GIULIANA SERAPHIM DE ARAUJO

Geochemical analyzes and ecotoxicological responses to assess the environmental quality in an estuarine protected area

Dissertation submitted to the Oceanographic Institute of São Paulo University, as part of the requirements to obtain the title of Master in Science, Oceanography Program, Biological Oceanography area.

Supervisor: Profa. Dra. Áurea Maria Ciotti

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Judged on: ___ / ___ / ____ by

Dra. Aurea Maria Ciotti	Concept
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Summary

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I dedicate this masters dissertation to nature,
which we try so hard to protect,
without being fully understood.

“O saber a gente aprende com os mestres e os livros,

a sabedoria se aprende é com a vida e com os humildes”

Cora Coralina

Acknowledgment

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Abstract

This study aimed to analyze the sediment quality of the Cananéia-Iguape-Peruíbe estuarine complex (CIP) by using biomarkers, geochemical and ecotoxicological analyses, in order to determine if metals from sediments were bioavailable to benthic organisms to the point of causing negative effects. Stations PT and PM presented the worst conditions, with chronic (PT and PM) and acute toxicity (PT), metal contamination and biomarkers induction. Worst condition tend to be associated to fines. Elements Pb, V, Co, Ni and Zn exceeded Sediment Quality Guidelines (SQVs) limits for stations PT and PM, Pb exceeding also TEL values. Biomarkers induction and tissue damage appears to be worse during winter. The different lines of evidence showed that the metal contamination from mining activities on upper Ribeira de Iguape River alters the estuary. Toxicities were attributed mainly to metals suggesting that the anthropic factors are capable of producing ecological risks. Additionally, such as summer toxicity are present in the northern estuarine portion and during winter, this same toxicity appears to be in the southern portion, this study evidenced that depositional areas appears to move along the estuary, which in its turn depends on the freshwater inputs associate to seasonality.

Key words: *Callinectes danae*, metals, biomarkers, sediments, toxicity.

Resumo

Este estudo teve como objetivo analisar a qualidade dos sedimentos do complex estuarino de Cananéia-Iguape-Peruíbe (CIP), utilizando Biomarcadores, análises químicas e ecotoxicológicas, a fim de determinar se os metais nos sedimentos estariam biodisponíveis para os organismos bentônicos. As estações PT e PM apresentaram as piores condições, com toxicidade crônica (PT e PM) e aguda (PT), concentração de metais no sedimento e maior indução dos biomarcadores. As piores condições tendem a estar associadas aos sedimentos finos. Os elementos Pb, V, Co, Ni e ZN ultrapassaram os limites de "*Sediment Quality Guidelines*" (SQVs) nas estações PT e PM, Pb ultrapassou também os limites de TEL. A indução dos biomarcadores analisados e danos nos tecidos apresentaram maiores valores durante o inverno. As diferentes ferramentas utilizadas mostram que a contaminação por metais, advindas das atividades de mineração do Alto do Rio Ribeira de Iguape estão influenciando o estuário. A toxicidade encontrada foi atribuída principalmente aos metais, sugerindo que os fatores antrópicos produzem riscos ecológicos. Além disso, como a toxicidade durante o verão se apresenta mais na parte superior do estuário e no inverno essa mesma toxicidade se apresenta nas estações mais ao sul, esse estudo evidenciou que as áreas de deposição parecem mover-se ao longo do estuário, o que depende da quantidade da entrada de água doce no sistema, que se apresenta associada a sazonalidade.

Palavras-chave: *Callinectes danae,* Metais, Biomarcadores, Sedimento, Toxicidade.

Chapter 1 – Introduction

A Protected Area (PA) is considered as an area of land and/or sea especially dedicated to the protection of biological diversity, and of natural cultural resources, and managed through legal or other effective means (IUCN, 1994). IUCN also states that marine and estuarine protected areas (MPAs and EPAs, respectively) have been established with varied objectives, which include protecting fish stocks, vulnerable habitats, endangered species, and breeding areas, and reducing the anthropic impacts. The use of marine protected areas has its popularity increased as they complement and strengthen traditional fisheries management (Agardy, 2000).

In Brazil, PAs are regulated by the National System of Protected Areas (known as SNUC), that was created by the federal act 9.985/00 (BRASIL, 2000). According to the SNUC, the Brazilian PAs are divided in two main groups: the Strict Protection group, which include those PAs with the main purpose of preserving the nature and prohibiting the direct use of natural resources; and the Sustainable Use group, which joins the PAs with purposes of conserving the nature in harmony with the sustainable use of their natural resources.

The coastal zone of São Paulo State presents an extensive network of PAs, which forms a large ecological corridor, encompassing terrestrial, marine and estuarine areas. The Cananéia-Iguape-Peruíbe Protected Area (CIP-PA) is inserted in this system; this PA was established by the Federal decrees 90.347/84, and 91.892/85 (BRAZIL, 1984; 1985). The CIP-PA is managed by the federal agency *Instituto Chico Mendes de Biodiversidade* (known as ICMBio). The PA falls into the category of Environmental Protected Area (EPA), which is a sustainable-use PA dedicated to the protection of natural and cultural heritage together with the improvement of quality of life to the resident population.

The CIP-PA is located in the area known as Estuarine Complex of Iguape and Cananéia, which is, in its turn, placed on the south coast of São Paulo state (southeastern Brazil). This region is formed mainly by barrier islands and estuarine channels, which form a complex net of water bodies, which banks are occupied by well-developed mangrove forests (Schaeffer-Novelli *et al.* 1990). The water circulation in its estuarine portion is predominantly generated by semidiurnal tides (Miyao & Harari, 1989), but it is also influenced by the contribution of a wide net of rivers, especially the Ribeira de Iguape River (RIR), and, in a smaller scale by the wind (Miranda *et al.* 1995). The RIR is the main contributor of both nutrients and contaminants to the CIP estuarine complex, especially metals (Mahiques *et al.* 2009), due to the mines located in upper RIR.

This region has experienced some significant changes over the past 150 years, receiving part of the contribution of the RIR, especially after the construction of “Valo Grande” channel, which connects the river to the inner portion of the estuary (Mahiques *et al.* 2009). This channel caused approximately 60% of RIR water flux to flow toward the estuary, modifying the estuarine freshwater-saltwater balance and discharging increased inputs of large amounts of suspended solids in the estuary.

However, residues from old mines are deposited on the upper portion of the RIR (Guimarães and Sígolo 2008; Kummer *et al.* 2011), causing unknown amounts of metals to be continuously introduced into the river. The mining activities along the RIR basin were intensified in 1943, when the company Plumbum S.A. installed a metallurgy plant for processing and producing Pb in the region (near the “*Panelas*” mine), coming into operation in 1945. The extraction and beneficiation of ores in the upper RIR were often rudimentary, with no control over the environmental impacts resulting from such processes.

Since the beginning of mining activities in the region, the tailing and metallurgical slags of blast furnace, rich in As, Ba, Cd, Pb, Cu, Cr and Zn, were directly dumped into the Ribeira de Iguape River (Guimarães & Sigolo 2008). Cassiano (2001) estimated that about 5.5 tons/month of residues were discharged into the river along the mining period. This situation persisted until early 1990’s decade, and afterwards, the material started to be disposed directly on the ground, on the river banks, exposed to the weathering (Guimarães & Sigolo 2008). In the early 2000’s, about 89,000 m^3 of metal rich residues were kept deposited on the ground, close to the river (Franchi 2004); in recent visits to such sites we could confirm that the residues are still deposited on the river banks at the current days.

Metal influences in water bodies can occur naturally by geochemical processes and weathering; or by anthropogenic activities (Melo *et al.*, 2012). Mining is a known source of metals to aquatic systems (Yabe & Oliveira, 1998; Rietzler *et al.*, 2001; Bosso *et al.*, 2008). The release of metals into the environment may occur through leaching of stored tailings, erosion and dam disruption. Once in water bodies, metals may be transported, causing contamination in areas distant from the respective pollution sources (Paolielo & Chasin, 2001).

In the aquatic environment, most anthropogenic chemicals and waste materials frequently tend to remain for a limited time in the water column, precipitating into the bottom (Seriani *et al.*, 2006) and accumulating in sediments (Ingersoll *et al.*, 1997). Therefore, sediments may become orders of magnitude more contaminated than the adjacent water column (Ingersoll, 1995), and thus sediments may be considered as a major repository for many of the more persistent chemicals that are introduced into the aquatic environments. However, metals can be released back to the water column and organisms due to biological, physical and chemical processes (Hortelani *et al.*, 2008; Santana *et al.*, 2007; Burgess & Scott, 1992).

Metal accumulation in sediments depends on the distribution of sources and on the geochemical performance of geochemical carriers that bind to metals by adsorption processes (Perin *et al.*, 1997). Baptista Neto *et al.* (2006) observed that fines (<0.63mm) and organic matter rich sediments form an important geochemical substrate to heavy metals released in ecosystems, regulating the amount of bioavailable metals in the sediment and, therefore, in the metal exchange between the water the biota (Perin *et al.*, 1997).

Previous studies found metal contamination in the RIR (Guimarães & Sígolo, 2008a; Morais *et al.,* 2013; Abessa *et al.*, 2014). Higher values of Cu, Cr and Zn were found in sediments from Iguape, near the river mouth, evidencing that the transport occurs associated to the suspended particles (mostly fines), which tends to deposit in areas of lower energy, favoring the deposition of heavy metals around the mouth of Valo Grande, near the city of Iguape (Guimarães & Sígolo, 2008a). Mahiques *et al.* (2009) reported that Pb concentrations in sediments from the CIP-PA (close to Iguape) were comparable to those found in sediments from Santos estuary, one of the most degraded and polluted estuarine areas of Brazil.

Pb concentration displays concerning values for some sectors of the estuary as well as the occurrence of levels above the reference levels in bivalves (Guimarães & Sígolo, 2008b). Moraes *et al.* (2004) found high concentrations of heavy metals in RIR sediments, including those from the CIP-PA, and suggested that the Pb concentrations found in the Ribeira de Iguape sediments were influenced by mining activities. Sediments from CIP-PA exhibited apparently increasing toxicity and metal contamination, probably as a result of different sources, and this contamination could be capable of producing risks to the aquatic biota (Cruz *et al.*, 2014; Mahiques *et al.*, 2013).

The capacity of estuaries to act as a 'sink' for pollutants and, in particular heavy metals, is related to many factors (Kehrig *et al.*, 2003). This is due to the relatively limited circulation present in these water bodies, reducing their capacity to dilute and disperse contaminants that end up accumulating mainly in the sediments, even more because CIP-PA is considered a partially mixed and weakly stratified estuarine system (Miranda *et al.*, 1995).

The CIP-PA overlaps with the 3 municipalities that comprise the Southern coast of São Paulo State: Cananéia, Iguape and Ilha Comprida. These cities have deficiencies in their structures of basic sanitation (Nascimento *et al.*, 2009), part due to incomplete coverage of the system, and part due to the failure of the drainage system, which causes the discharge of wastes in rivers, groundwater or directly into the sea. According to Morais & Abessa (2014), the basic sanitation structure of this area is insufficient and the enforcements taken are not consistent with the local demands. Thus, the economic gains have not been effectively translated into improvements in quality of life and environment. However, according to the State Environmental Agency (CETESB, 2010), there was an improvement in the bathing conditions of the south coast of the state of

São Paulo in the last ten years. The water quality of this region is considered good, mainly due to the small resident population and the incipient tourism developed in these municipalities; industrial activity is not relevant to the region (CETESB, 2001).

However, some recent reports have evidenced that the CIP-PA is getting impacted by metals and that further studies must be conducted (Cruz, 2014; Mahiques *et al.*, 2009), especially because it is a preserved area. Sediment contamination constitutes a threat to aquatic organisms and since contaminants may be accumulated by the biota and transferred through the trophic chain, ecological risks for both ecosystems and public health may occur.

Sediment quality has been used as an important indicator of the health of aquatic ecosystems (Power and Chapman 1995). In this sense, chemical and ecotoxicological approaches have been widely used aiming to evaluate the environmental contamination and its biological effects (Adams *et al.* 1992, Araújo *et al.* 2013). They provide information on the environmental quality considering the effect of the discharged contaminants and help to define critical and/or priority areas for protection or recovery (Abessa *et al.* 2008).

The present study has the purpose of evaluating the contamination of the CIP-PA by metals, as well as detecting possible effects to the biota, relying on the fundamentals of ecotoxicology, which appeared by the end of the 1980s in Brazil, as a response to marine pollution. Ecotoxicology aims to evaluate the effects that biologically active substances (contaminants and complex effluents) may produce to the ecosystems. Marine ecotoxicology can then be understood as the science that studies the biological effects caused by the release of anthropic pollutants in the ocean and seas, using tools that enable such investigations (Samollof & Wells, 1984). Ecotoxicology integrates data information and environmental assessment, combining ecology and toxicology (Chapman, 2002), involving knowledge of areas such as chemistry, physiology, biochemistry, geology, statistics, and others.

Aquatic biota may be exposed to contaminants either by food pathway (ingestion of sediment, organic matter, living organisms), dermal contact or respiratory surface. The benthic invertebrates feed on particles containing organic matter, which may contain high levels of contaminants levels associated to them. Accordingly, benthic and epibenthic organisms are potentially more susceptible to contamination, representing good models to assess the biological effects and exposure of environmental contamination (Araújo *et al.*, 2008).

Toxicity tests constitute a classical approach and have been used to assess the potential effects of sediment-associated contaminants (Abessa *et al.*, 2008). They can be employed with a range of organisms, in order to evaluate responses at long and short-terms, and their results generally present good correspondence to the field. Embryo-larval development tests have been widely used to characterize a variety of toxicants, including sediment elutriates, solid phase and interstitial water (Bryn *et al.*, 1998). In general, such experiments have been accepted internationally as appropriate for evaluating toxicity (U.S. EPA, 1995; Environment Canada, 1997; CETESB, 1999; ABNT, 2006). Benthic copepods are broadly applied in chronic and acute toxicity evaluation of environmental samples and specific contaminants (Sousa *et al.*, 2012). These organisms are very useful because of their high reproductive potential (high fecundity) and fast life cycle (Souza-Santos *et al.*, 2006). Amphipods constitute a group of benthic organisms that has also been used to accomplish ecotoxicological studies (Abessa *et al.*, 1998; Fenili, 2012; Melo & Nipper, 2007; Araujo *et al.*, 2013), especially when sediments are considered.

Another frequent approach that has been used in environmental studies is the assessment of bioaccumulation of chemicals in the soft tissues of potentially exposed organisms. Biomonitors are species that accumulate xenobiotic substances in their tissues and therefore can be used to monitor the bioavailability of these substances in a particular environment (Wagner & Boman, 2004). Some elements, such as trace metals, are generally more concentrated in marine organisms than in the surrounding water. Marine organisms are obligate accumulators of metabolically essential metals like Cu, Zn and Fe but may also accumulate non-essential metals such as Cd and Pb, possibly via transport pathways common to essential metals (White & Rainbow, 1987).

Ecological studies, bioaccumulation and toxicity tests have been employed in order to detect and/or assess the effects of the introduction of xenobiotics into the marine environment. However, these methods are limited to detecting the effects once they are expressed at higher levels of biological organization (such as population and/or community); therefore they are often less sensitive than other methods that can provide *early warning* about the effects occurring at biochemical, cellular and physiological levels. Biomarkers often provide suitable information on the toxic and metabolic effects of contaminants (Pereira *et al.*, 2011), at lower levels of biological organization. According to Walker *et al.*, (1996), biomarkers are biochemical, physiological or histological indicators of exposure or effect of xenobiotic chemicals in the environment at organism or "sub-organism" level. Biomarkers may provide information on the responses from molecular/biochemical levels, which can potentially be used as an early sign of toxic effects, based on the fact that the starting point of all xenobiotics damages involves disruption of biochemical and molecular processes in the cells, resulting subsequent effects at higher organizational levels. (Cheung *et al.*, 1998), such as illness, death or population changes (Bresler *et al.*, 1999; Fishelson *et al.*, 1999).

