized at a percentage of around 40% after both 6 and 24 h exposures. The 24 h expected recolonization was very similar to the observed recolonization for the concentrations of 25 and 50%; however, it was overestimated for the two highest concentrations (Figure 6).

Los Esteros

During the first 30 min of exposure, organisms did not avoid the tested sediment concentrations. Yet, after 6 h of exposure the avoidance response reached values between 25 and 50%, while at the concentration of 100% no avoidance was observed, indicating a displacement of the organisms towards that section. This avoidance behaviour was maintained after 24 h of exposure. After this same exposure period, a recolonization response of 100% was observed for the concentration of 25%, while for the concentrations of 50 and 100% the recolonization percentage was around 40%. The 24 h expected recolonization was again overestimated for the three highest concentrations and underestimated for the concentration of 25% (Figure 7).

Río Muerto

All sediment concentrations were avoided, resulting in a 60 to 80% avoidance in the first three concentrations (25, 50 and 75%), but for the 100% sediment concentration it decreased to 40%. Basically, organisms only recolonized the concentration of 25%. Therefore, the recolonization percentage predicted for the 24 h recolonization was close to the observed recolonization for the concentrations of 25 and 50% and was again underestimated for the highest concentrations (Figure 8).

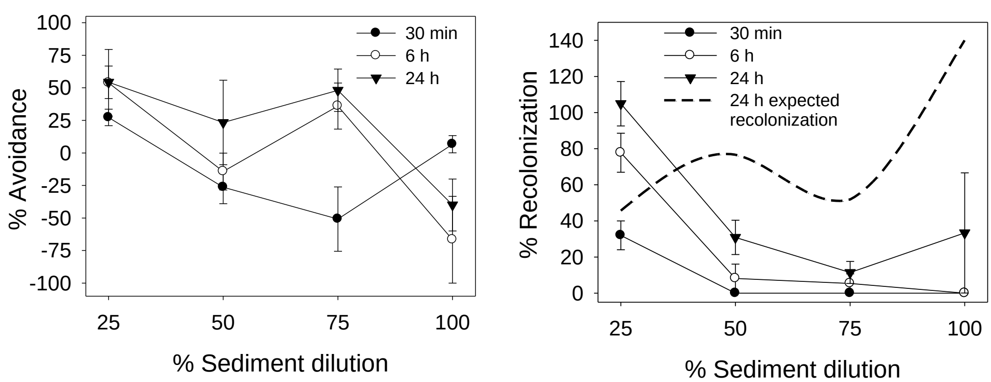

Figure 7. Avoidance (left) and recolonization (right) percentages of the snail *Olivella semistriata* exposed to different dilutions of the sediment from Los Esteros.

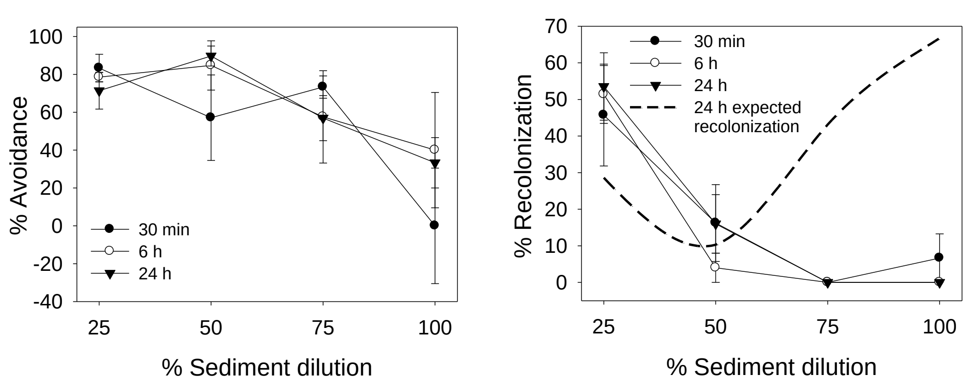

Figure 8. Avoidance (left) and recolonization (right) percentages of the snail *Olivella semistriata* exposed to different dilutions of the sediment from Río Muerto.

AC50 and RC50 Values

AC_{50} values for El Puerto and Los Esteros were estimated at >100% as there was no avoidance. For the other sediment samples, the AC_{50} values were <25%. With regard to recolonization, sediment from El Puerto and Los Esteros were more intensively colonized, with RC_{50} values of 55.2 and 51.4%, respectively. For La Poza, Río Burro and Río Muerto the RC_{50} values were lower: 27.6, 21.8 and 26.9%, respectively. Confidence intervals were not calculated for any of the samples.

Discussion

Given the diverse avoidance and recolonization responses observed for the different tested sediments, results of both avoidance and recolonization assays for each sediment sample are discussed separately.

El Puerto

As no avoidance was observed, the expected recolonization was much higher than that observed in the recolonization assays. Although the recolonization percentage clearly increased with exposure time, it did not reach the expected value (Figure 4). A 24 h exposure time was possibly not sufficient for the colonization of the most extreme sections. This explanation is not contradictory to the 100% recolonization observed in the reference sediment (El Murciélago beach) after the same exposure time. When organisms were exposed to reference sediment in the recolonization assays there was no additional or different stimulus (i.e., differences in the biological, chemical and physical composition of the sediment), as the sediment present in all sections of the system was similar (El Murciélago sediment sample). However, in the recolonization assays with the El Puerto sediment organisms received different stimulus at each sediment concentration (e.g., El Murciélago beach and El Puerto sediments differed in TOC, OM and particle size distribution). Therefore, their decision to stay, return or move on might have required more time.

La Poza

Curiously, in the section with 25% of La Poza sediment there was avoidance (ca. 60%) after 6 and 24 h but there was also more than 80% recolonization after 24 h of exposure, whereas for the other sediment concentrations neither avoidance nor recolonization was recorded. The absence of avoidance at the highest sediment concentrations could be related to temporal avoidance (retraction inside their shell, a behaviour known to occur in response to contamination; Hellou et al., 2009; Araújo et al., 2012) and, therefore, spatial avoidance (displacement towards reference sediment) is not observed. Furthermore, as those same concentrations are not favourable to be inhabited by the snails they were not colonized.

Another effect that could have triggered this response is the incidence of moribundity (decrease or even loss of the ability to avoid contamination), preventing the organisms' displacement (Gutierrez et al., 2012; Araújo et al., 2014).

The observed results also suggest the possibility that other factors than contamination might have influenced both avoidance and recolonization responses. For instance, the organic matter in the sediment from La Poza is much higher than that in other sediments, including the reference El Murciélago beach sediment (see Table 1). For the lowest La Poza concentration (25%) this content of organic matter could have been an attractive factor in the recolonization assays since organisms moved from reference sediment with low organic matter content. This recolonization did not increase with the increase of the organic matter probably because the levels of other chemical elements, such as metals (see Table 1), also increased and might have triggered an avoidance response. Possibly due to an attractive stimulation caused by the organic matter (mainly microphytobethos, a major food source for snails; Lopez and Levinton, 1987; Coelho et al., 2011; Araújo et al., 2015) present in the sediment section of 25%, recolonization increased with the exposure time (% of recolonizers increased). However, it seems not to have been sufficient to maintain the population for long, since 60% of the snails escaped that section (Figure 5).

Río Burro

This sediment was avoided by a high percentage of the exposed snail population, even though avoidance decreased slightly (from ca. 60 to 50%) at the highest sediment concentration. The observed recolonization was in accordance with the avoidance response but only for the lowest concentration (25%): avoidance of 60% and recolonization of 40%. This was because, in general, there was no recolonization for the other sediment concentrations. The less intense avoidance observed in that sediment concentration can be explained by a decline in snail activity, which was indeed visually observed for organisms recorded in the 100% section already after 6 h of exposure. In sediments triggering a strong avoidance, organisms might initially opt for a temporary avoidance (e.g., retraction inside their shell; Hellou et al., 2009; Araújo et al., 2012), and only after a spatial avoidance might then occur, if moribundity does not take place.

Los Esteros

The avoidance observed was not concentration-dependent. For the first three concentrations it varied between 25 and 50%, but for the 100% sediment concentration it was negative (organisms moved towards that section). This result is corroborated by the trend of the snails to colonize after 24 h of exposure the highest sediment concentration whose colonization percentage was of 33%. Therefore, it is possible to consider that organisms do not avoid this sediment concentration; however, the attraction to colonize it is not immediate.

Río Muerto

The avoidance response observed in this sediment was similar to that occurring in the sediment from Río Burro; snail avoidance was between 60 and 80% at the three lowest sediment concentrations but was reduced to 40% at the 100% concentration. Equally to the Río Burro sediment, recolonization was restricted to the first sediment concentration (25%), suggesting the lowest avoidance in the 100% section can be explained by temporary avoidance and/or moribundity. If the reduction in activity of the snails is deliberate and moribundity did not occur, then a longer exposure time could trigger a spatial avoidance.

Ecological Implications

Avoidance assays with snails have only been recently employed in ecotoxicology to assess the ability of organisms to escape from contaminated sediments, moving to most favourable areas (Marklevitz et al., 2008a,b; Araújo et al., 2012, 2016). However, this response deserves special attention due to the effects that it can cause on the spatial distribution of snail populations. Additionally, in the present study, recolonization was used to verify the ability of the organisms to move from reference towards test sediments, a scenario likely to occur in aquatic systems under recovery. The ecological relevance of this approach consists in predicting the contamination-triggered spatial displacement of organisms. In fact, according to our visual observations, snails are not frequently found in all the sampling areas of the present study: for instance, populations of *O. semistriata* have been found in a very high density in the El Murciélago beach (reference point), with lower frequency in El Puerto and Los Esteros, and sporadically in La Poza, while no records were made in Río Burro and Río Muerto. As there is no physical barrier preventing the displacement of the organisms between those areas (except for organisms from El Murciélago that are isolated from all other points by a fishing port), it can therefore be hypothesized that organisms avoid inhabiting sediments in La Poza, Río Burro and Río Muerto. Results of the laboratory assays attested that organisms avoided sediment from Río Burro and Río Muerto, even at low concentrations. These results seem to reflect the spatial distribution observed in the field and the role that contamination seems to play as a chemical barrier preventing the displacement among areas. As the ability to avoid contaminants allows organisms to escape before effects at the individual level occur, the consequences of the avoidance response fall on the ecosystem (e.g., pond, stream, estuary, beach) and landscape (e.g., river basin, coastal ocean) levels by disturbing their structure and functioning.

For the recolonization assays, results were neither in accordance with avoidance experiments nor with visual field observations, except for the reference sediment (El Murciélago beach), in which recolonization was of 100%, as expected. It is important to consider that characteristics of the sediment other than contamination may play an important role in the decision of the organisms to avoid or recolonize an area (Cardoso et al., 2013). Cardoso et al. (2013) observed that density, biomass and growth productivity of *P. ulvae* increased along a mercury gradient in a shallow coastal lagoon (Ria de Aveiro, Portugal), and that factors other than toxicity, such as resource availability, presence of refuges, etc. also seemed to determine the spatial distribution of the organisms.

The findings of the present study allow hypothesizing that the higher the avoidance response the lower is the expected recolonization, although the required time seems to be sediment dependent. This pattern can also be seen by analyzing the AC_{50} and RC_{50} values, with the first parameter being always lower than the second. Due to the design of the assays, for the highest concentration of a sediment sample (placed in the section furthest from the reference sediment), organisms in both avoidance and recolonization assays are not expected to take the same time to avoid the most contaminated sample and to possibly colonize it. Therefore, a 24 h exposure might not have been sufficient for a complete recolonization, i.e., the difference in the intensity of both responses can be directly related to exposure time. Finally, it is crucial to take into account that the decision to avoid, stay or colonize a habitat might be based on multiples factors. The intensity of the avoidance is directly related to how repulsive is the risk (Harper et al., 2009), and thus recolonization will be faster and more intense the more attractive the environment is.

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Chapter 16

POLLUTION IN COASTAL AREAS: AN INTEGRATED PERSPECTIVE IN ENVIRONMENTAL RISK STUDIES

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ABSTRACT

Transitional water systems such as estuaries and subestuaries are areas of great concern in ecotoxicology due to urban and industrial settlement, being key areas of productivity and biogeochemical exchange between ocean and land. The ecotoxicological risk of trace metals and organic pollutants in the environment depends on whether they can be accumulated by organisms and this, in turn, depends on the uptake from food and water, as well as the efflux. Chemicals are often sequestered in estuarine sediments and thus likely to be accumulated, affecting the benthic communities directly. The surface oxic sediments include the biofilm, and its associated biota which are ideal models to understand the ecological risk in aquatic environments. South American estuaries along the Atlantic coast show different levels of human impact. We reviewed the status of environmental pollution and ecotoxicology of these estuaries and highlighted the potential sources of novel pollutants.

Keywords: Estuaries, aquatic pollution, trace metals, POPs

POLLUTION IN ESTUARINE AREAS

Transitional water systems such as estuaries and subestuaries are highly productive environments where biogeochemical exchanges between ocean and land occur. These ecosystems support nursery and recruitment areas for many species (including commercial

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fish) due to the presence of key prey species, such as dense populations of invertebrates (McLusky and Elliott, 2004; Dauvin, 2008).

Estuaries are also naturally stressed areas due to the natural dynamic of mixing fresh and marine waters. For this reason, estuarine organisms should be adapted to cope with this complex environment. Probably most of the organisms living in transitional water systems contain a strong defense battery to fight the naturally occurring oxidative stress. The presence of these defense mechanisms, which are similar responses upon exposure to pollutants, imply that these complex environments could be sources of misinterpretation if a putative toxic response is trying to be associated with aquatic pollution. However, estuaries are areas of great concern in ecotoxicology due to the urban and industrial settlement.

The ecotoxicological risk of trace metals and organic pollutants to the environment depends on whether they can be accumulated by organisms, and this in turn depends on their uptake from food and water, as well as the efflux. As estuaries are characterized by fine grained and organic-rich sediments, they act as a sink for contaminants and are therefore priority areas for identifying potential bioindicator species and biomarkers of pollution (Ducrotoy, 2010). We reviewed the status of environmental pollution and ecotoxicology of several South American estuaries and highlighted potential sources of novel pollutants.

Sources of Contamination in Estuarine Environments and New Pollutants

The most problematic chemical pollutants are the persistent, bioaccumulative and toxic (PBTs), which include inorganic (trace metals) and some organic compounds. Inorganic pollutants as trace metals are natural components of the Earth’s crust. Metals are released from the erosion of rocks and appear as dissolved forms or associated with particulate material, although they could occur in the atmosphere in particles, aerosols or gases (see Luoma and Rainbow, 2008). Some metals have an essential biochemical function in a metabolic pathway due to their affinity to sulfur and nitrogen, and this affinity is the one that turns all metals into potentially toxic in high concentrations (Rainbow et al., 2006; Luoma and Rainbow, 2008). Anthropogenic metal sources include punctual ones such as industries and diffuse ones such as agriculture and street runoff. The recent surge of nanotechnology has meant a rise of emerging contaminant sources. Metal based nanoparticles are produced for a wide range of commercial and industrial products, generating a novel pollutant, with poorly understood long-term effects on the environment (Luoma, 2008). The most used metal based nanoparticles are composed of silver and copper, which can be internalized by organisms, generating toxic effects in estuarine benthic communities (Khan et al., 2012; García-Alonso et al., 2014).

The entry of metals to estuaries occurs mainly via freshwater, and upon reaching brackish water they may suffer changes in chemical speciation that affect their partitioning and their bioavailability.

Metals can bond to organic compounds forming organometals such as tributyltin (TBT) and methylmercury. TBT was used as one of the main components of anti-fouling paint to prevent the accumulation of marine organisms on vessels. This compound was slowly released to the environment, ending up with tens of thousands of tons of TBT discharged into aquatic ecosystems from boat bottoms before its ban in the 1970s (Luoma and Rainbow, 2008). TBT is used as a fungicide, bactericide, insecticide, and as a preservative in textiles, paper, leather, electrical equipment and plastics (Clark et al., 1988). During the 1970s, the accumulation of TBT in the coast of France nearly resulted in the collapse of commercial oyster fisheries. TBT interferes with the larval settlement and proliferation of chambers in oyster shells of *Crassosstrea gigas* (Alzieu, 2000) and is also highly accumulated by aquatic organisms (Meador, 2000). Mollusks are known to accumulate high levels of TBT. For instance, female gastropods develop male sexual characters called imposex upon exposure to TBT (Gibbs and Bryan., 1986). Estuarine harbors around the world are still contaminated by TBT and imposex is still recorded in gastropods from South American estuaries (Penchaszadeh et al., 2001).

Methylmercury is a potent toxic compound that is bioaccumulated and biomagnified through the food web, ending up in top predators and humans. Neurotoxicity is one of the major negative effects that this compound generates. Mercury is released by industrial emissions and generally deposited in soil or water near the source (Renner, 2005). Methylmercury is naturally produced by bacteria in water; therefore release of non-bioavailable Hg elements to the environment must be avoided or reduced.

Organic pollutants are in general man-made compounds that are not natural components of the earth's crust, most of them containing a primary carbon structure. Among organic pollutants, some of the most relevant are those that can persist in the environment (UNEP/GPA, 2006). These chemicals called persistent organic pollutants (POPs) are the most problematic chemicals since several of them are also PBTs and thus bioaccumulate and biomagnify in the biota. There are many kinds of POPs such as plastic derivatives, surfactants and biocides, among others. Endocrine disrupting compounds (EDCs) are POPs grouped by the effect they generate and not by its chemical structure. EDCs interfere with the normal endocrine function. Some plastic derivatives such as PCBs and bisphenols, as well as detergents such as alkylphenols, act as xenoestrogens. These are EDCs that act as estradiol, a sex steroid hormone involved in the behavior and reproductive cycle control of females, but also in the proliferation of estrogen-dependent cancer cells. Different levels of responses to EDCs could be useful for their detection in estuaries. Early warning effects of EDCs could be used as the induction of detoxification enzymes (e.g., glutathione-s-transferase) (Ayoola et al., 2011) or the expression profile of female-specific proteins in males (e.g., vitellogenin or coriogenin) (García-Alonso et al., 2006). Endpoints such as morphological feminization or presence of ovotestis (gonadal histology), developmental toxicity (García-Alonso et al., 2011a), fecundity, and long term exposure recording local extinctions (Kidd et al., 2007) are good tools to indicate the presence of EDCs in the environment.

FATE OF CONTAMINANTS IN ESTUARINE ENVIRONMENTS

As the environmental behavior of pollutants depends on the complex interaction of many factors, any significant environmental alteration is likely to affect their distribution and fate. In the case of transitional waters "The legacy lies in the sediments" is the title of a note made by Rainbow and Luoma (2010), as chemicals are often sequestered in estuarine sediments and thus likely to be accumulated and affect the associated organisms (bacterial communities of the biofilm and benthic infauna). In Figure 1, a conceptual scheme represents the entry of pollutants by diffuse inputs (e.g., runoff) and punctual release (e.g., urbanisation, industries, ships). Pollutants are trapped in sediments of intertidal mudflats and at the transitional areas of estuaries where the salt front acts as a barrier.

Geomorphology and hydrology of the system drives the transport, precipitation and accumulation of sediments (de Souza-Machado et al., 2016). This is why sediment analysis is particularly useful in the detection of contamination sources and the selection of critical sites for routine analysis, as they could evade detection if measured in water (Páez-Osuna, 2005). There is a surface layer on the sediment called biofilm, which is rich in organic matter and composed of small sediment grains, detritus, bacteria, fungus and micro-organisms. The accumulation of pollutants in this interphase is probably the major source of the toxicity process in the associated biota. Contaminants in the anoxic and compacted sediments a few centimeters below the biofilm are probably not near the biota and do not exert any ecotoxicity reaction.

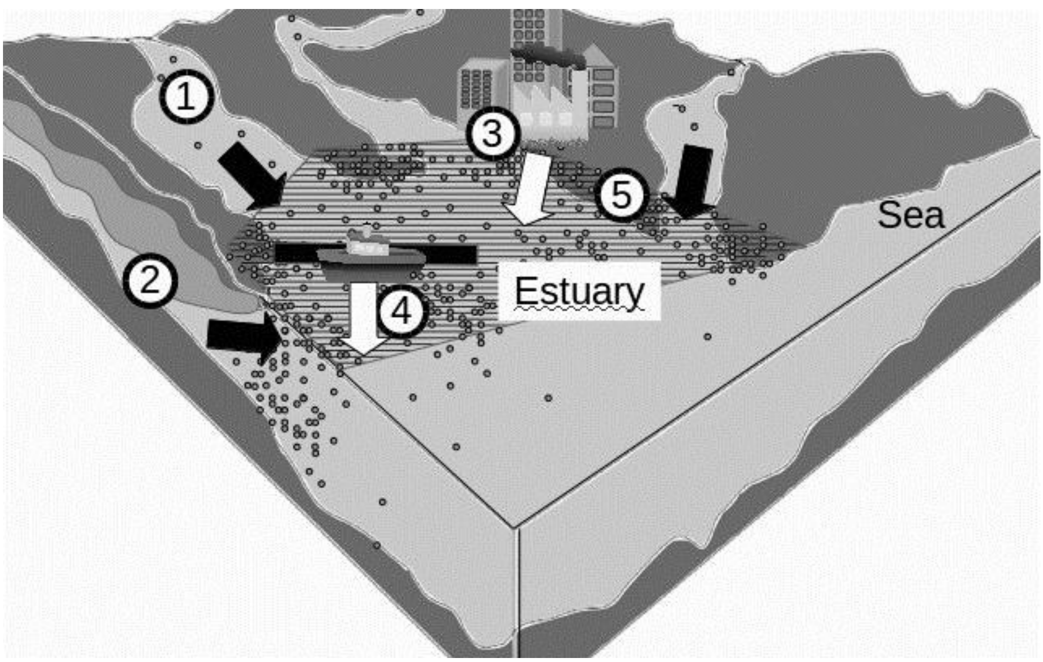

Figure 1. Conceptual model of pollutant fate and accumulation in estuarine systems. Black arrows denote diffuse inputs from rivers and ground-water, while white arrows denote punctual inputs from ships, urban sewage and industrial discharge. Pollutants (small circles) tend to accumulate in the tidal flood-plain and bottom sediments of the estuarine zone where the salt-front occurs. 1, Diffuse inputs; 2, Subterranean water inputs; 3, Urban and Industrial inputs; 4, Fluvial transport and harbor inputs; 5, Intertidal mudflats. Dashed area denotes the transitional water zone including the salt front. Adapted from de Sousa-Machado et al., (2016).

The physicochemical characteristics of the environment affect the partition of contaminants. In the case of metals, for an approximately neutral pH, metal partition between the solid phase and solution greatly favors particulate material, which means that sediments and this particulate material are the greatest metal reservoirs in the water body. Metals such as Ag, Cd, Zn and Cr are assimilated more efficiently from suspended particulate material or from sediments with high organic matter content and, in general, more efficiently from organic ligands than inorganic ones (Harvey and Luoma, 1985; Decho and Luoma, 1994; Lee et al., 1998). Greater metal concentrations can be found in particles with sizes below 63 µm, while the fine sand fraction tends to accumulate less due to the dominance of quartz components (Kersten, 2002). Another important variable is salinity, as the stress generated by high temperatures combined with low salinity raises the toxicity of metals (Bryan and Langston, 1992). Also, when pH and dissolved oxygen are high (around 8.0 and 8.0 mg/L, respectively), or even at a neutral pH, the oxidation rates are faster and therefore the precipitation of metals rises. At low pH (5.5-6) the most important ionic metal liberation occurs; nevertheless, low pH occurrences are very rare in natural environments (Atkinson et al., 2007).

Organic pollutant properties in the environment differ in particular from metals. The octanol/water coefficient classifies molecules as polar (<1) and non-polar (>1). The lipophilic properties of a chemical drive the fate of POPs. Partition in different matrices of aquatic systems (e.g., water column, sediment, and biota) depends on the octanol/water coefficient of the contaminant and the sediment-water distribution of hydrophobic organic chemicals plays a key role in their food-chain transfer. Increasing lipid content and biomagnification of POPs are believed to be responsible for the higher bioconcentration factors that can be observed with increasing trophic levels (Jones, 1991). Substances that bioaccumulate and biomagnify are of great concern, as they can potentially attain toxicologically significant loads in higher trophic-level species such as predatory fish, birds, and mammals (including humans) (Kelly et al., 2004).

ESTUARINE BIOTA AT RISK AND ECOTOXICOLOGICAL STUDIES

Estuaries are very productive ecosystems, important nursery and recruitment areas for terrestrial and aquatic organisms, presenting a high density of estuarine invertebrates, which act as key prey species for higher animals (McLusky and Elliott, 2004; Dauvin, 2008). As estuaries are characterized by transitional waters and fine grained, organic-rich sediments, they can trap contaminants and are therefore priority areas for studies of potential biomoitoring and bioindicator species as well as the development of pollution biomarkers (Ducrotoy, 2010).

The naturally stressed estuarine environments are places where species which have successfully adapted have developed different strategies to cope with this complex environment, thus the interest in euryhaline species as models to study pollution tolerance. Additionally, biological responses to the presence of contaminants in estuaries may have some influence on population tolerance, survival or adaptation (e.g., detoxification activity). Many of such responses are already used in organic and metal risk analysis of estuarine polychaetes (e.g., Glutathione-*S*-transferase) (Ayoola et al., 2011; García-Alonso et al., 2011b).

Biofilm communities and deposit-feeding organisms are ideal models to understand the ecological risk in coastal areas. Thus, understanding the shift of bacterial communities in impacted estuarine sediments allows the determination of a basal biological level effect. Metagenomics is a novel tool based on high-throughput massive sequencing analysis of environmental samples. Advances in sequencing strategies such as pyrosequencing have made possible a more sensitive and accurate detection of bacterial diversity, which happens to be one to two orders of magnitude greater than previous estimates. The use of high-resolution approaches together with a thorough analysis of environmental variables, helps to discriminate between assemblages corresponding to the natural estuarine variability and those shaped by anthropogenic impact (Piccini and García-Alonso, 2015).

Human impacted estuaries contain different bacterial communities in comparison to pristine zones of the same estuary (Sun et al., 2012). It is assumed that some members of microbial communities are always present but vary in population size depending on environmental conditions, such as those found in estuaries (Caporaso et al., 2012). Recent studies using metagenomics in sediments of intertidal areas of Rio de la Plata showed a clear relationship between the prokaryotic communities and the level of impact (Piccini and García-Alonso, 2015), becoming a useful tool for ecological risks assessment.

Several ecotoxicological studies have been performed using vertebrate model organisms such as fish (as reviewed by Scholz et al., 2013). However, there is an increasing public pressure to avoid the use of vertebrates. The 3Rs idea of reduction, refinement and replacement of vertebrates should be considered for future strategies on ecological risk and impact studies. Furthermore, many vertebrates such as fish are in constant movement and do not reflect the exact spatial situation of the environment. Micro and macrobenthic estuarine communities are useful organisms in ecotoxicology and more studies should be developed to avoid the sacrifice of vertebrates.

The South American polychaete, *Laeonereis acuta*, that inhabits intertidal mudflats, is an interesting model organism in ecotoxicology since it lives in close contact with the oxic biofilm layer of the sediment. Detoxification by enzymes such as glutathione-*S*-transferase (GST), super oxide dismutase (SOD), among others, have been analyzed in polychaetes exposed to different sorts of pollutants such as metals and POPs (Geracitano et al., 2004; Díaz-Jaramillo et al., 2016). Bioavailability of trace metals from sediments to *L. acuta* using a weak (HCl) digestion has proven to be a good approach to obtain the bioavailable fraction of metals in sediments and thus their potential toxicity in the environment (Castiglioni, 2015).

INTEGRATED APPROACHES

In order to maintain the sustainability of the surrounding environments, several integrated approaches should be performed including managers, researchers, fishermen, agriculturist, engineers, architects and policies makers. Latin American countries present weak environmental legislation and poor conservation management, plus the pressure to improve the economic situation of the regions. Due to their advantages, estuaries are becoming attractive places for “dirty” industries to settle. Baseline studies on non-impacted estuaries are the best start to generate the discrimination capacity when a human impact occurs. Thus basic research should be made to support any *a posteriori* decisions on environmental management, fishery politics, biodiversity and aquatic conservation policies, and urban and socio-economical parameters.

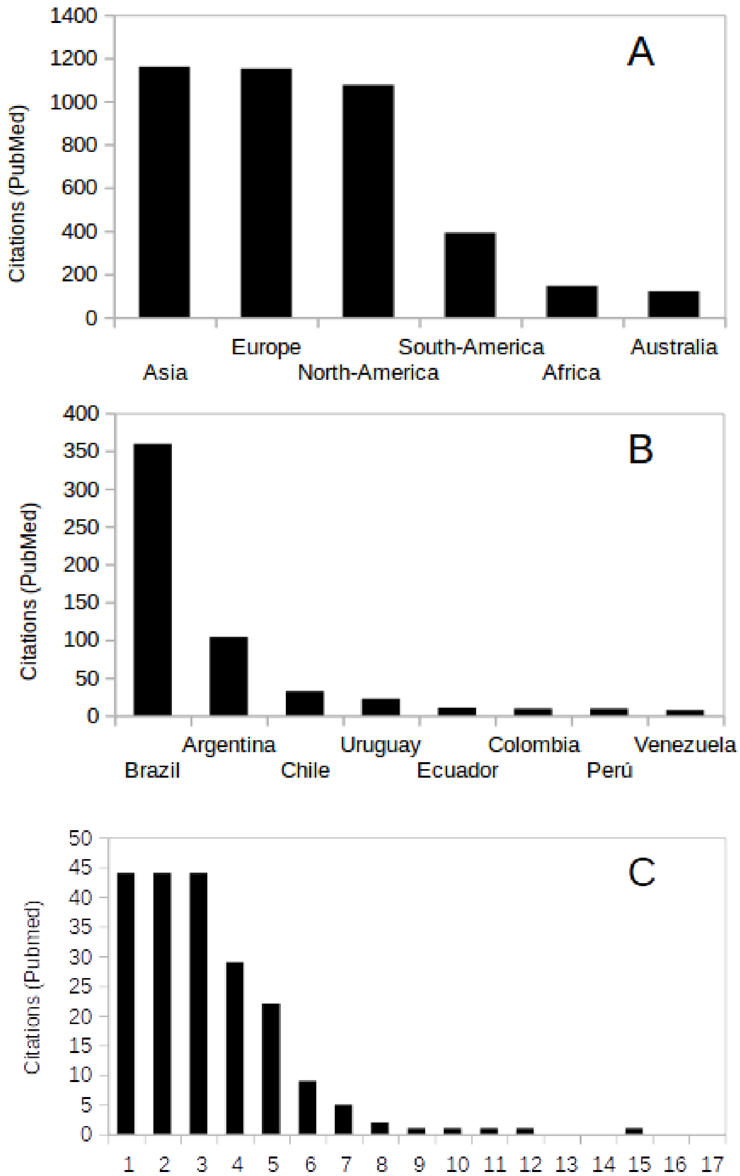

Figure 2. Number of "estuary" citations in Pubmed (NCBI) database as of February 2016. Comparison among continents (A), South American countries (B) and South-American estuaries (C). 1, Rio de la Plata; 2, Patos Lagoon; 3, Bahia Blanca; 4, Amazon; 5, Parnaiba; 6, Lenga; 7, Valdivia; 8, Orinoco; 9, Dagua; 10, Biobio; 11, Rio Negro; 12, Paranaiba do Sul; 13, Rio Gallegos; 14, Colorado; 15, Aconcagua; 16, Maipo; 17, Rapel.

A multidimensional approach to an environmental problem is the best way to cope with ecological risk, being very robust disciplinary pillars the best strategy. Using a single tool to estimate ecological risks requires several assumptions (e.g., toxicological predictions of metals based on analytical approaches assume equal effect of the environment on its potential toxicity), while integrative measures require fewer assumptions (e.g., adjusting the whole-toxic effect in relationship to environmental conditions such as pH, conductivity, synergism with organic pollutants). Interdisciplinary approaches generate synergy and as a result of this interaction a new level of results emerges. For instance, in ecotoxicological research, biologists, chemists and statisticians should interact in order to better interpret what is occurring. Multivariate approaches on estuarine pollution have helped to model estuarine risk environments (García-Alonso et al., 2011b), but further horizontal thinking is needed to integrate the research with environmental management and policy makers.

Integrative approaches in environmental monitoring and management require multidisciplinary research studies and therefore multivariate statistic tools. These are suitable strategies for a sustainable management in critical areas where conservation status might be at risk, setting fundamental bases for good models that can help to avoid environmental weakening. New strategies to approach environmental monitoring studies are in demand mainly including the application of multivariate statistical tools to verify the association between pollutants, toxicity and physical variables.

Specifically for South American estuaries, no studies have been published that integrate interdisciplinary approaches on ecological risks. Furthermore, there is a huge difference in water quality criteria around the world, reflecting the lack of an integrative approach to determine, for example, at which level a potential chemical would become toxic. An international effort should be made to standardize protocols that would fit natural complex systems such as estuaries.

STATUS OF SOUTH AMERICAN ESTUARIES

The study of contaminants in estuarine systems is of great importance as these areas are highly productive and receive large amounts of pollutants from terrestrial drainage. In a global context, South American estuaries appear to have relatively few ecotoxicological studies in comparison to other regions of the world (Figure 2). Within South American countries more studies have been performed in Brazil, followed by Argentina. The most cited estuary is the Río de la Plata, followed by Patos Lagoon and Bahía Blanca. Several important estuaries in South America suffer the impact of urbanization and industrialization (Muniz et al., 2015), so we described briefly the status of some of them (Figure 3). However, many small estuaries of the Neotropical continent have been studied, such as Santos, in Brazil (Eça et al., 2013) and Lenga, in Chile (Diaz-Jaramillo et al., 2013), among several other small systems.

Orinoco

The Orinoco basin covers an area of 880000 km^2. The estuary comprises a huge floodplain area including the “Delta del Orinoco Biosphere Reserve” (UNESCO, 2011). The estuary is well preserved as it is not greatly urbanized (i.e., Guyana City) and has no diffuse contamination inputs. Moreover, the Orinoco estuary is probably the only South American estuary inhabited by native people, the nomadic Warao.

The Warao population reached a total of 36028 individuals by 2001, of which 83% lived in the estuary, and 85% in traditional communities (INE, 2002). Few studies exist on human impacts on this system and no literature was found on ecotoxicology. One of the most relevant impacts is the construction of a dam in the 1960s which generated a reduction of the mangrove area (Colonnello and Medina, 1998).

Trace metal levels do not show evidence of a negative impact on the biota, even at the discharge of red mud containing high amounts of some metals (Mora et al., 2015). Due to the low level in relation to the sea (reaching areas with one meter below sea level), predictive climate change models suggest that the Delta may be submerged by 2100 (Vegas-Vilarrúbia et al., 2015).

Amazon

The Amazonia estuary receives freshwater from the biggest water basin on Earth, the Amazon River. It covers 7050000 Km^2 which represent 40% of the total surface of South America. The Amazon Estuary, located in northern Brazil, includes also the discharge of the Tocantins River, resulting in an annual mixture of approximately 6300 km^3 of river water carrying 93108 tons of sediments with the waters of the Atlantic Ocean (Meade et al., 1979). New integrated approaches are being developed in order to improve the water quality of the estuary (Monteiro et al., 2016). Trophic transfer and bioaccumulation studies of trace metals have been performed in pearl oyster of the Amazon estuary (Vilhena et al., 2016).

Parnaíba

The Parnaíba River watershed (34400 km^2) is characterized by a low industrial development, while its estuary presents low inputs of contaminants and prevalence of diffuse pollution sources (de Paula Filho et al., 2015). This relatively pristine transitional water system on the Atlantic coast of South America presents an extensive mangrove, with very high primary production in the estuary and its coastal plume. Being a unique, complex and tropical estuary, this environment integrates an important global conservation area (MMA, 2006): since 1996 the delta was declared an Environmental Protection Area, spreading over three states in Northeastern Brazil (Ceará, Maranhão and Piauí). Background levels of potential toxic metals have been recently described (da Paula Filho et al., 2015), indicating relatively low levels of these metals in the estuarine sediments.

Patos Lagoon

On the south coast of Brazil, Patos Lagoon and Rio Grande estuary system is another huge transitional water area with the presence of urbanized regions and extensive agriculture. It also receives the discharge of the Merin lagoon basin from an area covering the east part of Uruguay. The Patos Lagoon estuary receives local inputs from urban (e.g., Rio Grande city), industrial and harbor activities which have enriched the sediments with metals such as Cu, Pb, Zn (Geracitano et al., 2004) and polycyclic aromatic hydrocarbons (PAHs) (Filho et al., 2012). There are many ecotoxicological studies on estuarine biota, such as morphological and physiological changes in the polychaete *L. acuta* (Geracitano et al., 2004; Diaz-Jaramillo et al., 2016) and in fish such as the white croaker *Micropogonias furnieri* (Amado et al., 2006a) and flounder (Amado et al., 2006b).

Río de la Plata

On the Atlantic coast, one of the biggest estuaries is the Rio de la Plata, located at 35°S. This system is a highly productive area, sustaining valuable fisheries of Uruguay, Argentina, and international fleets (Martinez and Retta, 2001; Acha et al., 2008). Specifically on the Montevideo coastal area exists one of the most polluted harbors of the region (Danulat et al., 2002; Muniz et al., 2004). Gómez et al. (2009) found that microbenthic communities of the Río de la Plata were governed by two gradients, the first one determined by anthropic factors and the second one by conductivity and turbidity. In the case of bacterioplankton communities, Alonso et al. (2010) showed that bacterial abundance and diversity patterns based on ARISA data were highest at the frontal zone, where turbid waters from Paraná and Uruguay rivers mix with the Atlantic Ocean (Nagy et al., 2008). Regarding the organic pollutants, there is evidence of natural and anthropogenic (petrogenic and pyrogenic) contribution to the Uruguayan coastal portion of the Río de la Plata Estuary (Venturini et al., 2015). Montevideo Bay, one of the most urbanized areas of the estuary, presents chronic oil pollution with the occurrence of hydrocarbons derived from both crude petroleum and petroleum combustion. Also, metal concentrations and biochemical markers assessed in the area indicate that adverse effects to the biota are likely being generated (Muniz et al., 2004; Venturini et al., 2015).

Figure 3. Map of South America showing the main Neotropical water basins associated with the most known estuaries in term of ecotoxicological status. Dashed squares represent the approximate area of estuarine influence in the marine environment.

Bahía Blanca

The Bahía Blanca estuary is located in the south-eastern section of Buenos Aires province, Argentina. It is a coastal environment with a city that exceeds 350,000 inhabitants, whose pre-filtered effluents are directly introduced into the estuarine waters. In particular, it presents intense anthropogenic activity at the north shoreline, including oil, chemical and plastic factories, two commercial harbors and a fishing fleet, thus requiring regular dredging (Ferrer et al., 2006; Arias et al., 2010). The inner part of the estuary presents low urbanized rural lands, a tourist area and an artisanal fishing recreational port (Arias et al., 2010). A 33% of saltmarshes ecosystem dominated by *Spartina perennis* have been replaced by human land uses (Pratolongo et al., 2013) and an integrative strategy to manage the estuary threatened by climate change and pollution has been recently proposed by researchers (Kopprio et al., 2015).

Metals such as copper, lead and zinc that are present in surface sediments in this estuary have concentrations comparable to those reported as natural background for similar regions. However, cadmium concentrations were higher than those previously reported from the same environment, and similar to concentrations reported from other industrialized areas. The same authors addressed the potential toxicity of these elements to the native crab *Neohelice granulata* (Simoneti et al., 2011). Young crabs of this species were considered potentially dangerous agents for transfer of metals along the associated trophic network, due to their relative elevated resistance and capacity to bioaccumulate trace metals in their tissues (Ferrer et al., 2006). A recent assessment of POPs in surface sediments indicated that some individual POPs exceeded the low effect range as well as the median effect range in this area (Oliva et al., 2015). POPs monitoring has also revealed a wide recent use of some currently forbidden insecticides such as Heptachlor and Mirex, banned in 1993 and 1999 respectively (Arias et al., 2010).

General Discussion

Although several of the South American estuarine systems are suffering some type of human impact, few of those with an important drainage area remain pristine or have no recorded data. In general, scarce information exists on ecotoxicological approaches to understand the ecological risk of pollution in transitional waters of this continent. Although European and North American macrotidal estuarine model systems as references help as a source of basic information, Neotropical transition waters are naturally different with a wide range of hydrological regimes that covers tropical, sub-tropical and temperate climates. For future directions aimed at conservation status and impact assessments of South American estuaries, baseline integrated monitoring studies to allow early prediction of human impacts are becoming urgent.

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Chapter 17

ECOTOXICOLOGY IN THE MARINE ENVIRONMENT: BIOACCUMULATION AND BIOCONCENTRATION FACTOR OF POLYCYCLIC AROMATIC HYDROCARBONS

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ABSTRACT

The bioaccumulation of polycyclic aromatic hydrocarbons (PAHs) was determined for *Anadara similis* and *A. tuberculosa* (Mollusca: Bivalvia), the latter used as a marine environmental quality bioindicator organism on the Colombian Pacific coast. For each species a total of 120 organisms were exposed to a mixture of 16 PAHs (1.50×10^{-2} µg/mL) considered priority pollutants by the US Environmental Protection Agency. The concentrations tested were those considered safe for aquatic life by the Canadian Council of Ministers of the Environment. The exposure lasted a total of 45 days for *A. tuberculosa* and 30 for *A. similis* (due to higher mortality rates). The accumulation of the tested PAHs was lower for the compounds with high molecular weight (HMW) compared to the compounds with low molecular weight. However, the bioconcentration factors for HMW compounds were higher due to their increased stability and persistence. After exposure, no significant differences in the levels of bioaccumulated PAHs between species were demonstrated (Mann-Whitney U Test, $p=0.24$).

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Keywords: bioaccumulation, bioconcentration factor, polycyclic aromatic hydrocarbons, bivalves

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) make up the most toxic part of petroleum and therefore have the greatest ecotoxicological interest. Their presence in aquatic environments makes them bioavailable to organisms, being therefore incorporated through bioaccumulation processes and consequently biomagnified throughout the food web.

Sixteen PAHs are considered of interest as environmental contaminants by the US Environmental Protection Agency-US EPA (Achten and Hofmann, 2009; Rey-Salgueiro et al., 2009), the US Agency for Toxic Substances and Disease Registry (ATSDR, 2015) and the International Agency for Research on Cancer (IARC, 1983), mainly because of their mutagenic and carcinogenic effects (Meador et al., 1995; Potin et al., 2004). These compounds can disperse in the water column and build up in sediments and biota (Bihari et al., 2007). Bioaccumulation levels are a good estimate of xenobiotics in the wild, where the sessile or sedentary organisms, with little capacity for metabolic degradation, are the most exposed to the entry of contaminants (Fernández and Freire, 2005).

Filtering organisms such as bivalve mollusks incorporate and accumulate PAHs compounds in their tissues at concentrations considerably higher than those in the surrounding water (Neff, 2002). It is for this reason that bivalves have been used as biomonitors of pollutants in aquatic environments (Chęć et al., 2008).

Biomonitoring has great importance in monitoring programs to evaluate the quality of aquatic environments. Although *in situ* biomonitoring provides information regarding the quality status of a water body, it is important to assess the accumulation of contaminants and the effects on organisms caused by such accumulation.

Bioaccumulation of chemicals in organisms represents an important criterion for ecotoxicological risk evaluations (Ivanciuc et al., 2006), allowing to identify the quality of the aquatic environment. This is how the Colombian Pacific bivalve mollusk *Anadara tuberculosa* has been used as a biomonitor for assessing water quality in presence of oil derivatives. This species and *A. similis* were exposed to a mixture of PAHs to determine bioaccumulation, bioconcentration factors (BCF) and differences that can make one species a better biomonitor than another.

METHODS

Test Species

A. tuberculosa is the most exploited mollusk in Colombia; its distribution covers the area between Lake Whales (South California) to Tumbes (Peru) (Keen, 1971). It has been used to predict exposure, effects and susceptibility in the presence of anthropogenic substances in the monitoring of marine pollution (such as the *Mussel Watch* program; Sericano et al., 1995) and by some countries in the American Pacific (De la Cruz, 1994; Beliaeff et al., 1997; Jara-Marini et al., 2012).

A. similis shares the same habitat with *A. tuberculosa* in the mangrove ecosystem and has a geographical distribution from Corinto (Nicaragua) to Guayaquil (Ecuador) (Keen, 1971). Both species bury in the sediment, and features such as being filter-feeders and sessile make them ideal in biomonitoring programs. Their economic importance constitutes 96% of the total production of bivalves in Colombia (Instituto Colombiano de Desarrollo Rural-INCODER, 2007).

The Test Solution

The PAHs mixture was added to the test aquaria to obtain a final concentration of 1.50×10^{-2} µg/mL. Concentrations of compounds in the mixture were established using the maximum levels recommended by the Canadian Environmental Quality Guidelines of the Canadian Council of Ministers of the Environment-CCME (2010). For some compounds that do not have a reference level established by the CCME, a lower concentration than that stipulated for anthracene (the most restrictive among this group within the guide) was employed.

Acenaphthylene concentration was determined taking into account its similarity in structure and molecular weight (MW) with acenaphthene; for chrysene was considered the similarity in MW with pyrene. For indeno[1,2,3-cd]pyrene was established a lower concentration with respect to the remaining compounds due to their complex structure and high MW (Table 1).

Table 1. Composition of the PAHs mixture used in the test (µg/mL) based on the maximum levels recommended by the Canadian Council of Ministers of the Environment-CCME (2010) (modified from Zambrano et al., 2012a)

Compound	CCME guideline	Test concentration
Anthracene	1.2×10^{-5}	1.2×10^{-5}
Pyrene	2.5×10^{-5}	2.5×10^{-5}
Benzo[k]fluoranthene	-	1.0×10^{-5}
Benzo[g,h,i]perylene	-	1.0×10^{-5}
Benzo[a]pyrene	1.5×10^{-5}	1.5×10^{-5}
Benzo[b]fluoranthene	-	1.0×10^{-5}
Benzo[a]anthracene	1.8×10^{-5}	1.8×10^{-5}
Indeno[1,2,3-cd]pyrene	-	5.0×10^{-6}
Dibenzo[a,h]anthracene	-	1.0×10^{-5}
Fluoranthene	4.0×10^{-5}	4.0×10^{-5}
Chrysene	-	2.5×10^{-5}
Naphthalene	1.4×10^{-3}	1.1×10^{-3}
Acenaphthylene	-	5.0×10^{-3}
Acenaphthene	5.8×10^{-3}	5.8×10^{-3}
Fluorene	3.0×10^{-3}	3.0×10^{-3}
Phenanthrene	4.0×10^{-4}	4.0×10^{-4}
Test concentration	-	1.5×10^{-2}

Bioassays

The development of bioassays was based on standard methodologies of the American Public Health Association (APHA, 2005) and the US EPA (2000a). Live organisms were acquired on the open market in Tumaco (Colombia) and transported to the laboratory in the Center for Pacific Oceanographic and Hydrographic Research-Cccp where the bioassays were performed. They were acclimatized in 250 L tanks (permanent aeration) for 10 days during which physicochemical variables (temperature, salininity, pH and dissolved oxygen–DO) were recorded using a WTW Multi 340i multiparameter equipment. Organisms were fed 1 L/day of $3x10^6$ cel/mL diatomaceous cultivation (semi-intensive, not specific for a particular species). After acclimatization, 10 organisms of each species were sacrificed in order to determine baseline PAHslevels. The remaining organisms were moved to 20 L volumetric glass aquariums (10 organims/aquarium). 120 organims of each species (12 aquariums/ specie) were exposed to a mixture of 16 PAHs ($1.5x10^{-2}$ µg/mL) and every 5 days accumulation of PAHs was evaluated. The PAHs mixture was totally renewed every 24 h and food ($3x10^6$ cel/mL) was provided daily. Exposure period was of 45 days for *A. tuberculosa* and, due to high mortality, only 30 days for *A. similis*.

Two types of controls were used, one containing only seawater and the other with the solvent (acetone) used to prepare the mixture in a volume equal to that used to deliver the compounds in solution (15 mL). Controls were tested in triplicate.

Aeration was not provided during due to the volatility of some compounds. The water used to prepare the replacement solutions was aerated during several hours in order to saturate it with DO.

Laboratory Analysis

For determination of PAHs levels, tissues (±10g; all the body) of 10 organisms (composite samples) were homogenized and analysed by using gas chromatography and mass spectrometry (GC/MS; gas chromatograph - Agilent Technologies 6890N Network - coupled to a mass selective detector - MSD-Agilent Technologies 5973 Series Network) following the methodologies described by the US EPA (1996a) and Russell et al. (2002); under the method of selective ion monitoring (SIM) and using standard analytical high purity (Supelco trademark) for identification and quantification. Likewise, the fats and oils content was determined using Soxhlet extraction with ethyl ether as solvent (US EPA, 1996b). Data are provided in dry weight.

Bioconcentration Factor (BCF)

Since BCF indicates the ratio between the concentration of a chemical in an organism and in the water (US EPA, 2000b), calculations were performed by dividing the concentration of PAHs in tissues by the concentration of the compound in water.

Statistical Analysis

Statistical differences in PAHs bioaccumulation between species for each sampling period were checked by the Mann-Whitney U Test. Data analysis were performed in SPSS program (version 24) with a significance level of 95%.

RESULTS

Physical Chemical Variables

The physicochemical variables in the vessels during testing showed values between 23.30 and 25.70ºC for temperature, 7.91 and 8.24 for pH; 29.30 and 30.00 psu for salinity and 3.25 and 7.80 mg O_2/L for DO.

Mortality

Survival of 100% occurred during the acclimation period. Throughout the test, both species exposed to PAHs recorded a mortality reaching 43.50% in *A. tuberculosa* (between 2 and 29 days) and 48.50% in *A. similis* (between 2 and 21 days, with the highest percentage between days 2 and 12). In the control vessels, *A. tuberculosa* mortality reached 33% (between 13 and 33 days) and 53% in *A. similis* (between 7 and 18 days). In the treatment containing only acetone, a mortality of 56.60% for *A. tuberculosa* (on days 16 and 22) and 66.66% for *A. similis* (between days 5 and 13) was recorded.

Fats and Oils

As for the content of oils and fats, the highest percentage in *A. tuberculosa* was determinated at the start of the test and at 15 days (1.86%); the lowest percentage was found on day 30 (0.04%) (Table 2). For *A. similis* the highest percentage was determined 15 days after the start of the test (2.70%) and the lowest percentage was found at 5 days (0.08%) (Table 3).

Bioaccumulation and BCF

The incicial PAH concentration observed before the experiment was of 1.40 µg/g in *A. tuberculosa* (Table 2) and $2.00x10^{-2}$ µg/g in *A. similis* (Table 3). *A. tuberculosa* after 45 days showed a tendency to bioaccumulate in greater proportion HMW compounds (Figure 1). Acenaphthene recorded lower concentrations followed by fluorene, phenanthrene and acenaphthylene; while the highest concentrations were recorded by the acenaphthene, fluorene, acenaphthylene and phenanthrene.

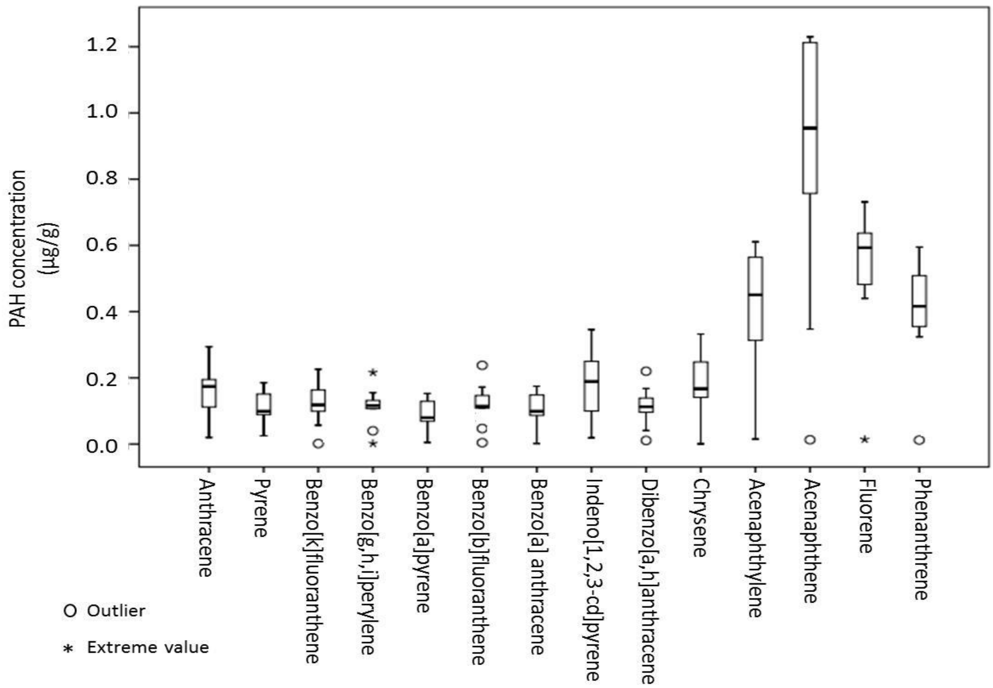

Figure 1. PAH concentration distribution incorporated in *Anadara tuberculosa* during the test (45 days).

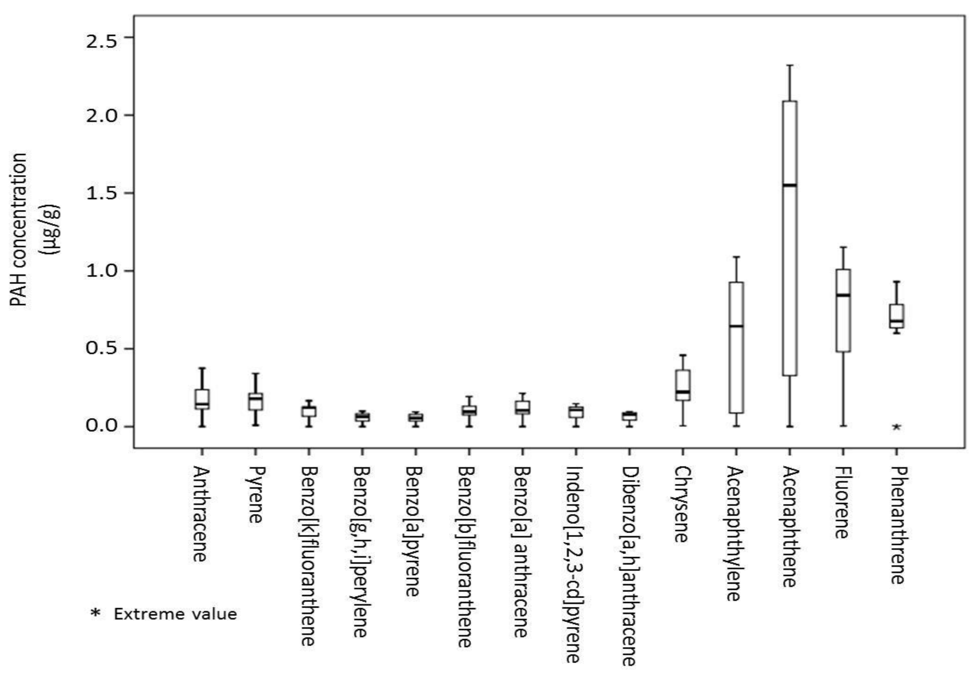

Figure 2. PAH concentration distribution incorporated in *Anadara similis* during the test (30 days).

A. similis exposure covered a period of 30 days, presenting high levels of compounds with low molecular weight (LMW) such as acenaphthene, fluorene and acenaphthene, and low concentrations of benzo[a]pyrene, benzo [g,h,i]perylene and dibenzo[a,h]anthracene, compounds with HMW (Figure 2).

The BCF in *A. tuberculosa* indicated that indeno[1,2,3-cd]pyrene reached the highest value (69200) after 35 days, followed by anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene, whose maximum values were recorded between 30 and 35 days of exposure. Lower values for the recorded BCF of acenaphthylene ranged between 40 and 122 (Table 4).

The BCF determined in *A. similis* reached higher values for anthracene, followed by byindeno[1,2,3-cd]pyrene, benzo[b]fluoranthene, chrysene, benzo[k]fluoranthene, pyrene and benzo[a]anthracene; acenaphthylene showed the lowest BCF (Table 5). With respect to the mixture, the last recordings were obtained at 25 (500)and 45 days (365) in *A. similis* and *A. tuberculosa*, respectively, while the highest values recorded for *A. similis* (>300) were observed after 15 days (Table 5), showing a higher affinity for these compounds in relation to *A. tuberculosa.*

There were no statistical differences between the total PAHs levels accumulated in both species (Mann-Whitney U Test;p=0.24). The dispersion in terms of incorporation was higher in *A. similis* due to the differences of the concentration recorded for each compound in particular (Figure 3).

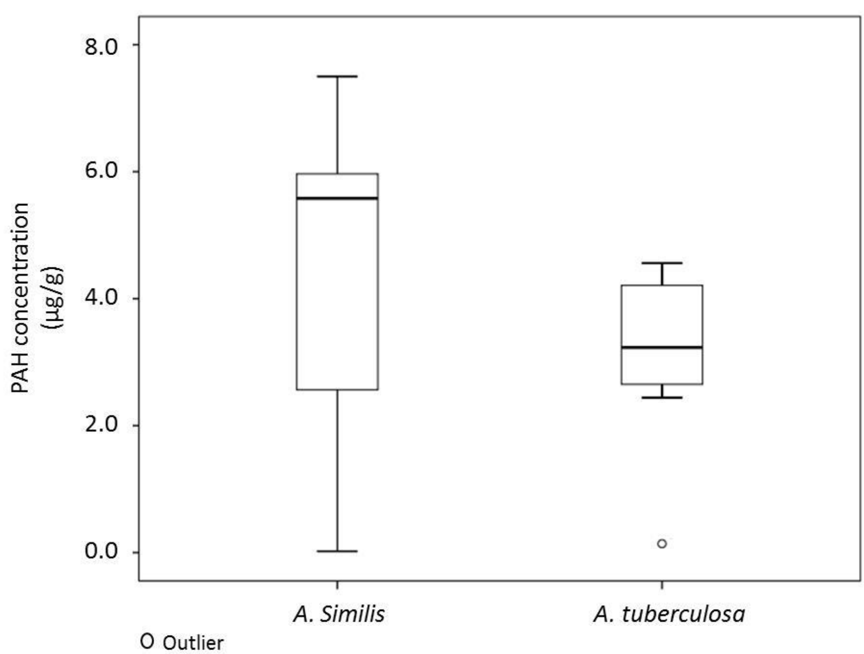

Figure 3. Distribution of PAHs in organisms during the bioaccumulation test.

Table 2. PAHs concentrations and percentages of fats and oils in *Anadara tuberculosa* during the test (μg/g dry weight) (modified from Zambrano et al., 2012a)

Compounds	Exposure days									
	0	5	10	15	20	25	30	35	40	45
Anthracene	$2.00x10^{-2}$	$1.02x10^{-1}$	$2.18x10^{-1}$	$1.14x10^{-1}$	$1.93x10^{-1}$	$1.12x10^{-1}$	$2.94x10^{-1}$	$1.55x10^{-1}$	$1.94x10^{-1}$	$1.95x10^{-1}$
Pyrene	$2.50x10^{-2}$	$6.70x10^{-2}$	$1.85x10^{-1}$	$9.00x10^{-2}$	$1.31x10^{-1}$	$9.80x10^{-2}$	$1.65x10^{-1}$	$8.90x10^{-2}$	$1.51x10^{-1}$	$1.00x10^{-1}$
Benzo[k]fluoranthene	$1.60x10^{-3}$	$5.70x10^{-2}$	$1.10x10^{-1}$	$1.14x10^{-1}$	$1.63x10^{-1}$	$1.22x10^{-1}$	$1.72x10^{-1}$	$2.26x10^{-1}$	$9.90x10^{-2}$	$1.33x10^{-1}$
Benzo[g,h,i]perylene	$1.70x10^{-3}$	$4.00x10^{-2}$	$1.40x10^{-1}$	$1.20x10^{-1}$	$1.18x10^{-1}$	$1.10x10^{-1}$	$2.16x10^{-1}$	$1.55x10^{-1}$	$1.07x10^{-1}$	$1.32x10^{-1}$
Benzo[a]pyrene	$4.70x10^{-3}$	$3.20x10^{-2}$	$8.50x10^{-2}$	$6.90x10^{-2}$	$1.29x10^{-1}$	$7.50x10^{-2}$	$1.53x10^{-1}$	$1.42x10^{-1}$	$7.50x10^{-2}$	$9.50x10^{-2}$
Benzo[b]fluoranthene	$4.30x10^{-3}$	$4.70x10^{-2}$	$1.10x10^{-1}$	$1.09x10^{-1}$	$1.47x10^{-1}$	$1.18x10^{-1}$	$1.72x10^{-1}$	$2.38x10^{-1}$	$1.10x10^{-1}$	$1.39x10^{-1}$
Benzo[a]anthracene	$1.70x10^{-3}$	$5.40x10^{-2}$	$1.00x10^{-1}$	$8.70x10^{-2}$	$1.58x10^{-1}$	$9.80x10^{-2}$	$1.48x10^{-1}$	$1.75x10^{-1}$	$9.80x10^{-2}$	$1.23x10^{-1}$
Indeno[1,2,3-cd]pyrene	$1.90x10^{-2}$	$6.50x10^{-2}$	$1.00x10^{-1}$	$2.05x10^{-1}$	$2.50x10^{-1}$	$1.73x10^{-1}$	$3.08x10^{-1}$	$3.46x10^{-1}$	$1.45x10^{-1}$	$2.24x10^{-1}$
Dibenzo[a,h]anthracene	$1.10x10^{-2}$	$4.10x10^{-2}$	$1.37x10^{-1}$	$1.07x10^{-1}$	$1.12x10^{-1}$	$9.60x10^{-2}$	$2.20x10^{-1}$	$1.68x10^{-1}$	$1.13x10^{-1}$	$1.39x10^{-1}$
Fluoranthene	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$	$<2.00x10^{-4*}$
Chrysene	$5.00x10^{-4}$	$1.02x10^{-1}$	$1.41x10^{-1}$	$1.61x10^{-1}$	$2.73x10^{-1}$	$1.73x10^{-1}$	$2.48x10^{-1}$	$3.32x10^{-1}$	$1.50x10^{-1}$	$2.13x10^{-1}$
Naphthalene	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$	$<6.00x10^{-4*}$
Acenaphthylene	$1.50x10^{-2}$	$3.13x10^{-1}$	$2.01x10^{-1}$	$5.64x10^{-1}$	$5.92x10^{-1}$	$3.64x10^{-1}$	$3.71x10^{-1}$	$5.41x10^{-1}$	$5.30x10^{-1}$	$6.11x10^{-1}$
Acenaphthene	$1.30x10^{-2}$	$7.57x10^{-1}$	$3.47x10^{-1}$	1.23	1.21	$8.38x10^{-1}$	$7.92x10^{-1}$	1.15	1.07	1.23
Fluorene	$1.40x10^{-2}$	$4.40x10^{-1}$	$4.82x10^{-1}$	$5.75x10^{-1}$	$6.29x10^{-1}$	$5.06x10^{-1}$	$7.04x10^{-1}$	$6.37x10^{-1}$	$6.11x10^{-1}$	$7.31x10^{-1}$
Phenanthrene	$1.20x10^{-2}$	$3.24x10^{-1}$	$5.09x10^{-1}$	$3.60x10^{-1}$	$4.07x10^{-1}$	$3.55x10^{-1}$	$5.95x10^{-1}$	$4.44x10^{-1}$	$4.25x10^{-1}$	$5.23x10^{-1}$
ΣPAHs	$1.40x10^{-1}$	2.44	2.86	3.91	4.51	3.23	4.56	4.80	3.87	5.48
Fats and oils (%)	4.64	0.73	0.13	1.86	1.00	1.34	0.04	1.30	1.26	1.46

* Detection limit.

Table 3. PAHs concentrations and percentages of fats and oils in *Anadara similis* during the test (µg/g dry weight) (modified from Zambrano et al., 2012a)

Compounds	Exposure days						
	0	5	10	15	20	25	30
Anthracene	7.00x10^{-4}	1.14x10^{-1}	1.44x10$^{-1°}$	1.45x10^{-1}	2.80x10^{-1}	3.75x10^{-1}	1.94x10^{-1}
Pyrene	8.70x10^{-3}	4.90x10^{-2}	1.79x10^{-1}	2.04x10^{-1}	1.66x10^{-1}	3.41x10^{-1}	2.21x10^{-1}
Benzo[k]fluoranthene	<2.00x10^{-4*}	1.31x10^{-1}	9.30x10^{-2}	1.22x10^{-1}	4.10x10^{-2}	1.22x10^{-1}	1.66x10^{-1}
Benzo[g,h,i]perylene	<2.00x10^{-4*}	8.40x10^{-2}	5.50x10^{-2}	6.40x10^{-2}	1.80x10^{-2}	8.20x10^{-2}	1.00x10^{-1}
Benzo[a]pyrene	<2.00x10^{-4*}	6.80x10^{-2}	4.60x10^{-2}	5.60x10^{-2}	2.70x10^{-2}	9.40x10^{-2}	8.90x10^{-2}
Benzo[b]fluoranthene	<2.00x10^{-4*}	9.50x10^{-2}	9.60x10^{-2}	1.21x10^{-1}	5.60x10^{-2}	1.40x10^{-1}	1.93x10^{-1}
Benzo[a]anthracene	<4.00x10^{-4*}	8.40x10^{-2}	1.04x10^{-1}	1.20x10^{-1}	8.10x10^{-2}	2.14x10^{-1}	2.06x10^{-1}
Indeno[1,2,3-cd]pyrene	<6.00x10^{-4*}	1.07x10^{-1}	8.30x10^{-2}	1.15x10^{-1}	3.60x10^{-2}	1.36x10^{-1}	1.47x10^{-1}
Dibenzo[a,h]anthracene	<6.00x10^{-4*}	8.30x10^{-2}	5.90x10^{-2}	7.70x10^{-2}	2.40x10^{-2}	9.60x10^{-2}	9.60x10^{-2}
Fluoranthene	<2.00x10^{-4*}	<2.00x10^{-4*}	<2.00x10^{-4*}	<2.00x10^{-4*}	<2.00x10^{-4*}	<2.00x10^{-4*}	<2.00x10^{-4*}
Chrysene	5.00x10^{-3}	1.60x10^{-1}	2.22x10^{-1}	3.15x10^{-1}	1.76x10^{-1}	4.08x10^{-1}	4.58x10^{-1}
Naphthalene	<6.00x10^{-4*}	<6.00x10^{-4*}	<6.00x10^{-4*}	<6.00x10^{-4*}	<6.00x10^{-4*}	<6.00x10^{-4*}	<6.00x10^{-4*}
Acenaphthylene	3.40x10^{-3}	1.35x10^{-1}	4.00x10^{-2}	8.14x10^{-1}	1.04	1.09	6.45x10^{-1}
Acenaphthene	<3.00x10^{-4*}	5.04x10^{-1}	1.53x10^{-1}	1.93	2.25	2.32	1.55
Fluorene	4.40x10^{-3}	5.36x10^{-1}	4.25x10^{-1}	8.79x10^{-1}	1.14	1.15	8.43x10^{-1}
Phenanthrene	<4.00x10^{-4*}	6.00x10^{-1}	6.78x10^{-1}	7.02x10^{-1}	8.65x10^{-1}	9.30x10^{-1}	6.69x10^{-1}
ΣPAHs	2.00x10^{-2}	2.75	2.38	5.66	6.27	7.50	5.58
Fats-oils (%)	0.44	0.08	1.80	2.70	1.36	0.68	1.15

* Detection limit.

Table 4. Bioconcentration factors for PAHs incorporated by *Anadara tuberculosa* during the test

Compounds	Exposure days								
	5	10	15	20	25	30	35	40	45
Anthracene	8500	18167	9500	16083	9333	24500	12917	16167	16250
Pyrene	2680	7400	3600	5240	3920	6600	3560	6040	4000
Benzo[k]fluoranthene	5700	11000	11400	16300	12200	17200	22600	9900	13300
Benzo[g,h,i]perylene	4000	14000	12000	11800	11000	21600	15500	10700	13200
Benzo[a]pyrene	2133	5667	4600	8600	5000	10200	9467	5000	6333
Benzo[b]fluoranthene	4700	11000	10900	14700	11800	17200	23800	11000	13900
Benzo[a]anthracene	3000	5556	4833	8778	5444	8222	9722	5444	6833
Indeno[1,2,3-cd]pyrene	13000	20000	41000	50000	34600	61600	69200	29000	44800
Dibenzo[a,h]anthracene	4100	13700	10700	11200	9600	22000	16800	11300	13900
Chrysene	4080	5640	6440	10920	6920	9920	13280	6000	8520
Acenaphthylene	63	40	113	118	73	74	108	106	122
Acenaphthene	131	60	213	209	145	126	199	185	212
Fluorene	147	161	192	210	169	235	212	204	244
Phenanthrene	810	1273	900	1018	888	1488	1110	1063	1308
PAHs mixture	163	191	261	301	215	304	320	258	365

Table 5. Bioconcentration factors for PAHs incorporated by *Anadara similis* during the test

Compounds	Exposure days					
	5	10	15	20	25	30
Anthracene	9500	12000	12083	23333	31250	16167
Pyrene	1960	7160	8160	6640	13640	8840
Benzo[k]fluoranthene	13000	9300	12200	4000	12200	16600
Benzo[g,h,i]perylene	8400	5500	6400	9000	8200	10000
Benzo[a]pyrene	4533	3067	3733	1800	6267	5933
Benzo[b]fluoranthene	9500	9600	12100	5600	14000	19300
Benzo[a]anthracene	4667	5778	6667	4444	11889	11444
Indeno[1,2,3-cd]pyrene	21400	16600	23000	72	27200	29400
Dibenzo[a,h]anthracene	8300	5900	7700	24	9600	9600
Chrysene	6400	8880	12600	7040	16320	18320
Acenaphthylene	27	8	163	208	219	129
Acenaphthene	87	26	333	387	400	268
Fluorene	179	142	293	381	384	281
Phenanthrene	1500	1695	1755	2163	2325	1673
PAHs mixture	183	159	378	418	500	372

DISCUSSION

According to the obtained results, each species presented different responses for each type of pollutant. Bioaccumulation and biomagnification occur when the contaminants do not interfeer with the life processes of the organism or when concentrations are sufficiently low to allow tolerance, acclimatization or adaptation (Baqueiro-Cardenas et al., 2007). Bio-accumulative contaminants are chemically hydrophobic compounds whose elimination is very slow and difficult, so they tend to accumulate in tissues and organs such as those consisting of fatty tissue (Fernández and Freire, 2005). Fat solubility is a determining factor for the absorption of toxic compounds, such as PAHs that are difficult to eliminate.

The lipid content is influenced by factors such as age, species, feeding and spawning, which changes during the year (Perugini et al., 2007). During testing it was observed that the content of fats and oils showed variations, with an initial level that then significantly declined and that may be related to the process of adapting to a new environment, such as when moving from the acclimation tanks to the test vessels. Thereafter ascents and descents that are not related to an increase or decreased concentration of of PAHs were presented.

PAHs levels absorbed by the organisms exposed to the PAHs mixture were high considering the low exposure concentrations, which indicates the great potential of bivalves to be used in assessment of the quality of marine environments.

PAHs with LMW were supplied at higher concentrations (for their low stability in water) with respect to HMW, this increased is related to greater concentrations determinated in organisms for LMW compounds.

Bioaccumulation was proportionally superior to the compounds of HMW, taking into account the exposure levels and accumulated concentrations. This can be explained by the slow decline of those compounds, that remain in the water column for extended periods (Yamada et al., 2003). LMW compounds showed an inverse behavior, due to their greater availability and lower persistence, that allows for a greater mobility between environmental compartments.

The persistence and the potential to bioaccumulationare higher for HMW compounds; therefore, compounds such as benzo[a]pyrene are highly persistent and bioaccumulative, characteristics that classify it as an aquatic pollutant of concern (Binelli and Provini, 2003).

The high accumulation observed also for LMW compounds could be not only due to the greater exposure concentrations, but also because the solvent made them more stable in the environment and therefore more available; PAHs are non-polar, soluble in organic solvents, and difficult to mobilize due to their low water solubility (El-Motaium et al., 2009). However, the bioaccumulation of most of these compounds showed large variations. Given that such compounds canbe excreted more efficiently, the variability in the accumulation levels may have been caused by constant absorption and excretion at short periods (Calero and Zambrano, 1997; Albaigés, 2005; Zambrano et al., 2012b; Zambrano Ortiz, 2015).

A. similis has greater capacity to incorporate PAHs reaching 7.50 µg/g after 25 days of exposure and a maximum of 5.48 µg/g in *A. tuberculosa* after 45 days, despite the fact that the differences in terms of incorporation between species are not significant.

Since the concentration of the PAH mixture used in the tests was below the range estimated as lethal (concentration at which a mortality of the exposed organisms has reached 50%; LC_{50} of $3.50 x 10^{-2}$ µg/mL; NOAA, 1993), the mortality observed may be associated with factors other than the PAHs (e.g., the duration of the acclimatization period).

Regarding the commercial importance that a large number of bivalves have and due to the fact that trail species represent an important source of food for the populations settled on the Colombian Pacific coast, the presence of toxic compounds may represent risks for the health of the marine environment and to human consumers. Therefore, taking into account the regulations of the European Union (Official Journal of the European Union, 2011), it was possible to establish that the specified concentrations in the test organisms where only safe for human consumption prior to starting the test after several days or weeks of acclimatization, as has been observed in others studies (Farrington et al., 1982; Lee et al., 2010; El-Gamal 2011), because after exposure to the mixture both the level of benzo[a]pyrene ($5.00 x 10^{-3}$ µg/g) and the sum of indicator compounds ($3.00 x 10^{-2}$ µg/g for the sum of chrysene, benzo[a]pyrene, benzo[a]anthracene and benzo[b]fluoranthene as markers or indicators of toxicity) exceeded the recomended limit.

The bioconcentration process is favored by the lipophilicity and measured through the octanol-water partition coefficient (Kow). Polar substances have low Kow values, whereas for hydrophobic contaminants Kow values are very high. Bioconcentration of these compounds in water by marine organisms is directly proportional to the log Kow (Neff, 2002); LMW PHAs are generally more volatile, slightly soluble in water and less lipophilic than HMW. These physicochemical characteristics largely determine their behavior in the environment; therefore, bioconcentration of HMW is much higher with respect to LMW (Pruell et al., 1986). The volatile PHAs of LMW (less than four rings) tend to be soluble in water and have low affinity to be adsorbed to particles (Log Kow <5); PHAs with HMW are generally insoluble in water and have a very strong affinity to be adsorbed on the surfaces of particles suspended in air and water (log Kow>5) (López Geta et al., 2008). Generally, substances with log Kow >6 are considered highly lipophilic (ONU, 2005).

The PHA levels obtained allowed to determine the highest BCF in *A. tuberculosa* for indeno[1,2,3-cd]pyrene followed by anthracene, benzo[b]fluoranthene, dibenzo[a,h]anthracene and benzo[g,i,h]perylene. Both species registered the lowest BCF with phenanthrene, acenaphthene and acenaphthylene, all with LMW and low logKow. High concentrations recorded for LMW compounds canbe associated to the factors previously mentioned for bioaccumulation: high concentrations of compounds used in the experiments, and higher availability due to the use of a solvent. The BCF evidenced a high potential for bioconcentration by obtaining readings from an order of magnitude of >3 and 4 for HMW compounds, contrary to HMW acenaphthylene, acenaphthene and fluorene which varied between orders of magnitude of 1 to 2, therefore classifying these compounds with a “medium”potential to bioconcentration according to Repetto and Sanz (1995).

A. similis presented higher BCF with a maximum of 500.33 after 25 days; this is how at 15 days trial this species with 378 exceeded the maximum obtained for *A. tuberculosa* (365), which was recorded after 45 days. *A. similis* BCF values remained higher until the end of the exposure, a situation associated with the differences in the BCF in different species (Rezaie-Boroon et al., 2014) as was reported by Wang (2012) for bivalves exposed to metals; certainly for fat-soluble compounds, levels of fats and oils in organisms play a key role, these being slightly higher in *A. similis* during testing.

As filter feeders, bivalves are heavily exposed to the accumulation of pollutants reaching high BCF for metals and organic compounds in contaminated areas (Phillips, 1995). BCF is a useful tool to evaluate risk of exposure to xenobiotics and estimate the consequences of long term exposure tohealth to establish permissible legal levels (Repetto and Sanz, 1995). In this context, ecotoxicology has great importance in assessing water quality before the onset of environmental damage and in establishing safe levels of toxic compounds aimed at ensuring the quality of ecosystems and protecting and preserving marine fauna.

