Faculty of Science and Technology

Biomonitoring and risk assessment tools to manage impact of diesel oil in tropical coastal habitats

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BIOMONITORING AND RISK ASSESSMENT TOOLS TO MANAGE IMPACT OF DIESEL OIL IN TROPICAL COASTAL HABITATS

ADRIANA E. SARDI

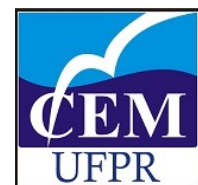## THESIS FOR THE DEGREE OF PHILOSOPHIAE DOCTOR

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"La felicidad es como un mosquito al cual uno quiere matar... primero lo ves, no lo pierdes de vista y luego comienzas a seguirlo pero si lo pierdes de vista te enojas contigo mismo porque no lo has matado y con certeza el mosquito te picará de nuevo; pero, si lo matas, te sientes como un héroe indestructible, en fin, el más fuerte e inteligente de la batalla.

Así veo la felicidad. Creo encontrármela por ahí volando, la persigo sin cesar, pero cuando la atrapo me doy cuenta de que como los mosquitos no es la única que se encuentra por ahí volando, hay mas…

En fin, ¿cómo se cual es el "mosquito" que me dará mas satisfacción matar?"

— Myself (August, 2007)

Creo que nunca podría responder esta pregunta existencial, pero estoy segura de que este trabajo es uno de los mosquitos, ahora a buscar y matar al siguiente…

SUMMARY

The focus of this work is in developing biology–based tools for environmental monitoring and risk assessment associated with diesel oil contamination in tropical coastal habitats. Prediction of impacts is generally conducted via environmental monitoring, in which environmental quality over time and space is assessed by repeated observations. Prediction of risk is included within the risk assessment process, which is the procedure that estimates the likelihood or the actual adverse effects caused by anthropogenic activities on ecosystems. During the past decades, oil production has increased, and so has the risk of oil pollution, either through produced water discharges, accidents, or other diffuse sources. This risk is notably high in tropical and subtropical areas, which represent around 60% of total global oil production. Petroleum is composed of a mixture of various mono– and polycyclic–aromatic hydrocarbons, toxic chemicals consisting of two or more fused benzene rings. The mode of action of PAHs is classified as narcotic, meaning that PAHs are expected to penetrate cell membranes and alter the lipid bilayer, ultimately disturbing the normal function of cells. On average, nearly 85% of the total petrogenic PAH input to the marine environment origins from petroleum consumption or diffuse sources. Among marine coastal habitats, tropical and subtropical coastal regions are home to speciose and highly productive ecosystems. Estuaries are among the most productive of marine ecosystems and are areas with high economic and ecological importance. Being economic centers for coastal communities that harvest biotic resources, tropical and subtropical estuarine intertidal environments (i.e. mangroves, salt marshes, and unvegetated tidal flats) are particularly susceptible to anthropogenic disturbance. Specifically, chronic diesel oil contamination that leaks from marine vessels poses a real risk to the species inhabiting the Paranaguá Estuarine System (PES) in southern Brazil, which host the third largest harbor of Brazil, and receives around 200 ships per month. Oil contamination from such diffuse sources, is an untraceable chronic source of contamination that can occur anywhere a ship travels and may have different effects, depending on the physical–chemical characteristics of the environment into which the oil is released. Therefore, tools for biomonitoring the effects of short and long–term exposure to diffuse oil contamination are much needed. The general objectives of this work are to validate the use of antioxidant biomarkers as tools for biomonitoring coastal estuarine habitats in Brazil, as also to compare the sensitivity and risk assessment metrics from species distributed from subtropical, temperate and Arctic regions exposed to a toxic PAH.