Thus, biomarkers have been indicated for environmental assessment programs because (among other reasons) they are highly sensitivity and evidence short-term responses (Pereira, 2003). Biomarkers can be classified as being of exposure or effect (Monserrat *et al.*, 2003), which means, respectively, that they are specific (organ or toxicant) or nonspecific (antioxidant enzymes). The most used biomarkers include the activity of antioxidant enzymes, which play a crucial role in maintaining cellular homeostasis, and their use has been proposed as indicators of oxidative stress in a wide variety of marine organisms (Winston, 1991).

Biological models frequently used in biomarkers' studies include bivalves (Domingos, 2006; Franco, *et al.*, 2006; Almeida, *et al.* 2007; Souza, 2010; Zanette *et al.*, 2006; Pereira, 2003), fish (Domingos, 2006; Rossi, 2008; Travasso, 2011; Brito, 2010; Lins *et al.*, 2010), crustaceans (Moser, 2011; Togni, 2007; Brown *et al.*, 2004; Lavradas *et al.*, 2014; Bordon, 2013), among others. The potential of marine organisms to accumulate contaminants (especially invertebrates) justify their use as biomonitors for estuaries and coastal environments (Phillips, 1980; Bryan *et al.* 1980).

Some crustaceans have been shown to be affected by environmental contaminants (Fingerman *et al.*, 1998), constituting excellent organisms to accomplish biomarkers analyzes (Engel & Brouwer, 1987). Beyond being ecologically important, some of them are also important as a fishery product (Bordon *et al.*, 2012a). Other species such as shorebirds, seabirds, and fish also feed of this crabs, evidencing that carbs play an important role in the transfer of pollutants to other trophic levels (Lavradas *et al.*, 2014).

Considering the presented context, this study aimed to evaluate if the CIP-PA is contaminated by metals at levels that are capable to negatively affect the biota. To accomplish that, this study was divided in more two chapters. The following chapter (Chapter 2) attempted to evaluate the sediment quality of CIP-PA trough geochemical analyses and ecotoxicological tests (chronic toxicity to *Lytechinus variegatus* and *Nitocra* sp. and acute toxicity to *Tiburonella viscana*). In the Chapter 3, we identified the adverse biological effects (at sub-organismal level) related to the environmental contamination, through the use of biomarkers (Glutathione S-transferase (GST), glutathione peroxidase (GPx), acetylcholinesterase (AChE), Metallothioneins (Met), DNA damage and Lipid peroxidation (LPO)) and the condition factor, using the blue crab *Callinectes danae*.

Chapter 2 – Chemical and Ecotoxicological approach

Metal contamination in sediments from a Protected Area assessed by chemical and ecotoxicological approaches.

1. <u>Introduction</u>

Marine protected areas have been established with different objectives, including protecting fish stocks and reducing anthropic impacts (IUCN, 1994). On the coastal zone of São Paulo there is a mosaic of protected areas (PAs), in which the Cananéia-Iguape-Peruíbe Protected Area (CIP-PA) is inserted. The CIP-PA was established by the Federal decrees 90.347/84, and 91.892/85 (BRAZIL, 1984; 1985) and is located in the area known as Estuarine Complex of Iguape and Cananéia.

Previous studies showed that metals are the major contaminants for this estuarine complex (Mahiques *et al.*, 2013; Guimarães & Sígolo, 2008a/2008b), as a consequence of the disposal of mining residues, at the upper areas from the Ribeira de Iguape River (RIR) basin. The mining activities started in 1943 (Moraes, 1997), with several mines of Pb operating along the RIR basin, producing residues that were discharged directly into the river, producing contamination of sediments from downstream RIR. After the mining activities have ceased, the residues from the former mines were still deposited along the river banks (Guimarães and Sígolo 2008; Kummer *et al.* 2011), representing sources of metals to the river. The RIR mouth is located in the North part of the CIP-PA, consequently the estuarine area has been influenced by contamination from mining (Amorim *et al.*, 2008). The influence was intensified after the construction of the “Valo Grande” channel, which caused approximately 60% of RIR water flux to flow toward the estuary (Mahiques *et al.*, 2013), and making this river to became the major contributor of nutrients and contaminants to the CIP estuarine complex (Barcellos *et al.*, 2005; Mahiques *et al.* 2009).

The sediments from CIP-PA present metal concentrations (especially Pb) that are comparable to those observed in polluted industrial areas, such as the Santos Estuarine System (Mahiques *et al.*, 2009). The presence of uncontrolled contamination sources of metals in the upper RIR (mines, ore and slags dumpsites and metallurgy plants) and the evidences of environmental contamination (Abessa *et al.*, 2014; Barcellos *et al.*, 2005; Guimarães & Sígolo, 2008a/2008b; Mahiques *et al.*, 2009; Cruz *et al.*, 2014) suggest that metals are impacting CIP-PA and producing risks to the biota.

As sediments accumulate contaminants along time, the investigation of their quality constitutes a reliable strategy for evaluating environmental quality and risks due to contamination. Chemical analyses are useful to identify and quantify the contaminants, whereas ecotoxicological approaches may evaluate potential effects on aquatic biota (Costa *et al.*, 2008).

Aquatic pollution is one of the reasons that PAs have been frequently ineffective in accomplish its goals (Gubbay, 2005; Kelleher *et al.* 1995; Jameson *et al.* 2002). However, few studies have been focused on pollution as an important factor threatening biodiversity within protected areas. Considering that the CIP-PA may be under influence of contamination by metals from external sources, and that the contamination may threat this PA, this study aims to evaluate the sediment quality in CIP-PA using geochemical and ecotoxicological approaches, in order to detect if metals are causing risks to biota and consequently to evaluate if this PA is effectively protected from external impacts.

2. <u>Materials and methods</u>

2.1 <u>Study area</u>

The CIP-PA comprises an area of 565,200ha and is located on the southern coast of the São Paulo State (Brazil), between the latitudes 23º45’-25º15’S, and longitudes 46º45’-49º30’W (Figure 1). The region comprises the lower course of Ribeira de Iguape River and the islands of Cananéia, Cardoso, Comprida and Iguape. The area is limited by the Juréia ridge at North and by the state of Paraná down south (SMA, 1997). Anthropic occupation is more intense in the northern portion of the system, especially near the urban centers of Iguape and Ilha Comprida cities and in the lower valley of RIR (Barcellos *et al.*, 2005).

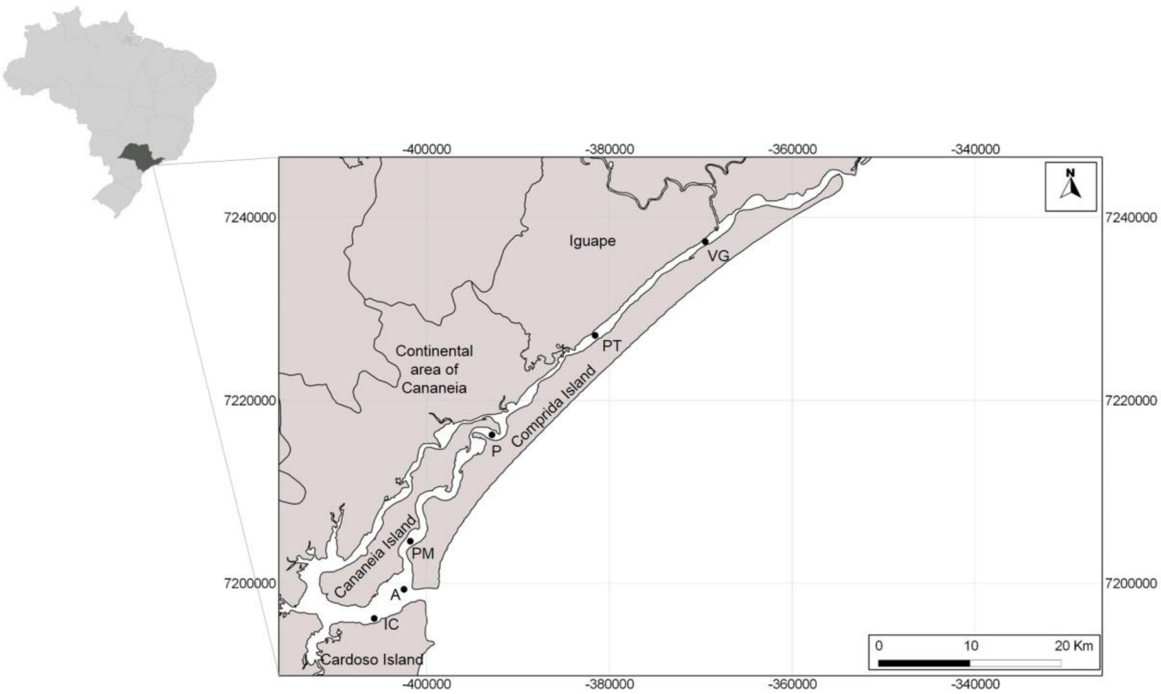

Figure 1: Map showing the sediment collection stations around the study area, within CIP-PA, Brazil.

The Iguape-Cananéia estuarine system is the major estuarine complex of São Paulo coast, which in addition to RIR forms the largest coastal plain of the State. As previously mentioned, currently this system receives a considerable part of the RIR drainage; after the construction of the Valo Grande channel (mid-nineteenth century), at least 60% of the RIR flow was diverted to Mar Pequeno, altering the hydrological regime in the estuarine system (Mahiques *et al.*, 2013). Then, the RIR became the main contributor of sediment and nutrients to this system, especially in its central and northern portion (Freitas *et al.*, 2006). Additionally, rainstorms have a major role in removing surface soils and contaminants along the RIR basin (Costa *et al.*, 2009). The regional climate is subtropical humid. According to Silva (1984), rainy season extends from December to April and the dry season from May to November; March being the rainiest month.

2.2 Sediment sampling

The sediment sampling was conducted along 6 stations distributed along the estuary, from the vicinities of the Valo Grande channel (where the influence of contaminant sources is expected to be more intense) to the Cardoso Island (control area), as shown in the Figure 1 and the Table 1. The sampling surveys were conducted in September 2013 (wintertime). Sediment samples were collected with a 0.036 m² stainless steel Van Veen grab sampler, conditioned on plastic vessels and placed on ice until the arrival at the laboratory, then, aliquots for toxicity tests were separated and kept refrigerated at 4ºC, while those for geochemistry were stored frozen at -20ºC. To better understand local seasonality, results from this investigation were compared with results obtained by Cruz (2014) to the previous summer period (March 2013).

Table 1: Geographic coordinates of the sampling stations for sediments within the CIP-PA.

Station	Latitude	Longitude
VG	24°44.509'S	47°35.984'W
PT	24°49.294'S	47°42.186'W
P	24°54.124'S	47°48.547'W
PM	25°00.167'S	47°54.104'W
A	25°02.689'S	47°54.880'W
IC	25°04.281'S	47°55.839'W

2.3 Sediment properties

Sediment grain size distribution was analyzed based on the protocol proposed by Mudroch and MacKnight (1994). About 30g of previously dried sediment were wet sieved through a 0.063µm mesh to separate fine particles (mud and silt). The material retained on the sieve was then dried and weighed. Initial and final weight differences demonstrated the mud fraction. Subsequently, the sandy material retained on the 0.063µm mesh was sieved into different meshes (ϕ scale) in order to separate different classes of sands, and the results were further classified based on the Wentworth scale. The calcium carbonate ($CaCO_3$) contents in each sample were measured using the method described by Hirota & Szyper (1975), which consists of separating fractions of 5g of each sediment sample and then adding 10ml of hydrochloric acid (5N HCl) for 24 hours to eliminate calcium carbonates. Next, samples were washed with distilled water and dried at 60°C. The difference between initial and final weights showed the amount of $CaCO_3$ in the samples. Organic matter (OM) contents in the sediment samples were estimated using the ignition method (Luczak *et al.*, 1997), in which 5g aliquots of dry sediments were separated and incinerated in a muffle (500°C) for 4 hours. Organic Matter contents were established by calculating the difference between initial and final weights.

The analysis of the Simultaneously Extracted Metals / Acid Volatile Sulfides (SEM/AVS) on summer sediments was conducted by Cruz (2014). This method describes the procedures for the determination of acid volatile sulfides (AVS) and selected metals that are solubilized by a weak acidification (simultaneously extracted metals, SEM). The AVS is one of the factors that control the bioavailability of metals in anoxic sediments (DiToro *et al.*, 1990).

2.4 Inductively coupled plasma mass spectrometry

The determination of trace elements was conducted in the Spectrometry Laboratory (Labspectro), of the Pontifícia Universidade Católica do Rio de Janeiro (PUC-RJ). Metals were analyzed by inductively coupled plasma mass spectometry (ICP-MS) without using reaction cell. The ICP-MS used to the analyses consisted of a model ELAN DRC II (Perkin Elmer-Sciex, Norwalk, CT, USA). The sample introduction in plasma system followed the standard system supplied by the ICP-MS manufacturer, and consists of a Meinhard nebulizer type with twisted cyclonic chamber. During the analysis, the ^{103}Rh was used as an internal standard at a concentration of 20 μg L^{-1} to monitor the nebulization process and the plasma stability. The elements analyzed were Al, V, Cr, Fe, Co, Ni, Cu, Zn, Cd, Ba, Hg, Pb, U. The accuracy of the analytical procedure was checked with procedural blanks and by the parallel analysis of certified reference materials given by the National Research Council of Canada (DORM-4, dogfish muscle tissue), in duplicate.

2.5 Ecotoxicological assays

2.5.1 Chronic toxicity of Sediment-Water Interface to Lytechinus variegatus

The chronic test with sediment-water interface (SWI) followed the method described by ABNT (2006), adopting the reduced volumes proposed by Cesar *et al.* (2004). Adult sea urchin individuals, from the species *Lytechinus variegatus*, were obtained by snorkeling at Palmas Island, Guarujá, SP, and used as broodstock. About 2g of sediment were transferred to glass tubes (15 mL) and then 8 mL of filtered seawater were added (4 replicates/sample). To prevent direct contact between the embryos and the sediment, a mesh (45 µm) was introduced in each tube and placed on the sediment surface. The tubes were allowed to stabilize for 24 h before exposure. The same procedure was made with control tubes, which contained clean seawater (control water), or a 45 µm mesh and water (control mesh).

The gametes were obtained by osmotic induction (0.5 M KCl), and then the ovules were fertilized by adding an aliquot of sperm solution containing activated sperm cells. Afterwards, the fertilization success was checked, by observation of control samples under microscope, and >90% fertilization was achieved. For the toxicity test, approximately 500 eggs were added to each chamber and then incubated for 24 h with constant temperature (25 ± 2°C) and a 16h light/ 8h dark photoperiod. Afterwards, the content of each chamber was transferred to other vessels to preserve the larvae (500 mL formaldehyde). Then, 100 embryos were counted for each replicate, and the percentage of normal embryos was calculated. Normal plutei were identified based on typical larval development, considering the branch symmetry, shape and size of the skeleton (Perina *et al.*, 2011).

2.5.2 Chronic toxicity of whole-sediment to Nitocra sp.

The whole sediment chronic toxicity assay with the benthic copepod *Nitocra* sp., cultured in the laboratory, was based on the protocol developed by Lotufo & Abessa (2002). Four replicates were set up for each sample, and 15ml of high density polyethylene flask were used as test-chambers; they were filled with 2ml of sediment and 8ml of filtered sea water. Ten healthy ovigerous females were introduced into each chamber. The entire test system was incubated at 25±2ºC for 7 days with photoperiod of 16:8 light:dark and salinity

17. Then, the content of each replicate was fixed with formaldehyde (10%) and Rose-Bengal dye (1%). Finally, the numbers of adult females and their offspring (nauplii and copepodits) were counted using a stereomicroscope. The reproduction rate was given by dividing the number of offspring by the number of females of each replicate.