CONCLUSION

The levels of PAHs incorporated by both bivalvespecies studied here suggest a potential risk to the health of consumers regarding organisms collected in areas with direct or indirect exposure to hydrocarbons. Even when exposed to sublethal concentrations, the bioconcentration capacity allows them to reach PAHs levels that are unfit for human consumption. As *A. similis* showed a greater tendency to bioaccumulate more PAHs and thus present higher bioconcentration factors than *A. tuberculosa*, it has a potential as biomonitor for PAHs contamination within the Colombian Pacific.

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Chapter 18

SOIL ECOTOXICOLOGY IN ENVIRONMENTAL RISK ASSESSMENT: A CASE STUDY IN A METAL CONTAMINATED SITE IN BRAZIL

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ABSTRACT

The majority of the ecological risk assessment (ERA) studies carried out in Latin America are based just on chemical characterization, focused on limit values of contaminants in soils and water, usually not including biological and ecotoxicological considerations. This chapter presents the experience of the application of a tiered ERA framework to tropical environments, and evaluates the feasibility and usefulness of different assessment tools to be used in different tiers within a triad approach. The case study was carried out in an abandoned lead smelter in Santo Amaro (Bahia, Brazil). The preliminary investigation included the problem formulation phase and the collection of the scientific information available about the study area, that resulted in the conceptual model and the analysis plan for the risk assessment. A tiered approach was proposed integrating information from three lines of evidence (LoE): chemical, ecotoxicological and ecological. Aims and actions of each phase of ERA were established to include the ecological and ecotoxicological perspectives, focusing on the soil compartment. The analysis plan included two tiers using the triad approach: tier 1, the screening phase, and tier 2, the detailed risk assessment. Besides chemical analysis, the evaluations on soil ecotoxicology included laboratory and field parameters: avoidance behavior and reproduction of soil invertebrates; bait lamina test; vegetation structure; soil ground

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running invertebrates; decomposition of organic material on litter bags; and some microbial parameters, to include ecosystem function data. The results aimed to characterize the ecological risk, ranking sites in the study area, thus supplying information to indicate remediation measures.

Keywords: soil ecotoxicology, contaminated sites, soil invertebrates, triad

INTRODUCTION

Relevance of ERA (Ecological Risk Assessment) in Contaminated Sites Management

The protection of soils, its diversity and ecological functions, has become an objective of environmental agencies around the world (Bone et al., 2010). In this context, the ecological risk assessment (ERA) process has been recognized as a powerful tool for the decision-making process in the management of contaminated sites or sites suspected of contamination (USEPA, 1998; Suter et al., 2000).

ERA is a complex process of collecting, organizing and analyzing environmental data to estimate the probability of adverse effects due to contamination, using data from different environmental compartments collected from different lines of evidence (LoE) and different sources of information, such as chemical analysis of contaminants, physical properties of the environment, biological surveys, and ecotoxicity tests. Although a general trend of biological responses can be expected in contaminated areas, the relationship between sources, exposure, and effects on organisms are complex and often specific to a particular site, a set of environmental conditions, and a specific receptor organism. This calls for a site-specific ERA to support decisions about risk management and remediation (Jensen and Mesman, 2006).

In general, management actions on soil protection rest on two basic approaches: (i) optimization of biodiversity and (ii) protection of ecosystem services (and their underlying biological/ecological processes) essential for the survival of mankind. Soil is seen as a multifunctional unit, supplying provisioning (food, water, fuel), regulating (soil erosion, flood control), cultural (recreation, spiritual value, sense of place) and essentially supporting (soil formation, nutrient cycling, oxygen from photosynthesis) services simultaneously (Millennium Ecosystem Assessment, 2005). In this sense, an ERA process should include indicators both of ecosystem structure and functions (Burger et al., 2007).

Main Phases of an ERA Process

Although different schemes of ERA are proposed and applied in different countries, the components of the ERA process are similar among them, generally including the following phases (see Figure 1):

Preliminary investigation: this phase includes the problem formulation of the assessment, including the collection of all the scientific information available for the area; the development of a conceptual model; and an analysis plan for the risk assessment (e.g., Pereira et al., 2004; Weeks et al., 2004). The conceptual model is built involving what is currently known about the site, geographical limits, source and type of contamination, historical use and activities in the site, current pathways of exposure, and observation of perceptible risks and ecological receptors at risk (Weeks, 2004; Jensen and Mesman, 2006; Ashton et al., 2008).

Exploratory investigation: this is an optional phase to confirm the existence of unacceptable contamination in the study area by performing a preliminary field sampling, followed by chemical analysis and comparison with soil quality guidelines.

Main investigation: this phase is performed in case a contamination is confirmed and a potential ecological risk can occur. This phase is usually done using a tiered approach where in each tier different assessment tools belonging to different LoE are applied, including more detailed chemical analysis, bioassays and ecological field surveys (Jensen and Mesman, 2006; Swartjes et al., 2008). At the end of the assessment, the results are integrated to describe the risk.

Several schemes of ERA are available in different countries, e.g., US (USEPA, 1998), Canada (CCME, 1996), UK (Weeks, 2004). In The Netherlands, the EU country with most experience in the application of site-specific ERA, a practical triad approach has been developed (Rutgers and Den Besten, 2005; Mesman et al., 2007) which has been adapted and successfully applied in Europe (Jensen and Mesman, 2006; Critto et al., 2007). In Latin America(LA), studies involving ERA are incipient. In Brazil, the ABNT (Brazilian National Standards Organization) started a discussion in 2014 about which methodology will be adopted to conduct ERA processes in this country (Niva et al., 2016). The respective guideline is under construction.

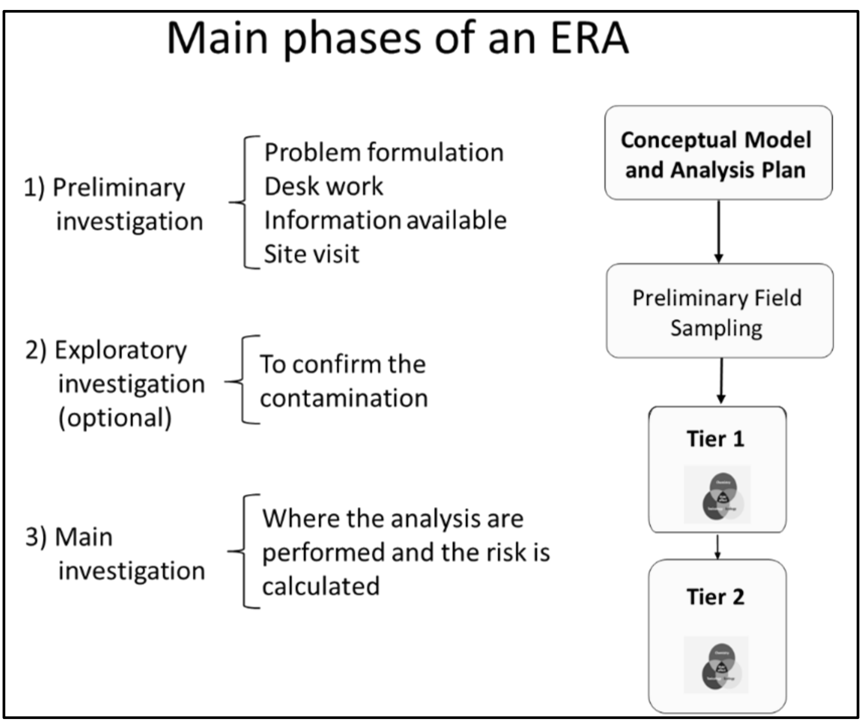

Figure 1. General scheme of an Ecological Risk Assessment process.

The Triad Approach

Consisting of three LoE (chemical, ecotoxicological and ecological), the triad approach is usually applied within a tiered system, i.e., information from each LoE is collected at each tier following a stepwise cost-effective process (Jensen and Mesman, 2006). The tiered approach is designed to be efficient in excluding extreme sites, i.e., either sites that pose no risk to ecosystems or sites that pose a high risk and where remediation actions are needed (Figure 2). The triad approach relies on the concept of weight-of-evidence (WoE), which is the process of combining information from multiple LoE to reach a conclusion about an environmental system or stressor, as such an approach minimizes the chance of false positive and false negative conclusions (Burton et al., 2002).

Tier 1 is essentially a screening phase, aiming to produce a first spatial representation of the risk and to determine whether a site can be excluded from higher tiers and of further testing (either because it is unlikely to pose a risk to the relevant ecological receptors or because a high risk is detected and there could be a need for immediate mitigation actions), or if it needs to be further evaluated at Tier 2. Thus, the tools used in tier 1 to collect information from each LoE should not only be able to indicate effects, but also be rapid, easy to apply and cost-effective (Jensen and Mesman, 2006).

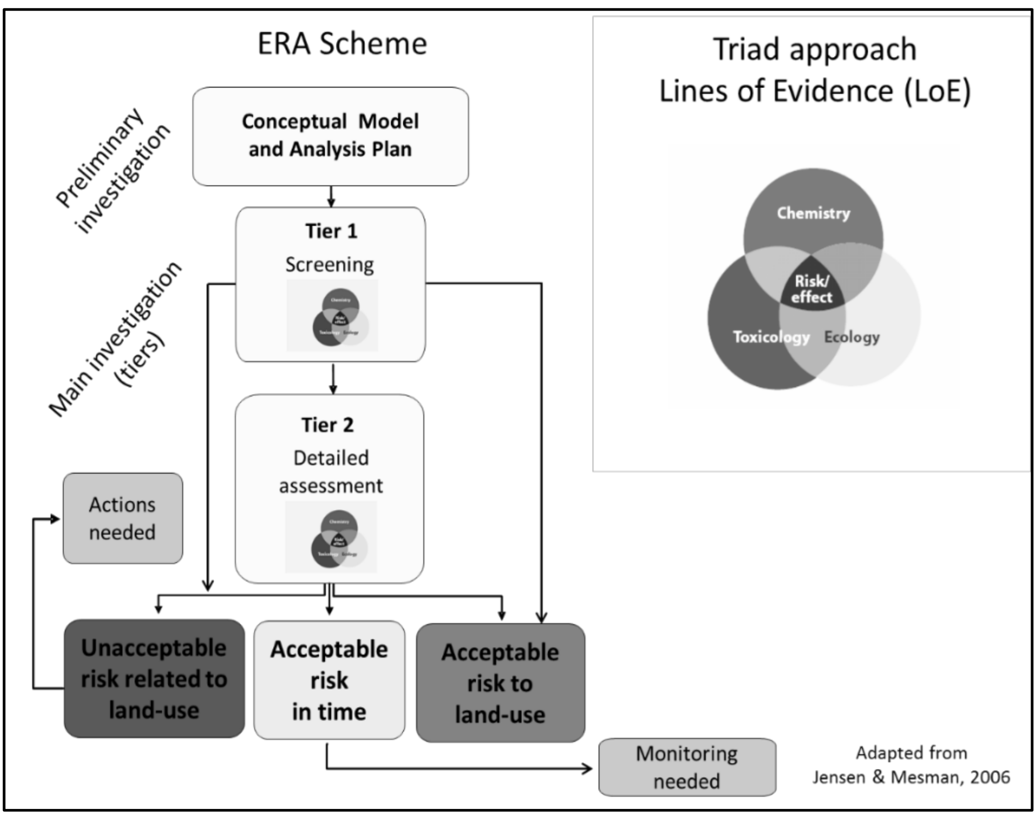

Figure 2. General scheme of Environmental Risk Assessment process organized in tier phases. Detail: triad approach composed by chemical, toxicological and ecological lines of evidence.

Tier 2 (and tier 3, if necessary) is performed to reduce uncertainties about the actual risk. In tier 2, the chemical LoE can comprise extraction techniques to assess the bioavailable fraction of pollutants in soil. This should be complemented with information derived from ecotoxicological tests (ecotoxicological LoE) and ecological surveys (ecological LoE) (Jensen and Mesman, 2006). The ecotoxicological LoE usually comprises long-term studies focusing on chronic endpoints such as reproduction and growth, and some mineralization processes, since these effects can occur at intermediate levels of pollution. The ecological information at tier 2 is collected to get more details about the possible impact on microbial communities and flora and fauna populations *in situ*, thus assessing effects at higher hierarchical levels of organization (Weeks et al., 2004; Critto et al., 2007).

THE STUDY CASE OF SANTO AMARO, BAHÍA, BRAZIL

An ERA process was carried out by Niemeyer et al. (2010, 2015) in the abandoned lead smelter of Plumbum metallurgy that was operational between 1960 and 1993, located adjacent to the urban area of Santo Amaro (BA, Brazil), about 150 mm away from Salvador, state capital (Figure 3). The area presented a high health risk to cattle and humans due to high levels of metals in soil and water, as well as by tailings and airborne dust from atmospheric deposition through chimney emissions while the smelter was operational. The study aimed to answer the following questions:

1) Does the metal contamination in the smelter area still pose some ecological risk to the soil habitat and retention functions 17 years after the closure of the smelter? How is the spatial extension of the risks posed by the smelter area?

Figure 3. Smelter area of Plumbum metallurgy, Santo Amaro, Bahia, Brazil.

Figure 4. Smelter area of Plumbum metallurgy, Santo Amaro, Bahia, Brazil. A) Evidence of runoff and erosion in a pile of furnace slag (gray material) in the smelter area during the rainy season. B) The aspect of the smelter area in the dry season (dust and lack of vegetation).

2) Are the detected effects directly associated to the presence of the metals (direct toxicity) or to an indirect stressor (habitat disruption)?
3) Is a tiered ERA framework suitable to be applied in contaminated sites with this typology of contamination?
4) Which type of biological (ecotoxicological and ecological) parameters are more sensitive to detect risk? Are they able to discriminate different levels of risk? And at which tier should they be used?

Preliminary Investigation: Building the Conceptual Model and Analysis Plan of an ERA Process

Based on the available information about the site, including data obtained during the site visits (Figure 4) and the chemical pre-sampling, a conceptual model (Figure 5) and an analysis plan for an ERA were developed.

The primary contamination source identified was soil contaminated by furnace slag deposition or by aerial deposition (wind-blown dust or past chimney emissions). The principal source of potential exposure to the ecological receptors is the contaminated soil, through ingestion, cellular absorption, aerial deposition (wind-blown particles) and root uptake. Metal contaminants can be available, posing potential risk to primary receptors such as plants, soil invertebrates and soil microbial communities. In addition, other species can be linked to contaminants through the terrestrial food chain, such as invertebrates feeding on plants, and vertebrates, such as birds (seed-, plant-eating and invertebrate-feeding species), small mammals, amphibians, reptiles and raptor species.

Table 1. Assessment and measurement endpoints. Adapted from Weeks et al. (2004)

Receptor	Relevance for the ecosystem functioning	Assessment endpoints	Measurement endpoints
Plant community	Food and habitat supply for animal species. Maintenance of soil structure. Supply of nutrients.	Habitat function that sustains plant germination, growth, biomass and species richness.	Determination of vegetation cover *in situ* (1). Plant toxicity test with monocotyledonous and dicotyledonous species (2). Determination of vegetation richness species *in situ* (2).
Soil invertebrate community and activity	Food supply for animal species. Predation of microfauna. Decomposition of organic material. Maintenance of soil structure.	Habitat function that sustains diverse and active invertebrate populations.	Avoidance behavior tests with earthworms and springtails (1). Feeding activity evaluated by bait lamina test *in situ* (1) Reproduction tests with earthworms, springtails and enchytraeids (2). Composition of soil surface dwelling macroarthropod community collected with pitfall traps (richness, diversity index, differences in community composition) (2). Decomposition rate of organic material in litter bags *in situ* (2).
Soil microbial community	Nutrient supply to support plant growth. Important in maintaining microagregate soil structure.	Habitat function that sustains viable and functional microbial populations.	Soil basal respiration (1). Soil microbial biomass of carbon and nitrogen (2). Microbial enzymatic activity (dehydrogenase, acid phosphatase and asparaginase) (2). Soil nitrification and ammonification rate (2). Organic material breakdown in litter bags *in situ* (2).
Microorga-nisms and algae	Primary production. Recycling of nutrients.	Retention function of soil to evaluate risks to aquatic receptors (in this case particularly via groundwater contamination).	*Vibrio fischeri* (bacteria) luminescence test (1). Algae growth test (2).
Aquatic invertebrates	Aquatic food web.	Retention function of soil to evaluate risks to aquatic receptors (in this case particularly via groundwater contamination).	Cladoceran lethal tests (1). Cladoceran reproduction tests (2).

(1) Tier 1; (2) Tier 2.

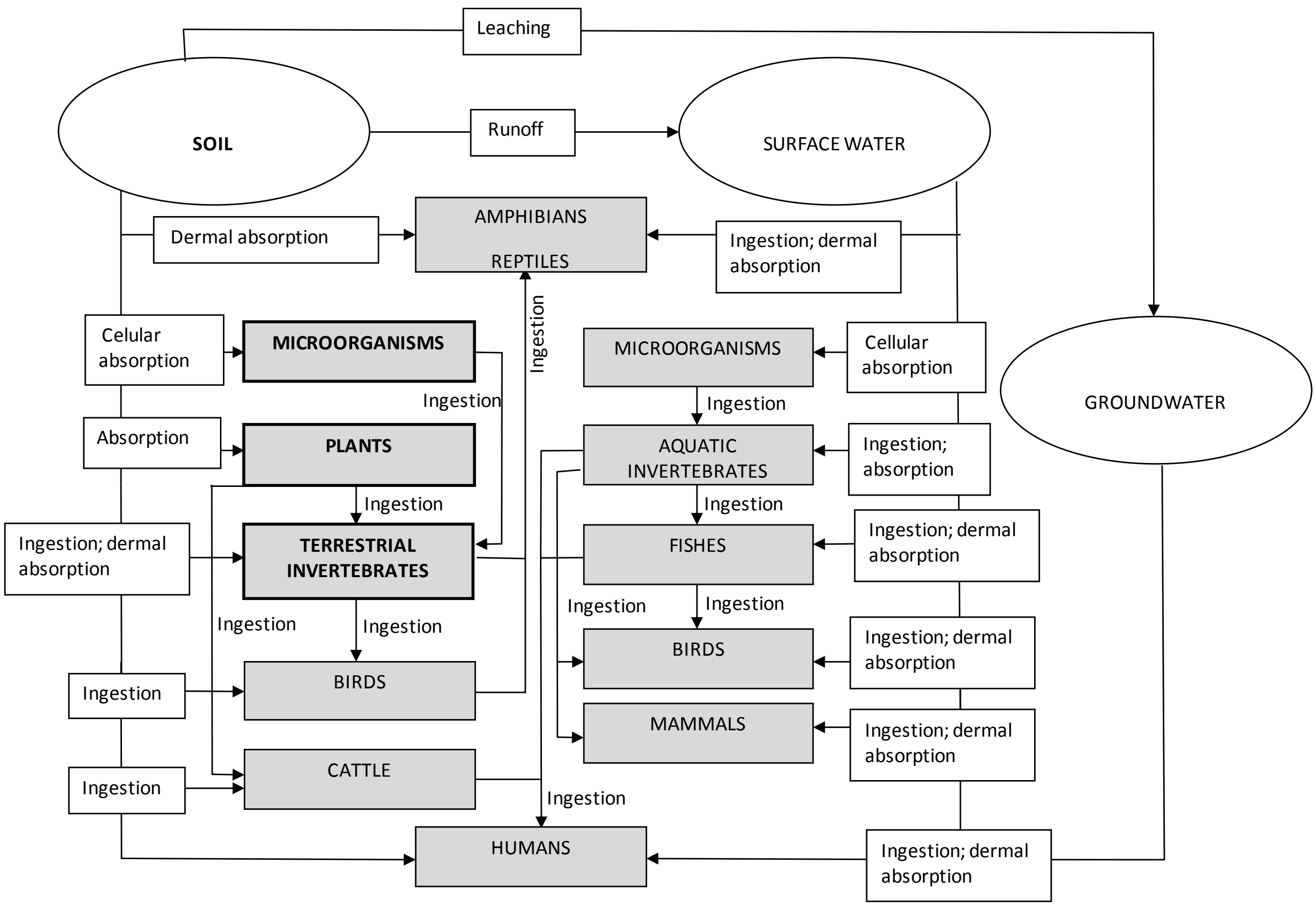

Figure 5. Conceptual model for risk assessment in the contaminated area in Santo Amaro, Bahia, Brazil. Environmental compartments are ellipses, exposure pathways are arrows and ecological receptors are represented by rectangles. The soil is the main source of contaminants (tail deposits and aerial deposition). Receptors in bold were those considered for evaluation in the study. Niemeyer (2012).

The analysis plan for the site-specific risk assessment was focused on the soil compartment, addressing indirectly the risk to groundwater and freshwater systems. Direct effects on non-soil invertebrates, vertebrates and water systems were not covered by this study. A summary of the assessment and measurement endpoints is shown in Table 1. Details about sampling strategy are described by Niemeyer et al. (2010). Due to the heterogeneity of the soil among the different sampling points, it was necessary to work on a multi-reference basis. Therefore, soils were assembled into three groups based on a Factorial analysis. Each group differed mainly in terms of texture, organic matter content and pH. To find matching reference soils, soil from several points in the surroundings of the area were screened, analyzed for metals and soil properties, and three reference soils (the best possible for each identified group of soils) were selected at 9 km (Ref. 1) and 3 km (Refs. 2 and 3) from the site. Details on this process (grouping of soils and finding the reference soils) can be found in Niemeyer et al. (2010).

The analysis plan included two tiers. In tier 1 (screening), the chemical LoE comprised of the calculation of the toxic pressure (Rutgers and Jensen, 2011) based on the comparison of the total concentrations of metals of the study site with soil screening levels, $HC50_{cor}$ (Dutch $HC50_{EC50}$ values; Rutgers et al., 2008) corrected for sampling site-specific differences, taking into account the organic matter and the clay content of each soil. The ecological information at tier 1 was collected through a quick vegetation survey and by assessing easy measureable functional parameters, such as soil respiration (Jensen and Mesman, 2006) and soil faunal feeding activity using bait lamina sticks (Von Törne, 1990). Regarding the ecotoxicological LoE, short-term cost-effective bioassays evaluating both the habitat and retention functions of the soil were carried out. The latter was evaluated using soil extracts (eluates) in tests with a cladoceran species (*Daphnia magna* acute test) and with the luminescent bacteria *Vibrio fischeri* (Van Gestel et al., 2001; Loureiro et al., 2005). Soil samples were used to evaluate the loss of habitat function through avoidance tests with earthworms and collembolans (Natal-da-Luz et al., 2004). Details on this phase can be found in Niemeyer et al. (2010).

Tier 2 (detailed assessment) was performed to reduce uncertainties about the actual risk shown by tier 1. The chemical LoE comprised the calculation of the toxic pressure based on total metals in habitat function (as done in tier 1) and the analysis of extractable metals using 0.01 M $CaCl_2$ solution to assess the soil retention function (mainly with the aim of evaluating potential of ground-water contamination). The ecotoxicological LoE comprised standardized chronic tests with Collembola (ISO, 1999) and Oligochaeta (ISO 1998, 2004), which were performed to evaluate sub-lethal effects of soil matrix on reproduction of soil invertebrates. Effects towards plants were evaluated by performing plant growth tests using standard species (one monocotyledonous and one dicotyledonous species) following ISO 11269-2 (ISO, 2005). In addition, soil extracts (eluates) were used to perform widely established tests with cladocerans (OECD, 2008) and microalgae (OECD, 1984) to evaluate the retention function of soil, thus assessing the indirect risk to the aquatic compartment (mainly groundwater) (Jensen and Mesman, 2006; Chelinho et al., 2009).

Regarding the ecological LoE at tier 2, information was collected to obtain more details about the possible impact on selected ecological receptors. Changes in diversity and community composition of plants (by field survey), soil surface dwelling invertebrates (pitfall trapping), as well as several functional processes were evaluated. Among them, microbiological soil-quality indicators considered in this study were microbial biomass, substrate-induced respiration, enzymatic activity and nutrient transformations. These are proxies for important processes related to soil fertility and can be used as bioindicators of soil stressed by contamination (Castaldi et al., 2004; Gulser and Erdogan, 2008), or to indicate suitable management and restoration practices (Nogueira et al., 2006).The ecological evaluation was complemented with the assessment of effects on organic matter (litter) decomposition in litter bags, a functional parameter by excellence, which can be used as indicative of negative effects on the soil microbial community, soil fauna or both (Knacker et al., 2003; Römbke et al., 2003).

For the data analysis differences among contaminated soils and the respective reference soil in ecotoxicity tests were in general evaluated by one-way analysis of variance (ANOVA), followed by one-tailed Dunnet's test when necessary. Prior to all analysis, normality and homoscedasticity were checked via the Shapiro-Wilk's test and Bartlet test, respectively. When homoscedasticity was not fulfilled, an equivalent non-parametric test was used, namely the Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons test. Ecological data were analyzed using ANOVA, t-tests, or ANOSIM approaches according to the parameters. Such analyses are described in Niemeyer et al. (2010, 2012a,b, 2015).

All parameters measured for each LoE were used for risk calculations where risk values were expressed in a scale ranging from zero ("no risk") to one ("highest risk") assuming that the risk value of reference soils is zero. For each sampling point, risk values were calculated following three steps, according to calculations presented by Jensen and Mesman (2006): (1) scale the results (between 0 and 1) of each test/parameter within each LoE; (2) integrate all scaled information of all parameters within each LoE; (3) integrate the information from the three LoEs and calculate the integrated risk. In the present study, the integrated risks to the soil habitat and retention function were calculated separately. More details about risk calculations are presented in Niemeyer et al. (2010, 2015).

Results from Tier 1 and Tier 2

Chemical LoE from Tier 1 pointed to high risk due to metal concentrations, mainly Pb, Cd, Cu and Zn, in superficial soil from the smelter area and surrounds. Despite the low extractability of metals in $CaCl_2$ 0,01M solution, high risk levels were indicated by ecotoxicity tests and ecological evaluations at the site. Avoidance tests indicated that earthworms and collembolans avoided contaminated sites when compared to reference soils. The lack of these groups of organisms can affect important soil functions related to nutrient cycling, structure and fertility of soil, besides impacts on food chain level (Römbke et al., 2005). Eluates evaluated by aquatic tests with cladocera and bacteria showed a compromised retention function at sites corresponding to residue deposits, which point to some risk of migration to surface and groundwaters. The low vegetation cover inside the smelter area and low feeding activity of soil fauna in bait lamina test evidenced impact to plants and soil invertebrates on the site. In general, integrated risk of Tier 1 pointed to high risk values inside the smelter area (more details in Niemeyeret al., 2010).

Results of the tier 2 evaluation confirmed the high risk levels in the smelter area already indicated in tier 1, and associated with tailing deposits, but with a further reduction of uncertainties. Locations outside the smelter area demonstrated lower or acceptable risks. As in tier 1, the low toxicity in eluate tests indicated high adsorption of metals in soil, probably favored by content and type of clay, ageing and neutral pH, and consequently negligible risk due to the high retention capacity in most sampling points, except for residue deposits. Moreover, the results indicate that the current cover of the tailing deposits failed to restore the site by not creating appropriate conditions for the establishment of plant (revegetation), microbial and animal communities inside the area. So, besides the direct effects of metal contamination, also indirect effects are visible due to the presence of these contaminants, compromising the functioning of the ecosystem inside the smelter area. Detailed results are presented and discussed in Niemeyer et al. (2012ab, 2015).

Recommendations for the Studied Site

High risk values in habitat function inside the smelter area indicate the need to proceed with some remediation action, such as an improved encapsulation of tailing deposits and recovery of the vegetation (Figure 6). These actions could not only improve soil conditions and ecosystem functioning, but could mainly avoid the transport of contaminants to other environmental compartments, namely via dust dispersal to outside the area, or via surface runoff to the existing temporary ponds and the Subaé river.

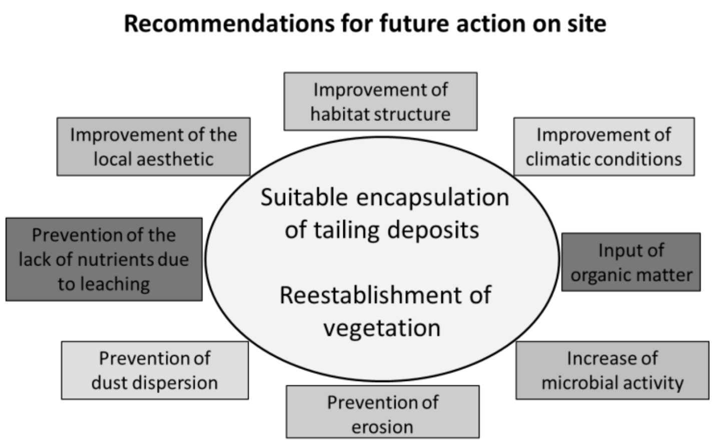

Figure 6. Suggested actions and expected ecological improvements according to the Environmental Risk Assessment results.

The improvement of the vegetation cover can be effective in providing the necessary surface stability to prevent wind-blow of contaminated soil particles, preventing erosion, and reducing water pollution by interception of a substantial proportion of incident precipitation (Tordoff et al., 2000; Wong, 2003). Furthermore, the choice of appropriate vegetation is crucial to remediate the adverse physical and chemical properties of the site and to reestablish ecosystem functioning (Wong, 2003), besides the aesthetical improvement of the site. Organic matter, soil nutrients and species diversity generally increase with community development during succession (Wang et al., 2011). It is important to consider the use of locally adapted species which are tolerant not only of physical and chemical conditions of tailings, but also to the climatic conditions of the site. Furthermore, plant species with different traits can increment the heterogeneity of soil habitats (Podgaiski and Rodrigues, 2010), and can thus support soil communities that demand different requirements of food and shelters (Wardle et al., 2004).

Sensitivity of Ecotoxicological and Ecological Parameters for Risk Assessment

Aiming at evaluating how sensitive and cost-effective the different ecotoxicological and ecological parameters in the risk assessment could be, a sensitivity analysis was conducted taking into account not only the ability of each parameter to detect differences between contaminated and non-contaminated points (outside the affected area), but also their ability to detect a gradient of contamination. For this purpose, individual parameters were analyzed based on observed significant differences in comparison to reference points (using ANOVA). For soil fauna abundance and taxonomic richness, the sensitivity analysis was based on significant differences between sampling points outside the area (non or low contamination) and points inside the area (high contamination): high ($p<0.001$); medium ($p<0.01$) and low ($p<0.05$) sensitivity. Ability to differentiate the level of contamination was based on significant correlations with metal loadings (Widianarko index): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$). The time necessary to obtain the parameter was also estimated. Results are presented in Tables 2 and 3.

Table 2. Valuation of the sensitivity of each ecotoxicological parameter assessed at the smelter area. grey - parameters selected based on sensitivity criteria (see text for details)

Organism group	Category	Parameter	Significant response in contaminated sites (1)	Ability to differentiate the level of contamination (2)	Days needed to obtain the parameter (time of exposure)	Use in ERA (Tier)
Soil inverte-brates	Habitat function for soil invertebrates	Reproduction of *Eisenia andrei*	High	High	56	2
		Reproduction of *Enchytraeus crypticus*	High	High	28	2
		Reproduction of *Folsomia candida*	No differences	No	28	2
Plants	Habitat function for plants	Growth of *Avena sativa*	Low	Medium	14-21	2
		Dry weight of *A. sativa*	No	No	14-21	2
		Growth of *Brassica rapa*	No	No	14-21	2
		Dry weight of *B. rapa*	No to medium	No	14-21	2

(1) Information based on observed significant differences against reference points (ANOVA): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$).

(2) Information based on significant correlations with metal loadings (Widianarko index): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$).

Table 3. Summary of the sensitivity of each ecological parameter assessed at the smelter area. Light grey - parameters derived from multivariate analysis based on individual parameters from the corresponding organism group; Dark grey - parameters selected based on sensitivity criteria (see text for details)

Organism group	Category	Parameter	Significant response in contaminated sites (1)	Ability to differentiate thelevel of contamination (2)	Days needed to obtain the parameter (estimated number of working days)	Use in ERA (Tier)
Micro-organisms	Community activity	Microbial respiration	Low to high	High	8 (4)	1-2
	Community structure	Microbial biomass C	Low to high	High	2 (2)	1-2
	Community activity	Dehydrogenase activity	Low to medium	No	1 (1)	1-2
	Community activity	Acid phosphatase activity	Low to high	Medium	1 (1)	1-2
	Biological process	Nitrification rate	Low to high	Medium	21 (3)	1-2
		Multivariate analysis with all microbial parameters	Medium			1-2
Inverte-brates	Community activity	Feeding activity (bait lamina)	High	Medium	14 (4)	1
	Community structure	Abundance	Group dependent; low	No	37 (30)	2
	Community structure	(Morpho) Species richness (3)	Group dependent; low	No		2
	Community structure	Shannon diversity index (3)	No differences	No		2
	Community structure	Pielou evenness index (3)	No differences	No		2
	Community structure	Margalef richness index (3)	No differences	No		2
	Community structure	Berger-Parker index (3)	No differences	No		2
	Community structure	Changes in species composition (3)	High			2
Plants	Community structure	% of vegetation cover	High	Low	1 (1)	1
	Community structure	Species richness (3)	No differences	No	2 (2)	1-2
	Community structure	Changes in species composition (3)	High			2
Microorga-nisms and invertebrates	Ecosystem function	Litter breakdown (decay rate)	High	Low	140 (25)	2

(1) For individual parameters, information based on observed significant differences against reference points (ANOVA); for soil fauna abundance and taxonomic richness and for integrated multivariate analysis (ANOSIM), based on significant differences between sampling points outside the area (non or low contamination) from points inside the area (high contamination); high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$).

(2) Information based on significant correlations with metal loadings (Widianarko index): high ($p<0.001$); medium ($p<0.01$); low ($p<0.05$).

(3) Parameters that require specific taxonomic knowledge.

The ability of the avoidance behavior to detect toxicity within a short test period and at low cost makes this test suitable for use in decision processes. However, some care should be taken in the choice of reference soils (similar in properties except for the contamination level). If finding matching reference soils becomes a difficult task, models are available to correct for the influence of soil properties (Chelinho et al., 2011).

The results demonstrated the high sensitivity of oligochaeta reproduction tests to evaluate the contaminated sites; however, results obtained with *Folsomia candida* were not sufficient for an adequate assessment of metal contaminated soils. As soil invertebrate species are affected in a different way, it is recommended for a suitable evaluation of the risk the use of several species from different ecological groups, representing distinct routes of exposure to contaminants.

The microbial community was highly impaired by metal contamination. However, only basal respiration, microbial biomass (C), acid phosphatase activity and nitrification presented a high to medium capacity to distinguish the level of soil contamination. Since the first two parameters were highly correlated, assessing only one is enough to give information relative to microbial activity. Bacterial parameters related to community structure (not assessed in this study) and bacterial growth/biomass were highly rated by Critto et al. (2007) as parameters to assess in all triad tiers, mainly due to their rapidity and low cost. Based on these findings only one parameter seems not to be sufficient to give information about general microbial activity, and it should be complemented with other parameters related to microbial genetic diversity (e.g., DGGE) or metabolic diversity (e.g., Biolog), in addition to other specific activity parameters if processes involving particular nutrients are of interest.

The high sensitivity of feeding activity of soil fauna, allied to the fact that several studies showed the relation between bait-lamina data and abundance of several microarthropod groups and lumbricids (Birkhofer et al., 2011), make the bait-lamina test a definitive parameter to include in the ecological LoE in site specific assessments. Due to its ease of use and practicability, allowing to process the information from a large number of sampling points over a short time, it is a parameter to use in tier 1 of a triad approach. Regarding abundance and normal biodiversity descriptors of surface dwelling arthropods, results did not show very promising results in distinguishing different levels of contamination. However, community composition could be a more promising parameter. Although not performed in this study, identification of the family and/or trophic group, including the use of vulnerability traits, could be more useful to better decipher the true risks to this group of organisms. Another aspect, also not contemplated in this study, is to sample not only surface dwelling organisms but true soil dwellers that could have a completely different response.

Vegetation cover and changes in plant composition were able to detect differences between points inside and outside the smelter area. However, their ability to detect gradients of contamination was not met. Critto et al. (2007) presented a low rank for vegetation survey related parameters in tier 1, mainly due to their cost. However, in higher tiers (tiers 2 and 3) these parameters presented higher ranking mainly related to their site specific relevance.

Plant litter decomposition showed a high sensitivity to contamination and derived habitat disruption, but presented a low capacity to differentiate the level of contamination. In this case, it gave a similar information as the bait lamina test ($r=0.83$, $p<0.01$), thus not being a priority parameter to integrate in a tiered scheme (also due to the long time needed to obtain results).

FINAL CONSIDERATIONS

According to Rutgers and Jensen (2011), although many tools for a triad approach in ERA are available, the increasing number of triad-based risk assessment will demand for improved, new, standardized, robust and cost-effective tools. This is specially applied to Latin America, where more adequate experimentation design should be pursued to provide more realistic scenarios taking into consideration native species and environmental conditions (such as temperature and soil types). As pointed out by Filser et al. (2008), ecotoxicologists should make better use of basic ecology when establishing new tests or risk assessment schemes and convince regulatory authorities of the necessity of such an approach. Experience can be acquired by testing the triad basic approach in practical situations, at a number of characteristic sites, aiming to provide important information to help the regular use of the ERA schemes in supporting site restoration and reclamation decisions in Brazil and Latin America.

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Chapter 19

ECOTOXICOLOGICAL ASSESSMENT OF DREDGED SEDIMENTS FROM GUANABARA AND SEPETIBA BAYS (RIO DE JANEIRO, BRAZIL) USING BIOASSAYS WITH EARTHWORMS

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ABSTRACT

The limits for metal concentrations in soil and terrestrial disposal of dredged sediments established by Brazilian law are based on Canadian and Dutch legislations. To evaluate the adequacy of such threshold values, dredged sediments were collected from polluted aquatic systems of Rio de Janeiro state: Saco do Engenho River (Sepetiba Bay), Meriti River (Guanabara Bay) and São Francisco River (Sepetiba Bay). The sediments were mixed with an artificial soil in proportions between 0% (pure artificial soil) and 30% for performing acute bioassays with *Eisenia andrei*. The earthworm median lethal

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concentration (LC50) for the Saco do Engenho River was 16.26% while for Meriti River it was 17.46%. No significant mortality was detected for the sediment from São Francisco River. The highest toxicity observed for the sediment from Saco do EngenhoRiver is in agreement with the higher metal levels among the sediments studied. The organisms increased their biomass when exposed to low dosages of sediments from Saco do Engenho River and Meriti River, while those exposed to sediment from São Francisco increased their biomass with increasing sediment dose. The bioconcentration factors (BCF) for zinc and copper generally decreased with increasing concentrations of sediment, indicating the existence of internal regulating processes. The BCFs for lead, chromium and cadmium were <0.3. The highest BCF values were found for nickel in mixtures with the sediment from Saco do Engenho River. The bioassays performed were adequate for a preliminary evaluation of the damages associated with disposal of dredged sediments in soil systems.

Keywords: bioconcentration factor, *Eisenia andrei*,ecotoxicity, acute tests, metals

INTRODUCTION

Over the last decades large amounts of domestic and industrial wastes have been discharged into aquatic systems of South America without an adequate treatment. This situation is particularly critical along the coastal area of Rio de Janeiro state (RJ; southeastern Brazil), especially in the Guanabara and Sepetiba bays where concentrations of metals, hydrocarbons, pathogenic microorganisms and domestic wastes are abnormally high (Junior et al., 2002, Machado et al., 2002, Cesar et al., 2014). In the RJ state, the dredging of contaminated sediments is often required to keep these water-ways navigable and to avoid their eutrophication (Cesar et al., 2015a). Given the fact that the transport of dredged sediments is relatively expensive (about 50-60% of total costs of the dredging), the disposal of these sediments in adjacent soils is a common practice in Brazil and can cause serious damages to soil ecosystems (Cesar et al., 2014, 2015b).

The limits for metal concentrations established by Brazilian legislation for terrestrial disposal of dredged sediments is based on Canadian and Dutch guidelines, which were idealized for temperate environments and dredging of ports areas. Thus, the application of these values in tropical regions (e.g., in Brazil) should not be recommended. Furthermore, the dredging of estuarine and fluvial systems (and not only port areas) of RJ state is also required to attend for safe practice of water sports and fishing for human consumption, which include the dredging of sediments and decontamination of water and bottom sediments from Guanabara Bay.

Most studies on metal contamination in tropical soils and bottom sediments have been traditionally based on total chemical analysis, which generally includes selective metal analysis and geochemical speciation (DePaula and Mozeto, 2001; Gleyzes et al., 2002; Cesar et al., 2011). Even though those analyses are extremely relevant, they are not sufficient to predict metal ecotoxicity and to prevent damages on soil ecosystems if not complemented with bioassays using representative soil organisms. In this context, earthworms have been widely used as test organisms in bioassays, since they are abundant in both tropical and temperate soils, ingest large amounts of soil and are sensitive to contaminants (Hinton and Veiga, 2002; Nahmani et al., 2007). Moreover, the determination of metal concentrations in tissues of surviving organisms from bioassays provides relevant information on their bioavailability levels in the soil, which may be related to the occurrence of adverse effects on soil biodiversity (Straalen et al., 2005).

The acute toxicity test with *Eisenia andrei* made part of a ring test to define a test battery to evaluate the ecotoxicity of three representative waste types (Moser and Römbke, 2009). Those assays were performed by different European laboratories, and showed that the acute bioassay with *E. andrei* is an adequate test for characterizing the risks of waste disposal in terrestrial systems (Moser and Römbke, 2009). Cesar et al. (2014) studied the ecotoxicity of dredged sediments from Cunha Channel (Guanabara Bay basin, RJ) using acute bioassays with *E. andrei* and showed that this assay may be a robust method for evaluating the effects of the dredged sediment to soil fauna.

The present chapter aims to make a preliminary ecotoxicological evaluation of dredged sediments from Guanabara and Sepetiba bays (RJ) using survival, biomass changes and bioaccumulation of metals as endpoints. To attain this purpose, acute bioassays with *E. andrei* were applied in artificial soils treated with different doses of dredged sediments from Guanabara and Sepetiba bays. The working hypotheses are: (i) the acute bioassay with *E. andrei* is an appropriate method to evaluate the ecotoxicity of dredged sediments from Guanabara and Sepetiba bays to soil biota; (ii) the current threshold metal concentrations defined by Brazilian legislation are not adequate to prevent noxious effects on soil organisms; and (iii) the organic matter provided by the sediment is a potential source of food for the test organisms.

METHODS

Sampling of Dredged Sediments

Three samples of dredged sediment were obtained from three different collecting stations (one sample from each collecting point) in September 2012 by using a Van Venn Grab Sampler. Two sampling points are located at Sepetiba Bay (mouths of Saco do Engenho River and São Francisco River), while the other one is located at Guanabara Bay(mouth of Meriti River) (Figure 1). The samples were dried at 40°C and ground for ecotoxicological evaluation.

The basins of Guanabara and Sepetiba bays are characterized by high concentrations of metals, petroleum hydrocarbons and domestic wastes in sediments, water and biota (Machado et al., 2002; Bidone and Lacerda, 2004; Silva et al., 2007; Silveira et al., 2010; Rodrigues et al., 2011). Meriti River basin receives high amounts of domestic wastes and effluents containing heavy metals, especially mercury. The mouth of Meriti River is often silted which disturbs natural water circulation in the Guanabara Bay. The Saco do Engenho River has been receiving high amounts of heavy metals (lead, cadmium, zinc and copper) due to an old (and nowadays not active) metallurgical industry located at its adjacent areas. The mouth of Saco do Engenho River is often dredged to support the activities of the Sepetiba Port. The São Francisco River basin contains high amounts of domestic wastes and some of its tributaries drain industrialized areas. Its mouth is also frequently silted.

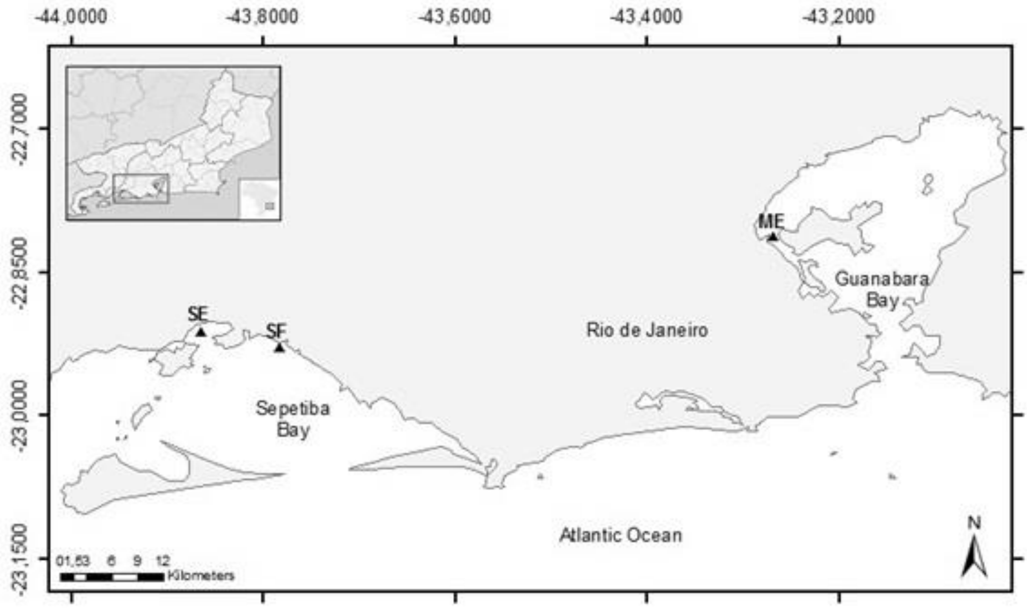

Figure 1. Location of sampling sites for dredged sediment in the Guanabara and Sepetiba bays (Rio de Janeiro, Brazil). ME: Mouth of Meriti River; SE: Mouth of Saco do Engenho River; SF: Mouth of São Francisco River.

Acute Toxicity Test with *Eisenia andrei*

The acute earthworm test was performed according to the ASTM (2004) protocol with some adaptations. Five replicates per test concentration were prepared, each one consisting of a cylindrical plastic box (3.8 cm diameter and 6.4 cm height) with 500 g (dry weight equivalent) of uncontaminated soils or mixtures of soil and dredged sediment. Ten adult organisms (previously acclimated in the natural soils for more than one day, washed with distilled water and weighed) were added to each replicate. Earthworms with an average weight of 0.548±0.18 g (average ± standard deviation, n=300) were used in the test. Moisture content of test mixtures was previously adjusted to 50% of its water-holding capacity. The test was performed under constant illumination and controlled temperature (20±2ºC). Additional replicates with pure tropical artificial soil were also prepared. The artificial soil was prepared according to Garcia (2004) consisting of 70% of quartz sand, 20% of kaolin and 10% of coconut shells dust. After 14 days of exposure, mortality and biomass change of the surviving earthworms were determined. After being counted, washed and weighted, surviving organisms were maintained in moistened absorbent paper for 24 h to purge the gut before being stored for metal content determination. The validity criterion of acute tests assumed earthworm mortality lower than 10% in replicates with pure artificial soil.

Chemical Characterization

Organic carbon contents were quantified by elementary analysis, using a LECO SNS-2000 equipment. The total concentrations of Al, Cd, Cu, Cr, Fe, Ni, Pb, Zn and Mn in the dredged sediment and test mixtures were determined through the solubilisation of 1 g of homogenized sample in 40 ml of an acid mixture of HF:HCl:HClO4 (2:1:1). The concentration of metals in soil extracts was determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES; Horiba JobinYvon, Ultima 2). Vanadium (V) concentrations were obtained by using the same procedure used for metals. Detection limits were 0.4 mg/kg, 0.1 mg/kg, 0.2 mg/kg, 0.01 mg/kg, 0.4 mg/kg, 0.01 mg/kg, 0.2 mg/kg, 0.2 mg/kg, 1.4 mg/kg and 0.4 mg/kg, for Al, Cd, Cu, Cr, Fe, Ni, Pb, V, Zn and Mn, respectively.

Metal concentration obtained in the sediments was compared to the threshold values established by Brazilian law for dredged sediment disposal (CONAMA 454): the limits of low (Level I) and high (Level II) probability of adverse effects on biota. Metal concentrations determined in mixtures of soil and sediment were compared to the limits defined by Brazilian law for soil quality (CONAMA 420 and CETESB 2005): the reference limit (soil geochemical background for Sao Paulo state), prevention limit (based on the risks on soil ecological receptors) and intervention limit (based on the risks on human health).

After being frozen, lyophilized and triturated, total metal concentrations in tissues of surviving earthworms were determined by using the same procedures applied to the dredged sediment. Metal bioavailability levels were evaluated with the bioconcentration factor (BCF), a ratio between total metal content in the organisms and total metal content in the soil. When earthworm metal concentration was lower than the detection limit, the BCF was determined according to its respective detection limit value.

Total metal concentrations in the dredged sediment and test mixtures were compared to the limits established by Brazilian legislation for terrestrial disposal of estuarine sediments (CONAMA, 2012) and Brazilian soil quality (CONAMA, 2009). Metal concentrations measured in the dredged sediment were used to calculate the Geoaccumulation Indices (IGEOs) for each metal, using background values determined by other researchers in sediment cores collected in Guanabara and Sepetiba bays (Tables 1 and 2). When such values were not available, the values determined in standard shale (Müller, 1979) were used and a factor of 1.5 was applied to account for regional differences. Fe, Al and Mn are generally abundant metals in tropical soils and, due to this reason, the IGEO is not applicable to such elements. Vanadium (V) is not a metal, a thus the application of IGEO is also unfeasible. Because of that, Fe, Al, Mn and V backgrounds are not mentioned in the Table 1. The IGEO can be calculated applying the following equation, where *Me* means metal concentration obtained in the field, while NBN_{ME} means the background value for a specific metal:

$$IGEO = Log_2 \, Me / NBN_{Me} \times 1.5 \quad (1)$$

Statistical Analysis

Earthworm weight change and mortality found in the test mixtures of soil and sediment were compared to that in the respective controls (uncontaminated soils - 0%) by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. The number of surviving earthworms in the acute toxicity tests was used to calculate the earthworm median lethal concentration (LC50) through Probit analysis (using the software PriProbit 1.63; Sakuma, 1998).

Table 1. Background metal concentrations obtained in sediment cores from Sepetiba bay (Gomes et al., 2009), Guanabara bay (Monteiro et al., 2012) and in the standard shale (Turekian and Wedepohl, 1961). *=metal concentration obtained in the standard shale

Metal	Sepetiba bay (mg/kg)	Guanabara bay (mg/kg)
Cd	0.34	0.30*
Cr	90*	90*
Cu	8.04	2
Ni	8.32	60
Pb	20	15
Zn	54	6.1

Table 2. Geoaccumulation Indexes (IGEO) of heavy metals in sediments of theRhine River(Germany). Adapted from Müller (1979)

Intensity of pollution	Metal accumulation in the sediment (IGEO)	IGEO class
Very strongly polluted	>5	6
Strongly to very strongly polluted	>4-5	5
Strongly polluted	>3-4	4
Moderate to strongly polluted	>2-3	3
Moderately polluted	>1-2	2
Low to moderately polluted	>0-1	1
Practically non-polluted	<0	0

RESULTS

Dredged Sediment and Test Mixtures

The organic matter contents measured in the dredged sediments were 1.80, 4.90 and 0.93%, for the month of Saco do Engenho River (Sepetiba bay), Meriti River (Guanabara bay) and São FranciscoRiver (Sepetiba bay), respectively (Table 3). According to the current Brazilian legislation (CONAMA, 2004), the only sediment whose Cd concentration was above the second level (high probability of adverse effects on biota) was the Saco de Engenho. On the other hand,Cd concentrations were lower than the detection limits for the other sediments. In the Saco do Engenho River, Zn, Cd and Pb concentrations were extremely high. In the Meriti River, all heavy metal concentrations were above maximum values established by the Brazilian law (generally higher than the first level), except Cd. On the other hand, in the São Francisco River, Zn was the only metal whose concentration was above the Brazilian threshold concentration (higher than the first level).

The estimated IGEOs indicated absence of Cr pollution (class 0) for all tested sediments (Table 3). In the Saco do Engenho River, the sediment was "strongly to very strongly" polluted by Cd and Zn (class 5), while the Ni pollution was classified as "moderate to strongly polluted" (class 3). The sediment of the Meriti River was "very strongly polluted" (class 6) by Zn and Cu, and "moderate to strongly polluted" (class 3) by Pb. The sediment from the São Francisco River showed the lowest values of IGEO classes, and the highest one was observed for Zn (class 2; "low to moderately" polluted) (Table 3).

For the sediments from Saco do Engenho River and São Francisco River, the pH of the test mixtures increased with increasing sediment dosage in soil (Tables 4 and 5). However, water holding capacity (WHC) decreased for high dosages of sediment application. For the Meriti River, the pH decreased with the increasing doses of sediment, while the WHC increased with the increasing sediment dose in soil (Table 6).

Table 3. Metal concentrations (mg/kg), organic matter content (OM; %) and IGEO classes obtained for the dredged sediments collected in Sepetiba and Guanabara bays

Metal	CONAMA 454 (Brazilian law)		Saco do Engenho River (Sepetiba Bay)		Meriti River (Guanabara Bay)		São Francisco River (Sepetiba Bay)	
	Level I[a]	Level II[b]	mg/kg	IGEO	mg/kg	IGEO	mg/kg	IGEO
Cd	1.2	7.2	7.2**	5	<0.1	0	<0.1	0
Cr	81	370	133*	0	111*	0	43.5	0
Cu	34	270	15	1	111*	6	11.1	1
Ni	20.9	51.6	58.4**	3	42.9*	0	13	1
Pb	46.7	278	36.8	1	90.9*	3	30.2	1
V	-	-	<0.2	-	<0.2	-	<0.2	-
Zn	150	410	1200**	5	926**	6	206*	2
OM (%)	-	-	1.8	-	4.9	-	0.93	-
Al (%)	-	-	8.2	-	9.4	-	8.2	-
Fe (%)	-	-	4.9	-	3.9	-	3.4	-
Mn (%)	-	-	275	-	196	-	290	-

a – limit of low probability of adverse effects on biota; b – limit of high probability of adverse effects on biota (CONAMA 2012). */**=value higher than CONAMA level I/II (CONAMA 454), respectively. CONAMA 454: threshold values established by Brazilian legislation for disposal of dredged sediments.

Metal concentrations measured in the test mixtures were lower than the intervention limits (the limit of occurrence of adverse effects on human health considering agricultural land uses) defined by Brazilian law (CETESB, 2005; CONAMA, 2012) for all the sediments (Tables 4, 5 and 6). In the Saco do Engenho River, the concentrations of Zn and Cd were higher than the prevention limits for ecological receptors in some test mixtures (18, 24 and 30%) (Table 4). In fact, the concentrations of Zn and Cd were always higher than the reference limits (CETESB, 2005; soil geochemical background for São Paulo state) in all test mixtures (Table 4). In the Meriti and São Francisco Rivers, metal concentrations determined in the test mixtures were generally lower than the prevention limits (Table 5). The exception was Zn for the highest dose of sediment application from the Meriti River. In this respect, in the Meriti River the concentrations of Zn and Pb were also always higher than the reference limits for all test mixtures, while in the São FranciscoRiver such concentrations were higher than the reference limits only in some test mixtures (Table 6).

Earthworm Acute Toxicity Test

The validity criterion of the acute earthworm test was fulfilled since no mortality was found in replicates with pure artificial soil (0%). The mortality observed in the mixtures of artificial soil and sediments from Saco do Engenho River and Meriti River showed dose-response relationships for treatments of both sediments (Figure 2). No mortality was found for the treatments using the sediment from the São Francisco River. Mortality found in mixtures with the sediment from Saco do Engenho River and Meriti River was significantly higher than that in the control for concentrations ≥15% and 18%, respectively (Figure 2). The estimated values of LC50 (and 95% confidence interval) were 16.26% (15.72-16.78) and 17.46% (16.71-17.68), for the Saco de Engenho and Meriti River, respectively. The biomass loss was higher in mixtures with the sediment from Saco do Engenho River compared to the other sediments. The organisms increased their biomass levels at low doses of sediment application from Meriti River (6 and 12%), followed by a biomass reduction at higher doses (18%). The organisms exposed to mixtures with the sediment from São Francisco River significantly increased their biomass levels (Figure 2).

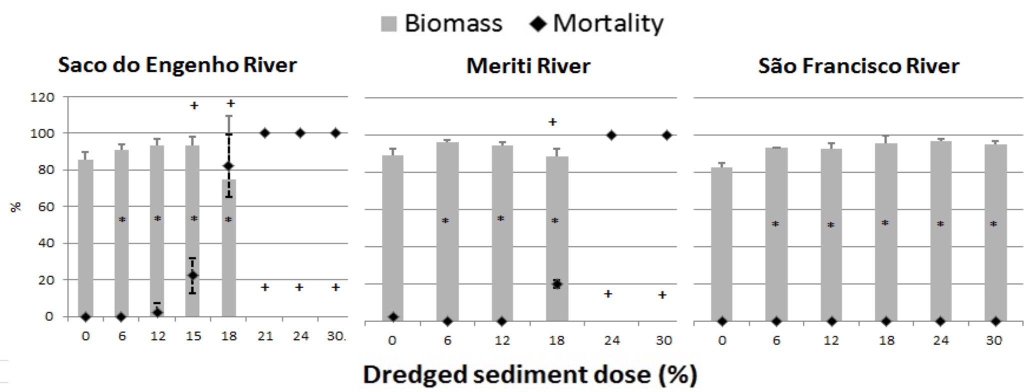

Figure 2. Mortality (diamonds) and biomass change (columns) (average % ± standard deviation; n=4) of *Eisenia andrei* exposed to mixtures of artificial soil with dredged sediments from the mouth of Saco do Engenho River (Sepetiba bay), Meriti River (Guanabara bay) and São Francisco River (Sepetiba bay). * Biomass change significantly different from control ($p \leq 0.05$). + Mortality significantly different from control ($p \leq 0.05$).

Earthworm Bioconcentration Factors

Cr, Cd and Pb concentrations measured in the earthworms exposed to the test mixtures were the lowest among the measured metals and their BCF values were <0.3 for mixtures of both test soils (Table 7). The earthworm Pb concentrations were lower than the Pb detection limits in all test mixtures. BCF values of Ni were also <1 in mixtures with the sediments from Meriti and São Francisco, while such values were >1 for some test mixtures with the sediment collected in the Saco do Engenho River. BCF values of Cu were generally <1, except for the 30% treatment with the sediment from São Francisco River, whose values increased with the increased dredged sediment dose (Table 7). On the other hand, Cu concentrations and its BCF values decreased with the increasing sediment dose from Saco do

Engenho River. Earthworm Zn concentrations were the highest among the measured metals with BCFs >1 for the all sediments studied, decreasing with the increased dredged sediment dose for the sediments collected in the Meriti River and Saco do Engenho River. The treatments with the sediment from São Francisco increased their BCF values of Zn with increased dredged sediment dose (Table 7).

Table 4. Water holding capacity (WHC), pH and metal concentrations (mg/kg) determined in mixtures of artificial soil with dredged sediments from Saco do Engenho River (Sepetiba bay, Rio de Janeiro). 0%: pure artificial soil

Doses (%)	WHC (%)	pH	Cr	Cu	Ni	Pb	Zn	Cd
0	84.2	5.74	7.20	-	2.90	13.00	48.60	0.100
6	82.4	5.88	14.75	2.05	6.23	14.43	117.68*	0.526*
12	78.8	6.37	22.30	3.10	9.56	15.86	186.77*	0.952*
15	82.0	6.72	26.07	3.62	11.23	16.57	221.31*	1.165*
18	79.4	6.73	29.84	4.14	15.62*	18.45*	312.50**	1.378**
24	76.1	6.46	37.39	5.19	16.22*	18.71*	324.94**	1.804**
30	73.9	7.01	44.94*	6.24	19.55*	20.14*	394.02**	2.230**
Reference*			40	35	13	17	60	<0.5
Prevention**			75	60	30	72	300	1.3
Intervention***			150	200	70	180	450	3.0

* – pedogeochemical background levels of the São Paulo state, Brazil (CETESB, 2005); ** – limit of occurrence of adverse effects on soil ecological receptors; *** – limit of occurrence of adverse effects on human health (CETESB, 2005; CONAMA, 2009).

Table 5. Water holding capacity (WHC), pH and metal concentrations (mg/kg) determined in mixtures of artificial soil with dredged sediments from São Francisco River (Sepetiba Bay, Rio de Janeiro). 0%: pure artificial soil

Doses (%)	WHC (%)	pH	Cr	Cu	Ni	Pb	Zn
0	75.7	5.64	7.20	6.80	2.90	13.00	48.60
6	79.2	5.67	9.38	7.06	3.51	14.03	58.04
12	74.5	5.80	11.56	7.32	4.11	15.06	67.49*
18	76.0	6.00	13.73	7.57	5.21	16.94	84.68*
24	73.5	6.04	15.91	7.83	5.32	17.13*	86.38*
30	72.9	6.11	18.09	8.09	5.93	18.16*	95.82*
Reference*			40	35	13	17	60
Prevention**			75	60	30	72	300
Intervention***			150	200	70	180	450

* – values representing pedogeochemical background of the São Paulo State, Brazil (CETESB, 2005); ** – represent the limit of occurrence of adverse effects on soil ecological receptors; and *** – represent the limit of occurrence of adverse effects on human health (CETESB, 2005; CONAMA, 2009).

Table 6. Water holding capacity (WHC), pH and metal concentrations (mg/kg) determined in mixtures of artificial soil with dredged sediments from Meriti River (Guanabara bay, Rio de Janeiro). 0%: pure artificial soil

Doses (%)	WHC (%)	pH	Cr	Cu	Ni	Pb	Zn
0	74.8	5.85	7.20	6.80	2.90	13.00	48.60
6	83.3	5.13	13.43	13.05	5.30	17.67*	101.24*
12	85.1	4.87	19.66	19.30	7.70	22.35*	153.89*
18	89.0	4.93	25.88	25.56	12.07	30.85*	249.70*
24	92.1	4.96	32.11	31.81	12.50	31.70*	259.18*
30	91.1	4.60	38.34	38.06*	14.90*	36.37*	311.82**
Reference*			40	35	13	17	60
Prevention**			75	60	30	72	300
Intervention***			150	200	70	180	450

* – values representing pedogeochemical background of the São Paulo State, Brazil (CETESB, 2005); ** – represent the limit of occurrence of adverse effects on soil ecological receptors; and *** – represent the limit of occurrence of adverse effects on human health (CETESB, 2005; CONAMA, 2009).

DISCUSSION

The mortality and biomass loss observed in the acute earthworm tests agree with the results of the metal determination. The highest metal concentrations and the highest toxicity (expressed in LC50 values) were detected for the sediment collected in the Saco do Engenho River, followed by Meriti River. The highest organic matter content in the sediment from Meriti River (4.9%) was an important aspect to toxicity, since organic matter plays an important role in the reduction of metal mobility and bioavailability by decreasing metal concentration in pore water (Vijver et al., 2005; Lukkari et al., 2006). This fact may also explain the lower toxicity levels found for the sediment from Meriti River compared to the sediment from Saco do Engenho River. Last but not least, metal contents measured in the sediment collected in the São Francisco River were the lowest among the sediments studied and their low IGEO classes also support the absence of earthworm mortality and of biomass loss found in all the treatments.

Metal concentrations in the pure sediments were generally higher than the limits for the first level of contamination (low probability of adverse effects on biota), and some metals were even higher than the limits established for a high probability of adverse effects on biota (CONAMA, 2004), especially the sediments from Saco do Engenho River and Meriti River. Those levels of contamination were confirmed by the toxicity found in the bioassays.

Table 7. Bioconcentration factors (BCF) measured in three composite samples (average, n=4) obtained from the surviving earthworms (40 individuals in treatments without mortality) after 14 d of exposure in mixtures of dredged sediment with artificial soil. SE: Saco do Engenho River (Sepetiba Bay); ME: Meriti River (Guanabara Bay); SF: São Francisco River (Sepetiba Bay); UD: unavailable data. BCF of Cd was not calculated for MR and SFR because the concentrations in the sediment were lower than the detection limits

Dredged sediment dose (%)	Cr			Cu			Ni			Pb			Zn			Cd
	SE	MR	SF	SE	MR	SF	SE	MR	SF	SE	MR	SF	SE	MR	SF	SE
0	0.24	0.03	0.03	0.79	0.37	0.37	0.05	0.10	0.10	0.15	0.15	0.02	1.58	1.87	1.89	0.10
6	0.12	0.10	0.02	0.75	0.38	0.38	0.03	0.06	0.09	0.14	0.11	0.01	0.51	0.92	1.72	0.19
12	0.22	0.20	0.02	0.67	0.36	0.40	2.30	0.04	0.07	0.13	0.09	0.01	1.09	1.04	1.52	0.11
15	0.20	UD	UD	0.51	UD	UD	1.30	UD	UD	0.12	UD	UD	0.87	UD	UD	0.09
18	0.19	0.25	0.02	0.48	0.33	0.55	1.24	0.22	0.06	0.11	0.09	0.01	0.25	0.79	1.66	0.07
24	UD	UD	0.03	UD	UD	0.51	UD	UD	0.08	UD	UD	0.01	UD	UD	1.94	UD
30	UD	UD	0.02	UD	UD	1.00	UD	UD	0.28	UD	UD	0.01	UD	UD	1.68	UD

Zn and Cd determination in test mixtures with the sediments from Saco do Engenho River and Meriti River revealed that only the respective doses of ≥18 and 30% were higher than prevention limits (for ecological risk). However, significant mortality levels and biomass loss were found for lower dosages of sediment application onto soil. Therefore, toxicity data are not in agreement with the fact that metal concentrations in the test mixtures were lower than metal limits established by CETESB (2005) and CONAMA (2009) for soil quality. In this respect, other tests with tropical local soils are highly recommended to confirm such results, since soil properties play a crucial role in the toxicity and bioavailability of heavy metals.

Cesar et al. (2015a), when performing chronic bioassays with *E. andrei* using mixtures of artificial soils and dredged sediments from Cunha Channel (Guanabara bay), foundmuch lower toxicity (e.g., no mortality for 20% dose) compared to the sediments collected in the Saco do Engenho River and Meriti River. Cesar et al. (2013), when studying the ecotoxicity of dredged sediments from rivers highly contaminated by domestic wastes in Belford Roxo Municipality (Guanabara Bay basin, RJ), also found much lower toxicity levels, since even the pure sediment did not cause significant mortality in acute bioassays with *E. andrei*. Such observations highlight the high toxicity of the sediments collected in the Saco do Engenho River and Meriti River, even compared to other dredged sediments from RJ state.

The bioassay with the sediment collected in the Saco do Engenho River and Meriti River showed that the organisms increased their biomass when exposed to low doses of dredged sediment (6 and 12%), while the earthworms exposed to the mixtures with the sediment from São Francisco increased their biomass in all test mixtures. The organic matter content found in the sediment of the Meriti River (4.9%; mainly from domestic wastes) was high and suggests that the organisms apparently recognized it as potential source of food. Carbonell et al. (2009) and Cesar et al. (2012, 2014), when performing bioassays with *E. andrei* using residues containing high organic matter contents from domestic wastes, also detected an increase of earthworm biomass. However, the organic matter concentrations in the other sediments (from Saco do Engenho River and São Francisco River) are low (<1%) and do not explain such increase of biomass, especially in the test mixtures with the sediment from the São Francisco River. On the other hand, it is important to note that these aquatic systems are often impacted by domestic wastes, which usually contain high concentrations of hormones (whose concentrations were not quantified for this chapter). Some of these compounds are endocrine disruptors and can induce earthworm biomass changes. Cesar et al. (2015a) studied the ecotoxicity of mixtures of an artificial soil with dredged sediment from Cunha Channel (Guanabara Bay) using chronic bioassays with *E. andrei*, and suggested that endocrine disrupters could play an important role in the biomass changes.

The increase of pH with the increasing dose of sediment from Saco do Engenho River and São Francisco River is most probably due to the presence of carbonated minerals in the sediment, that are common in estuarine sediments. On the other hand, an opposite behavior was detected for the sediment from Meriti River, i.e., reduction of pH and increase of WHC values with the increasing dose of sediment. Meriti River sediment is much more impacted by domestic wastes compared to the other sediments, which explains its high organic matter content (4.9%) and the decrease of pH due to anaerobic conditions. It is thus important to highlight that the variations of pH may influence the oxidation and geochemical availability of metals in the environment (Peijnenburg and Jager, 2003).

For Ni, while the BCFs estimated with sediments from Meriti and São Francisco Rivers were <0.3, the BCFs with the sediment from Saco do Engenho River were >1 (12, 15 and 18% doses). This fact evidences that Ni uptake happened only in mixtures with the sediment from Saco do Engenho River, which agrees with the respective LC50 that is lower than that estimated for the other sediments. On the other hand, the BCFs found for Zn and Cu at higher doses of sediment reflect the constant Zn and Cu concentration in earthworms and no tendency of increased BCF with increasing Zn concentration in soil, except for Cu with sediment from São Francisco, where the BCFs increased with the increasing sediment dose. This is possibly due to the ability of the earthworms to internally regulate these metals, since these elements are essential for their physiology (Lukkari et al., 2005). In this respect, Zn plays an important role in the cell metabolism, and in the development, growth and regeneration of some tissues, while Cu participates in the transport of substances among cells and tissues (Lukkari et al., 2005).

Pb, Cr and Cd are highly toxic metals and well-known for their ability to cause serious noxious effects on biota, even at low concentrations (Katz et al., 1993, Paoliello et al., 2002). Given the fact that the earthworm BCFs found for Pb and Cd in the present study were low, apparently those metals were not highly available to the test organisms, not occurring in bioaccumulable forms (e.g., ionic forms and/or bound to geochemical supports).

CONCLUSION

The bioassays performed were appropriate for a preliminary assessment of the acute damages to soil biota associated with the disposal of dredged sediment in soil. The most toxic sediment was the one collected in the Saco do Engenho River (Sepetiba bay), followed by Meriti River (Guanabara Bay) and São FranciscoRiver (Sepetiba Bay). Furthermore, some toxic metals (e.g., Ni) were highly bioavailable for the earthworms when dredged sediments were mixed with artificial soil. More ecotoxicological tests (e.g., reproduction tests) using other test species (e.g., collembolans, predatory mites, etc.) are required to confirm and further investigate these findings. Also, bioassays using local soils are extremely recommended, since soil properties play a crucial role in metal and dredged sediment toxicity (Cesar et al., 2014). The data obtained suggest that the limits established by Brazilian law are not adequate to prevent the occurrence of adverse effects on soil fauna. The development of metal threshold values that reflect the variety of Brazilian tropical soils and biomes is also urgent and will more effectively support decision-makers in actions of environmental control and soil biodiversity preservation.

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Chapter 20

SOIL ECOTOXICOLOGY: CHILEAN STUDIES WITH SOILS POLLUTED BY COPPER MINING

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ABSTRACT

Chile has had a long mining history and is currently considered the most important copper (Cu) producer in the world. The environmental problems historically associated with Cu mining are widely known in north and central areas of the country, particularly in relation to the contamination of agricultural soils by metals and metalloids. Copper is the dominant contaminant in polluted soils of Cu mining areas in Chile. Arsenic is also present in polluted soils as it is a common secondary element found in Cu ores. In this chapter, we discuss the importance of using field-contaminated soils, instead of metal-spiked soils, in ecotoxicological studies. We also derive thresholds of Cu toxicity to ryegrass and arsenic toxicity to *Eisenia fetida*. Finally, we discuss the necessity of future studies using toxicity tests with microbial properties/processes.

Keywords: copper, arsenic, ryegrass, *Eisenia fetida*, microorganisms

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SCOPE OF THE CHAPTER

Soil ecotoxicology studies the effects of chemicals on organisms in the soil environment (van Gestel, 2012). The field of soil ecotoxicology is very wide and includes studies with different pollutants (organic and inorganic), biological receptors, and soil types. Sensitivity of organisms to metals is species-dependent (Bierkens et al., 1998) and reactivity of organisms to metals varies according to soil type (Carporale and Violante, 2015). Therefore, several ecotoxicity tests need to be performed in order to give a clear view of toxic effects of metals in soils for organisms (ISO 17402, 2008; ISO 17616, 2008). In this chapter, we limit the discussion on Chilean studies with soils polluted by Cu mining (inorganic pollutants), and studies on toxicity tests with ryegrass (plant) and *Eisenia fetida* (earthworm). We also discuss the necessity of future studies using toxicity tests with microbial properties/processes.

RELEVANCE OF THE CHAPTER

Chile has had a long mining history and is currently considered the most important copper producer in the world (Comisión Chilena del Cobre, www.cochilco.cl). The environmental problems historically associated with copper mining are widely known in north and central areas of the country, particularly in relation to the contamination of agricultural soils by metals, such as copper (Cu), zinc (Zn), lead (Pb), and cadmium (Cd), and metalloids such as arsenic (As; henceforth referred to as "metal" for convenience) (González et al., 1984; Ginocchio, 2000; De Gregori et al., 2003). Copper is the dominant contaminant in polluted soils of Cu mining areas in Chile (Goecke et al., 2011). Arsenic is also present in polluted soils as it is a common secondary element found in Cu ores (O'Neill, 1995). Copper is an essential micronutrient to all organisms that becomes toxic above a certain threshold, while As is non-essential and potentially toxic for all organisms, particularly for animals (McBride, 1994; Adriano, 2001).

Currently, Chile does not have any legislation on the maximum permissible concentrations of metals in soils nor regulations to protect soil quality. However, the government has developed in the last decades a national policy for evaluating how likely is that the human health and the environment may be impacted in suspected contaminated sites, as a result of exposure to environmental stressors such as metals (CONAMA, 2009). The main emphasis of the policy was human health protection but ecological risk assessment was afterwards incorporated for crop and wildlife protection. In this context and for the proper implementation of the policy, we consider that it is important to distinguish between soils where metals are present but do not represent an ecological risk (i.e., high total metal concentrations but low metal bioavailability) from those that, at similar total metal concentrations, do represent significant ecological risks (i.e., high metal bioavailability). It is also critical to keep track that background soil metal levels in Chile would be much higher than elsewhere given the underlying geochemistry of the Andes range (Badilla-Ohlbaum et al., 2001).

The concept of metal bioavailability has been extensively discussed in the literature (Ginocchio et al., 2006, 2009; Carporale and Violante, 2015) and will be not developed here. However, it is relevant to consider that metal bioavailability may be assessed in two complementary ways (ISO 17402, 2008): (1) biological methods which expose organisms to metal enriched soils in order to monitor effects, and (2) chemical extraction methods which determine different fractions of a contaminant in the soil. These analytically-determined values need to be correlated with biological effects (Adriano, 2001). If a correlation between the resulting chemical values and effects has been demonstrated, soil quality criteria might be obtained (Sauvé et al., 1998).

USE OF METAL-SPIKED SOILS VERSUS FIELD-CONTAMINATED SOILS

Early versions of the protocols of soil quality tests with plants and earthworms proposed the use of artificial soils (made of peat, clay, and sand) or natural non-contaminated soils spiked with solutions of metals at increasing concentrations (ISO 11268-2, 1998; ISO 11269-2, 2005; OECD 208, 2006; OECD 222, 2004). However, it is well known that soil solubility of metals, and thus their potential toxicity, is greater in artificially-contaminated soils in comparison to field-collected soils (Spurgeon and Hopkin, 1995; McBride et al., 2009; Smolders et al., 2009; Hamels et al., 2014). This difference in solubility is explained by aging processes in soils, which are very slow and occur during several years (Ma et al., 2006; Martínez and McBride, 2000). Thus, metal-spiked soils cannot adequately represent real environmental contamination conditions (Davies et al., 2003). As a result, the latest version of the protocols of soil quality tests with plants and earthworms (ISO 11268-2, 2012; ISO 11269-2, 2012) also consider field-collected soils, instead of artificially-contaminated soils.

We therefore argue that metal-spiked soils have been relevant for understanding basic scientific phenomenon but have limited relevance from an environmental point of view. Accordingly, we emphasize the importance of using field-collected soils for plant- and animal-based toxicity tests. However, the use of field-collected soils presents several difficulties. First, in areas near Cu mining activities in Chile, soils have high concentrations of a mixture of several metals (Cu, Pb, Zn, Cd and As, among others) (De Gregori et al., 2003; Ginocchio et al., 2004). In this case, it might be difficult to distinguish between the effects of different metals on organism responses. Second, the intrinsic physical-chemical characteristics of the soil - such as pH, salinity, texture and organic matter content - are known to affect metal uptake and toxicity for plants (Adriano, 2001; McBride, 1994; Rooney et al., 2006) and earthworms (Ma et al., 1983; Spurgeon et al., 2006; Owojori et al., 2008, 2009a). Finally, distribution of earthworm populations is known to be affected by soil organic matter (Jordan et al., 1999; Leroy et al., 2008; Fonte et al., 2009; Monroy et al., 2011), salinity (Valckx et al., 2009), texture (Klok et al., 2007; Holmstrup et al., 2011), and pH (Klok et al., 2007); however, less attention has been paid to the effects of soil chemical and physical properties on earthworm responses in bioassays. Likewise, differences in soil nutrient availability and soil chemical and/or physical properties may also affect plant responses, in addition to metal toxicity (Ginocchio et al., 2009; Verdejo et al., 2016).