Biomarkers are defined as measures of exposure or effect expressed at the sub–organism level (i.e. biochemical, cellular, physiological or behavioral) in taxa under environmental stress. We proposed the use of antioxidant biomarkers as sub-lethal measures of exposure at the sub-organism level. Before implementing antioxidant biomarkers in biomonitoring programs, several conceptual and methodological issues needed to be addressed. Namely, it is important to determine their basal levels of activity, to select an appropriate sentinel species for their measurement, and to determine the best group of biomarkers for a multi–biomarker approach. Also, it is necessary to establish a correlation between the presence of diesel oil contamination and the activity of selected biomarkers. This work addresses these points, first by conducting a seasonal baseline of biomarker values, and then by performing experimental manipulations both in the lab and the field. Because the activity of antioxidant enzymes is involved in cell homeostasis, they are expected to vary in relation to reproductive cycles, food availability, and environmental drivers. Thus an initial screening in the activity of 5 different subtropical species was conducted at two seasons (austral winter and austral summer) at two different locations that have different levels of organic and PAH contamination. Then, experimental manipulations that tested the correlation between the antioxidant response and diesel oil exposure were conducted. The first experiment characterized the antioxidant biomarker response in two common species under laboratory conditions; while in the second experiment, the antioxidant biomarker response in the clam species *Anomalocardia flexuosa* was evaluated after chronic exposure to diesel oil *in situ*. The significant changes in the biomarkers activities following exposure suggested a causal relationship between biomarkers and diesel oil contamination, with the activities of GST and SOD being the most sensitive to experimental manipulations. These cause–effect relationships indicate that it is possible to use these biomarkers as tools in biomonitoring programs at PES. However, it was noticeable that natural variability is a major confounding source of variation, which in our experiments was handled by including appropriate control treatments for comparing the response from the experimental treatment with that from natural conditions. As part of the outcomes of this work, a guiding framework for selecting biomarkers and testing their causal relationship to contamination and specific recommendations for designing experiments for biomonitoring purposes are provided. Briefly, well–designed experiments have a clear hypothesis to test, for which the measurement of environmental parameters at an adequate sampling intensity is feasible, given financial and logistic constraints. The statistical power of the design must be

considered before starting sampling and the design should include spatial and temporal variability. Regarding differences in risk assessment metrics following the exposure to 2–Methylnaphthalene, our results indicate that No–Effect Concentration (NEC) values —concentration thresholds use to assess species sensitivity to toxic exposure— were not significantly different among the studied species and differences among regions were not identified. However, when defining sensitivity as the time to observe an effect —a metric that includes the NEC and a toxicokinetic parameter like the elimination rate— differences in sensitivity among regions were detected. In summary, species from Arctic to subtropical regions have similar NEC thresholds, but the time they need to reach that threshold varies, and this variation is related to taxonomy and trophic level. Arctic species had on average shorter times for starting to show an effect, followed by subtropical and finally temperate species. Our results suggest that assuming that species sensitivities from Arctic, and temperate regions is sufficiently similar to those from subtropical regions might be incorrect. We suggest that in in the search for metrics for safeguarding the marine ecosystem, attention should not be given only to concentration thresholds. Concentration thresholds might be providing assessors an inaccurate metric for species sensitivity, which is ultimately underestimating the risk to marine and estuarine ecosystems.

CONTENTS

LIST OF PAPERS

Paper I

Sardi, A. E., Renaud, P. E., Lana, P., C. & Camus, L. (2016). Baseline levels of oxidative stress biomarkers in species from a subtropical estuarine system (Paranaguá Bay, southern Brazil). Marine Pollution Bulletin, 113(1-2), 496-508.

Paper II

Sardi, A. E., Sandrini-Neto, L., da S. Pereira, L., Silva de Assis, H., Martins, C. C., Lana, P. C. & Camus, L. (2016). Oxidative stress in two tropical species after exposure to diesel oil. Environmental Science and Pollution Research, 23(20), 1-11.

Paper III

Sardi, A. E., Renaud, P. E., Morais, G. C., Martins, C. C., Lana, P. C. & Camus, L. Effects of an *in situ* diesel oil spill on oxidative stress in the clam *Anomalocardia flexuosa*. Manuscript.

Paper IV

Sardi, A. E., Augustine, S., Morais, G. C., Olsen G. H. & Camus, L. Exploring species sensitivity to a model hydrocarbon, 2–Methylnaphthalene, using a process–based model. Manuscript.