2.5.3 Acute toxicity of whole-sediment to Tiburonella viscana

The acute sediment toxicity assay with *T. viscana* (Thomas & Barnard, 1983) was conducted following the protocol described by Melo & Abessa (2002) and ABNT (2008). The amphipods used in this assay were collected at Engenho d'água Beach, Ilhabela – São Paulo (23°48'S – 45°22'W). In this test, three replicates of each sediment sample were prepared in 1L-polyethylene test chambers that contained a 2-cm layer of sediment and 750ml of dilution seawater. After 24 hours, ten healthy adult and non ovigerous amphipods were introduced into each test chamber. The experiment lasted 10 days, and was kept under constant lightning and aeration, and 25±2ºC temperature. At the end of the test, the contents of each test chamber were sieved and the surviving organisms were counted. Missing organisms were considered dead.

2.6 Statistical analyses

The results of the ecotoxicological tests were first checked for homoscedasticity and normality by Bartlett's and Shapiro-Wilks tests, respectively. Then, all samples were compared to the reference sediment using unpaired Student-t'-test, using the R software package. When significant differences in the toxicity endpoints were detected between the references and the test-samples, sediments were considered toxic ($p<0.05$); absence of statistical difference indicated non-toxic sediments.

Geochemical and ecotoxicological data were transformed trough logarithmic and arcsin equations. Arcsin transformation is used to allow the comparison between continuous data. Log transformation reduces the magnitude of the different variables, it was used log10(x+1). Then, data were integrated using principal component analysis (PCA).

3. Results

3.1 Sediment properties

Cruz (2014) found that samples from VG, P, A and IC were predominantly sandy (>80% sand), whereas sediment from PT and PM were muddy (>50%). The winter campaign produced similar results; however, the sample from PM presented differences between collections, as in the winter sampling, the sediment texture has changed from muddy to sandy. In both seasons, sediments presented low $CaCO_3$ contents (between <QL and 8.80%), whereas OM amounts ranged from 0.20% to 16.80% (Table 2).

Table 2: Contents of Calcium Carbonate, Organic Matter, Sand and Mud, in percentage, in sediments from CIP-PA (2013). Summer values are from Cruz (2014).

	Summer				Winter			
Stations	$CaCO_3$	OM	Sand	Mud	$CaCO_3$	OM	Sand	Mud
VG	0.00	0.20	95.44	4.55	0.00	0.28	98.87	1.12
PT	8.20	13.80	1.83	98.16	6.35	14.4	3.84	96.15
P	1.60	0.60	91.46	8.53	0.00	0.82	96.74	3.25
PM	8.80	0.60	40.65	59.34	1.32	6.06	77.21	22.78
A	5.00	16.80	83.55	16.44	2.21	1.00	92.45	7.54
IC	1.83	2.00	89.84	10.15	2.53	2.05	89.62	10.37

3.2 Sediment Chemistry

3.2.1 Summer

Metal concentrations in summer sediment samples are from Cruz (2014) and are presented in Table 3. Higher concentrations of metals occurred in sediments from PT and PM, the only two stations that were predominantly muddy. Sediments from VG exhibited intermediate values, in comparison to the other stations. For some elements (Cd, Cr, Cu, Fe, Pb, Mn and Zn) the highest concentrations were observed for PT. It is noteworthy that the concentrations of lead in sediments exceeded TEL at station PT (TEL = 30.20 mg/kg; CCME, 2001), even considering that Cruz (2014) used a weaker acid extraction than that used to derive the Canadian SQGs, and therefore the TEL exceedance may indicate a more severe situation of contamination by Pb.

Table 3: Metal concentrations on sediments from CIP-PA during summer (mg/kg) (Cruz, 2014). Bold values are above TEL.

	Sampling Sites					
	VG	PT	P	PM	A	IC
Cd	0.02	0.16	0.01	0.03	0.01	0.01
Cr	1.14	8.58	0.39	3.84	2.42	1.30
Cu	0.87	7.98	0.29	1.60	0.02	0.16
Fe	2846.75	17537.94	1772.21	6472.68	2679.88	2460.76
Mg	87.34	221.20	16.13	149.24	34.73	26.02
Pb	8.27	**32.83**	2.23	8.67	1.51	1.83
Zn	5.06	32.61	4.14	14.40	14.15	11.62

3.2.2 Winter

Metal concentrations in sediment samples collected during wintertime are presented in Table 4. Once again, higher concentrations of metals occurred in sediments from PT and PM. These results may be related to the sediment texture, as PT was predominantly muddy and PM presented fines >20%; these sediments presented also the higher amounts of OM. Sediments from A and IC exhibited intermediate values, in comparison to the other stations. It is noteworthy that the concentrations of Pb in sediments exceeded TEL at station PM (TEL = 30.20 mg/kg; CCME, 2001) and also sediment quality guidelines (SQVs), for North and South Atlantic littoral zones (Choueri *et al.*, 2009) for stations PM and PT. Based on the classification proposed by Choueri *et al.* (2009), V achieved levels of moderately polluted in PM, Co and Ni for PT and PM, Ni achieving levels of highly polluted. PM was considered moderately polluted regardin Zn.

Table 4: Metal concentration in sediments from CIP-PA during winter (mg/Kg). Bold values are above limits (CCME, 2001; Choueri *et al.*, 2009).

	Sampling sites					
	VG	PT	P	PM	A	IC
Al	411.30	9642.41	622.81	13630.66	1895.896	1401.80
V	0.83	22.49	2.41	**40.49**	5.27	3.62
Cr	1.07	20.38	1.53	31.97	5.41	3.67
Fe	907.39	15432.70	1722.79	26661.31	3827.72	2813.91
Co	0.43	**4.64**	0.82	**10.02**	1.63	0.97
Ni	0.31	**7.20**	0.47	**11.70**	1.76	1.37
Cu	0.28	5.47	0.46	16.27	0.80	0.68
Zn	1.65	23.32	2.71	**45.18**	6.21	3.87
Cd	0.0097	0.07	0.01	0.14	0.02	0.01
Ba	3.50	15.17	2.17	72.95	3.55	2.58
Hg	0.015	0.06	0.01	**0.12**	0.02	0.01
Pb	0.83	**13.08**	2.93	**40.24**	3.08	2.04
U	0.19	1.02	0.19	2.53	0.24	0.37

3.3 Ecotoxicological assays

3.3.1 Lytechinus variegatus

In the chronic toxicity test of SWI, physical-chemical parameters of the overlying water into the test chambers remained within acceptable ranges (ABNT 2006; Cesar *et al.*, 2004) (Appendix A). Salinities ranged from 31 to 35; pH levels were between 5.84 and 7.66 and DO levels ranged from 2.77 to 4.22 mg.L^{-1}. In the winter, the normal embryonic development of organisms exposed to sediments from stations PT, P, PM and A was significantly altered (Figure 2). Sediments from Cruz (2014) showed toxicity also for VG, PT and P (Figure 2). Being PT the most toxic station for both seasons.

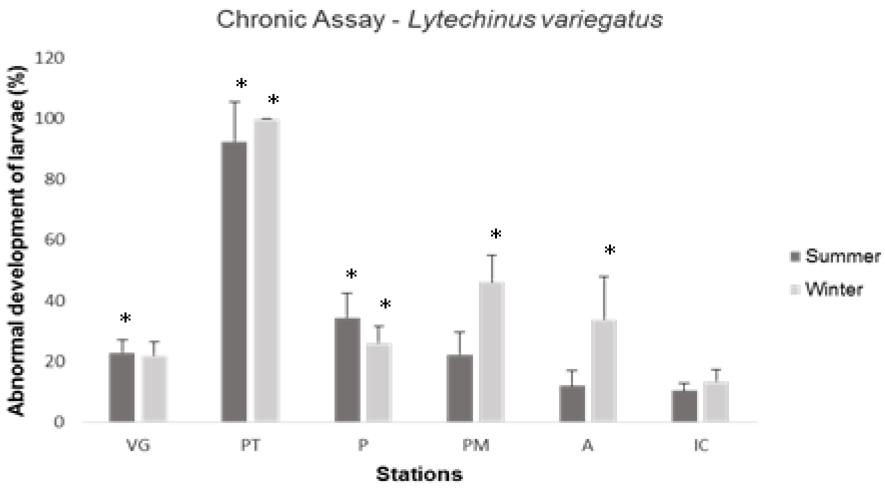

Figure 2: Abnormal development of larvae of *Lytechinus variegatus* exposed to sediments from the CIP-PA. Summer samples are from Cruz (2014). Asterisks (*) indicate significant differences relative to the control (IC) ($p<0.05$).

3.3.2 Nitocra sp.

In the test with copepods, the measured parameters of overlying waters were within acceptable levels in the most of samplings, except for PT (Appendix B), according to Lotufo & Abessa (2002). Salinities ranged from 15 to 20; pH levels were between 6.24 and 8.01, except for PT that had lower pH levels (4.14 – final summer and 4.65 – initial winter). Acidification in sediment/water systems occur when hydrogen ions are generated during oxidation (Hong *et al.*, 1991), so this acidification in the sediment (pH<6) probably was due to their high levels of Fe and sulfides that when oxidized produce H^+ ions. Also, DO levels ranged from 1.56 to 6.45 mg.L^{-1} and low values were observed in all test samples of summer, in the beginning of the test (2.46<values<1.56). However, at the end of the test, values were greater than 3.62 mg/L. Sediment from PT was considered significantly toxic for both seasons. In summer, sediment from P were considered significantly toxic when compared to the reference sediment (IC) and in winter sediments from PM were considered toxic (Figure 3).

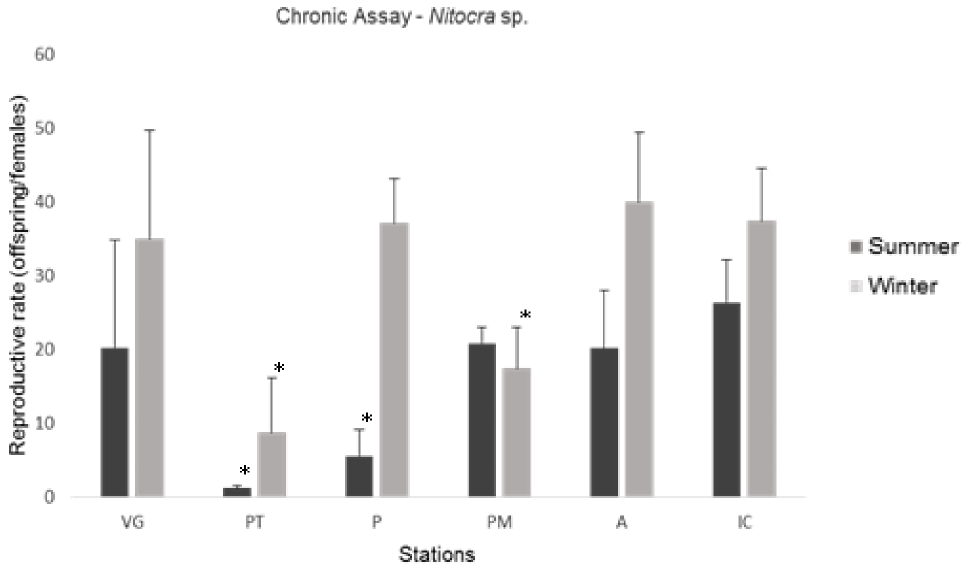

Figure 3: Reproductive rate of *Nitocra* sp. exposed to sediments from CIP-PA. Summer samples are from Cruz (2014). Asterisks (*) indicate significant differences relative to the control (IC) ($p<0.05$).

3.3.3 *Tiburonella viscana*

During the acute toxicity test, physical and chemical parameters of the overlying water within the test chambers remained within acceptable ranges (Melo & Abessa 2002; ABNT 2008) (Apendix C). Salinities ranged from 30 to 36; DO levels ranged from 4.61 to 6.34 $mg.L^{-1}$. The pH values ranged between 6.61 and 8.25, except for PT in the summer, which presented lower final pH values (4.09). Sediments from station PT were significantly toxic in both seasons, producing 100% mortalities among the exposed amphipods (Figure 4).

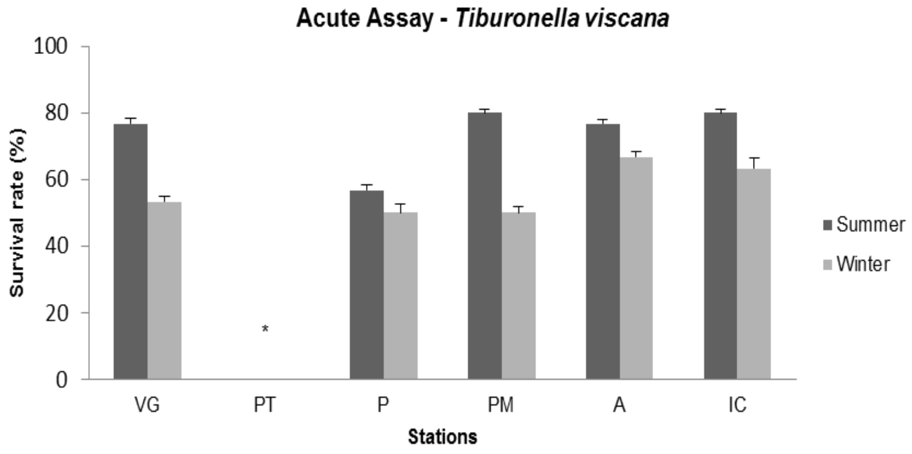

Figure 4: Survival rate of *Tiburonella viscana* exposed to sediments from the CIP-PA. Summer samples are from Cruz (2014). Asterisks (*) indicate significant differences relative to the control (IC) ($p<0.05$).

3.4 Integrative approach

3.4.1 Summer

As mentioned, the results obtained in the summer campaign were analyzed by Cruz (2014), and was associated by the use of a PCA. In that campaign, the first two axes of PCA explained 89.96% of variances (Table 5, Appendix E). The 1st PC joined most of geochemical factors, as O.M. and the metals analyzed. The 2nd axis correlated some geochemical carriers (mud, O.M. and calcium carbonates) and toxicities (*L. variegatus* abnormal larvae, low *Nitocra* sp. fecundity rate and *T. viscana* mortality). In that PCA, PT station correlated strongly (3.89) with 2nd axis, showing high toxicity, mud, O.M. and $CaCO_3$ values.

Table 5: PCA eigenvalues integrating sediment properties, chemistry and Biomarkers, for summer samples from CIP-PA (Cruz, 2014). Bold indicate significant associations.

Axis	Eigenvalue	%Variance
1st Factor	7.335	56.419
2nd Factor	4.360	33.542

	1st Factor	2nd Factor
Sea Urchin_abnormality	0.4238	**0.8643**
Copepod	0.3126	**0.8992**
Amphipod_mortality	0.3056	**0.9101**
Mud	0.3722	**0.8643**
O.M.	**-0.5624**	**0.7632**
Calcium carbonate	-0.0446	**0.6781**
Cd	**-0.9901**	0.0947
Cr	**-0.9531**	0.2497
Cu	**-0.993**	0.0566
Fe	**-0.9915**	0.1082
Mn	**-0.927**	-0.01
Pb	**-0.9924**	0.0168
Zn	**-0.8972**	0.3177

3.4.2 Winter

During the winter, the first two axis of PCA explained >95% of variances (Table 6, Appendix F). The 1st axis joined toxicities (*L. variegatus* abnormal larvae, low *Nitocra* sp. fecundity rate and *T. viscana* mortality) with geochemical factors and metals. The 2nd axis showed association between toxicities. In that PCA, PT (4.5) and PM (5.5) stations correlated strongly with 1st axis, showing high toxicity, mud, O.M., $CaCO_3$ and metal concentration.

Table 6: PCA eigenvalues integrating sediment properties, chemistry and toxicity for winter samples from CIP-PA. Bold indicate significant associations.