Despite these arguments, in this chapter we present evidence that detailed characterization of soil properties and of metal concentrations in plant/earthworm tissues would allow discrimination between confounding factors and metal-toxicity factors, and thus better estimation of metal toxicity thresholds for plants and earthworms.

USE OF SOILS SPIKED WITH CU-RICH SOLID MINE WASTES

Chemical forms of metal contaminants released to the environment by mining operations may be very different from metal salts used for soil spiking in standard organism toxicity tests. In one of our studies (Ginocchio et al., 2006), we determined Cu solubility and phytotoxicity using soils spiked with different types of Cu-rich solid mine wastes (i.e., tailings, slags, smelter dust) and Cu-concentrate. A Cu salt (copper sulfate, $CuSO_4$) was also included for comparison. We found that mine materials pose different levels of risk to plants at the same total Cu concentration due to their different chemical/mineralogical characteristics. The following general tendency for Cu solubility and possible phytotoxicity, from high to low, was found: $CuSO_4$, smelter dust, acidic tailing sand >> sulfidic Cu ore, Cu concentrate, smelter slag. This tendency shows Cu salt-based toxicity tests represent the worst-case scenario and may be only useful for testing acute toxicity effects of Cu in soil organisms but not exemplifies real environmental effects. It also represents a short-term contamination event since no aging was considered in the study of Ginocchio et al., (2006). With regards to long-term contamination, we performed another study (Mondaca et al., 2015) on Cu solubility and partitioning in two areas polluted with different Cu-rich mine wastes (smelter dust versus tailings sand). We argued that the type of Cu-rich mine waste might not have a major influence on soil chemical degradation once it has had a long time to equilibrate in the field.

Finally, in an additional study of our group (Ginocchio et al., 2009), we spiked soils with two Cu-rich mine wastes (oxidized tailings and smelter dust) in order to determine the relevance of confounding factors on metal toxicity, such as secondary soil acidification. Addition of these mine wastes resulted in soil acidification and thus high concentrations of soluble Cu and Zn. Neutralization of experimental soil mixtures with buffers resulted in marked decrease in soluble Cu and Zn, and significantly reduced metal toxicity in barley (*Hordeum vulgare* L.). Thus, we stress the importance of considering confounding effects on derivation of phytotoxicity thresholds when using short-term laboratory tests.

THRESHOLDS OF CU PHYTOTOXICITY

In this chapter, we present results of one of our studies (Verdejo et al., 2015) on emergence and early growth (21 days) of perennial ryegrass (*Lolium perenne* L.) in agricultural soils historically contaminated by mining activities (smelting or metal processing) in central Chile (Puchuncaví valley and Aconcagua river basin). Ryegrass is recommended by the ISO and OECD methods for testing toxicity of compounds in soils (ISO 11269-2, 2005; OECD 208, 2006) and has been used often as a toxicity bioindicator for metals in soils contaminated by mining activities (Arienzo et al., 2004; Stuckey et al., 2009; Goecke et al., 2011). When using field-collected soils rather than artificially-contaminated soils for metal phytotoxicity tests, other soil characteristics relevant for plant growth are also expected to vary, thus posing confounding effects in test results. In the study of Verdejo et al., (2015), selected soils showed variation in metal contents and in a range of other soil characteristics, such as nutrient availability (Table 1). Results of the study demonstrated, however, that ryegrass responses were unaffected by soil nutrient availability, suggesting that ryegrass is a good bioindicator of Cu toxicity in contaminated soils with different nutrient availability. In contrast, other crops (such as lettuce, maize and tomato) have limited applicability for metal toxicity assessment in metal-contaminated soils with different nutrient availability, due to sensitivity of their responses to nutrient deficiencies (Verdejo et al., 2016).

In terms of Cu toxicity, the study found that total Cu content in soils was the best predictor of ryegrass responses. The effects of Pb, Zn, and As on plant responses were not significant, suggesting that Cu is a metal of prime concern for plant growth in soils exposed to Cu mining activities in central Chile. Shoot length of ryegrass was found as a robust response variable for metal toxicity assessment in selected contaminated soils. Therefore, effective concentration (EC) values (EC_{10}, EC_{25} and EC_{50}) of total soil Cu were estimated for shoot length response variable (Table 2). The EC_{10}, EC_{25} and EC_{50} values for Cu shoot concentrations in ryegrass were also estimated, using shoot length as response variable (Table 2). Derived values are higher than the Cu concentrations considered normal for ryegrass (11 mg kg^{-1}) (Davis and Beckett, 1978). Similarly, the EC_{10} found in the study (22 mg kg^{-1}) is very comparable to the lowest observed effect concentration of foliar Cu of 21 mg kg^{-1} reported for ryegrass by Davis and Beckett (1978).

Table 1. General physicochemical properties (mean and range values; n=27 samples) of soils historically contaminated by mining activities in central Chile (Puchuncaví valley and Aconcagua river basin). Based on Verdejo et al. (2015)

Soil characteristic		Mean	Range
Total metal (mg kg^{-1})	Cu	418	82 - 1295
	Pb	46	25 - 97
	Zn	160	86 - 345
Soluble metal (mg L^{-1})	Cu	0.22	0.04 - 0.71
	As	0.04	0.002 - 0.18
Texture (%)	Sand	54	21 - 95
	Clay	18	5 - 37
	Silt	28	0 - 43
General	pH	7.0	5.7 - 7.6
	pCu^{2+}	8.6	6.8 - 9.8
	OM (%)	3.1	0.7 - 5.8
	EC (dS m^{-1})	2.6	0.7 - 10
Nutrient availability (mg kg^{-1})	N	33	4 - 134
	P	48	8 - 123
	K	302	78 - 1143

pCu^{2+}: negative logarithm of the activity of free Cu^{2+}; OM: soil organic matter; EC: electrical conductivity of saturated paste extract.

In the study of Verdejo et al. (2015), the derived 95% confidence intervals for EC_{10}, EC_{25} and EC_{50} values of total soil Cu were rather widespread (Table 2), not allowing a robust assessment of metal toxicity for agricultural crops, based on total soil Cu concentrations. Therefore, plant tests might need to be performed for metal toxicity assessment. These results are in agreement with other studies (i.e., Hamels et al., 2014). Another point that should be taken into consideration is that the 50% inhibition represents a drastic impact on agricultural productivity. Although this might be deemed acceptable for an industrial site, it would certainly not be acceptable from an agricultural perspective where even a 10% yield reduction would generate serious financial difficulties.

Table 2. Effective concentration (EC_{10}, EC_{25} and EC_{50}) of total Cu (mg kg^{-1}) content in soils and in plant tissues for shoot length in ryegrass; 95% confidence intervals in parenthesis. Based on Verdejo et al. (2015)

Matrix	Shoot length		
	EC_{10}	EC_{25}	EC_{50}
Soil	327 (94 - 559)	735 (575 - 896)	1144 (874 - 1413)
Tissue	22 (16 - 28)	31 (27 - 35)	39 (32 - 47)

Effect of Different Cu Fractions on Plant Responses

Generally, it is considered that total metal content is not a good indicator of soil metal toxicity (McBride, 1994; Adriano, 2001) compared to metal soluble fractions (Kabata-Pendias, 2004; McBride et al., 2009). Furthermore, the free Cu^{2+} ion is considered the main bioavailable form of copper in soils and the best indicator of copper phytotoxicity (Oliver et al., 2004; Sauvé et al., 1998). However, other ions, principally H^+, Ca^{2+} and Mg^{2+} according to the Terrestrial Biotic Ligand (Thakali et al., 2006), and soil components, such as organic matter and dissolved organic carbon (i.e., Ginocchio et al., 2009), compete with Cu^{2+} and, therefore, affect its toxicity. Toxicity is correlated only to the fraction of the total biotic ligand sites occupied by Cu^{2+}. For this reason, soluble copper concentrations and/or free Cu^{2+} activities in a soil extract are not suitable indexes for Cu phytotoxicity (Zhao et al., 2006) and Cu uptake by plants (Zhang et al., 2001).

Consistent with these arguments, total Cu concentration was a better predictor of ryegrass responses (Table 3), in comparison to soluble Cu and activity of free Cu^{2+} (pCu^{2+}) (Verdejo et al., 2015). Likewise, the shoot Cu concentration was correlated with total Cu concentration in soils, while soluble copper and pCu^{2+} were not significant in explaining shoot Cu concentration. These findings are consistent with results of another study on Cu uptake by vegetable crops grown in Chilean metal-polluted soils (Ginocchio et al., 2002). We demonstrated that Cu concentration in plant tissues depends not only on the availability of free Cu ions in soil solution but also on other soil Cu pools that supply the element to the soil solution.

Table 3. Determination coefficients (R^2) of regressions between different Cu fractions in the soil and ryegrass responses ($p < 0.05$). Based on Verdejo et al. (2015)

Shoot length			Root length			Dry shoot mass			Dry root mass		
CuT	CuS	pCu^{2+}	CuT	CuS	pCu^{2+}	CuT	CuS	pCu^{2+}	CuT	CuS	pCu^{2+}
0.58	0.24	ns	0.40	0.40	0.33	0.34	ns	ns	0.35	ns	ns

CuT: total soil Cu concentration; CuS: soluble Cu; pCu^{2+}: $-\log[Cu^{2+}]$; ns: not significant.

The findings of our studies are consistent with arguments of Zhang et al. (2001), that resupply from the solid phase due to local depletion is the dominant controlling process for Cu uptake by plants. In other words, the process of Cu uptake by roots depends on the buffering capacity of the soil to resupply pCu^{2+} (Sauvé, 2002; Zhao et al., 2006). For these reasons, Cu uptake by ryegrass and Cu toxicity to ryegrass in the study of Verdejo et al. (2015) depended on the total soil Cu pool that was capable of supplying Cu to the soil solution at the same time as plant roots locally depleted the ions through active uptake.

The technique of diffusive gradients in thin films (DGT) has shown to be better at predicting Cu phytotoxicity and Cu uptake by plants, in comparison to soluble copper concentrations and/or free Cu^{2+} activities in a soil extract (Zhang et al., 2001; Zhao et al., 2006). It has been demonstrated that plant bioavailability of Cu in soil depends on Cu speciation, interactions with protective ions (particularly H^+, Ca^{2+} and Mg^{2+}), and the resupply from the solid phase. Those studies concluded that the DGT measurement provides an integrated measurement of both intensity (free activities of Cu^{2+} and H^+) and resupply. One can argue that the DGT-measured Cu would be a better predictor of plant responses, in comparison to total soil Cu, but a controversy is still discussed in the literature (i.e., Hamels et al., 2014). We have not used the DGT technique in our studies yet.

THRESHOLDS OF ARSENIC TOXICITY TO *EISENIA FETIDA*

As a result of the key-ecological importance of earthworms (Blouin et al., 2013; Edwards and Bohlen, 1996), they have been adopted as indicator organisms for the assessment of soil quality (ISO 11268-2, 2012; OECD 222, 2004). These tests use the cosmopolitan earthworm *E. fetida* because it can be easily cultivated in the laboratory (OECD 207, 1984; OECD 222, 2004).

In this chapter, we present results by Bustos et al. (2015) on earthworm reproduction of *E. fetida* in soils affected by Cu mining activities (smelting or metal processing) in central Chile (Table 4). Results of this study indicated that As was a metal of prime concern for *E. fetida*, while Cu exhibited a secondary effect, contrary to what one could expect in soils affected by Cu mining activities. Perhaps this difference is due to the fact that the assimilation (and thus toxicity) of Cu by *E. fetida* can be regulated by homeostatic mechanisms of Cu excretion (Spurgeon and Hopkin, 1999). In contrast, no elimination of As by *E. fetida* has been reported (Lee and Kim, 2013). As a consequence, the bioconcentration factor for As was considerably higher than that for Cu (Table 4), which may imply higher toxicity. Lee and Kim (2013) reported no elimination of As by *E. fetida* during its exposure to clean soil, probably due to a formation of As-thiol complexes in earthworm tissues. This absence of an elimination pattern of As is similar to other non-essential metals (Cd and Pb), while rapid excretion is known for Cu and other essential metals (Spurgeon and Hopkin, 1999).

We were able to estimate the EC_{10} [8 (-6-21) mg kg^{-1}], EC_{25} [14 (7-22) mg kg^{-1}] and EC_{50} [22 (17-26) mg kg^{-1}] of total soil As for responses of cocoon production. Lee and Kim (2013) reported that As(III) showed higher toxicity to *E. fetida* than As(V). In the studied soils of central Chile, As(V) and As(III) represented 75±12% and 12±6% of the total soil As (Vargas et al., 2015). Thus, derived thresholds correspond mainly to As(V). Based on our literature review, we noted a lack of data on thresholds of As toxicity to *E. fetida* in field-collected soils. Thus, findings of the present study provide new data for estimating thresholds of As toxicity to *E. fetida*.

Table 4. Bioconcentration factor (BCF) of Cu and As by *Eisenia fetida* and *E. andrei* in field-contaminated soils. Average and range of values are shown

Earthworm	pH	BCF_{Cu}	BCF_{As}	Reference
E. fetida	6.97 (5.70–7.57)	0.15 (0.04–0.40)	3.21 (1.43–5.87)	Bustos et al., (2015)[a]
E. andrei	6.49 (5.26–7.17)	0.43 (0.15–1.25)	0.76 (0.12–3.31)	Janssen et al., (1997)[b]

[a]For soils with electrical conductivity ≤ 0.29 dS m^{-1} (measured in 1:5 soil:water extract), which corresponds to EC_{50} of salt toxicity for *E. fetida* (Owojori and Reinecke, 2009).

[b]For a sub-sample of soil with pH > 5, to avoid direct effects of low pH, as suggested by Janssen et al., (1997).

Results of the present study differed from previously-reported thresholds of As toxicity for *E. fetida* in artificially-contaminated soils. Specifically, Lee and Kim (2009) reported a LC_{50} of 5.9 mg kg^{-1} for total soil As, in a spiked soil, for an exposure time of 4 weeks (as used in the present study), while there were no lethal effects observed at this concentration in the present study. Similarly, Fischer and Koszorus (1992) reported a LC_{10} of 50 mg kg^{-1} of added potassium arsenate, expressed on wet basis (70% of water content). This value is equivalent to 85 mg kg^{-1} of potassium arsenate expressed on dry basis, which in turn is equivalent to 25 mg kg^{-1} of total soil As expressed on dry basis. However, there were no lethal effects observed at this concentration in the present study which is based on field-collected soils and without spiked As. Moreover, the exposure time was of 8 weeks in the study of Fischer and Koszorus (1992), and the values of lethal concentration increase with decreasing exposure time (Lee and Kim, 2009). Thus, one would expect a higher value of LC_{10} for an exposure of 4 weeks used in the present study, making even greater the discrepancy between the results of the present study and the findings of Fischer and Koszorus (1992). Likewise, Lock and Janssen (2002) reported EC_{50} of 10.8 mg kg^{-1} for total soil As, using cocoon production as a response variable, while a considerably higher value (22 mg kg^{-1}) was found in the present study for the same response variable.

Based on a direct determination by electron probe microanalysis (Ávila et al., 2007), iron oxides and Cu sulfides are the As-bearing phases in agricultural soils affected by mining activities in the Aconcagua river basin, central Chile. On the other hand, potassium/sodium arsenate was added in the above-mentioned studies with artificially-contaminated soils. Thus, the discrepancy between the previously-reported thresholds of As toxicity to *E. fetida* in artificially-contaminated soils with those reported in the present study could be explained by differences in solubility of As-bearing phases. Findings of the present study indicate that metal-spiked soils cannot adequately represent real environmental conditions and, consequently, have limited relevance from an environmental point of view (i.e., Hamels et al., 2014).

We were also able to estimate the EC_{10} [38 (24-53) mg kg^{-1}], EC_{25} [47 (38-56) mg kg^{-1}] and EC_{50} [57 (51-62) mg kg^{-1}] of earthworm tissue As for cocoon production. Thresholds of earthworm tissue As obtained in the present study are consistent with previously-reported thresholds of tissue As in *E. fetida*: least-observed effect concentrations of 18 mg kg^{-1} (Lock and Janssen, 2002), non-lethal concentrations of 80 mg kg^{-1} (Leduc et al., 2008) and near the lethal concentrations of 900 mg kg^{-1} (Fischer and Koszorus, 1992). Likewise, we were able to determine a no-observed effect As concentration in tissue As of *E. fetida* of 24 mg kg^{-1} (95% confidence interval of -2–49 mg kg^{-1}). The previously-reported no-observed effect concentration of 10 mg kg^{-1} (Lock and Janssen, 2002) is within the 95% confidence interval of our study. Thus, findings of the study of Bustos et al., (2015) provide new data for establishing thresholds of As bioaccumulation by *E. fetida*.

In the study of Bustos et al., (2015), soil electrical conductivity was another toxicity factor, besides metal toxicity. These results are consistent with other reports of reproduction sensitivity of *E. fetida* to salts (Owojori and Reinecke, 2009; Owojori et al., 2009b). Multiple regressions revealed that the interaction between As and soil electrical conductivity was not statistically significant ($p > 0.05$), thus suggesting that these two toxicity factors are independent of each other. Thus, the challenge of interpreting results of the study of Bustos et al., (2015) was in discernment between metal and salt toxicity factors. Therefore, the earthworm reproduction test might have a limited applicability in soils with high electrical conductivity because salinity-induced toxicity will hinder the interpretation of results. In order to isolate the effect of soil salinity on earthworm reproduction, one could consider only soils with electrical conductivity ≤ 0.29 dS m^{-1} (measured in 1:5 soil:water extract), which corresponds to the reported EC_{50} of salt toxicity for *E. fetida* (Owojori and Reinecke, 2009).

NECESSITY OF FUTURE STUDIES WITH MICROBIAL PROPERTIES/PROCESSES

Soil microbial properties are being increasingly used as indicators of soil quality since they provide a direct measure of soil functioning (Garbisu et al., 2011). Also, there is strong evidence that soil microbes are more sensitive to metals than animals or plants (Sauvé et al., 1998; Giller et al., 1999).

We are currently comparing the sensitivity of different microbial properties/processes to metals in Chilean agricultural soils polluted with metals. We will determine the most suitable microbial properties/processes to monitor the quality of metal-polluted soils. We will also determine threshold limits of metal concentrations corresponding to X% of inhibition of biological response (i.e., microbial number and biomass, microbial diversity, nitrification rate, nitrogen mineralization rate, and soil bacterial diversity).

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Chapter 21

INTER-LABORATORY CALIBRATION OF THE OSTRACODTOXKIT F ASSAY USING THE CRUSTACEAN HETEROCYPRIS INCONGRUENS ON PAH-CONTAMINATED SOILS

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ABSTRACT

A short inter-laboratory calibration exercise was conducted by the Laboratory of Environmental Toxicology and Aquatic Ecology (LETAE), Ghent University with the participation of the Department of Marine Biology and Limnology, University of Panama and the Institute for Agrobiotechnology in Tulln, Austria. The purpose was to identify the precision and repeatability of the Ostracodtoxkit F assay using the species *Heterocypris incongruens*, when PAH-contaminated soils have to be assessed. Six soil samples were collected from a known site polluted with polyaromatic hydrocarbons (PAHs) in Austria and were packaged, homogenized and shipped to each laboratory. In each laboratory the Ostracodtoxkit F assay was performed and an additional bioassay named Lumistox inhibition assay based on bacteria was performed in Austria. Chemical analysis in the soils were also performed in order to determine the concentration for 13 PAHs. All laboratories reached a successful hatching of ostracod cysts as well as successful validity conditions of the Ostracodtoxkit F assay. The outcome for the lethality of the Ostracods exercise showed that the intra-laboratory precision (coefficient of variation) varied

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between 0 and 19% and the inter-laboratory precision between 0 and 11%. Reproducibility was attained when both laboratories found similar results for mortality ($r=0.98$; $p<0.05$). The Ostracodtoxkit assay showed to be sensitive to soil contaminated with PHAs, and in particular to acenaphthene according to the statistical correlation ($r=0.82$; $p=0.046$). These results were also found at a similar statistical significance with the second bioassay, the Lumistox assay.

Keywords: maintenance free tests; ostracod *Heterocypris incongruens*; soil; Lumistox; polyaromatic hydrocarbons

INTRODUCTION

The solid phase sediment tests with aquatic invertebrates are complex, time-consuming and costly. The search for a user-friendly alternative in the Laboratory of Environmental Toxicology and Aquatic Ecology (LETAE, Belgium) eventually led to the development of a new "cyst-based" free microbiotest (independent of the year-round culture costs) with the ostracod *Heterocypris incongruens*. The new 6 days microbiotest is based on two endpoints: mortality and growth inhibition. The measurement of sublethal effects is particularly useful in samples in which no mortality is detected.

A first study was hence undertaken on 16 soil samples obtained from former zinc smelters in Flanders, Belgium and from sites on which contaminated river sludges had been spread. The sensitivity of the 6 days *H. incongruens* microbiotest (Chial and Persoone, 2002) was compared to the 28 days reproduction inhibition assay with the springtail *Folsomia candida* (Lock and Janssen, 2001). The outcome of the investigations revealed that for most samples the ostracod assays were substantially more sensitive than the springtail tests.

Leachates were subsequently prepared with the same soils to determine to what extent the detected toxicity was due to the "soluble" compounds that partitioned to the leachates or to the chemicals that remained absorbed to the soil particles after leaching. The ostracod tests performed on both the solid and liquid fractions indicated that the mortality of the organisms was still very high in leached-soil contact tests, whereas it was low in most leachates (Chial and Persoone, 2002). The latter findings clearly confirm the necessity of direct contact tests for contaminated soils, as has already been demonstrated for sediments (Burton et al., 1996; Vangheluwe et al., 2000).

This chapter reports on a short laboratory intercalibration exercise carried out by LETAE in Ghent Univesity and the Institute for Agrobiotechnology in Tulln, Austria, with the Ostracodtoxkit F assay on PAH-contaminated soils. Finally, the sensitivity of this assay was compared with the acute Lumistox luminescent bacteria test.

METHODS

The soil used was excavated from a historically PAH contaminated site, a former gas plant, in the east of Vienna, Austria. The contamination was determined for five different soil samples coded as WGalpha, WGbeta, BZ 10, TA 14-16 and TA 4-6. The soil samples were collected, homogenized and packaged. One part of the sample was tested in Austria and the other in Belgium. The inter-calibration exercise consisted of undertaking a whole soil bioassay with the Ostracodtoxkit using the specie *H. incongruens* on this soil contaminated with PAHs in both mentioned laboratories. The test was run twice for each soil sample in each country. The second inter-calibration exercise, run in Austria was performed 2 months after the first one due to the lack of material. In addition to the Ostracodtoxkit bioassay, a second available bioassay named Lumistox inhibition assay was performed in Austria without repetition. The concentrations of acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(g,h,I) perylene and indeno(1,2,3-c,d)pyrene PAH known to be present, were determined in the soil.

Ostracodtoxkit Bioassay

The 6-d solid phase ostracod microbiotests with *H. incongruens* were applied in accordance with the methodology described in Chial and Persoone (2002), which is the basis for the Standard Operational Procedure of the ostracod microbiotest (Ostracodtoxkit F^{TM}, 2001). The method consists of adding freshly hatched ostracod neonates to multiwell plates (10 organisms per test well in 6 replicates) with 2 ml synthetic freshwater (US EPA, 1991), 300 µL soil and $3x10^7$ algal cells (*Raphidocelis subcapitata* renamed *Pseudokirchneriella subcapitata*) as complementary food. Calibrated reference sand is used as a control sediment (Ostracodtoxkit F^{TM}, 2001).

The 300 µL soil samples were obtained as follows: in a plastic test tube filled with the sediment sample wetted previously with synthetic freshwater, a large-mouth syringe without the plunger was inserted and pushed until reaching the bottom. The syringe was pulled out with the sample and then the plunger inserted. The plunger was then pushed down until it reached the sediment core in the syringe tube, and the sediment core was compacted. For very smooth sediments, the syringe had to use the plunger to suck up the sediment by pulling the plunger. Finally, 300 µL was dispensed into each testing well to expose the ostracods. The same procedure was repeated for the replicates of the reference sediments. A reference soil, Ifa-acker from Austria (Department of Environmental Biotechnology, Institute for agrobiotechnology, Konrad Lorenz Str. 20, A-3430 Tulln, Austria) was also tested. This second reference soil was used in order to identify other possible sources of control sediment.

After 6 days incubation at 25 °C in darkness, the ostracods were retrieved from the substrate (soil in this case) and the mean percentages of mortality and growth inhibition were determined. The percentage of growth inhibition in the test samples is calculated with the formula: *% growth inhibition=100 – (Linc test sed/Linc control sed) x 100*, in which "Linc test sed" is the mean growth (length increment) of the organisms in the test sample after 6 days (i.e., their length at the end minus the length at the start, in µm), and "Linc control sed" the mean growth of the organisms in the control sediment (calibrated reference sand) after 6 days.

The validity criterion of the ostracod assays is a minimum survival of 80% and a minimum length increment of 400 µm of the test organisms in the control sediment.

Lumistox Inhibition Assay

The Lumistox test was conducted with the soil elutriates and according to the manual procedure using the Luminometer, reagents and *Aliivibrio fischeri* (formerly known as *Vibrio fischeri*) luminescent bacteria (DANETV Test Environmental Technology Verification Program, 2011).

Chemical Analysis of PAHs

To perform the PHA analysis, an elutriate from each soil was obtained (soil:distilled water=1:3.5). Liquid chromatography was performed with an HP 1050LC (Hewlett-Packard, USA) equipped with an HP1100 series three-dimensional fluorescence detector. Injection volume was set to 20 µl for standards and samples on a 1050 HP autosampler. For separation, an ODS Hypersil guard column (20x4 mm, particle size 5 µm) followed by a C/18 Grace Vydac separation column (250x4.6 mm, particle size 5 µm) was used. The column was heated at 26 °C and operated with an eluent flow rate of 1.5 mL/min. The eluent gradient profile was set up as follows: 50% acetonitrile (ACN)/50% Milli-Q- water for 2.5 min, followed by a linear gradient of 9.5 min up to 90% acetonitrile and a linear gradient of 8 min up to 100% acetonitrile, held for 2.5 min. Subsequently, a linear gradient (2.5 min) was set back to initial conditions for column conditioning before the next run. The 3d.FLD was operated at four different excitation/emissions wavelength pairs: excitation was set to 260 nm, emission wavelengths were 350, 420, 440 and 500 nm, respectively.

Data Treatment

In order to compare the data obtained by both laboratories for each soil sample, the mean percentages of mortality and inhibition growth were calculated. The intra and inter-laboratory variability (precision) was estimated by means of the coefficient of variation (CV).

The degree of correspondence between the assays and the PAHs was subsequently determined by general Pearson pairwise product-moment correlations between percentages of mortality and growth inhibition, and the concentration of the PAHs to estimate the reproducibility of the test regarding the level of significance.

RESULTS

In both laboratories the ostracods hatched properly from cysts within 52 h and the validity conditions of the Ostracodtoxkit F assay were reached (control mortality <20% and a minimum mean growth increment of 400 µm).

Table 1 displays the concentration of PAHs, mortalities and growth inhibition for each soil sample. The PAHs determined in elutriates, displaying a differential concentration of the PAHs where the maximum amount is observed in the soil WG alpha. However, acenaphthene increased accordingly with the known contamination of the soil samples: WG alpha=WG beta < BZ 10 < TA 14-16 < TA 4-6.

The two Ostracodtoxkit F assay repetitions and the Lumistox assay were performed with each soil sample in both laboratories (Belgium and Austria) showed that the Ostracodtoxkit F as well as the Lumistox assay detected three soil samples (BZ 10, TA 4-6 and TA 14-16) presenting a high toxicity range for mortality and inhibition endpoint (between 91 and 100%) among the laboratories. The coefficient of variation of the studied endpoints was 0% for all Ostracodtoxkit results, while it was between 2.6 and 3.7% for acute Lumistox and all other Ostracodtoxkit results. The mortalities detected in the other soils (Ifa-acker, WG beta and WG alpha) were in the range considered non toxic (11 to 19%), with the exception of the soil sample WG alpha when tested with the Ostracod assay in Austria, in which case the mortality was 32%.

Table 1. Concentration of poliaromatics hydrocarbons (PAHs) in soils coded and results of the bioassays obtained in each country with the Lumistox acute and Ostracodtoxkit test

PAHs	Soils					
	WG alpha	WG beta	BZ10	TA 14-16	TA 4-6	Reference soil (Ifa-acker)
Acenaphthene (2 rings)	0	0	16	54	27	0
Fluorene (3 rings)	51	10	20	70	29	0
Phenanthrene (3 rings)	219	27	40	150	55	0
Anthracene (3 rings)	171	12	10	39	36	0
Fluoranthene (4 rings)	909	74	8	42	54	0
Pyrene (4 rings)	771	78	4	29	31	0
Benzo(a)anthracene (4 rings)	408	34	1	5	9	0
Chrysene (4 rings)	342	31	1	6	9	0
Benzo(b)fluoranthene (5 rings)	281	30	0	2	4	0
Benzo(k)fluoranthene (5 rings)	190	20	0	1	2	0
Benzo(a)pyrene (5 rings)	398	41	0	1	4	0
Dibenz(a,h)anthracene (5 rings)	0	0	0	0	0	0
Benzo(g,h,i)perylene (6 rings)	163	18	0	0	3	0
Indeno(1,2,3-c,d) pyrene (6 rings)	299	30	0	1	3	0
Sum of PAHs	4202	405	101	400	266	0
Bioassays by country and endpoint	Results					
Lumistox acute-Austria (% inhibition)	13.8	12.3	93.7	91	93.5	0
Ostracods-Austria (% mortality to calibrated reference sand)	32	12	100	100	100	17
Ostracods-Austria (% growth inhibition to reference soil Ifa-acker)	51.8	34.8	100	100	100	0
Ostracods-Austria (% growth inhibition to calibrated reference sand)	79	67	100	100	100	54
Ostracods-Belgium (% Mortality)	20	17	100	100	100	11
Ostracods-Belgium (% growth inhibition)	58	47	100	100	100	47

Mortality data obtained in Belgium indicates that the coefficient of variation between repetitions for five (Ifa-acker, WG beta, BZ 10, TA 4-6 and TA 14-16) of the six soil samples ranged from 0 to 7% and one soil sample (WG alpha) reached 64% CV. In Austria, the CV between repetitions ranged from 0 to 19% for all soil samples.

The inter-laboratory coefficient of variation between the mean mortalities ranged from 0 to 11% for five of the six soil samples, and 49% for one soil sample (WG alpha).

Growth inhibition data obtained after six days in each laboratory is presented in Table 1. The Ostracodtoxkit F assay showed growth inhibition in all soil samples tested (Ifa-acker, WG beta and WG alpha) in both laboratories for which no significant mortalities (less than 20%) were observed. The growth inhibition obtained in Belgium ranged from 47 to 58% with a CV between repetitions from 4 to 10%. In Austria, the growth inhibition ranged from 54 to 79% and was not possible to estimate the CV because only a single data was recorded. In both laboratories the lowest inhibition corresponded to the Ifa-acker soil in which PAH concentrations were not detected.

The interlaboratory variability for growth inhibition obtained in Belgium and the single value recorded in Austria ranged from 6 to 17%, being the lowest value for Ifa-acker soil obtained in Austria.

The correlation analysis between mortality found in both laboratories with the Ostracodtoxkit F assay indicates good correspondence ($r=0.98$; $p<0.05$), as well as with the acenaphthene concentrations ($r=0.82$; $p=0.046$) and not with other PAHs. A similar correlation was found with the same PAH and the Lumistox inhibition assay data ($r=0.80$; $p=0.055$).

DISCUSSION

The low intra-laboratory variability observed for the Ostracodtoxkit F assay indicates a reasonably good precision when mortality was the endpoint considered, according to the commonly "~30 %" acceptable variation of toxicity (USEPA, 1991; EPS, 1995). A possible weak point could be the apparent high variability results observed in the soil sample WG alpha performed in Belgium. This variability could be due to the soil heterogenity since it was not observed in Austria.

The inter-laboratory variability also showed very good precision, with the exception of soil sample WG alpha. This variability for WG alpha caused by an increasing mortality in the second repetition of the soil assay performed in Austria might be due to the fact that it was performed ca. 2 months after the first test. The storage conditions and time could have influenced the physical-chemical parameters in such a way that it increased the availability of the PHAs, resulting in a higher toxicity response. This toxicity could be due to the differential solubility of PHAs, reported in previous studies (Guzzella, 1998; Jennings et al., 2001; Scherr et al., 2008).

Moreover, the inter-laboratory precision could be verified when taking into consideration that the reference field soil, Ifa-acker, had an intra-laboratory CV lower than 10%, while the inter-laboratory precision was 11%. Besides, good accuracy of the assay was apparent because the results did not produce false positives for this natural, non-polluted soil.

Substantial information can be found in the literature regarding the factors affecting intra and inter-laboratory test results (Dorn et al., 1987; Persoone et al., 1993; EPS, 1995; Burton et al., 1996). The success of the present study was due to the two most important factors: the analyst experience and test organism health were maintained homogeneous in both laboratories since the operators were previously trained and the organisms were obtained from the same cysts batch. Therefore, any significant variability is most probably due to test conditions and soil storage, i.e., handling conditions.

The significant statistical correlation found between the mortality endpoints produced in the soils once the Ostracodtoxkit F assay was performed in both countries is indicative of its good reproducibility ($r=0.98$; $p<0.05$). Furthermore, the toxicity response of the Ostracodtoxkit F could also be corroborated when a significant statistical correlation was found with the light inhibition signals obtained when the Lumistox assay was performed ($r=0.997$; $p<0.05$).

The Ostracodtoxkit F assay demostrates a sensitivity to PHAs, in particular to acenaphthene according to the significant correlation found. The correlation with acenaphtene might be indicating the known chemical-species toxicity dependence or the differential availability of PAHs (Guzzella, 1998; Jennings et al., 2001; Scherr et al., 2008).

Although the additive toxicity of PAHs does not seem to play an important role in the toxicity caused to the ostracods and bioluminisce bacteria, final conclusions have to be drawn with caution because the final PAH concentrations were not measured in the supernatant nor were the organisms exposed separately to each PAH concentration. The growth inhibition (calculated on the basis of the calibrated reference sand), identified with the Ostracodtoxkit F assay for these soils indicated a fairly acceptable intra-laboratory precision, thus the operational procedures should now be standardized in order to obtain comparable results. It seems that the Ifa-acker soil itself presents a growth inhibition to the ostracods, producing interlaboratory variability when taken as the reference soil to estimate the growth inhibition.

Considerations are taken by the University of Panama through the Department of Marine Biology and Limnology to continue this research and implement it in Panama in order to evaluate sediment and soil pollution from different sources. This and other bioassays will complement the traditional chemical analysis performed until now in the country and obtain a more realistic evaluation of pollutant effects on wildlife. The adoption of this approach in our region will add a more scientific support to protect biodiversity, while generating new toxicity information from tropical regions.

CONCLUSION

Reasonably good intra and inter-laboratory precision has been demonstrated by the Ostracodtoxkit F assay for soils contaminated with PAHs.

The new ostracod solid phase sediment microbiotest showed also to be applicable to detect soils contaminated with PAHs on the basis of lethality and growth inhibition.

Growth inhibition of ostracods seems to be an important alternative to detect toxicity using the ostracod toxicity test when other than acenaphthen PAH has to be detected in soil.

More knowledge of the sensitivity of the ostracod assay to the PAHs could be obtained if PAH concentrations are also determined in the supernatant of the test during the assay.

Analyst experience and the use of similarly aged and healthy organisms obtained from the dormant stage (cyst) probably contributed to the success of this short inter-laboratory intercalibration.

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Chapter 22

MINING IN VENEZUELA: ITS EFFECTS ON THE ENVIRONMENT AND HUMAN HEALTH

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ABSTRACT

Factors that affect water quality and river habitats of the Orinoco River Basin have been a source of constant concern and their study has become a priority for the Venezuelan scientific community. Due to economic pressures, the old and isolated artisanal gold mining has exploded uncontrollably, become more industrialized and has extended to almost all river basins of the Venezuelan Guiana Shield. Precious mineral prospection and extraction has become a serious threat to aquatic ecosystems by destroying the surrounding protective forests and riverbanks, by polluting the water with mercury and other heavy metals that upon entering the food chain cause serious deleterious health effects to local inhabitants. The diminishing quantity and quality of food, as well as the health problems due to mercurial toxicity have created drastic changes in the social structure of these communities. We summarized studies made on mercurial contamination in fish and how it is being transferred to humans. Finally, their overall impact on the native indigenous population is presented. The actions controlling gold extraction and other minerals cannot be limited to a single country and require their consistent and vigorous application within all nations of the Amazonian, Guianian and Orinoquian river basins.

K**eywords**: Guiana Shield, gold, biodiversity, human health

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INTRODUCTION

The conservation and environmental services offered by the biodiversity in tropical areas in favor of human health has been a matter of recent consideration in academic, economic, social and political grounds. The action of natural or anthropic factors as well as the responses, norms and necessary regulations are equally considered in workshops, symposia and scientific papers (Lasso et al., 2010, 2011; Machado-Allison et al., 2011; Machado-Allison, 2015). An unlocked wealth remains hidden within the tropics' biodiversity considering its enormous potential for new food production, pharmacological derivatives, green-energy and industrial derivatives. Equally important is to understand how this immensely varied biodiversity maintains a delicate balance in terrestrial and aquatic ecosystems, in order to avoid major perturbations, and providing the most abundant and un-intervened sources of clean air and potable water necessary to maintain several native human groups as well as a rich wildlife. The world's tropical regions will be the basis for global sustainable development in the 21st century and beyond (Machado-Allison, 2015).

Venezuela is located at the northern tip of South America and constitutes an area of great biotic importance. One of its geographical features is the Guiana Shield, dated from the Pre-Cambrian (1.5-2 x 10^3 million years of age), and is one of the most ancient geological rock formations on Earth. Numerous processes of biotic and abiotic evolution have produced an amalgam of organic ecosystems and communities, many of them unique. The water bodies and their aquatic communities do not escape this fabulous history, which continues to evolve to this day (Machado-Allison, 2014).

In contrast to this natural history, the social and economic reality is quite different. Despite the country's immense wealth in resources (petroleum, gas, iron, hydroelectric power, fertile land, agriculture), a significant inequality gap persists with many living in extreme poverty and some dying of hunger. This has created a social structure and environmental deterioration that has reached levels never seen in the past. More recently, the development of the Orinoco Bituminous Belt and oil operations alongside the Orinoco River in addition to the artisanal and industrial exploitation of gold (such as Mining Arch) in some of its headwaters have created a substantial threat to Venezuela's main aquatic environments.

The purpose of this chapter is to discuss some of the gold extraction operations occurring in Venezuela and their impact on the environment and local human populations. At the end we discuss the importance of guiding human activities that allow both environmental sustainability and a closure of the social inequality gap.

GEOGRAPHIC AREA: GENERAL CHARACTERISTICS

The Venezuelan "Guiana" includes an area occupied by Amazonas, Bolívar and Delta Amacuro states located to the south of the Orinoco River and extends approximately 620,000 km². Contained within this area are the headwaters of numerous rivers (Aro, Autana, Caroní, Caura, Cuchivero, Cuyuní, Paragua, Ventuari) and the Orinoco itself. Within this basin there are "clear," "white" and "black" rivers (Sioli, 1965, 1975), which have significant heterogeneity in their physical-chemical and biotic characteristics. Besides representing the main source of drinkable and irrigation water for most residents living on their banks, they also constitute one of the greatest hydro-electric reserves of the world with an estimated generation capacity that will save 750000 barrels of oil per day once all projects are completed (Caroni and Paragua rivers).

The landscape is covered by an exuberant and luxurious tropical wet evergreen forest, which grows on nutrient poor soil. Tree growth is very slow and nutrient extraction is critically dependent at root level of complex biological associations called microrhizae. The lowland forest is broken by high mountain "tepuis" on top of which special and unique plant communities have been able to adapt and survive. In between and at different altitudes there is a variation of evergreen, deciduous forest and grasslands. Over nine thousand plant species have been described from these different biota including grasses, ferns, shrubs, evergreen and deciduous trees, "palm tree morichales," bromeliads, orchids, and other highly endemic flora living on of the "tepuis" (Hubber and Alarcon, 1988; Hubber, 1990; Fernández et al., 2010). Importantly, the vegetation that lives in close association to water bodies is the main source of organic nutrients and food (seeds, fruits, insects) for many aquatic organisms such as the fruit-eating piranhas *Pygopristis* sp. (Marrero et al., 1997; Machado-Allison et al., 2010).

Terrestrial fauna is immensely diverse and constitutes the last and only wild reserve for a great number of endangered species such tapirs, jaguars, dears, toucans, monkeys and macaws (Aguilera et al., 2003; Rodríguez and Rojas-Suárez, 2008). The aquatic fauna is abundant and equally varied, containing an important list of fishery species (for food production or aquarium enthusiasts) and that is the primary source of protein for the human communities in the area.

Humans have been present in these areas for over 6-9 thousand years. At least 15 autochthonous tribes live in Venezuelan Guayana (Figure 1), and generally reside close to large water bodies (rivers and lakes), where they obtain resources for housing, wood for canoes, fiber for hammocks, food and water.

Recognizing its biological, anthropological and geological importance, several regions have been assigned as "Áreas Bajo Regimen de Administración Especial" (ABRAE) (Special Protection Areas) and are protected by national environmental laws. These include some of the world's largest national parks (Parima-Tapirapeco 39000 km^2, Canaima 30000 km^2, La Neblina 13600 km^2, Duida-Marahuaca 3800 km^2, Jaua-Sarisariñama 3300 km^2, Yapacana 3200 km^2), and several important biosphere and forest reserves. All tepuys are also considered under protection (Bevilacqua et al., 2006).

MERCURY: THE PROBLEM

Conflicting interests have not allowed a consensus among international, national and regional governmental and non-governmental agencies, indigenous communities, research groups and economic groups with regards to the sustainable use of the resources of the Venezuelan Guayana, including the exploitation of precious minerals (gold and diamonds) and how to mitigate its effect unto the environment. Table 1 summarizes some of the most important developments in the last 30 years (in addition see notes from Machado-Allison, 2014).

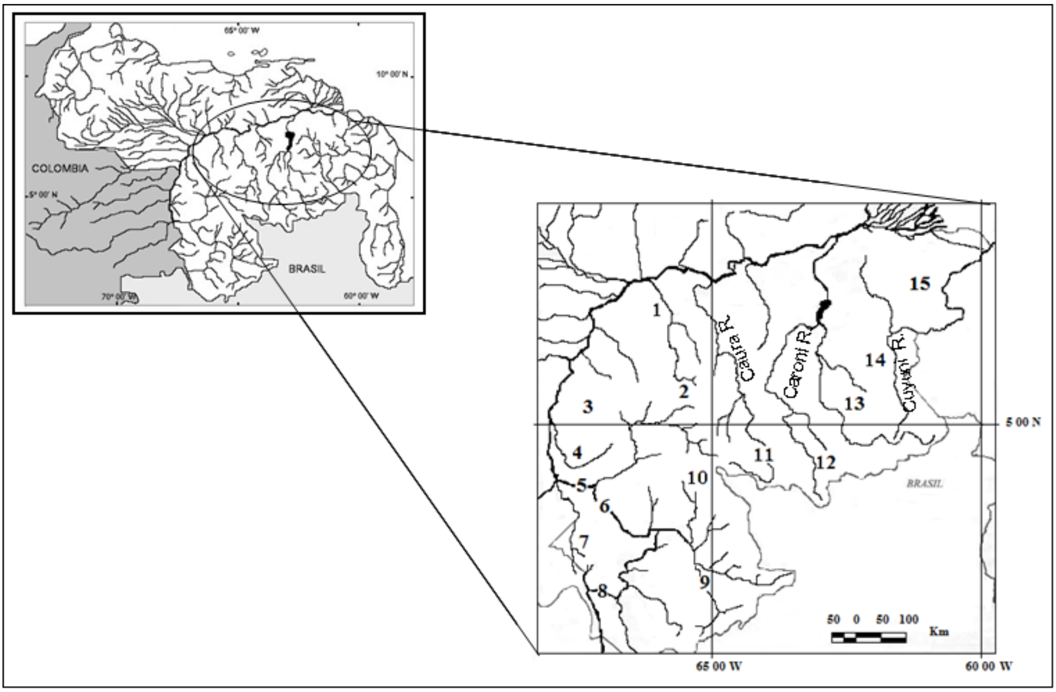


Figure 1. Distribution of local indigenous communities in Venezuelan Guayana: 1. Panare, 2. Hoti, 3. Piaroa, 4. Puinave, 5. Piapoco, 6. Baniva, 7. Kurripaco, 8. Bare, 9. Yanomami, 10. Yekuana, 11. Sanema, 12. Niamn, 13. Pemon, 14. Kapon, 15. Kariña.

Despite severe environmental disasters, the loss of wildlife and threats to public health, uncontrolled mineral extraction continues and has recently shown even more worrisome figures (USEPA, 1984, 1989; Nico and Taphorn, 1994; Veiga et al., 1995; Veiga, 1997; Machado-Allison et al., 2000; Machado-Allison, 2005; Farina et al., 2009; Trujillo et al., 2010; Milano, 2014; SPDA, 2014). In general, "artisanal" gold extraction is characterized by:

- The use of elemental metallic Hg for gold separation from sediments and amalgamation. Mercury is later evaporated by heating and released into the atmosphere.
- Approximately 45% of this Hg enters directly into the water column where microorganisms convert it into highly toxic methyl-mercury (MeHg). Sediments are then dragged by white water currents, favoring its dispersion.
- The other 55% of evaporated Hg remains in the atmosphere in the form of ethyl-mercury, being latent up to 24 months in dry zones, but rapidly dropping into the soil in regions with high rainfall (Veiga, 1997).
- MeHg is quickly incorporated into the food chain (Figure 2), accumulating mostly in top predators such as: fish, aquatic reptiles, birds and mammals (Rosas and Lehti, 1996; Gutleb et al., 1997). This causes serious effects on diversity as predators may die off and is a high risk to people that ingest this contaminated food (e.g., fish and reptiles) (USEPA, 1984, 1989; Fréry et al., 2001; Porto et al., 2005; Castilhos et al., 2006; Trujillo et al., 2010.

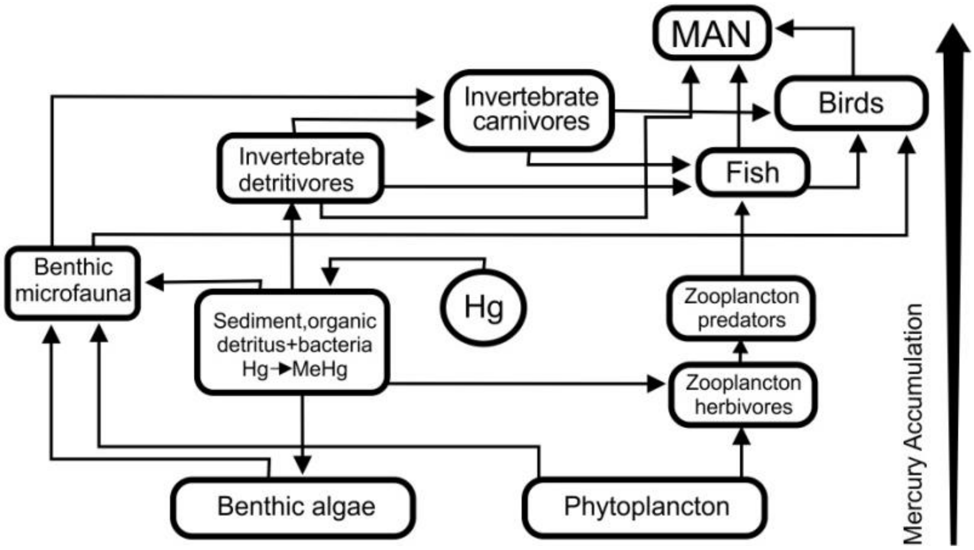
Table 1. Historical reports and confused data in studies performed in the Caroni (1) and Cuyuni (2) River Basins (see Figure 1)

Source	Area	Conclusion
Norodin and Falcon (1989)	1	The river system is fragile; great impact due to removal of soil and large amount of sediments in water resulting in change of transparency; removal of vegetation; mining activities must be regulated.
PLEXUS (1990)	1	69% of miners and 37% of the nearby population were found contaminated with Hg.
Bermúdez (1993)	1	Levels of Hg in water below 1.0 µg/L; main river channel with low productivity; material removed from the bottom with low environmental impact; carnivore fish with less than 0.5 µg/kg of Hg.
CVG (1995)	1	Sediments samples showed a variation from 0.0052 to 7.02 µg/g.
		Lower Caroni River with 7.01 Hg/g (source of potable domestic water system). Yuruari River with 0.0133 Hg/g.
		Water values below 2 µg/L; sediments above 20 µg/L; fish contaminated but values below 500 µg/kg; Hg was 45 to 80 times higher compared to a non-contaminated river.
		Water with <1 µg Hg/L; sediments and suspended soils (>0.5 µg Hg/g); fish contaminated but below 0.5 µg Hg/g except in those coming from Amarillo Creek.
		Beaches close to mining camps with 36 µg/g of Hg; rivers with 350 times higher than the background natural concentration; fish with Hg but not reaching dangerous limits.
CVG (1995)	2	Water and sediment samples with high mercury concentrations of 13.1 µg/L and 42.2 µg/g, respectively.
	2	High Hg in sediments downstream from mining site. Hg in fish above 0.5 µg/g.
Machado-Allison et al. (2000)	2	High sedimentations and forest destruction in Amarillo Creek and Cuyuni River; elimination of fish, macroinvertebrates and water plants in both rivers; modification of river bottom and changes in water quality; high impact on river banks and main channel over several kilometers.

DAMAGES TO ENVIRONMENT AND BIODIVERSITY

Machado-Allison et al. (2000) produced one of the first reports showing the environmental and biodiversity deterioration produced in the Amarillo Creek part of the Cuyuní river basin in Venezuela, which is the main tributary of the Esequibo River in Guyana. This study revealed how the devastation of the Amarillo Creek (near Km 88/Las Claritas, Bolivar State) was affecting fish diversity both up and downstream from its discharge into the Cuyuní River (Table 2 and Figure 3).

Furthermore, Nico and Taphorn (1994), Farina et al. (2009), Trujillo et al. (2010), and Milano (2014) showed Hg present in fishes captured in the Orinoco River Basin and made a public statement of it to be considered a problem of national concern. Table 3 depicts the summary on the data obtained from these studies.



Modified from: Machado-Allison, 2015.

Figure 2. Incorporation and accumulation of MeHg in the food chain.

Table 2. Ecological parameters taken from the Cuyuní River

Parameters	Field Stations											
	Downstream (1-6)						Upstream (7-12)					
	1	2	3	4	5	6	7	8	9	10	11	12*
Temperature (°C)	24.0	24.0	24.0	24.0	26.5	26.5	26.5	26.5	26.5	26.5	25.0	26.5
Transparency (cm)	50	50	10	10	20	10	0	10	5	20	>50	0
pH	5-6	5-6	6-7	6-7	5-6	5-6	7	5-6	7	5-6	5-6	>7
Aquatic vegetation (%)	100	100	25	25	25	0	0	0	0	25	25	0
Terrestrial vegetation (%)	100	100	50	50	50	25	25	50	25	50	75	0
N° species	87	30	9	15	42	17	14	21	16	9	51	0
N° specimens	1762	180	103	175	348	64	74	165	58	187	531	0
Diversity (D)	40.0	15.4	2.7	3.6	18.8	8.6	7.7	10.5	11.1	7.2	5.1	0
Equitativity (E’)	0.79	0.80	0.53	0.39	0.77	0.78	0.84	0.79	0.90	0.67	0.79	0

Source: Machado et al., 2000.
[*] Amarillo Creek.

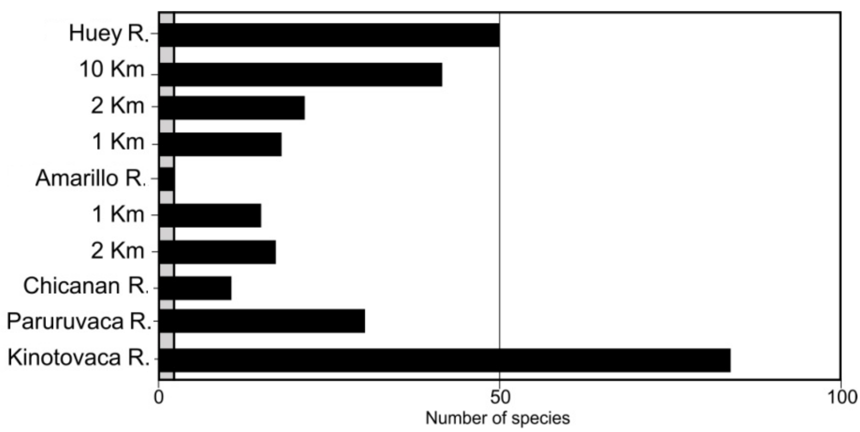
HUMAN HEALTH

For the last 25 years, contamination by Hg has become an increasingly worrisome public health problem. A significant number of studies have revealed high levels of Hg in blood samples of people living in these mining zones (Romero et al., 1987; Herrero et al., 2004; Silva et al., 2004; Veiga et al., 2004; Alvarez and Rojas, 2006; Carrasquero-Duran, 2006; Milano, 2014). These authors found that Hg concentrations in samples coming from miners working in “El Callao” are the highest in the world (>2.7 µg/g). It is not surprising that there has been a steady increase in the incidence of severe neurological degenerative disease among miners and their families.

Table 3. Mercury level in carnivorous fish tissue from the Cuyuní River

Species	Hg_{total} (µg/kg)	MeHg (µg kg)	MeHg (µg/100 g)	HQ*
Plagioscion squamosissimus	4105.7	3900.5	390.1	55.7
Electrophorus electricus	3935.8	3739.0	373.9	53.4
Cynodon septenarius	2352.1	2234.4	223.4	31.9
Cynopotamus essequibensis	2158.4	2050.5	205.0	29.3
Pimelodus ornatus	1769.3	1680.8	168.1	24.0
Ageneiosus inermis	1618.8	1537.9	153.8	22.0
Acestrorhynchus microlepis	1456.2	1383.4	138.3	19.8
Serrasalmus rhombeus	968.6	920.2	92.0	13.1
Crenicichla lenticulata	785.3	746.1	74.6	10.7
Crenicichla johanna	527.5	501.1	50.1	7.2
Hoplias macrophthalmus	493.0	468.3	46.8	6.7
Synbranchus marmoratus	221.1	210.0	21.0	3.0
Sternopygus macrurus	203.3	193.1	19.3	2.8
Acestrorhynchus falcatus	184.2	175.0	17.5	2.5

* HQ: Hazard Quotient (USEPA 1989).
Modified from Farina et al., 2009.



Source: Machado-Allison et al., 2000.

Figure 3. Relative number of fish species (up and downstream) from Amarillo Creek, Cuyuní River.

Hair and urine samples obtained from indigenous communities of Brazil and Venezuela living along some of these rivers have also shown toxic concentrations of mercury. Their levels tend to be higher than those obtained from miners coming from the same locality, especially if fish is their main source of protein (Rojas, 2010). Fetuses and children of all ages are particularly susceptible to Hg, presenting birth defects and impaired neurological development (WHO, 1990). Among children from native communities we have found values from 0.07 to 2.7 µg/g (normal is <0.01 µg/g). Close to 92% of women in reproductive age examined along the Caura River (Sanema and Yek'uana tribes) had levels higher than 0.5 µg/g, that are considered a high-risk exposure. Importantly, some of these effects can be detected as far as 200 km from the source of pollution. Disturbances in fertility and child development can cause a significant impact on demographic growth for these populations, completely disrupting social structures and even existence for some of these tribes.

In parallel, Milano (2014) has made several observations of the mining communities of Las Claritas (1996), Santo Domingo (1996), El Manteco (2002), El Callao (2005), and the Low Caroní River (2007). They found that 68% of children and 87% of women showed mercurial exposure with higher concentrations than those considered safe by the WHO. Miners, on the other hand, had highly variable exposure with 25 to 84% having abnormal values. However, these percentages went up to 90% if miners were directly involved in the gold refining process. Their studies also confirmed the correlation between the incidence of different neurological diseases with the degree of exposure and toxicity.

LEGAL AND INSTITUTIONAL ASPECTS

The legal framework related to mineral extraction in Venezuela is quite extensive and is even mentioned in several articles of the national Constitution (1999). These include a series of national laws, executive decrees and regulations that protect the environment and human indigenous rights, and regulate the rational exploitation of mineral resources. The most important laws are: Environmental Penal (Criminal) Law, Criminal Code, Water Law, Indigenous Population Rights Law and different Mining Laws.

In Milano (2014) we can find a recent chronology with regards to different gold mining regulatory agencies and institutions that until 1999 were under the central control of the Ministry of Energy and Mines and the Environmental and Natural Resources Ministry. These include:

- 1999, Decree 3091: allowing the mining metal giant Guayana Venezuelan Corporation (CVG) to provide permits for gold exploration and exploitation, bypassing those that were originally given by the Environmental and Natural Resources Ministry (MARNR).
- 2001, Decree 1234: Official Gazette 37155; The National Institute of Geology and Mining is created and is responsible for research and scientific expertise in: geology, mineral resources, geo-physics, geo-chemistry, and geo-technology.
- 2005, creation of the Popular Ministry for Basic Industries and Mining (MPPIBAM) that incorporates the CVG and takes over policy-making and control of the mining sectors.
- 2011, Decree 8683: declaration of all gold mines within Venezuela by the national government as "Public Domain," reserving its exclusive right for their exploitation. This operational task is now assigned to the Venezuelan Petroleum Company (PDVSA) replacing the MPPIBAM.
- 2013, Mining Venezuela Corporation (CVM) is created as part of PDVSA-Industrial, to control, organize and develop gold mining.

However, Milano (2014) states that *"...nevertheless all manifestations of 'the state' done through codes, laws, decrees and other normative (regulation) with goals to avoid, contain and minimize the negative impacts of mining activity have achieved inefficient results.*"

Table 4. Summary of proposed actions by Red Ara (2013)

Area	Actions
Public Health	- Develop programs to allow fast determination of contamination or intoxication. - Guarantee permanent medical attention. - Establish a permanent educative program on environmental and human health risks due to mercury contamination - Establish programs allowing capacity building in all sectors related to public health and environmental management - Establish actions that guarantee production and intake of uncontaminated food in human rural indigenous settlements - Develop epidemiological evaluations.
Social and Development Management	- Promote consulting processes and participation in decision-making actions with all the actors - Promote permanent programs of social action and economic support for a better quality of life. Close surveillance of human rights. - Establish dialog processes to show development alternatives. - Establish effective measures to control illegal activities.
Environment	- Train miners in a better use of techniques of low environmental impact. - Enforce Venezuelan environmental legislation. - Establish continuous monitoring programs of the mercury levels in the environment. - Establish continuous programs for evaluating contamination, degradation and mitigation of soils. - Promote research programs directed to establish new techniques friendly with the environment. -Integrate international/cooperative programs with neighboring countries. - Promote programs of adequate mercury disposal, bioremediation and restoration of degraded areas including rivers. - Promote interchange of scientific information and knowledge.

DISCUSSION AND RECOMMENDATIONS

In order to achieve a reasonable balance between economic development and nature, it is necessary to understand how environments and ecosystems behave, from the biotic, abiotic and social points of view. It is also important to determine their significance in terms of natural and economical resources and prioritize their real benefits for both local and broader human communities. Every effort must be made to maintain aquatic ecosystems as a renewable and sustainable resource. It is naive to think that future economic development in tropical countries will spare casualties and avoid damage to the aquatic ecosystems without the loss of aquatic floral and faunal species. However, what we need is to encourage the use of modern techniques avoiding the unnecessary extension and irreversibility of environmental impacts and allowing the recovery of an ecosystem once mineral extraction is finished.

All countries have the moral obligation to be responsible and preserve their aquatic resources. The maintenance of high quality, pollutant free water may be critical for the survival of human settlements downstream. Even though rivers flow in only one direction, an environmental impact can spread in all directions.

As previously mentioned, the environmental contamination by mercury is a dynamic process that affects the entire ecosystem and has accumulating effects in upper echelons of the food chain. Many of the top predators such as the great catfishes of the genera *Brachyplatystoma* and *Pseudoplatystoma* (that accumulate Hg) happen to be migratory fish. It is thus not surprising that mercurial exposure can be detected far away from its source of origin due to the consumption of these “prize catches.”

Unfortunately, despite multiple and well-documented evidence of environmental deterioration, hazardous exposure to local population and serious threats to public health, the policy-makers and regulatory agencies have done very little to reduce the use of mercury for gold processing. On the contrary, data indicates that its use is at a historical high (Veiga et al., 2004).

We subscribe the very important efforts from different NGOs such as the Red ARA (Red Organizaciones Ambientales No Gubernamentales de Venezuela – Network of Environmental NGOs of Venezuela) that continue to bring these issues to the forefront of discussion by organizing meetings, workshops and connecting with the mass media. One of these workshops, “Mercury Contamination in the Venezuelan Guiana Shield: A Proposal and Dialog for Action” (Red Ara, 2013) calls for important action plans that could be implemented (Table 4).

CONCLUSION

Recognizing the importance of this environmental problem, several international organizations such as the United Nations-Development Program and United Nations-Development Bank, the World Wildlife Fund (WWF) and members for the Convention on Biological Diversity met in May 2014 to engage in a conversation of all nations within the Guiana Shield. The “Guiana Shield Action Plan to Facilitate Biodiversity, Corridors, Achieve Aichi Targets” calls for governments (Brazil, Colombia, Guyana, Suriname, French Guayana and Venezuela) and partner organizations to:

1. Support national efforts to better manage and monitor small and medium-scale gold mining, including best practices for biodiversity conservation and water resources management.
2. Encourage the further development of trans-boundary protected areas in the Guiana Shield.
3. Create synergies with existing global and regional platforms in order to take advantage of efficiencies of scales, efforts and momentum.
4. Develop and strengthen linkages with collaborative connectivity projects.
5. Organize a technical database that allows identifying and prioritizing trans-boundary corridors

6. Strengthen and facilitate academic research (university research cooperation and other research institutions) into a more cooperative science.

Gold mining and the “sustainable development” of other resources within ecosystems of the Guiana Shield should not be considered a utopian dream. However, it does require a conscious and collective effort to understand and maintain their delicate balance. The real wealth present in these forests and rivers is not the value of the metals and diamonds that are found once in a lifetime, but in the biodiversity present in them that could represent the next revolutionary food, drug and energy source that could save mankind in this century and beyond.

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Chapter 23

ENVIRONMENTAL RISK OF LEAD ACCUMULATION IN CROPS IRRIGATED WITH WATER FROM THE MANTARO RIVER, JAUJA SECTOR, PERU

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ABSTRACT

Lead is an environmental pollutant highly toxic to human health. This chapter deals with the hazard index and environmental risk of lead accumulation in soil and agricultural products irrigated with water from the Mantaro River on the left bank of the Mantaro Valley in Jauja, the Junín region, Peru. The methodology applied was that recommended by the Environmental Protection Agency (EPA) and the Environmental Ministry of Peru (MINAM). Soil, water, corn and potato samples were analyzed and 35 people who are frequently exposed to lead contamination were interviewed including males and females from 4 to 83 years old. The results indicate that lead concentrations in corn and potato exceeded the maximum allowed levels by between 5 and 30 times. The hazard index between 0.1163 and 0.1186 corresponds to a low risk to the health of the population exposed. The environmental risk level for agricultural areas irrigated by the Mantaro River was high (0.67). Overall, the probability of damage to humans and the environment and at a socio-economic level due to contaminated food intake and lead accumulation in agricultural soils and groundwater was very high.

Keywords: environmental risk, hazard index, lead accumulation, Mantaro River, Peru

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INTRODUCTION

High levels of heavy metals in irrigation water are a problem for agriculture, biodiversity and human health. Heavy metals and metalloids in irrigation water are dangerous due to their non-biodegradable nature. Consequently, they can accumulate in agricultural soils and exert toxicity on different crops (Garcia and Dorronsoro, 2005).

The water resource in the Mantaro Valley of the Junín Region, Peru, is becoming scarce, and there are 25 important districts in the region where the irrigation of agricultural soils depends on the Mantaro River. In the study area (Jauja sector, province of Jauja, Junín region), farmers have been irrigating their agricultural crops (corn, potatoes, beans, carrots, artichokes) and pasture land with water from the Mantaro River for more than 70 years, with high contents of heavy metals (Cr, Cu, Fe, Hg, Pb, Cd, Ni, Zn) and metalloids (As). These have originated from the different mining operations in the region and the La Oroya metallurgical plant (Proyecto Mantaro Revive, 2008). As a result, the soils present high concentrations of these elements, which cause a deterioration of the quality of the soil and aquifers (Porta et al., 2003). There are more than 17,060 irrigation users that irrigate 7,754 acres of soil (36% of the total surface irrigated; Ministerio de Agricultura, 2011a) with contaminated water. In 2005, research conducted by Saint Louis University in Missouri and the Archdiocese of Huancayo determined that in the provinces of Oroya and Concepción (near the province of Jauja), about 25% of the child population of Concepción from zero to six years old exceeded the 10 µg/dl limit of lead in the blood (Saint Louis Missouri University, 2005).

Lead is a high-density heavy metal (Manahan, 2013) that persists in the soil for from 1,000 to 3,000 years (Duran, 2010), is assimilated by organisms, is transferred through the food chain and represents a threat to human health and ecosystems (Calderon and Maldonado, 2008). The adsorption of these toxic substances by soil particles (Duffus, 1983) affects the soil quality due to their high persistence and difficult degradation (Porta et al., 2003; Duran, 2010). According to the Agency for Toxic Substances and Disease Registry (ATSDR), the World Health Organization (WHO), the Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC), lead is considered a dangerous and highly toxic substance, and they have determined that it is probably carcinogenic for humans. Lead exposure causes neurological, renal, endocrinal, gastrointestinal, cardiovascular, reproductive and developmental damage, which in some cases, is irreversible (ATSDR, 2007). The portion of lead that is not excreted is distributed via the blood to soft and mineral tissues (bones and teeth): in adults, 95% of the body burden is stored in bones (Saldívar Osorio et al., 1997; World Health Organization, 2000) with a half-life of 10 to 20 years (ATSDR, 2007).

Evans et al., (2003) and Zuck and Ize (2010) define risk as the probability that negative consequences (physical, chemical, biological and cultural) may occur due to exposure to a hazard. The probability of a risk is expressed in values between 0 and 1: the closer the value is to 1 the higher the certainty that there is a risk (Tyler Miller, 2002). Although the probability theory plays an important role in making decisions, future events cannot be predicted with absolute certainty (Beck, 1998).

The exposure to heavy metals by inhalation, dermal contact or ingestion is associated with different effects on the population's health. The risk assessment methodology constitutes a useful tool in the decision making related to public health (Hernández, 2012).

The present chapter presents an estimation of the hazard index and environmental risk due to the accumulation of lead in the soil and agricultural products irrigated with water contaminated by flooding upstream of the Mantaro River water basin.

METHODS

Study Area

The study area of approximately 13.50 km^2 is located on the left bank of the Mantaro River, province of Jauja, Department of Junín, located between 3370 and 3350 m.a.s.l., 40 km from the city of Huancayo. The irrigation system starts in "Siclachaca," 250 m north of Stuart Bridge at the entrance of the Mantaro valley with a river flow of 400 m^3/s in the rainy season and of 100 m^3/s in times of low water (Ministerio de Agricultura, 2011a).

The water samples were collected in August (2013) at six points in each one of the secondary channels of the irrigation main channel: Sausa, Ataura, Huamalí, The Mantaro, San Lorenzo and Apata. Each sample was placed in 1 L plastic bottles with nitric acid and sent in a thermal box (cooler) (Ministerio de Agricultura, 2011b) to the laboratory of soil, plant, water and fertilizer analysis of the Universidad Nacional Agraria La Molina.

Twelve soil samples, six from corn crops and six from potato crops, were taken from the six sampling sites in September (2013). Six to nine 1 kg soil sub-samples were collected from a depth of 0-30 cm using an auger and according to the square grid sampling technique. The sub-samples were then pooled and homogenized to form a composite sample (MINAM, 2014). In the laboratory, each composite soil sample was dried, sifted and stored in 500 g polyethylene bags until further analysis.

Six corn (variety "cuzqueado") samples were collected in December (2013). Each corn sample was composed of 16 corncobs taken at random from each plot; they were peeled and 500 g of corn grains were dehydrated in a drying oven in the laboratory and placed in plastic containers. Six potato (variety "yungay") samples were selected in March (2014). Each sample was randomly composed of six units, washed and dried, then separated into 500 g of fresh potato and placed in plastic containers. Subsequently the potato samples were dehydrated in a drying oven in the laboratory. The collection of soil samples, corn and potatoes were performed by farmers who agreed to the collection at the six sampling sites. Soil and agricultural crop samples originated from the same selected plot.

Irrigation users from 115 families were interviewed in the six towns to know about their irrigation practices, the frequency of corn and potato intake, economic activity and income. The population at risk was represented by children, adults and some older adults (35 people in total) that consumed corn and potato on a daily basis.

Risk Assessment Methodology

The methodology used to estimate the hazard index on the population's health was based on the US Environmental Protection Agency (EPA, 1998), and the environmental risk assessment procedure that was established by the Environmental Ministry of Peru according to the Spanish standard UNE 150008:2008 (MINAM, 2010; 2011).

The hazard index for the population's health was studied in four stages:

i. Hazard Identification

Lead concentrations in the irrigation water, soil and agricultural products (corn and potato) were determined via the atomic absorption spectrophotometer according to 3111B: direct method of air flame – acetylene (APHA, AWWA, WPCF, 1992).

The lead reference levels in water and soil were those established by the Environmental Quality Standards (EQS) of the Environment Ministry of Peru: EQS of 0.05 mg/L for vegetable irrigation and animal drinking water and of 70 mg/kg for agricultural soil (MINAM, 2008; 2013). The permissible levels of lead in agricultural products were those recommended by the Codex Alimentarius Commission: 0.10 mg/kg for potato and 0.20 mg/kg for corn (Codex Alimentarius Commission, 2013).

ii. Exposure Assessment via the Average Daily Dose (ADD)

The *ADDs* were calculated for each agricultural product and individually for each person studied, using the following expression:

$$ADD\ (\text{mg/kg/day}) = \frac{C_i * I_i}{W_i}$$

where C_i is the lead concentration in the food (mg/kg), I_i the daily food (corn or potato) intake (kg/day), and W_i the human body weight (kg).

Corn and potato *ADD* were compared between age groups and genders via analysis of variance with a significance level of p=0.05. The means comparison of corn and potato *ADD* according to gender and age groups were performed using Tukey's test. Data processing was performed using the statistical software R (Kuhnert and Venables, 2005; De Mendiburu, 2009).

iii. Risk Characterization via the Hazard Index (HI) or Endangerment Coefficient (HQ)

The *HI* was calculated from *ADD* data for each location using the following expression:

$$HI = \frac{ADD\ (\text{mg/kg/day})}{RfD\ (\text{mg/kg(day)})}$$

where *RfD* is the theoretical reference dose.

If the *HI* is greater than 1, then the metal *ADD* exceeds the *RfD*, which indicates that there is a potential risk associated with the metal. The *HI* is a conservative index and is related to small responses of the organism when exposed to a metal (Teuschler et al., 1999). The probabilistic hazard index (*HIp*) was calculated with the data of probabilistic *ADD*

(*ADDp*) by applying 10000 Monte-Carlo simulations (Rodríguez, 2011) to the lead concentration data using the statistical software R (Kuhnert and Venables, 2005; De Mendiburu, 2009).

iv. Risk Assessment

The following 4-step procedure was adopted (MINAM, 2011):

a) Hazard Identification

The risk factors derived from the lead accumulation in agricultural soils were identified. Additionally, the effects on the human and natural environments, as well as on the socio-economic conditions were considered:

- Natural environment indicators (water, soil and crops).
- Human environment indicators (population health, affected population).
- Socio-economic indicators (economic activities such as income and crop yield)

b) Occurrence Probability Estimation

The probability of the occurrence of risks that were threatening the health of the population were estimated as well as the quality of the environment and the socio-economic environment as a result of exposure to lead. The intake frequency of corn and potato, the irrigation frequency of crops, the frequency of low crop yields and low prices were identified. Values of probability of occurrence for the three environments were assigned as those established by the MINAM (2011): very probable (5), highly probable (4), probable (3), slightly probable (2) and very slightly probable (1).

c) Gravity Estimation

This consisted in estimating the damage that the risk scenarios may cause. The gravity was applied to the values suggested by the MINAM (2011) with the following expression:

- Human environment gravity=amount + 2 dangerousness + extension + affected population
- Natural environment gravity=amount + 2 dangerousness + extension + environmental quality
- Socio-economic environment gravity=amount + 2 dangerousness + extension + productive capital

The "amount" refers to the quantity of pollutant (lead) in the risk scenario; "dangerousness" refers to the lead toxicity level; "extension" refers to the area affected by the pollutant; "affected population" refers to the number of people affected by the contaminant; "environment quality" refers to the damage that the pollutant causes to natural resources; and "productive capital" is related to the soil's productivity level. The gravity assessment assigned to each one of the aforementioned components is from 1 to 5. The resulting gravity estimation levels (and values) are: critical (5), severe (4), moderate (3), mild (2) and not relevant (1).

d) Environmental Risk Assessment

The environmental risk was determined by multiplying the given value of the occurrence probability by the gravity value.

RESULTS

Lead Concentrations at Sampling Sites

The concentration of lead in water from the Mantaro River exceeded the Environmental Quality Standard for crop irrigation water and animal drinking water in the six sampling sites. The agricultural soils of Huamali and Apata had lead concentrations above the national reference value, while the lead content in corn and potato samples exceeded permissible levels recommended by the Codex Alimentarius Commission of the Food and Agriculture Organization of the United Nations and World Health Organization at all six sampling sites (Table 1).

Assessment of Lead Exposure per Inhabitant

There were significant differences in the food intake between males and females regarding corn ($F=6.19$, $p=0.018$) and potato ($F=9.33$, $p=0.005$), with males consuming more than females (Figure 1). Significant differences in food consumption according to age groups for corn ($F=28.19$, $p=0.000$) and potato ($F=35.42$, $p=0.000$) were also observed. The Tukey test indicated that seniors, aged above 50 years old, registered a higher consumption of corn (150.0 g/day in females and 236 g/day in males) and potato (200 g/day in females and 290 g/day in males). The corn and potato intake in children was lower than adults (56 g/day of corn in girls and 56 g/day in boys and 80 g/day of potato in girls and 78 g/day in boys).

Table 1. Lead concentration in water, soil, corn and potato irrigated with water from the Mantaro River

Sampling site	Lead concentration			
	Irrigation water (mg/L)	Agricultural soil (mg/kg)	Corn (mg/kg)	Potato (mg/kg)
Sausa	0.117	46.41	0.80	0.50
Ataura	0.115	55.50	1.00	0.50
Huamalí	0.115	78.94	1.85	2.50
El Mantaro	0.114	67.88	5.00	1.60
San Lorenzo	0.106	42.06	3.95	2.85
Apata	0.082	73.13	4.35	1.85
Reference level	0.050[a]	70.00[b]	0.20[c]	0.10[c]

[a]Environmental Quality Standards (EQS) for crop irrigation water and animal drinking water (D. S. N° 002-2008-MINAM, Peruvian standard, 2008); [b]Environmental Quality Standards (EQS) for agricultural soil (D. S. N° 002-2013-MINAM, Peruvian standard 2013); [c]Permissible maximum level of lead for human consumption (Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO): Codex Alimentarius, CODEX STAN 193-1995, 2013).

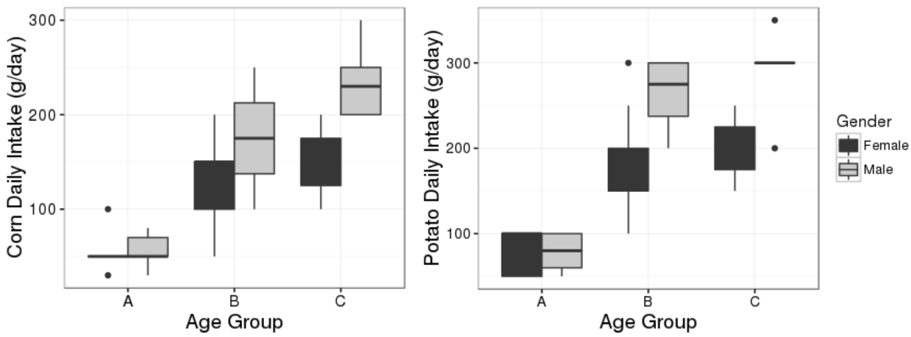

Figure 1. Daily corn and potato intake according to the age and gender of inhabitants of all six sampling sites (A: <13 years old; B: from 13 to 50 years old; C: >50 years old). Black dots indicate outliers of daily corn and potato intake in females and males.

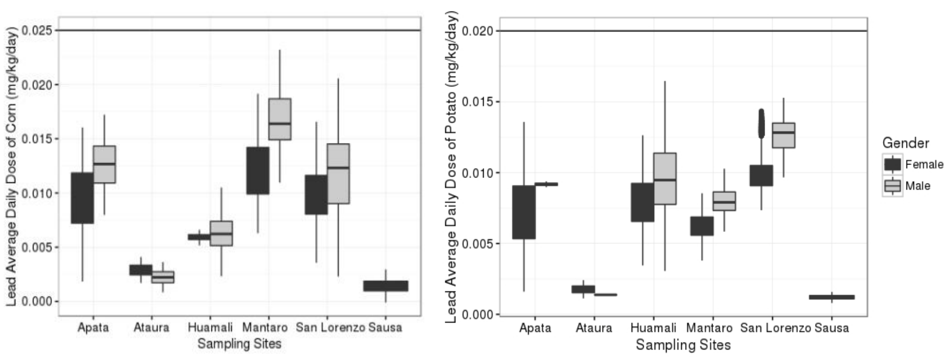

Figure 2. Average Daily Dose of lead (*ADD*; mg/kg/day) in female and male inhabitants consuming corn and potatoes from the six sampling sites irrigated with water from the Mantaro River. Black horizontal lines indicate 1/8 maximum allowed level for corn (0.2 mg/kg) and 1/5 maximum allowed level for potato (0.1 mg/kg) (CODEX STAN 193-1995, 2013). Outliers of *ADD* of potato in female of San Lorenzo are indicated with a thick dark vertical bar.

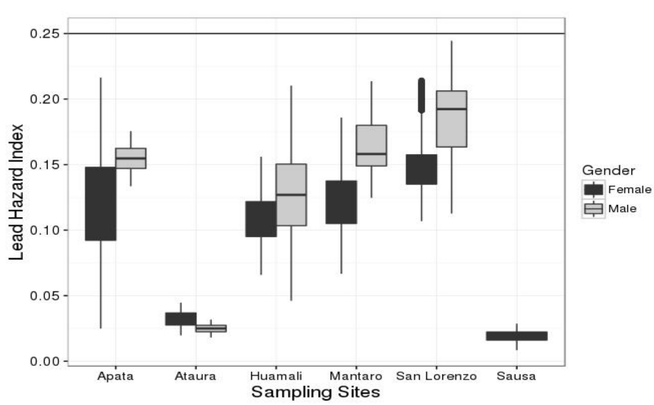

Figure 3. Lead Hazard Index (HI) in females and males from the six sampling sites regarding the daily consumption of corn and potato irrigated with water from the Mantaro River (1/4 HI (1)). Outliers of HI in females of San Lorenzo are indicated with a thick dark vertical bar.

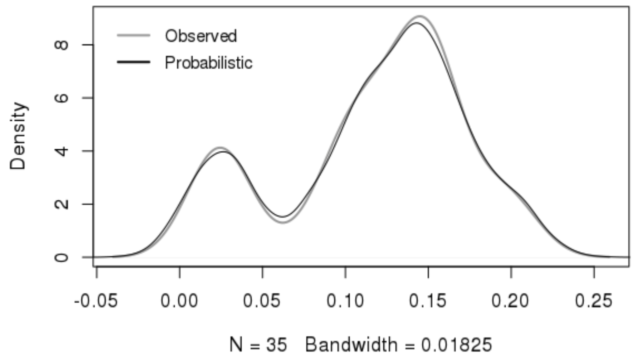

Figure 4. Observed and probabilistic data for the General Hazard Index (*HI*) for lead.

Average Daily Dose of Lead from Diet

The corn and potato *ADD* were lower than the maximum recommended level (0.1 mg/kg/day for potato and 0.2 mg/kg/day for corn; Figure 2). There were no significant differences in *ADD* between males and females regarding corn (F=3.002, p=0.093) and potato (F=2,973, p=0.095). The age groups showed no significant differences in *ADD* for corn (F=1,725, p=0.195) and potato (F=0.200, p=0.820).

Human Risk Characterization

The hazard index (*HI*) was below one in the males and females studied form all the sampling sites (Figure 3). Males presented higher deterministic and probabilistic *HI* values than females. The Huamali, El Mantaro, San Lorenzo and Apata sampling sites presented the highest *HI* values, but still below the maximum allowed level.

The *HI* probabilistic values were lower than one (the maximum reference level) and the result of 40% of the *HI* probabilistic data was between 0.1291 and 0.1642 (Figure 4).

Environmental Risk Assessment

The daily intake of corn and potato determined a risk occurrence probability of highly probable (4) in the human environment. The crop irrigation frequency and lead concentration in the soil and aquifers determined an occurrence probability for the natural environment of highly probable (4). The decrease in crop yield and the low prices of the agricultural products determined an occurrence probability of highly probable (4) in the socio-economic environment (Table 2).

The gravity of the damage identified in the study area was critical (5) in the human environment and severe (4) in the natural and socio-economic environment. This outcome was influenced by the high lead concentration in corn and potatoes i.e., lead toxicity, in more than 1000 acres of corn crops and 350 acres of potato crops. 750 farmers were affected and more than two natural resources (water, soil, crops) were affected as well as some moderately productive soils (Table 2).

DISCUSSION

Hazard Index

Water from the Mantaro River has been used to irrigate agricultural fields in the Sausa, Ataura, Huamali, Mantaro, San Lorenzo and Apata localities of the Jauja sector, every 15 or 20 days for more than 70 years, representing a potential hazard to population health and the natural ecosystems (Chávez et al., 2011). Agriculture is the main economic activity of the population studied. Irrigation water presented high levels of lead concentrations (considered a highly dangerous substance; Díaz-Barriga, 1999; ATSDR, 2007), among other heavy metals, because of the mining and metallurgical activity in the upper and middle basin of the Mantaro river (Proyecto Mantaro Revive, 2008).

The concentrations of lead in the irrigation water exceeded what is acceptable, according to water quality standards, at the six sampling sites, in agreement with data previously reported by Goetendia and Ruiz (1989), Tello (1996), Proyecto Mantaro Revive (2008) and Turco (2009).

The Huamali and Apata sites presented values for lead accumulation values in the agricultural soil above the maximum recommended level, constituting a hazard for human health and the environment (Porta et al., 2003).

Table 2. Probability, gravity and environmental risk to human, natural and socio-economic environments, in different lead (Pb) contamination scenarios

Scenario	Probability	Gravity	Environmental risk (%)
Human environment			
Irrigation of agricultural areas with heavy metals (Pb in corn and potatoes)	4	5	80
Irrigation of agricultural areas with heavy metals (Pb in soil and water)	4	4	64
Human environment average risk (%)			**72**
Natural environment			
Irrigation of agricultural areas with heavy metals (Pb in soil)	4	4	64
Irrigation of agricultural areas with heavy metals (Pb in groundwater)	4	4	64
Natural environment average risk (%)			**64**
Socio-economic environment			
Low income	4	4	64
Low crop yield	4	4	64
Socio-economic environment average risk (%)			**64**
Average risk characterization (%)			**67**

Contaminants may remain for a long time in soil. In the long run, this permanence is serious in the case of inorganic contaminants such as lead, which cannot be degraded (ATSDR, 2007; Calderon and Maldonado, 2008). In temperate soils lead remains present for 1,000 to 3,000 years depending on humidity and temperature (Duran, 2010). In general heavy metals in soil may experience different outcomes: be retained in the soil, be absorbed by plants and incorporated into trophic chains, or enter the atmosphere by volatilization and move to surface water or groundwater (García and Dorronsoro, 2005).

The lead concentration found in corn and potatoes exceeded the permitted maximum levels (as recommended by the Codex Alimentarius of the World Health Organization and Food and Agricultural Organization (2013) by between 5 to 30 times. The long-term persistence of lead in the soil contributes to the progressive accumulation and transfer to the food chain, thus constituting a danger to human health and natural ecosystems (ATSDR, 2007) at the six sampling sites studied.

More than 30% of the population interviewed was exposed to lead through corn and potato consumption. Males consume more than females and individuals above 13 years of age recorded a higher intake compared to the group aged below 13.

The lead *ADD* in the population studied is influenced by the variation of lead concentration in the agricultural products, the ingestion rate and the body weight of the individuals. The deterministic and probabilistic *ADD* was lower than the maximum allowed level in male and female inhabitants in the three age groups in the six towns. Males showed higher values of *ADD* (Figure 2). Furthermore, these values are below the *RfD*, these results match the assessment of the health risk constituted by consuming drinking water contaminated with heavy metals performed by Hernández (2012).

The *HI* for lead intake calculated deterministically and probabilistically in males and females is less than one, which represents a minimal risk to the health of the population exposed to it, in comparison to the *RfD*, in six locations of Jauja (Figure 3), with a range that varies between 0.1163 and 0.1186 of probabilistic *HI*. The probabilistic maximum values of lead *HI* distribution are, in general, higher than the deterministically calculated *HI*, where the maximum value of 0.2553 (in males) represents a low level of risk to human health due to lead exposure. The observed and simulated hazard indexes were higher in males and females from Huamali, Mantaro, San Lorenzo and Apata probably due to the higher lead concentration in corn and potatoes, although they were below the theoretical reference levels. These results coincide with those reported by Hernández (2012) who found a hazard ratio of below one concerning the consumption of drinking water, which is considered a minimal or not dangerous risk.

The Environmental Protection Agency (EPA, 2001) points out that some effects on human health, such as alterations to enzyme levels in the blood and neurological development deficiencies in children can occur at blood lead levels as low as those established only as threshold levels in studies concerning exposure to lead. The EPA also points out that current knowledge about lead pharmacokinetics indicates that the risk values obtained by standard procedures cannot indicate the potential risk, because of the difficulty which exists in accounting for the pre-existing levels of lead in the body: the metal bioaccumulates in the bones with a half-life of 10 to 20 years (Saldívar Osorio et al., 1997; World Health Organization, 2000) and is influenced by other variables such as age, health, nutritional status and any pre-existing levels of lead in the mother during gestation and lactation. It is therefore generally considered that the current information on theoretical thresholds is inadequate to decide the reference values for lead.

Environmental Risk

According to the risk assessment scale proposed by the MINAM (2011) the risk level estimated for the human environment is 72% (Table 2), which is characterized as high. This is explained by the high lead concentrations in foods such as corn and potatoes that exceed the maximum allowed levels suggested by Codex Alimentarius Commission (2013) by 5 to 30 times. Lead is immobilized in bones and does not contribute to the immediate toxicity, but it is a potential hazard (Saldívar Osorio et al., 1997; Rojas, 2002). Recent scientific research has discovered that damage caused by lead occurs at concentrations much lower than it was previously thought. Another form of exposure to lead for farmers in the six towns of Jauja is the dermal contact with the contaminant in the soil and irrigation water during agricultural activities such as: irrigation, planting, weeding and harvesting (potato). In general, heavy metals are considered very dangerous because of their high toxicity, and high tendency to be bioaccumulated (ATSDR, 2007).

The risk level for the natural environment is 64%, which is characterized as high; this is attributed to the lead concentrations in the soil and aquifers that affect their quality. The availability of organic matter containing nitrogen, phosphorus and potassium is low in the soils and there are also slight problems of salinity further, the pH varies from slightly acidic to mildly alkaline, the texture of the soils are sandy loam and clay loam. Soils with a high organic matter content lack elements such as Pb, Cu and Zn. Although the sandy nature of the soil favors infiltration by heavy metals they do not tend to be adsorbed and retained. Heavy metals do not usually become fixed in such soils, which results in their going underground and contaminating the groundwater (Calderon and Maldonado, 2008).

Absorption and accumulation of lead in agricultural products diminishes their quality thus precluding the production of crops for the purpose of export. This limits the destination of agricultural production to local and national markets and for self-consumption. The low income of farmers (more than 50% of the population interviewed have a monthly income of less than $300.00), the decrease in crop production in recent years and the lack of alternative crops for export are factors that have contributed to an increase in the rate of poverty in the region.

The overall average environmental risk estimated for the agricultural areas studied and irrigated with water from the Mantaro river containing lead on the left bank of the province of Jauja is high. This is attributed to the presence of lead which: affects population health, soil fertility, the biogeochemical cycle, degrades the ecological function of the soil, decreases the crop yield and changes the composition of the agricultural products.

Thus, it is recommended that the Local Water Authority of Junín build sedimentation pools in “Siclachaca” treated with *Sphagnum* spp., to reduce lead concentration in the irrigation water of the Mantaro River.

CONCLUSION

Lead concentrations in agricultural products (corn and potatoes) exceeded the maximum allowed level by between five and thirty times. In Huamali and Apata the lead concentrations in soil exceeded the limits established by the environmental quality standard for soil. The population studied is exposed to lead by its daily intake of corn and potatoes, with average daily doses in females and males below the maximum recommended levels.

The *HI* of the population exposed to lead through the consumption of agricultural products irrigated with the water from the Mantaro River, corresponds to a low risk, it does not represent a significant risk for the individuals studied.

The environmental risk level estimated from the accumulation of lead in soils and agricultural products was high on sites that are irrigated with water from the Mantaro River. The likelihood of damage to humans, the natural environment and the socio-economic environment~~al~~ was highly likely through the intake of food with lead levels that exceeded the maximum allowed level by lead accumulation in soil and aquifers. The severity of damage to the human environment was critical.

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Chapter 24

HUMAN HEALTH RISKS DUE TO AIR MERCURY EXPOSURE PRODUCED BY ARTISANAL AND SMALL-SCALE GOLD MINING ACTIVITIES IN PORTOVELO, ECUADOR

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ABSTRACT

Ecuador's main gold mining activities, located in the southern region, are characterized by large veins of ore that have been exploited since the colonial era. Mercury pollution generated during amalgamation and cyanidation in artisanal and small-scale gold mining processing centres threatens human health. The aim of this chapter is to assess the effects of mercury exposure on health in terms of toxic risk (hazard quotient,*HI*) in the El Pache (Portovelo district) mining community. This chapter presents data on atmospheric mercury concentrations from sampled areas around processing plants and when miners burn gold-mercury amalgams and zinc shavings after cyanidation. Sampled individuals were from the mining community living and working around processing centres, made up of administrative staff, miners, women, and children, who are exposed to mercury by air inhalation. Samples were taken in 2007, 2008, 2011, and 2012 during winter and summer. The most important specific conditions considered for the calculation of risk were concentration at the source, concentration at the point of exposure, target identification, pathways and exposure modes, and exposure time. Results show that human health risk due to mercury exceeds the limits of acceptability (*HQ* 1).

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Keywords: mining activities, mercury contamination, risk analysis, inhalation, hazard quotient

INTRODUCTION

In Latin America, mining activity dates back to precolonial times. It continued to be very important during colonization and has been developed up to the present. In Ecuador, artisanal and small-scale gold mining has had a great effect across the country. Gold potential is mainly located in the provinces of Zamora, Azuay, and El Oro, in the Zaruma-Portovelo, Nambija, and Ponce Enriquez deposits, respectively (Sacher and Acosta, 2012). The Portovelo mining district is the main gold mining processing centre in Ecuador.

In the district there are around 90 processing plants, which process around 4000 tonnes of ore per day. Approximately every year, 3000 miners visit the processing centres of the Portovelo mining district to rent equipment to recover the gold. Almost 40% of the processing centres are located in El Pache. Although ore processing and gold recovery is the main activity in El Pache, there are other types of business, such as gold buyers, mineral laboratories, restaurants, car washers, mining shops, and neighborhood stores.

Artisanal miners manipulate mercury to achieve the amalgamation process for gold recovery. Artisanal and small-scale gold mining is by itself the largest source of mercury pollution worldwide, with effects that can last several years even after the completion of mining activities. These activities have left a legacy of soil and water contamination of heavy metals, which pose a risk to the health of surrounding populations. The importance of studying the ecological health risk of heavy metals, including mercury, lies in their relation to the consumption of irrigated crops and fish, because of the risk of bioaccumulation and biomagnification in the food chain (Shiowatana et al., 2001). Mercury accumulation in water, soil, and sediments presents a danger to living beings.

The use, transport, marketing, and health risks for people related to mercury have become topics of discussion in the social policy agenda of national governmental authorities seeking measures to minimize emissions and mitigate the noxious environmental consequences. Since January 2013, Ecuador's Ministry of Environment (MAE) implemented the Zero Mercury Plan, in which the main objective was the gradual reduction of mercury utilization in various processes.

In January 2011 the MAE's Ministerial Agreement No. 003 defined the "List of Hazardous Chemicals of Severely Restricted Use" through which formulation, manufacturing, marketing, storage, use, and possession of mercury were restricted all over the country. On July 16, 2013, the Mining Act was amended with the prohibition of using mercury in mining operations, and the Transitional Law was enacted. In order to eradicate the use of mercury, these actions enforced upon natural or legal persons both national and foreign and holders of mining rights that they must apply alternative methods to eliminate mercury progressively in mineral recovery processes for a period of two years after the date of the Act. In April 2014 the regulations governing the procedures and requirements to obtain authorization for the transfer and use of mercury were implemented.

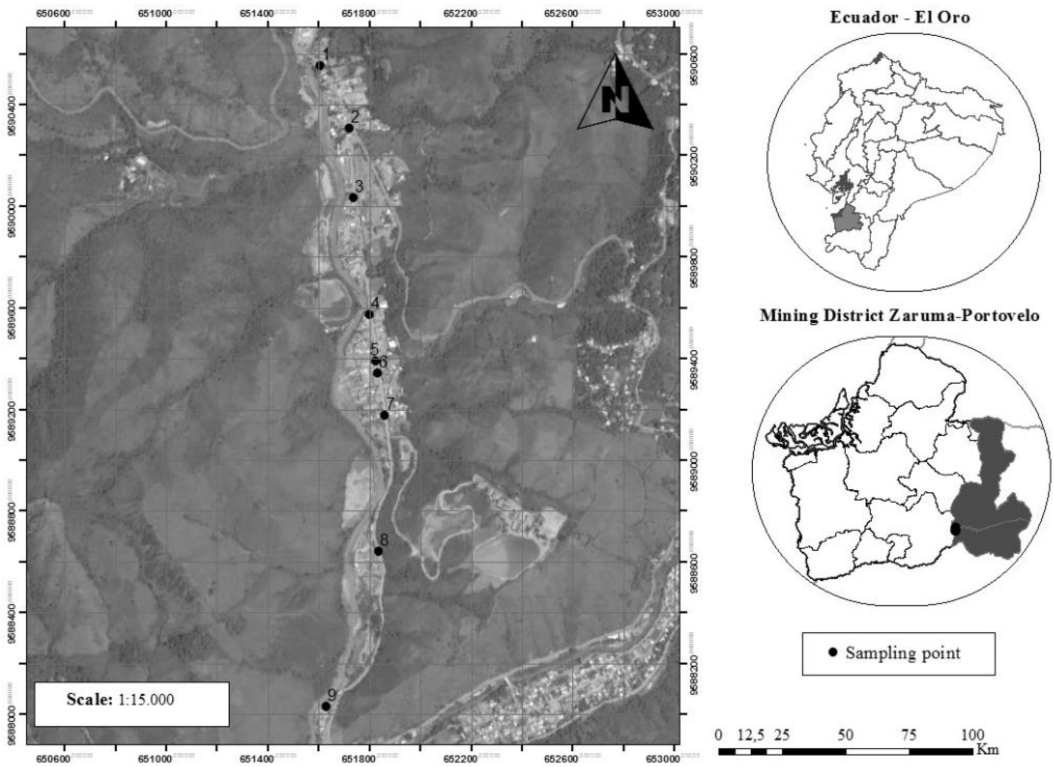

Figure 1. Selected sampling sites along the processing centres road in El Pache, Portovelo, southeastern Ecuador.

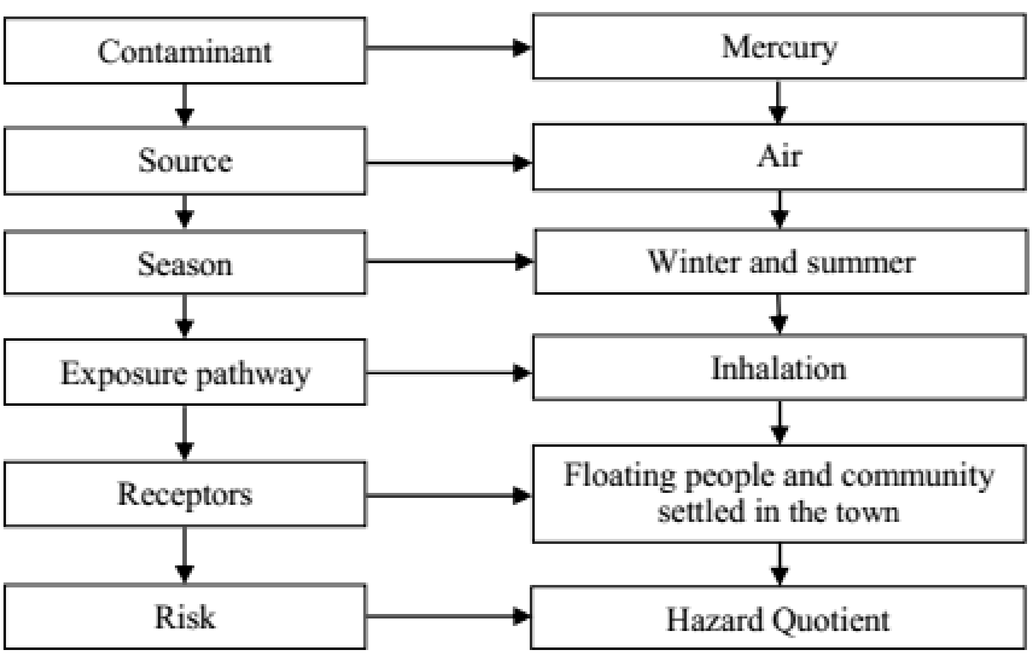

Figure 2. Mercury exposure scenario for the El Pache community in the Portovelo district.

This chapter analyzes the human health risk by quantifying the toxicological damage due to exposure to atmospheric mercury generated in the main mining processing centre of Ecuador, El Pache, Portovelo, where miners use mercury for gold recovery.

METHODS

Study Area and Monitoring

The assessment was carried out strategically in the El Pache area, at the Portovelo gold mining town (Figure 1). In Portovelo there are around 90 processing centres for gold extraction. About 30 processing centres are located in the El Pache mining community. For monitoring purposes, nine sites were selected along a 3 km stretch of the main road in front of the processing centres. Air mercury concentrations were monitored in the summer (dry) and winter (rainy) seasons in different years: in September, October, and November (summer) for 2007 and 2011, and in January and February (winter) for 2008 and 2012. A total of 603 samples were collected, 306 in the dry season and 297 in the rainy season. Specifically, 270 samples were taken in 2007, 36 samples in 2011, 270 in 2008, and 27 in 2012. Total mercury concentrations in ambient air were determined using the LUMEX portable atomic absorption spectrophotometer (RA-915+) with an optical length of 9.6 m and a detection limit of 1.0 ng/m^3.

Figure 2 describes the exposure scenario considered in the El Pache mining community case study. Two groups were assessed for risk assessment. The first group corresponded to the floating population (occasional visitor, administrative staff, and miners); and the second group corresponded to the community settled in the town—men, women, and children ages one to six years. 'Occasional visitors' are individuals entering the area sporadically and staying a limited amount of time. The miners at times visit the processing centres for ore processing that involves burning mercury. The settled population consists of processing centre administrators working in the contaminated environment and the population living in the area. The workers of the processing centres are exposed to gaseous elemental mercury and gold dust.

Quantitative Estimating of the Noncarcinogenic Hazard

The average daily dose (*ADD*; mg/kg/day) from inhalation of mercury was calculated as follows, in equation 1:

$$ADD=(C*IR*F) / BW \tag{1}$$

where C is the concentration at the point of exposure (mg/m^3), IR is the intake rate from the contaminated medium (m^3/day), F is the exposure factor (unitless), and BW is the body weight (kg).

The criterion used to select the representative concentration at the point of exposure is given by the precautionary principle. For this reason we used the so-called upper confidence limit (UCL). This procedure calculates the representative concentration through a data set obtained in situ. This value represents a highly conservative estimate of the true value of the average. To calculate statistical significance we used EPA's Pro Upper Confidence Level software (USEPA, 2013). The exposure factor was calculated via equation 2:

$$F=(EF*ED) / AT \tag{2}$$

where *EF* is the frequency of exposure (day/year), *ED* is the exposure duration (years), and *AT* is the average time (*ED* x 365 days/year). The exposure factor gives the dose averaged over the exposure period. Site-specific exposure values, namely the parameters that contribute to the determination of *F*, are published in the EPA's *Exposure Factors Handbook* (USEPA, 2011). The key to calculating the most accurate exposure dose is to identify values that relate specifically to the exposure situation being assessed. If site-specific information is not available, several conservative exposure assumptions can be applied. In order to more accurately calculate the dose of exposure related to a pollutant, it is important to gather proper data from the studied site.

Moreover, one must take into account that the dose-response relation describes the effect on an organism caused by the amount and the specific route of exposure to an agent (delivered dose) after a certain exposure time (Crump et al., 1976; Rivera-Velásquez et al., 2013). As the dose increases, the response tends also to increase. At low doses there may be no response, i.e., a body has a certain degree of tolerance upon exposure to a particular substance. A tolerable dose exists below which no adverse effects on human health are present for noncarcinogens (Davis and Svendsgaard, 1990; Daniels et al., 2000; Steenland and Deddens, 2004).

A reference dose (*RfD*) is a daily oral intake rate that is estimated to pose no appreciable risk of adverse health effects. From the *RfD*, the *HQ* assessment is calculated as follows in equation 3:

$$HQ=ADD/RfD \tag{3}$$

where *HQ* is the hazard quotient (unitless) and *RfD* is the reference dose (mg/kg/day). The standards for human protection defined by various international agencies provide the following acceptability criterion (APAT, 2008; USEPA, 2011): *HQ* (exposure to one or more substances) <1.0.

Health Risks Due to Air Mercury Contamination Produced by Mining Activities

The *HQ* was calculated for each sampling period. To calculate the representative concentration, the *ADD* (equation 1) was used for the whole series of samples corresponding to 603 *in situ* samples (306 in summer and 297 in winter). The approach taken to determine this value was 95% UCL. Two UCL values were calculated using ProUCL 5.0 software (USEPA, 2013).

The *HQ* was calculated by the deterministic method (Rivera-Velasquez et al., 2013) and evaluated assigning a single value in correspondence to the receptors considered.

For the *HQ* assessment, two different groups (and subgroups) of receptors were considered: the floating population (occasional visitors, miners, and administrative staff) and the settled community (men, women, children). The first group corresponds to people who visit the site for work or as casual visitors but that do not live in the area. The second group refers to people who conduct their entire daily activities within the studied area. The *BW* and *IR* values, considered in the *HQ* equation, were assigned according to the EPA database (USEPA, 2000, 2011). The other input values were obtained assuming site-specific characteristics, such as *ED* and *EF*.

RESULTS

Initially the concentration data obtained from the examined samples was subjected to a careful statistical analysis. Table 1 shows the mean, standard deviation, minimum, and maximum values of mercury concentration for each sampling point and each year: 2007 (n=270), 2008 (n=36), 2011 (n=270), and 2012 (n=27).

For the *HQ* model, mercury concentrations were computed using 95% UCL criteria corresponding to 0.004302 mg/m3 and 0.006428 mg/m3 for winter and summer seasons, respectively. For mercury inhalation, the RfD used in all cases was 8.571x10-5 mg/kg/day (Table 2).

Figure 3 shows the histogram of the *HQ* values for the six receptors considered. The histogram shows that the reference *HQ* is exceeded for almost all receptors with the exception of the nonresident people (floating population) in the winter season.

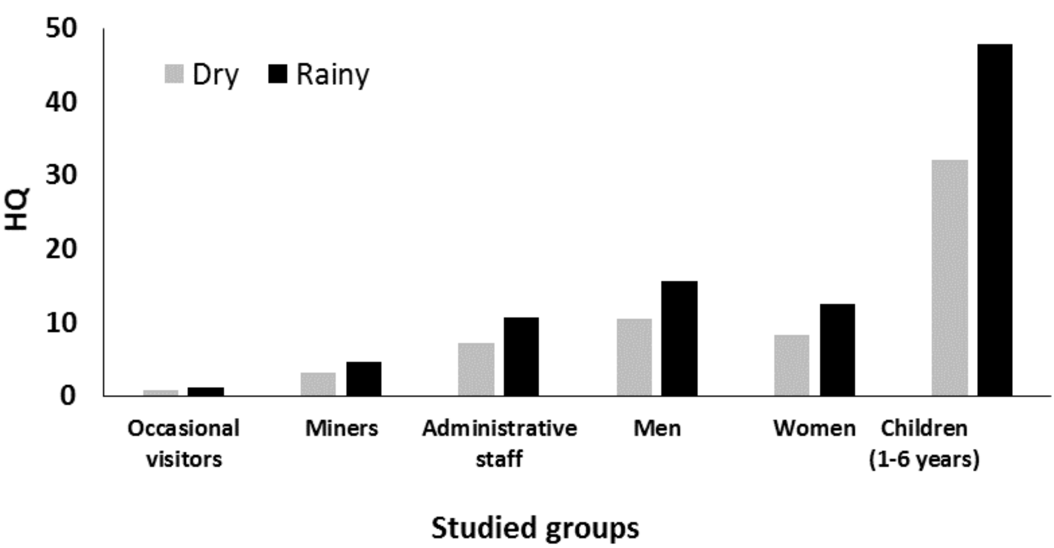

Figure 3. *HQ* values for the dry (winter) and rainy (summer) seasons for the El Pache mining community.

Table 1. Values for mercury air concentration (ng/m^3) detected in the El Pache zone

Site	*Winter 2007*				*Winter 2011*			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Site 1	1051	1306	198	5520	905	945	353	2317
Site 2	1029	798	168	3110	935	578	444	1746
Site 3	1003	448	301	1740	1165	448	683	1715
Site 4	2608	2840	584	10150	2300	2229	1038	5639
Site 5	8139	10581	766	35216	7550	8012	1491	19307
Site 6	10844	20616	891	111105	9636	7662	2033	19940
Site 7	10061	11090	960	48600	9991	5906	2254	16521
Site 8	2042	1758	665	6871	1820	1415	901	3919
Site 9	1242	603	211	2100	1142	486	665	1680
Mean ± SD	For winter:		4202±8935					
	Summer 2008				*Summer 2012*			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Site 1	320	143	120	773	320	41	280	362
Site 2	633	571	110	2185	633	495	181	1162
Site 3	450	129	148	692	451	32	428	488
Site 4	474	166	158	771	474	50	421	520
Site 5	1254	1705	298	5898	1254	1243	434	2684
Site 6	12815	19549	399	50000	12815	20795	591	36826
Site 7	4383	5621	580	22416	4383	5167	931	10323
Site 8	490	191	150	861	489	204	312	712
Site 9	391	130	193	587	391	149	220	494
Mean ± SD	For summer:		2357±7693					

SD: standard deviation; Min: minimum; Max: maximum.

Table 2. Hazard quotient and related parameters for floating and settled population in the El Pache mining community

Parameter	Floating population			Settled community		
	Occasional visitors	Miners	Administrative staff	Men	Women	Children (1-6 years)
IR (m^3/day)	15.2	15.2	15.2	15.2	11.3	10
EF (years)	24	104	240	350	350	350
BW (kg)	70	70	70	70	65	15
ED (years)	3	8	8	24	24	5
AT (years)	3	8	8	24	24	5
HQ (dry)	0.72	3.11	7.17	10.45	8.37	32.09
HQ (rainy)	1.07	4.64	10.71	15.62	12.50	47.94

IR: intake rate; *EF*: exposure frequency; *BW*: body weight; *ED*: exposure duration; *AT*: averaging time; *HQ: hazard quotient*. For calculation of *HQ* the value of *AT* is equal to *ED*.

DISCUSSION

Some studies indicate mercury as a carcinogen, although there is no definitive evidence. The EPA considers mercury as Class D, "Not Classifiable as to Human Carcinogenicity" (Burger and Gochfeld, 2012). The gold mining operation sets a good example of the possible exposure pathways for air mercury contamination. In the present case study, the risk from mercury in air was quantified via the *HQ*.

The values of Table 1 show that the highest concentrations of mercury were detected for sites 6 and 7, where more processing centres and gold shops are located. This means that mercury vapor comes from amalgamation and cyanidation gold processing and the amalgam refining process in gold shops (Velasquez-Lopez et al., 2011).

Moreover, seasonal variation of mercury concentration in the air was recorded during the study. In fact, as highlighted in Table 1, mean mercury concentrations were greater in summer (2357 ± 7693 ng/m^3; n=297) than in the winter season (4202 ± 8935 ng/m^3; n=306). Variation among the values in each season reveals the variation in mercury concentration among different sites. This variation demonstrates the existence of specific points of mercury emission within the study area. In Ecuador, the rainy season is characterized by having the highest temperatures, rain, and evaporation. The results of the study show that in the summer season, characterized by a dry environment and high temperatures, concentrations of mercury were higher than in the winter season.

Taking into account the assumption of the toxicological analysis, and placing particular emphasis on the *EF* and *ED*, in the floating population the values of these parameters are lower than in those of the community settled in the town. This leads to lower *HQ* values in the floating population in comparison to those of the settled community (Table 2).

The children are the most vulnerable target (settled community), largely exceeding the maximum recommended *HQ* in both summer and winter seasons. The least prone group is that of the "occasional visitors," considering that the maximum recommended *HQ* was only slightly exceeded in the summer. In increasing order of vulnerability are nonresidents, miners, administrative staff, men, women, and children (Table 2). Other studies carried out in

Portovelo reported mercury values around 1500 ng/m^3 in exhaled air from the breath of miners after the amalgamation process (Velasquez-Lopez et al., 2010; Gonzalez-Carrasco et al., 2011). This confirms that miners can uptake mercury directly when they are exposed to mercury vapors. Those miners are highly exposed, but temporarily, and the residents are more constantly exposed but to lower concentrations of mercury in the air that remain after burning mercury-contaminated products such as amalgams. Therefore, risk assessment can vary from one group of people to another according to their working conditions and the time of exposure.

Risk assessment for women and children is often calculated according to the sensitivity of the developing fetus and young children (ATSDR, 1999). The exposure limit established by the EPA to protect human health (0.2 ppm) probably does not protect the fetal and the neonatal brain (Landrigan et al., 2006). Mercury has neurobehavioral effects in adults and neurodevelopmental effects on the fetus (APAT, 2008; Burger and Gochfeld, 2012).

Mercury volatility increases with increasing ambient temperature. The equilibrium concentration in the air is 2.0 mg/m^3 at 25° C, increasing volatility eight times with increasing temperature from 20° C to 50° C (Mantyla and Wright et al., 1976). From this scenario, the risks due to mercury exposure in the rainy (summer) season should be of concern: the highest value recorded was 50,000 ng/m^3 in the dry (winter) season and more than double that (111,105 ng/m^3) in the summer. Other studies reported similar concentrations in Portovelo (Velasquez-Lopez et al., 2010) and artisanal gold mining communities in Colombia (Cordy et al., 2011).

According to topography, wind speed and direction, mercury can remain in the same site or it can travel long distances from the source of contamination (Hylander et al., 1994), increasing or reducing the risk on site. Therefore, risk due to mercury exposure will be determined by the time of exposure, its concentration, the topography of an area, and meteorological factors in the contaminated area. In this study it has been shown that many seasonal factors, such as temperature and rainfall, are very important environmental indicators of mercury pollution conditions.

It is believed that mercury concentration in the air will decrease, because the Ecuadorian government has recently enforced its elimination. However, it is known that miners are still using mercury and burning amalgams in their homes. Until complete mercury elimination and a new work model operates, it is important to emphasize the need to implement a monitoring campaign for mercury in the air, in an attempt to understand mercury dispersion and its behavior in the environment.

The recommended limit for public exposure to inorganic mercury vapors is 1.0 ng/m^3 (WHO, 2000). In environments where workers are subjected to long-term exposure to mercury vapors, the LOAEL (lowest-observed-adverse-effect-level) might be around 15,000-30,000 ng/m^3 (WHO, 2000). The recommended health-based exposure limit for metallic mercury is 25,000 ng/m^3 for long-term exposure—the time weighted average (TWA) concentration for an 8-hour day and a 40-hour work week during which workers can be repeatedly exposed without adverse effect. The normal atmospheric levels of mercury in rural areas are about 2-4 ng/m^3, and in urban areas about 10-20 ng/m^3 (Veiga and Baker, 2004). It has been reported that the usual levels of mercury in the air in El Pache, Portovelo are around 10,000 ng/m^3 in the dry season and around 5000 ng/m^3 in the rainy season (Gonzalez-Carrasco et al., 2011), and recent policy action of the government of Ecuador towards mercury elimination could have diminished such levels. A continuing monitoring of air mercury contamination in El Pache, Portovelo is suggested.

To protect the health of miners, processing plant workers, and the settled community, the authorities should recommend implementation of safety procedures and continuous monitoring of mercury levels, atmospheric conditions, and the health of the exposed population. Implementation of more frequent monitoring and decision making that emphasizes early detection and treatment of toxicological signs would help to guarantee safe occupational levels of mercury. The creation of educational programs on hazard management and risky behaviors in the contaminated environment is important. These recommendations can be implemented satisfactorily through a mercury reduction and elimination program. Although this study has focused on the risk of mercury from air contamination, it is also necessary to know the concentrations of mercury in the soil and in biota in the area. Intensive monitoring of populations at high risk from mercury poisoning in artisanal and small-scale gold mining communities is strongly recommended in order to provide evidence of adherence to existing regulatory criteria. The human health risk assessment methodology facilitates conducting of assessments at a contaminated site responsive to local and federal governments.

CONCLUSIONS

The survey carried out in the community of El Pache indicated that artisanal and small-scale gold mining activities present a high risk to human health, owing to pollution caused by mercury used for these activities. This community has been exposed to mercury levels above the recommended limits. New campaigns for sampling and monitoring are needed to confirm atmospheric mercury concentration and behavior over time, in order to know the effectiveness of the implemented governmental or nongovernmental measures.

From the results obtained, with only one exception (nonresident people in winter), the current concentrations of mercury vapor in samples of air collected in the El Pache district exceeded the maximum recommended levels of HQ=1, with relevant influence on the health of the surrounding community. Children are the most vulnerable group, with values three to four times higher in comparison to other exposed groups. The risk assessment in this study provides semiquantitative information to determine the potential damage to humans and provides an early warning of potential harm in order to help responsible public decision makers.

The high values of air mercury demonstrate urgency for implementing adequate practices regulating gold mining activities, in particular concerning artisanal and small-scale gold miners and owners of processing plants. Alongside government efforts to eradicate mercury, permanent environmental monitoring including health assessment of exposed people will increase awareness of mercury pollution not only in Portovelo but also in other regions where artisanal and small-scale gold mining operates. In order to prevent damage to the health of the community of El Pache, further studies should be performed to determine the point source, exposure pathways, concentration in all forms, and the concentration in the food web.

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Chapter 25

ENVIRONMENTAL IMPACT OF AGRICULTURAL ACTIVITIES ON HUMAN AND WILDLIFE POPULATIONS FROM PALIZADA, CAMPECHE, MEXICO

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ABSTRACT

The main impacts of agricultural activity are land use change and the use of dangerous pesticides. Approximately 25 million pesticide poisonings occur annually among agricultural workers in Southern countries. The state of Campeche is located in Southeastern Mexico. Here, more than 200,000 hectares are dedicated to agricultural activity. Many pesticides are employed on rice crops grown in the Palizada River system, mainly carbofuran, chlorpyrifos, glyphosate, monocrotophos, metomil, edifenfos, benomyl, among others. In order to assess the impact of agricultural activities on human and animal populations from the Palizada River system, mosquito fish (*Gambusia yucatana*) and black-bellied whistling duck (*Dendrocygna autumnalis*) were chosen as bioindicators for the analysis of biomarkers of exposure and effect to pesticides. Farmers and agricultural workers from the communities of El Juncal, Ich-Ek, Suc-Tuc and Castamay were also evaluated because they are exposed to pesticides during application.

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The integration of information generated in wild fish and exposed *in situ* reflected a concordance between percentages of acetylcholinesterase (AChE) activity inhibition during the pesticide application period. Results indicate that whistling ducks are exposed to pesticides (inhibition above 30% of AChE activity), at least during the season of intensive application. All agricultural workers are exposed to dangerous pesticides, and farmers from Suc-Tuc and Ich-Ek have a significant inhibition of AChE activity. It is recommended the use of pesticides that are less aggressive to the environment and human health. Further field studies incorporating a systematic monitoring of wildlife populations and investigation of population parameters are particularly important for local pesticide management.

Keywords: pesticides, aquatic ecosystem, biomarkers, agriculture, Mexico

INTRODUCTION

Agricultural activity has had an adverse effect not only on the physical environment but also on human and wildlife health, mainly due to habitat loss and alteration, and pesticide exposure (Tilman et al., 2011).

In 1984, the United Nations Environment Programme (UNEP), in conjunction with several national authorities and international organizations, prepared a list of priority chemicals/pollutants to focus on major environmental problems. Pesticides were included in this list because they enter in the environment in significant amounts as a result of human activities, widely distributed and in some regions they represent potential threats to ecosystems as well as to human health due to their toxicity and/or persistence.

Mexico is a great consumer of pesticides; the use of agrochemical in 2008 was around 93,000 tons (23% insecticides; 33% herbicides; 43% fungicides) (SEMARNAT, 2010). More than 1500 active ingredients have been registered as pesticides. Formulators mix these compounds with one or more of some 900 “inert” ingredients to create approximately 50,000 commercial products registered for use (PAHO, 1993).

In the majority of Southern countries, such as Mexico, insecticide use predominates, with a correspondingly higher level of acute risk. Although the use of pesticides in Southern countries is small in comparison to their use in Northern countries, it is nonetheless substantial and continuously growing. Pesticide use is particularly intense where export crops predominate.

As xenobiotics, pesticides suffer biotransformation in the body and thus chemical detection is not easy. Biomarkers are therefore determined to confirm and assess the exposure of individuals or populations, or the deleterious effect to a particular substance, providing a link between external exposures and internal dosimetry. Among the biomarkers used to assess the environmental exposure to pesticides are acetylcholinesterase (AChE, EC 3.1.1.7), glutathione-S-transferase (GST, EC 2.5.1.18) and lactate dehydrogenase (LDH, EC 1.1.1.28).

The fluvial lagoon system of the Palizada River (FLSPR; 18º19′04″/18º30′13″N; 91º44′36″/91º51′31″W) is located in the southwestern part of Terminos Lagoon in the State of Campeche, Mexico (Figure 1). The drainage basin is about 2460 km^2 (Benitez and Barcenas, 1996). With at least 64 fish species and four primary plant communities, Palizada River system (PRS) is one of the most important ecological lagoon-estuarine ecosystems in the southern Gulf of Mexico (Fuentes-Yaco et al., 2001).

Three climatic seasons exist in the FLSPR: the rainy season (from June to September), the "nortes" season (from October to January) and the dry season (from February to May). The rainy season is characterized by strong and intensive rain (mean of the season: 157.75 mm); the "nortes" season is characterized by a cold wind from the North (mean of the season: 68.7 mm) (Fuentes-Yaco et al., 2001); and the dry season is characterized by a relatively low rainfall, with a season mean of 24.1 mm.

In the study area, there are two main human activities with potential negative environmental impact: agriculture and cattle ranching. The most important activity is agriculture, with about 10,000 hectares of rice fields of which 5,000 are continuously irrigated by several small channels. At the beginning of the 90s, herbicides such as propanil, 2,4-D and molinate were identified as the pesticides most employed by farmers (Benitez and Barcenas, 1996). At present, carbofuran, chlorpyrifos, glyphosate, propanil, amines, monocrotophos, metomyl, edifenphos and benomylare intensively applied in these agriculture fields.

In this chapter the impact of the agricultural activity and the pesticide use in the FLPRS, and on local human populations is presented. To attain this general goal, four specific objectives were considered:

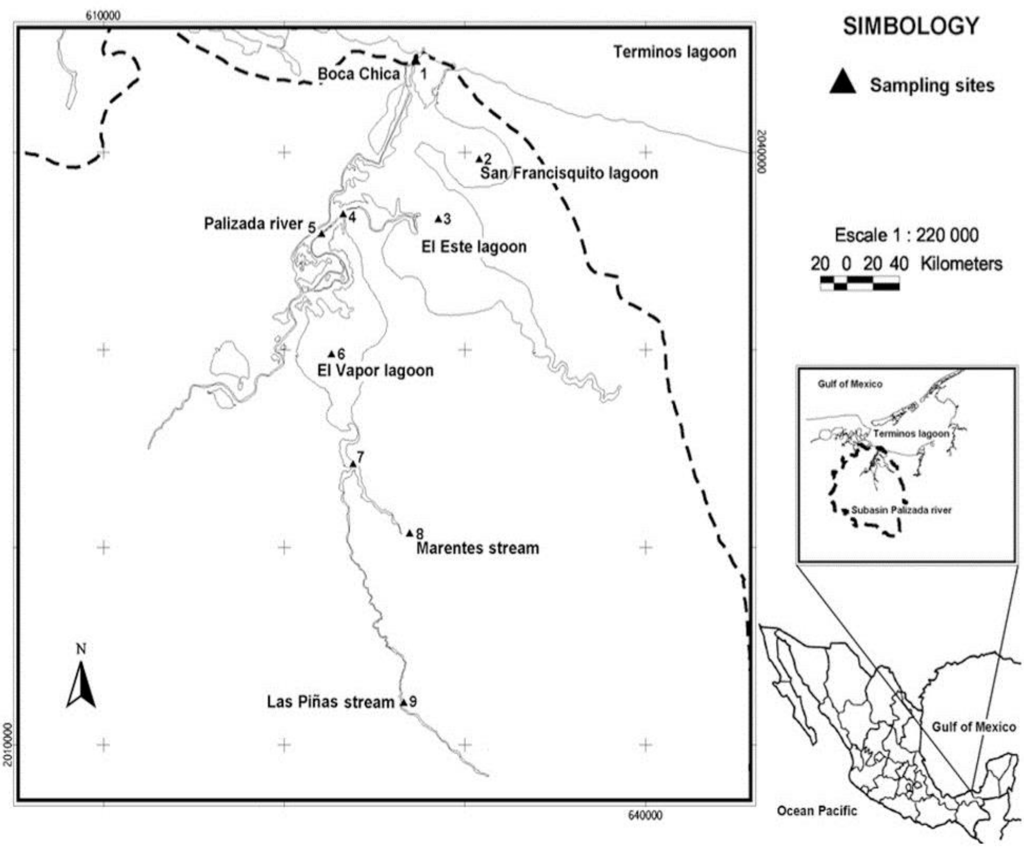

Figure 1. Map of fluvial-lagoon system of Palizada River (FLSPR) with indication of sampling sites (black triangles).

1) Study the effect of land cover on the FLSPR water quality.
2) Develop and validate an integrated approach (including a biomonitoring study, *in situ* assays and water quality parameters) with *Gambusia yucatana* to diagnose environmental contamination by chemicals.
3) Diagnose the exposure of wild duck populations (*Dendrocygna autumnalis*) to anticholinesterase pesticides.
4) Assess the effects of pesticides used in agriculture on farmers.

METHODS

Fieldwork was carried out every one or two months, during two years. Samples were collected in nine sites in the FLSPR watershed (Figure 1). Each sampling site was georeferenced in UTM and briefly characterized according to its ecological and vegetable characteristics (Table 1).

Table 1. Sampling sites and its georeference in UTM

Site	Location	Longitude	Latitude	Characteristics
1	Area of immediate influence of the Terminos Lagoon	627,319.85	2,044,602.11	Mouth of the Palizada River and connected to Terminos Lagoon. High salinity, surrounded by mangrove. Fishing activity.
2	San Francisco Lagoon	630,894.40	2,039,422.24	Lagoon without direct fluvial inflow with submerged hydrophytes and surrounded by mangrove. Poor fishing activity.
3	Este Lagoon	627,061.84	2,038,340.87	Lagoon with fluvial inflow without submerged hydrophytes. Surrounded by unforested wetland. Intense fishing activity.
4	Palizada River	623,377.28	2,037,007.77	Influence of wastes from Palizada town. Surrounded by unforested wetland. Intense fishing activity.
5	Confluence of El Vapor Lagoon and Palizada River	622,065.40	2,035,867.28	Unforested wetland. Surrounded by unforested wetland. Fishing activity
6	El Vapor Lagoon	623,685.85	2,030,189.96	Lagoon with fluvial inflow with very scarce submerged hydrophytes. Surrounded by unforested wetland. Fishing activity.
7	Confluence of Marentes and Las Piñas streams	623,544.47	2,024,307.84	Water body with unforested wetlands.
8	Marentes stream	626,932.26	2,020,713.36	Influence of incipient cattle ranching activity, surrounded by grassland. Scarce fishing activity.
9	Las Piñas stream	626,607.70	2,012,150.33	Strong agricultural activity in the area (rice crop). Scarce fishing activity.

Land Cover Changes in the FLSPR Basin (1980 – 2000)

The watershed boundaries of FLSPR were established using topographic information and 1:50,000 scale maps (INEGI, 2000). The FLSPR basin is a geographic area of land bounded by topographic features and land altitude that drains waters to a shared destination. This area is a large temporally flooded plain; therefore, the division of the basin was traced according to the drainage or crestlines that mark the hydrologic map. In order to determine the land cover change, differences between data of each land use cover type reported in the National Forest Inventory 2000 and data from 1980 reported by Benítez et al. (1995) were calculated in Arc/View V.3.2 (ESRI, 1999).

Variables Analyzed and Analytical Methods

Sixteen water parameters were determined: temperature, dissolved oxygen (DO), pH, redox, salinity, conductivity, depth, transparency, hydrogen sulfide, ammonium, nitrites, nitrates, phosphorus, silicates, sulphates and hardness.

The indexes presented in the results section were calculated with the values obtained during the whole sampling period. A Water Quality Index (WQI) (Rendón-von Osten et al., 2006) was obtained using five of the mentioned variables as follows:

WQI=(pH + Dissolved Oxygen + Ammoniun + Nitrites + Phosphorus)/5,

where 5 is the number of variables considered for index calculation and has been introduced to obtain a WQI value ranging from 0.0 to 1.0.

In Situ Assays

For the *in situ* assays, mosquito fish were cultured under controlled conditions in a container filled with 1,000 L of local well water (renewed every 15 d) with submerged vegetation. Containers were maintained in outdoor facilities with natural photoperiod and temperature. These fish were considered as control in order to compare the results of each studied biomarker.

In situ assays were performed using test chambers (Rendón-von Osten et al., 2006) with ten *G. yucatana* fish in each chamber. Chambers with fish were exposed during 21 days in three sampling sites: Palizada River (site 5), Marentes stream (site 8) and Las Piñas stream (site 9) (Figure 1).

After 21 days, mortality was recorded and the surviving fish were weighed, body size measured and sacrificed by decapitation. Decapitation is an acceptable method, provided the procedure is performed quickly and accurately (Nickum et al., 2004). Immediately after decapitation of the animals, head, gills and a piece of dorsal muscle were removed and prepared for biochemical determinations. Physical and chemical water parameters were monitored at the beginning and at the end of the *in situ* study as described by Rendón von Osten et al. (2006).

In situ assays were performed in each of the three selected sites in different periods according to climatic seasons: at the beginning and at the end of the dry season (February and

May 2000), during the rainy season (July 2000) and during the "nortes" season (November 2000). In 2001, fish were exposed in February (beginning of the dry season) and in August (middle of the rainy season) to complete the information obtained in the previous year.

Biomonitoring

A total of 310 native *G. yucatana* were evaluated, being 232 male and 78 female. Of these, 95 fish were from the stream in the area with cattle ranching activity (site 8), 116 from the stream near the agricultural area (site 9) and 99 from the Palizada River (site 5) (Figure 1). Immediately after decapitation of the animals, head, gills and a piece of dorsal muscle were removed and prepared for biochemical determinations.

Black-Bellied Whistling Duck Sample Collection

To assess the impact of rice crop pesticide used on natural populations of *D. autumnalis*, 26 ducks were sampled during a period of intensive agrochemical use (November) and 23 ducks during a period with presumably no use (July). All the ducks used were obtained from local inhabitants who hunted them for meat consumption. After decapitation, the forebrain was immediately removed and put in ice-cold phosphate buffer until analysis of acetylcholinesterase (AChE). Duck sex, body weight, body length, and other morphometric parameters were recorded. To obtain information about the range of AChE brain activity in non-exposed black-bellied whistling ducks, seven adult individuals from a local duck farm were used as reference group (data from Rendón von Osten et al., 2005). These ducks were considered as references because the duck farm is located 110 km from the agricultural area.

Biochemical Determinations in Mosquito Fish and Ducks

Activity of AChE in mosquito fish muscle and duck brains was determined by the method described by Ellman et al. (1961). Gill GST activity was determined by the method of Habig et al. (1974). The activity of LDH in muscle was determined by the method of Vassault (1983). All the methods were adapted to microplate. A Lab system Multiskan EX microplate reader was used for all determinations.

Human Blood Sample Collection and Analysis of Erythrocyte AChE

Blood samples from a fingertip of farmers and agricultural workers were taken during the period of insecticide application. Samples were taken immediately after the end of the workday. In order to minimize the bias of AChE reactivation from carbamate exposure, a Test-Mate OP field Kit was used to determine erythrocyte cholinesterase levels immediately after blood sampling (Rendón von Osten et al., 2004).

A questionnaire about the possible symptoms of pesticide poisoning experienced by farmers was performed at the end of the workday in which several variables were considered (socio-demographic variables such as education levels; unsafe pesticide practices such as eating and smoking in the field, type of poisoning and symptoms classified as mild, moderated and severe). The interview included the communities of El Juncal, Ich-Ek, Suc-Tuc and Castamay.

Data Analysis

Analysis of variance (ANOVA) was used to compare different treatments in the experiments with selective inhibitors and pesticides, as well as to compare AChE activity among reference ducks, birds collected during the application period, and ducks captured when no pesticides were applied in the field. If ANOVA indicated statistical significant differences among treatments, Tukey's HSD multiple comparisons test was carried out to identify significantly different treatments. Student t-test was used to compare AChE activity between males and females.

RESULTS

At present, the surface of FLSRP dedicated to agricultural activity is 30,075 ha (12.2% of the water basin surface). At the beginning of the 80s, the agricultural activity in FLSPR occupied only 2,450 ha (1.0%). At the beginning of the 90s the entire agricultural surface was dedicated to rice, totaling 20,580 ha (8.4% of the water basin surface) (Benítez et al., 1995).

The main change in the vegetation cover was due to agriculture, which had an increment of more than 1,200% during a period of 20 years. Reduced vegetation covers, possibly changed to agricultural areas, were mangrove, popal-tular and forest, that decreased from 169,050 ha in 1980 to 145,158 ha in 2000, a reduction of almost 16%. In 2000, the natural vegetation present in the basin occupied 28.5% of its surface, while an area of 169,050 ha (36% unforested wetland, 25% forest, 4.0% mangrove and 4.0% of disturbed forests) was reported in the 80s and an area of 142,100 ha (approximately 58.0% of basin surface) was reported for the 90s by Benítez et al. (1995).

Water Quality in the FLSPR

Of the obtained data records, 27.8% failed to fulfill DO requirement, 74.1% were above ammonium limit value, 9.3% of the data were above nitrite limit concentration and all the records were above the phosphorous limit according to the DOEU (2006).

The WQIs ranged from 0.49 (July 2000) to 0.70 (March 2000) in Marentes, from 0.44 (March 2000) to 0.73 (November 2000) in Las Piñas, and from 0.54 (September 2001) to 1.00 (May 2001) in Palizada.

Statistically significant differences among sites were found ($F_{8,162=}3.82$; $p < 0.0001$). The lowest WQI value (WQI=0.57), which was significantly different from all the others, was determined for site 8, under the influence of cattle ranching activity. The low WQI is mainly due to low DO values. From the remaining sites, the lowest WQI was calculated for site 9 (WQI=0.71), located in the agricultural area. However, differences among these sites were not statistically significant.

Significant differences in WQI mean values were found among different months ($F_{13,\ 112}$=12.31; $p < 0.0001$). The lowest values were found in June 2000 (WQI=0.59) and September 2001 (WQI=0.58), in the rainy season. OD and nitrites were the variables that influenced the WQI behavior.

During the 1999-2000 climatic cycle, WQI was higher during the rainy season with exception of site 8 which had in the three seasons a very low value. However, during the 2000-2001 cycle the highest WQI values were found in the dry season, with the exception of site 8 that had low WQI values in all the seasons.

Mortality of mosquito fish recorded in each site during the in situ assays is shown in Figure 2 and weight alterations are shown in Figure 3. In February and May 2000 (dry season), no mortality occurred in fish exposed in the Palizada River. In addition, these fish gained 6% in body weight. After this period, mortality was 60% in July 2000 (rainy season), 30% in November 2000 (nortes season) and 100% in August 2001(rainy season). Surviving fish had a loss of body weight of 12% in July and of 20% in November. In the site with cattle ranching activity, 20% of mortality was found in February 2000 (nortes season), with a loss of 22% in the body weight, and 100% mortality was observed in all the other months. In the site with agricultural activity, the mortality was always equal to or higher than 80%, except in November 2000 where a low mortality (10%) and only an 8% reduction of body weight were found.

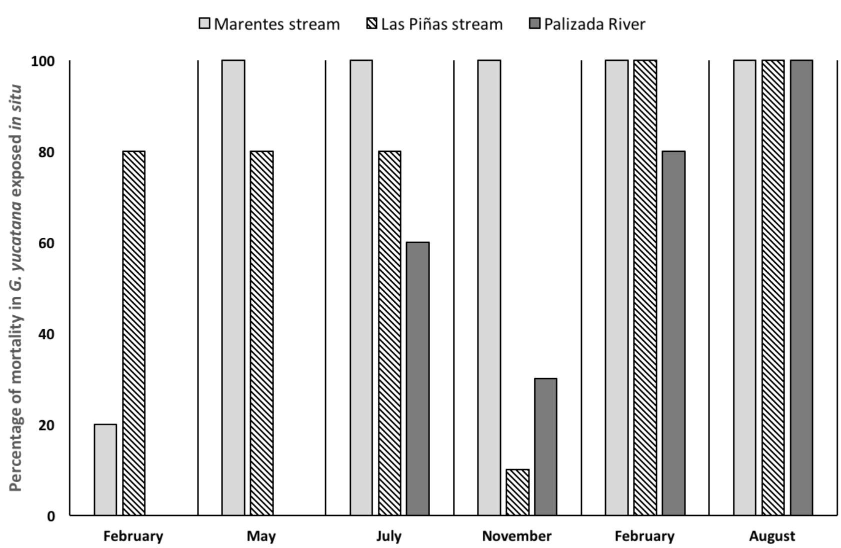

Figure 2. Percentage of mortality of *Gambusia yucatana* recorded per month in *in situ* assays at each sampling site.

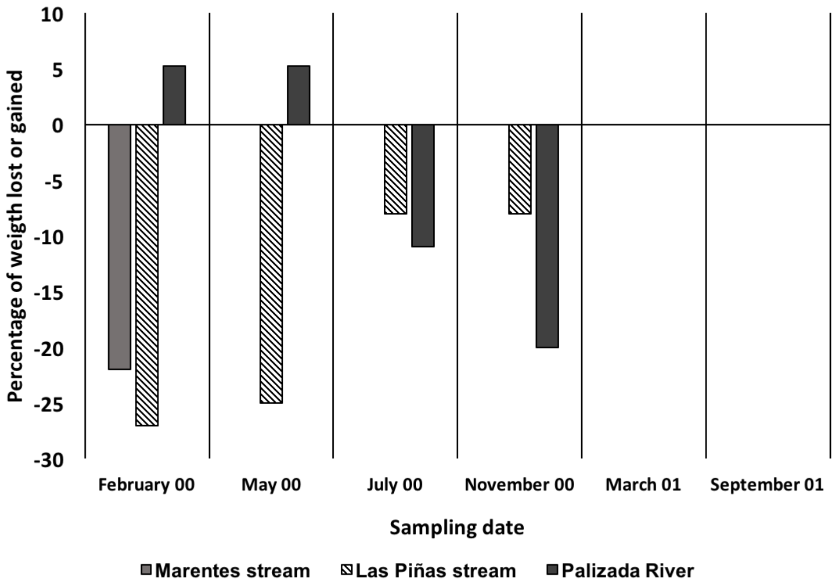

Figure 3. Monthly *Gambusia yucatana* weight variations in *in situ* assays at each sampling site.

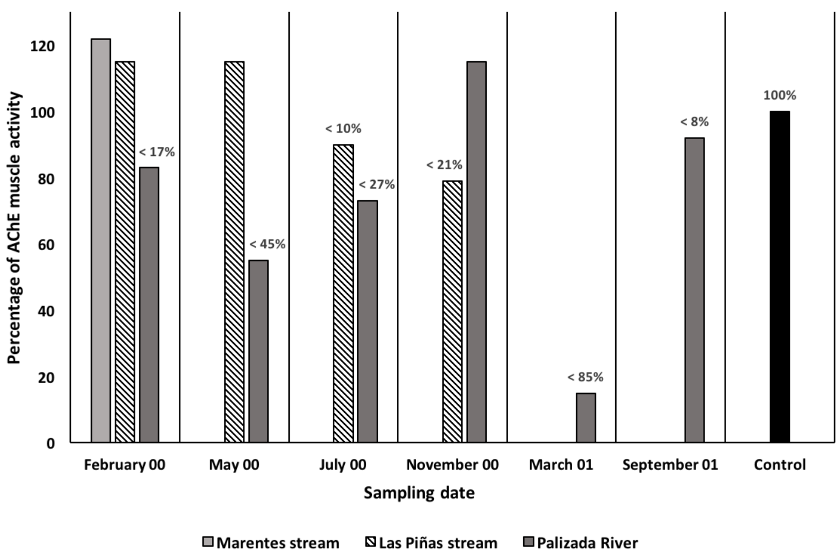

Figure 4. Percentage of AChE muscle activity measured in *Gambusia yucatana* that survived until the end of the 21-day *in situ* assay. Values above the columns represent the percentage of AChE inhibition.

Figure 4 shows the percentage of AChE activity in fish that survived the 21-day exposure relatively to the activity determined in correspondent time periods in fish from natural populations that had been maintained in the laboratory.

In Palizada River, only in November did the percentage of AChE activity in fish not show a decrease, while during May and July, and March and September fish exposed in the Palizada River showed an AChE depression.

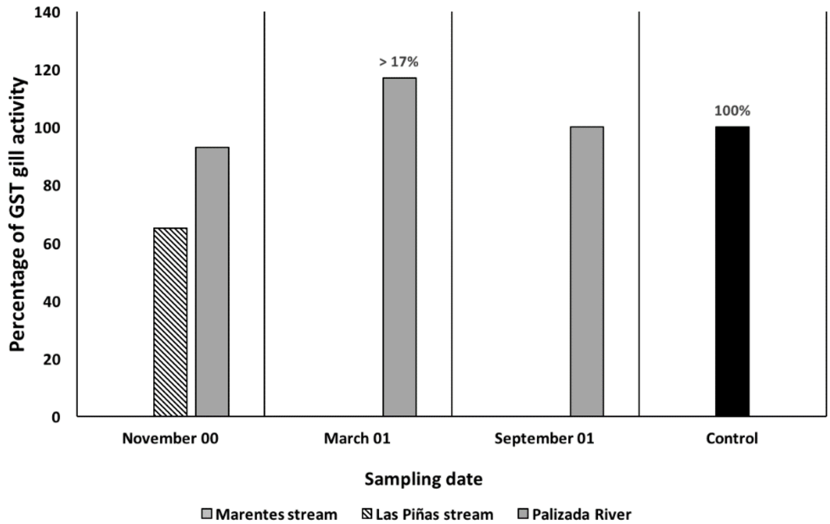

Figure 5. Percentage of GST gill activity measured in *Gambusia yucatana* that survived until the end of the 21-day *in situ* assay. Symbol > means the percentage of GST activation.

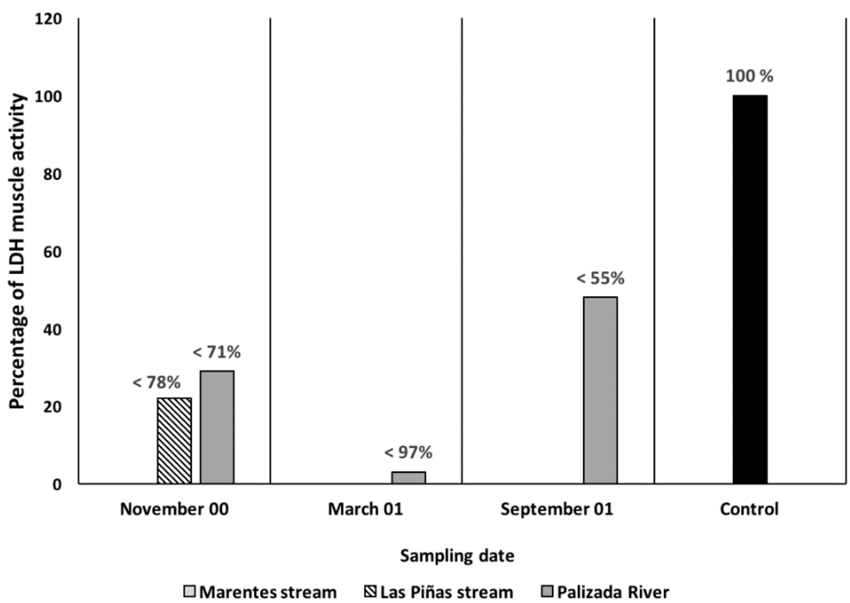

Figure 6. Percentage of LDH muscle activity measured in *Gambusia yucatana* that survived until the end of the 21-day *in situ* assay. Symbol < means the percentage of LDH inhibition.

Figures 5 and 6 show the percentage of gill GST and muscle LDH activity in fish exposed *in situ* relatively to the activities determined in laboratory animals. GST activities of fish exposed in the Palizada River were similar to control animals in November 2000 and September 2001, and a slight increase of activity was observed in March (about 17%). A high inhibition of LDH activity (above 78%) was found in November in fish exposed in both Las Piñas stream and Palizada River. Almost full inhibition was observed in fish exposed in the Palizada River in March and about 50% of inhibition was found in animals exposed in this site in September.

In general, fish from the Palizada River showed a pattern of AChE inhibition similar to that of fish from the Las Piñas stream, having in the most part of the months a higher depression (Figure 7).

In the case of GST, activity pattern variation from March 2001 until September 2001 was similar for all the sampling sites (Figure 8). Inhibition of GST activity relatively to laboratory fish was 8% in the site with agriculture activity and an induction of GST (41%) was observed in the Palizada River in March; an inhibition was recorded from 13 to 34% in May, and 20% in all sites in September.

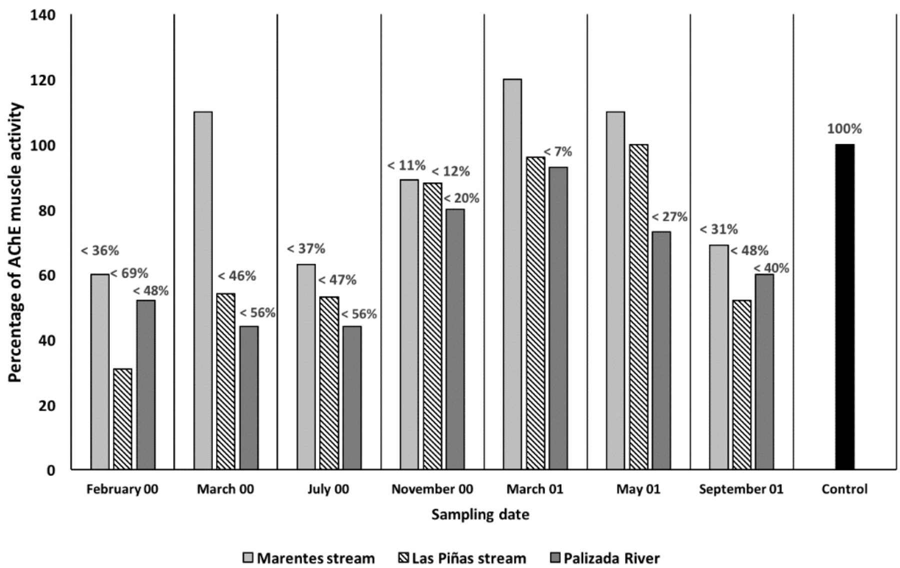

Figure 7. Temporal variation of percentage of AChE activity in muscle of native *Gambusia yucatana*. Symbol < means the percentage of AChE inhibition.

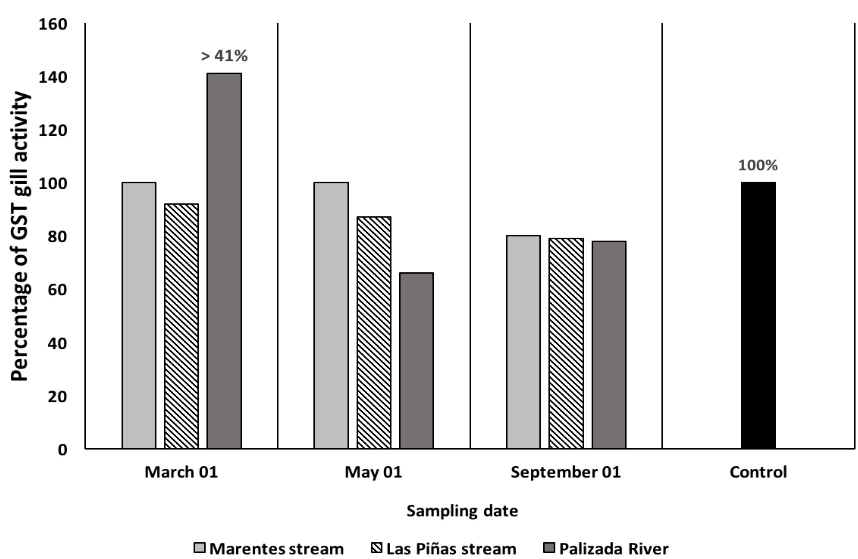

Figure 8. Temporal variation of percentage of GST activity in gill of *Gambusia yucatana* from three sampling sites. Symbol > means the percentage of GST activation.

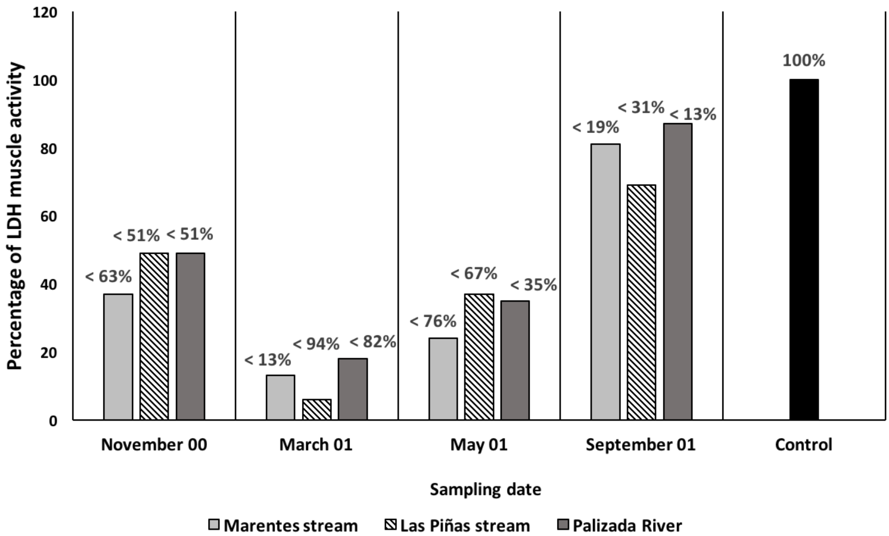

Figure 9. Percentage of temporal variation of LDH activity in muscle of *Gambusia yucatana*. Symbol < means the percentage of LDH inhibition.

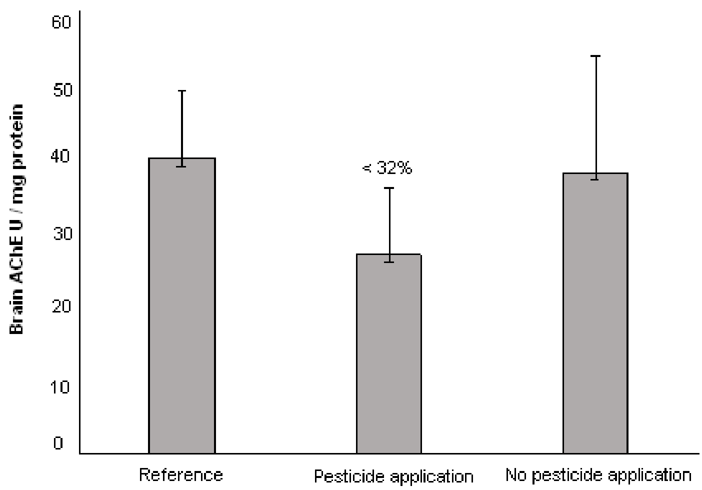

Figure 10. Brain cholinesterase activity in reference ducks and in wild ducks (*Dendrocygna autumnalis*) during pesticide application and when no pesticide was applied. Symbol < means the percentage of AChE inhibition.

The pattern of fish LDH variation from November 2000 until September 2001 was similar for all the sampling sites (Figure 9). Inhibition of LDH activity relative to laboratory animals (control) was from 51 to 63% in November, 82 to 94% in March, 65 to 76% in May and 13 to 31% in September.

In relation to the monitoring of ducks, significant differences in AChE activity ($F_{2, 54}$=6.21, p=0.003) were found among reference ducks (ducks reared on a farm), ducks collected during the season of intensive pesticide use (November), and ducks collected during the period without pesticide application (July) (Figure 10). Compared to the reference group, ducks captured during the pesticide application period showed a decrease of 34.2% of AChE activity.

According to all the farmers interviewed (n=121), the main mild symptoms associated with pesticide exposure were headache (15.7%), physical weakness (10.7%), nausea (8.3%) and low appetite (8.3%). Moderate symptoms most often reported were sweating (4.1%) and tiredness (4.1%). Tremor (2.5%) was the primary severe symptom reported.

In Ich-Ek, 22 farmers with AChE activity levels indicating poisoning due to pesticide exposure were found, threeof them with values compatible to severe poisoning. In addition, a case of poisoning in El Juncal, three in Castamay and four in Suc-Tuc were found. However, in the period of blood sample collection, none of the subjects from any of the four communities reported symptoms of pesticide poisoning.

DISCUSSION

The results indicated that during the last 20 years, in the FLSPR, the land use change has been drastic, mainly in agriculture cover that increased more than 1,000%; other land covers decreased in different percentages: unforested wetland decreased by nearly 25% and the grassland about 20%. It has been demonstrated that an increase of agricultural activity has an important environmental impact due to the use of agrochemicals, such as fertilizers and pesticides, and soil erosion (Neumann et al., 2002).

Land use can influence the water quality. Indeed, the results obtained indicate negative correlations between agriculture cover, pH and DO and sulphates concentrations. When the WQI was applied to the PRS, the results showed a clear differentiation between sites with diverse human activities. From the nine sampling sites studied in the PRS, Marentes (WQI=0.58) and Las Piñas streams (WQI=0.65) had a significantly lower WQI average than the remaining sites (WQI=0.68-0.73); these results indicate a lower water quality in sites associated with cattle ranching and agricultural activities, respectively. Water DO was the variable with a high weight in the WQI and showed a seasonal variability. In the region, between-year variations in the precipitation exist. It was observed that water quality depends on many environmental factors, but it is necessary to take into account the climatic seasons of the region.