Axis	Eigenvalue	%Variance
1st Axis	16.144	84.96
2nd Axis	2.06	10.88

	1st Axis	2nd Axis
Sea Urchin_abn.	**-0.7685**	**0.5432**
Copepod	**-0.8544**	0.4682
Amph._mort.	**-0.5967**	**0.7297**
Mud	**-0.8564**	0.432
O.M.	**-0.9113**	0.2873
$CaCO_3$	**-0.9102**	0.3926
Al	**-0.9822**	-0.0181
V	**-0.987**	-0.0802
Cr	**-0.9842**	-0.0638
Fe	**-0.983**	-0.0673
Co	**-0.9778**	-0.1974
Ni	**-0.9903**	-0.0861
Cu	**-0.9705**	-0.2161
Zn	**-0.99**	-0.1114
Cd	**-0.9303**	-0.3281
Ba	**-0.9211**	-0.3168
Hg	**-0.9174**	-0.3461
Pb	**-0.9624**	-0.2086
U	**-0.9278**	-0.328

4. Discussion

The past mining activities situated on the upper RIR represent the main sources of contamination to the RIR itself and to the CIP-PA (Moraes, 1997; Mahiques *et al.*, 2009). Still, some papers have reported that metals are mainly carried downstream associated with suspended particles (Guimarães & Sígolo, 2008a; Abessa *et al.*, 2014) and tend to precipitate close to the Valo Grande channel, within the estuarine system (Aguiar *et al.* 2008; Mahiques *et al.* 2009). According to Mahiques *et al.* (2013), the coarser grains tend to be deposited close to the areas with more energy (e.g., mainly along the upper and mid RIR regions), whereas the finer fractions tend to transported along longer distances within the estuarine channel.

Our results support such statement, as the high metal concentrations were observed in the sediments from the stations PT and PM, in both seasons. In these sediments, levels of Pb, V, Co, Ni, and Zn exceeded SQVs limits (Choueri *et al.,* 2009). Ni and Pb are considered chemicals of high toxicity, while Co is an essential chemical with toxic potential (Nunes, 2009). Pb in both seasons had the highest concentrations in comparison to the other metals analyzed, corroborating with other studies (Mahiques *et al.*, 2009; Guimarães & Sígolo, 2008b) and exceeding TEL during both seasons in samples from PT (summer) and PM (winter). Previous studies reported that Pb is the contaminant of main concern to the CIP-PA (Rodrigues *et al.*, 2012; Abessa *et al.*, 2014; Mahiques *et al.*, 2009; Moraes *et al.*, 2004; Mahiques *et al.*, 2013; Piedade *et al.*, 2014), and that its concentrations in sediments from CIP-PA were high (Mahiques *et al.*, 2009).

The worse conditions (metals and toxicity) tended to occur in muddy sediments (PT and PM), indicating that toxicity is strongly associated to fines, corroborating with other studies (Guimarães & Sígolo, 2008b; Cruz *et al*, 2014). Summer samples had higher metal concentrations occurring in sediments from PT, in comparison to the other stations, followed by PM (Cruz, 2014). During winter, these same stations presented higher concentrations of metals, with highest values in PM, followed by PT.

Our results showed that the suspended fine particles carried by the RIR are transported towards the south of CIP-PA and are also deposited at the midway between Iguape and Cananéia (Saito *et al.*, 2006), having a trend of greater concentration of fines between PT and the urban center of Iguape (Barcellos *et al.*, 2005). In addition to fine sediments (silt and clay), deposition areas tend accumulate organic matter as well (Moreira & Boaventura, 2003), thus sediment from PT and PM exhibited high O.M. contents. Aguiar *et al.*

(2008) reported higher concentrations of metals coinciding with higher levels of organic matter, corroborating our results. In this sense, contamination and toxicity tended to associate with the occurrence of fines and OM, since the two most toxic stations (PT and PM) were richer in O.M., presented fine sediments (>20%) and high metal concentrations during both seasons.

According to Cruz (2014), metals and ammonia were the main responsible for the sediment toxicity in CIP-PA (Cruz, 2014). Ammonia has been considered a confounding factor in ecotoxicological studies (Ankley *et al.* 1992; Ward *et al.* 2011), and in CIP-PA this compound is probably from natural origin. However, when in low concentrations, ammonia may interact with other contaminants (such as metals) resulting in additive or synergistic effects and consequently in toxicity increase (Ankley *et al.* 1990). In our study, although ammonia was not considered a main cause of toxicity, its role in the observed toxicity in PT and PM (the organically enriched sediments) cannot be ignored.

Climatic factors may have additional influence on the hydrological regime and the sediment transport in CIP-PA. According to Abessa *et al.* (2014) extreme rainfall episodes can contribute significantly to the supply of metals for CIP-PA (Melo *et al*., 2012). Furthermore, the RIR flow synchronously responds to rainfall variation achieving higher levels during the rainy period (DAEE, 2014). Some studies have related seasonality with hydrological regime changes in the RIR basin (Corsi & Landim, 2003; Cunha *et al.*, 2007; Costa *et al.*, 2009), which are mainly dependent of the rainfall precipitation and its interaction with the tidal cycles: during the summer (rainy season) a larger freshwater inflow is expected, pushing the riverine influence towards south, as well as the zone of maximum turbidity (e.g., where maximum siltation may occur). Thus, the areas with highest deposition may change throughout the year, which can promote a displacement of the areas under higher risk with possible consequences for the biota (Cruz, 2014). This phenomenon can be evidenced through the comparison of summer (Cruz, 2014) and winter samples. The present study shows a displacement of toxic stations regarding seasonality, through stations nearest to VG to stations closest to A. During summer, the three first stations

(VG, PT and P) presented chronic toxicity to *L. variegatus*, while during winter stations situated further south (PT, P, PM and A) presented this type of toxicity.

The integration of chemical and ecotoxicological data clearly shows that the CIP-PA is negatively impacted by the input of metals and their accumulation in the estuarine sediments, despite the protection status of the entire region. Jameson *et al.* (2002) stated that MPAs could be negatively influenced by external sources, since the fluid nature of their environment would not be efficient to retain pollutants outside the MPAs. Some examples indicated that MPAs could be affected by pollution (Araújo et a., 2013; Terlizzi el al., 2004). Regarding the CIP-PA, the area was used as reference site, due to the low anthropic influence (Azevedo *et al.,* 2011). However, based on the results obtained in the present study and the literature (Mahiques *et al*., 2009; 2013; Cruz *et al*., 2014; Guimarães & Sígolo, 2008a), we can conclude that the CIP-PA cannot be considered homogeneous, in terms of environmental quality: the area subject to the influence of RIR presents moderate environmental degradation (e.g., enriched levels of metals, toxicity). Therefore, the pollution by metals represents potential risk the local biota. The results also indicate that the CIP-PA is not being totally effective to protect this area from external impacts, and that policies are required to control the sources at the upper RIR.

Chapter 3 - Biochemical and Genetic Biomarkers

1. <u>Introduction</u>

About one third of the world's population lives on or close to the coastal zone (CZ), putting intense pressure on their ecosystems (UNEP, 2006). Urbanization increase leads to agricultural activity, industries and mining companies to meet the increasing demand of food and products (Ferreira, 2011). Mining activities are recognized as highly impactful (Benedicto *et al.*, 2008), being heavy metal contamination a cause of great concern (AMAP, 1998).

Marine organisms are normally exposed to and have potential to accumulate contaminants and present a set of biological responses due to such exposure, thus their use as biomonitors in estuaries and coastal environments is justified (Phillips, 1980; Bryan *et al.* 1980). Some crustaceans are affected by environmental contamination (Fingerman *et al.*, 1998) and may present ecological and economic importance. Crabs play an important role in the transference of nutrients and energy along the trophic chain, feeding on a variety of organisms and being predated by many species such as shorebirds, seabirds, and fish. Therefore, they may transfer pollutants to other trophic levels (Lavradas *et al.*, 2014). In addition, crabs are also important as a fishery resource, especially for artisanal fishers (Bordon *et al.*, 2012a), presenting thus social and economic relevance. Some species of crabs (for example *C. danae*) are recommended species to be used as environmental biomonitors (Bordon, 2013), providing biological responses as a result of contamination.

Marine crustaceans are suitable species to accomplish biomarkers assays (Engel & Brouwer, 1987; Moser, 2011; Togni, 2007; Brown *et al.*, 2004; Lavradas *et al.*, 2014). Biomarkers are defined as a change in a biological response, at a sub-individual level, ranging from molecular through cellular and physiological responses to behavioral changes (Peakall, 1994). Biomarkers can provide useful information in terms of knowledge of exposure or effects of toxicants (Pereira *et al.*, 2011), and they may detect responses in early stages before more severe disorders occur, such as illness, death or population changes (Bresler *et al.*, 1999; Fishelson *et al.*, 1999).

Biomarkers are useful to provide the first signs of negative effects due to an environmental stressor, including contaminants. Their importance resides in the fact that the presence of a particular chemical does not necessarily imply in visible signs of disturbance among the ecosystem components (Corsi *et al.*, 2005). In this sense, the use of an ecotoxicological approach capable to detect effects at molecular, biochemical and cellular levels can provide information to control the impacts before they spread to the upper levels of biological organization and produce ecological disruption (Adams *et al.*, 1989). Thus, biomarkers represent early warning of environmental stress (Jesus & Carvalho, 2008), and are particularly important in to assess environmental contamination in low to moderate contaminated areas, as for example the Marine Protected Areas (Corsi *et al.*, 2005; Bonacci *et al.*, 2007).

Coastal and estuarine areas provide suitable conditions to the establishment of urban structures and anthropic activities such as harbors, marinas mining and industrial centers (Miranda *et al.*, 2002; Ferreira, 2011), thus, the majority of human population inhabits coastal regions. Estuaries receive significant anthropic inputs from both point and diffuse sources located upstream or in their vicinities (Chapman & Wang, 2001). On the other hand, estuaries are among the most productive marine ecosystems in the world (Underwood & Kromkamp, 1999). In this sense, the contamination of estuarine sediments is a critical issue, due to the biological and ecological implications (Chapman & Wang, 2001).

The contamination in estuarine and marine ecosystems represents a main theme and has been studied all around the world (Abessa *et al*., 2008; Belzunce *et al*., 2001; Borja *et al*., 2000; Bouloubassi *et al*., 2001; Van Dolah *et al*., 1999; Weisberg *et al*., 1997). But surprisingly it has not been studied for Estuarine and Marine Protected Areas (Pozo *et al.*, 2009), although such areas can be affected by pollution (Jameson *et al*., 2002).

Along the coastal zone of São Paulo State (Southeastern Brazil), there is a wide mosaic of protected areas (PAs) including some terrestrial, estuarine and marine PAs. Among them, the Cananéia-Iguape-Peruíbe Protected Area (CIP-PA) is of main concern as it comprises the largest coastal plain of São

Paulo State (at the south coast of São Paulo) and includes a continuum of rainforest and mangroves, and the estuarine complex of Iguape and Cananéia. The CIP-PA was established by the Federal decrees 90.347/84, and 91.892/85 (BRAZIL, 1984; 1985) with the aim to protecting its natural and cultural heritage and improving the conditions for its inhabitants.

The Ribeira de Iguape River (RIR) is the main contributor to the CIP-PA. Historically, mining residues were deposited on the river banks from the upper portion of RIR (Guimarães and Sígolo 2008; Kummer *et al.* 2011), without any control, and nowadays they still constitute source of metals to the aquatic systems, via underground waters and surface drainage (Melo *et al.*, 2012; Cotta *et al.*, 2006) or even by leaching from the piles of slags and ores (Piedade *et al.*, 2014; Guimarães & Sígolo, 2008a). According to Morais *et al.* (2013) and Abessa *et al.* (2014), metals are carried downstream adsorbed on the suspended particles (fines and organic matter), reaching the CIP-PA. The input of metals to the region increased after the construction of the Valo Grande channel, as since its inauguration more than 60% RIR flux started to be discharged into the estuarine complex (Barcellos *et al.*, 2005; Mahiques *et al.* 2009). As consequence, some portions of CIP-PA present sediments with moderate to high concentrations of metals, which are related to the sources located in the upper RIR basin (Mahiques *et al*, 2009; 2013, Cruz, 2014).

After reaching aquatic systems, contaminants are spread and transported by currents and waves. However, they tend to remain in the water column for limited periods, because due to their properties, they often adsorb onto particles (fine sediments, organic matter, etc) and then precipitate to the bottom, accumulating in sediments (Ingersoll, 1995). Once deposited in the bottom environment, contaminants may be released back to the water column and the biota, thus sediments frequently have been reported as a secondary source of contaminants (Bordon *et al.*, 2012a, b; Lavradas, *et al.*, 2014; Virga & Geraldo, 2008). Sediment contamination may constitute a threat to aquatic organisms, since contaminants may cause toxicity or can be transferred through the trophic chain. Together with the biological components, sediment quality has been used as an important indicator of the health of aquatic ecosystems (Power & Chapman 1995).

In the case of the CIP-PA, which plays an important role in the conservation of marine/estuarine biological resources, there is a need to evaluate if the presence of metals is producing negative impacts on the local biota. Thus, this study aimed to identify the adverse biological effects related to environmental contamination by metals in the CIP-PA, through the use of biomarkers in the blue crab *Callinectes danae*.

2. Materials and methods

2.1 Study area

The region comprises the lower course of RIR and 4 islands (Cananéia, Cardoso, Comprida and Iguape).Together with its tributaries, the RIR forms one of the largest drainage basins of the S–SE Brazilian coast, with an area of about 25000 km^2 (Mahiques *et al.*, 2013) and a length of 470Km (Moraes *et al.*, 2014). The outflow of the river in its lower course varies from about 300 to more than 1200 m^3/s; this variation is strongly controlled by the climatic regime (Mahiques *et al.*, 2013). This area has a subtropical humid weather, with a rainy period from late November to April, being March the rainiest month, and a less rainy period from May to early November (Silva, 1984). The anthropic occupation is more consolidated and dense in the northern portion of the system, especially near the urban centers of Iguape and Comprida Island and in the lower valley of RIR (Barcellos *et al.*, 2005).

The estuarine complex of CIP-PA is located between the latitudes 23º45’-25º15’S, and the longitudes 46º45’-49º30’W, being limited by the Juréia ridge at North and by the state of Paraná down south (SMA, 1997). The tidal wave propagation is the main process that drives the hydrodynamic circulation of the system, promoting mixtures between oceanic and estuarine waters. The contribution of freshwater input and the wind action, although of secondary importance, also contribute to this process (Bonetti Filho *et al.* 1996). The “Valo Grande” channel was an artificial channel constructed to connect the RIR and the estuary with the purpose of reducing the navigation distance and the costs of trading the agricultural products that were exported from the former Port of

Iguape. However, this channel lacked any environmental impact assessment, and since its inauguration, most of the RIR flow started to be discharged into the estuary (Mahiques *et al.*, 2013). Then, the RIR became the main contributor of sediment, nutrients and contaminants to this system, especially in its central and northern portion (Freitas *et al.*, 2006; Mahiques *et al.*, 2009). Since then, sediments from the CIP-PA have exhibited increased levels of metals, specially of Pb (Rodrigues *et al.*, 2012).

2.2 Sediment and Blue Crab sampling

Sediment samples collection was described in item 2.2 of chapter 2.

Individuals of the species *Callinectes danae* were collected in the summer and winter campaigns, at the same six stations where sediment samples were collected (Figure 5, Table 7). The animals were collected by otter-trawling, each one lasting about 10-15 minutes at each station. When trawling was not possible, crab traps were used to collect the proper number of individuals. Eight organisms were collected in each station; and the animals were taken to the laboratory where they were conditioned for 12h in plastic buckets with estuarine salt water (with aeration).

Then, the organisms were immobilized and anesthetized by being placed on ice, once anesthetized they were sacrificed and then dissected to remove the organs utilized on biomarkers analyses. Based on other studies (e.g. Bordon *et al.*, 2012; Virga & Geraldo, 2008), muscle, gills and hepatopancreas tissues were removed to be further analyzed.

Table 7: Geographic coordinates of the sampling stations for sediments within the CIP-PA.