Regarding the *in situ* assays, 100% of mortality was observed in fish exposed in Marentes stream (cattle ranching activity) in all months except February 2000, when only 20% of mortality was found. The concentration of DO in this site was always lower than 3.5 mg/L. Parameters such as nitrites, nitrates and ammonia for fish mortality could contribute to this process, because even low concentrations of nitrites during short-term exposures can be toxic to fish (Martinez and Souza, 2002). Furthermore, a decrease in water DO may increase ammonia toxicity (Randall and Tsui, 2002). In some periods, in Marentes stream, a locally well-known phenomenon called the “barbasco” was observed. Barbasco is a group of natural rotenoids present in a tree of the genus *Lonchocarpus*. Rotenoids are potent fish killers (Metcalf and Müeller, 2000), and may have caused or contributed to the mortality of fish at the beginning of the rainy season.

In the Las Piñas stream, mortality was equal or above 80% in all months with the exception of November. AChE inhibitions lower than 20% were found in July and September but not in February and May. Considerable LDH and GST inhibitions were also found in November. Although the results of these parameters should be analyzed with precaution due to the low number of surviving fish, it is important to note that GST inhibition may be caused by carbofuran, which is applied in this zone, because *in vivo* gill GST inhibition was observed in *G. yucatana*.

The chambers used here allowed the survival of *G. yucatana* during 21 days in sites with low DO concentrations. In addition to mortality, it was possible to use other indicative parameters, namely weight variation, AChE, LDH and GST activities. The caging assays may overestimate the effects of contaminants in wild fish since these are able to move and avoid or reduce the exposure, while fish in closed chambers cannot.

LDH activity was found to be positively correlated with pH and ortophosphates and negatively correlated with salinity, ammonium, DO, and redox. Our results are in good agreement with Wu and Lam (1997) who found that LDH activity was significantly higher in mussels from sites with lower DO values. Gill GST activity was found to be positively and highly correlated with nitrates. Therefore, particular care with this parameter should be taken when measuring GST in field populations, because positive correlations of this enzyme with pH and negative correlations with sulfide and redox were also found. Sturm et al. (2000) reported for three-spined stickleback (*Gasterosteus aculeatus*) this same correlation, particularly with pH.

In the Las Piñas stream, where pesticides are continuously used, an AChE inhibition higher than 20% was found in February, March and July 2000 and September 2001, indicating exposure to anticholinesterase pesticides (Figure 4). Farmers apply insecticides incorrectly, mainly where the fields are continuously irrigated. In some conditions organophosphate pesticides may persist for considerable periods of time in soil and water (Bondarenko and Gana, 2004). A previous study in the area of Palizada river reported that chlorpyrifos residues in water were about 20 pg/L in April 2000 (Carvalho et al., 2009). This is a concerning situation because chlorpyrifos is applied mainly from August to October and was detected in April in several sites of Palizada River system. The induction of gill GST activity (Figure 8) and the inhibition of muscle LDH (Figure 9) found in fish collected in this month also suggest the presence of pollutants in the area.

AChE inhibition higher than 40% was found in March 2000 (Figure 7) but no inhibition was observed in March 2001 which confirms that farmers apply pesticides in different time periods from year to year, mainly according to climatic conditions, such as precipitation.

In Las Piñas stream, in November, when the WQI showed the highest value calculated during the period of the study, a low mortality of fish (10%) was recorded in the in situ assay, despite the LDH inhibition found in surviving fish. As a whole, these results suggest the presence of pollutants other than anticholinesterase pesticides. In this area, Σ-DDT (531 pg/L) and Σ-PCBs (4250 pg/L) have been found (Carvalho et al., 2009). PCBs are nucleophilic agents and GST participates in their detoxification (PCB 153) (Machala et al., 1998; Boon et al., 1989).

In relation to the biomonitoring of wild ducks, compared to reference ducks, a decrease of 34% in AChE activity was found in ducks captured in August during the pesticide application period, clearly above the 20% inhibition that has been used to diagnose exposure to anticholinesterase agents (Figure 10). On the other hand, no significant differences in

AChE activity were found between reference animals and ducks collected in July, when no pesticides were applied, indicating probably recovery of AChE levels in wild ducks.

The results suggest that wild *D. autumnalis* are exposed to pesticides at least during the season of intensive application. Inhibition above 30% of AChE activity found in wild ducks during the pesticide application period may indicate that effects on the neurological function have been induced. Therefore, it is likely that other physiological functions have been affected during the period when ducks experience low levels of AChE.

The results obtained from the interview to farmers indicated a considerable variation in education levels across the four communities studied. The non-use of protective clothing among the four communities is common and this behavior seems to be related with the local social connotation of the use of protective clothing as a weakness in the workplace (Tinoco and Halperin, 1997). Eating in the fields can be a route of pesticide exposure in farmers because over 30% of the farmers confirmed that they do this.

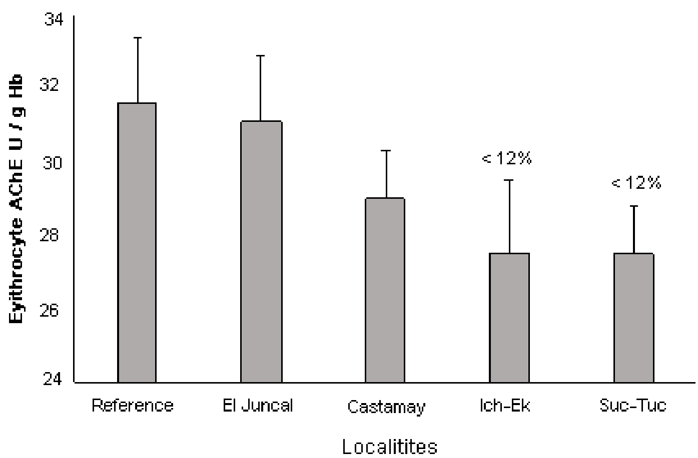

Figure 11. AChE activity in farmers from four communities of Campeche, Mexico. Symbol < means the percentage of AChE inhibition.

Twenty five per cent of El Juncal and Suc-Tuc farmers showed symptoms of mild poisoning that seem to be associated with carbofuran and methamidophos, respectively. According to pesticide use symptoms probably compatible with acute organophosphate and carbamate poisoning, mainly headache, weakness, nausea, sweating and tiredness were found in the communities.

The lower AChE activity levels in Ich-Ek and Suc-Tuc farmers relatively to the other groups seem to be due mainly to the use of organophosphate pesticides in these sites (Figure 11), since in El Juncal carbofuran, a carbamate insecticide, is intensively used and AChE activity was not significantly affected.

Depending on the pesticide used in a region, erythrocyte AChE inhibition provides a good biomarker of exposure to OP in field studies with human populations. However, for carbamate exposure this biomarker needs to be used with care because this enzyme can be reactivated in a short period of time (Nigg and Knaak, 2000).

CONCLUSION

In the FLSPR, the main change in land use occurred in the last 20 years and the agricultural area increased from 2,450.0 ha in the 80s to 30,075 ha in 2000. The implementation of a WQI in the Fluvio Lagoon System of Palizada River was useful to differentiate the environmental impact on sites with several human activities. Sites close to cattle ranching and agricultural activities have much lower WQI than other sites with different land use covers.

The *in situ* assays were able to discriminate levels of water contamination both in time and space, thus chambers and cabinets are suitable to in situ exposure. Biomonitoring studies with autochthonous organisms (*G. yucatana*) using enzymatic biomarkers present the advantage of diagnosing the exposure of natural populations to environmental contaminants or the effects of pollutants in wild populations.

The biomonitoring in the terrestrial compartment via the quantification of the inhibition of AChE activity in wild black-bellied whistling ducks (*D. autumnalis*) suggests that these organisms are exposed to pesticides at least during the season of intensive application. Due to the abundance and its widespread occurrence in wetlands, *D. autumnalis* can be used as a bioindicator for field studies incorporating a systematic monitoring of wild populations and investigating also population parameters in order to avoid irreversible adverse effects.

In farmers from the four localities studied, the main pesticides involved in any type of symptoms related to pesticide poisoning were carbofuran, methamidophos, methomyl, monocrotophos and methyl parathion. Erythrocyte AChE inhibition in farmers exposed to pesticides is a good biomarker of exposure to OP in field studies.

It was demonstrated that an integrated approach using *in situ* assays (based on mortality, growth and biomarkers), WQI and biomonitoring studies with aquatic and terrestrial organisms are of great utility to assess the effects of anthropogenic activities on the environment. The current pesticides used in the agricultural activity of the Palizada River system have a negative impact on human and animal populations from Palizada (Campeche, Mexico). The use of biomarkers in different ecological levels in the tropics is a cost-effective tool for an integrated risk assessment of wetlands.

ACKNOWLEDGMENTS

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Chapter 26

BIOACCUMULATION OF MERCURY IN YELLOWFIN TUNA (*THUNNUS ALBACARES*) FROM ECUADOR AND OTHER WORLDWIDE REGIONS: A COMPARATIVE STUDY

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ABSTRACT

Metals have been recognized as one of the main causes of pollution due to their persistence, toxicity and continuous accumulation in the environment. Metals can be incorporated into the trophic web and be bioaccumulated by organisms causing deleterious effects. Studies of metal accumulation in fish have had a significant increase, due to the risk of contaminated fish consumption by humans. Mercury (Hg) is one of the potentially toxic metals known to beaccumulated via the intake of contaminated fish found at the top of the trophic web. The present chapter aims at comparing reported Hg levels in yellowfin tuna *Thunnus albacares* collected or marketed in different regions of the Pacific Ocean and among other regions of the world. The collected information indicates that the highest levels of Hg found in individuals from the Eastern Pacific Ocean landed in Manta City, Ecuador. This can mean that Hg levels in yellowfin tuna vary depending on fish size, and the Pacific region where it is collected, given that

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specimens collected in this area are larger in size. Despite registering the highest levels of Hg in muscle tissue for this area, the yellowfin tuna from this region can be consumed with moderation.

Keywords: *Thunnus albacares*, mercury, bioaccumulation, human health

INTRODUCTION

Mercury (Hg) contamination is a problem of worldwide concern, since it is a persistent contaminant and is widely distributed in the environment, having either natural or anthropogenic sources (Ordiano-Flores et al., 2012). This metal usually enters the environment in an inorganic form as a result of natural processes (i.e., weathering and/or meteorization of rocks, degasification of the earth's crust, terrestrial and submarine volcanism), and from human activities (e.g., electric and measurement appliance industry, gold extraction, iron and steel industry, chloride-alkali production, fossil fuel burning, and production of fungicides made with Hg) (Wiener et al., 2003).

The World Health Organization (WHO) considers Hg among the top 10 chemicals of "major public health concern." The main form of Hg to which human populations are generally exposed to is methylmercury (MeHg). Once in the environment, Hg can be transformed by bacteria into MeHg, which then bioaccumulates in tissues of fish at the top of the aquatic food web (WHO, 2013). In human beings, MeHg exposure occurs predominantly via the consumption of seafood (including freshwater and marine varieties, and marine mammals) (WHO, 2013). MeHg is a particularly harmful neurotoxin to fetal brain development (UNEP, 2008). Extensive research has demonstrated relationship between exposure in utero and developmental neurotoxicity (e.g., deficits in fine motor skills, language and memory) among populations that consume seafood regularly (CTEM, 2000).

It has been widely recognized that fish ingestion is the main source of Hg exposure in humans. In the case of top predators such as large pelagic fishes, when exposed to metal contamination the risks tend to be exacerbated as many heavy metals tend to accumulate in their bodies at concentrations higher than those found in the environment and in their prey (Damiano et al., 2011; Onsanit et al., 2012). For commercially important fish, the bioaccumulation of metals can have serious implications for human health (Kojadinovic et al., 2006). Such studies have been used to develop health-based reference doses below which no appreciable risk of harm is thought to occur, including the provisional tolerable weekly intake (PTWI), established by the Joint Expert Committee on Food Additives (JECFA) of the Food and Agriculture Organization (FAO) and WHO (UNEP, 2008).

In Ecuador, fishing and fishery products are highly important economic resources and are considered the second most important activity in the country, accounting for almost 12% of total trade (Pro Ecuador, 2013). One of the most important harbors of Ecuador is located in the city of Manta (INP, 2015), known as the tuna capital of the country. The yellowfin tuna (*Thunnus albacares*) is consumed in high quantities and is a commercially relevant resource for national and international markets: Ecuador's tuna catch can reach up to 200,000 tons per year (IATTC, 2014). Given that this species is a top predator, the exposure and accumulation of metals via the food web tend to be intensified and could become a risk to human health (Storelli et al., 2005; Kojadinovic et al., 2007).

The present chapter aims at comparing reported Hg levels in yellowfin tuna *T. albacares* collected in Ecuador, with yellowfin tuna collected or marketed in different regions of the Pacific Ocean and among other regions of the world.

COMPARISON OF MERCURY LEVELS REPORTED FOR *THUNNUS ALBACARES* IN DIFFERENT SITES OF THE PACIFIC OCEAN

Data from all regions of the Pacific Ocean and other oceans around the world were considered in the current review. Regarding the Pacific Ocean, data from four different countries were considered in the chapter: Ecuador, Mexico, USA and Japan. We classified the data according to the different regions of the Pacific Ocean: Equatorial Eastern Pacific, Central Equatorial Pacific, North Eastern Pacific, and North Western Pacific. For all other regions of the Pacific there is a lack of data published. Only mean data of Hg determined in muscle tissue of *T. albacares* during the last ten years were considered, discarding studies in which only a few individuals were studied or those with historical data. Mean size data were also considered when available, due to evidence regarding the relation between Hg levels in muscle tissue and size (Cal et al., 2007; Drevnick et al., 2015).

Data from all regions of the Pacific Ocean demonstrate the tendency of the species *T. albacares* to accumulate high Hg concentrations in muscle tissue (Al-Busaidi et al., 2011) (Figure 1). In general, concentrations of Hg in the muscle tissue reported from the different regions of the Pacific Ocean presented levels below 0.5 ppm, far from the permissible intake limit of 1 ppm established by the European Commission Regulation-EC (2006). Only reported values from the *T. albacares* landed in the port of Manta (Ecuador) presented values above this permissible intake limit (Araújo and Cedeño-Macias, 2016); however, the reported values presented a wide range of deviation. The latter may be due to the wide range of sizes of the individuals sampled. It is therefore possible that the high values of Hg reported by Araújo and Cedeño-Macias (2016) in Ecuador correspond to the largest *T. albacares* individuals for the Pacific Ocean. This corroborates previous evidence indicating that the bioaccumulation of Hg in muscle tissue is correlated to the size of this species (Cal et al., 2007; Drevnick et al., 2015). An important number of studies performed in the Pacific Ocean did not report data of fish size (Figure 1) which makes it difficult to compare the ratio of bioaccumulation depending on the size range. We therefore recommend standardizing the method of collecting data such that all reports present not only the Hg values, but also the size and weight of animals sampled.

WORLDWIDE COMPARISON OF MERCURY LEVELS REPORTED FOR *THUNNUS ALBACARES* IN MANTA (ECUADOR)

Figure 2 compares the mean values of Hg in muscle tissue of *T. albacares* reported in Manta (Ecuador) (Araújo and Cedeño-Macias, 2016) to those reported in other regions of the world. Kojadinovic et al. (2007) reported high values of Hg (1.15 ppm) for the Reunion Island Region in the Indic Ocean. These values of Hg are similar to those reported by Araújo and Cedeño-Macias (2016) for the Pacific Ocean, being these two sites where Hg levels were found to be above the permissible intake limit established by the European Commission Regulation-EC (2006). Mean Hg values reported in the Atlantic Ocean for this species are similar among sites: 0.35 ppm (Besada et al., 2006); 0.30 ppm (Teffer et al., 2014); 0.25 ppm (Adams, 2014). In the Indic Ocean, the lowest mean value of Hg (0.30 ppm) was reported by Jinadasa et al., (2014), in the Sri Lanka region. Thus, the consumption of yellowfin tuna, in general, is safe for all the ports of the world considered here.

T. albacares fishery is a highly important economic resource worldwide, so Hg contamination in this species is an issue of special concern for human health. The risk associated to metal intake on a weekly basis was therefore estimated for each region of the world by calculating the Maximum Quantity for Weekly Consumption of *T. albacares* muscle (MQWC; grams per week; Araújo and Cedeño-Macias, 2016) via the following equation: *MQWC=PTWI* BW/*[Hg], where *PTWI* is the Provisional Tolerable Weekly Intake of 5 µg kg^{-1}, recommended by FAO/WHO (2010), *BW* is the Body Weight considered as 70 kg (Araújo and Cedeño-Macias, 2016), and [Hg] is the mean metal concentration determined in fish muscle. In order to calculate a value of PTWI for an average body weight of 70 kg, the mean value of Hg concentration was substituted by the Permissible Intake Limit (PIL) of 1 ppm established by the European Commission Regulation-EC (2006). This value obtained is 350 g of muscle per week (PTWI=70 kg* 5 µg kg^{-1}/1 mg kg^{-1}), and it was considered as a reference value to compare the rest of data from around the world(Figure 3).

Most of the data reviewed in the current chapter are under the PIL of 1 ppm established by the European Commission Regulation-EC (2006), as shown in Figures 1 and 2. Thus, consumption of *T. albacares* from most regions of the world is not considered a threat to human health. In order to consider the maximum quantities suggested for consumption per week by an adult of 70 kg, we only included data under 800 g per week, given that countries presenting high consumption of yellowfin tuna as Mexico, the average national consumption is only 200 g per week (Ordiano-Flores et al., 2011). Furthermore, values over 800 g per week are far enough from the 350 g per week recommended by FAO/WHO (2010) as PTWI value. In this way, the weekly quantity of the yellowfin tuna marketed in the Equatorial Eastern Pacific Ocean (landed in Mexico and Ecuador) and in the Western Indian Ocean (landed in Madagascar) considered safe for consumption according to the present study was less than that recommended by FAO/WHO (2010). Nevertheless, according to mean Hg values, the quantity per week for consumption in these regions is over the average national consumption in Mexico, thus even for these places, the moderate consumption under these limits offered in Figure 3 could be considered safe for human health. It should be noted that values of safe consumption for special populations such as children or pregnant women were not considered in the current chapter. Nevertheless, permissible limits should be considered as a suggested value and not as an absolute one.

In any case, *T. albacares* is known to migrate over large distances, thus metal levels found in muscle tissue might integrate contamination from several different areas and not only from the site of capture (Kojadinovic et al., 2007; Adams, 2009; Chen et al., 2014). Future monitoring studies in order to determine the levels of Hg in *T. albacares* from the Eastern Equatorial Pacific Ocean and the Western Indian Ocean are recommended, in order to identify the sources of these high levels.

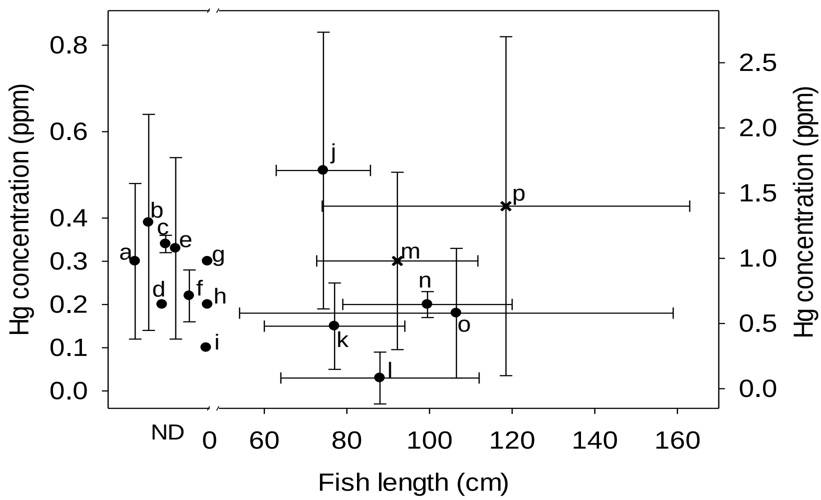

Figure 1. Average levels of Hg bioaccumulated in *Thunnus albacares* and respective fish length (when available) reported in the literature for different regions of the Pacific Ocean. Black dots correspond to the left Y axis, and x marks correspond to the right Y axis. ND means data of fish length not available. Vertical lines of each data represent the standard deviation for mean values of Hg (when available) and horizontal lines represent the standard deviation for mean size values (when available). a: Kaneko and Ralston, 2007; b: Yamashita et al., 2009; c: Drevnick et al., 2015; d, g, h and i: Ferris and Essington, 2011; e: Hisamichi et al., 2010; f: Boush and Thieleke, 1983; j and m: Ordiano-Flores et al., 2011; k: Ordiano-Flores et al., 2012; l: García-Hernández et al., 2007; n: Burger et al., 2011; o: Cal et al., 2007; p: Araújo and Cedeño-Macias, 2016.

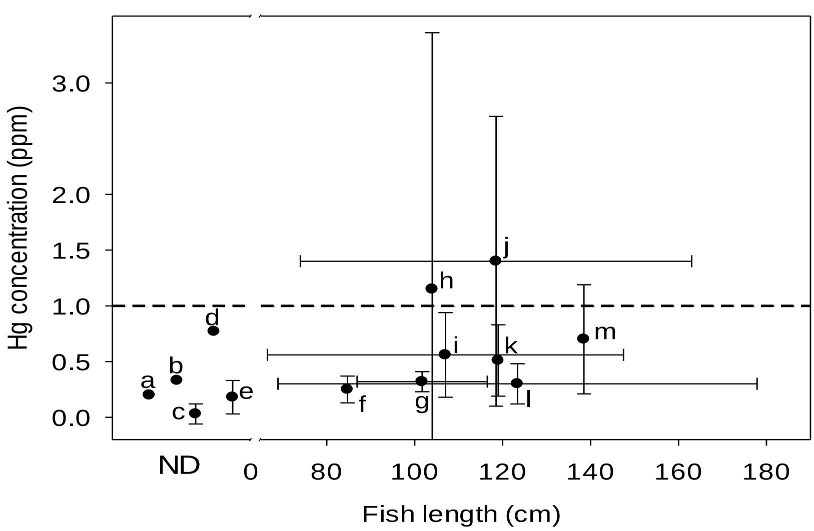

Figure 2. Average levels (black dots) of Hg bioaccumulated in *Thunnus albacares* and respective fish length reported in the literature for different regions of the world. ND means data of fish length not available. Vertical lines of each data means standard deviation for mean values of Hg (when available), horizontal lines mean standard deviation for mean size values (when available). Dashed line at 1 ppm is the Permissible Intake Limit of 1 ppm established by the European Commission Regulation-EC (2006). a: Burger and Gochfeld, 2011; b: Besada et al., 2006; c: Al-Busaidi et al., 2011; d: Bosch et al., 2015; e: Cal et al., 2007; f: Adams, 2014; g: Teffer et al., 2014; h: Kojadinovic et al., 2007; i: Kojadinovic et al., 2007; j: Araújo and Cedeño-Macias, 2016; k: Kojadinovic et al., 2006; l: Jinadasa et al., 2014, m: Kojadinovic et al., 2006.

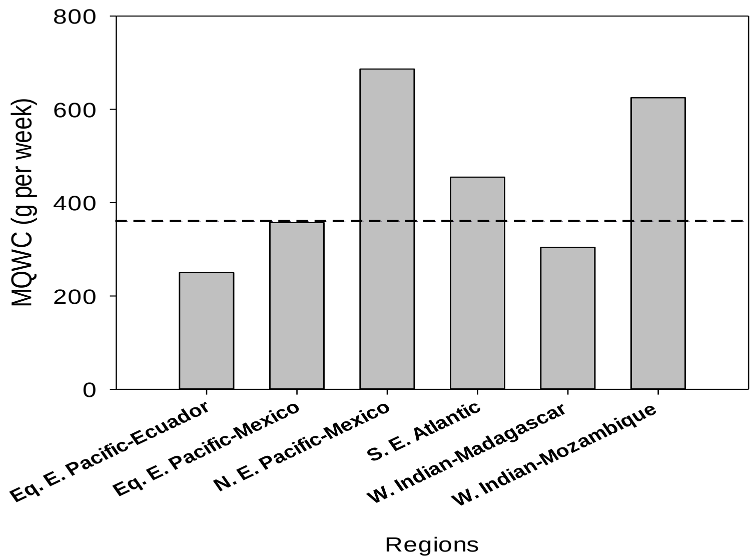

Figure 3. Maximum quantity for weekly consumption (MQWC, in g per week) of *Thunnus albacares* (yellowfin tuna) muscle by a 70 kg adult, calculated individually for each of the different regions considered: Equatorial Eastern Pacific-Ecuador (Araújo and Cedeño-Macias, 2016); Equatorial Eastern Pacific-Mexico and North Eastern Pacific (Ordiano-Flores et al., 2011); South Eastern Atlantic (Bosch et al., 2015); Western Indian-Madagascar and Western Indian-Mozambique (Kodajinovic et al., 2007). Dashed line at 350 g per week means the reference value recommended by FAO/WHO (2010).

CONCLUSION

In general, *T. albacares* landed in different regions of the Pacific Ocean can be safely consumed given that the reported Hg levels in muscle tissue are under the permissible intake limit of 1 ppm established by the European Commission Regulation-EC (2006). Only *T. albacares* landed in Ecuador (Araújo and Cerdeño-Macias, 2016) and in Madagascar (Kojadinovic et al., 2007) presented levels of Hg above this permissible intake limit, although it could be considered as safe for human consumption considering the provisional tolerable weekly intake (PTWI) value recommended by FAO/WHO. Future studies regarding bioaccumulation of Hg in Eastern Equatorial Pacific *T. albacares* landed in Ecuador are suggested in order to identify the reasons of the high levels of Hg found in this species.

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Chapter 27

LICHENS AND AIR QUALITY IN LATIN AMERICA

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ABSTRACT

Lichens can be used as bioindicators/biomonitors for research and control of air pollution. They are valid instruments for assessing air quality affected by emissions from mobile or fixed sources. In this context, this chapter gathers information about the progress of research on the use of lichens as biomonitors of air quality in Latin America (with emphasis on Venezuela). This review discusses the most important lines in the state of knowledge in this field, evaluating the methodological applications (sampling, physical and chemical treatment, instrumental analysis techniques and future prospects) of lichens, and their advantages/disadvantages compared to conventional research methods. The review is divided in three parts, summarizing the methodologies and experiences of using lichens as bioindicators/biomonitors, with special emphasis on the use of lichen physiology, their ability to accumulate heavy metals and polycyclic aromatic hydrocarbons (PAHs) over time and the analysis of lichen community, using Index of Atmospheric Purity and the Environmental Classification Factor.

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Keywords: bioindicators, biomonitors, Latin America, heavy metals, PAHs, IAP

INTRODUCTION: LICHENS AS BIOINDICATORS AND BIOMONITORS

Bioindicators are organisms that manifest particular symptoms in response to environmental changes, generally determined in a quantitative way. On the other hand, biomonitors refers to such organisms, their distribution or population studied over time and compared with standard values or base-line surveys, taking into account the deviations from the expected behavior (Hawksworth et al., 2005). Even though both concepts are different, in this chapter the word bioindicators will be used referring to both unless the term biomonitors is specified.

Since Grindon (1859) observed the gradual disappearance of lichens in Manchester (England), and then Nylander (1866) warned that lichens of the Jardin du Luxembourg (France) were disappearing probably due to air pollution, much research has been done concerning the use of lichenized fungi as bioindicators of air pollution.

Lichens are by definition a symbiosis of two organisms, a fungus (denominated mycobiont) and a photosynthetic organism (photobiont), which can be either a green algae or a cyanobacteria (Nash, 2008). Unlike higher plants, lichens have no roots or a well-defined cuticle, which emphasizes its applicability in monitoring purposes. These organisms are strongly dependent on dry depositions as moist mineral nutrients on the thallus surface. In addition, the lichen surface and structure facilitate interception and retention of particles (Szczepaniak and Biziuk, 2003).

Vareschi's publication (1953) is the first study on the use of lichens as bioindicators of air pollution in Latin America. By that time, the correlation between the presence of lichens and levels of air pollution was starting to be accepted worldwide. Since then, Latin America has demonstrated interest, and various institutions have committed to develop animportant amount of research on the subject.

This chapter gathers information about the progress of research on the use of lichens as bioindicators and biomonitors of air quality in Latin America with emphasis in Venezuela, evaluating lichen methodological applications and their advantages/disadvantages compared to conventional research methods.

PHYSIOLOGICAL RESPONSE OF LICHENS TO POLLUTANTS: WHAT HAS BEEN DONE IN LATIN AMERICA?

Because of their particular biological conditions, lichens are affected by numerous factors, especially atmospheric pollution whose physiological effects have been widely studied. Indeed, using lichen physiological responses as indicators of pollution has been a major resource for the assessment of air quality. Despite the fact that Latin America is one of the regions with less effort on this subject, in the last 20 years research teams from Argentina, Mexico and Brazil have been working to elucidate how lichen physiological parameters are influenced by pollutants.

Since 1994 in Argentina, studies involving transplanted lichens of the genera *Canomaculina* (González and Pignata, 2000; González et al., 2003), *Parmotrema* (Cañas et al., 1997), *Punctelia* (González and Pignata, 1994, 1997; González et al., 2003), *Ramalina* (Levin and Pignata, 1995; González et al., 1996, 1998, 2003; Pignata et al., 2004; Rodriguez et al., 2007; Bermudez et al., 2009) and *Usnea* (Carreras et al., 1998, 2005; Carreras and Pignata, 2002, 2007; Rodriguez et al., 2007; González et al., 2012), have been used to quantify and compare the effects of pollutants, especially heavy metals, produced from industrial activity and traffic. In Brazil, *Parmotrema tinctorum* and *Teloschistes exilis* have been used to assess how mutagenic and cytotoxic agents cause morphophysiological alterations in lichen thalli (Käffer et al., 2012). On the other hand, in Mexico the influence of chronic air pollution on tolerance and production of protective secondary metabolites in *Ramalina asahinae* and *Parmotrema stuppeum*, using *in situ* and experimental reactivity data have been studied (Valencia-Islas et al., 2007).

Most of these studies emphasize on measuring parameters such as chlorophyll and phaeophytin degradation, malondialdehyde (MDA), sulphur concentration, conjugated dienes, soluble proteins, phaeophytina/chlorophyll a, chlorophyll b/chlorophyll a and fresh/dry weight ratios; even some pollution indices have been calculated. These physiological parameters are usually compared with pollutant levels in the environment, accumulation of heavy metals, suspended particles and meteorological conditions, proving lichen efficiency as indicators of human health (Carreras and Pignata, 2001; Carreras et al., 2009a,b).

The general results of these studies showed that an increase of heavy metal concentrations negatively affects photosynthetic and non-photosynthetic pigment concentrations, as well as photosynthetic processes, synthesis of chlorophyll molecules and degradation of secondary metabolites. Also membrane integrity and selective permeability is affected by pollutants due to modifications of membrane lipids and protein structure. Other effects are reduction of percentage of live cell, carbon monoxide and chlorophyll values, as well as an increase of plasmolyzed cells, antioxidant activity, antiradical power of secondary metabolites and morpho-anatomical changes (e.g., chlorosis, necrosis, and medulla exposition).

LICHENS AS BIOMONITORS OF HEAVY METALS AND PAHS

Lichens incorporate environmental pollutants such as heavy metals and polycyclic aromatic hydrocarbons (PAHs), among others, and can be used as indicators of the bioavailability of such substances over time, allowing in some cases to compare pollution levels in different geographic areas (Conti and Cecchetti, 2001). In this sense, several countries (Argentina, Slovenia, Spain, USA, France, Italy, Portugal, among others) have continued to develop research in order to examine in detail the potential of lichens as bioindicators of the presence of heavy metals and PAHs in air (Jeran et al., 2002; Carreras et al., 2009a). Countries such as Brazil and Venezuela have recently joined the study, especially in urban areas (Saiki et al., 2006; Fernandez et al., 2011; Gomez et al., 2013a).

HEAVY METALS

A large number of studies have used lichens as bioindicators of heavy metals (e.g., Branquinho et al., 1999; Conti and Cecchetii, 2001; Carreras and Pignata, 2002; Jeran et al., 2002; Scerbo et al., 2002; Garty et al., 2003; Loppi et al., 2004; Tuncel et al., 2004; Williamson et al., 2004; Monnet et al., 2006; Bergamaschi et al., 2007; Brunialti and Frati, 2007; Basile et al., 2008; Godinho et al., 2008; Sorbo et al., 2009). Latin America has not been left behind in this area of research (Marcano et al., 1996; Carreras and Pignata, 2002, 2007; Carreras et al., 2005; Quijada, 2006; Bermúdez et al., 2009; Käffer et al., 2012; Hurtado et al., 2013).

The accumulation of heavy metals in lichen thalli is one of the aspects of lichen biology that has been most studied in Latin America (Carreras and Pignata, 2001; Carreras et al., 2009a,b) and worldwide. It usually depends on many factors such as morphology, ion exchange properties, type of reproduction, etc. (Carreras et al., 2009a,b). Therefore, the degree of tolerance to heavy metals is characteristic of each lichen species. A well-documented phenomenon is their ability to tolerate high elemental levels thanks to a number of mechanisms to avoid toxicity, although only a few have been thoroughly studied for some elements.

Research on the detection of atmospheric heavy metals using lichens in Latin America follows a similar methodology worldwide. The most important contributions from Latin America in this field have been the effects of heavy metals on the physiology of the lichenized fungi (e.g., Carreras and Pignata, 2001; Carreras et al., 2009a,b). For this reason more emphasis will be given to the use of lichens as biomonitors of PAH.

POLYCYCLIC AROMATIC HYDROCARBONS

Unlike the large number of studies which use lichens as bioindicators of heavy metals, little research has been reportedon their use as bioindicators of PAHs. PAHs are a group of organic compounds known for their carcinogenic, mutagenic and teratogenic effects. They are a particular family of corresponding hydrocarbon compounds containing a number of benzene rings fused by two or more carbon atoms. At an international level, up to 2013 there are less than 30 publications relating to the determination of this group of compounds in lichens (Gomez et al., 2013a), being eight pioneers in this research: Spain, Italy, India, Canada, Poland, Portugal, Sweden and Venezuela.

Sampling

In Venezuela, studies have taken place only in urban areas, taking as criteria for selecting sampling stations different levels of traffic flow on the main streets and avenues or proximities to main highways. Motor vehicles are one of the main sources of PAHs (Fernandez et al., 2011).

In passive monitoring, lichens are collected directly from where they are growing in the study area. This is the most common method until now, because most of these studies are preliminary assessments in which one of the objectives is to test the feasibility of using lichens as biomonitors of PAHs in the selected locations (Fernandez et al., 2011). Only one study in Latin America (Gomez et al., 2013b) has transplanted lichens from their natural ecosystem (reference locality) to an urban area.

Height at which the lichens are sampled or transplanted plays an important role. This generally varies between 1.0-3.5 m above the ground (Fernandez et al., 2011), seeking to avoid the influence of soil particles that probably also contains PAHs. Another reason is that lichens located just above the floor could be overprotected from atmospheric depositions by the existence of weeds.

In active monitoring (transplantation method), procedures should be standardized very well as many other factors can affect the response of lichens to pollutants in an area where lichens usually do not grow (Gomez et al., 2013b). In this sense, it is advisable to protect them once transplanted from direct solar radiation. It should be ensured that the exposed surface area of the biomonitors is as large and homogeneous as possible in all the studied locations (Gomez et al., 2013b).

Lichen Species Frequently Used

Foliose and fruticose lichens are the most used in environmental studies (Owczarek et al., 2001; Migaszewski et al., 2002; Guidotti et al., 2003, 2009; Blasco et al., 2006, 2007, 2008; Domeño et al., 2006; Naeth and Wilkinson, 2008; Augusto et al., 2009; Shukla and Upreti, 2009; Shukla et al., 2010; Fernandez et al., 2011; Gomezet al., 2013a) mainly due to the fact that they are easily removed from the substrate, compared to crustose lichens, which grow much more attached to their phorophyte. 24 different species of lichens have been used for PAH biomonitoring. In Venezuela only two species have been tested: *Pyxine cocois* (Sw.) Nyl. And *Parmotrema sancti-angelii* (Lynge) Hale (Fernandez et al., 2011; Gomez et al., 2013b).

Physical Treatment of the Samples

In the period of time between sampling and the physical and chemical processing in the laboratory, samples should be properly preserved at low temperature in order to avoid the maximum loss of the more volatile PAHs. They should also be protected from solar radiation to prevent photolysis of the compounds of interest. Different methods have been used to maintain these conditions. In some studies, lichens are kept wrapped in aluminum foil and at low temperatures, while in others the samples are kept at -20 °C in amber glass bottles until analysis (Fernandez et al., 2011).

The physical treatment of the samples has so far not been standardized, hence each author reports different steps for this stage of the analysis. Guidotti et al. (2003, 2009) dried the samples in an oven at 40 °C for 48 h; samples were cleaned by removing foreign materials such as dust, leaf and bark remnants, insects and pebbles using a microscope. They were then pulverized in an agate mortar; this procedure was also followed by Fernandez et al. (2011) and Gomez et al. (2013b), who additionally employed liquid nitrogen to pulverize the samples. Fernandez et al. (2011) rinsed the dried lichens using deionized water and gentle stirring. However, Gomez et al. (2013b) recommends not following this step since some significant PAHs may be lost in the process.

Despite there being no standardized protocol for the physical treatment of the samples, most authors include sample cleaning, pulverization or homogenization and drying of the samples. This is because the presence of materials other than the lichen thalli, sample heterogeneity and humidity can introduce a significant variance and/or error in the dry weight and the determination of PAHs.

Extraction

The extraction process is essentially based on placing the sample in contact with a suitable solvent. The mixture of the solid sample with the liquid solvent is then subjected to a series of treatments of varying intensity, ranging from simple stirring to critical pressure and temperature conditions, depending on the strength of the interaction between the analyte and the matrix (Blasco, 2008).

The conventional extraction method for PAHs in environmental samples has been the use of the Soxhlet extractor (Naeth and Wilkinson, 2008; Augusto et al., 2009; Shukla and Upreti, 2009; Shukla et al., 2010), taking as main reference the 3540 method of the United States-Environmental Protection Agency (US-EPA) (16-24 h, 300 mL of solvent).

Gomez et al. (2013b) and Fernandez et al. (2011) used a static ultrasound assisted extraction technique. The choice for this was made taking into account the advantages it offers in terms of time and amount of solvent used, compared to conventional extraction methods (Soxhlet). Fernandez et al. (2011) used 30 mL of cyclohexane: dichloromethane (4:1 v/v) as a solvent for 2 g of sample, employing an ultrasonic bath at room temperature for 30 min and performing the method twice with the same residual solid, subsequently combining the extracts. Gomez et al. (2013b) succeeded in reducing extraction time to 15 min by using a mixture of hexane:dichloromethane (3:2 v/v) as a solvent, for the same amount of solid and solvent employed by Fernandez et al. (2011).

The most significant contribution in terms of extraction time, quantity of solvent and sample mass required for quantification of PAHs was provided by Domeño et al. (2006), who in order to optimize the treatment step of the sample, compared three different organic extraction methods: DSASE (Dynamic sonication-Assisted Extraction Method), Soxhlet extraction and static ultrasound assisted extraction. The same amount of sample (0.2 g of lichen) and solvent (hexane) were used. They found that all three techniques have similar recovery rates for the 16 PAHs listed by the US-EPA in the priority pollutant list (US-EPA, 2000), and that the main advantage provided by the DSASE technique is in terms of time and solvent consumption. This solvent comparison was also done by Gomez et al. (2013b), agreeing with Domeño et al. (2006) in that hexane is more efficient in extracting the compounds of interest (aromatic), besides being much more selective than dichloromethane, which contributes to reducing interference at the moment of instrumental analysis.

Purification or Cleaning of the Aromatic Fraction

For the cleaning or purification step of PAHs, Gomez et al. (2013b) and Fernandez et al. (2011) used the method 3630 of the US-EPA (1996), which consists of preparing a silica gel column to purify nonpolar substances. These authors performed the purification or separation of the PAH from other organic compounds using activated silica gel as the stationary phase. This purification technique is by far the most efficient in terms of time and low consumption of solvent in comparison to the conventional method of column chromatography.

Instrumental Analysis Techniques

The characterization and quantification of PAH is performed by HPLC, coupled with fluorescence detector and/or UV-visible, and gas chromatography-mass spectrometry (GC/MS). Both techniques allow separation of a large number of molecules with similar structural features, which makes them ideal in this type of analysis. PAHs commonly quantified are the US-EPAs selection of 16 major pollutants: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, chrysene, benzo [a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h] anthracene, benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene.

Augusto et al. (2009), Shukla and Upreti (2009), Shukla et al. (2010), Fernandez et al. (2011), and Gomez et al. (2013b), used HPLC for the quantification of PAHs, based on the US-EPA 8310 method (Bursey, 1991).

Fernandez et al. (2011) and Gomez et al. (2013b) used the UV-visible detector for the quantification of all studied PAHs, selecting a wavelength of 254 nm as has been used by many others (Shukla and Upreti, 2009; Shukla et al., 2010). They also used a wavelength of 208 nm, because low molecular weight PAHs generally exhibit greater sensitivity at 208 nm and higher molecular weight at 254 nm. However, in the case of fluoranthene, dibenzo[a,h]anthracene and benzo[g,h,i]perylene, sensitivity was higher at 208 nm. It is important to say that detection by UV-visible has numerous disadvantages in terms of selectivity and sensitivity and in not being able to discriminate matrix interferences, especially in complex matrices such as lichens. Moreover, the fluorescence detector is more selective (many matrix components are not fluorescent) and sensitive. Gomez et al. (2013b) used an excitation wavelength of 340 nm and emission of 425 nm and Fernandez et al. (2011) an excitation wavelength of 375 nm and emission of 425 nm. However, not all commonly studied PAHs are fluorescent (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, chrysene and indeno[1,2,3-cd]pyrene are not fluorescent), so Augustus et al. (2009), Gomez et al. (2013b) and Fernandez et al. (2011) combined both detectors (UV-visible and fluorescence) for quantification of PAHs.

Gomez et al. (2013b) mention that one of the main advantages shown by HPLC with respect to GC/MS is analysis time, taking longer with GC/MS. On the other hand, greater selectivity and better resolution of the peaks is shown by GC/MS, facilitating the elucidation of isomers with much more efficiency, compared to the HPLC. Additionally, GC/MS allows the identification of PAHs that do not fluoresce from the ones that do using only one detector. In this sense, Gomez et al. (2013b) recommends instrumental techniques used jointly for this type of studies.

Comparison between the Cumulative Capacity of Different Species of Lichens and Other Environmental Samples

Foliose lichens show the highest levels of accumulation of PAH (Gomez et al., 2013a). It can also be said that more accumulation is seen in passive sampling than in lichens that were transplanted from a reference site to the study area, which may be related directly with the length of time during which these organisms have been exposed to the atmosphere being studied, as well as their adaptation to it (Gomez et al., 2013a).

Most studies indicate that lichens tend to have a profile of PAHs rich in compounds of 2, 3 and 4 rings associated with the gas phase, in contrast to compounds of 5 and 6 rings, associated with atmospheric particles (Owczarek et al., 2001; Migaszewski et al., 2002; Blasco et al., 2006, 2008; Augusto et al., 2009; Guidotti et al., 2003, 2009; Shukla and Upreti, 2009; Shukla et al., 2010; Fernandez et al., 2011). However, Blasco (2008), Shukla et al. (2010), and Gomez et al. (2013b) reveal that the profile will depend in many cases on the morphology of the lichen employed, noting that fruticose and crustose-squamulose lichen morphologies tend to preferably accumulate PAH associated with the gas phase, while those with foliose morphology tend to retain on their surface more of those PAHs associated to particles (monitoring the full spectrum of PAHs). This aspect has implications on choosing the appropriate species according to the specific objective of the study. In this sense, the most resistant lichens to air pollution will allow a monitoring of a more toxic mixture of PAHs.

Blasco et al. (2006) found that the concentrations of PAHs in atmospheric particles are 2 orders of magnitude higher than concentrations of PAHs in lichens. Blasco et al. (2006) and Augusto et al. (2009) agree that in both profiles (atmospheric particles and lichens) 4-ring PAHs are the most abundant. Blasco et al. (2006) conclude that there is a good correlation between the levels of PAHs found in lichens and in samples of air at different sampling stations.

Blasco et al. (2006) and Augusto et al. (2009) indicate a high content of 2 and 3 ring PAHsin lichens, while atmospheric particles have a higher content of 5- and 6-ring PAHs, which is directly related to 5- and 6-rings PAHs that are found in atmospheric particles, while the 2 and 3 ringsin the gas phase. Shukla et al. (2010) and Gomez et al. (2013b) indicate that the secondary metabolite biosynthesis by lichens (commonly depsids and depsidones) and other lichen substances consisting of -OH radicals-rich rings provide the hydroxy group for adduct formation. This would explain the high accumulation of compounds with 2 and 3 rings in lichens. The lichens used by Fernandez et al. (2011) and Gomez et al. (2013b) were *P. cocois* and *P. sancti-angeli*. *P. cocois* contains mainly lichexantone, which comes from the same acetate-polymalonate pathway as depsids and depsidones, and also contains $^{-}$OH radicals. *P. sancti-angeli* contains gyrophoric acid which is a depside. Thus, both species concur with the assumption of Shukla et al. (2010) and Gomez et al. (2013b).

Blasco et al. (2006) claim that both sampling techniques (biomonitoring and conventional monitoring without organisms) provide complementary information because lichens record information of air pollution for prolonged periods (in an integrated manner), whereas sampling of particles gives information generated during the sampling period (specific moment in time).

Blasco et al. (2008) determined the existence of a correlation between the Index of Atmospheric Purity (IAP) and PAH concentration. The combination of the two methods indicates that low values of the IAP and therefore little lichen biota, may be associated with the presence of mixtures of highly toxic PAHs (phenanthrene, fluoranthene, pyrene, dibenzo[a,h]anthracene and benzo[g,hi]perylene).

Future Prospects for This Kind of Environmental Studies

Although the last decade has shown much progress in the study of lichens as biomonitors of atmospheric PAH, there are still important issues to be clarified and researched. Some of these aspects can be listed as follows:

1. Standardize as rigorously as possible the sampling stage, with special emphasis on the care that should be taken in transplanting lichens from natural ecosystems to a contaminated area (active monitoring sampling).
2. Standardize methodologies for extraction and purification of PAHs in lichen matrices internationally.
3. Develop a certified reference material for PAHs in lichens.
4. Optimize conditions for separation and quantification of PAHs in lichens using HPLC and GC/MS techniques.
5. Conduct studies leading to the selection of at least one PAH pollution tracer.
6. Establish standards at regional, national and local scales for maximum permissible limits for such compounds in lichens.

BACK TO BASICS: USING LICHEN COMMUNITIES AS ECOLOGICAL INDICATORS OF ATMOSPHERIC QUALITY

A Latin American Perspective

In the preceding sections of this chapter, we explore the chemical and physiological way to evaluate how pollutants affect the inner performance and chemical behavior of lichen species. Contaminants not only affect the physiological processes within the individual, but also affect how individuals respond to the environment and thus affecting the species' local distribution. Therefore, the presence of pollutants can influence the composition of lichen communities. For this reason the study of lichen ecology and community composition is a well-used resource in assessment of atmospheric quality.

The lichen ecological approach on atmospheric quality assessment has two different lines. First, the observational and qualitative framework, which uses richness data, qualitatively established indicator species and general descriptions of lichen community. Second, the quantitative approach, based on a numerical index of air quality and statistical proceedings that allow a quantitative way of indicator species establishment and community composition evaluation (van Haluwyn and van Herk, 2002). The second line includes the characterization of lichen communities as indicators of some particular condition or pollution level, instead of one single indicator species.

LeBlanc and De Sloover (1970) formalized the first mathematical approach to calculate an IAP, in the form of a linear model that takes intoaccount the major factors that vary with the influence of pollution: lichen richness, a scale of frequency and cover and the sensitivity degree of each lichen species. This quantitative method has been widely used despite the major disadvantage of reduced precision due to the use of a scale of lichen frequency and cover data. Naturally, it has been modified several times in order to contextualize its application to each case (Nimis and Purvis, 2002). Nonetheless, the original model gives a good estimation of pollution levels.

In the tropics these kinds of studies were neglected for a long time. Nevertheless, it is noteworthy that even before the mathematical development of IAP, Vareschi (1953) used the richness, frequency and morphology of lichens to assess the influence of forests and urban parks on the air quality of Caracas, Venezuela. Twenty years later, Vareschi and Moreno (1973) made the same assessment once again in Caracas.

After the mid-80's other countries in Latin America began to apply this methodology: García and Rubiano (1984) and Rubiano (1986, 1987) made the first approach in Colombia. Other countries such as Argentina (González and Pignata, 1994) and Peru (Tovar and Aguinaga, 1994) began to consider lichens as a tool to assess the air pollution in the early 90's. The second half of the 90's saw an explosion in the use of lichens as bioindicators in Latin America, with a particular emphasis on chemicals and physiological protocols. The ecological approach was forgotten, although a few papers were published on this subject (Marcano et al., 1996; Estrabou, 1998).

In the early 2000's, the ecological approach was again prevalent with a high diversification of methods. Not only the richness and cover of lichen thalli were used, but also the type of reproductive structures and the morphological changes detected on the thalli (Monge-Nájera et al., 2002; Rubiano, 2002; Estrabou et al., 2004; García, 2004; Canseco et al., 2006; Rubiano and Chaparro, 2006; Santoni and Litjeroff, 2006; Anze et al., 2007; Martins et al., 2008; Riquelme, 2008; Calvelo et al., 2009; Litjeroff et al., 2009; Jaramillo and Botero, 2010), although some of them still remain as unpublished research.

In the last six years, there has been an increase of research in ecological assessment with the use of lichen communities as bioindicators of pollution in Latin America (e.g., Darré, 2011; Estrabouet al., 2011; Käffer et al.,2011; Neurhor et al., 2011, 2013; Simijacaet al., 2011, 2014; Quispeet al., 2013; Valois and Mosquera, 2014, Díaz et al., 2016), with an improvement in the use of statistical tools such as multivariate analysis to establish indicator species of every pollution level. One of the most important advances was the proposal of a new index to evaluate atmospheric quality. In order to increase the sensitivity for the detection and categorization of pollution zones, Käffer et al. (2011) proposed a mathematical factor that complements IAP. The environmental classification factor (ECF) assigns scales of cover to different growth forms, assuming a major sensitive scale of fructicose lichens, while crustose lichens seem to be more tolerant; the foliose lichens are somewhat moderately sensitive (Wetmore, 1981; Käffer et al.,2011).

The general sampling methods applied in Latin America vary between studies and are usually according to the particular conditions of each case. Although the most used index (i.e., IAP) only requires lichen richness and a scale of cover and frequency, most researchers prefer to measure lichen cover precisely, because it provides better elements for statistical treatment. Trees are the most typical substrate studied and free sampling, grids of different sizes and shapes, horizontal or vertical orientation rubber bands, have been applied. In almost every study developed in Latin America, the sampling unit is the tree and the number of sampling units varies according to every study. On the other hand, the ecological approaches of bioindication studies in Latin America have been assessed with both quantitative (e.g., IAP) and qualitative methods, with a major emphasis on richness and its negative correlation with pollution.

The pool of results is much more diverse than the methods applied, since every locality shows a different lichen community composition and different abiotic/biotic conditions. Even tolerant/sensitive taxonomic species identity, richness and the ranges of IAP vary between studies. In fact, the general idea of the sensitivity level of growth forms is in discussion, such that in some unpolluted areas great richness of crustose lichens was detected (Rubiano, 1987) and in other polluted areas fructicose lichens represented tolerant taxa (Rubiano, 1986).

This suggests that none of these parameters/factors are standard indicators. Composition of neighboring lichen communities, dispersion/colonization/establishment capability of species, lichen growth rate and, of course, different limiting factors besides pollution (e.g., latitude, altitude) are responsible for the overall species composition. This means that the complexity of the application of lichen community ecology to bioindication analysis can only be reliable if we have full understanding of every context, especially in tropical regions (van Haluwyn and van Herk, 2002).

However, two common patterns emerge. First, whatever the context is, it has a general decrease of lichen richness in polluted areas. Second, Physciaceae (e.g., *Hyperphyscia*, *Dirinaria* and *Pyxine*) and Parmeliaceae (e.g., *Canoparmelia* and *Parmotrema*) are the most common and richest families in polluted areas, especially in those with high influence of air pollution produced by intense traffic flow and industries. On the other hand, sensitive species are very different between studies.

Biomonitoring of Air Pollution in Venezuela: The Ecological Assessment

Despite that Venezuela was the pioneer country in the use of lichen communities as indicators of air quality in Latin America (Vareschi, 1953; Vareschi and Moreno, 1973), these methods were almost forgotten for more than 40 years, because of the emphasis on chemical evaluations. Even though in the last years great development has been made in the fields of taxonomy and ecology of lichens in the country, there is a major gap in national biomonitoring studies since the 70s.

The first two approaches of this nature done in Venezuela were exclusively based on richness analysis. Vareschi (1953) categorized Caracas into three zone types: some isolated areas with high air pollution (lichen deserts), a large area of low air pollution (almost the entire city) and normal, peripheral areas with a high level of air purity and exuberant lichens. Nevertheless, Vareschi and Moreno (1973) warned about the spread of lichen deserts to almost the entire city just 20 years after the first assessment. Since then, only chemical approaches have been made.

Currently, the application of lichen community ecology to biomonitoring of air pollution is a priority objective of Venezuelan researchers. In this context, the first national bioindication network was created, aiming at standardizing the protocols of sampling and monitoring air quality in Venezuela. At present, this project is being applied in Caracas, with more than 130 monitoring stations (and other cities such as San Cristóbal and Mérida), and will start in other cities such as Puerto Ordaz, Barquisimeto, Maracaibo, Maracay, and

Porlamar. The general purpose of this national network includes standardizing the following factors:

1. *Substrate: species and general conditions*. Identify those tree species with high frequency and good general conditions that allow the colonization and establishment of lichen communities, limiting the study to individuals with circumference higher than 40 cm at breast height.
2. *The protocol on measuring richness and cover*. It is recommended the use of the rectangular grid protocol (30 × 20 cm). The grid should be located between 1.5 and 1.8 m from the tree base and in the zone with highest lichen richness and cover.
3. *Use of quantitative methods.* The project emphasizes on the use of IAP (LeBlanc and De Sloover, 1970) and ECF (Käffer et al., 2011), using the mean cover percentage and frequency to assign the scales of cover and frequency.

Finally, the national network of bioindication intends to facilitate the general analysis of the local projects and visualization of national data in a free-access online national data base, providing online tools designed to calculate each index for atmospheric quality assess.

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Chapter 28

LICHENS AS BIOINDICATORS OF AIR QUALITY IN MINING AREAS OF LATIN AMERICA, WITH SPECIAL REFERENCE TO CATAMARCA, ARGENTINA

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ABSTRACT

The lichens *Parmotrema austrosinense* and *Canomaculina consors* were transplanted to a site within an open-pit mining project and to three localities potentially affected by mining emissions in the Western region of Catamarca, Argentina. In order to contribute to the interpretation of chemical response of these species to mining airborne pollutants and to analyse the feasibility of their use in air quality biomonitoring programmes, results of multielemental determinations by instrumental neutron activation analysis in the thalli are presented. The observed quali- and quantitative interspecific differences in the accumulative response of the transplanted lichens could be interpreted using the exposed/control ratio (EC ratio). The elemental accumulation of transplanted thalli could relate to mining airborne pollution only in *P. austrosinense*. Therefore, this species is the most suitable for biomonitoring air quality in areas with open-pit mines and environmental characteristics as those of Western Catamarca. In both species, the multielemental accumulation of the thalli reflected the geochemical characteristics of each transplantation site and local and regional environmental dynamics. In this regard, the results presented here contribute to establish environmental quality baselines for this region, which is in the process of exploiting its natural resources.

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Keywords: air pollution, biomonitoring, lichen, multielemental accumulation, open-pit mining

INTRODUCTION

Mining is one of the primary production activities that has a major impact on the environmental quality of a region and particularly on air quality (Moretton et al., 1996; Boamponsem et al., 2010). This is due to the extraction process itself, as well as crushing, grinding and deposits of sterile waste, among other associated activities. In particular, open-pit mining involves the removal of a large amount of rock materials, which can possibly be dispersed in an area beyond the mine itself.

Even though some mining projects have automatic continuous monitoring of atmospheric particulate matter levels, it is difficult to extend this methodology to areas that could be affected by pollution, due to economic and technical reasons, since simultaneous measurements of various pollutants at different points requires many sampling stations.

An alternative to traditional air quality monitoring methods, with low cost and relatively fast implementation and execution, is the use of lichens, which have been proposed as biomonitors both of accumulation and response (Arndt et al., 1995). Lichens can be used as accumulative biomonitors due to their ability to absorb and/or retain various elements from the atmosphere, combined with their longevity and resistance to environmental stress.

Biomonitoring assumes that the concentration of trace elements in lichens reflects the average concentration of particulate matter in the air, and both wet and dry deposition during a certain period (Steinnes, 1989). Furthermore, the metal content in certain lichen species allows to infer the proportion of these elements in the environment (Pignata et al., 2007).

As sensitive or reacting biomonitors, lichens function as integrators of stress caused by air pollutants and as preventive alarm systems. Due to the sensitivity of some species to certain substances or their combinations, biological response is indicative of air quality and provides information that cannot be obtained by conventional physical-chemical methods. Thus the biomonitor informs about the presence of certain substances in the air, and the effect of pollutants at a biotic level of the ecosystem.

At present, monitoring with lichens is widespread (Carreras et al., 2009; Koz et al., 2010; Loppi and Nascimbene, 2010), and there are various studies on the use of lichens as bioindicators of air quality in mining areas (Naeth and Wilkinson, 2008; Boamponsem et al., 2010; Sondergaard et al., 2010). In Latin America, this kind of studies are scarce and consist of floristic studies and analysis of heavy metals in lichens from some mining areas of Peru (Díaz Rivas Plata, 2006), list of species in areas of mining exploration in Ecuador (Carrasco Merchán and Guzmán Cárdenas, 2011), active monitoring in a mine of Venezuela (Hurtado et al., 2013), among others.

In Argentina, there have been studies of air quality related to mining areas in Catamarca province. For the last two decades approximately, mining has become the main economic resource for this province and the activity is mainly centred in the western region with prevalence of metalliferous ores extraction. In this region, within the Yacimientos Mineros Agua de Dionisio (YMAD) district, one of the most important open-pit mining projects of the country and of South America is placed.

Due to lichen scarcity in this semiarid region, active monitoring has been the chosen methodology for air quality studies, by transplanting lichens collected far away from the mining area, in slightly anthropized areas. The first researches were conducted in Belén (approximately 60 km southwest of the mine), with *Parmotrema austrosinense* (Zahlbr.) Hale as an accumulative and sensitive bioindicator. Within a study area of about 600 km^2, and in consecutive periods of time, lichen bags were transplanted to 29 monitoring sites, remaining exposed to the weather during three months. The concentrations of 25 elements were determined in the thalli by instrumental neutron activation analysis. Photosynthetic pigments, malondialdehyde (MDA) and sulphur were also quantified, and the pollution index (PI) proposed by González and Pignata (1994) was calculated. Preliminary results of this research were published by Mohaded Aybar et al. (2006) and Palomeque et al. (2006).

Regarding physiological parameters, photosynthetic pigments, defined by Cañas (2001) as biomarkers of air quality in urban-industrial areas, were those that better explained the variability of physiological data obtained in transplants to Belén during the study time. Also chlorophyll b/chlorophyll a and phaeophytin a/chlorophyll a ratios proved to be good explanatory variables (Ocampo et al., 2009). In general, pigments showed certain regularity in the response of this species transplanted during different periods of the year (Palomeque, 2008; Ocampo et al., 2009). Other parameters, such as sulphur content and MDA, depended on the exposure period (Mohaded Aybar et al., 2008a).

In general, *P. austrosinense* showed low elemental accumulation when transplanted to the study area in Belén. Nevertheless, a differential behaviour could be detected for this species, both for individual element retention and for multielemental accumulation at transplant sites. From the elemental analysis of *P. austrosinense*, it could be inferred that elements from soil and rock have an influence over air quality in the study zone. Multielemental composition of this species reflected natural geochemical associations, which are a characteristic of different lithologic formations present in the study area, as well as in the region where it is located. No emission sources other than natural ones have been detected, including wind blown soil and sediments from nearby areas, although they could be associated to overexploitation by shepherding or land clearing for agricultural purposes. No effect from the mine has been detected. This could be due to its location NE from the study area, while prevailing winds blow mainly from the SE (Palomeque, 2008; Jasan et al., 2011).

P. austrosinense showed a certain level of stress related to the total multielemental accumulation. In transplant sites where the accumulation in thalli was high, values of PI (in which parameters such as MDA content and phaeophytin a/chlorophyll a ratio are related with sulphur content as an accumulation parameter) were the highest (Palomeque, 2008). Due to these results, the author recommended *P. austrosinense* as a useful species for biomonitoring of airborne dust pollution, in areas with environmental characteristics similar to those of the study region.

Between October 2006 and January 2007, an air quality study with transplanted lichens was conducted within the Farallón Negro Volcanic Complex and its area of influence. The study established four transplant sites: Bajo de la Alumbrera mine and three localities potentially affected by mining emissions (Amanao, Andalgalá and Hualfin). In this case, *P. austrosinense* and *Canomaculina consors* (Nyl.) Elix& Hale were used as biomonitors. On the basis of physiological parameters, interspecific differences in lichen response to mining airborne pollution were established. For *P. austrosinense,* a higher stress level was detected in thalli that were transplanted within the mine boundaries; while for *C. consors,* the stress level was higher in thalli transplanted on the outskirts of Andalgalá city, far away from the mine (Mohaded Aybar et al., 2008b). Other parameters such as sulphur content and the PI, were accurate solely in evaluating air quality related to mining activity, with *P. austrosinense*, as for *C. consors* they did not show differences between transplant sites (Mohaded Aybar et al., 2010).

In this chapter, the results obtained from the multielemental composition analysis in *P. austrosinense* and *C. consors* samples are shown. These samples correspond to the previously mentioned study of air quality in Farallón Negro Volcanic Complex. The aims were to contribute to the interpretation of these species chemical response to mining airborne pollutants and thus to analyse the feasibility of their use in air quality biomonitoring programmes in Western Catamarca mining area. Methodological aspects and results of the research presented here could be considered for the evaluation of air quality by lichens in other mining regions of Latin America with similar environmental, geochemical and exploitation characteristics to those of this Argentine province.

METHODS

Study Area

The study area is located at the Central-Western region of Catamarca Province, in the extreme North of the morphostructural unit of the North-Western Pampean Ranges (Caminos, 1979). The area is characterized by the presence of narrow valleys and wide closed intermountain depressions (bolsones), alternating with uplifted basement blocks whose western slopes are usually steeper than the eastern ones. Geologically, it consists of a precambrian igneous-metamorphic basement, covered mainly by quaternary alluvial deposits. The climate is warm arid; precipitations are very scarce with only about 150 to 300 mm per year, mostlyin summer (about 60-70% of the total precipitation); prevailing winds blow from NE. Phytogeographically, it is located in the Monte Phytogeographic Province in the Chaqueño Domain of the Neotropical region (Morlans, 1995).

In the region, there are important Au, Ag, Cu and Mo deposits currently in operation. The first large scale mining operation of Argentina - Bajo de la Alumbrera - is located here. It is an open-pit copper-gold (Cu-Au) mine, which corresponds to a porphyry-type deposit included in Farallón Negro Volcanic Complex (Gutiérrez et al., 2006).This mine has been operating since 1997.

The study area is sparsely populated: the most important locality is Andalgalá (11,411 inhabitants), the third largest city of the province. There are also little villages such as Amanao (48 inhabitants) and Hualfín (993 inhabitants; INDEC, 2001). At these two localities, on the outskirts of Andalgalá and within the mining project, four transplant sites were established for this study (Figure 1). The three towns are located in intermountain depressions: Amanao and Andalgalá at the North end of the Bolsón de Pipanaco and Hualfin in the Hualfin Valley. Given their relative proximity to Bajo la Alumbrera, these human settlements are considered environmentally vulnerable. For this reason, they were chosen as monitoring sites for this study. Location data of each site are shown in Table 1.

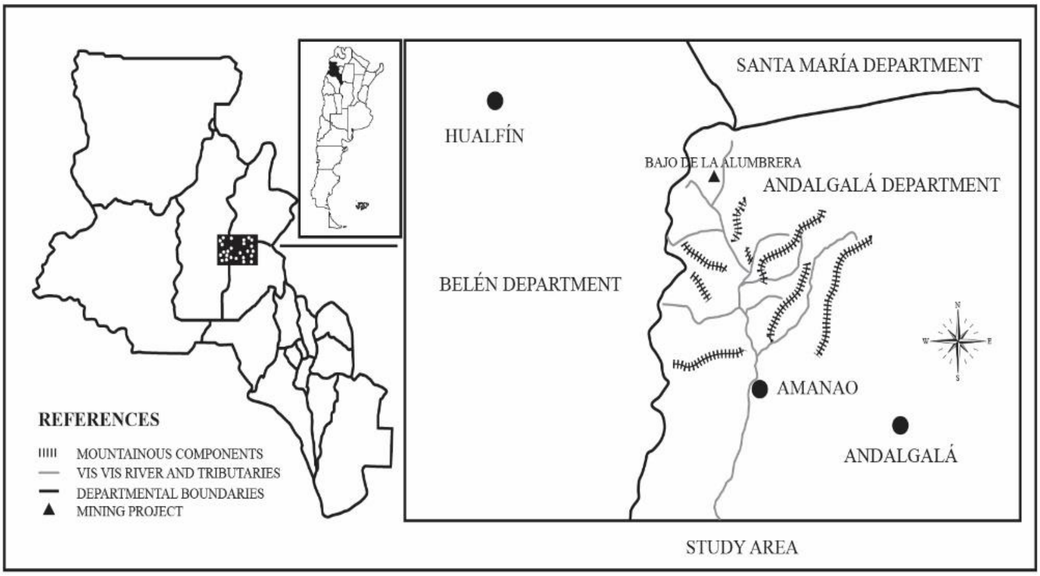

Figure 1. Location of the four sites to where lichens were transplanted in Farallón Negro Volcanic Complex and its area of influence, Catamarca, Argentina: Bajo de la Alumbrera mining project (La Alumbrera), Amanao, Andalgalá and Hualfín.

Sampling Procedure

In October 2006, thalli of the lichens *P. austrosinense* and *C. consors* were collected from a clean site near Coneta (central valley of Catamarca). Part of this freshly picked material was also analysed to obtain a baseline level with which to compare the transplant analysis results. Lichen bags were prepared and transplanted, following the methodology described by González and Pignata (1994). At each transplant site, three bags were exposed for four months. It is important to note that this study constitutes the first one in which *C. consors* is evaluated as a possible bioindicator of air quality in Catamarca province.

Table 1.Location of transplant sites in Farallón Negro Volcanic Complex and its area of influence, Catamarca, Argentina

Transplant site	Latitude	Longitude	Altitude (m.a.s.l.)	Distance to the open pit (km)
Bajo de la Alumbrera	27º 18' 00" S	66º 35' 10" W	2,406	3
Amanao	27º 32' 04" S	66º 30' 10" W	1,173	25
Andalgalá	27º 33' 52" S	66º 19' 23" W	1,123	39
Hualfín	27º 13' 28" S	66º 49' 52" W	1,845	25

Multielemental Analysis

Instrumental Neutron Activation Analysis (INAA) was used to determine elemental composition. Samples were ground in a Spex Centi Prep 6750 cryogenic mill and lyophilized for 24 h. About 300 mg of lyophilized material was pelletized and wrapped-up in aluminium foil for irradiation, together with two certified reference materials, NIST SRM 1633b Coal Fly Ash and IAEA Lichen 336, for calibration purposes. Irradiations were done at the RA-3 reactor (thermal flux 3 x 10^{13} cm^{-2} s^{-1}, 8 MW) of the Argentine National Atomic Energy Commission (Ezeiza Atomic Centre) for 4 h. Two measurements were performed after 7 and 30 day-decay using GeHP detectors (30% efficiency, 1.9 keV resolution for 1332.5 keV ^{60}Co peak), coupled to an Ortec 919E multichannel buffer module. Concentrations of As, Ba, Br, Ce, Co, Cr, Cs, Eu, Fe, Hf, La, Lu, Na, Nd, Rb, Sb, Sc, Se, Sm, Ta, Tb, Th, U, Yb and Zn were calculated using a software developed at the NAA laboratory. GBW07405 soil was used as control sample and the results obtained are summarized in Table 2. For all determined elements, experimental values showed good agreement with those in the material's certificate.

One way ANOVA was used to compare the elemental concentration of the two species in baseline conditions. A two way ANOVA was applied to transplanted sample data, being the factors species and transplant site.

Table 2. Quality control results (µg g^{-1}) obtained for the analysis of GBW07405 soil by Neutron Activation Analysis (NAA)

Element	Certified	Experimental
As	412 ± 24	407 ± 12
Ba	296 ± 40	316 ± 100
Ce	91 ± 15	97.9 ± 4.5
Co	12 ± 2	12.40 ± 0.39
Cr	118 ± 10	117 ± 6
Cs	15 ± 2	15.0 ± 0.8
Eu	0.82 ± 0.06	0.86 ± 0.16
Fe	9.0E+04 ± 2.0E+03	8.6E+04 ± 1.1E+03
Hf	8.1 ± 1.7	7.71 ± 0.98
La	36 ± 6	37.4 ± 0.5
Lu	0.42 ± 0.07	0.423 ± 0.047
Na	890 ± 30	886 ± 96
Rb	117 ± 9	121 ± 9
Sb	35 ± 7	34.7 ± 0.4
Sc	17 ± 2	17.4 ± 0.1
Sm	4.0 ± 0.6	4.54 ± 0.22
Ta	1.8 ± 0.3	2.03 ± 0.45
Tb	0.7 ± 0.2	0.755 ± 0.071
Th	23 ± 2	23.4 ± 0.6
U	6.5 ± 1.1	7.07 ± 0.97
Yb	2.8 ± 0.5	2.60 ± 0.55
Zn	494 ± 39	493 ± 35

n=10 (replicates).

Data Analysis

For lichens transplanted to each site, the ratio between the concentration of each element after exposure and that of the baseline sample prior to exposure (exposed/control ratio, EC ratio) was calculated according to Frati et al. (2005). EC ratios were explained by means of a 5-class interpretative scale, based on the deviation of the EC ratio from "normal" conditions, which refers to "natural" baseline concentrations of trace elements in lichens growing in unpolluted environments. Normal concentrations of trace elements show rather large variations due to the influence of many different factors (Markert, 1992), even in lichens collected at the same remote area. Metal accumulation depends not only on environmental availability of elements, but also on organism characteristics (species, age, state of health, type of reproduction, etc.) and on other parameters such as temperature, moisture availability, and substrate characteristics (Baker, 1983). Therefore, Frati et al. (2005) assumed a deviation of$\pm$ 25% of EC ratio equal to 1, when considering that an element concentration is at normal levels in an exposed sample. As a rule of thumb, this deviation should account for natural fluctuations in trace element concentrations in the biomonitor (Loppi et al., 2002).

On the basis of previous observations (Palomeque, 2008) and of those performed in this study, the deviation proposed is considered reasonable for *P. austrosinense* and *C. consors* in natural conditions, hence a 5-class interpretative scale has been chosen for the explanation of EC ratios in both species. According to Frati et al. (2005), the scale is as follows: (a) $0\leq$ EC ratio ≤ 0.25 (severe loss); (b) $0.25<$ EC ratio ≤ 0.75 (loss); (c) $0.75<$ EC ratio ≤ 1.25 (normal); (d) $1.25<$ EC ratio ≤ 1.75 (accumulation); (e) $1.75<$ EC ratio (severe accumulation).

RESULTS

Concentrations of elements analysed in both transplanted and baseline samples of *P. austrosinense* and *C. consors* are shown in Table 3. Similar concentrations were found in both species for most elements analysed, although higher contents of Br and Se were recorded in *P. austrosinense* and of Ba and Yb in *C. consors*.

In samples transplanted to the study area (Table 4), a significant interaction between species and transplant site was found for As, Ce, Cs, Eu, Fe, La, Lu, Rb, Sc, Se, Sm, Tb, and Yb. Cobalt showed the effect of the transplant site, regardless of species, and its content was significantly higher in samples transplanted to Bajo de la Alumbrera than in those of Amanao and Andalgalá. Bromine and U showed the individual effect of the two main factors. Bromine content was significantly higher in samples transplanted to Bajo de la Alumbrera than in those of Andalgalá and Hualfín. As could be observed in baseline samples, Br content in *P. austrosinense* was significantly higher than that in *C. consors*. Uranium content in samples transplanted to Amanao was significantly higher with respect to those of Bajo de la Alumbrera and Hualfín. Although U content did not show significant differences between species in baseline samples, it was higher in transplants of *C. consors* than in those of *P. austrosinense*. Chromium and Nd contents were significantly higher in *P. austrosinense*, as were those of Hf and Na in *C. consors*, regardless of the transplant site.

Table 3. Elemental concentration (mg kg^{-1}, dry weight) in *Parmotrema austrosinense* and *Canomaculina consors* transplanted to Farallón Negro Volcanic Complex and its area of influence (Catamarca, Argentina) and in baseline material. Baseline values in bold are significantly different at p<0.05 (ANOVA)

	P. austrosinense			*C. consors*		
	Range (min. - max.)	C.V.	Baseline value	Range (min. - max.)	C.V.	Baseline value
As	1.6 - 2.3	0.12	1.5	1.5 - 2.5	0.15	1.6
Ba	17 - 33	0.19	**17**	17 - 30	0.18	**23**
Br	3.5- 9.5	0.39	**4.6**	2.6 - 4.3	0.15	**3.1**
Ce	4.1 - 7.9	0.21	4.6	4.3 - 6.1	0.11	4.4
Co	0.54 - 1.69	0.39	0.54	0.61 - 1.03	0.17	0.62
Cr	6.7 - 48.1	0.40	30.0	8.0 - 19.3	0.29	27.1
Cs	0.50 - 0.71	0.12	0.49	0.54 - 0.80	0.13	0.56
Eu	0.07 - 0.14	0.21	0.08	0.08 - 0.12	0.13	0.08
Fe	1306 - 2263	0.18	1415	1485 - 2271	0.14	1610
Hf	0.14 - 0.28	0.21	0.18	0.19 - 0.33	0.20	0.22
La	2.0 - 3.2	0.14	2.1	2.1 - 3.2	0.11	2.1
Lu	0.026 - 0.037	0.12	0.024	0.027 - 0.043	0.11	0.027
Na	379 - 589	0.13	530	450 - 855	0.23	529
Nd	2.4 - 4.3	0.19	2,1	1.7 - 3.7	0.32	2.0
Rb	4.6 - 9.2	0.24	7.6	6.1 - 11.8	0.18	6.9
Sb	0.097 - 0.501	0.64	0.108	0.137 - 0.301	0.26	0.116
Sc	0.45 - 0.75	0.17	0.48	0.52 - 0.79	0.13	0.55
Se	0.24 - 0.55	0.24	**0.34**	0.21 - 0.36	0.16	**0.26**
Sm	0.47 - 0.77	0.18	0.43	0.36 - 0.79	0.22	0.47
Ta	0.03 - 0.73	1.66	0.06	0.06 - 0.10	0.21	0.05
Tb	0.055 - 0.136	0.31	0.059	0.040 - 0.070	0.14	0.053
Th	0.55 - 0.80	0.12	0.53	0.57 - 0.94	0.16	0.61
U	0.14 - 0.44	0.37	0.19	0.23 - 0.43	0.20	0.19
Yb	0.14 - 0.25	0.18	**0.15**	0.15 - 0.25	0.13	**0.17**
Zn	22 - 132	0.64	21	26 - 82	0.37	22

The elemental accumulation in thalli transplanted to the study area was estimated by means of the EC ratio. For *P. austrosinense*, results shown below had been already partially published by Mohaded Aybar et al. (2012). As shown in Table 5, for this species, Bajo de la Alumbrera showed higher average multielemental accumulation in transplanted thalli than other sites. Concentrations of Cr, Na, and Rb showed normal levels in all transplant sites, although a slight Rb loss was detected in lichens exposed in Hualfín and a slight Cr loss in those exposed in Andalgalá. Neodymium and Zn showed accumulation in thalli transplanted to all sites. The accumulation of Nd was severe in Amanao and Hualfín, while for Zn, it was severe in Bajo de la Alumbrera, Amanao, and Andalgalá. For other elements, concentrations increased in more restricted areas, such as Eu with high EC ratio values only for Bajo de la Alumbrera, and Hf and Se showing accumulation only in Amanao. The remaining analysed elements presented certain specific accumulation patterns. Thus, Ba and Sb showed an increase in thalli transplanted to Bajo de la Alumbrera, Amanao, and Andalgalá; As, Br, Ce, Cs, Co, Fe, La, Lu, Sc, Sm, Ta, and Yb did the same for transplants to Bajo de la Alumbrera and Amanao, Tb and Th in thalli transplanted to the latter two sites and Hualfín, and U in those transplanted to Amanao and Andalgalá.