Station	Latitude	Longitude
VG	24°44.509'S	47°35.984'W
PT	24°49.294'S	47°42.186'W
P	24°54.124'S	47°48.547'W
PM	25°00.167'S	47°54.104'W
A	25°02.689'S	47°54.880'W
IC	25°04.281'S	47°55.839'W

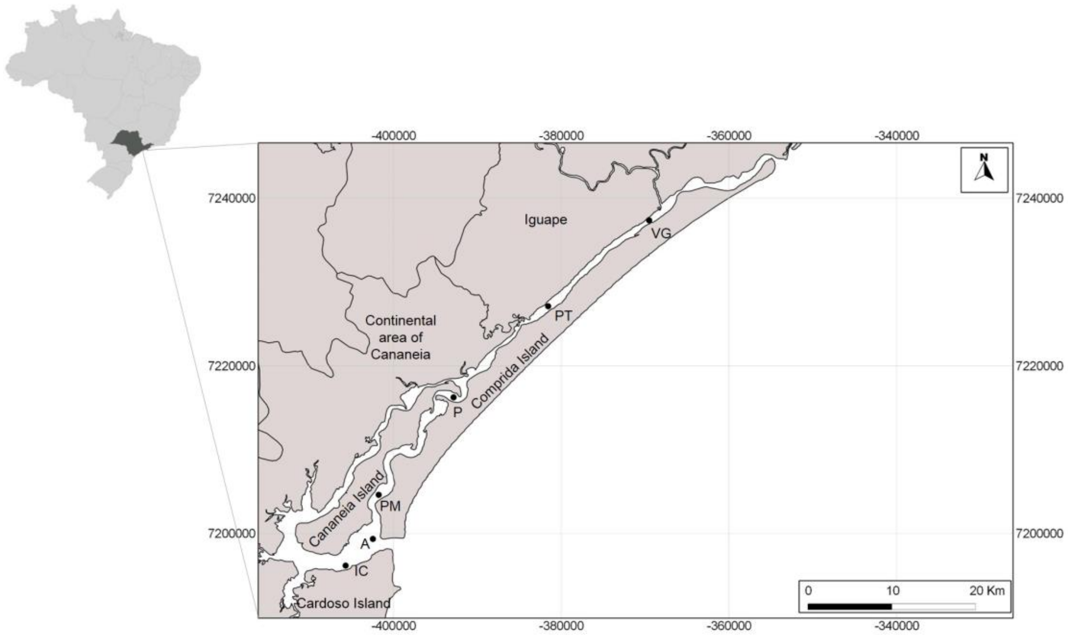

Figure 5: Map showing the sediment collection stations around the study area, within CIP-PA, Brazil.

2.3 <u>Sediment properties</u>

Described in the item 2.3 of Chapter 2.

2.4 <u>Inductively coupled plasma mass spectrometry</u>

Described on item 2.4 of Chapter 2.

2.5 <u>Biomarkers</u>

Biochemical biomarkers were evaluated in gills, muscle and hepatopancreas tissues of *C. danae*. After the acclimation, anesthetization and dissection, soft tissues were removed and kept frozen at -80ºC in ultrafreezer. Immediately prior to the biochemical analyses, samples were thawed on ice and homogenized with proper buffer solution (1:4), according to Lafontaine *et al.* (2000). Then, the extracts were centrifuged at 15.000g at 4ºC for 20 minutes, obtaining a supernatant fraction (S15), which was used for the enzymatic analyses.

The tissues were evaluated for the activity of glutathione S-transferase (GST) and glutathione peroxidase (GPx), following the protocol described by McFarland *et al.* (1999). Both enzymes have the function of promoting the conjugation of endogenous compounds and bio-transformed xenobiotics, making these compounds water-soluble, in order to facilitate their excretion (Keen *et al.*, 1976; Pereira, 2003). The intracellular levels of the reduced glutathione (GSH) tripeptide were also evaluated following the protocol described by Sedlak & Lindsay (1968). GSH also takes part in the cellular detoxification process providing substrate to the action of GST and GPx. The activity of the acetylcholinesterase (AchE) was analyzed according to Ellman *et al.* (1961), adapted to microplate. AchE has the function to decompose the acetylcholine neurotransmitter at synapses and neuromuscular junctions, preventing continuous nerve activation (Pereira, 2003). The amounts of metallothioneins (MTs) were also evaluated based on the protocol described by Viarengo *et al.* (1997). These proteins play roles in homeostasis and detoxification of both essential and non-essential metals. Because these proteins have thiols groups (-SH) in its structure, they can bind to excess of metals. Metallothionein production is induced by increasing the amount of metals within the cell, thus these are specific biomarkers of exposure to metals (Benson & Di-Giullio, 1992; Freire *et al.*, 2008). DNA damage (strand breaks) was analyzed by the alkaline precipitation assay proposed by Olive (1998), using fluorescence to quantify DNA traces (Gagné & Blaise, 1993). The analysis of lipid peroxidation (LPO) were done through thiobarbituric acid method, proposed by Wills (1987), in which the products of lipidic peroxidation were averaged and expressed as thiobarbituric acid reactive substances (TBARS). Results were expressed in µg TBARs/mg proteins. For DNA and LPO analyzes the samples were homogenized with proper buffer solution and were not centrifuged.

Results obtained to enzyme activities were normalized by the total proteins contents determined by the Bradford (1976) method. All the biochemical biomarkers were analyzed in the microplate reader band Bio Tek and model Synergy HT®.

2.6 Condition Factor

The condition factor (CF) indicates the degree of adjustment of a given species to the environment (Braga *et al.*, 1985; Wolff, 2007). It can be used as a quantitative indicator of the healthiness or “well-being” of the species in its environment (Vazzoler, 1996). CF is considered a biomarker, as it is a variable related to the nutritional state of a population, varying trough time. It can be associated to the fat accumulation, susceptibility to environmental changes or to the period of gonadal development (Barbieri & Verani, 1987; LeCren, 1951; Pinheiro & Fransozo, 1993; Froese, 2006). At laboratory, the specimens were measured and weighted at the carapace width (CW), without lateral spines, and wet weight (WW), respectively, by the use of a 0.05 mm precision caliper and a 0.1g precision balance. CF was described for each station (not divided by sex) through the equation $a=WW/CW^b$ (LeCreen, 1951), where WW is the wet weight, CW is the carapace width, b is the slope, and “a” the condition factor.

2.7 Statistical analyses

Biomarkers responses were first checked for homoscedasticity and normality by Bartlett’s and Shapiro-Wilks tests, respectively. Then, all samples were compared with each other and between seasons (summer-winter) by a 2-way ANOVA, $p<0.05$. Condition factor was evaluated by the coefficient of determination (r^2) obtained from its equation. An ANOVA was used to compare stations and seasons ($p<0.05$; Zar, 1996).

Prior to the analysis, data were transformed. Data expressed in % were firstly Arcsin transformed, allowing the comparison with continuous data, whereas log-transformation ($y = \log10_{(x+1)}$) was used to all data aiming to reduces the magnitude of the different variables. After data transformation, biomarkers and geochemistry data were integrated using principal component analysis (PCA).

3. Results

3.1 Sediment properties

The properties of sediments from the summer survey were analyzed by Cruz (2014). This author observed that the sediments from VG, P, A and IC were predominantly sandy (>80% sand), whereas sediment from PT and PM were mainly muddy (>59%). The winter survey showed similar results to most of samples, excepting sediment from PM, which was sandier (Table 8). In both seasons, sediments showed variable $CaCO_3$ and OM contents (Table 8).

Table 8: Contents of Calcium Carbonate, Organic Matter, Sand and Mud, in percentage, in sediments from CIP-PA. Summer values are from Cruz (2014).

	Summer				Winter			
Stations	$CaCO_3$	OM	Sand	Mud	$CaCO_3$	OM	Sand	Mud
VG	0.00	0.20	95.44	4.55	0.00	0.28	98.87	1.12
PT	8.20	13.80	1.83	98.16	6.35	14.4	3.84	96.15
P	1.60	0.60	91.46	8.53	0.00	0.82	96.74	3.25
PM	8.80	0.60	40.65	59.34	1.32	6.06	77.21	22.78
A	5.00	16.80	83.55	16.44	2.21	1.00	92.45	7.54
IC	1.83	2.00	89.84	10.15	2.53	2.05	89.62	10.37

3.2 Sediment chemistry

Summer sediments metal concentrations were analyzed by Cruz (2014). This study used weak acid extraction (SEM/AVS methodology), nevertheless Pb levels ranged from 0.857 to 51.628 mg/Kg, exceeding TEL in station PT (TEL = 30.20 mg/kg; CCME, 2001), none of the other metals presented values above Sediment Quality Guidelines (SQVs; Choueri *et al.*, 2009) nor TEL and PEL (CCME, 2001). Stations PT and PM presented higher metal concentrations and mud content.

Table 9: Metal concentration in sediments from CIP-PA during summer (mg.Kg^{-1}) (extracted from Cruz, 2014).

	Concentrations (mg.Kg^{-1}) in each sampling site					
	VG	PT	P	PM	A	IC
Cd	0.021	0.168	0.01	0.037	0.012	0.011
Cr	1.141	8.589	0.393	3.842	2.429	1.302
Cu	0.875	7.988	0.290	1.607	0.023	0.164
Fe	2846.755	17537.94	1772.216	6472.689	2679.882	2460.768
Mg	87.341	221.201	16.135	149.247	34.730	26.022
Pb	8.271	**32.839**	2.233	8.679	1.516	1.834
Zn	5.069	32.615	4.145	14.406	14.158	11.622

Wintertime metal concentrations in sediments are presented in Table 10. Stations A and IC had intermediate values of metal concentration, regarding other stations. Once again, the higher concentrations of metals occurred in sediments from PT and PM, which contained high amounts of O.M. Sediments from PT were predominantly muddy, while those from PM had more than 20% of fines in its composition. The only two stations that presented values of metal above limits (SQVs or TEL) were PT and PM. Sediment Quality Guidelines (SQVs) (Choueri *et al.*, 2009) demonstrated that Co and Ni were above limits regarding PT and that V, Co, Ni and Zn were above limits for PM. Ni achieved levels of highly polluted to PM. Pb exceeded TEL in PM (TEL = 30.20 mg/kg; CCME, 2001).

Table 10: Metal concentration in sediments from CIP-PA during winter (mg/Kg). Bold values are above TEL or SQVs (CCME, 2001; Choueri *et al.*, 2009).

	Concentrations (mg/kg)					
	VG	PT	P	PM	A	IC
Al	411.30	9642.41	622.81	13630.66	1895.896	1401.80
V	0.83	22.49	2.41	**40.49**	5.27	3.62
Cr	1.07	20.38	1.53	31.97	5.41	3.67
Fe	907.39	15432.70	1722.79	26661.31	3827.72	2813.91
Co	0.43	**4.64**	0.82	**10.02**	1.63	0.97
Ni	0.31	**7.20**	0.47	**11.70**	1.76	1.37
Cu	0.28	5.47	0.46	16.27	0.80	0.68
Zn	1.65	23.32	2.71	**45.18**	6.21	3.87
Cd	0.0097	0.07	0.01	0.14	0.02	0.01
Ba	3.50	15.17	2.17	72.95	3.55	2.58
Hg	0.015	0.06	0.01	**0.12**	0.02	0.01
Pb	0.83	**13.08**	2.93	**40.24**	3.08	2.04
U	0.19	1.02	0.19	2.53	0.24	0.37

3.3 Biomarkers

Biomarkers in posterior gills are shown in Figure 6. GPx for this tissue showed higher values in VG during both seasons, being different from IC only during winter; PT were also different from IC during winter. During summer, animals from P presented GST activity different from IC, and during winter only PM crabs showed statistical difference with the control. IC showed higher values of thiols (GSH and other thiols) during summer, presenting statistical difference with all other stations; during winter stations VG and PT were different from IC. Regarding metallothioneins, in the summer, animals from all stations presented similar quantities; otherwise during winter, animals from station P had higher MT expression, which was different from all the other stations, excepting A. During summer, LPO was greater among the crabs from the station A, but the measured value was not different from that observed among the animals from IC; during winter, crabs from PM presented higher significantly lipoperoxidation. Only DNA damage had statistically seasonal difference.

The responses of biomarkers in the anterior gillss are presented on Figure 7. GPx and GST had low induction during summer; and animals from stations PT, P and A exhibited higher activity of both enzymes during winter, presenting statistical difference in comparison to IC. GSH and other thiols had a different pattern, with higher values in the summer, but without any statistical difference in comparison to IC. In the winter, GSH values were low and similar among animals from the different sites. MTs had higher values during winter, and crabs from P presented MTs quantities different from IC in winter; in the summer, no differences were observed between stations. LPO presented similar values for animals of all stations during summer; however, in the winter, animals from P and IC presented the higher values; IC being different from VG, PT and A. DNA damage also had higher values during winter, with no difference to the control in any season. Anterior gills is the tissue that presented the clearest seasonal pattern, having statistical difference to GST, GSH, Metallothionein and DNA damage. All biomarkers had higher activity during winter, with the exception of the thiols.

Hepatopancreas responses are shown in figure 8. The animals from IC presented higher GPx activities during summer, which was different from the animals of A; winter analyzed showed no differences. Summer GSH presented no statistical difference between stations and the control; during winter this biomarker had values from station IC being different from stations with the lowest (VG) and the highest (PT) values. The highest values of thiols were observed in animals from PM; these values were different from those exhibited by the control animals only during summer. MT is more expressed in the animals from the station P during summer; during winter, the animals from PM presented the higher MT activities during winter, although both seasons showed no statistical difference between animals from the stations and IC. In the summer, LPO activity was higher among the animals from PM and A, being different from IC; animals collected in the winter exhibited higher LPO values, with the maximum values among animals from PT being different from all the other stations, including IC. DNA damage showed no difference between stations and IC, although it had difference between seasonality.

AchE in muscles (Figure 9) had high seasonality, being much lower during summer, reminding that this enzyme is inhibited in the presence of contaminants. AchE did not have any difference between stations and the control.

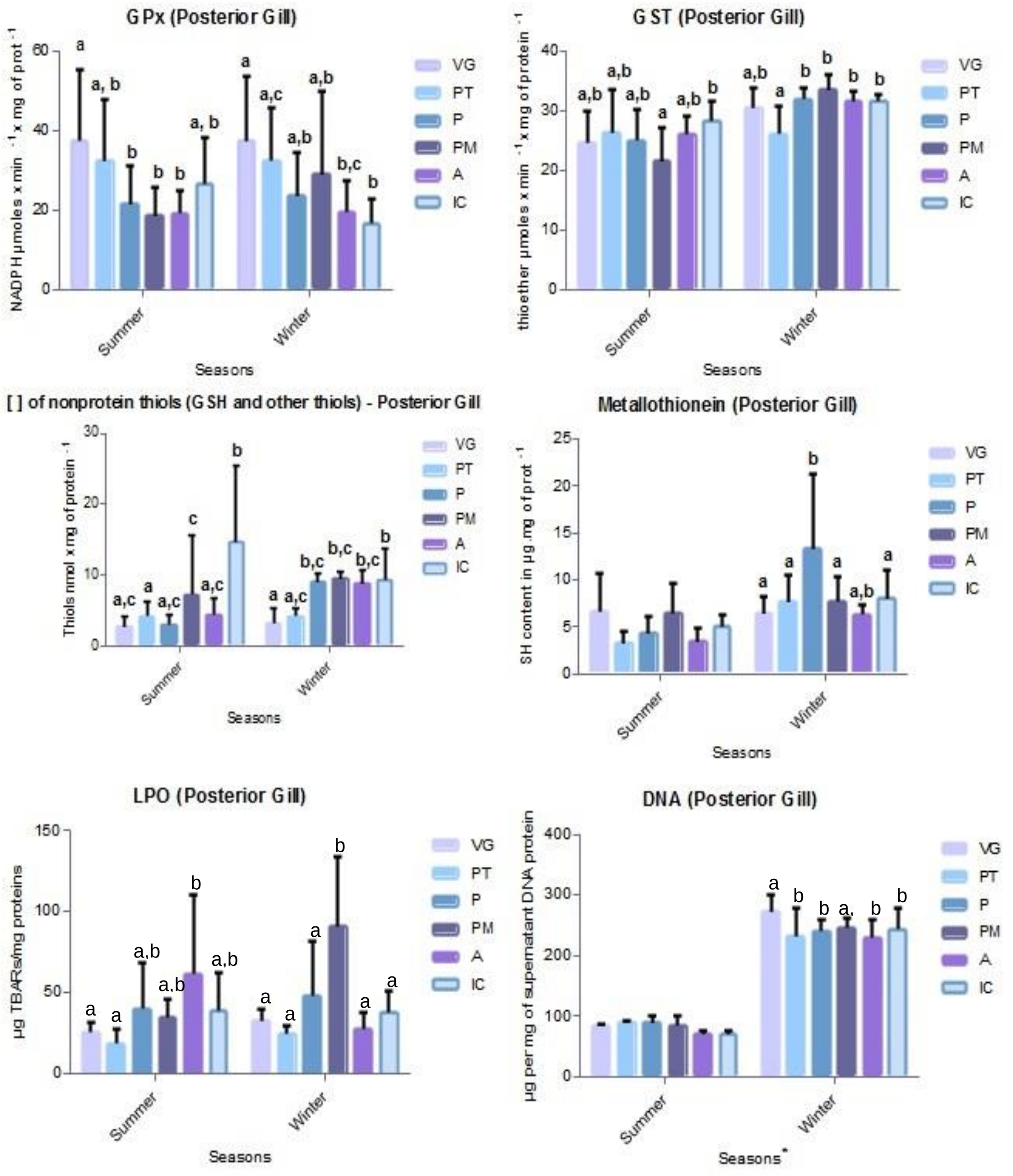

Figure 6: Biomarkers in Posterior Gill of *C. danae* from CIP-PA. Letters means statistical difference between stations and asterisk (*) towards seasons (summer-winter). p<0.05.

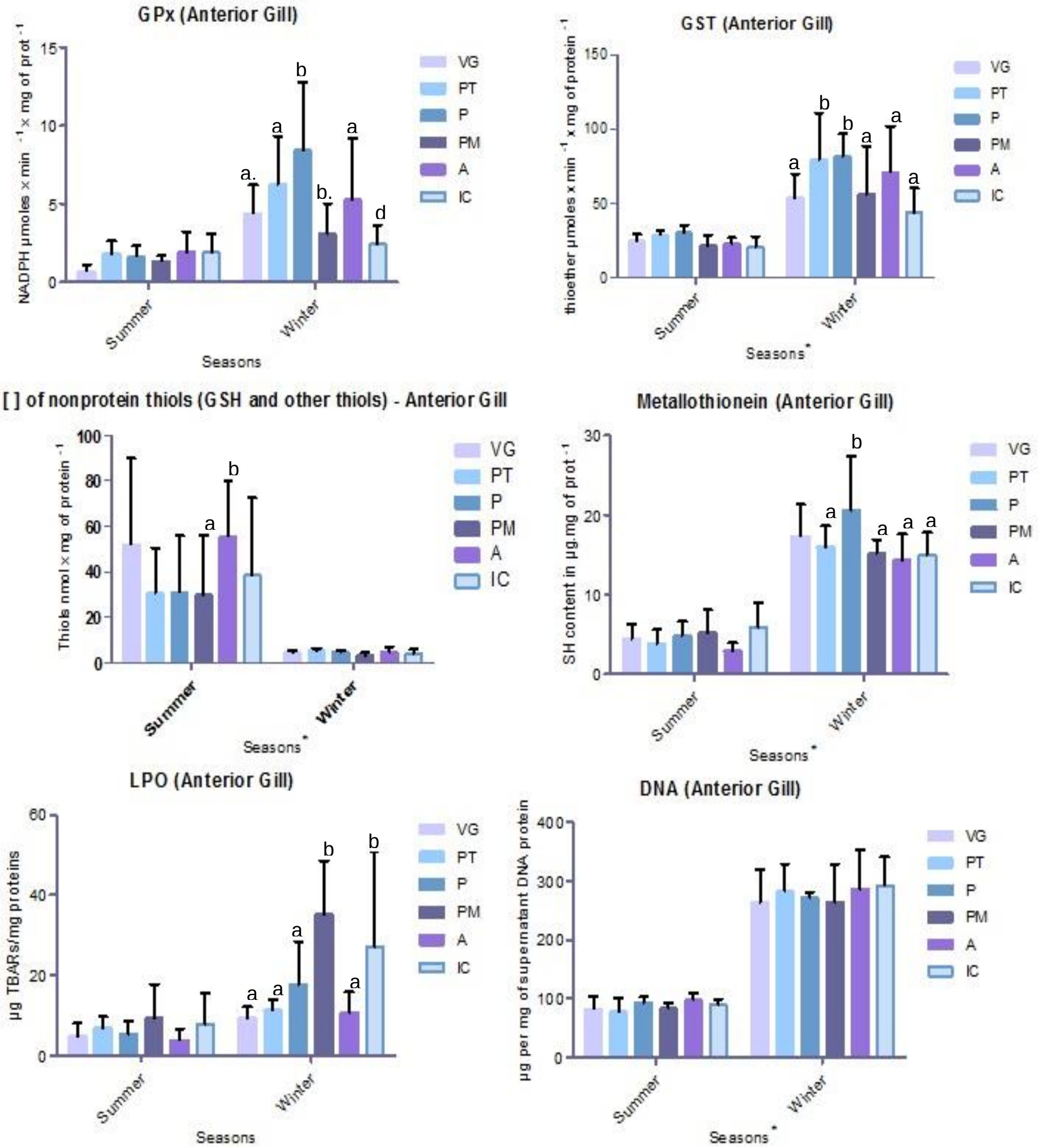

Figure 7: Biomarkers in Anterior Gill of *C. danae* from CIP-PA. Letters means statistical difference between stations and asterisk (*) towards seasons (summer-winter). p<0.05.

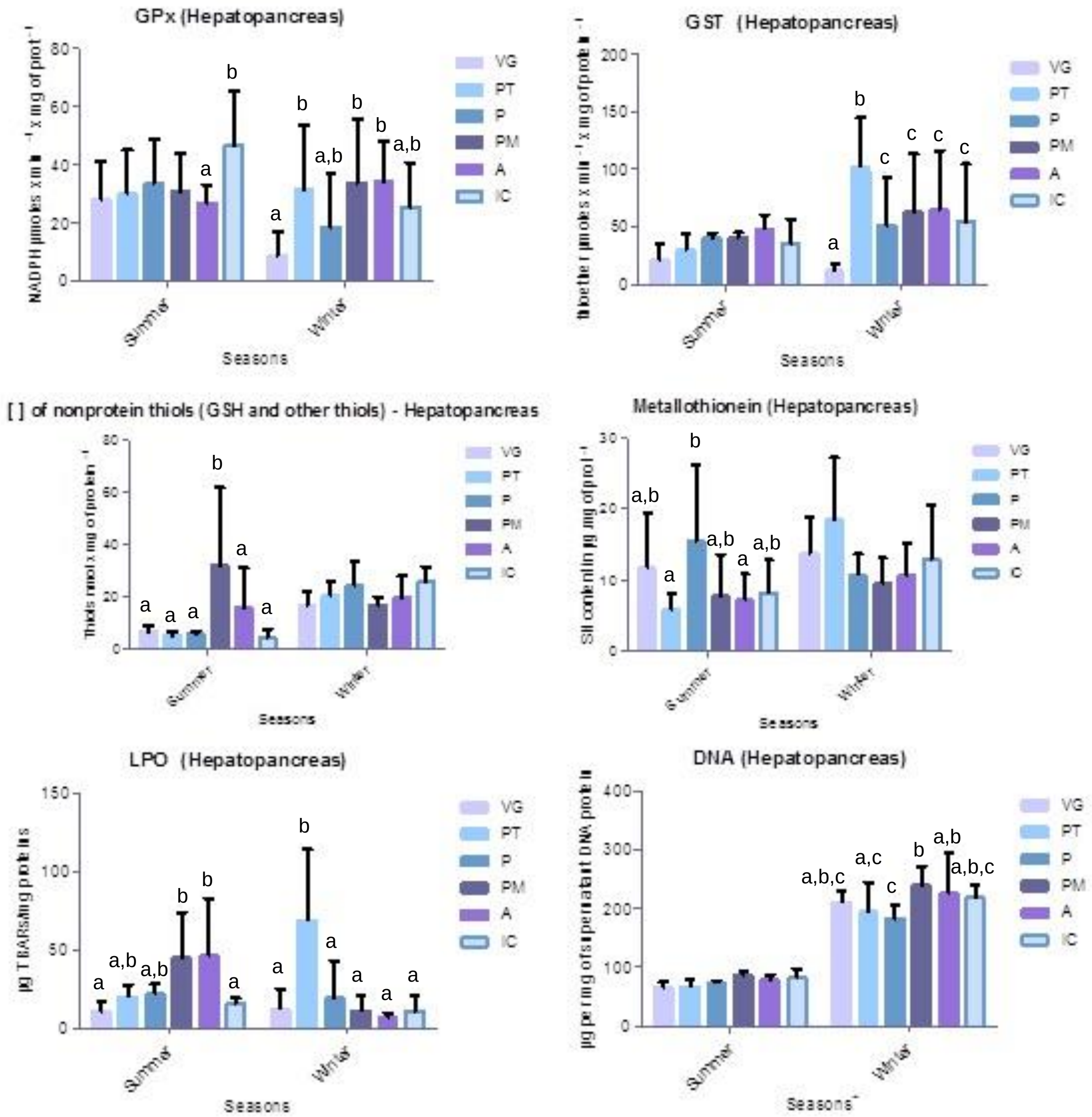

Figure 8: Biomarkers in Hepatopancreas of *C. danae* from CIP-PA. Letters means statistical difference between stations and asterisk (*) towards seasons (summer-winter). $p<0.05$.

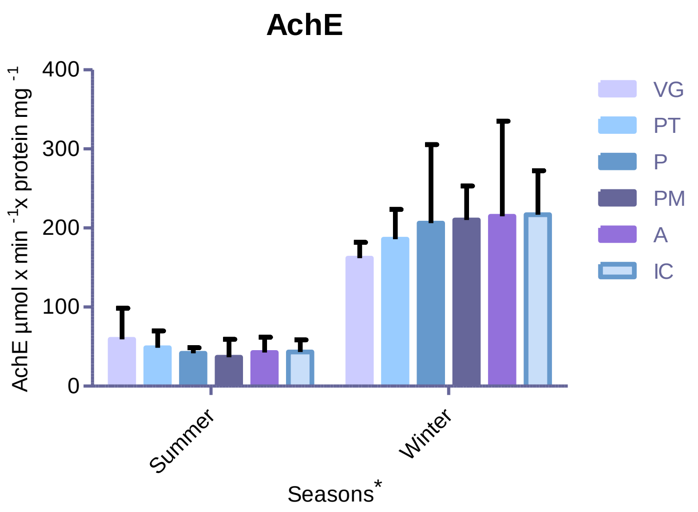

Figure 9: Biomarker Acetylcholinesterase (AchE) in muscle of *C. danae* from CIP-PA. Asterisk (*) means statistical difference towards seasons (summer-winter). p<0.05.

3.4 Condition Factor

Combining all obtained data, summer condition factor is statistically lower than winter (Table 11). Only stations PT and PM presented higher values in winter then during summer. Lower values of condition factor were for PT and IC during summer and for A and VG during winter. CF from all stations were not different to each other.

Table 11: Condition Factor for each station of CIP-PA.

	Summer	Winter
VG	0.5	0.06
PT	0.03	0.11
P	0.16	0.09
PM	0.22	0.09
A	0.11	0.03
IC	0.04	0.19

3.5 Integrative approach

3.5.1 Summer

The two first axes of summer PCA explained 87.12% of variances (Table 12, Appendix G). The 1st axis joined all metals, O.M. and LPO and DNA damage in posterior and anterior gills, respectively, GST in hepatopancreas and GPx in anterior gills. This axis showed inverse association to Metallothionein from both gills, DNA for posterior gills and LPO for anterior gills. The 2nd axis correlated the sedimentological factors (fines, OM and $CaCO_3$), GPx from anterior gills and LPO in hepatopancreas. This axis had inversely associated Metallothionein from posterior gills and hepatopancreas with mud showing an indication of oxidative stress (GSH) and AchE in anterior gills and muscle; condition factor appeared inversely associated to mud also.

Table 12: PCA eigenvalues integrating sediment properties, chemistry and Biomarkers, for summer samples from CIP-PA. Bold indicate significant associations.

Axis	Eigenvalue	%Variance
1st Axis	6.497	64.972
2nd Axis	2.216	22.155

Variable	1st Axis	2nd Axis	Variable	1st Axis	2nd Axis
Mud	0.2111	**-0.961**	DNA_PG	**0.519**	-0.089
O.M.	**-0.6121**	**-0.6923**	GPx_AG	**-0.543**	**-0.616**
Calcium carbonate	-0.255	**-0.8649**	GST_AG	-0.082	-0.038
Cd	**-0.947**	-0.0266	GSH_AG	-0.494	**0.523**
Cr	**-0.948**	-0.0136	Metallo_AG	**0.704**	0.243
Cu	**-0.9574**	0.0912	LPO_AG	**0.726**	-0.463
Fe	**-0.9898**	0.0902	DNA_AG	**-0.793**	0.333
Mn	**-0.9015**	0.1675	GPx_Hep	0.235	0.089
Pb	**-0.9189**	0.1343	GST_Hep	**-0.619**	-0.378
Zn	**-0.8181**	0.0350	GSH_Hep	-0.04	-0.264
GPx_PG	0.438	0.258	Metallo_Hep	-0.223	**0.785**
GST_PG	-0.411	0.06	LPO_Hep	-0.435	**-0.54**
GSH_PG	0.179	-0.273	DNA_Hep	-0.069	-0.243
Metallo_PG	**0.681**	**0.541**	AChE	0.09	**0.573**
LPO_PG	**-0.733**	0.225	Cond_Fac	0.355	**0.703**

3.5.2 Winter

The two first factors of winter PCA explained >95% of the data variance (Table 13, Appendix H). In the 1st axis, metals had a strong correlation with Mud, O.M. and $CaCO_3$; this axis also associated exposure biomarkers (GPx) in hepatopancreas. The 2nd axis correlated antioxidant enzymes (GST) with LPO and DNA in Posterior Gills, indicating that the enzymatic action is not being sufficient. Something similar occurred to AG, where there was an induction of

GSH causing also DNA damage. In hepatopancreas, GST, GSH and Metallothionein had high activity, leading to LPO. PT and PM were highly associated with 1st axis, revealing high metal contamination and fines.

Table 13: PCA eigenvalues integrating sediment properties, chemistry and Biomarkers, for winter samples from CIP-PA. Bold indicate significant associations.

Axis	Eigenvalue	%Variance
1st Axis	14.64	91.51
2nd Axis	1.16	7.25

Variable	1st Axis	2ndAxis	Variable	1st Axis	2ndAxis
Mud	**-0.8226**	**0.5667**	GPx_PG	-0.283	0.171
O.M.	**-0.8892**	0.4185	GST_PG	0.254	**-0.927**
Calcium carbonate	**-0.8738**	0.4509	GSH_PG	-0.075	-0.135
Al	**-0.9738**	0.1322	Metallo_PG	0.134	0.084
V	**-0.9934**	0.0498	LPO_PG	-0.341	**-0.795**
Cr	**-0.9896**	0.0738	DNA_PG	0.402	**-0.619**
Fe	**-0.9896**	0.0818	GPx_AG	0.147	0.26
Co	**-0.9909**	-0.1118	GST_AG	-0.175	0.321
Ni	**-0.9962**	0.0299	GSH_AG	0.334	**0.873**
Cu	**-0.9827**	-0.1726	Metallo_AG	0.407	0.185
Zn	**-0.997**	-0.0088	LPO_AG	-0.377	-0.318
Cd	**-0.9504**	-0.3067	DNA_AG	0.112	**0.78**
Ba	**-0.9386**	-0.3211	GPx_Hep	**-0.684**	0.348
Hg	**-0.9381**	-0.3387	GST_Hep	-0.644	**0.565**
Pb	**-0.9754**	-0.1405	GSH_Hep	0.315	**0.572**
U	**-0.9506**	-0.2727	Metallo_Hep	-0.104	**0.878**
			LPO_Hep	-0.433	**0.804**
			DNA_Hep	-0.284	-0.476
			AChE	-0.18	-0.342
			Cond_Fac	-0.095	0.148

3.6 Discussion

Crabs of the species *C. danae* from CIP-PA presented some differences in their health status, although the analyzed biomarkers have varied at lot. The multivariate approach showed that the metals in sediments are related to the blue crab responses. Crabs collected during winter generally presented higher enzymatic activity then those collected during the summer, especially regarding anterior gill, that had the clearest pattern of seasonal variability. All tissues showed significantly higher values during winter. However, when it comes to DNA damage, the same occurred to AchE (muscle). Higher DNA damage already were attended at low temperatures, being DNA repair mechanisms slower and less efficient leading to genetic damage (Beninca *et al.*, 2013; Buschini *et al.*, 2003). Since this values were presented for all stations analyzed (including IC), some of this responses are probably due to natural environmental factors related to seasonal variation.