For *C. consors*, the average multielemental accumulation was higher in thalli transplanted to Hualfín, although its value was similar to the accumulation observed in Andalgalá and Amanao. In all transplant sites, Ba, Br, Ce, Fe, Hf, Sc, and Tb concentrations were found at normal levels; while thalli showed loss of Cr and accumulation of Sb, Ta, and Zn. Zinc accumulation was severe in Amanao. Some elements showed specific patterns of accumulation, such as La, Lu, Nd, Sm and Yb, whose EC ratio values were high only in transplants to Andalgalá and Hualfín; and as As, Cs, Rb and U, whose EC ratio values were high in Amanao, Andalgalá, and Hualfín. Other elements showed an increase in more restricted areas, such as Co and Na whose EC ratio values were high only in thalli transplanted to Bajo de la Alumbrera; Eu and Se that showed accumulation only in Hualfín, and Th, whose accumulation was observed only in Andalgalá.

Table 4. Comparison of element concentration mean values (± standard deviation) (in mg kg^{-1}, dry weight) and its significance, between lichen species and among transplant sites

Factors	As	Ba	Br	Ce	Co
Species					
P. austrosinense	1.9 ± 0.2	23 ± 3	5.8 ± 1.8	5.9 ± 1.3	0.79 ± 0.26
C. consors	2.2 ± 0.3	26 ± 4	3.3 ± 0.3	5.5 ± 0.5	0.80 ± 0.10
Site					
Alumbrera	1.8 ± 0.1	25 ± 2	5.6 ± 2.6 a	6.1 ± 1.7	1.01 ± 0.22 a
Amanao	2.1 ± 0.1	23 ± 1	5.2 ± 3.0 ab	5.9 ± 1.1	0.74 ± 0.01 b
Andalgalá	2.1 ± 0.3	25 ± 4	3.8 ± 1.0 b	5.2 ± 0.4	0.64 ± 0.06 b
Hualfín	2.1 ± 0.6	25 ± 7	3.6 ± 0.4 b	5.5 ± 1.0	0.78 ± .18 ab
ANOVA					
Effects					
Species	**	ns	***	ns	ns
Site	**	ns	*	ns	*
Species x Site	***	ns	ns	***	ns
Factors	Cr	Cs	Eu	Fe	Hf
Species					
P. austrosinense	26.7 ± 7.7	0.62 ± 0.07	0.10 ± 0.02	1749 ± 324	0.21 ± 0.04
C. consors	13.4 ± 1.8	0.74 ± 0.13	0.10 ± 0.01	1995 ± 273	0.27 ± 0.04
Site					
Alumbrera	19.8 ± 9.5	0.63 ± 0.05	0.12 ± 0.02	2047 ± 105	0.23 ± 0.01
Amanao	24.9 ± 17.8	0.70 ± 0.02	0.10 ± 0.01	1882 ± 52	0.26 ± 0.01
Andalgalá	18.0 ± 2.6	0.64 ± 0.12	0.09 ± 0.01	1610 ± 235	0.20 ± 0.06
Hualfín	17.5 ± 7.5	0.73 ± 0.26	0.10 ± 0.02	1951 ± 618	0.26 ± 0.09
ANOVA					
Effects					
Species	***	**	ns	*	**
Site	ns	ns	**	ns	ns
Species x Site	ns	**	**	*	ns

Table 4. (Continued)

Factors	La	Lu	Na	Nd	Rb
Species					
P. austrosinense	2.6 ± 0.4	0.033 ± 0.004	480 ± 38	3.3 ± 0.5	6.7 ± 1.4
C. consors	2.8 ± 0.3	0.036 ± 0.003	671 ± 47	2.6 ± 0.5	9.8 ± 1.6
Site					
Alumbrera	2.8 ± 0.4	0.034 ± 0.003	578 ± 159	2.6 ± 0.9	7.9 ± 0.1
Amanao	2.8 ± 0.1	0.035 ± 0.001	605 ± 96	3.0 ± 1.0	8.8 ± 1.5
Andalgalá	2.6 ± 0.4	0.033 ± 0.006	536 ± 99	2.8 ± 0.1	7.7 ± 2.8
Hualfín	2.8 ± 0.5	0.035 ± 0.006	583 ± 186	3.4 ± 0.3	8.5 ± 4.7
ANOVA					
Effects					
Species	ns	ns	**	**	***
Site	ns	ns	ns	ns	ns
Species x Site	**	**	ns	ns	**
Factors	Sb	Sc	Se	Sm	Ta
Species					
P. austrosinense	0.184 ± 0.059	0.60 ± 0.11	0.36 ± 0.07	0.61 ± 0.11	0.12 ± 0.12
C. consors	0.179 ± 0.020	0.69 ± 0.08	0.30 ± 0.06	0.60 ± 0.10	0.08 ± 0.01
Site					
Alumbrera	0.208 ± 0.030	0.70 ± 0.04	0.30 ± 0.08	0.60 ± 0.16	0.18 ± 0.16
Amanao	0.154 ± 0.004	0.66 ± 0.00	0.36 ± 0.13	0.62 ± 0.10	0.08 ± 0.01
Andalgalá	0.218 ± 0.028	0.56 ± 0.09	0.31 ± 0.03	0.60 ± 0.11	0.06 ± 0.02
Hualfín	0.148 ± 0.047	0.67 ± 0.20	0.34 ± 0.07	0.59 ± 0.12	0.08 ± 0.03
ANOVA					
Effects					
Species	ns	*	*	ns	ns
Site	ns	ns	ns	ns	ns
Species x Site	ns	*	**	***	ns
Factors	Tb	Th	U	Yb	Zn
Species					
P. austrosinense	0.087 ± 0.023	0.70 ± 0.06	0.27 ± 0.09	0.19 ± 0.03	59 ± 28
C. consors	0.065 ± 0.015	0.78 ± 0.13	0.32 ± 0.03	0.21 ± 0.02	48 ± 12
Site					
Alumbrera	0.088 ± 0.040	0.68 ± 0.06	0.25 ± 0.04 b	0.20 ± 0.02	68 ± 40
Amanao	0.073 ± 0.026	0.76 ± 0.01	0.36 ± 0.05 a	0.21 ± 0.01	55 ± 13
Andalgalá	0.061 ± 0.002	0.71 ± 0.11	0.31 ± 0.07 ab	0.19 ± 0.04	50 ± 16
Hualfín	0.083 ± 0.006	0.81 ± 0.19	0.25 ± 0.08 b	0.20 ± 0.06	39 ± 11
ANOVA					
Effects					
Species	**	ns	*	*	ns
Site	ns	ns	*	ns	ns
Species x Site	*	ns	ns	**	ns

ANOVA results: ns=not significant; * $p<0.05$; ** $p<0.01$; ***$p<0.001$. Values in the same column followed by the same letter do not differ at $p<0.05$ (LSD Test).

Table 5.EC ratio for *Parmotrema austrosinense* and *Canomaculina consors* transplanted to four sites in Farallón Negro Volcanic Complex and its area of influence: Bajo de la Alumbrera (Al), Amanao (Am), Andalgalá (And), Hualfín (Hu). Accumulation in light grey; severe accumulation in dark grey; means were calculated from the four sampling sites results

Element	*P. austrosinense*					*C. consors*				
	Al	Am	And	Hu	Mean	Al	Am	And	Hu	Mean
As	1.26	1.50	1.25	1.17	1.29	1.04	1.29	1.43	1.46	1.30
Ba	1.50	1.30	1.26	1.15	1.30	0.97	1.00	1.21	1.16	1.08
Br	1.62	1.60	0.99	0.85	1.26	1.15	0.97	0.99	1.00	1.03
Ce	1.60	1.47	1.08	1.05	1.30	1.09	1.15	1.24	1.20	1.17
Co	2.18	1.37	1.12	1.24	1.48	1.33	1.19	1.08	1.21	1.20
Cr	0.88	1.25	0.66	0.76	0.89	0.46	0.45	0.59	0.60	0.52
Cs	1.36	1.40	1.14	1.12	1.25	1.03	1.26	1.27	1.32	1.22
Eu	1.55	1.24	0.97	1.03	1.20	1.25	1.23	1.19	1.33	1.25
Fe	1.50	1.36	1.02	1.07	1.24	1.18	1.13	1.09	1.15	1.14
Hf	1.19	1.44	0.88	1.07	1.14	1.07	1.21	1.08	1.12	1.12
La	1.48	1.39	1.09	1.18	1.28	1.15	1.24	1.33	1.30	1.25
Lu	1.47	1.50	1.20	1.25	1.35	1.11	1.19	1.31	1.30	1.23
Na	0.88	1.01	0.88	0.85	0.90	1.26	1.25	1.13	1.13	1.19
Nd	1.55	1.80	1.30	1.76	1.60	0.93	1.15	1.38	1.73	1.30
Rb	1.06	1.02	0.76	0.68	0.88	1.10	1.41	1.38	1.49	1.34
Sb	2.12	1.44	2.19	1.06	1.70	1.53	1.28	1.68	1.51	1.50
Sc	1.53	1.38	1.04	1.22	1.29	1.19	1.18	1.15	1.17	1.17
Se	1.06	1.34	0.99	0.87	1.06	0.93	1.01	1.11	1.38	1.11
Sm	1.67	1.59	1.22	1.17	1.41	0.99	1.15	1.42	1.43	1.25
Ta	5.09	1.35	0.76	0.91	2.03	1.47	1.70	1.54	1.51	1.55
Tb	1.97	1.53	1.04	1.33	1.47	1.06	1.00	1.09	1.15	1.07
Th	1.36	1.43	1.19	1.27	1.31	1.01	1.21	1.26	1.20	1.17
U	1.13	2.00	1.30	0.96	1.35	1.40	1.67	1.81	1.28	1.54
Yb	1.42	1.40	1.05	1.05	1.23	1.10	1.18	1.29	1.31	1.22
Zn	4.66	2.21	2.92	1.52	2.83	1.75	2.89	1.72	1.87	2.06
Mean	1.72	1.45	1.17	1.10	1.36	1.14	1.25	1.27	1.29	1.24

DISCUSSION

In general, concentration values detected in *P. austrosinense* have the same order of magnitude as those registered for this species transplanted to an area of Department of Belén, during the spring of 2005 (Jasan et al., 2011). Only Ce, Cs, Hf, Na, Rb, and Th contents decreased in the mining area respect tothose in Belén. There are no multielemental composition data for *C. consors* in Catamarca, due to the fact that the present study is the first one in which this species is evaluated as a possible bioindicator of air quality in the province.

In lichens transplanted to the mining area, and for 14 of the 25 elements analysed, a species-dependent spatial variation of concentration was observed (Table 4). Other elements such as Cr, Nd, Hf, Na, and U presented significantly different concentrations between species, regardless of transplanting site, even when their concentrations were similar in baseline samples. This might indicate a differential response of *P. austrosinense* and *C. consors* to being transplanted from their natural environment to the study area. Only Br, Co, and U showed a similar spatial variation of concentration in both species.

Interspecific differences in element concentration variation in exposed thalli with respect to baseline levels can be better interpreted by analysing elemental accumulation. Inorganic substances may occur in lichens as 1) particles (adsorbed onto the thallus surface or within intercellular spaces), 2) ions bound to extra- or intracellular exchange sites, and 3) soluble intracellular ions (Bargagli and Mikhailova, 2002). Elemental concentrations, as determined in this study, represent the sum of those fractions, and any variation in the elemental content of transplanted thalli can be considered as an accumulation or a loss with respect to the baseline level.EC ratio, which reports the enrichment degree of transplanted samples with respect to baseline samples, was calculated for determination of accumulation in lichen thalli, at different exposure sites.

In this sense, Cr (whose concentration, together with that of Nd, was significantly higher in *P. austrosinense* than in *C. consors*) showed EC values that correspond to concentration at normal levels for *P. austrosinense* (with the exception of thalli transplanted to Andalgalá); and EC ratios that also correspond to a Cr loss in thalli of *C. consors* (Table 5). Moreover, Nd enrichment was higher in *P. austrosinense* than in the other species, which accumulated this element in only two of the four exposure sites.

Regarding those elements with higher concentrations in *C. consors* than in *P. austrosinense*, EC ratio showed a slight Na accumulation in thalli of *C. consors* transplanted to Bajo de la Alumbrera, whilst the content of this element in *P. austrosinense* was at normal level in all transplant sites. Moreover, U accumulation was observed in thalli of *C. consors* transplanted to Amanao, Andalgalá, and Hualfín, and only at the first two sites for *P. austrosinense*. Considering EC ratio, the higher Hf content in transplants of *C. consors* in comparison to the other species, was not due to its accumulation, but to a higher concentration (even though not significant) in the original baseline material. In fact, *P. austrosinense* showed accumulation of this element in thalli transplanted to Amanao.

In general, elemental enrichment in *P. austrosinense* was higher than in *C. consors*, due to both the number of accumulated elements and the number of sites where a severe accumulation was determined. Thus, *P. austrosinense* showed a severe accumulation in the four transplant sites, whilst *C. consors*in only two. In addition, *P. austrosinense* showed a severe accumulation for seven elements (Co, Nd, Sb, Ta, Tb, U, Zn), and *C. consors* for only U and Zn (Table 5).

Certain elements such as Nd and Zn in *P. austrosinense*, and Sb, Ta and Zn in *C. consors* showed accumulation for all the exposure sites. The remaining elements, either presented EC ratios that correspond to concentrations at normal levels, or below normal (Cr, Na and Rb in *P. austrosinense*; Ba, Br, Ce, Cr, Fe, Hf, Sc, Tb in *C. consors*); or an accumulation restricted to certain transplant sites in the study area.

Regarding the accumulation at each transplant site (Table 5), *P. austrosinense* accumulated a higher number of elements in thalli exposed at Bajo de la Alumbrera and Amanao (19 and 21 elements, with severe accumulation of 5 and 3 elements in each site, respectively), followed by Andalgalá and Hualfín (5 and 4 elements, with severe accumulation of 2 and 1 element in each site, respectively). On the contrary, in *C. consors* a higher accumulation was observed in Hualfín and Andalgalá (14 and 13 elements, respectively; with severe accumulation of one element in the latter site), followed by Amanao and Bajo de la Alumbrera (7 and 5 elements respectively; with severe accumulation of one element in Amanao). Although for both species, high EC ratios were observed for Co, Sb, Ta, and Zn in thalli transplanted to Bajo de la Alumbrera. These EC ratios only corresponded to a severe accumulation in *P. austrosinense*.

It is necessary to clarify that the designation "severe" refers to neither the toxicity of the different elements towards lichen, nor to a comparison with air quality standards; but it refers to how marked the accumulation of some elements results, in thalli transplanted to certain sites.

A similar behaviour, with regard to the accumulation degree of each species at different transplant sites, is observed when considering the average EC ratios for each species (Table 5). *P. austrosinense* showed a higher average EC ratio in Bajo de la Alumbrera, followed by Amanao, Andalgalá, and Hualfín. This suggests a distance gradient with respect to the mining exploitation area itself. For Hualfin, these results could be related to the NW site location with respect to the mine. Although the region has NE prevailing winds, for the months during which this study was conducted, NW winds were very frequent. Similar results were reported by Mohaded Aybar et al. (2010) for sulphur content in these samples, since they observed a concentration gradient in thalli transplanted to Bajo de la Alumbrera>Amanao>Andalgalá, with significantly higher contents of this element in Bajo de la Alumbrera and Hualfín with respect to Andalgalá. These authors also observed higher values of PI in Bajo de la Alumbrera and Hualfín with respect to the other transplant sites. Given that PI has shown to be a good parameter for detecting the effect produced by airborne particulate matter in Belén (Palomeque, 2008), this could be indicating high levels of stress in lichens exposed to these kinds of pollutants at these sites.

Apart from the mining activity itself, atmospheric pollution in Bajo de la Alumbrera may originate from mobile sources (medium and heavy vehicles) within the mining project. In Hualfín, atmospheric pollution could have its origin from these mobile sources, given that National Route 40, along which heavy traffic moves to and from mining exploitations, crosses this town. Sulphur accumulation related to high levels of vehicular traffic has been previously observed in lichens in Argentina (Levin and Pignata, 1995; González et al., 1996; González et al., 2003). On the other hand, in an air monitoring study with automatic samplers and for the same period of lichen transplant, Herrero (2008) observed in Los Nacimientos, near Hualfín, moderately heavy traffic of trucks and cars on National Route 40. This road was then still under construction at this place, being a major source of particulate matter. The report stated that concentration values of respirable particulate matter (PM 10) were above the national standard reference (Ley Nacional Nº 24,585, 1995) in Bajo de la Alumbrera and Los Nacimientos, but not in Amanao and Andalgalá.

As can be seen in Table 5, the average EC ratio in *C consors* was higher in Hualfín, which may be related to particulate matter generated during road repairing. According to chemical-physiological analyses conducted on these samples (Mohaded Aybar et al., 2010), no significant differences were detected in sulphur content and PI of thalli transplanted to the four exposure sites. Considering the photosynthetic pigments content, Mohaded Aybar et al. (2008b) observed a certain stress level in thalli of *C. consors* transplanted to Andalgalá. Due to the fact that this transplanting site was located in the outskirts of the city, it appeared unlikely that the lichen response had been caused by the presence of urban pollutants. In this study and using photosynthetic pigments as biomarkers, a higher stress level was detected in *P. austrosinense* thalli transplanted to Bajo de la Alumbrera.

With the aim of determining the degree of correspondence between the elemental enrichment of lichens and the geochemical characteristics of different transplant areas, multielemental geochemical data of stream sediments reported by the Argentine Mining Geological Service - SEGEMAR (Ferpozzi et al., 2002) were considered. According to these data and taking into account only the elements considered in this study, Co, Fe, Sb, Sc, and Zn are abundant in Bajo de la Alumbrera area, which coincides with the EC ratio value of these elements obtained for *P. austrosinense* transplanted to this site. Particularly, geochemical data correspond to the EC ratios for Co, Sb and Zn (elements with EC ratios values above 1.75). *C. consors* also accumulated these three elements in thalli transplanted to Bajo de la Alumbrera.

Zn is characteristic of hydrothermal ore deposits such as Bajo de la Alumbrera and the rest of the ore deposits of Farallón Negro Volcanic complex. The high Zn, Co and Sb enrichments observed in *P. austrosinense* and in a lower degree in *C. consors* transplanted to Bajo de la Alumbrera could be also related to local pyrite oxidation phenomena in both natural state (ore deposit) as well as from tailings generated in the mining operation. On this site and by using automatic samplers, Herrero (2008) detected abnormal concentrations of Zn in atmospheric dusts, possibly correlated with (in other order of magnitude) geochemical anomalies of mineralized areas. The agreement of these findings with the results of the present study indicate that *P. austrosinense* may be an appropriate active bioindicator for this area.

P. austrosinense also showed severe accumulation of Ta and Tb. In contrast to Zn, Ta is very scarce in mineralized zones of Farallón Negro-Bajo de la Alumbrera; for this reason their accumulation in thalli transplanted to this area might indicate an origin from nearby granitic areas by physical dispersion phenomena. On the other hand, *C. consors* showed Ta and Na accumulation in thalli transplanted to Bajo de la Alumbrera. In this area, the largest Na availability consists of sulphate outcrops that fill fractures that affect the ore deposit. Na - Ba - K is a characteristic association in hydrothermal mineralizations, which are abundant in the region. Normally, they are also present in granitoid rocks.

For the Amanao area, geochemical data indicate an abundance mainly of Sb and Sc. The area where the town is located is extremely anomalous in Sb and several other elements, with the addition of certain elements from Quebrada de Vis Vis and Quebrada de Amanao ravines, both located to the North. In thalli transplanted to this site, *P. austrosinense* accumulated Sb and Sc, together with Hf and Se (which only showed an accumulation in this site for this species), Zn and rare earth elements. A severe accumulation of Zn, together with Nd and U, which are typical of the granitic domain of Quebrada de Amanao, was observed. Meanwhile, *C. consors* also accumulated Sb together with As, Cs, Rb, Ta, U and Zn, and the EC ratio of this last element was above 1.75. For both lichen species, Zn accumulation in Amanao might be explained by the presence of high contents of soluble sulphates in stream sediments, due to the contribution of some watercourses originating in strongly mineralized areas still unexploited.

For the Andalgalá zone, geochemical data show an abundance of As, Cs, Sb, Sm, Ta, Tb, Th, U, Yb and Zn; as well as an abundance of other elements to the north of this area, such as other rare earths (Ce, La, Lu, Nd) and U. *P. austrosinense* showed an accumulation of Ba, Nd and U in thalli transplanted to this site as well as an accumulation of Sb and Zn with EC ratio values above 1.75. *C. consors* showed accumulation of some of those abundant elements in that same area. Severe U accumulation resulted particularly important in thalli of this species transplanted to Andalgalá. In this site, EC ratio values obtained for U in both species could be reflecting the presence of important uranium sources (still unexploited) in the region where the study area is located. Radioactive granites present in the region have U and Th associated to rare earth elements such as Sm. On the other hand, Zn accumulation in thalli correspond to the high natural geochemical values of Zn in stream sediments in Andalgalá, which possibly come from the dispersion produced by Candado River, originated from copper porphyry deposits also unexploited. It is not excluded that, in this area, Zn can have an anthropogenic origin, apart from its natural origin. This element is present in certain agrochemicals used in fumigations of fruit trees, which are very abundant near the city of Andalgalá.

For the Hualfín zone, geochemical data show abundance of As, Cs, Rb, and Sb, with important concentrations of other elements such as Hf, Sc, Ta, U, Zn, and rare earth elements in nearby zones. For *P. austrosinense*, an accumulation of Nd, Tb, Th, and Zn was observed in thalli transplanted to this site, and the EC ratio of Nd was above 1.75. Due to the presence in the area of recent carbonate hydrothermal activity, the exclusive Nd accumulation in thalli transplanted to Hualfín would seem to indicate a selective accumulation of this element in *P. austrosinense*. In previous studies (Palomeque, 2008; Jasan et al., 2011), a similar behaviour of this species regarding Ta and Zn has been found. For this reason, it is likely that EC ratio values obtained in the present work are determined by geochemical characteristics of the transplant areas as well as by accumulation mechanisms of this lichen species.

C. consors accumulated As, Cs, Rb, and Sb in thalli transplanted to Hualfín; and Ta, U, Zn and rare earth elements (Eu, La, Lu, Nd, Se, Sm, Yb) in accordance with geochemical abundance of these elements in the area. Pyrite and arsenopyrite, containing As, are very abundant in the study area. In the West of Catamarca, As origin is attributed to the presence of volcanic ashes in the soil that have been carried from volcanic zones by aeolian forces; as well as to igneous, sedimentary and metamorphic rock meteorization, that can release arsenic compounds (arsenites, arsenates or arsenic trioxide) of great mobility which are able to migrate long distances from the source. For the province of Catamarca, it cannot be dismissed the possibility that As can be mobilised from different anthropogenic processes, such as mineral beneficiation that can lead to arsenic contamination phenomena (Vilches et al., 2005).

It is noteworthy that according to geochemical data, Cr is a slightly abundant element in the study area, which matched EC ratio values obtained in thalli of both lichen species transplanted in all exposure sites.

CONCLUSION

P. austrosinense and *C. consors* exhibit a differential response of multielemental accumulation due to their transplant to different sites within the study area. These quali- and quantitative interspecific differences could be interpreted using the EC ratio.

P. austrosinense was the species with a higher elemental enrichment in general terms, particularly in Bajo de la Alumbrera and in Amanao, which is located to the South of the mine. Elemental accumulation in thalli transplanted to these sites can be related to mining airborne pollution.

In *C. consors,* elemental accumulation was higher in thalli transplanted to Hualfín and Andalgalá. This can be related to emission sources other than open-pit mining exploitation present in the study area.

In both species, multielemental accumulation in thalli reflected the geochemical characteristics of each transplanting site, and somehow, also the local and regional environmental dynamics. Therefore, it is considered that *P. austrosinense* and *C. consors* are suitable for detecting variations of air quality, which might be produced due to natural or anthropogenic factors in the region. In this regard, the results of this study contribute to establish environmental quality baselines for a region that is in the process of exploiting its natural resources.

Even though *P. austrosinense* and *C. consors* allowed determining for the first time the atmospheric availability of different elements in the study area, it will be necessary to inquire about a possible selective biogeochemical behaviour of each species. That will allow a better interpretation of results obtained in air quality studies by means of these species as biomonitors.

Results presented here allow concluding that, of the two species, *P. austrosinense* is the most suitable one for biomonitoring air quality in areas with open-pit mines and environmental characteristics similar to those of the Western region of Catamarca.

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Chapter 29

DYNAMICS OF PRIORITY POLLUTANTS AND ADEQUACY OF WASTEWATER TREATMENTS IN THE LAKE MARACAIBO BASIN (VENEZUELA)

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ABSTRACT

The origin and dynamics of priority contaminants in Lake Maracaibo (Venezuela) will be discussed in this chapter, as well as the potential of different treatment technologies for domestic and industrial effluents discharged in this basin which has received little or no pretreatment. These wastewaters serve as a major source of pollution to this aquatic ecosystem, coupled with other anthropogenic activities such as extraction and transportation of oil, agriculture, tanneries, petrochemical and food industry, etc. Ecotoxicological studies with native organisms are scarce and only in recent years have a few been made using some bacteria, ciliate protozoa and bivalve mollusks, showing high resistance to heavy metals. Maracaibo Lake's current trophic level indispensably drives the enforcement of recovery plans and environmental quality monitoring, these geared towards ensuring the survival of the native species and to perpetuate the existence of an alternate source of water for the human population.

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Keywords: biotechnology, contaminant mobility, eutrophication, organic matter, wastewater treatment

INTRODUCTION

Watershed of Lake Maracaibo

Lake Maracaibo is a partially mixed hypertrophic tropical estuary, located in Western Venezuela (Zulia). It consists of four different water bodies: Maracaibo Lake, Maracaibo Strait, Tablazo Bay and Gulf of Venezuela, in addition to its 135 tributaries (Figure 1) (Parra-Pardi, 1979; Rodríguez, 2000).

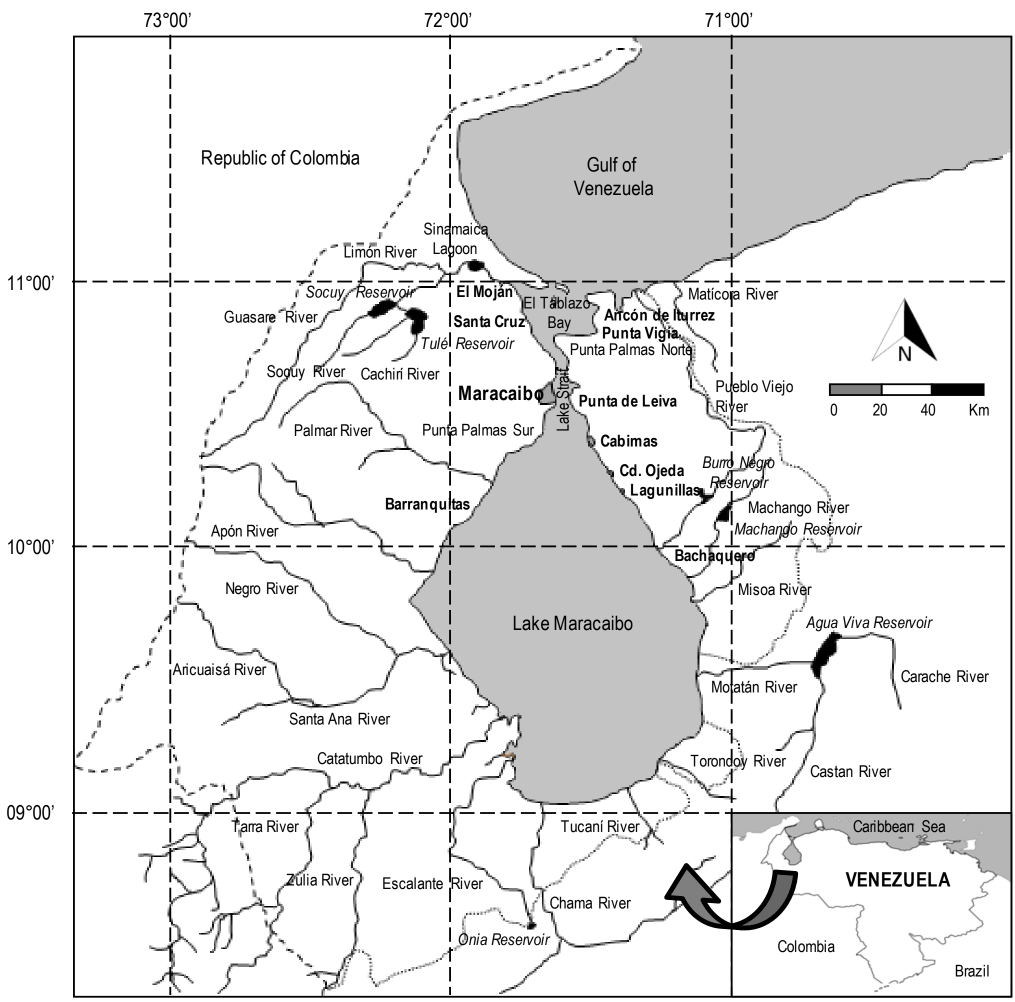

Figure 1. Map of Lake Maracaibo showing its main tributaries (normal font), reservoirs (italic font), coastal cities and villages (bold font).

The lake's watershed has an area of approximately 121,823 km^2; of which 12,400 km^2 correspond to the lake and 1,120 km^2 to Maracaibo Strait and Tablazo Bay that connect it with the Gulf of Venezuela. It is located between 70°30' and 73°24' West longitude and between 8°22' and 11°51' North latitude. This lake has four basic limnological features: i) hypolimnetic cone in the center; ii) counterclockwise movement of water masses from epilimnion around the cone; iii) influence of Catatumbo river; and iv) intrusion of seawater. Between 1953 and 1963, a navigation channel was dredged across the strait (100.6 km) to connect the Gulf of Venezuela with Lake Maracaibo and allow the entry of large ships (Parra-Pardi, 1979). This channel influences the salt wedge and therefore the distribution of nutrients and salinity in the system.

This basin has the largest oil reserves in South America and one of the largest in the world, with a total amount of 33 billion barrels extracted between 1914 and 1995, most of which come from the bottom of the lake. Besides oil exploitation, many other activities take place in the basin, including the petrochemical industry, gas processing, transportation of hydrocarbons, exploitation and transportation of coal in opencast mines, metal-mechanical industries, tanneries, pharmaceutical factories, food companies like slaughterhouses and shrimp, among others (Rodríguez, 2000).

The main contributor of organic matter and inorganic nutrients is represented by the direct discharge of domestic wastewater into the lake (33,724 kg/d) containing fecal material, detergents rich in phosphates and nitrogenous substances, of which the highest proportion is discharged untreated into the Maracaibo Strait, from the cities of Maracaibo, Cabimas and Ciudad Ojeda (Rodríguez, 2000). The development of agriculture and livestock in the Northwest and South Lake (over 83,843 hectares) also represents an important source of organic and inorganic compounds, including pesticides, metals, nitrogen and phosphorus compounds (Rodríguez, 2000; Meléndez et al., 2005).

Adequacy of Wastewaters according to Venezuelan Discharge Regulations

Lake Maracaibo receives large amounts of wastewaters from domestic and industrial sources without treatment, infringing the Venezuelan legislation for quality control of water bodies and discharge of liquid effluents, which include permissible limits for: biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total suspended solids (TSS), nitrogen, phosphorus, metals, hydrocarbons, pesticides, coliform bacteria, among others (Decreto 883, 1995).

In order to propose alternatives for the treatment of wastewaters discharged into the lake, numerous studies have been made to adapt the quality of the final effluent to Venezuelan law (Decreto 883, 1995). Some of the technologies that have been evaluated are: upflow anaerobic sludge blanket reactors (UASB), rotating biological contactors (RBC), sequential batch reactors (SBR), constructed wetlands, adsorption with activated carbon, microbial consortia, coagulation-flocculation, among others.

In this chapter, previous studies of different researchers are described and discussed, concerning: i) the origin and dynamics of priority pollutants in Lake Maracaibo; and ii) treatment of domestic and industrial wastewaters conducted with effluents generated in the watershed of Lake Maracaibo, aimed to counteract both the progress of the eutrophication process as well as of the modification of ecological dynamics to native communities. Recent reports of ecotoxicological tests with autochthonous species are also presented.

ANALYSIS OF PRIORITY POLLUTANTS AND NUTRIENTS

Carbon, Nitrogen and Phosphorus

Numerous studies have analysed C, N, P contents in the water column and sediments of Lake Maracaibo basin, including tributary rivers. Determinations have included both organic and inorganic forms, by applying standard methods (Parra-Pardi, 1979; APHA et al., 1998; Gardner et al., 1998; Ledo et al., 2003; Rivas et al., 2005, 2009; Torres et al., 2010; Polo et al., 2014).

Mejías et al. (2014) applied water quality indices to describe the trophic state and environmental condition of Lake Maracaibo, specifically in its coastal zone. They used data from 42 sampling stations around the Lake during rainy and dry periods in 2012. The indices were: index of the National Sanitation Foundation-USA (INSF), weighted arithmetic water quality index (WQI_{WA}), geometric-weight multiplicative water quality index (WQI_{GWM}), unweighted arithmetic water quality index (WQI_{UWA}) and unweight geometric-multiplicative water quality index (WQI_{UWGM}). The authors assessed index applicability by comparing contamination degree via principal component analysis (PCA).

Pesticides

Twelve organochlorine pesticides - α-BHC (α-benzene hexachloride), β-BHC, γ-BHC (lindane), heptachlor, aldrin, endosulfan, DDE (dichlorodiphenyldichloroethylene), dieldrin, TDE (1,1-dichloro-2,2-bis(4-chlorophenyl)ethane), endrin, DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane) and metoxichloride - were analysed in samples of water, sediment (Urdaneta, 1989) and muscle fatty tissue of 14 fish species (Urdaneta et al., 1994) from pisciculture ponds located in the Limón river basin (Guasare and Socuy rivers) by gas chromatography with electron capture detector (Urdaneta, 1989; Urdaneta et al., 1994).

Moreover, Sánchez et al. (2005) determined glyphosate concentrations in 144 water samples of the Catatumbo river basin (six stations in Tarra, Socuavo, Zulia and Catatumbo rivers), using a liquid-liquid extraction with dichloromethane and ion chromatograph equipped with a conductivity detector.

Hydrocarbons and Phenols

Table 1 describes the analytical methods used to determine concentrations of hydrocarbons and phenols in environmental samples from Lake Maracaibo.

Table 1. Analytical methods and other study variables for hydrocarbons and phenols in Lake Maracaibo

Parameter	Method	Sample	Location in Lake Maracaibo	Reference
n-alkanes, methylnaphthalenes, total saturate hydrocarbons, total aromatic hydrocarbons and toxicity	Gas chromatography and 24-hour median tolerance limit (TLm24)	Water, fish and shrimp	North, center and South	Templeton et al. (1975)
Total hydrocarbons; 1,2,4, trimethylbenzene; 1,3,5 trimethylbenzene; diphenylmethane; anthracene and pyrene	Extraction with carbon tetrachloride, analysis by infrared spectrometry and gas chromatography	Water	Samples taken 50 and 250 m from the shore in 7 sites	López and Márquez (1991)
Phenol; *o*; m; and *p*-cresol; 2-isopropylphenol; *o* and *p*-ethylphenol; 2,3; 2,4; 2,5; 2,6; 3,4 and 3,5-xylenol; and 2,3,5-trimethylphenol	Liquid-solid extraction with graphitized carbon cartridges and gas chromatography	Water	38 samples taken at different depths in 13 stations	Burger (2000)
16 polycyclic aromatic hydrocarbons	Liquid-solid extraction disks C18 (500 mg octadecylsilane) and gas chromatography	Water	48 superficial samples in 5 stations	Ulacio et al. (2000)
Benzene, toluene, ethylbenzene, *m*-xylene and *o*-xylene	CS_2 extraction and gas chromatography	Sediment	2 samples from 9°51´17" N latitude and 71°31´41" W longitude)	Faría et al. (2005)
Total hydrocarbons	Standard gravimetric method	Water	Port of Toas island (Tablazo Bay)	Alburgue (2015)

Metals

The content of heavy metals in Lake Maracaibo has been determined in samples of water, sediment and biota (aquatic birds, fish, macrophyte *Lemna* spp. and bivalve *Polymesoda solida*), using the techniques of atomic absorption spectrometry with graphite furnace and cold vapour, and inductively coupled plasma mass spectrometry (ICP-MS) (Prieto and García, 1988; Barco, 1989; Urdaneta, 1995; de Bautista et al., 1999; Hermoso and Márquez, 2005; Pirela and Casler, 2005; Ávila et al., 2007, 2010; Rojas, 2012; Marín et al., 2014). The most studied metals were cadmium (Cd), chromium (Cr), vanadium (V), lead (Pb), mercury (Hg), copper (Cu) and nickel (Ni).

Assessment of Wastewater Treatments

The sequential batch reactor (SBR), rotating biological contactors (RBC), constructed wetlands and upflow anaerobic reactors (UASB) were evaluated at laboratory or pilot scale for the treatment of different types of wastewater (Behling et al., 2003, 2008, 2012a; Vera et al., 2010; Díaz et al., 2012a; Carrasquero et al., 2015). These researches were carried out using domestic and industrial wastewater effluents considered pollutants to Lake Maracaibo. The parameters measured to determine the efficiency of treatment systems were defined according to standard methods (APHA et al., 1998).

Microalgae and autochthonous bacteria were isolated from water samples of Lake Maracaibo and from oil pit located in the eastern shore of this ecosystem, and were tested individually, in mixed cultures (coexistence of different species) and microbial consortia (dependent association between different species) regarding their ability to degrade hydrocarbons. For this, feasibility and treatability assays were performed in order to establish bacterial efficiency in nutrient and hydrocarbon fraction removal (analysis of saturates, aromatics, resins and asphaltenes, SARA) (Araujo et al., 2002; Díaz et al., 2012b, 2013; Alburgue, 2015).

The physicochemical treatments have also been evaluated regarding removal efficiency of priority pollutants in Lake Maracaibo, such as organic matter, nutrients, heavy metals and hydrocarbons (Rojas et al., 2008). The processes include coagulation-flocculation (Carrasquero et al., 2014a; Pardo et al., 2014) and adsorption (Contreras et al., 2008).

ECOTOXICOLOGICAL BIOASSAYS

Data on ecotoxicological bioassays with organisms of Lake Maracaibo are scarce or nonexistent. Tests with heavy metals in bacteria isolated from macrophyte *Lemna* spp. (Díaz et al., 2007; Marín et al., 2011), pelagic bacteria (Castro et al., 2015) and ciliated protozoa (Rincón, 2014) have been carried out in recent years. The minimum inhibitory concentration (MIC) (bacteria), inhibitory concentration for 50% of population (IC_{50}, 24 h) (bacteria) and lethal concentration for 50% organisms (LC_{50}, 1 h) (protozoa) were determined.

Rojas (2012) performed acute and chronic toxicity bioassays with the bivalve mollusc *P. solida* exposed to metals Cd^{2+}, Cr^{6+}, Pb^{2+} and V^{5+}, determining incorporation and elimination rates of metals and individual LC_{50} (96 h) values.

PHYSICOCHEMICAL CHARACTERISTICS OF SURFACE WATER

A physicochemical characterization of surface water in the different zones of Lake Maracaibo is presented in Table 2. These features are influenced by the input of freshwater from the South (tributaries) and the entry of seawater forming a salt wedge on the North (Gulf of Venezuela). The dissolved oxygen concentrations decrease from North to South, due to intensification of organic matter oxidation processes entering from natural and anthropogenic sources.

Carbon, Nitrogen and Phosphorus

Studies have shown that the high productivity of Lake Maracaibo coupled with the elevated level of nutrients in local areas of high biological production and discharges have contributed to the acceleration of the eutrophication process. The N/P ratio is low (<5), resulting from an excess of P in the system (Parra-Pardi, 1979; Gardner et al., 1998; Polo et al., 2014).

The accumulation of N (5,471.86 mg/kg dry weight) and P (2,505.51 mg/kg dry weight) in sediment from thecentre of the lakeand mobility of nutrients across the sediment-water interface are relatively high. This highlights the importance of sediments for nutrient dynamics in this ecosystem, based on dissolved oxygen levels of the hypolimnetic cone (Ledo et al., 2003; Torres et al., 2010). Tributary rivers contribute significantly to nutrient loading and eutrophication of the lake (Rivas et al., 2005, 2009).

Water quality indices applied to Lake Maracaibo explain the trophic state and environmental condition of the water in the coastal area. The temporal-spatial gradient shows high variability between sampling sites and seasonal periods (dry and wet), as well as influence of local phenomena on water quality (Mejías et al., 2014). The PCA indicated that INSF responds better regarding lake characteristics; however, other indices used (WQI_{WA}, WQI_{GWM}, WQI_{UWA} and WQI_{UWGM}) can also describe the water condition when applied together, becoming a versatile tool to know about ecological state or environmental condition of this water body.

Pesticides

The presence, mobility and persistence of pesticides in Lake Maracaibo basin have been poorly studied. The literature reflects scarce current data for these contaminants in waters of tributaries or adjacent zones to vegetable cultivation plots in the Northern and Southern areas of the lake.

Table 2. Physicochemical characteristics of surface waters from Lake Maracaibo

Area	Temperature (°C)	pH	Dissolved oxygen (mg/L)	Salinity (psu)	Electric conductivity (mohm/cm)	Reference
Gulf of Venezuela	26-27			30-34		Rodríguez (2000)
Tablazo Bay	30.7 ± 3.1[a]	8.20-8.72[b]	7.8-10.9[b]	8.13-13.09[b]		[a]Polo et al. (2014), [b]Rojas (2012)
Lake Strait	31.3 ± 1.2[a]	7.95-8.81[b]	6.01-10.5[b]	2.92-5.77[b]		[a]Polo et al. (2014), [b]Rojas (2012)
Lake	30.19-31.17	7.09-8.20	4.38-8.22	3.8-4.4	7,500-8,540	Gardner et al. (1998)
Tributary rivers	28.8 ± 1.84	6.28-8.04			0.161 ± 0.083	Rivas et al. (2005)

An analysis of organochlorine pesticides made between 1987 and 1992 in water, sediment (Urdaneta, 1989) and fishes (Urdaneta et al., 1994) of aquaculture ponds in Limón river prairie (Northwest basin area) showed the following concentrations: between 0.0001 (DDE) and 0.9000 (α-BHC) µg/L in water, between 0.0002 (endrin and dieldrin) and 0.4123 (α-BHC) mg/kg in sediment (Urdaneta, 1989) and in cultured fish fat tissue between 0.0058 µg/g of aldrin in *Pimelodus clarias* and 2.8729 µg/g of metoxichloride in *Roeboides dayi* (Urdaneta et al., 1994).

Recently, Sánchez et al. (2005) did not detect the presence of glyphosate (herbicide) in the Catatumbo river basin (detection limit: 1.24 µg/L), suggesting that the basin was not affected by its use for eradication of poppy crops in the neighboring Republic of Colombia for the sampling period.

Monitoring of the presence and persistence of pesticides in water and sediments of Lake Maracaibo needs to be carried out. This is of particular importance regarding rivers running through lands of vegetable cultivation and animal husbandry in the Northern and Southern areas of the basin, in order to detect their incorporation into aquatic and benthic food chains, and possible ecological consequences for the ecosystem.

Hydrocarbons and Phenols

A two-year study conducted by Templeton et al. (1975) showed low concentrations of hydrocarbons in Lake Maracaibo water (<1 mg/L) and no detectable accumulation of petroleum in muscle tissue of selected commercial fish (<1 µg/g; lake's center). Nonetheless, in the South area of the lake, hydrocarbon concentrations were high in some species: 38.4 µg/g in *Prochilodus reticulates* and 11.3 µg/g in *Anodus laticeps*, probably as a result of differences in diet and metabolism of these species. Toxicity studies with fish and shrimp *in vivo*, also developed by Templeton et al. (1975), indicated that relatively high concentrations of oil are required to cause mortality, with TLm24 (24-hour median tolerance limit) of 15,000 mg/L for shrimp *Penaeus schmitti* and of 2,400 mg/L for fish *Mugil curema*, using Tía Juana medium crude oil (Tía Juana geological formation).

In surface waters of the Northeastern area of the lake, between El Hornito and Los Coquitos sectors, López and Márquez (1991) reported concentrations of total hydrocarbons between 1.22 and 9.97 mg/L. These values are 1.54 times higher than those found in 1973 for this same area, which could be related to increase in oil production operations. The presence of 1,2,4-trimethyl benzene, 1,3,5-trimethyl benzene, biphenyl methane, anthracene and pyrene was also detected.

Surface waters from Port of Toas Island (Tablazo Bay) exhibited a total hydrocarbon concentration of 6,211.67 ± 2,972.83 mg/L (Alburgue, 2015). This value exceeds the permissible limit of 20 mg/L established by Venezuelan law for discharge to a marine-coastal environment (Decreto 883, 1995), which can seriously affect biological processes of the lake. Ulacio et al. (2000) reported concentrations of polycyclic aromatic hydrocarbons between 0.4 and 4.4 µg/L for water samples from five sampling stations.

Regarding the content of phenols, a high presence of methylphenols was found in water samples from the coast of Maracaibo city (66-212 µg/L), with concentrations of phenol 84 ± 15; 2-isopropylphenol 59 ± 15; 3,4-xylenol 42 ± 14 and *o*-cresol 17 ± 8 µg/L (Burger, 2000) above those allowed by Venezuelan law (2 µg/L, Decreto 883, 1995).

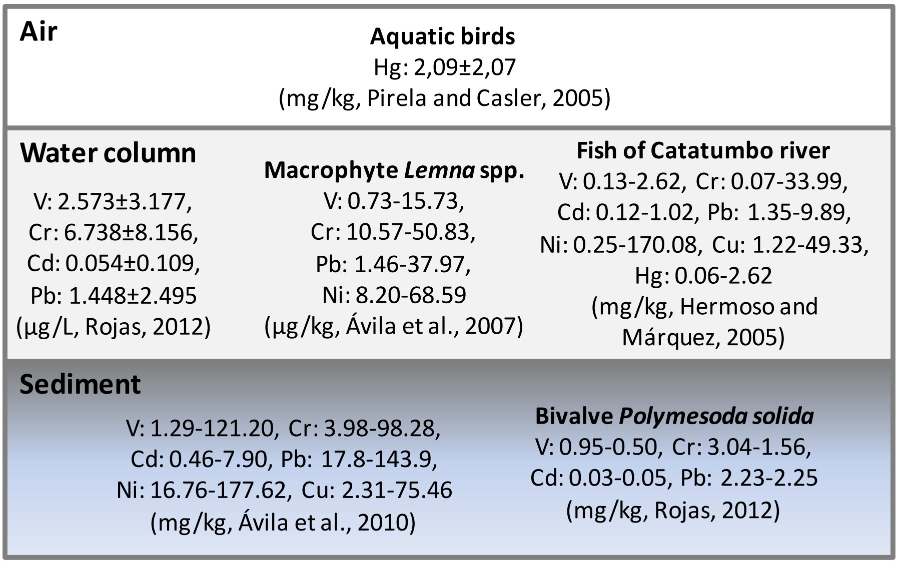

Figure 2. Current data regarding heavy metal concentrations in water, sediment and biota of Lake Maracaibo and fish of Catatumbo river.

In the case of sediments, Faría et al. (2005) did not detect residues of aromatic hydrocarbons in samples from the lake center, indicating possible interference during analysis due to the presence of moisture in the samples as a result of extraction with CS_2 (insoluble in water). Detection limits were: 260 (benzene), 92 (toluene), 90 (ethylbenzene), 120 (*m*-xylene) and 87 µg/kg (*o*-xylene).

Metals

Historically, metal concentrations in Lake Maracaibo have been very low, becoming undetectable during the 1970s and '80s. However, Prieto and García (1988) found 17.5-70.8 ngHg/L in proximity to Tablazo Petrochemical Complex. Barco (1989) reported mean concentrations of 93.10 ± 4.96 µgPb/L and 0.53-17.67 µgHg/L for the Lake Strait, while de Bautista et al. (1999) found 2.8 (surface) to 19.76 mgHg/L (bottom) in this area (Figure 2).

The presence of heavy metals in sediment and biota has been reported in several studies (Barco, 1989; Urdaneta, 1995; Pirela and Casler, 2005; Hermoso and Márquez, 2005; Ávila et al., 2007, 2010; Rojas, 2012; Marín et al., 2014), showing that their concentrations increase through the aquatic food web (Figure 2).

The dynamics of heavy metals in Lake Maracaibo show increasing levels from North (Tablazo Bay) to South (Lake) as a result of metal load provided by tributaries. Concentrations in sediments meet the Canadian sediment quality guidelines for protection of aquatic life (CCME, 1999). However, the content of metals found in the clam *P. solida* (maximum 1.267 mgCd/kg dry weight and 38.412 mgPb/kg dry weight; Marín et al., 2014) is not considered acceptable for human consumption, according to the maximum tolerable weekly limit ingestion per body weight, established by the Food and Agriculture Organization of the United Nations (WHO/FAO, 1999).

Table 3. Characteristics and removal percentages of some effluents treated by sequential batch reactor (SBR)

Wastewater	Operational sequence	HRT (h)	CRT (d)	VOL (kgCOD/m^{3}d)	C_o (mg/L)	% removal	Reference
Domestic	An/Ae/Ax	24	20	0.331	BOD: 130 COD: 276 TKN: 99 TP: 8	BOD: 92 COD: 85 TKN: 52 TP: 67	Cárdenas et al. (2012)
Dairy factory	An/Ae/Ax/ Ae/Ax	24	20	15.34	COD: 12,784 TKN: 99 TP: 37	COD: 88 TKN: 90 TP: 57	Angulo et al. (2015)
Shrimp industry	Ae/Ax	10	15	1.271	COD: 530 TKN: 68 TP: 8	COD: 83 TKN: 78	Díaz et al. (2012a)
Slaughterhouse	An/Ae/Ax	15	25	26.569	COD: 16,606 TKN: 646 TP: 11	COD: 95 TKN: 90 TP: 44	Carrasquero et al. (2015)
MOPW	Ae	15	15	1.27	SCOD: 794 Phenol: 1.47 TH: 104 O+G: 148	SCOD: 65 Phenol: 87.5 TH: 77 O+G: 56	Díaz et al. (2005a)
LOPW	Ae	16	15	1.598	SCOD: 1065 Phenol: 19.3 TKN: 34 TP: 1.07	SCOD: 88 Phenol: 97	Díaz et al. (2005b)
HOPW	Ae	16	15	0.46	SCOD: 307 Phenol: 2.7 TKN: 10 TP: 2.68	SCOD: 63 Phenol: 83	Díaz et al. (2005b)
Tannery (soak liquour + dyeing)	Ax/Ae	12	15	54.926	COD: 27,148 TKN: 2,696 TP: 21 Cr: 14	COD: 47 TKN: 30 TP: 65 Cr: 62	Pire et al. (2010a)
Tannery (Tanning+ dyeing)	Ax/Ae	12	15	12.048	COD: 6,024 TKN: 644 TP: 13 Cr: 174	COD: 47 TKN: 30 TP: 75 Cr: 79	Pire et al. (2010b)
Food industry	Ae	6	15	3.520	BOD: 880 COD: 1,254 TN: 14 TP: 8	BOD: 83 COD: 92	Carrasquero et al. (2014b)
Tannery	Ae/Ax	15.6	25	3.089	COD: 2,008 TKN: 229 TP: 7,7 Cr: 2,3	COD: 49 TKN: 63 TP: 28	Carrasquero et al. (2014c)

HRT: Hydraulic retention time; CRT: Cellular retention time; VOL: Volumetric organic loading; C_o: Initial concentrations; SCOD: Soluble chemical oxygen demand; O+G: Oil and grease; TH: Total hydrocarbons; COD: Chemical oxygen demand; BOD: Biochemical oxygen demand; TP: Total phosphorus; TN: Total nitrogen; TKN: Total Kjeldahl nitrogen; MOPW: Medium oil production water; LOPW: Light oil production water; HOPW: Heavy oil production water; An: Anaerobic condition; Ae: Aerobic condition; Ax: Anoxic condition.

Table 4. Characteristics and removal percentages of some effluents treated by rotating biological contactors (RBC), upflow anaerobic sludge blanket (UASB) combined with RBC and anaerobic rotating biological contactors (AnRBC)

Wastewater	Reactor	HRT (h)	OL (g/m^2d)	Initial concentration (mg/L)	% removal	Reference
Slaughterhouse	RBC	24	7.7 12.3 18.4 24.6	COD: 12,250	COD: 92 88 86 77	Behling et al. (2003)
Shrimp industry	RBC	24 16 12 8 6	4.0 2.3 6.7 6.3 16.4	COD: 973	COD: 74.5 71.9 92.3 73.3 44.6	Behling et al. (2008)
Synthetic ww (glucose)	RBC	24		COD: 1,000-2,000	COD: 97-99	Behling et al. (2005)
Synthetic ww (glucose+ phenol)		12		COD/Phenol 1,000/6 1,000/20	COD: 97.5 Phenol: 99.7	
Synthetic ww (sucrose+urea)	RBC (3-stage)	24 12 6	6.10 11.68* 23.97	COD: 1,096 TN: 226.6	COD: 96.25 TN: 62.95	Behling et al. (2012b)
Synthetic ww (glucose+ phenol)	UASB+ RBC	UASB RBC 10.12 18.97 10.12 18.97 15.15 28.41	UASB RBC 9.42 8.38 9.73 5.5 6.55 3.45	COD: 4,097.8 TKN: 200 PO_3^{4-}: 40.6 Phenol: 17.6	COD: 95.84 TKN: 80.71 PO_3^{4-}: 81.03 Phenol: 99.6	Behling et al. (2012a)
Synthetic dairy industry (sucrose+milk)	AnRBC	24	24.67	COD: 2,500	Continuous† reactor: COD: 82.7 Batch† reactor: COD: 93.6	Behling et al. (2013)

* Best treatment. Synthetic ww: Wastewater prepared in the laboratory; HRT: Hydraulic retention time; OL: Organic load; RBC: Rotating biological contactors; UASB: Upflow anaerobic sludge blanket; AnRBC: Anaerobic rotating biological contactors; COD: Chemical oxygen demand; BOD: Biochemical oxygen demand; TP: Total phosphorus; TN: Total nitrogen; TKN: Total Kjeldahl nitrogen; PO_4^{3-}: Orthophosphate; †: Flow regime.

PERFORMANCE OF WASTEWATER TREATMENTS

Sequential Batch Reactor (SBR)

SBR have been successfully used in the treatment of domestic and industrial wastewater, demonstrating the flexibility of SBR systems to adapt to effluents with different physicochemical characteristics (Table 3). The SBR system promotes the removal of nutrients using an anaerobic/aerobic/anoxic (An/Ae/Ax) sequence in the treatment, resulting in removal of carbon, nitrification, denitrification, anaerobic phosphorus release and uptake of excess phosphorus during the aerobic phase. Cárdenas et al. (2012) used this sequence for simultaneous nutrient removal from domestic sewage of the city of Maracaibo, yielding an effluent that meets the current Venezuelan regulatory standards of discharges to water bodies.

Waters associated with oil exploitation represent one of the main pollutants of Lake Maracaibo. During oil production, a certain amount of effluent known as production water (OPW) is coproduced. Díaz et al. (2005a,b) used laboratory reactors for treatment of light oil production water (LOPW), medium oil production waters (MOPW) and heavy oil production water (HOPW). They concluded that OPW with total phenol concentrations between 1.40 ± 0.27 and 19.36 ± 2.02 mg/L can be successfully treated by SBR reactors (Table 3).

SBR have also been used successfully to simultaneously remove nutrients and organic matter from effluents of cattle slaughtering processes (Carrasquero et al., 2015) and from shrimp processing (Díaz et al., 2012a).

Pire et al. (2010a,b) removed COD (chemical oxygen demand), TKN (total Kjeldahl nitrogen) and TP (total phosphorus) from tannery effluent with a chromium concentration of 174 mg/L. These researchers showed that the strongest removal of compounds occurred in the early hours of treatment, which was attributed to the anoxic conditions that allowed the decomposition of structures of the complex organic matter. Besides, Carrasquero et al. (2014c) successfully treated the mixed settled effluents produced in a tannery. The operational sequence Ae/Ax in combination with a cell retention time of 25 days allows for the best efficiencies for the simultaneous nutrient and COD removal.

From effluents with nutrient deficit, such as those from vegetable processing industries, average removal values for COD and BOD of 80 and 92%, respectively, were obtained. These removals were achieved without needing to add macronutrients (nitrogen and phosphorus) to the wastewater (Carrasquero et al., 2014b).

Rotating Biological Contactors (RBC)

RBC has been tested at laboratory scale showing high efficiency, low cost, and ease of operation and maintenance (Table 4). Behling et al. (2003, 2008) evaluated the behavior of a RBC for different types of wastewater. In the case of effluents from meat and shrimp industries the system was able to remove a high amount of organic matter. Particularly for the shrimp, effluent, COD and pH were reduced to levels that comply with the limits established in the Venezuelan regulations. In another study, RBC achieved 97% removal of phenolic compounds (Behling et al., 2005).

Moreover, Weffer (2010) and Behling et al. (2012a) evaluated the performance of a combined system treatment (UASB+RBC) using a synthetic effluent (glucose+phenol), and they obtained a high overall efficiency of phenol degradation (99.6%) and removal of organic matter (95.8%). Weffer (2010) concluded that the UASB followed a first-order kinetics Monod model and RBC followed the Kincannon and Stover model.

Behling et al. (2012b) studied the influence of organic load (OL) on efficiency of the aerobic 3-stage RBC to treat a synthetic industrial effluent: the greater removal of C and N was reached in the first stage of reactors, meeting the Venezuelan discharge limit for COD (<350 mg/L). The authors observed that nitrification increased the concentration of inorganic nitrogen in the final effluent. At the same time, Marín et al. (2012) found that the abundance of bacteria was affected by both organic loads and the position in the reactor chambers. They also noted simultaneous occurrence of the nitrification-denitrification processes, evidenced by the detection of both bacterial groups in the biofilm.

Finally, Behling et al. (2013) conducted tests in an anaerobic RBC (AnRBC) using a synthetic effluent with similar characteristics to that generated in the dairy industry (sucrose+milk). Significant removals of COD were achieved, but without meeting the discharge limits in the Venezuelan regulation. Remaining organic matter content in the AnRBC effluent was due to the contribution of suspended solids; they therefore recommended the installation of a secondary settler after the reactor.

Constructed Wetlands

Constructed wetlands have been tested successfully for the treatment of domestic, industrial (water deriving from oil production) and mining wastewater, removing contaminants from the effluents through different ways (absorption, adsorption, sedimentation). For instance, Vera et al. (2010) evaluated the removal of nutrients and organic matter from domestic sewage using a free water surface (FWS) wetland planted with *Typha dominguensis*, and found that it was more efficient in removing organic matter in relation to the control (without emergent vegetation); nevertheless, the nutrient removal was higher in the control treatment. Results showed the important role of this macrophyte during removal of suspended solids and organic matter. However, more studies are needed to determine the importance of the macrophyte in nutrient removal (Table 5). On the other hand, Vera et al. (2010) determined that eight weeks of growth were the optimal time for *T. dominguensis* pruning.

In another study, Núñez et al. (2007) observed the synergistic effect of the combination of macrophytes (*Lemma* sp. and *T. dominguensis*) on removal of TN and COD over the system that operated without vegetation (control). They also observed the important activity of bacteria and algae during nutrient removal from wastewater.

Araujo et al. (2014) evaluated the influence of surface coverage of the FWS wetland with *Pistia* sp. on the removal efficiency of nutrients from domestic effluent. They obtained the largest removals of TN and TP when the wetland surface was completely covered with macrophytes. However, in the treatment where no coverage with *Pistia* sp. was used, a significant removal of nutrients was obtained due to the presence of the microalgae *Chlorella* sp. ($401.2x10^4$ cells/mL).

On the other hand, Blanco-Fontalvo (2008, 2011), treating effluents from oil production industry in a SSF wetland, obtained higher removal of phenols, hydrocarbons, COD, SS, Cl^-, S^{2-} and metals (Cr, Cu, Ni, Pb and Zn) when using the combination of macrophytes (Table 5). The results suggest that SSF wetlands are efficient in removing contaminants from this kind of effluents, and such efficiency depends on the macrophytes used.

Simultaneously, Paz-Palacios (2008) and Paz et al. (2012) evaluated the efficiency of pilot-scale FWS wetlands for the treatment of wastewater from oil production. The removal of TP, hydrocarbons, S^{2-}, phenol, and TN showed no significant differences between the wetland planted with *Cyperus luzulae*, *C. fertile*, *C. ligularis*, *Paspalum* sp. and *T. dominguensis*, and the control (without emergent vegetation). Particularly, they could not demonstrate the influence of these macrophytes in the removal of S^{2-} and phenolic compounds.

Marín et al. (2006) observed a marked trend towards the reduction of the content of heavy metals (Pb, Zn, Fe, Al and Ni) as the hydraulic retention time (HRT) was increased during the treatment of coal mine drainages in wetlands. Sediment resuspension from the pit, secretion by plants and atmospheric deposition material mechanisms could be related to the mobility of metals.

Table 5. Characteristics and removal percentages of some effluents treated by different types of constructed wetlands: free water surface (FWS) and subsurface flow (SSF)

Wastewater	Wetland	Organism	% removal	Reference
Domestic	FWS	*Pistia* sp.	TN: 100 TP: 77.7	Araujo et al. (2014)
		Chlorella sp.	TN: 97 TP: 70.7	
Domestic	FWS	*Typha domínguensis*	COD: 30.3-30.5 BOD: 34.6-31.6 TSS: 43.9-52.4 VSS: 42.9-60.8 NH_4^+: 45-55 TKN: 48-50	Vera et al. (2010)
Domestic	FWS	*T. domínguensis* + *Lemma* sp.	COD: 42 TKN: 84 $N\text{-}NH_4^+$: 93.4 $N\text{-}NO_2^-$: 54 $N\text{-}NO_3^-$: 41	Núñez et al. (2007)
Water from oil production	SSF OL=29.4 g/m^2d HRT: 7d	*T. domínguensis* + *Paspalum* sp. *Cyperus luzulae, C. fertile, C. ligularis*	Phenol: 90.69 Hydrocarbons: 76 COD: 31.8 TSS: 50.8 Cr: 35.5, Cu: 47.4, Ni: 52.6, Pb: 52.9, Zn: 27.0	Blanco-Fontalvo (2008)
Water from oil production	SSF	*T. domínguensis, C. luzulae, C. feraz, C. Ligularis*	Cu: 58 Pb: 53	Blanco-Fontalvo et al. (2011)
Water from oil production	FWS HRT: 7 d	*C. luzulae, C. feraz, C. ligularis, Paspalum* sp., *T. domínguensis*	TP: 11 Hydrocarbons: 68 S^{2-}: 66 Phenol: 57 TN: 72 Cr: 91, Ni: 20 Cu: 91, Zn: 28 Pb: 7	Paz-Palacios (2008)
Water from oil production	FWS HRT: 7 d	*C. luzulae,* *C. ligularis* *C. feraz, Paspalum* sp., *T. domínguensis*	S^{2-}: 62-66 Phenol: 61-71	Paz et al. (2012)
Coal mine	SSF HRT: 1, 3, 5, 7, 9 d	*T. domínguensis*	$SO_4^{2-} > 80$	Marín et al. (2007)

HRT: Hydraulic retention time; OL: Organic load; COD: Chemical oxygen demand; BOD: Biochemical oxygen demand; TP: Total phosphorus; TN: Total nitrogen; TKN: Total Kjeldahl nitrogen; $N\text{-}NH_4^+$: Ammoniacal nitrogen; $N\text{-}NO_2^-$: Nitrite; $N\text{-}NO_3^-$: Nitrate; TSS: Total suspended solid; VSS: Volatile suspended solid, S^{2-}: Sulfides; SO_4^{2-}: Sulfates.

In a parallel study, Marín et al. (2007) evaluated the behavior of sulfates, which are one of the main problems in the liquid effluents of the coal industry. The results showed that concentrations of SCOD, TKN and TP remained below the permissible limit set by the Venezuelan regulations. The variability of the parameters studied is determined by the type of support material employed in the wetlands and HRT. The system was highly efficient in reducing the sulfate content in a biological way.

Anaerobic Biological Treatment Systems

Recent research has demonstrated the applicability of anaerobic biological treatment system of wastewater containing high organic loads and toxic or inhibitory compounds, simulating the wastewater discharged in the Lake Maracaibo basin (Table 6). Caldera et al. (2007) demonstrated the efficiency of a UASB reactor treating slaughterhouse effluents. Besides, Gutiérrez et al. (2007) evaluated the treatability of water oil production in batch reactors, showing that the biodegradability of LOPW under thermophilic conditions presented no difference compared with mesophilic conditions (78 and 80%, respectively); however, results were different for MOPW and HOPW, where the increase in temperature improved biodegradability.

Other biological treatments have been evaluated for domestic effluents discharged in the basin of Lake Maracaibo. Cárdenas et al. (2002) used aerated ponds for the treatment of municipal wastewater from the city of Maracaibo. They demonstrated the efficiency of this treatment in tropical weather (removal of 91% SCOD, with a HRT of one day).

Microbial Consortia and Native Microorganisms

Native bacteria and microalgae isolated from surface waters of Lake Maracaibo have demonstrated high efficiency in hydrocarbons removal. Fertilized and aerated cultures of bacteria from the lake were able to remove up to 89% of total hydrocarbons present in lubricating oil (initial concentration of 7,000 mg/L) (Araujo et al., 2002), while microalgae mixed cultures of *Coenochloris* sp. and *Chlorococcum* sp., isolated from an oil pit, in consortium with its associated bacteria, efficiently degraded kerosene to 68% at an initial concentration of 0.5%, and to between 43 and 57% with kerosene initially at 3% (Díaz et al., 2012b), highlighting the remediation potential of the established microbial consortium.

Kerosene (1%) hydrocarbons were removed up to 60% in 30 days by mixed cultures of bacteria *Yersinia rohdei*, *Pantoea agglomerans*, *Sphingobacterium thalpophilum* and *Actinobacillus capsulatus*, isolated from an oil pit in the Zulia State. *S. thalpophilum* and *A. capsulatus* were equally efficient in total hydrocarbons removal in axenic cultures (Díaz et al., 2013). Thus, microbial consortia and native microorganisms can be efficiently used in the biological treatment of hydrocarbon contaminated water before being discharged into Lake Maracaibo.

Table 6. Characteristics and removal percentages of some effluents treated by anaerobic reactors: double chamber anaerobic reactor (DCAR), bacth reactor (BR), upflow anaerobic sludge blanket (UASB)

Wastewater	Reactor	HRT (h)	VOL (kgCOD/ m^3d)	T	Co (mg/L)	% removal	Biogas (L/d)	Reference
Synthetic ww (glucose)	DCAR	12-2	0.9-6.0	M	COD: 467-508	COD: 69-85	0.89-2.56	Rincón et al. (2013)
Synthetic ww (glucose+Ni)	BR	24	1.0-3.0	M	COD: 1,000-3,000 Ni: 20-50	COD: 86-16	0.2-4.5	Martínez et al. (2012)
Synthetic ww (glucose+ phenol)	UASB	24	0.9-5.6	M	COD: 903-5,596 Phenol: 20	COD: 82 Phenol: 34 2-chlorophenol: 77 2-nitrophenol: 55 2,4 dinitrophenol: 77	2-6	Vacca et al. (2008)
Tannery	UASB	24	1.0-3.0	M	COD: 1,000-3,000	COD: 92-94	0.31-0.85	Behling et al. (2004)
Slaugtherhouse	UASB	12	17	M	COD: 8,500	COD: 87	15.5	Caldera et al. (2007)
LOPW MOPW HOPW	BR	24	1.28 8.64 9.33	Tm	SCOD: 1,280 SCOD: 864 SCOD: 933	SCOD: 71 SCOD: 60 SCOD: 62	-	Gutiérrez et al. (2007)
LOPW	UASB	24-6	1.4-5.6	Tm M	SCOD: 1,400	SCOD: 79-53 SCOD: 75-40	-	Gutiérrez et al. (2006)
Clarifier (a mix of LOPW+MOPW+ HOPW)	BR	24	0.55-0.84	M	SCOD: 553-848 Naphthalene: 7.91 Acenaphthylene: 1.25	SCOD: 20-60 Naphthalene: 99.5 Acenaphthylene: 0	-	Mas y Rubí et al. (2011)
Shrimp industry	UASB	32-11	1.43-6.1	M	COD: 1,902-2,849	COD: 50-83	0.34-3.38	Pérez et al. (1999)

DCAR: Double chamber anaerobic reactor; Synthetic ww: Wastewater prepared in the laboratory; HRT: Hydraulic retention time; VOL: Volumetric organic loading, Co: Initial concentrations; T: Temperature; BR: Batch reactor; COD: Chemical oxygen demand, UASB: Upflow anaerobic sludge blanket; SCOD: Soluble chemical oxygen demand; M: Mesophilic; Tm: Termophilic; MOPW: Medium oil production water; LOPW: Light oil production water; HOPW: Heavy oil production water.

Physicochemical Treatment of Effluents

Carrasquero et al. (2014a) used chitosan (obtained from crab waste) as a coagulant for treating wastewater from the flour processing industry. They found removals of 98.9 and 80.2% of turbidity and COD, respectively. Besides, the ability of chitosan to form complexes with metal ions was explored by Pardo et al. (2014), obtaining a 45% removal of chromium during the pretreatment of tannery effluent.

Activated carbon prepared from sugarcane bagasse has a high surface area and a porous structure suitable for adsorption of complex organic compounds during wastewater treatment, being able to remove 2-chlorophenol, 2-nitrophenol and 2,4-dimethylphenol: removal percentages obtained from the adsorption of these compounds on activated carbon obtained from sugar cane bagasse were 49.8, 66.3 and 57.6%, respectively (Contreras et al., 2008).

RECENT ECOTOXICOLOGICAL BIOASSAYS

Ciliated protozoa *Euplotes* sp., *Uronema* sp. and *Loxodes* sp. (Rincón, 2014), bacterial strains (Castro et al., 2015) and the plant *Lemna* ssp. (Díaz et al., 2007; Marín et al., 2011) from Lake Maracaibo, showed high resistance to heavy metals, with very high MIC, LC_{50} andIC_{50} values, particularly in bacteria (to the order of 10^8 mg/L), as a result of pollution prevailing in the ecosystem (Castro et al., 2015).

Toxicity bioassays with bacteria indicated that the most toxic metal was Cr^{6+}, with the following sensitivity pattern $Cr^{6+} > Cd^{2+} > Cr^{3+} > Ni^{2+} > Pb^{2+}$ (Castro et al., 2015). In the case of ciliated protozoa, results showed that *Euplotes* sp. was the most sensitive to Cr^{6+} and Cr^{3+}, while *Loxodes* sp. exhibited major susceptibility to Cd^{2+} and V^{5+} (the sensitivity order to the tested metals was $Cd^{2+} > V^{5+} > Cr^{3+} > Cr^{6+}$). *Euplotes* sp. exhibited the greatest response to all the tested heavy metals, showing LC_{50} of 14.35 mgCr^{6+}/L, 4.82 mgCr^{3+}/L, 0.82 mgCd^{2+}/L and 2.13 mgV^{5+}/L, indicating that this species is a potential bioindicator of metal contamination in Lake Maracaibo (Rincón, 2014).

The concentrations of heavy metals in soft tissues of *P. solida* (Cr > Pb > V > Cd) did not correlate with levels found in water and sediment. Toxicity tests showed LC_{50} (96 h) values of 15.94 mgCr^{6+}/L, 77.79 mgPb^{2+}/L, 128.25 mgCd^{2+}/L and 401.96 mgV^{5+}/L. Bioassays of incorporation and elimination of Cr^{6+} and V^{5+} were characterized by a rapid uptake and increased sensibility to Cr^{6+}. Chromium (VI) accumulation was higher but V^{5+} was retained for a longer time (Rojas, 2012; Rojas et al., 2015). Thus, *P. solida* is also recommended as bioindicator for heavy metal pollution in Lake Maracaibo estuarine system due to its ability to incorporate, tolerate and accumulate metals in concentrations higher and proportional to exposure levels.

CONCLUSION

The hydrodynamic conditions of Lake Maracaibo basin are particularly complex due to the simultaneous interaction of different factors such as tides, winds, stratification, saline intrusion, and hydrologic and hydraulic effects. A high accumulation of C, N, P and heavy metals in bottom sediments has been identified, which, depending on oxygenation conditions of the water-sediment interface, maybe retained in the water column. Bioaccumulation processes and high resistance to heavy metals have also been identified in the biota of this aquatic system.

Treatment technologies tested at laboratory and pilot scale seem to be viable alternatives for depuration of wastewaters discharged into Lake Maracaibo.

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Chapter 30

IMPORTANCE OF BACTERIAL BIOFILM FOR ZN RETENTION IN THE SEDIMENT-WATER INTERFACE: THE ROLE OF BACTERIAL ZN RESISTANCE

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ABSTRACT

Bacterially-mediated processes have a pivotal role in determining the cycling of trace elements in sedimentary environments, while the potential role of microbial biofilms developed under heavily-contaminated conditions on this cycling is less known. Microcosm experiments were performed to test the hypothesis that metal resistance

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induced by exposure to elevated metal levels can result in the production of bacterial biofilms more able to affect the zinc (Zn) cycling in the sediment-water interface of a tidal creek that drains a mangrove ecosystem. After 4 h of flooding by tidal water with high Zn concentration (50 mg L^{-1}), creek sediments with Zn-resistant biofilm showed significantly different HCl-soluble Zn concentrations (62% higher) and inventories (56% higher) than control sediments. A significant correlation between HCl-soluble Zn and Fe occurred for most data, with the exception of results from the uppermost sediment layers treated with Zn-resistant biofilm, evidencing that this treatment overwhelmed the general positive effect of Fe on Zn concentration. A positive feedback between enhanced Zn resistance and Zn sorption capacity of sediments was observed. This finding may contribute to a better comprehension of the Zn cycling at sites in which Zn resistance is developed and sites that receive Zn-resistant biofilm associated to suspended particulate matter transported from nearby contaminated areas. Since the short flooding time (4 hours) simulated in these experiments evidenced a significant biofilm effect on Zn accumulation, longer periods of evaluation are recommended to test the duration of the observed effect (e.g., throughout complete tidal cycles).

Keywords: bacterial biofilm, zinc resistance, sorption, sediments, microcosm experiments

INTRODUCTION

Bacterially-mediated processes have a pivotal role in determining the cycling of trace elements in sedimentary environments, driving diagenetic reactions that produce and degrade geochemical compounds (Shaw et al., 1990; Borch et al., 2010), and affecting sediment physical properties, such as sediment particles cohesion (Decho, 2000). The assemblages of bacterial populations attached by extracellular polymeric substances (EPS) to aquatic system substrates (i.e., bacterial biofilms) can constitute important trace element-binding surfaces in such systems, considering the metal sorption by bacterial surfaces (Fein et al., 2001; Guiné et al., 2006) and by the EPS secreted by bacterial consortia (González et al., 2010; Kenney and Fein, 2011). When a dissolved or paticulate substance transported by the water phase meets a biofilm, it encounters a heterogeneous hydrogel with very different sorption sites, including: charged groups (e.g., $-COO^-$, $-SH^-$, etc.), apolar groups (e.g., aromatics, alifatics, etc.), cell walls, cytoplasmic membrans and cytoplasms; while the EPS consist of polysaccharides, proteins and lipids (Flemming and Leis, 2002). The EPS composition seems to be not constant, but is influenced by growth conditions and environmental stress (Flemming and Leis, 2002).

The biofilm sorption of trace metals has been widely evidenced in sediments, soils, natural waters and wastewater (Borrok et al., 2004; Toner et al., 2005; Hockin and Gadd, 2006; Ha et al., 2010). This biofilm role can occur in different ways, for instance, by ion exchange reactions, complexation and precipitation, as passive binding (e.g., electrostatic interactions, ion exchanges, precipitations and complexations with biofilm components already present and not formed in response to the bound substance) or active binding (i.e., through the excretion of binding, chelating or precipitating substances in response to the presence of the dissolved substance) processes (Flemming and Leis, 2002). Biofilm coatings can often enhance metal sorption by sandy sediments, actively participating in binding dissolved metals from both overlying and pore water (Schlekat et al., 1998), while elevated metal exposure can induce bacterial toxicity and increased EPS production to cope with such effect, resulting in increased metal immobilization by EPS (Yang et al., 2013). Therefore, considering that metal resistance is the ability of microorganisms to continue growing when exposed to the considered metal (Harrison et al., 2007), it may be hypothesized that the metal sorption by sediments can be significantly affected by biofilms with amplified metal resistance.

Possible effects of bacterial biofilm with amplified Zn resistance on the sorption of Zn from overlaying water by tidal creek sediments from the Itacuruça mangrove ecosystem (Sepetiba Bay, SE Brazil) were evaluated in microcosm experiments. Although it is known that biofilms can affect the metal sorption capacity of sandy sediments (Schlekat et al., 1998; Yang et al., 2013), evaluations of this biofilm role in intertidal tropical ecosystems were not found in the literature. Moreover, enhanced metal sorption capacity by the influence of bacterial biofim with improved metal resistance (in response to previous metal exposure) may be an additional factor affecting metal accumulation in these environments.

METHODS

Zinc-resistant biofilm was obtained after a treatment of surface sediments (0–3 cm depth) collected from the mouth of the Saco do Engenho creek (Figure 1), Sepetiba Bay (22°55′12″S, 43°49′6″W). This creek is recognized as the major source of Zn to the bay due to past drainage from a large Zn smelt plant refuse pile (Barcellos et al., 1991; Molisani et al., 2004). Zinc emissions from this source were estimated as 3,660 t yr^{-1}until 1997, when the plant was closed (Molisani et al., 2004).

The procedures adopted to obtain the bacterial biofilm with amplified Zn resistance were those described by Pennafirme et al. (2015), which employed the same exposure material used in this work. Resistant bacteria were isolated from Saco do Engenho creek sediments and multiplied in a sterilized cultivation media of filtered sea water (<0.45 μm) with bacto-peptone (2 g L^{-1}), urea (2 g L^{-1}), glucose (6 g L^{-1}) and Zn (50 mg L^{-1}), during 30 days. In April 2013, surface sediments and surface tidal water were collected in the tidal creek that drains a mangrove forest dominated by the red mangrove (*Rhizophora mangle* L.) located in Itacuruçá, Sepetiba Bay (22°55′19″S, 43°53′09″W) to proceed with the experiments. There are previous evidences that after the sorption of ^{65}Zn from overlying water by sandy sediments from Itacuruçá creek, this radiotracer presented a partial release back to overlying water, while adjacent mangrove-colonized, muddy sediments presented more efficient ^{65}Zn retention (Machado et al., 2008). In order to test the potential role of Zn-resistant biofiolm in improving the Zn sorption from tidal water, such sediments with lower Zn sorption capacity were chosen for the present study. From the studied sampling site (Figure 1), surface sediments (0–5 cm depth) were collected using a plastic spatula and stored in a plastic bag under refrigeration until processing in laboratory, while surface (~5 cm depth) tidal water was sampled at the same moment during an ebbing tide, by using a 25-L plastic container. The experiments were performed ~2 h after the sampling.

Plexiglas tubes were used to contain experimental microcosms (Figure 2), which were composed of 6 cm height sediment columns covered by 120 mL of local tidal water spiked with Zn at a concentration of 50 mg L^{-1}. The experimental apparatus was adapted from Petersen et al. (1998), according to Machado et al. (2008). Since natural variability in sediment composition and benthic organisms activity can affect trace element sorption from overlying water (e.g., Suzuki et al., 2013), sampled sediments were homogenised prior to tube preparation to improve data comparability.

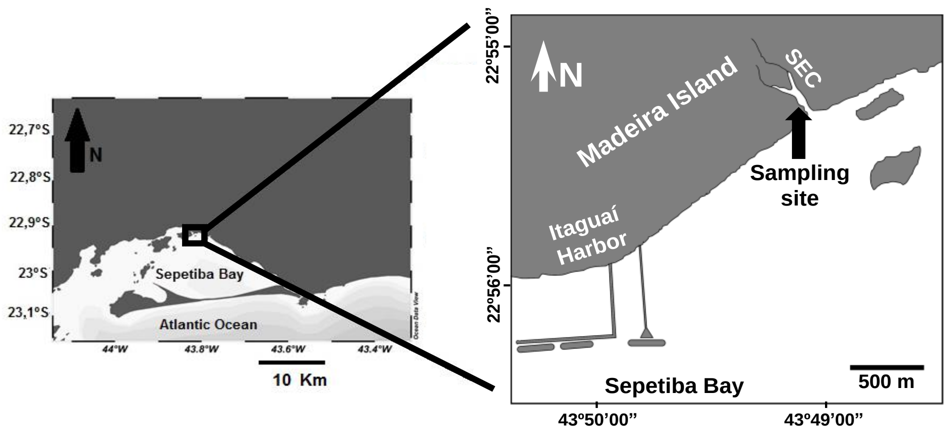

Figure 1. Location of Saco do Engenho creek (SEC), Sepetiba Bay, Brazil, and the study sampling site at the SEC mouth.

One set of triplicate cores received 1 mL of culture medium containing Zn resistant biofilm (with 1.13×10^9 cells cm^{-3}), while the other set of other triplicate cores were used as controls. After a 12-h acclimation period, all cores were slowly covered (minimizing sediment re-suspension) with Zn-spiked tidal water to start the experiments, which were carried out at room temperature (~26°C). Tidal water pH (8.0) and salinity (28) were measured using a Hanna pH meter and a Shibuya refractometer, respectively. Oxygen saturation of overlying water was induced by air pumping. After 4 h (simulating a short flooding period), the overlying water was removed and the sediments were extruded and sliced at 2 cm depth intervals. After agitation of sediments in 1 mol L^{-1} HCl for 16 h (Huerta-Diaz and Morse, 1990), reactive Zn (Zn_{HCl}) concentrations in sediments were determined, considering that this extraction has been employed to estimate potential metal bioavailability for benthic organisms (e.g., Sokolowski et al., 2007; Sabadini-Santos et al., 2014a,b). This procedure extracts metals associated to carbonates, Fe and Mn (oxy)hydroxides, labile organic matter and metal sulfides, besides metal adsorbed to the surface of sediment particles. Possible associations of Zn with organic matter and reactive Fe (Fe_{HCl}) and Mn (Mn_{HCl}) contents were evaluated. Metal concentrations were determined in duplicates by ICP OES, showing reproducibility better than 10%. Organic matter contents were estimated by the loss-on-ignition (LOI) method (450°C, 24 h). Dry sediment density was estimated by weighing a known volume of wet sediment after drying at 50°C for 72 h, ranging from 1.59 to 1.72 g cm^{-3}, which is consistent with the coarse nature of sediments from this site (generally showing over 60% sand; Silva et al., 2003). Inventories of Zn_{HCl} were calculated as the product between Zn_{HCl} concentrations (mg kg^{-1}), sediment density (g cm^{-3}) and sediment depth intervals (cm). Bacterial cell number was determined by epifluorescent microscopy, using acridine orange method and UV-radiation (Kepner and Pratt, 1994). A Mann-Whitney *U* test was used to compare results from the Zn-resistant biofilm treatment with the control data, while a Pearson correlation analysis was performed to evaluate possible statistical associations between the variables. A significance level of 0.05 was accepted for all statistical tests.

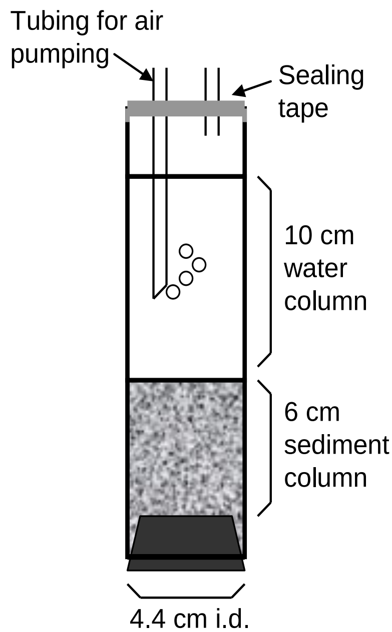

Figure 2. Used experimental apparatus, as modified from Petersen et al. (1998). “i.d.”=inner diameter. Note the presence of a tubing for air pumping and a smaller tubing communicating the “head space” with the atmosphere.

RESULTS

The evaluated concentrations of potential geochemical carriers (LOI, Fe_{HCl} and Mn_{HCl}) of trace metals did not present statistically significant differences between control sediments and sediments with Zn-resistant biofilm in the different depth intervals (Figure 3a). An exception was Fe_{HCl} in the uppermost depth interval, which was significantly lower in sediments treated with Zn-resistant biofilm.

Concentrations and inventories of Zn_{HCl} did not present significant differences between control sediments and sediments with Zn-resistant biofilm at different depth intervals, with the exception of significantly higher values observed in the uppermost layers of sediment cores treated with Zn-resistant biofilm (62% and 56% higher in average, respectively) than in control sediments (0-2 cm depth data; Figure 3b). Although there was a significant correlation between Zn_{HCl} and Fe_{HCl} for most data ($r=0.61$; $p<0.05$), data from the uppermost layers treated with Zn-resistant biofilm did not follow this trend (Figure 4), which evidenced that this treatment overwhelmed the general positive effect of Fe_{HCl} on Zn_{HCl} concentration. This explains the higher Zn_{HCl} accumulation under conditions of lower Fe_{HCl} levels found in the uppermost layers treated with Zn-resistant biofilm. Therefore, a change in the geochemical controls on Zn_{HCl} retention within sediments was evidenced, in response to Zn-resistant biofilm effects on surface layers. On the other hand, there was no significant correlation of

Zn_{HCl} with the very low contents of LOI (essentially below 0.8%) and Mn_{HCl} (essentially below 9 mg kg^{-1}) observed in the studied sediments (r=-0.19 and -0.39, respectively).

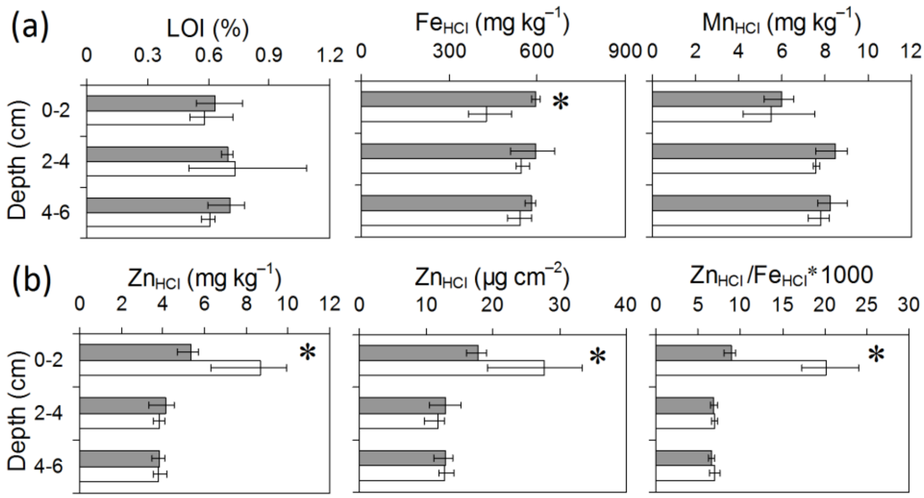

Figure 3. Depth variability of (a) LOI, Fe_{HCl} and Mn_{HCl} contents, and (b) Zn_{HCl} contents, Zn_{HCl} inventories and Zn_{HCl}/Fe_{HCl} ratios in control sediments (grey bars) and sediments with Zn-resistant biofilm (white bars). Bars represent mean values. Error bars represent the ranges of triplicate analyses. Depth intervals with significant differences between control sediments and sediments with Zn-resistant biofilm are indicated by an asterisk (Mann-Whitney *U* test, $p < 0.05$).

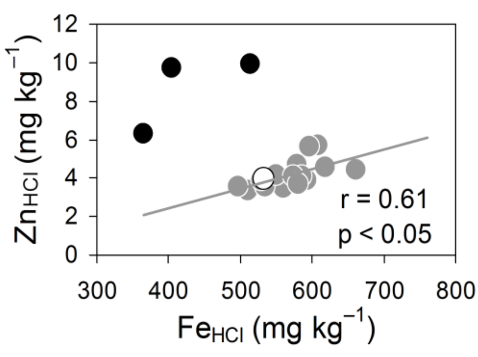

Figure 4. Relationship between Zn_{HCl} and Fe_{HCl} levels. The white symbol represents the average of duplicate data from used sediments before the experiment. Black symbols represent surface layers with Zn-resistant biofilm. Grey symbols represent all other data (irrespective of depth and treatment) obtained at the end of the experiment, for which correlation analysis trendline and results are presented.

Considering the reactive (HCl-extractable) Fe compounds as major binding phases for trace metals (e.g., Fe (oxy)hydroxides), Morse et al. (1993) used a Zn_{HCl}/Fe_{HCl} ratio to evaluate anthropogenic Zn_{HCl} enrichments in Galveston Bay (USA) sediments. In an analogous approach, Zn_{HCl}/Fe_{HCl} ratios were used in the present study to evaluate the extent in which Zn-resistant biofilm affected Zn_{HCl} accumulation, in addition to the evaluation of absolute Zn_{HCl} concentration differences. Figure 3b demonstrates that Zn_{HCl}/Fe_{HCl} ratios presented the same trend observed for Zn_{HCl} concentrations. There was a significant difference in the uppermost depth intervals for these ratios, corresponding to an average value 2.2-times higher in the Zn-resistant biofilm treatment, in comparison with the control sediment data. In fact, most Zn_{HCl}/Fe_{HCl} ratios were essentially near those found in the sediments before starting the experiments (averaging 7.4×10^{-3}), whereas ratios ranging from 17.3 to 24.0×10^{-3} were observed for the uppermost layers treated with Zn-resistant biofilm (Figure 3b).

There was no statistically significant change (as indicated by Mann-Whitney U tests) in the bacterial cells number in relation to control sediments at the end of the experiment (Table 1). This observation evidenced that higher Zn sorption by bacterial consortia adapted to high Zn exposure and/or enhanced binding of Zn to EPS secreted by this adapted consortia occurred without dependence on changes in bacterial cell number.

Table 1. Depth variability of bacterial cell number (No. cm^{-3}) in sediments after 4 h experiments

Depth (cm)	Control			Zn-resistant biofilm addition		
	Mean	Minimum	Maximum	Mean	Minimum	Maximum
0-2	2.86×10^8	2.77×10^8	2.96×10^8	2.97×10^8	2.74×10^8	3.31×10^8
2-4	2.14×10^8	1.67×10^8	2.41×10^8	1.85×10^8	1.73×10^8	2.10×10^8
4-6	2.05×10^8	1.75×10^8	2.30×10^8	2.78×10^8	1.93×10^8	3.43×10^8

DISCUSSION

Although the used extraction procedure can extract metals adsorbed to sediment particles and bound to carbonate, hydrous aluminosilicates and amorphous and crystalline oxyhydroxides (Huerta-Diaz and Morse, 1990), a predominance of Fe_{HCl} oxidized phases is expected, considering that oxygenated tidal water generally covers the sediments from the Itacuruçá creek (Lacerda et al., 1988), which are coarse-grained and periodically exposed to air. Since Fe-reducing bacteria are ubiquitous in coastal sediments (Borch et al., 2010), a possible explanation for the observed decrease in Fe_{HCl} contents within the uppermost depth interval is an enhanced consumption of the Fe(III) previously accumulated, used as a terminal electron acceptor in the bacterial respiration after Zn-resistant biofilm addition.

The observed Zn sorption trends by Saco do Engenho creek sediments are probably a net result of cell-EPS-mineral aggregate effects (Hao et al., 2013). The results were significantly affected by a short (4 h) time period under Zn-resistant biofilm influence, as evidenced by evaluating Zn_{HCl} and Fe_{HCl} concentrations and concentration ratios. This indicates that even a single, short flooding period can be sufficient to promote such significant changes in the reactive Zn behavior for the evaluated sandy sedimentary environment. While Machado et al. (2008) evidenced experimentally that Zn sorption by these sediments during the tidal flooding may be a reversible process, it was observed that the Zn-resistant biofilm influence may contribute to limiting this reversibility. The results, therefore, supported the hypothesis that a metal-resistant biofilm coating can often enhance the metal sorption capacity of sandy sediments, as found for Zn. Longer periods of evaluation are necessary, however, to test the duration of the observed effect (e.g., throughout complete tidal cycles).

It is well known that coupled effects of living and nonliving organic matter, metal (Fe, Mn and Al) oxides, and clay minerals can control the binding of trace metals to aquatic sediments (e.g., Chapman et al., 1998), but the form and extent in which biofilms can affect the involved mechanisms deserve further attention. A positive feedback between enhanced Zn resistance and Zn sorption capacity of studied sediments is suggested by the observed results. This may contribute to a better comprehension on the Zn cycling across sediment-water interface in sites in which Zn resistance development occurs and sites that receive bacterial biofilm with enhanced Zn resistance transported from adjacent areas (e.g., attached to particulate matter released from contaminated sites).

Considering the known ubiquity of biofilms in the environment (e.g., Costerton et al., 1995), the metal-binding properties of biofilms (e.g., Decho, 2000) and the results from the present study, the suggested role of biofilms with enhanced Zn resistance can be an important mechanistic aspect involved in determining Zn cycling near the sediment-water interface in contaminated coastal zones. The relatively rapid effect observed after Zn-resistant biofilm addition to the sediment surface, often affecting the role of Fe_{HCl} compounds as binding phases for Zn_{HCl}, evidences that such an effect may be significant along with typical tidal flooding periods. This experimental data suggests that potential cumulative effects of Zn-resistant biofilm on Zn accumulation during larger time periods deserve investigation to evaluate to what extent the suggested feedback mechanism can affect the Zn sorption capacity of sediments.

The implications of these processes in determining the Zn cycling across the sediment-water interface could also be related to the protective role that the biofilms may have towards microphytobenthos communities against metal toxicity, since EPS-imbedded mature microbial biofilm consortia act as biogeochemical barriers (Ivorra et al., 2000). These microphytobenthos communities may also have pivotal functions in the trace metal biogeochemistry in coastal systems, e.g., by affecting the accumulation of these elements within the sediments (Harbison, 1986). However, the concurrent ability of biofilms to trap metals may be strongly dependent on the historical exposure to these elements, as exemplified here for Zn.

Coastal microbial mats are naturally able to favor trace metal accumulation within thin uppermost sediment layers, although a large knowledge gap exists (e.g., Glavaš et al., 2015). This is also the case in relation to polluted systems, in which the metal toxicity to the benthic bacterial consortia can possibly affect this role. However, there is consistent evidence in the literature that the sensitivity of benthic bacteria derived from sites previously exposed to trace metals is lower than that observed for bacteria derived from clean conditions, which may result in a change in the microbial community towards a metal-resistant consortia (e.g., Admiraal et al., 1999). This evolution has, therefore, important implications for the metal cycling in the colonized environments.

However, the replacement of sensitive microorganisms by resistant species may have strong ecological implications, since the community adaptation to a pollutant can often result in failure of the maintenance of ecological functions (van Beelen and Doelman, 1997). For instance, a number of specific geochemical and mineralization reactions mediated by microbial consortia are often performed by specialized bacteria that cannot be easily replaced by other species, while bacterial toxicity can affect biomass production and taxonomic diversity (van Beelen and Doelman, 1997). Consequently, these potential losses of ecological roles from bacterial communities may often affect other processes involved in determining the sedimentary cycling of the toxic trace elements (as positive or negative feedbacks in relation to metal accumulation by biofilms).

Biofilm formation is a useful strategy used by microorganisms to survive an exposure to adverse conditions, such as contact with various toxic elements (Harrison et al., 2007). Multiple impacts are frequent in the coastal zone that can receive refuses from multiple anthropogenic sources, making difficult the prediction of metal behavior derived from experiments carried out with a single pollutant. For instance, there is more information regarding benthic microalgal responses to concurrent exposure to multiple contaminants, including toxic metals (e.g., Ivorra et al., 2002). Since this multiple-contaminant exposure is a situation likely observed in many coastal systems at a worldwide scale, evaluations on the role of biofilm resistance in the trace metal cycling in response to multiple-contaminant exposure are recommended to clarify potential synergisms and antagonisms for different elements. Further research is requested for assessing the net effects of the discussed processes for different environmental conditions and trace elements.

CONCLUSION

Experiments on the potential effects of bacterial biofilm with amplified Zn resistance on the Zn sorption by intertidal sandy sediments from Sepetiba Bay (SE Brazil) showed that significantly different HCl-soluble Zn concentrations (62% higher) and inventories (56% higher) were induced after 4 hours of Zn-resistant biofilm addition. This treatment was evidenced as a factor affecting the geochemical controls on Zn retention, since a significant correlation between Zn_{HCl} and Fe_{HCl} was observed for data from most sediment layers, with the exception of data from the uppermost layers treated with Zn-resistant biofilm. A positive feedback between enhanced Zn resistance and Zn sorption capacity was suggested, which may contribute towards a better comprehension of the Zn cycling across sediment-water interfaces at sites where Zn resistance development occurs and sites that receive bacterial biofilm with enhanced Zn resistance transported from adjacent areas. These findings imply an important role of bacterial biofilm resistance to determine biofilm ability in affecting the Zn behavior, since biofilms may act as biogeochemical barriers at the sediment-water interface. However, the experiments were performed under nearly-steady conditions, and the extent to which strong natural disturbances (e.g., intense benthic fauna bioturbation, storm-induced resuspension, etc.) and anthropogenic disturbances (e.g., dredging, trawling, etc.) of sediments can affect this biofilm role remains unknown. Further research on these aspects of the bacterial biofilm role in the water-sediment interface are necessary in order to elucidade potentially different responses for different trace elements, according to variable environmental conditions, considering longer evaluation periods (e.g., throughout tidal cycles).

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Chapter 31

OVERVIEW OF THE OCCURRENCE, EFFECTS AND RISKS OF REGULATED AND EMERGING CONTAMINANTS TO FRESHWATER ORGANISMS IN LATIN AMERICAN WATERSHEDS

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ABSTRACT

This chapter offers an overview of the occurrence and effects of both regulated and emerging contaminants to freshwater organisms in Latin American watersheds, the sentinel wild fish that have been proposed, and the ecological risk assessment methodology in use. Freshwater ecosystems in Latin America are threatened by natural events and human activities including overpopulation, dams, deforestation, agrochemicals use, inefficient wastewater treatments and offshoring and outsourcing of large production companies. Many of the thousands of existing chemicals are emerging contaminants, making it necessary to estimate the overall effects of their mixtures through ecotoxicity testing. Moreover, under the influence of climate change, the complexity of the analysis of effects increases. If the end-points of bioassays are developmental, reproductive or other sub-lethal effects, special considerations must be taken to derive dose-response relationships, as they act at very low doses and via not completely elucidated mechanisms of action. Even though some investigations in Latin America have described the problem, there is still scarcity in databases for the Southern Hemisphere to substantiate risk assessments. The ecoregion approach is proposed to assess the risk at the Latin American level. A case study is presented concerning an integrated risk assessment of endocrine disruptors in the Uruguay River as an example of multiple stressors released from multiple sources. Tools included *in vitro* screens,

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biomarkers of endocrine disruption, tissue residues, biomonitoring, and bioassays with wild fish and standard species.

Keywords: Latin America, ecotoxicity, risk assessment, endocrine disruptors, emerging contaminants

INTRODUCTION

This chapter reviews the occurrence and effects caused by exposure of freshwater species to contaminants and ecological risk assessment (ERA) methodologies used to evaluate the risk of different stressors in selected watersheds in South America and the Caribbean.

Freshwater ecosystems are increasingly threatened by human activities and natural events. Therefore, there is a need to implement measures to ensure environmental sustainability (Iscan, 2004). The hazards to ecosystem health should be identified, and the impact of chemicals on organisms and on the interactions of organisms with each other and with their environment (Bilgen and Sarıkaya, 2015). Risk management measures should then aim at decreasing the continuous inputs of contaminants to diminish the probability of harming aquatic organisms, in order to guarantee the survival of future generations (Smital, 2008).

An ERA produces a risk characterization by combining both the exposure profiles and the exposure-effect characterization, to identify a level at which harmful effects occur on plants and animals. The systematic steps for performing an ERA are outlined in Figure 1.

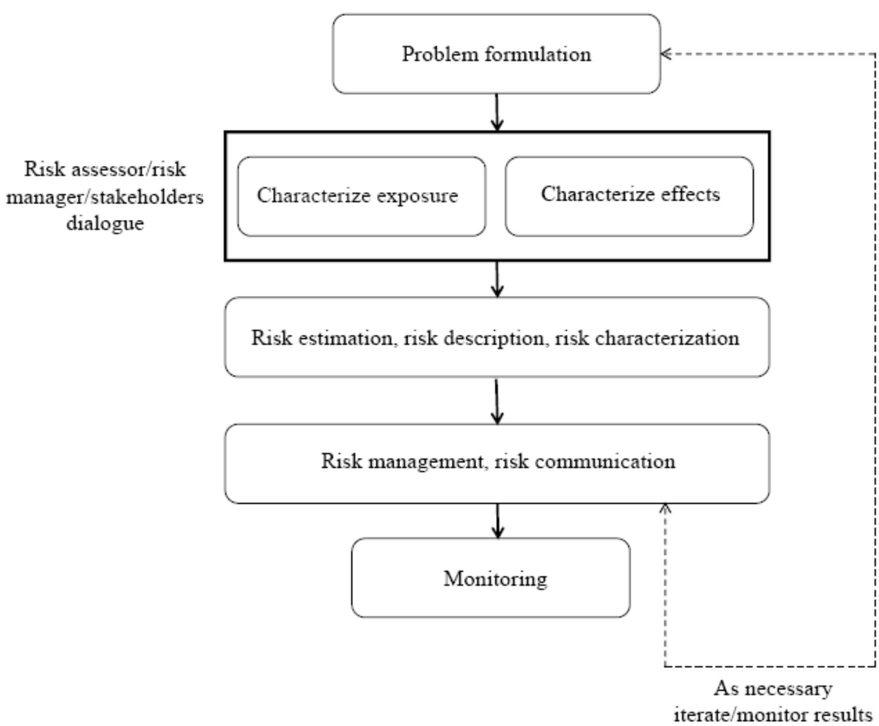

Figure 1. Ecological Risk Assessment framework (adapted from USEPA, 2015a).

In part due to offshoring and outsourcing of South America locations to large production companies (Crook, 2015), the situation has changed from pristine conditions to somewhat altered conditions. Moreover, under the influence of climate change, ecosystems are subject to variations that add complexity to the analysis of the stressor effects (Giarratani et al., 2013). The potentially existing chemicals in the environment add up to more than eighty thousand man-made substances, but only about 200 priority substances have been regulated (Dudgeon, 2014). Many of these substances are “emerging compounds”, meaning “any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment, but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects” (USEPA, 2015b). Their effects are still not completely known, making it necessary to estimate the overall effects of a mixture of chemicals through ecotoxicity testing.

METHODS

A proposal for a systematic methodological approach in the case of regional stressors would be to focus on the main shared river basins. However, another one could be to divide the continent into ecoregions to consider the differences in wildlife receptors. Therefore, the threats, hazards, and risks in the main freshwater basins, as well as some of the more prevalent pollutants and the most used species found in the literature for exposure and effects assessment in some ecoregions of the continent are described.

Scope

Geographical Scope

The focus of this chapter is Latin America, a highly diverse subcontinent, in terms of landscape, culture and biological species.

Wildlife and Human Receptors

The influence of biodiversity loss, contaminated fish through bioconcentration and biomagnification, and deteriorated water quality, eventually also affect human receptors. This implies that some biomarkers that are common to humans and animals can be used in order to be more conservative regarding any receptor within the environment. The ERA is turned into an integrated risk assessment, to include both.

Hazard Identification

Anthropic Activities and Threats in Latin American Watercourses and Ecoregions

Anthropic pressures displace creatures from their natural pristine environments due to constructions or cultures feeding an ever increasing population. Domestic, agricultural and industrial sources produce multifactorial stressors that combine to produce a cocktail of substances that eventually enter freshwater aquatic environments.

Some of the threats are common to the different ecoregions, while others are more critical due to the high biodiversity, or the presence of endangered species of fauna and flora. The geographical description within the following information was extracted from the Freshwater Ecoregions of the World online database (FEOW, 2015).

Deforestation, River Transportation, Pollution and Mining

Deforestation implies threats to biodiversity in several ecoregions such as the North Andean Pacific Slopes – Atrato River, or the Amazonas River Basin and Rainforest **-** because of logging for timber and pastures for cattle grazing. The Paraná/Paranaíba inner forests are also experiencing similar effects.

The Uruguay River has several dams diverting the watercourses that affect the natural habitats, and natural forests are being converted to eucalyptus plantations for pulp production, soy crops and large scale pulp mills. The Paraná River also presents dams and dykes, while hunting, urban expansion, and pollution represent threats to wildlife. Similar problems are found along the delta region of the Orinoco River, because of oil extraction and overhunting.

The São Francisco River suffers the effects of urban expansion, timber extraction, and of pollution by agrochemicals (Britto et al., 2015). The Northeastern Mata Atlântica suffers from urban expansion, while the Paraíba do Sul from illegal timber extraction and forest conversion into agricultural lands.

The Chaco region is altered due to cattle grazing. The Pantanal is the largest wetland in South America, and the largest in the world not substantially modified by humans, but pesticide runoff in the Paraguay River Basin and gold mining are threats. The temperate grasslands of the Pampas have amphibian populations at risk from the increasing use of agrochemicals in soy fields (Lajmanovich et al., 2010).

Megacities

According to the World Meteorological Organization (WMO, 2015), in 2010, 83% of the population of South America resided in cities, of which 20% inhabited the largest cities, some of them megacities of more than 10 million people (Buenos Aires, Rio de Janeiro, Sao Paulo, and Belo Horizonte) or near that value (Bogota, Lima and Santiago). The increase of built environments leads to the displacement of terrestrial and aquatic natural ecosystems.

Climate Change

The impact of pollution enhanced by climate change is one of the main stressors to aquatic biota. Changes in fish susceptibility to abrupt changes in temperature have been linked to the presence of pollutants that affect their immunity and decrease their ability to overcome adverse environmental conditions, exacerbating fish mortality events. Climate change combined with pollution can also increase the threats to freshwater ecosystem goods and services (Munang et al., 2010).

Transgenic Crops and Pesticide Use

The world´s population in 2015 estimated by the United Nations was 7,349.472 inhabitants. It is projected to increase by more than one billion people within the next 15 years, reaching 8.5 billion in 2030, and further to 9.7 billion in 2050 and 11.2 billion by 2100 (United Nations, 2015). This implies that more resources will be needed to feed the population, more land used for crops and more water abstracted for drinking water production and other uses. Land use has drifted from native woods to cereals and in Argentina, Brazil, Bolivia, Paraguay, and Uruguay to genetically modified soybean cultivations able to withstand the herbicide glyphosate, other herbicides, co-adjuvant surfactants, and insecticides. Several investigations show that glyphosate cause toxicity in frogs (Lajmanovich et al., 2010), in fish (Rocha et al., 2015) and in periphyton communities (Vera et al., 2010).

Eutrophication, Cyanobacterial Blooms and Floating Plants

Excess phosphates and nitrates produce eutrophication, leading to cyanobacteria overgrowth under certain conditions of light and temperature. *Microcystis aeruginosa* produces microcystin-LR and about 80 other toxins. The increase in frequency of algal blooming events is due to agricultural, municipal and industrial inputs of nutrients, and nitrogen fixation from the air. Losses in ecosystem services (such as tourism) and in water quality can threaten human health via intake of contaminated drinking water, fish or mollusks. Macrophytes can also be a problem, as is the case of Maracaibo Lake that suffers from overgrowth of *Lemna obscura* that blocks light passage and kills other plants and algae (González, 2004).

Urban and Industrial Discharges and Challenges to Conventional Wastewater Treatment Processes

The presence of increasing amounts of conventional and emergent contaminants in effluents requires enhanced treatments. Some cities do not have secondary or tertiary treatment to treat the organic matter, nutrients and recalcitrant compounds that human activities produce.

RESULTS

Exposure Assessment

Regulated Contaminants

In Latin America, most countries routinely perform surveillance monitoring to control the compliance to environmental regulations, with different established limits among each nation.

i. Heavy metals
   Fernández Severini et al. (2011) found lead (Pb) in dissolved and particulate phases and in zooplankton from the Bahía Blanca estuary, Argentina, in samples collected between March and December 2005. They linked the origin to industrial and sewage discharges. Mean concentrations of dissolved Pb were 2.15 ± 0.46 µg/L and of particulate Pb 13.52 ± 3.07 µg/g dry weight (dw). In copepods the concentration was 13.38 ± 4.41 µg/g dw, while in mysids it was 9.81 ± 1.89. In the Negro River, Uruguay, the reported median concentrations of Pb in sediments were 7.7 $\pm$ 3.8 µg/g at Baygorria site, and less than the quantitation limit (<5 µg/g) at Bonete site (Míguez et al., 2012).

ii. Pesticides and herbicides

Endosulfan, a highly toxic persistent organochlorine pesticide, was shown to bioaccumulate in fish and sediments (Boleda et al., 2011; Míguez, 2013).

Campanha et al. (2015) investigated pyrethroid residues and toxicological effects in *Jenynsia multidentata*, correlating integrated biomarkers with total pesticide levels. At higher temperatures the survival rate of fish larvae of *Odontesthes bonariensis*, "pejerrey", exposed to cypermethrin decreased (Carriquiriborde et al., 2009). Its metabolites were identified in the bile of the before-mentioned fish (Carriquiriborde et al., 2012). Hunt et al. (2016) determined 17 insecticides in sediments from 53 streams in soy production regions (Argentina in 2011-2014, Paraguay and Brazil in 2013) during peak application periods, finding toxicity towards *Hyalella azteca*.

Glyphosate, one of the most used herbicides, causes skeletal malformations in zebrafish and affects human placental cells, even at concentrations below regulatory levels (Mesnage et al., 2015).

iii. Persistent organic pollutants (POPs)

POPs can exert toxic effects on human and environmental health because of their potential to bioaccumulate and biomagnify along the food chain. There are regulated compounds, such as dioxins, furans, PCBs, and PAHs, and unregulated chemicals, which lay within the category described in the next subchapter. For example, studies carried out in several Chilean lakes, revealed that sediment PCB concentrations ranged from 1.2 ± 1 to 64 ± 30 ng/g dw (Pozo et al., 2007).

Emerging Contaminants

Not many references on the occurrence of emergent contaminants in South America exist given that the analytical techniques required to determining their concentrations are complex and still too expensive for some countries.

i. Endocrine disruptors

These chemicals do not share a similar molecular structure, but act on the homeostasis and endocrine systems, causing reproductive, immunological, or developmental effects (Diamanti-Kandarakis et al., 2009). Studies carried out at a location on the Uruguay River, subject to multiple stressors from agricultural, industrial (pulp mill), and domestic municipal discharges, showed the presence of several of the prioritized chemicals with potential or demonstrated endocrine disruptive action (Míguez, 2013). Later on, their occurrence was also detected in the Santa Lucía Basin, as pharmaceuticals such as ibuprofen resisted depuration within a wastewater treatment plant. Natural estrogens and the synthetic estrogen ethynilestradiol used in contraceptive pills were also present, as well as nonylphenol (NP) and its ethoxylates; NP was found at the final discharge site, which justifies the application of a tertiary treatment (Astigarraga, 2015). In another project carried out from September 2012 to September 2014 in the Uruguay River and Plate River, microcystin-LR and other non-regulated toxins were found in these watercourses (Kruk et al., 2015).

Given that the sources of water abstraction and of contamination inputs can influence drinking water quality, it is important to determine the existing compounds in these matrixes. Surface drinking water supplies receive large amounts of raw sewage inputs, introducing emerging contaminants that could reach consumers. In Campinas, Brazil, endocrine disruptors were found in tap water samples, namely bisphenol A quantified at a concentration of 0.16 ± 0.03 µg/L (Sodré et al., 2010).

ii. Stimulants and drugs of abuse

Stimulants and drugs of abuse have also been recently detected in environmental samples. Sodré et al. (2010) found caffeine at a concentration of 0.22 ± 0.06 µg/L in drinking water supplied to Campinas, Brazil. In South American countries and in Panamá, Rosa et al. (2011) reviewed the concentrations of drugs of abuse in drinking water at levels below 1 ng/L. The most prevalent compounds, cocaine and its main metabolite, were quantified at 0.6 and 4.5 ng/L, respectively; caffeine and nicotine were found at frequencies of 81 and 88% and at concentrations of 38 and 40 ng/L, respectively.

iii. Pharmaceuticals and personal care products

Pharmaceuticals and personal care products (PPCPs) are yet another category of chemicals of the emerging contaminants group. Pharmaceuticals, hormones, and triclosan were analyzed in surface water in São Paulo State quantifying caffeine, paracetamol, and atenolol at $12.96x10^4$, 3.42×10^4 and $0.82x10^4$ ng/L, respectively, while hormones estrone and 17-β-estradiol were detected at levels up to 14.8 ng/L (Campanha et al., 2015). Some of the most commonly prescribed pharmaceuticals (atenolol, carbamazepine and diclofenac, among others) were detected in the Suquía River basin (Córdoba, Argentina), and found to bioaccumulate in *Gambusia affinis*, a widely distributed fish species inhabiting the river basin (Valdés et al., 2014).

iv. Emerging hydrophobic organic pollutants

Among this class, brominated flame retardants (BFRs) such as polybrominated diphenyl ethers (PBDEs) bioaccumulate and biomagnify. The European Union has banned many of them but due to their persistence they can still pose a risk to human and environmental receptors (European Food Safety Agency, 2015). BFRs were in higher concentrations than UV filters (UV-F) in Chilean and Colombian sediments: BFRs at up to 2.43 and 143 ng/g dw of PBDEs in Chile and Colombia, respectively, and UV-F at up to 2.96 and 54.4 ng/g dw in Chile and Colombia, respectively (Baron et al., 2015).

Effects Assessment

Even when the international framework for risk assessments is broadly applicable to Latin America, it needs further refinement in terms of extrapolation from the Northern Hemisphere to local conditions and native species (Carriquiriborde et al., 2014), and from single species and single compound toxicity tests to multiple species and mixtures of contaminants.

Acute Toxicity

Ecotoxicological information considered for regulatory measures is in general obtained via acute toxicity testing. As an example of investigations that used this end-point, the crustacean *Daphnia similis* experienced high toxicity after exposure to the wastewater treatment plant of a hair care products company that produces shampoos, conditioners, moisturizers and hair vitamins in Brazil (Dias de Melo et al., 2013).

Sub-Lethal Effects

Giusto et al. (2012) exposed *Hyalella curvispina* amphipods in chronic ten-day bioassays, finding a decrease in survival and growth at 11.25 mg Cd/L in liquid phase and at 5.6 mg Cd/Kg dw in solid phase of samples of water and sediments taken from the Pampasic region of Argentina. Also in Argentina, Carriquiriborde et al. (2009) found effects on growth after exposing specimens of the fish species *Odontesthes bonariensis* in the laboratory to pyretroid pesticides. In Uruguay, in the Negro River the highest sub-lethal toxicity experienced by *H. curvispina* was found with samples from the Baygorria site (83%) by comparison to Bonete site (94%), showing a possible link to the increased concentration of lead in the clay fraction (Míguez et al., 2012). In Brazil, avoidance, a behavioural sub-lethal effect, was observed in fish exposed to a commercial formulation containing 300 g/L of pyrimethanil collected from outdoor mesocosm systems (Araújo et al., 2014).

Chronic, Carcinogenetic, Developmental, Immunotoxic, Neurotoxic and Reproductive Effects

Other potential effects of metals relate to cancer, developmental and reproduction effects. Genotoxicity was observed in model organisms exposed to environmentally relevant concentrations of cadmium (Pereira et al., 2016). Neurotoxicity of organophosphate pesticides such as azinphos-methyl has been studied in the native snail *Chilina gibbosa*, deeming it applicable as sentinel species for Argentina and Chile (Bianco et al., 2014). Castro et al. (2015) found that azinphos-methyl affected cell viability and phagocytic activity in hemocytes of this freshwater snail.

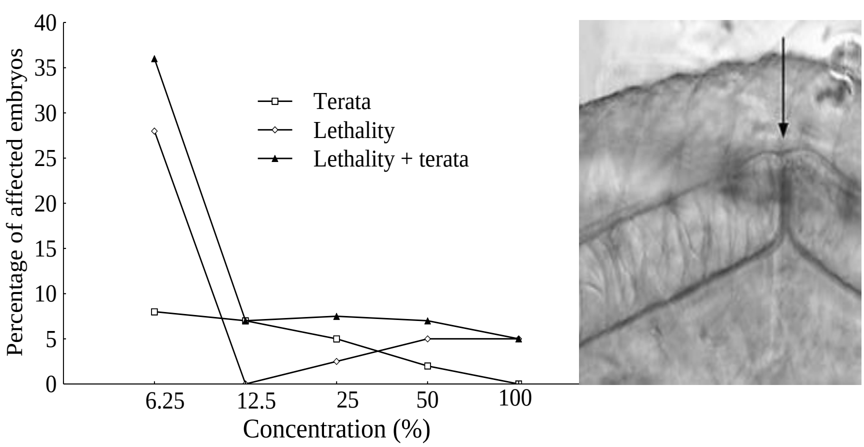

Figure 2. Non-monotonic kinetics of larval spine malformations of *Pimephales promelas* exposed to sediment elutriates. The photo on the right illustrates a typical example of spinal-cord malformation in an exposed embryo; arrow indicates the vertebral malformation observed. Sediments were dredged from a site close to industrial effluents into the lower Uruguay River, Uruguay; n=8 (Míguez et al., 2011; Míguez, 2013).

Aquatic frogs can also be subject to reproductive effects. The genus *Telmatobius* (*T. ceiorum*, *T. laticeps* and *T. pisanoi*) has historically been present in streams of Tucumán Province, Argentina, but *T. pisanoi* populations have declined since 1968 from 1-36 specimens, to 0-3 in 2005 (Barrionuevo and Ponssa, 2008).

Non-monotonic responses are paradoxical kinetic behaviors, meaning that the highest effects are seen at the lowest tested concentrations. In laboratory embryo development tests with the reference fish species *Pimephales promelas* exposed to elutriates of sediments dredged near the outfall of a pulp mill, the kinetics followed non-monotonic behavior regarding bone alterations (Míguez et al., 2011). Lethality in samples from the upstream Nuevo Berlín site was 21.5% while for those from the Fray Bentos site it was 7.5%. While the upstream site showed no developmental anomalies in exposed embryos, the sample elutriate from Fray Bentos lead to 3.3% spinal-cord malformations (Figure 2).

Endocrine Disruption

Endocrine disruption was observed in carp fish exposed to sediments of industrial areas of the Uruguay River (Rivas-Rivera et al., 2014). Endocrine disruptive effects were also observed using *P. promelas* fish and *Astyanax fasciatus* as a sentinel wild fish from a large river (Míguez, 2013). This characid fish, widely distributed from Central America to the Río de la Plata Basin of western Uruguay, has been used before to evaluate hepatic porphyrins (Carrasco-Letelier et al., 2006). Other fish species successfully tested as sentinel are *Cichlasoma dimerus* (Teleostei, Perciformes), "chanchita", a South American fish of streams of the Paraná River basin, the Paraguay River drainage in Brazil, Bolivia and Paraguay, and the Paraná River drainage of Argentina (FishBase, 2015), *Odontesthes bonarienses* (Table 1), and *Cnesterodon decemmaculatus.* The responses of the latter were compared to those of *P. promelas* in effluents and reference toxicants, being in agreement for effluents and potassium dichromate, but not for sodium dodecyl sulfate (Saona et al., 2015).

Table 1. Effects experienced by native model fish species in Latin America

Chemical(s) and/or sample origin	Species	Effects	Reference
Environmental pollutants, Lower Uruguay River Basin	*Astyanax fasciatus*	Endocrine disruption	Míguez (2013)
Furnas Reservoir, Grande River and Paraguay-Paraná basin	*A. fasciatus*	Endocrine disruption	Prado et al. (2011, 2014)
Endosulfan	*Cichlasoma dimerus*	Gonadal steroidogenesis disruption	Piazza et al., 2015
Endosulfan	*C. dimerus*	Effects in GnRH cells	Da Cuña et al., 2013
17β-Estradiol (E2) and synthetic 17α-ethynilestradiol (EE2)	*C. dimerus*	Decrease in sperm motility, fertilization, and embryo and larval survival	Meijide et al., 2016
17β-estradiol	*C. dimerus*	Vitellogenin in male fish and other endocrine disruptive effects	Moncaut, 2003
Octylphenol	*C. dimerus*	Non monotonic kinetics	Genovese et al., 2014
Estradiol and ethynilestradiol	*Odontesthes bonariensis*	Estrogenicity and low sperm quality, fertilization and embryo-larval survival	Gárriz et al., 2015

GnRH: Gonadotropin-releasing hormone.

Frameworks for Exposure and Effects Assessment

i. Caged fish in the river and laboratory exposure assays
In Chile, Chiang et al. (2015) exposed male and female juvenile rainbow trout to pulp mill effluents in laboratory experiments, both showing significantly higher levels of plasma vitellogenin (Vtg) than the control. The concentrations of Vtg were comparatively higher in fish exposed to *Eucalyptus globulus* than to *Pinus radiata*-based effluent. Moreover, male fish showed intersex characteristics in all exposure assays. Furthermore, similar estrogenic responses were observed in caged fish in the river.

ii. Tiered approach for exposure and effects assessment
An integrated risk assessment of endocrine disruptors in the lower Uruguay River was carried out by Míguez (2013). The experimental design of the exposure and effects assessment relied on a tiered approach that combined both *in vitro* and *in vivo* tests, chemical analyses, molecular biomarkers and lab exposure of standard species and field studies with a native fish (Figure 3).

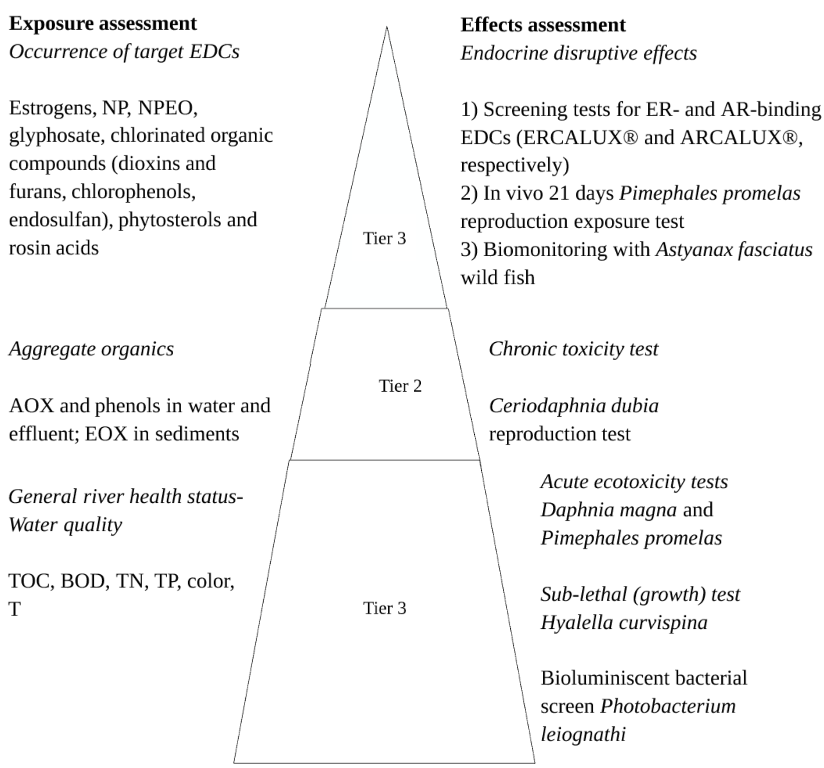

Figure 3. Tiered methodology for exposure assessment of endocrine disruptors in the Uruguay River (Míguez, 2013). Adsorbable organic halogens (AOX), extractable organic halogens (EOX), nonylphenol (NP), nonylphenol ethoxylates (NPEO), total organic carbon (TOC), biochemical oxygen demand (BOD), total nitrogen (TN), total phosphorus (TP), temperature (T), estrogen receptor (ER), androgen receptor (AR).

The first tier assessed the river health status by measuring general water quality parameters, while effects were assessed by developing acute toxicity tests with crustaceans, fish, amphipods and bioluminescent bacteria. The second tier analyzed AOX and phenols in river water and effluent and EOX in sediments, while effects were evaluated via chronic toxicity tests for reproduction with the cladocer crustacean *Ceriodaphnia dubia*. In the third tier, the specificity increased to measure the occurrence of endocrine disruptors and to determine endocrine disruption effects. Firstly, luciferase tests ER-CALUX® and AR-CALUX® were applied to screen the presence of ER- and AR-binding compounds in the watershed, which resulted positive. *In vivo* analyses were then performed in the laboratory to assess the effects in fish gonads at molecular, tissue and functional levels: *P. promelas* were exposed to both pulp mill effluent and stream water subject to municipal wastewater discharges during 21 days each. A set of biomarkers of estrogenicity by PCR, vitellogenin (Vtg) by ELISA, histological analysis of the gonads and fecundity (egg counts) were then measured. Moreover, field biomonitoring with more than 1000 specimens of the native fish *A. fasciatus* was carried out at sites located upstream and downstream from the pulp mill and city discharges. The occurrence of prioritized chemicals was determined in the water catchment to then determine their bioaccumulation in fish, crustaceans and other organisms, including microorganisms, plants and additional invertebrates. Complex mixtures related to anthropic activities were found in sediments, water and biota. Among the pesticides, endosulfan was also found in fish, and the herbicide glyphosate in sediments.

Risk Estimation and Risk Characterization

From a holistic stand point, the harm to wildlife and human beings living in the watershed should be evaluated in all dimensions and compartments, with a basin approach, taking into account the water cycle and food web interactions. In a study performed by Míguez (2013), a decrease in fecundity was observed after exposure of *P. promelas* to an industrial effluent, but no intersex appeared in this case nor in assays with samples of a stream receiving municipal discharges. The estrogenicity of the latter was demonstrated by the results of estradiol, *zona radiata* biomarker, estrogen receptor 1 and IGF-I expressions in the exposed fish in the laboratory, along with the positive results of the luciferase receptor-binding screen ERCALUX®, and the occurrence of estrogens and nonylphenol. On the other hand, anti-estrogenicity or androgenicity was suggested to be caused by the industrial effluent. Biomonitoring of wild *A. fasciatus* revealed changes in gonad size at sites near the outlet from a pulp mill, and differences in condition factor in areas influenced by municipal wastewater. The risks of endocrine disruption to humans via fish and water ingestion were characterized as low, and from low to moderate to freshwater biota. However, hotspots of higher risk were identified for risk management.

CONCLUSION

There are no complete databases that are ecologically relevant to Southern Hemisphere ecosystems for the development of risk assessments that support informed risk management decisions. Unlike the Northern Hemisphere, there are currently no protocols describing which species to use to assess the risks of contamination of Southern Hemisphere water bodies, in part due to the scarcity of data reporting the responses of native species. The experiments described in this chapter concerning wild fish can be used to derive internationally standardized guidelines through comparison of the responses among standard model fish and native fish, to build databases and derive sound risk assessments.

Threats to freshwater ecosystems do exist nowadays in Latin America and may act additively, synergistically or antagonistically. Natural conditions such as climate change and extreme events (drought or flooding) are not the only causative agents as human interventions, overpopulation and overconsumption of natural resources (e.g., for crop production or production of luxury items) are some of the most influential factors that have altered many pristine areas around the world. At the macro scale, the ecoregion approach could be applied to the study of Latin America ecosystems, within the framework of integrated water resources management (IWRM), coupled with a study of drivers, pressures and ecosystem services that consider every water use. On the other hand, at a detailed scale, ecologically relevant bioindicators could be chosen by joint research teams to enlarge databases, for which increased international collaboration is needed. Further research must be done to determine the effects of not only regulated pollutants but also of emerging contaminants, including the most recent target agents such as nanoparticles, microplastics and endocrine disruptors.

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Chapter 32

NANOMATERIALS AS CECS: FROM ENVIRONMENTAL INTERACTION TO REGULATORY EFFORTS FOR A SAFE USE IN LATIN AMERICA

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ABSTRACT

The term "contaminant of emerging concern" (CEC) is applied to non-regulated anthropogenic material recently found in the environment that may represent environmental or public health risks. In the present chapter, general aspects regarding nanomaterials (NMs) are addressed, from their role as CECs to the potential deleterious effects on organisms and the environment. Five NMs are considered as examples: silver nanoparticles representing metallic NMs, ceria and titanium dioxide representing metallic oxides, and carbon nanotubes and fullerenes representing carbon NMs. An overview of the processes determining the lifetime of nanoparticles in the environment is given. A perception survey was conducted amongst Costa Rican high-ranking authorities from the environmental and health sectors, and researchers from public universities as representatives of the academic sector. The results show that even though awareness regarding the relevance of CECs exists, the participants of the survey perceive an important lack of regulatory tools and of effective governmental actions. An overview of the current situation of regulatory efforts and accomplishments regarding NMs in

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Argentina, Brazil, Costa Rica and Mexico is given, showing advances mostly in Mexico and Brazil, where alliances with European counterparts seems to have helped.

Keywords: CEC, ENM, nanomaterial, regulation, Latin America

INTRODUCTION

The term CECs was a much-needed precision of the still commonly used terms "emerging contaminants" and "emerging pollutants." CECs can be applied to non-regulated anthropogenic material recently found in the environment that may represent environmental or public health risks. This term comprises a wide variety of substances and materials; some are intentionally produced, like pesticides, antibiotics, drugs, plasticizers, some nanomaterials (NMs), and some are unintentionally produced (PCDF/Ds, PAHs, some aerosols, some NMs). Their importance is increasing in the scientific community, because of their bioaccumulation, biomagnification, widespread exposure, potential persistence, and environmental and health implications. Some CECs are suspected or well known carcinogens or endocrine disrupters, and there are others for which effects are still to be discovered.

CECs can be classified based on how long they have been known as contaminants (Sauvé and Desrosiers, 2014). Some are in fact new, emerging substances or materials, which are recognised as contaminants. Other CECs such as arsenic, although considered contaminants for centuries, are subject of renewed interest. Engineered nanomaterials (ENMs) have made some significant contributions to CECs, mostly as new materials with previously unsuspected interactions with the environment and organisms. These interactions arise mostly from a combination of size and chemical composition at the surface. This chapter deals with NMs, namely ENMs, in view of their potential as CECs. Our approach aimed to help safeguard the exploitation of this technology by promoting the questioning and environmental health assessment methodologies. The safe use of NMs is the prevailing objective while mindfulof preventing governmental overregulation or negative public perception.

Nanomaterials General Aspects

NMs are the materials occurring naturally in the size range of 1 to 100 nm (frequently called nanoscale) while ENM are intentionally manufactured materials, containing highly uniform structures with at least one critical dimension in the nanoscale. According to the US National Nanotechnology Initiative (NNI), NT is allowing the development of modern advances in different fields of knowledge including chemistry, physics, biology, medicine, and materials engineering (NNI, 2000). NM represent a bridge between atomic or molecular structures and bulk materials, due to the large surface area per unit of volume, and the quantum effects that appear at the nanometer scale, which consequently lead to a significant improvement of particular intrinsic properties of the substance. The potential market value for NT-related products between 2011 and 2015 was estimated to be up to US$1 trillion per annum (Navarro et al., 2008).

Variations in NM specific surface area can render these materials much more reactive, and thus more toxic than their larger homologues (Ozin and Arsenault, 2005; WHO, 2011).

Even substances known to be chemically passive can become reactive at the nanoscale (e.g., gold, silica), posing unsuspected risks if assessed traditionally (WHO, 2011). This can be enhanced by the potential of ENM particles to translocate body barriers, penetrate cell membranes, accumulate in mitochondria, and deposit in specific organs.

Numerous reports have suggested that due to their smaller size, ENM can easily bypass water treatment plants, air pollution control equipment, or be released during handling of waste (European Commission, 2011; OECD, 2016); nonetheless, current research has shown considerable variability on ENM removal efficiency (Andersen et al., 2014). It has thus become necessary to develop new assessment methods for treating these materials (see Ozin and Arsenault, 2005; Ponce, 2010; OECD, 2012; Savolainen, 2013), and assessing the risk of NMs in penetrating various animal tissue membranes (Savolainen, 2013) and bioaccumulating or causing damage to tissues or DNA. Individual experts have called for such regulation of risk assessment procedures for NMs (El Universal, 2011), while at the same time suggesting that a balance must be struck between technology developments and protecting the public (Foladori and Záyago, 2011).

Nanotechnology and the Environment

There are various ways in which NT can improve environmental technology and provide benefits to the environment. For example, by contributing with products that enhance energetic efficiency, improve energy storage, provide potable water or reduce waste.

In the case of NT, the environment will be increasingly exposed to affectations by pollution from manufacture, use, and disposal of ENM. For instance, a three-fold increase has been identified for Europe since 2006, especially after considering the number of products containing nanoparticulate materials, including consumer products (Wijnhoven, 2011).

Currently, there are a widespread variety of regulations in place in manufacturing countries, especially in the European Union, including those regarding consumer and environmental protection. NT has also been spurred in Latin American countries such as Brazil, Argentina, Chile, Mexico, Cuba, and Costa Rica. These advances are considered promising from a quality of life standpoint. Despite this, very few Latin American countries have regulatory frameworks that specifically address the right-to-know, as a mechanism for assuring consumer knowledge of ENM content and the consequent risks in products containing them (Foladori, 2006). Moreover, nano-content is not necessarily disclosed appropriately to manufacturers and consumers. It is even less reflected in waste handling regulations regarding content of discarded products or in specific regulatory and collection schemes for this special waste (OECD, 2016).

Differences in toxicity between bulk materials and particles having at least one dimension in the nanoscale have shed some light on modes of ecotoxic action of NMs. The factors that can influence the ecotoxicity of NMs can be divided into two types: those concerning the NM itself, and those concerning the medium and the associated ecological receptors.

The size of a NM (regularly the only parameter considered), the surface properties of the particles, and the physicochemical environmental conditions, such as pH, temperature, redox conditions, ionic strength, and natural organic matter, affect its mode of interaction with the environment. Knowledge regarding physicochemical interactions of NMs with the environment has been increasing; meanwhile their potential impact may be affecting biogeochemical cycles due to considerable mobility and toxicity (OECD, 2016).

The level at which most of the current research on ENM has been performed ranges from effects on unicellular organisms - assessing microbial toxicity in order to extrapolate the observed effects of chemicals to organisms of higher levels of organization – (Mortimer, 2011; Elsaesser and Vyvyan, 2012), to that of whole multicellular organisms, in order to compare the susceptibility of organisms at various trophic levels.

Research has shown that ENMs may lead to unforeseen health risks due to their ability to bypass protective biological mechanisms, including the blood brain barrier, or negatively affect the environment due to their antibacterial properties (OECD, 2016). Owing to the risks and uncertainties, a precautionary approach must be followed and countries should embark on a "mission" to experiment with novel schemes of extended producer responsibility and right-to-know activities, hopefully reflected in regulatory instruments.

Bioavailability and Cellular Uptake of ENM

The bioavailability of an ENM affects dose and exposure duration and is generally determined by physicochemical factors of the material, amount and stability of the substance, and receptor sensitivity. Most inorganic ENMs such as TiO_2, Ag, CeO_2, ZnO, and quantum dots are likely to be found in the environment due to their increasing production and application in consumer products. According to Gottschalk et al. (2013), in studies referring to the environmental concentration of these ENM, the most studied material was nano-TiO_2, followed by nano-Ag fullerenes and CeO_2. Furthermore, nano-Ag and TiO_2 lead the list of the most ingested NMs (Woodrow Wilson International Center, 2006; Illuminato et al., 2007). It should be considered that physicochemical properties may affect the potential toxicity of NMs. For this there are several extensive lists of properties that are applicable to NMs, such as the list proposed by the Organisation for Economic Co-operation and Development (OECD, 2010).

Herein, environmental, health-relevant results for five ENMs are presented. Considering that particle composition is probably the element that plays a primary role in the cytotoxic effects of different nanoparticles (NP), the main difference among the five selected ENMs (CeO_2, fullerenes, Ag, TiO_2, and CNTs) is the composition. These ENMs will be named criteria NMs, as they represent substances of generalized use of a certain category, sharing characteristics that influence their intrinsic hazard. First, inorganic ENMs (CeO_2, Ag and TiO_2) will be addressed, followed by the carbon ENM products (fullerenes and carbon nanotubes).

Inorganic Criteria ENMs

Metallic ENMs

Nano-sized silver is the most used and representative ENM for this category. Different forms, including NPs, silver wires and oxides, are currently being widely used for their antimicrobial activity in fabrics and personal care and medical products.

Human oral and pulmonary administration of AgNPs has shown silver bioavailability, being spleen, liver, and kidney the main target organs for deposition after systemic availability. Moreover, exposure to ionic silver and AgNPs has been shown to interfere with gene expression (SCENIHR, 2014).

For the aquatic ecosystem, tests with zebrafish embryos have reported decreased hatching rates, weak heart beats, edema and abnormal notochords after exposure to 10-20 nm Ag-NPs for 48 h in concentrations of 10-20 μg/L (Yeo and Pak, 2008).

Regarding prokaryotic cells, it has been found that Ag-NPs affect bacterioplankton metabolism, production, community composition and the emergence of rare bacteria phylotypes (Das et al., 2012).

Metal Oxides

Due to its diverse range of applications, CeO_2 is a widely used material, representative of metal oxides, similarly to TiO_2. Current applications of nano-CeO_2 range from catalyst to insulator on silicon substrates and as electrolyte material of solid oxide fuel cells. Ceria NPs have attracted attention for the study of their ecological and health effects. In vitro tests have suggested that nano-CeO_2 could be a potential exogenous source of reactive oxygen species (ROS) (Park et al., 2008). Cell death and tissular damage of lung epithelial cells when exposed to different sizes of nano-CeO_2 (from 15 to 45 nm) have been reported (Park et al., 2008).

Oxidative stress damage and ROS accumulation was observed in *Caenorhabditis elegans* in vivo testsat different concentrations (1-100 nM), causing significant decrease of nematode mean lifespan upon exposure with low and environmentally relevant concentrations such as 1 nM. This decrease was presumably due to oxidative damage induced by ROS accumulation (Zhang et al., 2011).

Fine and nanosized TiO_2 powders have been extensively manufactured worldwide. Their application ranges from pigments and food additives to their use as photocatalysts. The latter has allowed the use of TiO_2 in the oxidation of organic compounds, especially in water decontamination and the production of H_2 as a fuel using solar energy. As a result of the wide range of applications, there are also several exposure pathways, making dermal penetration, ingestion and intravenous injection possible. However, toxicity studies have primarily focused on dermal penetration and inhalation (Zuniga and Quesada, 2015).

TiO_2 particles larger than NPs are generally considered to be biologically inert for living beings. However, cytotoxicity effects for TiO_2 have been found, although very low as compared with other materials (ZrO_2, Al_2O_3, Si_3N_4) (Yamamoto et al., 2003).

Although size alone is not an effective predictor of cytotoxicity, it is the only parameter considered in NM cytotoxicity studies. However, this simple general aspect is lacking in product labels regarding the specification of the properties of commercial TiO_2 powders (Zuniga and Quesada, 2015). This increases the risk for workers and consumers upon exposure, and precludes the establishment of appropriate security measures.

Another especially important parameter for cytoxicity studies of this material is the crystalline structure. For photocatalytic applications, the anatase structure is more effective than the rutile (although some applications may require mixtures and proper toxicity analysis). An aspect of significant importance is that paints are the main end use market of TiO_2,(ICIS, 2010), conferring risks mainly due to inhalatory and dermal exposure. The average retention time of TiO_2NP in rat lungs was of 541 days, 4.62 times longer than the average time for 250 nm-sized NPs. Studies in rats exposed to TiO_2 rutile structure fine powders, at a dose of 12.5 mg/kg, showed systemic translocation. Higher accumulation was found for 80 nm-sized NPs, which remained mainly in the liver; the 25 nm NPs and fine particles were not retained in the liver but found to be accumulated in lung tissue, kidney and spleen (Wang et al., 2007).

Carbon ENM Products

This category basically includes allotropes of carbon such as fullerenes, carbon nanotubes (CNTs), and some others with a specific targeted market such as carbon black, hydrochar and graphene wires. A special feature that must be considered is the wide range of materials of this group, an aspect that hinders establishing comparisons and the development of appropriate regulations in order to mitigate potential risks. Manufacture of these materials is also occurring in increasing amounts for different applications, ranging from cosmetics to drug delivery, as is the case of the C60 fullerene.

Fullerenes

This material (also called buckyballs, short for buckminsterfullerene) presents a molecular structure with sixty carbon atoms. Some of the market available variations of this ENM are fullerols and carboxyfullerenes, more soluble derivatized molecules that imply an increasing potential of interaction with organisms.

Water-soluble fullerene (nC60) had shown to induce lipid peroxidation (LPO) in brain of juvenile largemouth bass (Oberdörster, 2004). *Daphnia magna* exposure for 21 days to 2.5 and 5 ppm of nC60 caused a delay in molting and a decrease in offspring production. Furthermore, tetrahydrofuran-solubilized-fullerenes effects on two fish species were compared to water-stirred fullerenes. A 100% fish mortality was found between 6 and 18 h exposure in tetrahydrofuran-solubilized-fullerenes, while in water-stirred fullerenes no physical effects after 48 h were detected (Zhu et al., 2006).

Tests on mice have shown suppression of P450-cytochrome levels (Ueng, 1997) and blood-brain-barrier penetration (Yamago et al., 1995).

Bactericidal activity was observed in prokaryotic cells exposed to aqueous-C60 suspensions, metallofullerenes and carboxyfullerenes (Kamat, 2000; Tsao et al., 2002; Lyon et al., 2005), while oxidative eukaryotic cell damage was caused by hydroxylated fullerenes (Tsao et al., 2002).

Carbon Nanotubes

CNTs are arrangements of carbon atoms in a crystalline tubular graphene form. The main categorization considers the number of layers or tubes within a tube, distinguishing single and multi-walled carbon nanotubes.

It is questioned if there are analogous mechanisms to other fibrous particles, such as asbestos, which could enable CNT lung tissue penetration. Features such as biopersistence, *ratio between width and height,* and the fibrogenic character of CNTs are important characteristics that may influence the occurrence of adverse health effects. Some studies have confirmed CNT asbestos-like or even more harmful effects, e.g., greater fibrotic potency of single-walled CNT (SWCNT) on mice 1 year after aspiration (Shvedova et al., 2013).

The *in vitro* and *in vivo* exposure to CNTs revealed the potential of this ENM to cause DNA strand breakages, oxidative damage, mitotic spindle formation and mutations (Darne, et al., 2014). Another study found SWCNT to be the ENM to induce most breakage in DNA strands in fibroblasts, compared to carbon black, silicon dioxide (SiO_2), and zinc oxide (ZnO) NPs (Yang et al., 2009).

It has been found that multi-walled CNT (MWCNT) was internalized in vacuoles of human epidermal cells. Furthermore, down-regulation of membrane scaffold protein and cell cycle inhibition has also been observed (Montero-Riviere et al., 2005).

Differences in bioactivity between SWCNT and MWCNT are apparent, as well as other differences regarding relevant physicochemical properties that may affect their potential toxicity (Oberdörster et al., 2015). For CNTs, the most relevant properties are: the synthesis method, surface properties, impurities, density, and shape. In terms of their toxicological effects, characteristics such as shape, length, width, morphology and dispersion are considered determinant, as has been observed in asbestos and glass fiber toxicological analysis (Broaddus et al., 2011).

Metallic NP such as Fe, Co, Ni, and Mo are commonly used as catalysts for CNT growing in a synthesis process (Hoyos-Palacio et al., 2014). Morphological effects, efficiency of the synthesis process and toxicity are aspects that can be influenced by the selected catalyst. Iron, one of the most common catalyst-substrates for CNT production (Dundar and Karatepe, 2011), has been found to interfere with bacterial and fungal cell reproductive processes (Chaves et al., 2013). The latter was seemingly due to nanotube endocytosis mediated by siderophores, which are iron-sequestering molecules produced by the microorganisms. Fungal and microbial cell saturation with siderophore containing nanotubes were observed after 30 min, leading to cell death because of interference with electron transport (Chaves et al., 2013).

Hydrochar

Hydrothermal carbonization (HTC) is an emerging technology that promises to be widely implemented in coming years for the treatment of wastewaters highly loaded with suspended and dissolved organic matter (Quesada et al., 2016; Zuniga and Quesada, 2016), as well as of very moist biomassic waste materials (Libra et al., 2011; Sevilla et al., 2011; Xiao et al., 2012). Hydrochar, the solid product of this process, is comparable to charcoal in some macroscopic aspects. The wastewater from the HTC process contains some soluble organic compounds and all the soluble mineral components of the original waste material (Libra et al., 2011); uses as a foliar fertilizer or directly on the soil have been mentioned, but release into water bodies may become frequent. In the wastewater from the HTC process, nanoparticulatehydrochar can remain suspended and therefore may be also released (Quesada et al., 2016).

Sink Processes and Persistence Mechanisms

ENMs can be released via several pathways to the environment: transformation of the ENM into another material by dissolution or by chemical reactions; or permanent immobilization by being irreversibly aggregated or deposited and/or incorporated into sediment and thus being transformed into another material. The combination of both processes can also occur, when aggregated NPs, usually in sediments, are subjected to changing physicochemical conditions leading to dissolution or reaction.

Experimentation with pure ENMs and environmentally unrealistic concentrations or settings have been criticized (Lowry, 2008; Olson and Gurian, 2012; Gottschalk et al., 2013; Judy and Bertsch, 2014; Mitrano et al., 2015). Risk-assessment (Wiesner et al., 2009) and life-cycle approaches (Mitrano et al., 2015) have been proposed along with some prioritization mechanisms (Wagner et al., 2014).

Generally, the environmental impact of an ENM is considered to be determined by the material's persistence. Thus our major concern here is focused on ENMs that may lack dramatic acute effects, but do show some degree of persistence.

Processes Affecting Released ENMs

ENMs can be expected to form part of a product that is distributed, used, and eventually disposed of in waste streams. A pure ENM will reach a waste stream as a result of an accidental or exceptional event. However, it will usually be subject to processes occurring in the waste stream, after which it will undergo further processes at the final receptor site. Even though experimentation with pure ENMs has been questioned as to its relevancy regarding real scenarios, it has led to knowledge concerning the primary processes that NP can undergo in different media.

Processesin the Atmosphere

The fate of nanoparticulate materials emitted to the atmosphere is predictable from the aerosol science standpoint, with major concerns regarding exposure near the source, for example of workers, and regarding interactions of the particles with the environment after deposition (Klaine et al., 2008; Lin et al., 2010). Particles in the atmosphere, whether solid or liquid, have been subject of very relevant studies for decades. NPs in the atmosphere undergo a series of processes, ending up deposited in water bodies or on solid surfaces, with variable degree of transformation.

Atmospheric deposition of particles can be dry or wet; particles and aggregates may collide with surfaces and be trapped, or be carried in a depositing water droplet to a surface. Particles collide with each other to form aggregates. Gravitational deposition or sedimentation becomes important for diameters above 10 µm. Atmospheric particles and aggregates can be trapped by collision with water droplets, but they can also nucleate atmospheric water droplets and undergo cloud processing. Water droplets may evaporate leaving behind modified particles, usually conglomerates, or they may be removed by rainout. Atmospheric solid particles in the accumulation mode, with diameters from about 50 nm to about 2 µm, which may be single or agglomerates, have longer atmospheric lifetimes, in the order of weeks, as opposed to other size ranges which last minutes or days at most (Finlayson-Pitts and Pitts, 2000; Seinfeld and Pandis, 2006).

Processes in the Condensed Phases

ENMs released to the environment will eventually reach a water body or the vadose zone (Berkowitz et al., 2008; Wagner et al., 2014), and will thus be exposed to processes taking place in water. Longer life times can lead to their presence in underground water. Composition and arrangement at the surface of the NPs determine their behaviour in aqueous media, where the most relevant processes they may undergo are:

- Dissolution and reaction

Dissolution and reaction of ENM in aqueous media lead to their transformation into other materials, occasionally new NMs. Dissolution of a material is less likely near saturation, a condition to be rarely expected in ecosystems. When dissolved species are formed, either due to dissolution or to reaction, as in the cases of some AgNP oxidizing to Ag^+ or ZnO dissolving in water, these species will have their own interactions with the surroundings; for example, Ag^+ may react with Cl^- to form AgCl or may undergo uptake into living tissue, among other possibilities. Transformation by dissolution can also lead to formation of other crystalline phases with the same composition, occasionally with similar sizes, or to the formation of an outer layer with a different composition (Judy and Bertsch, 2014).

Reactivity and solubility of the base materials, combined with composition of the media, are determinant factors. The kinetics are determined by exposed surface area, presence of species that can adsorb onto the particles or else stabilize the reaction products (chiefly dissolved organic matter (DOM)), ionic strength, pH and concentrations of other reactants (Aiken et al., 2011; Wagner et al., 2014). Coatings on NPs determine their solubility and reactivity, especially if chemically bonded to the surface, often rendering them inert and thus extending their lifetime (Jarvie and King, 2010).

- *Aggregation and deposition*

NPs suspended in aqueous media form colloids and may stay in a colloidal state for considerable time spans, even in soil-pore water (Whitley et al., 2013). Aggregation among suspended NPs is driven by Brownian motion, differential settling and fluid motion (Wagner et al., 2014). The primary tool to understand and predict aggregation behavior of colloids is the DLVO theory (named after those that progressively developed it: Boris Derjaguin, Lev Landau, Evert Verwey and Theodor Overbeek). The DLVO theory is a quantitative description for stable aqueous dispersions, which uses van der Waals (vdW) and Coulombic forces to predict the aggregation or else repulsion of suspended particles.

It applies well to most cases of colloids, including bare ENMs (Hotze et al., 2010; Hartmann et al., 2014; Wagner et al., 2014) though not if the particles are coated with layers that stabilize them via steric and electrosteric interactions. Additionally, when such outer layers are chemically bonded, persistence increases.

The DLVO theory predicts highest aggregation rates when the charge on colloidal particles due to the electrical double layer (EDL) approaches zero. Charge is determined by the interactions at the particles' surface and can be varied by changing pH, ionic strength, or by ions with high charge density. The destabilization of colloids produced by ions with higher charge density can be predicted as well. As known by experience (consider river deltas), high salinity and especially higher valence cations (consider water treatment plants) lead to colloid destabilization. Aggregation of particles of the same material (homoaggregation) is the simplest case and the most often considered in published studies. In real settings, heteroaggregation (different materials) is several orders of magnitude more likely than homoaggregation or deposition to surrounding surfaces. Particles of different materials show different charges in the same conditions, so opposite electrostatic charges may occur leading to destabilization and aggregation (Hartmann et al., 2014; Wagner et al., 2014).

Hydrophobic interactions, steric repulsion, polymer bridging and magnetic and hydration effects can also influence aggregation/deposition, and are termed non-DLVO processes. Coatings on NPs determine their colloidal behavior as well as solubility and reactivity, especially if chemically bound to the surface, often enhancing their mobility far beyond that of bare NPs, due to non-DLVO processes. The behavior of NPs below 30 nm has been shown to diverge from DLVO predictions as well, showing longer lifetimes (Wagner et al., 2014; Hartmann et al., 2014).

Aggregates in a colloid tend to grow and eventually depose, and NPs become thus immobilized. Forces between aggregated particles are often strong enough to avoid that the aggregate redisperses (peptization). Judy et al. (2012) hypothesized that the formation of aggregates should reduce the bioavailability of the ENMs, for which confirmatory evidence was produced. Notwithstanding, aggregates may in specific circumstances undergo peptization, whereby previously immobilized ENPs can again become available.

- Adsorption

Additional to the EDL, which is adsorbed on the surface of a particle, nonionic solutes may be adsorbed onto a suspended particle's surface (physisorption) and driven mostly by vdW forces. Generally, longer molecules with more interacting sites can adsorb more effectively than shorter ones. Molecules adsorbed onto NPs change their interaction with the surroundings, often increasing their colloidal stability by solvation and by conferring them a negative charge, or by electrosteric stabilization (Hartmann et al., 2014). Adsorbing molecules may be long enough to produce bridging among particles, leading to aggregation, as flocculants do (Hartmann et al., 2014; Wagner et al., 2014).

- Interactions with dissolved organic matter (DOM) and other dissolved species

DOM comprises a variety of organic compounds including small to macro-molecules, among them polymeric substances, proteins, humic and fulvic acids, carboxy-compounds, amines, and thiols, all with a great variety of heteroatoms lending them excellent water solubility and, at the same time, excellent possibilities to establish electrostatic as well as vdW interactions (Aiken et al., 2011; Lowry et al., 2012). NPs with non-polar surfaces as CNTs, as well as those with polar interactions, are commonly stabilized by DOM in the colloidal state. DOM can form a coating, stabilizing them as colloids; these coated particles may not behave according to the DLVO theory. For example, coagulation induced by Ca^{2+} will be inhibited in the presence of DOM, which is not predicted by DLVO theory (Hartmann et al., 2014; Wagner et al., 2014). With high concentrations of cations, the DOM can be desorbed from the surface, if not chemically bonded to the surface of the NPs, facilitating destabilization and eventually aggregation of the NPs.