Stations PT and PM presented higher sediment metal concentrations, fines (>20%) and O.M. content during both seasons, which could lead to oxidative stress (read by biomarkers). The station PT is closer to the Valo Grande, receiving RIR drainage, whereas PM is closer to the city of Cananéia. Sediments from PM already presented toxicity (Cruz *et al.*, 2014). PM was one of the stations in which the crabs presented higher biomarker response during summer, whereas the organisms from PT, during winter, presented similar qualitative results.

Summer data suggests that metals are causing effect (LPO and DNA) on blue crabs gills (posterior and anterior gills, respectively); although this effect was negatively correlated to MTs expression, which already has been showed by other studies (Mieiro *et al.*, 2011). Low activity of MT associated with metal causing LPO and DNA in gills probably shows a redox imbalance, which leads to LPO and DNA damage (Gravato *et al.*, 2006; Brennan *et al.*, 2012).

Animals collected at muddy and organically rich sediments (PM and PT) presented signs of oxidative stress (Anterior Gills) and lipoperoxidation (Hepatopanreas) during summer. These results may be influenced by hypoxia, as muddy estuarine sediments often present low levels of dissolved oxygen (Chapman & Wang, 2001; Paerl *et al.*, 1998), and induce the formation of reactive oxygen species (ROS). Under such conditions, peroxidation of membrane lipids and damage of cellular components can occur (Lima *et al.*, 2006; Silva *et al.*, 2012), causing LPO and DNA strand breaks (Gutteridge, 2001; Geihs *et al.*, 2014). Hypoxia is a feature of some estuaries and can decrease metal availability (Zoumis, 2001), as the soluble phases may be immobilized by the AVS; on the other hand, hypoxia is a multiple stressor condition with undefined toxic values, challenging the organisms living in the estuarine environment (Hypes, 1999) and inhibiting immune responses (Brunett & Stickle, 2001). Using physiological adaptations, many aquatic crustaceans can tolerate moderate hypoxia and maintain oxygen uptake, such as to increase the flow of water over the gills by elevating scaphognathite beat frequency, which results in an increased oxygen supply to the tissues (Hypes, 1999).

The PCA axis showed some biomarkers inversely associated to mud (MT, GSH, Ache and condition factor). This is probably due because metals are present in moderate concentrations but are less bioavailable in muddy sediments (David *et al.*, 1994). In this study, the higher metal concentrations were observed in the muddier and organically richer sediments (PT and PM); in this case, hydrogen sulfides and humic acids are important chelators that may regulate the amount of free or biologically available metals in sediments (Perin *et al.*, 1997), reducing then the bioavailability to the blue crabs (Luoma, 1983; Champan & Wang, 2001).

The winter results also showed that metals and muddy sediments are related to the increased activity of GPx in *C. danae*. The association of some biomarker responses to the contents of fines may indicate the indirect influence of the sediment properties on the biomarker responses. Hypoxia and the geochemical chelators may have influenced on the data. Generally, biomarkers response in crabs collected during winter were much higher than those collected during summer, showing that seasonality strongly influences on the responses of the biomarkers in crabs from CIP-PA.

Animals from the stations PM and A (summer) and VG, PT and P (winter) presented higher biomarkers expression. Posterior gills tended to present a higher activity of antioxidant enzymes when compared to anterior gills. A similar result was noticed in the estuarine crab *C. granulata* (Oliveira *et al.*, 2005). Most of regulating crabs have clear functional differences towards anterior and posterior gills, being the first one responsible for respiration (gas exchange), and the second for transport of salt (osmoregulation) (Péqueux, 1995). Thus, enhanced activities of these enzymes could be expected in posterior gills, given the probable increased metabolism for hyper-osmoregulation (Van Horn *et al.*, 2010), especially in the summer, when the salinities were very low in most of the sampling sites. However, during winter both gills had almost the same pattern. Freire *et al.* (2011) also did not find a higher activity of antioxidant enzymes in the posterior gills when compared to anterior gills. In aquatic organisms, as crabs, gills represent the first barrier against xenobiotics.

GPx activity appears to be essential for marine animals, which have a large amount of unsaturated fatty acids in the composition of their biological membranes (Joseph, 1982). To avoid oxidative damage of tissues and lipids, *C. danae* maintains high activity of antioxidants enzymes, presenting high levels of GPx activity (Togni, 2007). In both seasons, this enzyme was associated to the metals and to LPO levels; such association may suggest that contamination by metals is activating the anti-oxidant system and causing lipid peroxidation in the tissues of *C. danae*. Studies have shown that, in response to metal exposure, there is an increased formation of ROS (Stohs and Bagchi, 1995; Silva *et al.*, 1999), which can provoke widespread damage to cell such as lipoperoxidation and genotoxicity. Therefore, the induction of antioxidant enzymes like GPx and GST is an important protective mechanism to minimize cell oxidative damage in polluted environments.

GSH is a major intracellular antioxidant in living organisms and the first line of defense against oxidative stress. It is also a central component in the cellular detoxification system that constitutes an important mechanism for cellular protection against agents, such as metals, that produce ROS (Lavradas *et al.*, 2014). This enzyme was associated with mud in all PCAs and caused lipoperoxidation in hepatopancreas in station PM. Hypoxic environment tend to diminish GSH levels, indicating that LPO in this case was not caused by low oxygen levels (Hauser-Davis *et al.*, 2014). Although, this enzyme has also the capacity to chelate metals (Sterling *et al*., 2010; Wang *et al*., 2008), inhibiting the detoxification activation system.

Togni (2007) reinforce the anti-xenobiotic function of GST, since only abiotic factors variation, without the presence of contaminants, tend to not affect the activity of GST. This was also reported by Fonseca *et al.* (2011), who discussed the effects of temperature and salinity and suggested that these biomarkers have higher specificity to chemical exposure, rather than variability due to environmental dynamics. In the winter, GST activity was increased in anterior gills (PT and P) and hepatopancreas (PT) of crabs from CIP-PA. GST activity can be induced by PAHs and PCBs exposure (Van der Oost *et al.*, 2003), but may respond to metals (Maria *et al.*, 2009), especially Cu and Cr, which are able to produce free radicals (Ercal *et al.*, 2001).

The present study showed AChE inhibition during summer, in comparison to winter results, for the animals of all the studied sites. AChE inhibition can reveal health impairment (Thi Tu, 2009) and has also the potential to serve as a biomarker of heavy metal pollution (Devi & Fingerman, 1995; Rodrigues & Pardal, 2014). There is also the possibility that the AChE depression observed could be related to difference in hormonal control, metabolic rate or animal movement (Payne *et al.*, 1996). Some authors suggest that complex mixtures of contaminants could be important sources of AChE-inhibiting compounds in the aquatic environment (Van der Oost *et al.*, 2003; Payne *et al.*, 1996).

Regarding crustaceans, specifically crabs, the excess of accumulated heavy metals tend to be eliminated to the environment by physiological processes (excretion) or during the molting process (Virga & Geraldo, 2008), as observed a decrease on Cd levels after molting process in shrimps (Keteles & Fleeger, 2001). *C. danae* performs ecdysis throughout the entire year, with higher activity in spring and summer; periods that are influenced by temperature and salinity (Shinozaki-Mendes *et al.*, 2014). Oxidative stress levels enhance thought the molt cycle and the risk of harmful effects of ROS is higher in the post molt (Vieira & Gomes, 2010). Thus the molting cycle can create metabolic stress (Kuballa *et al.*, 2011). In this sense, some differences observed for biomarkers between summer and winter could be due to this natural factor. Thus, for the proper use of crabs as biomonitors, further studies to understand how the molting cycle affects the biochemical responses are required.

Molting and reproductive cycles also may lead to low condition factors (CF) (Pinheiro and Taddei, 2005), since blue crabs do not feed during the period around the ecdysis (Hines *et al.*, 1987), or change their metabolism during reproduction. Studies found higher CF during the spring and lower from summer to winter, probably due to a higher energy intake as a preparation to the spawning period (Golodne *et al.* 2010; Pinheiro & Taddei, 2005). The blue crab has an extensive reproductive cycle, coincident with warmer periods (Branco, 1991). *C. danae* has continuous reproduction and molting process, thus CF of organisms with such characteristics appears not to be so influenced by reproduction and molt (Branco *et al.*, 1992). In general, the present study found higher values of CF during summer (March), except for PT and IC, supporting the idea that reproduction and molt does not meet in organisms with continuous process.

According to our results, blue crabs from the CIP-PA are capable to respond to metal contamination, trough different paths and tissues. Although the strong influence of seasonality on the biochemical responses, it is possible to observe that the biomarkers activity was more evident during winter, indicating a worsened environmental quality. Animals from PT and PM presented the worst conditions, reproducing the conditions in sediments, as these two sites were the most contaminated. Crabs from CIP-PA presented responses to GPx, GST, LPO and DNA damages, indicating that the levels of metals in CIP-PA are enough to produce negative responses in the native biota. Biomarkers analyses were capable to detect signs of environmental degradation in a moderately contaminated area.

General Conclusion

The CIP-PA region has been considered as a reference for many years by different authors. However, using different tools, it can be seen that the region has undergone trough some changes in their physical characteristics, exhibiting signs of metal contamination. The combination of biomarkers, toxicity tests and chemical analyses showed that the region is being affected by the input of metals trough the RIR. Sediments exhibit higher levels of metals, especially in the north of the CIP-PA, and also caused toxicity in laboratorial tests. In addition, some biomarkers of *C. danae* responded positively to the contamination, indicating that the native biota is being effected by the pollutants. As the contamination was associated to fine particles and organic matter, this study reinforces the evidences that the fine particles are the main carrier of metals from the RIR to the estuarine area. Additionally, this study evidenced that depositional areas appear to move towards south, within the estuary, depending on the freshwater inputs and the hydrological regime. Seasonality also affects the biochemical responses, and individuals collected in the summer tended to exhibit a better health. Geochemistry, toxicity and biomarkers agreed to indicate the stations PT and PM as those more affected. The results are of main concern, as the study area is a legally protected site, which is under risk due to the contamination originated from external sources. Then, designating an area as a PA cannot always ensure its protection and actions are needed to ensure control of both internal and external sources of contaminants for the CIP-PA.

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Appendix

Appendix A: Physical-chemical parameters of *Lytechinus variegatus*

Appendix A. Data of salinity, pH, dissolved oxygen (DO) and temperature (T°C) in the *Lytechinus variegatus* test. DO was measured at mg/L.

Summer				
	Salinity	pH	DO	T°C
VG	34	7.32	4.17	23±2
PT	34	6.40	3.50	23±2
P	35	7.40	3.39	23±2
PM	35	7.30	4.22	23±2
A	35	7.65	3.63	23±2
IC	35	7.66	3.92	23±2

Winter				
	Salinity	pH	DO	T°C
VG	35	5.84	4.16	23±2
PT	35	6.17	3.6	23±2
P	31	6.31	2.77	23±2
PM	35	6.33	3.34	23±2
A	35	6.43	3.51	23±2
IC	35	6.57	3.32	23±2

Appendix B: Physical-chemical parameters of *Nitocra* sp

Appendix B. Data of salinity, pH, dissolved oxygen (DO) and temperature (T°C) in the *Nitocra* sp. test. DO was measured at mg/L.

Summer								
	Initial				Final			
	Salinity	pH	DO	T°C	Salinity	pH	DO	T°C
VG	16	7.10	2.46	23±2	16	7.21	5.05	23±2
PT	15	6.24	1.97	23±2	15	4.14	3.65	23±2
P	17	6.98	2.45	23±2	16	7.47	4.30	23±2
PM	19	7.31	1.66	23±2	19	7.81	3.62	23±2
A	18	7.22	1.64	23±2	19	7.71	4.92	23±2
IC	18	7.29	1.56	23±2	19	8.01	4.42	23±2

Winter								
	Initial				Final			
	Salinity	pH	DO	T°C	Salinity	pH	DO	T°C
VG	17	7.66	4.69	23±2	18	7.36	6.45	23±2
PT	17	4.65	4.47	23±2	16	7.43	4.9	23±2
P	18	6.89	4.34	23±2	17	7.52	5.65	23±2
PM	19	7.04	3.15	23±2	19	7.3	4.1	23±2
A	19	7.09	3.84	23±2	19	7.26	5.2	23±2
IC	19	7.01	3.15	23±2	20	7.21	4.71	23±2

Appendix C: Physical-chemical parameters of *Tiburonella viscana*

Appendix C. Data of salinity, pH, dissolved oxygen (DO) and temperature (T°C), in the *Tiburonella viscana* test. DO was measured at mg/L.

	Summer							
	Initial				Final			
	Salinity	pH	DO	T°C	Salinity	pH	DO	T°C
VG	31	7.82	4.61	25°±1	31	7.82	5.75	25°±1
PT	31	7.80	5.38	25°±1	32	4.09	6.21	25°±1
P	32	7.60	6.08	25°±1	35	8.02	6.28	25°±1
PM	35	7.77	5.31	25°±1	37	7.89	6.34	25°±1
A	35	7.69	5.13	25°±1	35	8.05	6.09	25°±1
IC	35	7.56	4.86	25°±1	36	8.25	5.84	25°±1

	Winter							
	Initial				Final			
	Salinity	pH	DO	T°C	Salinity	pH	DO	T°C
VG	31	7.67	6.07	25°±1	34	8	6.23	25°±1
PT	30	7.36	5.42	25°±1	30	6.61	5.75	25°±1
P	32	7.61	5.85	25°±1	32	8	6.28	25°±1
PM	32	7.54	5.61	25°±1	34	8.19	6.21	25°±1
A	33	7.31	5.85	25°±1	34	7.99	6.09	25°±1
IC	34	7.62	5.78	25°±1	34	7.99	5.94	25°±1

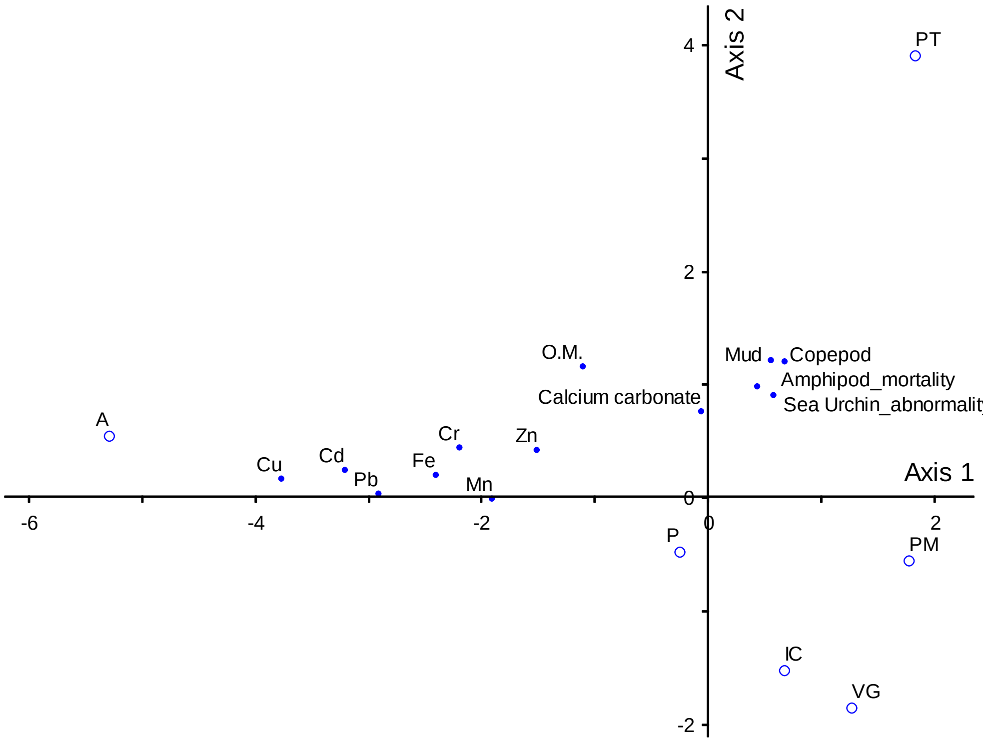
Appendix D: Biometric data of *Callinectes danae*

Appendix D. Data of carapace width, weight and sex of collected *C. danae* for biomarkers analyzes. CW means carapace width, W is weight and S is for sex, being M for male and F for female.