- Interaction with the soil and other porous media

In the case of the soil media, interaction with sediments and their constituents, especially those with high specific surface areas such as clays and organic matter, may bind NM, changing their bioavailability as compared to that of their suspensions in pure water (NPCA, 2008; Rana and Kalaichelvan, 2013). NPs in porous media such as soils may persist as colloids for considerable timespans, but can also depose onto the surfaces. As surface area increases, the likelihood of deposition to the surface also increases and that of aggregation decreases (Wagner et al., 2014). Deposition is driven by the same interactions as aggregation, but physical entrainment due to pore size may also occur, as well as size exclusion from pores, in spite of colloidal stability.

- Uptake by organisms

While ENM are known to undergo uptake by organisms such as plants and worms (Nyberg et al., 2008; Tourinho et al., 2012; Hartmann et al., 2014), where biotransformation may take place, trophic transfer, bioaccumulation and biomagnification have also been shown to occur. Uptake by plant roots has been proven to depend on factors that are extrinsic (pH, ionic strength, temperature, surface of the NP, irrigation, and so on) as well as intrinsic (cell wall pore size, root exudates, hydraulic conductivity) to the plants (Judy and Bertsch, 2014; Sharma et al., 2014). Although uptake of ENMs into organisms could be assumed to be at the end of their lifetime in the environment, trophic transfer, bioaccumulation and biomagnification may actually prolong it (Judy and Bertsch, 2014). As the research hereby implied can undoubtedly take significant efforts for each ENM studied, it is well worth considering an approach that includes an initial screening step for persistence regarding those materials more likely to reach the environment, before assessing uptake and subsequent processes in organisms, as proposed by Olson and Gurian (2012). Subsequently, attention should be given first to ENMs with persistent coatings and those lacking a natural equivalent (Wagner et al., 2014).

The End of the Lifetime of a Released ENM

ENMs released to the environment should reach an end to their lifetimes as soon as possible, causing minimum impact. In general, when not by dissolution/reaction, ENMs will be led to the end of their lifetimes by aggregation, deposition, sedimentation and subsequent processes, which may involve dissolution/reaction or simply permanent immobilization.

PERCEPTION SURVEY TO COSTA RICAN AUTHORITIES

As a mean to preliminarily identify the needs, weaknesses and positioning of the local authorities regarding ENM and their potential as CECs, some results of a survey undertaken in Costa Rica are herein presented. The study was performed in order to pursue the safe use of this promising technology, while protecting public health. The survey aimed at contrasting the perception of national authorities from the health, environment, and academic sector on four key issues that are described below.

High-ranking authorities were selected as respondents representing three different sectors (environmental, health and academic sector~~s~~) and no volunteers were considered. In all cases, the survey was developed indicating the specific interest in CECs but not specifically in NMs, to avoid bias. Although nine categories of pollutants were included in the survey form as answer options of potential CECs, only the section on NMs was considered here.

Knowledge About the Term CECs

Most of the surveyed authorities (93.3%) ensured to know the concept, and when asked to give a definition for the term. The major similarities in the concepts were regarding their novelty (35.7%), implications of toxicity or harm for living beings (28.5%), and the absence of regulation or standards (21.4%).

Extent of Agreement Regarding Current Local Affectation of the Environment and Public Health by CECs

Respondents were asked to indicate their perception of the current local affectation by CECs to the environment and to the public health. First, the surveyed authorities were invited to express their extent of agreement with the phrase: *Currently in Costa Rica, CECs affect local environmental conditions.* The total of the surveyed high-ranking authorities (n=15) chose to express their extent of agreement by selecting the option *"totally agree"* in a closed-ended question.

The perception on the affectation of public health due to CECs was evaluated with the phrase: *Currently in Costa Rica, CECs affect the health of the country's inhabitants.* The results are illustrated in Figure 1.

A significant variation of opinion was found within and between sectors with respect to public health affectation. None of the surveyed authorities selected the options "Totally disagree" and "Moderately disagree," although both of these were available options along with the other three reflected in figure 1. The academic sector was the only group where most respondents totally agreed with the statement. However, it was also in this group where more than two options were selected as an answer. Generally, the health sector tended to show more moderate positions than those found in the other two groups.

Surveyed authorities were asked to express their opinion on the importance that should be given to CECs considering the contrasts that were exposed above. The latter was performed by giving a qualification of 1 to 10 in which 10 was the most important. Mean values for the given qualifications are illustrated in Figure 2.

Differences between perceptions of the same and different public sectors related to the influence of CECs on current local affectations are a key element for environmental health policy development.

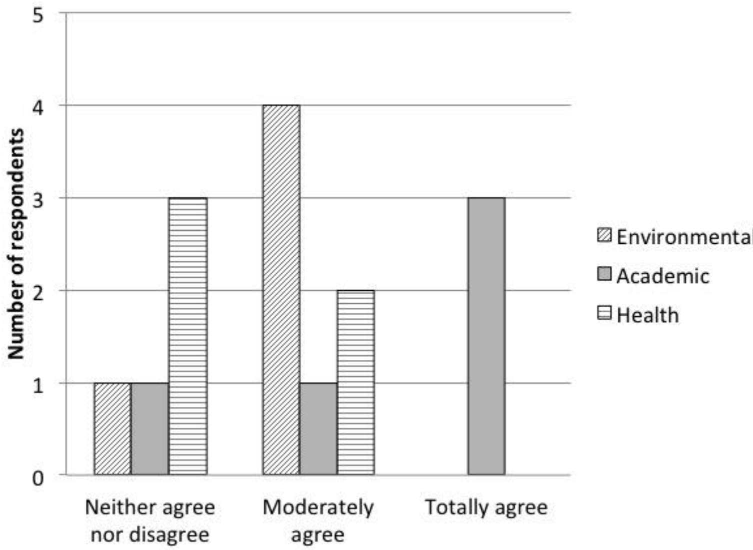

Figure 1. Extent of agreement of each of the three surveyed groups with current local affectation of the population health by CECs; n=15.

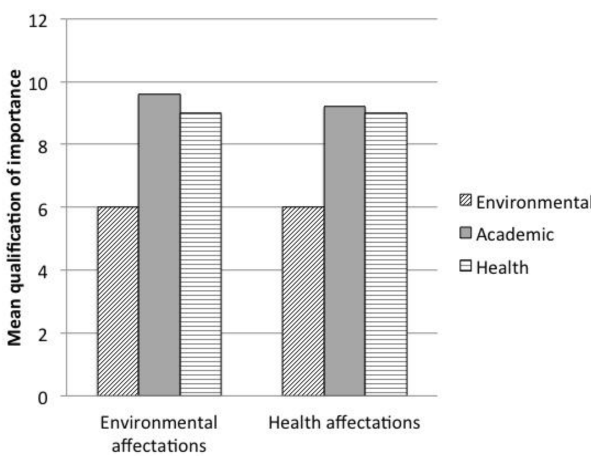

Figure 2. Agreement of each of the three surveyed groups on the extent towhich the CECs deserve attention in contrast to other affectations; n=15.

Perception of ENM as CECs

The results regarding the choice of substances, from the given pollutants list, that respondents considered ENM to be CECs or not, are shown in Figure 3. Previous to this question, an official definition of CECs was read to all respondents, regardless of the answer given to having knowledge or not of what is a CEC. Furthermore, the authorities were informed of the existence of a one-page glossary available for their consultation from that moment, in which the definition of ENM was included.

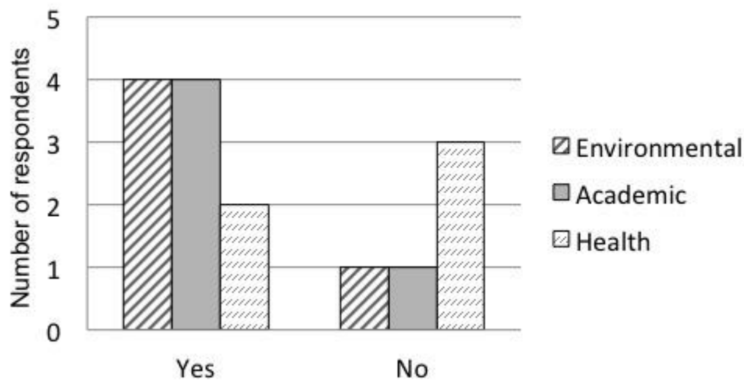

Figure 3. Selection of ENM as CECs from the given pollutants list by the three groups of respondents; n=15.

The perceptions were different between the three sectors. Most of the respondents from the environmental and academic sector do consider ENM as a CEC, contrary to what the health authorities did. However, the coefficient of variation of the answers of the health sector (%CV=34) was the highest in comparison to that of the other two sectors (%CV=47) which had the same deviation.

Furthermore, respondents were asked to express their opinion on the importance that should be given to the contaminants included in the same pollutant list provided, by giving a qualification from 1 (the most important) to 10 (the less important). The values resulting for ENM on the list of each authority are exposed in Table 1.

Table 1. Qualification of importance given to ENM by respondents among a pollutant given list; n=15

Surveyed sector	Minimum	Maximum	Mean	Coefficient of variation (%)
Environmental	1	8	5.0	55
Academic	3	7	5.2	30
Health	4	9	7.2	34

ENM were given a higher mean qualification, and thus importance, by respondents of the health sector followed by the academic sector. The environmental sector presented the smallest mean value, minimum qualification, and also a higher coefficient of variation (%CV=55), than in the other two groups and than in the entirety of the results (%CV=40). This shows that although the authorities seem to be the ones who consider it slightly less important to give attention to ENM, this opinion is variable within the authorities of this sector compared to their counterparts in the health sector as well as the surveyed researchers.

Difficulties and Weaknesses in Addressing CECs

Authorities were asked to indicate if they considered that Costa Rica is having difficulties for an effective response and action in situations that may be associated with the CECs. With the exception of the health sector, the different authorities perceived unfavourable conditions. Respondents who considered that such a situation is occurring, were asked to detail which are the weaknesses. The answers were mainly linked to lack of information about specific risks and difficulties for proper generation and systematization. Lack of political interest and of knowledge in the population was also mentioned.

An important aspect is that this answer was given considering CECs under general terms which could represent a scenario even less favorable to ENM.

This preliminary survey suggests that a thorough review of current regulatory schemes for registration/import of products including ENM into and within Latin America is necessary, including defining the main product types and risk assessment methods.

STATE OF LEGISLATION AND REGULATORY SCHEMES IN ARGENTINA, BRAZIL, MEXICO AND COSTA RICA- MINIMIZING THE CONSEQUENCES OF UNFORESEEN HEALTH RISKS

Regulation of nanotechnologies in Latin America has lagged behind its technological development. While consensus on defining nanotechnology and nanomaterials (NMs) has been convergent (OJEU, 2011), there is still no general recognition or consensus of the issues to be regulated world-wide. It is evident that health issues seem more urgent than environmental concerns. However, the risk of liberating NMs to the environment eventually becomes a health issue, as they can be reincorporated into the food chain (WHO, 2011).

Regional initiatives prompted by WHO (2011) concern for health and food safety and the current growth and use of nanotechnologies, purport a wide array of areas that need to be regulated: development of nanostructures to enhance or modify food textures, solubilization or encapsulation of nutrients, nanosensors to detect food degradation, improved food containers, use of nanofiltration to remove unwanted ingredients, use of nanochemicals to improve production or application of human and veterinary drugs, pesticides and other agrochemicals. A very complete assessment of regulatory needs has been undertaken by the European Commission (2011), covering existing environmental regulation and anticipating future trends and existing gaps. The impact of these products on the post-market phase must also be addressed, since they currently end up in the general waste stream. It is important to analyze the impact at recycling facilities, incinerators, landfills, wastewater treatment plants and other waste handling operations, in view of Best Available Technologies (BATs), which have shown limited success as effective barriers for their liberation to the environment. NMs can end up in waste streams (ordinary solid and hazardous waste, wastewater, gas emissions) as well as drinking water, due to their ubiquitous presence in various consumer products, ranging from cosmetics to automobiles (European Commission, 2011):

> "This understanding drew on possible environmental exposure pathways for specific nanomaterials and for nanomaterials in general and on data on the possible hazards associated with specific nanomaterials that were identified in the literature. Although a wide range of possible exposure pathways were identified, concrete evidence of releases was only found to support some of these pathways, notably releases of treated wastewaters into surface waters and into soil through sewage sludge and treated effluent from sewage plants. For other

pathways, either the very limited number of studies or the complete lack of studies made the identification of possible exposure more speculative (Conclusions, p.203)."

However, the only solid piece of legislation to disclose ENMs content in consumer products is linked to the European Union's initiative to inform the public of these substances in cosmetics (Savolainen, 2013), through the Cosmetics Legislation (Regulation No. 1223/2009), which for products manufactured after July 2013 requires labelling the presence of ENM. According to Savolainen (2013), no specific mention of ENMs is made in the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) legislation (OJEU, 2008), the broad definition of "substance" therein contained can be interpreted to include ENMs. As a result, the European Chemicals Agency (ECHA) has updated its guidance on safety data sheets in order to include information requirements and safety assessment of NMs. The EU has updated its biocidal products regulation (EU 528/2012) so that approval of an active substance will not include the NM form, unless NM have been specifically assessed for their risk to human and environmental health. Other regulatory initiatives include plastic materials (EU 10/2011) and other articles that may come in contact with food. In addition, a number of concrete decisions on 'ecolabelling' contain specific considerations regarding NMs (2011/383/EU, 2011/381/EU). France has initiated a registry of NMs, and other countries in Europe may follow suit (OECD, 2014).

OECD's work in this area is performed through a Working Party on Manufactured Nanomaterials, periodic meetings with country advances and round-tables, and its testing program. This program includes: a) NMs information/identification, b) physical-chemical properties, c) environmental fate, d) environmental toxicology, e) mammalian toxicology and f) material safety. The Recommendation of the Council on the Safety Testing and Assessment of Manufactured Nanomaterials (OECD, 2013), provides a strong incentive for OECD members to adhere to the organization's tools and guidelines for testing of ENMs, which include recommendations for sample preparation and dosimetry, exposure measurement and risk assessment. Other topics researched by OECD include exposure assessment and mitigation, workplace exposure assessment, guidelines for personal protective equipment in the workplace, specific guidelines for laboratory exposure assessment and control, lifecycle assessment of NMs, genotoxicity of NMs, methods and models for exposure assessment, and development of interspecies variability factors for human health risk evaluation. OECD is a Europe-based economic organization, which also includes countries such as the USA, Mexico, Chile, Australia, South Korea and Japan, and which for the topic of NMs partners with the Inter-Organisation Programme for the Sound Management of Chemicals (IOMC), FAO, United Nations Institute for Training and Research (UNITAR) and WHO. It is to be seen whether other regions of the world, such as Latin American countries, some of which are discussed below, are able to take advantage of these developments.

Regional Initiatives in the Americas

Initiatives in the Americas include the Strategic Approach to International Chemicals Management, henceforth the Strategic Approach to International Chemicals Management (SAICM) (Foladori et al., 2013), UNITAR (2011) and work pursued by the Inter-American Development Bank (IADB, 2015), all of which are fostering partnerships between experts and creation of professional and government networks, which point towards regulatory programs in the mid and long term.

The SAICM work is conducted through a series of technical workshops geared to foster knowledge on the "state-of-the-art" in nanosciences and the risks posed by substances or waste containing NMs, and has partnered in joint activities with UNITAR and OECD. SAICM aims to work with the Latin American and Caribbean countries and has proposed a life-cycle approach (cradle-to-grave) to chemicals' management, thus encompassing health and environmental issues related to nano-containing consumer products. Proceedings from a 2011 SAICM Workshop in Panama, acknowledge that the use of NMs has increased in the last decades, while there is a lack of evidence on risks posed by these materials, to which must be added the lack of national and international regulatory instruments of such substances. SAICM also recognizes that these substances may harbor important risks to vulnerable groups, such as children (including embryos) and the elderly, and thus calls for the formation of expert groups and capacity building to analyze such risks while appealing to manufacturers to disclose the nano-content of their consumer products through appropriate registration and labeling schemes (Foladori et al., 2013). SAICM also calls for integration of this precautionary principle into public policies related to chemicals' management and scientific/technical information exchange, including consultation to workers' associations, and limiting of waste transfers to countries that lack the capacity to manage them adequately (Foladori et al., 2013).

UNITAR has specifically addressed capacity building more directly, and has developed an 85-page manual with the Swiss Government (UNITAR, 2011), alerting on public health, environment and worker´s health, as areas to be covered when developing national programs on NT. The manual further goes into governance based on current national legislation and international agreements, priority setting, and keeping these priorities at a manageable level through adequate coordinating mechanisms and capacity building. UNITAR has partnered with the Latin American Network on Nanomaterials and Society (RELANS), which has achieved a considerable number of joint publications with other international and national networks (Invernizzi, 2015).

The IADB cites the following countries as having important activities in nanoscience: Brazil, Mexico, Argentina, Colombia, Chile, Venezuela, Peru, Uruguay, Dominican Republic, Costa Rica, Cuba, Guatemala, El Salvador, Ecuador and Panama (Foladori and Invernizzi, 2012), while only Brazil is currently part of NanoReg, an international endeavor, sponsored by a group of European countries and which also includes Australia, Canada, the Republic of Korea, the USA and Japan.

Prominent environmental and awareness-raising organizations have also voiced their concerns at a regional level, promoting workshops and meetings aimed at analyzing the risks of technology deployment, promoting consumer right-to-know schemes and the application of the precautionary principle when regulating NMs (ETC Group, 2008, 2010). Although advances in the regulatory field have been limited, countries such as Argentina, Brazil, Mexico and Costa Rica have prompted efforts in this area.

Argentina

Current efforts in Argentina have been made, but there is to date no specific regulation of NT that includes risk assessment to health and the environment. Work has been done by ANMAT (National Authority for Medicine, Food and Medical Technology), through an "Observatory," with the aim of promoting information exchange and networking, and in the longer term to have an adequate diagnostic of scientific and regulatory issues (ANMAT, 2015; Cid Pharma Packaging, 2012). The Ministry of Science, Technology and Productive Innovation, through Law 26.338 of 2007 (a modification of the Law of Ministries) is responsible for the promotion of NT. This has been implemented through the Fundación de Nanotecnología created through Decree Nº 380/05 (Infoleg, 2015) under the supervision of the Ministry of Economy and Production.

Although criticized by the Comité Nacional de Ética en la Ciencia y la Tecnología for allowing loopholes in the handling of national budgets (CECTE, 2005), the Decree establishes clear responsibilities and functions for this Foundation, which is focused only on NT promotion, capacity building and market creation, with no mention of risk assessment or regulation, nor research in these key areas. Other efforts have been made to establish a General Framework Law for Development of Micro and Nanotechnologies (Argentinian Senate and Congress, n.d) proposed to Congress, based on Law 25.467, General Framework Law for Science, Technology and Productive Innovation, in an effort to repeal Decree Nº 380/05. Nonetheless, this proposal offered no guidance on risk management and regulation of nanotechnologies in the health and environmental areas, and was never enacted (Foladori, 2006).

Brazil

Similarly to Argentina, Brazil has had important developments in national networks since the year 2000 and experts have joined international and regional networks such as SAICM or RELANS (Foladori, 2006; Foladori et al., 2013; Rocha, 2014). Brazil, however, has granted greater attention to toxicology research and workers' health (Arcury, 2010). Through legislation enacted by the Ministry of Science, Technology and Innovation (Law 510 of 2012), Brazil created an Inter-Ministerial Committee on Nanotechnology (CIN), encompassing 10 Ministries, in charge of the Brazilian Nanotechnology Program (PBN) and a Network of Strategic (government) and Associate (university) Laboratories for Nanotechnology (SisNano). Aims of the CIN include management of the Nanotechnology Program, integrating financial resources, promoting networks, international cooperation and program evaluation. Toxicology research has been fostered, and there are networks in: aquatic toxicology of NPs, cytotoxicity and genotoxicity of nanostructured composites, toxicology of NPs in the oil and gas industry, occupational and environmental nanotoxicology (regulation and risk evaluation), toxicity of NPs in biological systems, and toxicity of materials applied to medicine and agriculture using in vivo and in vitro methods (Rocha, 2014). Although health and environmental areas are considered in terms of toxicology research, and a firm governmental basis exists for possible regulation of technologies, there is no formal regulatory mechanism for NM.

Brazil partners with NanoReg, 14 European countries with the common goal of establishing a firm basis for regulatory testing of NMs. This, as well as an official decision to join regulatory efforts in Europe (Nanosafety Cluster), and aligning its environmental health and safety programs in NT with those of Europe and the USA, will undoubtedly promote North-South exchange and will allow harmonization of standards and mutual data acceptance (Rocha, 2014). Brazil has recognized international efforts for health and safety regulation, and its highly developed scientific networks will benefit from insertion into global initiatives such as the Nanosafety Cluster and NanoReg.

Brazilian NT efforts are "ahead of the game" in terms of anticipating ill-effects of technology deployment. Nonetheless, a project for legislation in 2005 calling for policies and a fund for work in nano-safety (including establishing a multisector National Technical Commission), as well as a project voted in 2013, pushing for disclosure of NM content in food, cosmetics and pharmaceuticals, were never enacted (Portal Brasil, 2013). The Inter-Ministerial Committee on Nanotechnology, based on a single piece of legislation that created it, is steering Brazil´s work on NT.

Mexico

Since the year 2000, Mexico´s Special Program on Science and Technology, as part of the National Development Plan 2001-2006, gave a strategic positioning to NT development. A specific program for NT was drafted, a steering committee was instituted, and a National Network for Nanoscience and Nanotechnology (NNNN) was created in 2009 (Foladori et al., 2013). Funding, however, has not been strongly supported by the government and most researchers and enterprises have been financed by partnering with larger firms abroad (Foladori, 2006). This, however, has not hampered participation of scientists in the network, which by 2010 totalled 500 researchers and 60 universities (Foladori and Zayago, 2011).

Although solid regulations have not been in place in Mexico, commercial exchange with the US, under NAFTA, has facilitated adherence of Mexican policy makers to US guidelines for NT and NM regulation.

Recently, and based on an OECD survey comparing NT policies in 24 countries, the Mexican case was studied (Foladori et al., 2015). This survey concludes that Mexico does not have a national strategy for NT development; even though it considered its importance since 2001. Additionally, Mexico has not promoted public participation in NT; yet it does have institutionalized mechanisms to encourage participation on other topics in the scientific agenda. Scientific policy does not include exposure and risk of manufactured NPs, even though Mexico has access to OECD Decisions and Recommendations, which must be reflected in policy and regulation. Mexico participates in SAICM but has scarcely translated its recommendations or decisions into legislation. Not until 2013 was a Mexican Technical Committee on NMs constituted, based on the Federal Law on Metrology and Standardization. The Committee established its rules of operation, and is empowered to create standards for NT. It actively participates in setting international standards for NT through the International Organization for Standardization (ISO) within the Technical Committee ISO/TC 229 *Nanotechnologies* (Foladori et al., 2015).

Costa Rica

Costa Rica has envisioned NT as a long-term strategy and has considered it, along with biotechnology, a cornerstone for development towards 2050 (Estrategia Siglo XXI, 2006; MICITT, 2015), including it in its National Plan for Science, Technology and Innovation 2015-2021. In 2011, the official newspaper La Gaceta published Decree 36567, by which research in NT and its applications, as performed in institutions of the Science and Technology Sector and public universities, is declared of public interest; research is considered an instrument of economic and social development (La Gaceta, 2011).

Additionally, the decree calls all public and private institutions to contribute, according to their means and regulatory frameworks, economic, logistic and technical resources, to improve scientific research in NT and its applications. The preamble to the decree recognizes work already initiated by the Ministry of Science and Technology (MICyT), the Rectory Council for Public Universities (CONARE), the National Center for High-Tech (CENAT) and the National Laboratory for Nanotechnology (LANOTEC), and places NT as an emerging and convergent technology that can foster scientific development plans considered within the National Development Plan 2011-2014 (La Gaceta, 2011). This, however, is the only regulatory instrument which, although visionary, does not provide sound funding for research activities within a coordinated national plan for nanotechnology, as other countries in the Americas have managed to propose. In spite of these constraints, the National Plan for Science, Technology and Innovation 2015-2021 considers that Costa Rica should promote work in nanomedicine, nanopharmaceuticals, nano-biotechnology, nano-microelectronics, environmental applications of NT, development of nano and microsensors, nanocatalysts; without disregarding intellectual property, ethical, and social aspects of NT. The aforementioned comments in the National Plan, proposed by the LANOTEC director, end with a recommendation to draft, alongside this National Plan, a technical report that charters the route for development and sustainability of NT in Costa Rica.

CONCLUSION

While the concern of this chapter is the potential of the ENM as contaminants, there are several benefits (environmental technology, remediation, medical and commercial applications) that humanity can obtain from NT products, if developed in a sustainable way.

As the fields of applications of ENM and products containing them grow, it is pivotal to give more attention to the field of nanotoxicology from an ecosystemic point of view.

Research on the presence of CECs, their risk and their quantification in the environment, should be of special interest for Latin America, which possesses the richest diversity of species and ecoregions in the world and holds one third of the world's renewable water resources and close to 30% of the world's total runoff (ECLAC, 2002).

Latin America has lagged in providing populations with proper regulation for NMs. In these fields many NGOs have raised concerns and called for risk assessment and control. Though inter-governmental initiatives such as SAICM and OECD are providing some capacity building efforts, most countries have started this technological journey by providing merely a technology-development and promotion infrastructure, with varying degrees of success.

Common Regulatory Approaches in Leading Countries

The countries portrayed in this brief overview of regulatory capacities show varying strategies in response to their specific political and scientific context. A common response has been the promotion of policies for NT development, network creation, alliances and public participation at the national and international levels (spurred by regional and international organizations). This response, however, has not been enough to promote strong NT regulation and protect consumers. In most countries one concludes that awareness of the risks is not strong enough to overcome industries' fear of regulation by the legislative/executive branches of the government.

Closing the Gap

Latin American countries aim to partner with international agencies or intergovernmental initiatives, share knowledge and build capacities for both technology development and risk assessment, with a sound foundation on toxicology, perhaps the "new nano-toxicology." This knowledge will create risk awareness and set the basis for its reflection in regulatory instruments, avoiding "re-inventing the wheel" and optimizing resources worldwide. These regulatory means will be focused on the worker and the population's right-to-know, particularly with regard to food products, cosmetics, pharmaceuticals, chemicals and agrochemicals, coupled with the precautionary principle. All must be based on a sound risk assessment throughout the life-cycle, focused on size-dependent properties, and the recognition that assessment of the micro and larger homologues bears little, if any, relationship to the nano-scale version.

Some topics that the countries in Latin America should consider in their legislation include governance and technology innovation, regulatory authorities´ responsibilities, definitions of NMs, material testing (physical-chemical, ecotoxicology and human health testing -acute and/or chronic when appropriate-, metrology), manufacturing reports by industry, disclosure of NM content in products of environmental or health concern, workers' protection in laboratories, scale-up plants and industry, life-cycle risk assessment, and mechanisms for information exchange between experts and authorities.

Even though this work has been limited thus far, development of knowledge and regulations in the Americas seems promising, and the growing networks of experts will certainly play an important role in bridging this gap.

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Chapter 33

PHARMACEUTICAL AND PERSONAL CARE PRODUCTS (PPCPS) IN THE ENVIRONMENT: LATIN AMERICAN OCCURRENCES, ADVERSE EFFECTS AND PERSPECTIVES

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ABSTRACT

This chapter presents a review on pharmaceuticals and personal care products (PPCPs) occurrence and adverse effects performed by researchers in Latin America. Recent data confirm that PPCPs occur widely in freshwater, soils and marine and coastal environments in Brazil, Argentina, Colombia, Chile and Mexico. Unfortunately, Latin America has a common and very serious environmental health problem: the failure or absence of wastewater treatment plants. In contrast, the Latin American pharmaceutical market represents approximately 25% of global pharmaceutical sales. Therefore, studies regarding occurrence and assessment of potential risk to biota need further efforts. Because PPCPs are bioactive compounds being designed to interact with specific physiological pathways at low doses, it is important to focus on modes of action and to address the molecular, cellular and individual levels on species from Latin America. In addition, new polices on environmental risk assessment, waste management, and publiceducation must be enforced locally.

Keywords: personal care products, pharmaceuticals, Latin America

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INTRODUCTION

In recent decades, the impact of chemical pollution has focused almost exclusively on the conventional "priority" pollutants, such as metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and pesticides, especially those that cause acute toxicity, considered carcinogenic and persistent in the environment (Daughton and Ternes, 1999). Pharmaceuticals are considered emerging pollutants, and include different kinds of substances varying in physicochemical and pharmacological properties, modes of action, fate in the environment and degradation. Since the human body does not completely metabolize pharmaceuticals they are excreted slightly transformed or even unchanged, and end up in water systems. For these reasons, the study of the behaviour and possible effects of these compounds in the environment is complex. A vast amount of information has been generated during the last 20 years in Europe, North America, and Asia regarding the environmental occurrence and possible adverse effects of pharmaceuticals on biota. The European and North American legislation include guidelines for assessing environmental risk caused by exposure to pharmaceutical substances. In these guidelines, environmental risks are recommended as part of the approval process of new substances. It is especially important to determine the issue of pharmaceutical compounds to the environment and ecotoxicological assessment of possible adverse effects. Unfortunately, information available for Latin America is still very limited.

Pharmaceuticals and personal care products (PPCPs) have been recently considered an environmental problem. This issue is of increasing scientific concern (Kümmerer et al., 2010; Heberer, 2002), represented by the growing number of scientific papers on concentrations of pharmaceuticals in environmental matrices (Pereira et al., 2016). Studies on possible adverse effects on the biota also increased in recent years, focusing mainly on the acute toxicity of pharmaceuticals (Santos et al., 2010). The main studies are on growth, reproduction and mortality (Santos et al., 2010). However, few research groups have developed studies to understand more deeply the bioavailability of these compounds and possible chronic effects on aquatic communities. In addition, the existing literature is restricted almost entirely to works developed in temperate environments.

In Latin America, there is a rich diversity of ecosystems which may be differently affected by emergent pollutants such as PPCPs. Latin America houses the richest biodiversity in the world, including biomes such as mangroves, pampas, tropical forests, deserts, cerrado, among others. Focusing on aquatic environments, few studies have been performed to determine the environmental concentration of pharmaceutical products. This lack of knowledge may be due to the fact that environmental toxicology and chemistry in Latin America is a relatively young discipline (Carriquiriborde and Bainy, 2012). A quick analysis of studies published in 2011 by researchers based in Argentina, Brazil and Chile, the three countries of the Latin-American branch of the Society of Environmental Toxicology and Chemistry (SETAC-LA) with the greatest number of contributions, revealed subtle differences in the relative development of environmental disciplines (Carriquiriborde and Bainy, 2012). In Brazil, 2.1% of major environmental issues tackled by researchers were about PPCPs, while Chilean researchers published 3.4% of scientific work on PPCPs (Carriquiriborde and Bainy, 2012). Beek et al. (2016) also elucidated that peer-reviewed publication of environmental data in universities located in developing countries such as

Brazil is relatively uncommon as a result of language barriers, publication costs and limited time frames for graduation procedures.

The world consumption of PPCPs has risen drastically in the last decade, also increasing the excretion of their metabolites in their original form. Pharmaceutical consumption is often associated with longevity and stressful lives, particularly in urban areas. Advances in medicine, the significant growth of chemistry and pharmaceutical companies, and changes in social conditions have all led to the more widespread use of pharmaceuticals and psychotropic substances (Borova et al., 2014). Between 2000 and 2010, the consumption of antibiotic drugs increased by 36% (Van Boeckel et al., 2014). Brazil, Russia, India, China and South Africa accounted for 76% of this increase.

Latin America is a diverse, multi-coloured and dynamic region, boasting the highest life expectancy among developing regions (Valverde, 2014). Unfortunately, Latin America has a common and very serious environmental health problem: the failure or absence of wastewater treatment plants (WWTPs). The Latin American pharmaceutical market represents approximately 25% of global pharmaceutical sales (Valverde, 2014). In consequence, high pharmaceutical concentrations are expected in Latin American environmental matrices. The constant release of PPCPs elucidates the importance and urgency of the determination of environmental concentrations of different PPCPs, as well as the potential environmental effects, including acute and chronic toxicity, and bioaccumulation data.

PPCPs Concentrations Found in Environmental Matrices in Latin America

Sewage outfalls, WWTPs and wastewater without proper treatment are the main sources of PPCPs to the environment. Even in places supported by WWTPs, it is known that conventional WWTPs are not able to entirely degrade complex organic molecules to reduce their toxicity and improve their biodegradability. For this reason new technologies, i.e., advanced oxidation processes, are being developed to handle this demand (Borghi and Palma, 2014).

Most of the efforts to assess PPCPs occurrence and potential impacts on non-target organisms in aquatic environments have focused on freshwater rivers and streams heavily impacted by wastewater effluent (Pereira et al., 2016). These observations were mainly made in Brazil (São Paulo, Rio de Janeiro and MatoGrosso do Sulstates), Argentina, Colombia and Mexico. Concentrations varied from $ng \cdot L^{-1}$ to $\mu g \cdot L^{-1}$, indicating significant inputs of PPCPs into these regions.

The first studies about PPCPs occurrence in Brazil were in the state of Rio de Janeiro published by Ternes et al. (1999) and Stumpf et al. (1999). The first article was about the quantification of estrogens in sewage samples varying from 0.021 to 0.040 $\mu g \cdot L^{-1}$(Ternes et al., 1999). Stumpf et al. (1999) published about the detection of pharmaceutical residues such as lipid regulators, anti-inflammatories and some metabolites in raw sewage, treated waste water and river water. The median concentrations in the effluents of sewage treatment plants (STPs) of most pharmaceuticals investigated ranged from 0.1 to 1 $\mu g \cdot L^{-1}$. After the STP, median concentrations ranged from between 0.02 and 0.04 $\mu g \cdot L^{-1}$ n river water, whereas the maximum values were observed to be up to 0.5 $\mu g \cdot L^{-1}$. Caldas et al. (2013) also found 18 PPCPs and 33 pesticides in surface and drinking waters in the state of Rio de Janeiro.

Sodré et al. (2010) evidenced the occurrence of PPCPs in drinking water of the city of Campinas, Brazil. Seasonality was observed, since some pharmaceuticals were detected only in one season. Stigmasterol showed the highest average concentration (0.34 ± 0.13 $\mu g \cdot L^{-1}$), followed by cholesterol (0.27 ± 0.07 $\mu g \cdot L^{-1}$), caffeine (0.22 ± 0.06 $\mu g \cdot L^{-1}$) and bisphenol A (0.16 ± 0.03 $\mu g \cdot L^{-1}$) (Sodré et al., 2010). Acetaminophen, acetylsalicylic acid, diclofenac, ibuprofen, caffeine, 17β-estradiol, estrone, progesterone, 17α-ethynylestradiol, levonorgestrel, diethylphthalate, dibutylphthalate, 4-octylphenol, 4-nonylphenol and bisphenol were detected in surface waters from the same region, in the Atibaia watershed (São Paulo state, Brazil) (Montagner and Jardim, 2011). The lowest concentration determined was 0.096 $\mu g \cdot L^{-1}$for diclofenac, whereas caffeine showed concentrations as high as 127 $\mu g \cdot L^{-1}$ (Montagner and Jardim, 2011). Antibiotics amoxicillin, ampicillin, cefalexin (CEF), ciprofloxacin (CIP), norfloxacin (NOR), sulfamethoxazole, tetracycline (TET) and trimethoprim were quantified also in surface water samples from the Atibaia watershed (Locatelli et al., 2011). In the Atibaia River, sample concentrations ranged from 29 $ng \cdot L^{-1}$ for CEF to 0.5 $ng \cdot L^{-1}$ for NOR. In a sewage-affected stream, however, concentrations up to 2.422 $ng \cdot L^{-1}$ CEF were found.

A preliminary risk assessment of Triclosan (TCS) was conducted for Brazilian surface waters in accordance to an approach for the prioritization of organic toxicants (von der Ohe et al., 2011), which has been used to assess the priority of TCS in Europe (von der Ohe et al., 2012). Of the 71 samples analysed, 32 contained TCS at concentrations above the limit of quantification, ranging from 2.2 to 66 $ng \cdot L^{-1}$, and with six out of seven sites exceeding the lowest "Predicted no-effect concentrations" (PNEC) (Montagner et al., 2014).

The occurrence as well as the spatial and temporal variations of some commonly prescribed pharmaceuticals was determined in water samples from the Suquía River basin (Córdoba, Argentina) (Valdés et al., 2014). Eight out of 15 pharmaceuticals studied were found in river waters: ciprofloxacin, enalapril, estrone, dihydrotestosterone, oxcarbazepine, carbamazepine, atenolol and diclofenac. Among all compounds analysed, atenolol, carbamazepine and diclofenac were the most frequently detected (reaching high levels of $\mu g L^{-1}$).

Estrogens have been identified as the main contributors to the estrogenic activity of STP effluents. They are considered as endocrine disrupting chemicals (EDCs) that can interfere with the normal functioning of the endocrine system in wildlife and humans. Valdés et al. (2015) investigated for the first time the concentrations of estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) in sewage effluents and receiving waters of the "Rio de la Plata" estuary and neighbouring areas (Argentina). E2 and EE2 showed concentrations ranging between 122 and 631 $ng \cdot L^{-1}$ and between 65 and 187 $ng \cdot L^{-1}$ in effluents, while these estrogens were detected at 369 $ng \cdot L^{-1}$and 43 $ng \cdot L^{-1}$ in surface waters, respectively.

Bertin et al. (2011) measured the levels of one synthetic (EE2) and three natural (estrone, 17β-estradiol, estriol) estrogens in coastal sediment samples collected from nine locations of central-southern Chile. Steroid estrogens were found in every sample. Remarkably high levels of 17α-ethinylestradiol were detected, reaching up to 48.14 $ng \cdot g^{-1}$ dry weight. The studied coastal region houses a large variety of fish and seafood of commercial value such as flatfish, hake, red and pink cusk-eels, drumfish, crabs, mussels, abalone and clams (Sernapesca, 2008). Thus, the contamination of marine sediments by steroid estrogens should not be neglected, particularly in areas which host edible fish and seafood, as this may turn out to be a significant health issue for humans (Bertin et al., 2011).

The first study on the presence and distribution of PPCPs in Colombia was published by Gracia-Lor et al. (2012). Seventy three surface water samples (rivers, lakes and reservoirs and effluent wastewater) showed that the large majority of the target analytes were present in the samples analysed. Target analytes were two anti-inflammatories (ibuprofen, diclofenac), a lipid regulator agent (clofibric acid), two angiotensin II antagonists (losartan, ibersartan), two antiepileptic drugs (gabapentin, carbamazepine) and a diuretic (furosemide). Among personal care products, four preservatives (methyparaben, ethylparaben, propilparaben, butylparaben) and five UV filters were included (benzophenone, benzophenone-1, benzophenone-2, benzophenone-3, benzophenone-4).

Hernández et al. (2015) made alargescreening in Colombia of around 1,000 emerging contaminants, focused on PPCPs, illicit drugs and their metabolites. This survey was made in urban wastewaters (both influent and effluent) and surface waters from the area of Bogotá. The main compounds detected were acetaminophen/paracetamol, carbamazepine and its dihydro-dihydroxylatedmetabolite, clarithromycin, diclofenac, ibuprofen, gemfibrozil, lincomycin, losartan, valsartan, the two metabolites of metamizole (4-acetamido-antipyrine and 4-formylamino-antipyrine), sucralose, cocaine and its main metabolite benzoylecgonine. Also caffeine, the sweetener saccharin, two hydroxylated metabolites of losartan and lidocaine were identified in the samples analysed.

In Mexico, environmental studies concerning pharmaceuticals are mainly focused on soils irrigated with wastewater. One of the main objectives of Siemens et al. (2008) was to characterize the flow of pharmaceutically active substances through the Mezquital Valley wastewater irrigation system (Mexico City, Mexico). Trimethoprim, erythromycin, naproxen, ibuprofen and diclofenac in sewage equalled or exceeded the US FDA (US Food and Drug Administration, US Department of Health and Human Services, 1998) action limit of 1 $mg\cdot L^{-1}$ for detailed environmental risk assessment (ERA).

Gibson et al. (2010) reported the persistence and leaching potential of a group of acidic pharmaceuticals, carbamazepine, and three endocrine disruptors in soils from the Tula Valley in Mexico, one of the largest irrigation districts in the world that uses untreated wastewater. Ibuprofen (742-1,406 $ng\cdot L^{-1}$), naproxen (7,267-13,589 $ng\cdot L^{-1}$) and diclofenac (2,052-4,824 $ng\cdot L^{-1}$) were consistently present while clofibric acid, gemfibrozil and ketoprofen were below limits of detection (LOD) in all samples (100, 50 and 50 $ng\cdot L^{-1}$, respectively) and were not considered further. Concentrations of ciprofloxacin, enrofloxacin, sulfamethoxazole, trimethoprim, clarithromycin, carbamazepine, bezafibrate, naproxen and diclofenac, as well as the occurrence of *Enterococcus* spp. and *sul* and *qnr* resistance genes in soil irrigated with wastewater were observed by Dalkmann et al. (2012).

There is a lack of knowledge about PPCP concentrations and adverse effects in marine environments. The most recent study concerning the occurrence of pharmaceuticals, illicit drugs (cocaine) and its metabolite (benzoylecgonine) in the marine environment was published by Pereira et al. (2016). Pharmaceuticals and cocaine were assessed in a subtropical coastal zone (Santos, São Paulo state, Brazil) in five sampling sites around the sewage outfall. Acetaminophen (17.4-34.6 $ng\cdot L^{-1}$), caffeine (84.4-648.9 $ng\cdot L^{-1}$), diclofenac (19.4 $ng\cdot L^{-1}$), ibuprofen (326.1-2094.4 $ng\cdot L^{-1}$), losartan (11.8-32 $ng\cdot L^{-1}$) and valsartan (10.8-75 $ng\cdot L^{-1}$) were quantified in seawater samples. Ibuprofen showed the highest concentrations in the order of $\mu g\cdot L^{-1}$. Cocaine and benzoylecgonine were both quantified in all of the samples analysed at concentrations that ranged from 12.6 to 537 ng·L^{-1} and 4.6 to 20.8 ng·L^{-1}, respectively.

Beretta et al. (2014) detected PPCPs in marine sediment from Todos os Santos Bay (Bahia, Brazil). The highest concentrations were found for the fragrances galaxolide (52.5 ng·g^{-1}) and tonalide (27.9 ng·g^{-1}), followed by caffeine (23.4 ng·g^{-1}) and pharmaceuticals ibuprofen (14.3 ng·g^{-1}), atenolol (9.84 ng·g^{-1}), carbamazepine (4.81 ng·g^{-1}), erythromycin (2.29 ng·g^{-1}), diclofenac (1.06 ng·g^{-1}) and diazepam (0.71 ng·g^{-1}).

Elorriaga et al. (2013) published the first survey of pharmaceuticals in municipal wastewaters discharging into fresh and estuarine waters from areas with varying degrees of urbanization of Argentina. Pharmaceutical concentrations were in μg·L^{-1} which may be considered higher than the average concentration of such compounds in the environment that are normally at then ng·L^{-1} level. Concentrations were within the range of 0.9-4.2 μg·$L^{-1}$and 0.4-13.0 μg·L^{-1} for caffeine and ibuprofen, and at the lower levels of 0.2-2.3 μg·L^{-1}, 0.2-1.7 μg·L^{-1} and 0.03-1.2 μg·L^{-1} for carbamazepine, atenolol and diclofenac, respectively.

ADVERSE EFFECTS ON BIOTA AND ENVIRONMENTAL RISK ASSESSMENT

Pharmaceuticals represent a versatile group of chemical compounds. In general, chemicals can be harmful and toxic because of many different mechanisms. For example, chemicals can bind to molecules such as hormones, DNA and RNA, lipid membranes and proteins. Recent studies have been performed in Latin America concerning adverse effects of PPCPs on biota. Godoy et al. (2015) assessed the ecotoxicity of propranolol hydrochloride and losartan potassium, both individually and combined, by using the *Lemna minor* growth inhibition test. The risks associated to the effects of both pharmaceuticals on non-target organisms were quantified through the measured environmental concentration (MEC) / predicted-no-effect concentration (PNEC) ratios. For propranolol, the total frond area was the most sensitive endpoint (effect concentration - EC_{50}=77.3 mg·L^{-1}), while for losartan there was no statistically significant difference between the endpoints. Losartan is only slightly more toxic than propranolol. Both concentration addition and independent action models overestimated the mixture toxicity of the pharmaceuticals at all the effect concentration levels evaluated. The joint action of both pharmaceuticals showed an antagonistic interaction for *L. minor*. Derived risk quotient (RQ) assumed lower values for propranolol than for losartan. The MEC/PNEC ratios showed that propranolol may pose a risk for the most sensitive aquatic species, while acceptable risks posed by losartan were estimated for most of aquatic matrices.

TCS (Triclosan- 5-chloro-2-(2,4-dichlorophenoxy) phenol) is an antibacterial compound widely employed in PPCPs. Cortez et al. (2012) observed effects on reproduction (fertilization and embryo-larval development) and physiological stress (lysosomal membrane stability - LMS) in the marine sentinel organism *Perna perna*. The mean inhibition concentrations for fertilization ($IC_{50=}$0.490 mg·L^{-1}) and embryo-larval development ($IC_{50=}$0.135 mg·L^{-1}) were above environmental relevant concentrations (ng·L^{-1}) given by previous studies. On the other hand, a significant reduction of LMS was found at 12 ng·L^{-1}, demonstrating the current risk of the continuous introduction of TCS into aquatic environments.

Borghi and Palma (2014) draw attention to the fact that there are other compounds in addition to the bioactive substance of the drugs, which can also cause adverse effects to biota. One example is the antibiotic tetracycline which has high world consumption, representing a human consumption of about 23 kg·day^{-1} in Brazil in 2007. At the moment, research is being made to develop new tetracycline that incorporate metals (Hg, Cd, Re, Pt, Pd) to their structures in order to increase their bactericidal effect.

Chronic adverse effects due to the antidepressant fluoxetine (FLX, 0.54 μg·L^{-1}) and the synthetic estrogen, 17 α-ethinylestradiol (EE2, 5 ng·L^{-1}), alone and in combination, were observed in malegoldfish *Carassius auratus* after 14-days of exposure (Assis et al., 2013). The results showed an increase in estrogen receptor alpha (*esr1*) and vitellogenin (VTG) gene expression by 1.9-2.4 fold in the FLX and EE2 groups.There was a significant increase in the number of plasma proteins that were related to endocrine system disorders in the FLX and FLX plus EE2 groups. The level of VTG protein was increased in the plasma from goldfish exposed to EE2, FLX, and FLX plus EE2. This study demonstrates that low concentrations of FLX and EE2 in a simple mixture produce strong estrogen-like effects in male goldfish.

Ribas et al. (2014) published a work about toxic effects of non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac (0.2, 2, 20, 200 and 2000 ng·mL^{-1}), ibuprofen (0.1, 1, 10, 100, and 1000 ng·mL^{-1}) and acetaminophen (0.025, 0.25, 2.5, 25 and 250 ng·mL^{-1}) evaluated on primary culture of monocytic lineage of *Hoplias malabaricus* anterior kidney. NSAIDs influenced lipopolysaccharide (LPS)-induced nitric oxideproduction and caused DNA damage in monocytic cells, suggesting that these substances can induce immunosuppression and genotoxicity.

Effects of diclofenac and dexamethasone on hematological parameters and immune response were observed also in the fish species *H. malabaricus* after trophic exposure (Ribas et al., 2016). Intraperitoneal inoculation with diclofenac (0, 0.2, 2 and 20 μg·kg^{-1}) or dexamethasone (0.03, 0.3, and 3 μg·kg^{-1}) was performed in 20 doses. Although some fish responses were variable for different compounds, the results suggested that trophic exposure to diclofenac and dexamethasone can lead to hematological changes and immunotoxic effects.

The gonad and liver from the same fish were collected to calculate gonadosomatic and hepatosomatic indices (Guiloski et al., 2015). Antioxidant enzyme activity and biotransformation were also evaluated in liver and gonads. Both diclofenac and dexamethasone reduced the levels of testosterone, causing impairment in reproduction. Guiloski et al. (2015) suggested that diclofenac and dexamethasone caused oxidative stress and reduced testosterone levels with negative impacts in fish.

Biochemical, genetic and hematological biomarkers were assessed in the liver, kidney, and blood of juvenile *Rhamdia quelen* fish exposed to diclofenac for 96h at concentrations of 0.2, 2, and 20 μg·L^{-1}. No oxidative stress was observed in liver. In kidney, the superoxide dismutase activity increased in all concentrations, suggesting an alteration in the hydrogen peroxide production. Diclofenac exposure increased the red blood cells number at concentrations of 0.2 and 2 μg·L^{-1}, and monocytes and neutrophils at 2 and 20 μg·L^{-1}, respectively. Ghelfi et al. (2016) suggested that acute exposure to diclofenac caused hematologic and renal enzymatic alterations.

The first report on pharmaceuticals in superficial waters of Argentina as well as the first report on the bioaccumulation of atenolol in whole body fish was reported by Valdés et al.

(2014). The bioconcentration of carbamazepine and atenolol was studied under controlled conditions in *Gambusia affinis*, a widely distributed fish species inhabiting the Suquía River basin (Córdoba, Argentina). Estimated bioconcentration factors (BCFs) were: 0.13 and 0.08 $\mu g \cdot kg^{-1}$ upon exposure to 100 and 1000 $\mu g \cdot L^{-1}$ atenolol in water, respectively; while BCFs were 0.7 and 0.9 $\mu g \cdot kg^{-1}$ when exposed to 10 and 100 $\mu g \cdot L^{-1}$ carbamazepine, respectively.

Estrogen concentrations are able to disrupt the endocrine system of aquatic vertebrates (Sumpter and Jobling, 2013). E2 concentrations as low as 50 $ng \cdot L^{-1}$ were effective in impairing the expression of brain aromatase and the sexual behaviour of the South American fish *Jenynsia multidentata* (Guyón et al., 2012) and EE2, fiftytimes more potent, was able to disrupt the expression of the gonadal aromatase andthe sex ratio of *Odontesthes bonariensis* (Pérez et al., 2012), an emblematic species of the "Pampas" streams and shallow lakes. According to Valdés et al. (2014), the measured concentration of estrogens in the receiving waters of the ''Pampas'' region with low dilution capacity, would represent a risk for the reproductive successes of local fish, and probably other aquatic organisms.

Magdaleno et al. (2014) performed toxicity and genotoxicity assessment with effluents from a STP serving around 10 million inhabitants in Buenos Aires (Argentina). Toxicity assays showed that 55% of the wastewatersamples from a public hospitalwere toxic to the green algae *Pseudokirchneriella subcapitata* and genotoxicity for 40% of the samples tested with the *Allium cepa*. Nevertheless, the sample fromthe STP was not genotoxic to *A. cepa* but toxic to the algae, showing that sewage treatment was not totally effective.

González-González et al. (2014) exposed common carp *Cyprinus carpio* to water from Madín Reservoir (Mexico) for 96 h. Water samples contained contaminants such as metals and NSAIDs. Results showed that contaminants (metals and NSAIDs) present in the water induced oxidative stress.

PERSPECTIVES

Environmental risk and impacts of PPCPs have been largely assessed via acute toxicity assays, which are likely to be underestimating real impacts, since such compounds have been found at low concentrations in surface waters ($ng \cdot L^{-1}$), but are being continually released into ecosystems. This steady flow contributes to their persistence and potential adverse effects (Alygizakis et al., 2016; Andreu et al., 2016). Nowadays, it is well known that these bioactive compounds might cause mainly chronic toxicity with serious consequences for the environment. Chronic exposures and specific mode of action of PPCPs need to be considered as major aspects for future scientific investigation in Latin America.

Knowledge on the transformation of pharmaceuticals in WWTPs and aquatic ecosystems is rather fragmented. Several studies have assessed attenuation (net balance between removal and release to and from the water column) of pharmaceuticals in either WWTPs or freshwater ecosystems, but none have assessed attenuation of pharmaceuticals and their transformation products including both systems within the studied system boundaries and using the same approach (Aymerich et al., 2016). The conventional WWTPs are not able to promote significant PPCP load reduction. Thus, the main step to minimize the PPCPs contamination should be the development ofeffective treatments for wastewater discharges. Implementation of mitigation actions andthe development of environmental tools to monitor PPCPs contamination and effects are also crucial to preserve aquatic biodiversity.

Concerning specifically illicit drugs and their breakdown products, occurrences in regions of production and use and areas with insufficient wastewater treatment are not well studied (Rosi-Marshall et al., 2015). Several studies have found cocaine and its metabolites in wastewater, surface water, sediment and organisms, and adverse effects have been observed (Zuccato et al., 2008; Binelli et al., 2012; Klosterhaus et al., 2013; Pereira et al., 2016). These substances must be considered of environmental concern since illicit drugs are a growing social and public health problem. The most recent World Drug Report estimates that the highest rates of cocaine use (including crack cocaine) occur in North, Central, and South America (UNODC, 2014). Illicit drug use has increased dramatically in the past decades in developing countries of South America (Johnson et al., 2013). The consumption and trafficking of cocaine in South America has become more prominent, particularly in Brazil, due to factors such as geographic location and the increasing populations clustered in urban centres.

New polices on ERA and environmental monitoring are necessary. According to Aymerich et al. (2016), for instance, the anti-inflammatory diclofenac, the synthetic hormone ethinylestradiol (EE2) and the antibiotics erythromycin, clarithromycin andazithromycin have recently been included in the ‘Watch List’ of priority substances under the European Water Framework Directive (Decision 2015/495/EU). In addition, in 2015 the US Environmental Protection Agency included pharmaceuticals such as erythromycin and EE2 in the Water Contaminant Candidate List 4, a list of contaminants that are currently not subject to any proposed or promulgated national primary drinking water regulation but are known or anticipated to exist in public water systems. Developing countries in Latin America should follow such examples to reduce the amount of PPCPs waste released into aquatic environments, since the populationsare growing, aging, and becoming more concentrated in metropolitan areas.

Public education must be enforced and should include both health professionals (prescriptions) and consumers (release) in order to reduce PPCPs in aquatic ecosystems.

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Chapter 34

RESTORATION AND CONSERVATION ACTIONS: CHILEAN STUDIES ON PHYTOREMEDIATION OF METAL-POLLUTED, ACIDIC SOILS

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ABSTRACT

The topic of restoration and conservation actions is very broad. In this chapter, we limit the discussion to phytoremediation of soils polluted by copper mining, focusing on two remediation options: phytostabilization and phytoextraction. We present Chilean studies for each of these two options of remediation of metal-polluted, acidic soils in a site located in proximity of a copper smelter. Specifically, the Puchuncaví Valley, located on the coast of Central Chile, received strong atmospheric depositions from the Ventanas smelter from 1964 to 1999. The soils of the valley are currently acidic, with scarce vegetation. They are severely eroded and contain high total concentration of metals, such as Cu, Zn, Pb, and Cd, and metalloids, such as arsenic. Currently, a large-scale remediation is needed to reverse the strong historic degradation of polluted soils around the Ventanas industrial complex. In this chapter, we demonstrate that phytostabilization is a technique that could be effective for large-scale remediation of the metal-polluted, acidic soils of the Puchuncaví Valley. On the other hand, we conclude that the phytoextraction technique is unfeasible because of the long time necessary for cleaning up soil metals with plants, besides other restrictions.

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Keywords: phytostabilization, phytoextraction, revegetation, earthworms, microorganisms

IN SITU METAL IMMOBILIZATION AND PHYTOSTABILIZATION

The *in situ* immobilization of metals in polluted topsoils has been suggested as a cost-effective method of soil remediation (Mench et al., 2000; US EPA, 2007). A plant-based approach for i*n situ* metal immobilization in polluted soils is phytostabilization. Phytostabilization has been defined as the use of metal-tolerant plants (metallophytes) that accumulate metals in underground tissues (excluder metallophytes). Excluder metallophytes contribute to *in situ* metal immobilization through root absorption and sorption (Berti and Cunningham, 1999; Bouwman and Vangronsveld, 2004) and the exudation of organic compounds that chelate metal ions in the rhizosphere (Meier et al., 2012). Metal accumulation in roots and immobilization in the rhizosphere stabilize polluted soils and reduce or eliminate the risk of movement of toxic elements from the contaminated soil to the environment (e.g., Macnair et al., 2000). Thus, metals are not removed from the soil but are reduced to less-soluble forms that are not available for biological actions, such as root uptake and phytotoxicity in less tolerant and/or ecologically or commercially relevant species (Vangronsveld and Cunningham, 1998; Macnair et al., 2000).

Phytostabilization can be used in combination with organic and/or inorganic soil amendments, being then named aided phytostabilization (Mench et al., 2010). It consists of reducing metal concentrations in the soil solution through the incorporation of organic and/or inorganic soil amendments into polluted topsoil. Sorption and/or precipitation reactions induced by soil amendments decrease the concentration of bioavailable metals in the soil. Soil amendments also improve other soil limiting conditions for plant establishment and growth derived from soil degradation, such as macronutrient deficiencies, microbial malfunctions, and physical limiting conditions (US EPA, 2007). Aided phytostabilization thus allows revegetation and ecosystem remediation on contaminated sites. Vegetation recovery reduces or even prevents the dispersal of soil contaminants via wind and water erosion and improves the esthetical value of formerly bare areas (Vangronsveld and Cunningham, 1998; Macnair et al., 2000).

ACTIVE VERSUS PASSIVE REVEGETATION OF METAL-POLLUTED SOILS

Two options have been used for restoring plant covers in degraded soils once plant-growth restrictions have been removed from the site (e.g., by the use of soil amendments). The first, active revegetation, employs seeding and/or outplanting of plant species (Arienzo et al., 2004; Clemente et al., 2005; Alvarenga et al., 2008). The second, passive revegetation, consists in the spontaneous development of plants from the natural soil seed bank, seeds dispersed by natural vectors, and/or vegetative reproduction of remaining vegetation (Álvarez et al., 2003; Madejón et al., 2006; Conesa et al., 2007). Both practices have been extensively studied and used in the field of ecological restoration (e.g., Van Andel and Aronson, 2012;

Zahawi et al., 2014) but not in the field of aided phytostabilization (e.g., Velayoudon et al., 2014), where plants have been actively introduced.

A field study performed by our work team (Córdova et al., 2011) compared aided versus passive revegetation in a metal-polluted, acidic site in the Puchuncaví Valley from the approach of aided phytostabilization. Our results showed that plant cover and aboveground biomass were the same under active and passive revegetation regimes, suggesting that plant cultivation was unnecessary. Likewise, under the assisted revegetation regime, both the aboveground biomass and plant cover of all spontaneous species were significantly higher than those of cultivated species (Table 1). We concluded that the remaining native soil seed bank of the study site may be sufficient to recover the plant cover and biomass production of original vegetation, after application of proper soil amendments. Stimulation of spontaneous recolonization from remaining propagules though proper soil management practices would reduce the costs that are inherent of cultivation practices.

Table 1. Plant cover and aboveground biomass of cultivated and the accompanying spontaneous species under aided phytostabilization, seven months after soil amendment application. Based on Córdova et al. (2011)

Plant species type	Plant cover (%)	Aboveground biomass (kg ha^{-1}, dry weight)
Cultivated	9 ± 8 a	246 ± 369 a
Accompanying spontaneous	55 ± 17 b	1387 ± 1083 b

Average data and standard deviation (n=6) are shown. Different letters in the same column indicate significant differences among the plant species type ($p<0.05$).

Table 2. Chemical characteristics of topsoil (0-5 cm) in experimental plots five months after amendment application. Average data and standard deviation are shown (n=3). Based on Ulriksen et al. (2012)

Characteristic	Treatment			
	Control	Lime	Compost	Lime+compost
pH	4.5 ± 0.1 a	8.1 ± 0.0 b	7.3 ± 0.1 b	8.0 ± 0.1 b
pCu^{2+}	4.4 ± 0.2 a	9.1 ± 0.7 b	9.4 ± 0.8 b	9.8 ± 0.7 b
Cu_{ex},mg kg^{-1}	33 ± 16 a	0.3 ± 0.2 b	1.4 ± 0.6 b	1.5 ± 0.5 b
OM, %	1.0 ± 0.3 a	0.8 ± 0.3 a	2.8 ± 1.0 b	3.0 ± 0.5 b
N, mg kg^{-1}	15 ± 1.0 a	13 ± 2.4 a	42 ± 12 b	42 ± 5.4 b
P, mg kg^{-1}	30 ± 3.6 a	29 ± 6.6 a	114 ± 33 b	117 ± 17 b
K, mg kg^{-1}	0.4 ± 0.0 a	0.3 ± 0.0 a	1.9 ± 0.2 b	1.6 ± 0.2 b
WHC, %	33 ± 0.6 a	33 ± 0.6 a	39 ± 2.4 b	39 ± 0.6 b

Different letters in the same row indicate significant differences ($p<0.05$). pCu^{2+}: $-\log[Cu^{2+}]$, where $[Cu^{2+}]$ is activity of Cu^{2+} ion in the soil solution; a higher value of pCu^{2+} implies lower activity of Cu^{2+} ion. Cu_{ex}: exchangeable copper determined in an extract of 0.1 M KNO_3 (at a 1:2.5 soil:liquid ratio). OM: organic matter. WHC: water holding capacity.

LIME AND COMPOST PROMOTE PLANT RECOLONIZATION OF METAL-POLLUTED, ACIDIC SOILS

In this chapter, we present field results on the use of lime and compost in promoting plant recolonization of metal-polluted, acidic soils in our study site at the Puchuncaví Valley (Córdova et al., 2011; Ulriksen et al., 2012). We found that the application of lime and/or compost decreased the Cu^{2+} ion activity in the soil solution and the exchangeable Cu in the soil, showing effective Cu immobilization in the topsoil (Table 2). Likewise, incorporation of compost into degraded soil significantly increased the organic matter content, concentration of available nutrients (NPK), and the water-holding capacity of the soil (Table 2).

Lime application had no effect on plant productivity in comparison to unamended control, while application of either compost or lime+compost increased plant cover and aboveground biomass (Table 3). The regression analyses revealed that plant productivity (in terms of either the aboveground biomass or plant cover) significantly and positively responded to the nutrient availability and water-holding capacity of the soil (Córdova et al., 2011; Ulriksen et al., 2012). Other soil chemical characteristics, including total concentrations of Cu, Zn, Pb, and As, organic matter content, pCu^{2+}, soluble and exchangeable Cu, and pH, did not significantly correlate ($p>0.05$) to plant response variables. These findings are in agreement with the study of Ginocchio (2000), who determined the soil characteristics that best explained the changes observed in the plant species richness and abundance in the Puchuncaví Valley. This author reported that the low nitrogen availability was the main soil stress factor limiting plant establishment and growth, whereas soil acidification and soil Cu enrichment were the second and third stress factors, respectively.

Although the Cu^{2+} activity and the exchangeable Cu were markedly lower in the amended soils than in the unamended control, the shoot Cu concentration was similar among most combinations of plant species and amendments (Table 4). Thus, the Cu accumulation in the aboveground tissues of the *Lolium*s pp. and *Eschscholzia californica*- dominant plant species in the study area - did not depend solely on Cu availability in the soil. These species - not responsive to the soil Cu concentrations, with constant Cu concentrations in the aboveground tissues - would be classified as excluders according to the classification of Baker (1981). However, to confirm these phenotypes in the present study, it would have been necessary to determine the Cu concentrations in the underground tissues.

In general, a concentration of 20 mg kg^{-1} is considered the threshold Cu concentration in plants, not considering highly sensitive and tolerant species (Kabata-Pendias and Pendias, 1992). However, the same authors reported that the majority of plant species can accumulate more than 20 mg kg^{-1} of Cu when growing in soils with elevated Cu concentrations. In accordance with this observation, shoot Cu concentrations of *Lolium* spp. were slightly higher and those of *E. californica* were considerably higher than the threshold value of 20 mg kg^{-1} (Table 4). Likewise, the shoot concentrations of Zn, Pb, and As were above the background values for the shoots of plants growing in unpolluted soils (Benton, 1983; Kabata-Pendias and Pendias, 1992; Adriano, 2001; Römheld, 2012; Alloway, 2013) (Table 4). Nevertheless, plants were able to complete their life cycles: they flowered and produced seeds even when growing on the unamended control soil. It may therefore be stated that the plant development in all of the treatments was independent of elevated soil metal concentrations.

Table 3. Plant cover and aboveground biomass of treatments at the end of the first plant growing season. Average data and standard deviation (n=3) are shown. Based on Ulriksen et al. (2012)

Treatment	Plant cover, %	Aboveground biomass, kg ha^{-1}
Control	36 ± 22 a	337 ± 247 a
Lime	56 ± 16 a	582 ± 28 a
Compost	95 ± 8.1 b	2516 ± 377 b
Lime + compost	99 ± 2.3 b	2630 ± 767 b

Different letters in the same column indicate significant differences between the treatments ($p<0.05$).

Table 4. Shoot concentration of Cu, Zn, Pb, and As in dominant plant species of the study area. Average data and standard deviation (n=3) are shown. There were no significant differences between treatments with regards to shoot metal concentration of the same species. Based on Ulriksen et al. (2012)

Species	Treatment	Total concentration, mg kg^{-1}			
		Cu	Zn	Pb	As
Lolium spp.	Control	28 ± 10	69 ± 17	7.6 ± 3.1	6.4 ± 1.0
	Lime	30 ± 2.1	65 ± 2.5	11 ± 0.7	7.6 ± 0.8
	Compost	29 ± 9.6	71 ± 21	10 ± 3.8	7.0 ± 0.6
	Lime+compost	27 ± 8.1	60 ± 2.8	9.2 ± 3.1	5.2 ± 0.5
Eschscholzia	Control	46 ± 9.5	57 ± 11	7.2 ± 3.7	5.0 ± 2.1
californica	Lime	73 ± 36	64 ± 6.2	11 ± 5.7	4.5 ± 0.9
	Compost	70 ± 11	70 ± 14	12 ± 3.3	4.8 ± 1.7
	Lime+compost	71 ± 22	41 ± 9.0	8.8 ± 1.9	5.3 ± 0.1

EARTHWORMS AS BIOINDICATORS FOR ASSESSMENT OF THE EFFECTIVENESS OF *IN SITU* METAL IMMOBILIZATION

The effectiveness of *in situ* soil immobilization treatments can be assessed by toxicity tests/assays with organisms. In one of our field studies (Neaman et al., 2012), we used earthworms as bioindicators for assessing the effectiveness of lime and compost treatments for *in situ* immobilization of trace elements in polluted soils of the Puchuncaví Valley. Lime application had no effect on earthworm reproduction in comparison to the unamended control, whereas application of compost increased cocoon and juvenile production (Table 5).

Table 5. Results of chronic toxicity tests with earthworms for different treatments. Number of cocoons and number of juveniles were used as endpoints. Based on Neaman et al. (2012)

Treatment	Cocoons	Juveniles
Control	3.1 ± 3.6 a	5.0 ± 5.3 a
Lime	3.0 ± 2.8 a	5.1 ± 3.8 a
Compost	11.2 ± 6.7 b	16.7 ± 12.8 b
Lime+compost	4.8 ± 4.5 a	15.3 ± 10.7 b

Average data and standard deviation are shown (n=12). Different letters in the same column indicate significant differences among the treatments ($p<0.05$).

Table 6. Richness*, measured as number of ribotypes, of bacterial community in experimental soils based on T-RFLP and DGGE analysis. Based on Rojas (2013)

Treatments	T-RFLP (*MspI*)	T-RFLP (*HhaI*)	DGGE
Control	72 ± 11 a	89 ± 11 a	22 ± 0.5 a
Lime	115 ± 8.1 b	138 ± 6.3 b	24 ± 1.1 ab
Compost	146 ± 4.0 c	182 ± 4.6 c	29 ± 1.2 c
Lime+compost	162 ± 3.3 d	195 ± 2.3 d	25 ± 0.6 b

T-RFLP (*MspI*): Terminal restriction fragment length polymorphism; enzyme used *MspI*.

T-RFLP (*HhaI*): Terminal restriction fragment length polymorphism; enzyme used *HhaI*.

DGGE: Denaturing gradient gel electrophoresis.

* Richness is equal to the number of T-RFLP peaks or DGGE bands.

Values given for T-RFLP are the means of five replicates ± standard deviation and those for DGGE are the means of three replicates ± standard deviation. Values of the same letter in a column are not significantly different at $p<0.05$.

Spatial variability of soil properties exists within treatments in experimental field plots. This allowed the identification of which soil properties were actually having an impact on earthworm reproduction. For both cocoon and juvenile production, soil organic matter was a positive factor, i.e., more soil organic matter increased cocoon or juvenile production. The toxicity (negative) factor was total soil As. However, total Cu and total As were well correlated ($R^2=0.80$, $p<0.001$), hence some trends could have been masked. Thus, compost treatment was effective in improving the quality of soils of the Puchuncaví Valley, increasing earthworm reproduction.

MICROORGANISMS AS BIOINDICATORS FOR ASSESSMENT OF THE EFFECTIVENESS OF *IN SITU* METAL IMMOBILIZATION

Soil microbial properties are being increasingly used as indicators of soil quality since they provide a direct measure of soil functioning (Garbisu et al., 2011). Furthermore, there is strong evidence that soil microbes are more sensitive to metals than animals or plants (Sauvé et al., 1998; Giller et al., 1999). This is another reason to use soil microbial properties as indicators of soil quality.

In one of our field studies (Rojas, 2013), we assessed the effectiveness of the above-mentioned lime and compost treatments by using bacterial community richness and diversity as bioindicators of soil quality. Results indicated that the soil bacterial community responded to lime and compost treatments, changing their structure and increasing their richness and diversity (Tables 6 and 7), and thus improving soil quality. It has been reported that differences in diversity and composition of soil bacterial communities could largely be explained by a single variable, soil pH, with a higher bacterial diversity in neutral soils compared to acidic soils (Fierer and Jackson, 2006). Indeed, amendments used in our study increased soil pH, and this variable was significantly correlated to the Shannon diversity index, calculated using T-RFLP (*Msp*I) profile (Table 8). Likewise, the Shannon diversity index was significantly correlated to Cu bioavailability indices (exchangeable Cu and pCu^{2+}).

On the other hand, bacterial community richness was a less sensitive bioindicator, being only marginally correlated ($p<0.1$) to activity of the Cu^{2+} ion in the soil solution (Table 8).

Table 7. Shannon indices of diversity (H')* of bacterial community in experimental soils based on T-RFLP and DGGE analysis. Based on Rojas (2013)

Treatments	T-RFLP (*MspI*)	T-RFLP (*HhaI*)	DGGE
Control	3.4 ± 0.03 b	3.4 ± 0.04 c	3.0 ± 0.03 c
Lime	3.6 ± 0.10 a	3.8 ± 0.03 b	3.2 ± 0.04 b
Compost	3.6 ± 0.30 a	3.7 ± 0.09 a	3.5 ± 0.09 a
Lime+compost	3.6 ± 0.06 a	3.6 ± 0.10 a	3.6 ± 0.09 a

* Shannon index (H') was calculated as follows: $H' = -\Sigma p_i \ln(p_i) = -\Sigma(n_i/N)\ln(n_i/N)$, where pi is the relative abundance of a given T-RFLP peak, n_i is the intensity of band i as judged by its peak height and N is the sum of all peak heights in a given DGGE profile.

Values given for T-RFLP are the means of five replicates ± standard deviation and those for DGGE are the means of three replicates ± standard deviation. Values of the same letter in a column are not significantly different at $p<0.05$.

Table 8. Pearson correlation coefficients between richness/diversity of bacterial community and soil properties of experimental soils

Variable	Richness (number of ribotypes)			Shannon indices of diversity		
	T-RFLP (*Msp*I)	T-RFLP (*Hha*I)	DGGE	T-RFLP (*Msp*I)	T-RFLP (*Hha*I)	DGGE
pH	ns	ns	ns	0.978*	ns	ns
OM, %	ns	ns	ns	ns	ns	0.928**
pCu^{2+}	0.917**	0.908**	ns	0.994*	ns	ns
Cu_{ex},mg kg^{-1}	ns	ns	ns	-0.999*	ns	ns

*=$p<0.05$; **=$p<0.1$; ns=not significant ($p>0.1$); OM=organic matter. pCu^{2+}: $-\log[Cu^{2+}]$, where $[Cu^{2+}]$ is activity of Cu^{2+} ion in the soil solution; a higher value of pCu^{2+} implies lower activity of Cu^{2+} ion. Cu_{ex}=exchangeable Cu.

PHYTOEXTRACTION OF SOIL COPPER BY *OENOTHERA PICENSIS*

Phytoextraction is a soil remediation technique that uses plants, preferably native species, for the extraction of metals from polluted soils. This technique requires plants that are tolerant to relatively high concentrations of metals, and also capable of absorbing and accumulating large quantities of these elements within their aerial biomass. Ideally, such species are hyperaccumulators, capable of accumulating over 1000 mg kg^{-1} of Cu, Pb, As and Zn (Baker and Brooks, 1989).

To identify hyperaccumulator plants representative of the Chilean conditions, González et al. (2008) carried out a survey of plant diversity in the area affected by the emissions of the Ventanas smelter (92-872 mg kg^{-1} of total soil Cu) and in the area near a smelter slug pile (533-2790 mg kg^{-1} of total soil Cu). Copper concentrations in shoots of identified plants were determined. The species with the highest accumulation of copper (614 mg kg^{-1}) was

Oenothera picensis (Fragrant Evening Primrose). However, no hyperaccumulator species were found, i.e., species showing more than 1000 mg Cu kg^{-1} dry tissues. However, the population of *O. picensis* was considered as a good candidate for phytoremediation initiatives in Chile, because it is an easy to propagate perennial herb with high biomass production.

As we mentioned above, the success of the phytoextraction process is based on the capacity of the selected plant species to extract the metal of interest from the soil and translocate it to its aerial biomass (Chen et al., 2003). Metal bioavailability in a given soil (the fraction which is available to be absorbed by an organism) (National Research Council, 2003) is also an important factor, as it often happens that high total concentrations of such metals are found in a soil, and yet, the bioavailable fraction is low. Under such conditions, plants do not absorb much metal, even when they have the capacity for increased accumulation (Ernst, 1996). In this situation, chelates can be used to increase metal bioavailability in soil. In a laboratory study, González et al. (2011) evaluated the effects of the biodegradable chelate MGDA (methylglycinediacetic *acid*) on Cu extraction by *O. picensis* and on leaching of Cu through the soil profile. They used an *ex situ* experiment with soil columns of varying heights. They found that Cu extraction by *O. picensis* was increased 14 times by the addition of 6 to 10 mmol $plant^{-1}$ of MGDA. Although the application of MGDA did increase the leaching of Cu, this leaching was drastically diminished with depth, making it unlikely to contaminate groundwater. Thus, application of MGDA may be an effective and environmentally safe way to improve Cu extraction by *O. picensis* in these soils.

In another study, González et al. (2014) reported the effect of MGDA on Cu phytoextraction by *O. picensis* under field conditions. In the chelate treatment, Cu phytoextraction was 2.6 ± 2.1 mg $plant^{-1}$, which is equivalent to 212 ± 171 g ha^{-1} $year^{-1}$ at a planting rate of 8 plants m^{-2}. The Cu phytoextraction by *O. picensis* was greater than that by sunflower (59 g ha^{-1}) (Kolbas et al., 2011), but considerably smaller than that by *Elsholtzia splendens* (550-720 g ha^{-1}) (Jiang et al., 2004). The Cu phytoextraction by *O. picensis*is expected to be improved by increasing planting density.

The time necessary for phytoextraction of metals from the soil is the drawback of the phytoextraction technique. This time can be estimated by a simple calculation. Assuming soil bulk density of 1200 kg m^{-3}, total Cu concentration in the soil of 1000 mg kg^{-1}, and metal accumulation depth of 5 cm (Ulriksen et al., 2012), there is 600,000 g of total Cu in one hectare of soil. Assuming hypothetical high Cu extraction rate of 1000 g ha^{-1} $year^{-1}$, 300 years will be required to reduce total Cu concentration in the soil by half. Although the application of biodegradable chelates reduces the time necessary for phytoextraction of metal from the soil, the required time is still impractically long, making the phytoextraction technique unfeasible due to lack of Cu hyperaccumulator species in Chile and worldwide. On the other hand, as demonstrated above, phytostabilization is a technique that could be effective for large-scale remediation of the metal-polluted, acidic soils of the Puchuncaví Valley.

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