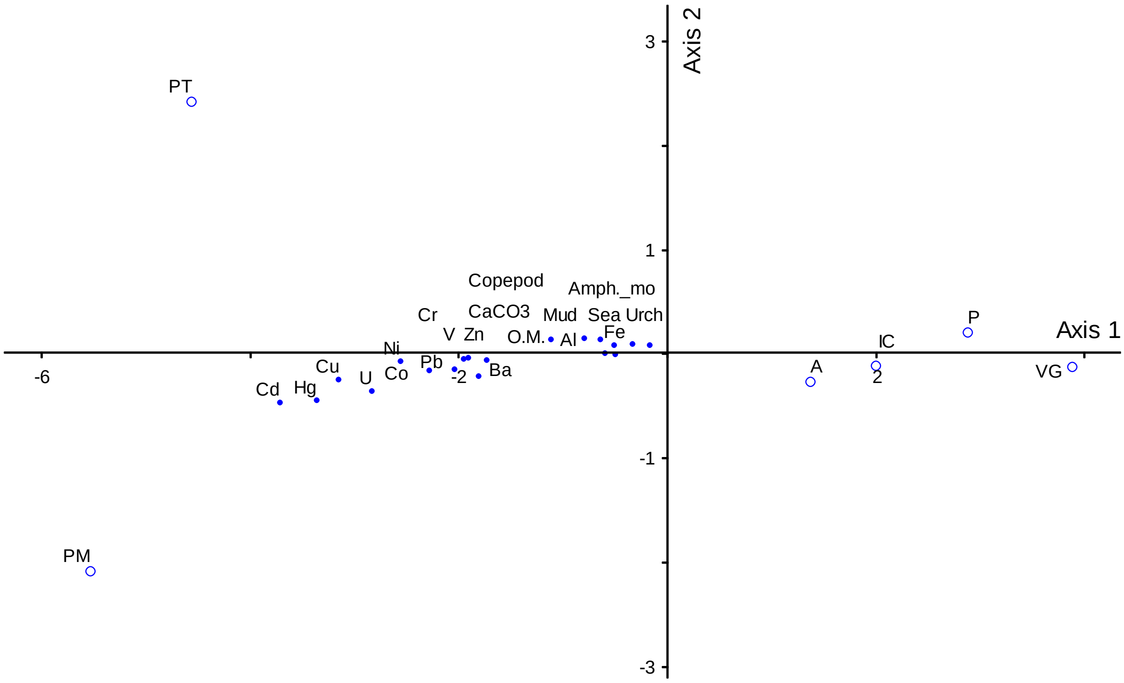
SUMMER								
	CW (mm)	W (g)	S	W/CW	CW (mm)	W (g)	S	W/CW
Number of individuals		VG				PM		
1	9.55	96.02	F	10.1	8.11	70.53	M	8.7
2	11.5	167.3	M	14.5	7.24	48.82	M	6.7
3	9.58	107.96	M	11.3	7.54	56.11	M	7.4
4	11	122.52	M	11.1	7.1	45.25	M	6.4
5	10.3	123.65	M	12.0	7.74	59.12	M	7.6
6	10.12	113.77	M	11.2	7.53	51.94	M	6.9
7	10.69	119.2	F	11.2	6.79	41.45	M	6.1
8	9.71	95.66	F	9.9	9.47	99.39	F	10.5
Average	10.31	118.26		11.41	7.69	59.08		7.55
Standart deviation	0.71	22.63		1.45	0.82	18.61		1.44
Number of individuals		PT				A		
1	9.44	110.92	M	11.8	7.36	59.19	M	8.0
2	9.12	97.08	M	10.6	6.7	41.05	F	6.1
3	8.73	76.97	M	8.8	7	44.14	F	6.3
4	7.9	59.01	M	7.5	6	28.56	F	4.8
5	8.26	57.04	F	6.9	6.14	32.35	M	5.3
6	7.4	46.27	M	6.3	7.1	41.26	F	5.8
7	7.97	56.71	M	7.1	6.5	31.49	M	4.8
8	7.13	38.1	F	5.3	6.08	28.74	F	4.7
Average	8.24	67.76		8.04	6.61	38.35		5.74
Standart deviation	0.81	25.25		2.21	0.51	10.38		1.12
Number of individuals		P				IC		
1	9.78	121.85	M	12.5	9.2	110.58	M	12.0
2	10.22	132.24	F	12.9	6.4	31.12	F	4.9
3	9.33	107.7	F	11.5	6.2	29.83	M	4.8
4	8.44	65.47	F	7.8	7	39.4	F	5.6
5	10.45	120.19	M	11.5	7.3	41.55	F	5.7
6	10.76	143.14	M	13.3	6.2	25.71	F	4.1
7	8.55	79.98	M	9.4	6.37	28.77	M	4.5
8	7.26	47.23	M	6.5	6.91	34.29	F	5.0
Average	9.35	102.23		10.67	6.95	42.66		5.83
Standart deviation	1.19	34.19		2.52	1	27.96		2.55

WINTER								
	CW (mm)	W (g)	S	W/CW	CW (mm)	W (g)	S	W/CW
Number of individuals		VG				PM		
1	8.1	54.51	M	6.73	6.9	40.24	F	5.83
2	7.6	46.44	F	6.11	6.16	26.7	F	4.33
3	10.1	132.7	M	13.14	6.14	25.64	F	4.18
4	9.64	105.83	F	10.98	7.1	33.33	M	4.69
5	11.2	156.4	M	13.96	8	63.43	M	7.93
6	9.95	117.78	M	11.84	5.55	21.01	F	3.79
7	8.1	55.94	M	6.91	7.88	64.17	M	8.14
8	11.1	148.46	M	13.37	6.4	30.78	F	4.81
Average	9.47	102.26		10.38	6.77	38.16		5.46
Standart deviation	1.39	44.38		3.28	0.87	16.82		1.7
Number of individuals		PT				A		
1	8.6	61.75	F	7.18	7.6	53.76	M	7.07
2	8	59.17	F	7.40	5.6	17.07	F	3.05
3	11.5	179.76	M	15.63	6.2	29.97	M	4.83
4	8.4	66	F	7.86	7	40.04	F	5.72
5	9.1	75.01	F	8.24	4.95	11.9	M	2.40
6	7.94	52.08	M	6.56	5.55	18.51	F	3.34
7	9.18	86.18	M	9.39	7.63	54.27	M	7.11
8	8	70.81	M	8.85	8.55	86.94	M	10.17
Average	8.84	81.35		8.89	6.64	39.06		5.46
Standart deviation	1.18	41.11		2.87	1.25	25.26		2.61
Number of individuals		P				IC		
1	7.75	55.99	M	7.22	7	38.58	F	5.51
2	8.4	74.32	M	8.85	5.5	20.57	M	3.74
3	8.66	82.17	M	9.49	6	25.94	F	4.32
4	5.3	16.75	F	3.16	5.58	18.32	F	3.28
5	5.3	16.09	F	3.04	6.3	25.9	F	4.11
6	5.95	26.04	M	4.38	6.5	28.38	M	4.37
7	11.6	188.77	M	16.27	5.84	23.52	F	4.03
8					4.5	11.17	M	2.48
Average	7.57	65.73		7.49	5.9	24.05		3.98
Standart deviation	2.28	60.58		4.68	0.75	8		0.88

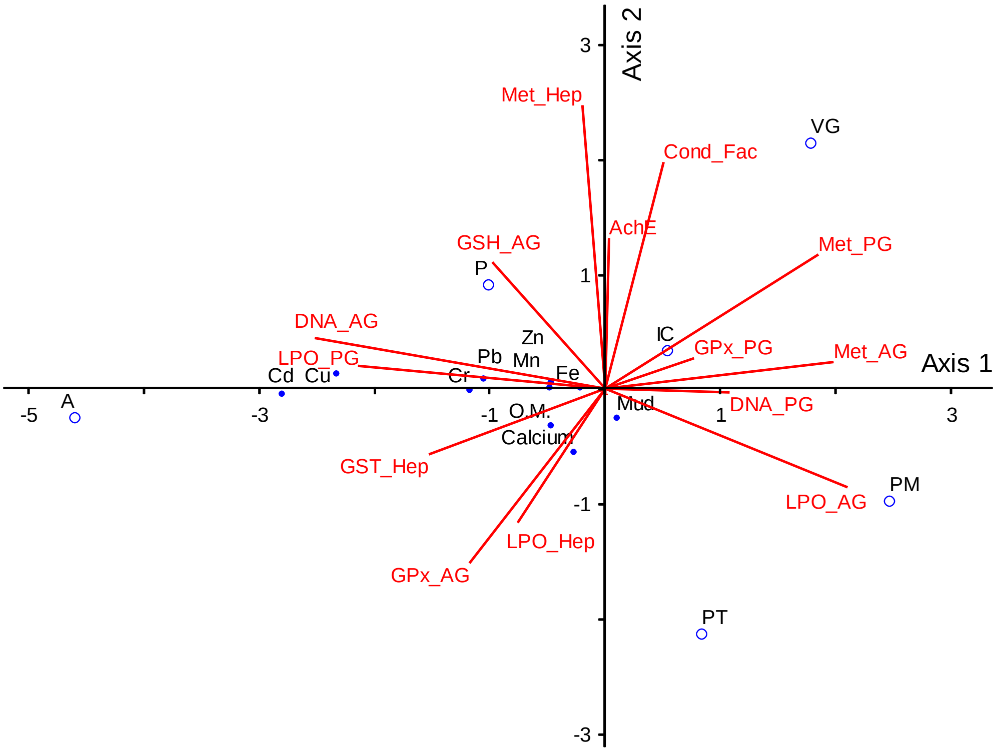
Appendix E: Bi-dimensional distribution of PCA results using geochemical and ecotoxicological data from summer analyzes (Cruz, 2014) from sediments from CIP-PA (PC1 x PC2).



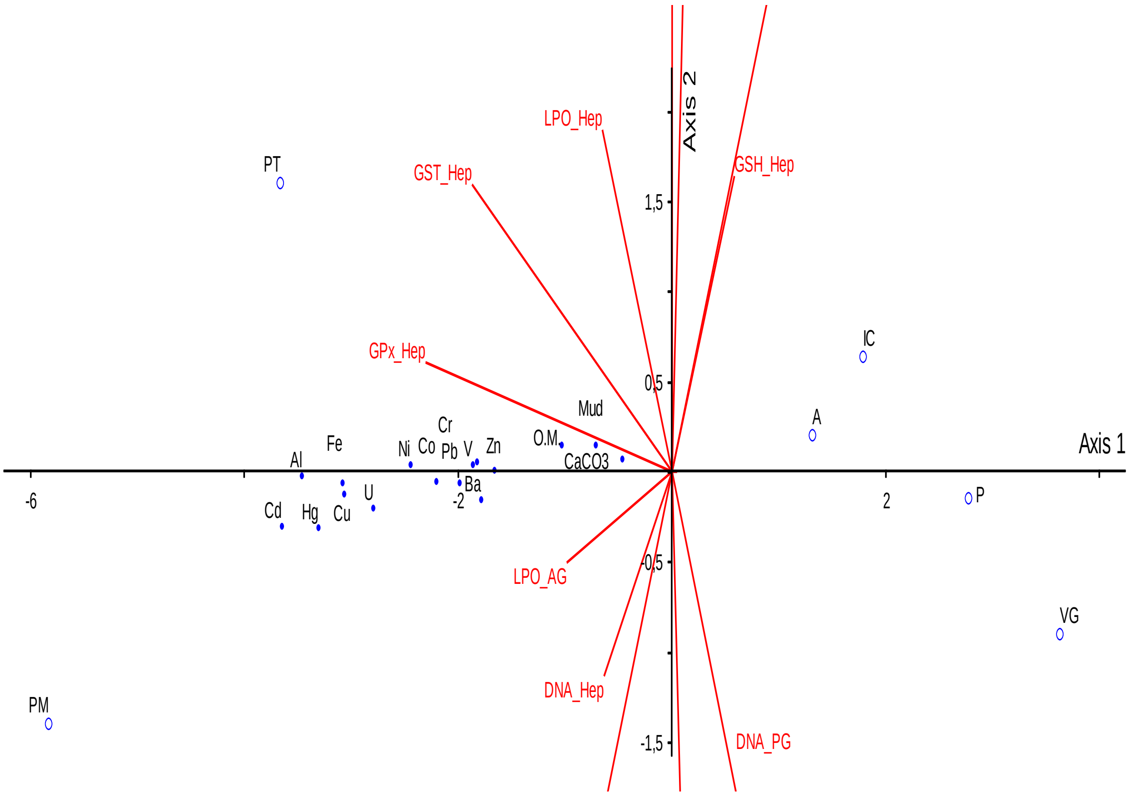
Appendix F: Bi-dimensional distribution of PCA results using geochemical and ecotoxicological data from winter analyzes from sediments from CIP-PA (PC1 x PC2).



Appendix G: Bi-dimensional distribution of PCA results using sediment geochemistry and biochemical biomarkers data from summer sediments from the CIP-PA (Axis 1 x Axis 2). Metals are from Cruz (2014).



Appendix H: Bi-dimensional distribution of PCA results using sediment geochemistry and biochemical biomarkers data from winter sediments from the CIP-PA (Axis 1 x Axis 2).



Appendix F: Decision Matrix - Data of biochemical biomarkers, metal concentration and toxicity from summer sampling.

Summer							
		VG	PT	P	PM	A	IC
Posterior gills	GPx						-
	GST			x			-
	GSH	x	x	x	x	x	-
	Metallo						-
	LPO						-
	DNA						-
Anterior Gills	GPx						-
	GST						-
	GSH						-
	Metallo						-
	LPO						-
	DNA						-
Hepatopancreas	GPx					x	-
	GST						-
	GSH				x		-
	Metallo						-
	LPO				x	x	-
	DNA						-
Summary Biomarkers	Biomarkers of exposure	1	1	2	2	2	-
	Biomarkers of effect	-	-	-	1	1	-
	Metal Concentration	Moderate	High	Low	High	Low	Low
	Acute Toxicity		X				
	Chonic Toxicity	X	X	X			

Appendix G: Decision Matrix - Data of biochemical biomarkers, metal concentration and toxicity from winter sampling.

Winter							
		VG	PT	P	PM	A	IC
Posterior gills	GPx	x	x				-
	GST				x		-
	GSH	x	x				-
	Metallo			x			-
	LPO				x		-
	DNA						-
Anterior Gills	GPx		x	x		x	-
	GST		x	x		x	-
	GSH						-
	Metallo			x			-
	LPO	x	x			x	-
	DNA						-
Hepatopancreas	GPx						-
	GST	x	x				-
	GSH						-
	Metallo						-
	LPO		x				-
	DNA						-
Summary Biomarkers	Biomarkers of exposure	3	5	4	1	2	-
	Biomarkers of effect	1	2	-	1	1	-
	Metal Concentration	Low	High	Low	High	Moderate	Moderate
	Acute Toxicity		X				
	Chonic Toxicity		X	X	X	X	