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1. GENERAL INTRODUCTION

In 2014, Brazil was the third–largest energy consumer in the Americas, following the United States and Canada. Additionally, Brazil's energy consumption has nearly doubled in the last decade as a consequence of sustained economic growth.[1] Brazil also contains significant oil reserves, which are estimated at 15 billion barrels of oil, the second–largest reserves in South America after Venezuela.[1] The accelerated increase in energy demands has launched the rapid development of the Brazilian oil industry and led to the long–term national goal of increasing domestic oil production. As a result, Brazil's oil production has grown 9.5% from 2013 to 2014, with a production of 2.95 million barrels per day (b/d). Brazil's consumption of petroleum and other liquid fuels continues to surpass its production, though projections from the U.S. Energy Information Administration (EIA) indicate that production in 2016 will exceed consumption for the first time since 2008. Recent increases in offshore petroleum production in Brazil has also increased ship traffic, and with it, the risk of oil spills in the coastal waters, especially in regions close to main harbors.[2,3]

The tropical and subtropical coastal waters, including those of Brazil, possess highly diverse and productive ecosystems, including mangrove forests, seagrass meadows, and coral reefs.[4] Estuaries are among the most productive of marine ecosystems[5] and are areas with high biodiversity that act as nursery habitats for many species. Also, they usually are economic centers for coastal communities that harvest biotic resources and profit from the numerous services provided by this ecosystem. Protecting land masses from the open ocean, disturbance regulation, nutrient cycling, recreation, and harvesting of food and raw material are among the services rendered by these important ecosystems.[4,6]

The variety of services provided by tropical and subtropical ecosystems highlights concerns that disruption of ecological processes affects not only the resident organisms; but also the human populations that depend on them for food, recreation, and protection. Estuarine intertidal environments (i.e. mangroves, salt marshes, and unvegetated tidal flats) are particularly sensitive to anthropogenic organic contamination, and their deterioration can have severe economic consequences, especially in areas vulnerable to tropical storms, hurricanes, or typhoons[4] stressing the necessity for developing effective monitoring tools for petrochemical contamination.

Petroleum is mostly composed of a mix of various mono and polycyclic aromatic hydrocarbons. Polycyclic aromatic hydrocarbons (PAHs) are chemicals made of two or more fused benzene rings. A variety of mechanisms may form PAHs. Pyrolysis is a rapid and incomplete combustion of organic materials that requires high temperatures (700 °C). Petrogenesis is a slow rearrangement and transformation of biogenic organic materials at moderate temperatures (100-300 °C). Diagenesis is the conversion of certain organic compounds in soils and sediments, and biogenesis refers to the direct biosynthesis of PAHs by organisms.[7]

Petroleum input to the sea can be categorized into four major groups, natural seeps, petroleum extraction, petroleum transport and oil consumption.[8] Natural seeps are responsible for 45% of oil entering waters worldwide. Among anthropogenic sources, extraction and transportation account for roughly 5% and 22% respectively. By 2003, the oil input from oil shipment was estimated to be 150,000 tons. Moreover, and contrary to spills occurring during extraction, contributions due to transport can occur anywhere tanker vessels travel and may have different effects that depend on the type of environment to which it is spilled.[8] Because toxicity of petroleum components greatly varies regarding water salinity and temperature, some environments may be more vulnerable to oil contamination than others.[7] Spills occurring during transportation activities may release a broad variety of oil products (i.e. gasoline, diesel, crude oil) each of which contains different concentrations of toxic compounds, like PAHs, and behaves differently in the environment.

The vast majority of petrogenic PAHs releases into the marine environment are due to petroleum consumption. Examples are, for instance, leakage from car and boat, discharges of treated or untreated ballast water, runoff from paved roads, and municipal sewage treatment plants.[7] On average, these diffuse sources contribute to nearly 85% of the total petroleum input accounted to anthropogenic sources.[8] Spills and other accidents at harbor terminals also contribute to coastal oil contamination and their risk of occurrence highlights the ecological and socio–economic problems inherent to petrochemical contamination in harbor areas.

Once in water, the behavior of PAHs is determined by their physical/chemical properties. PAHs of environmental concern span a broad range of solubilities, associated with their molecular weight and their degree of alkylation. The aqueous solubility of PAHs is approximated by the octanol/water partition coefficient (K_{ow}), a ratio that describes the

chemical concentration in the octanol phase to its concentration in the aqueous phase. K_{ow} varies with the PAH molecular weight and tends to increase with increasing molecular weight. Low molecular weight PAHs like naphthalene, alkyl naphthalenes, fluorene, and phenanthrene tend to evaporate from water, while the partition between water and colloidal phases of higher molecular weight PAHs depends on their organic carbon/water partition coefficient (K_{oc}s), which is proportional to the K_{ow}.[7]

PAHs often binds to colloids, small particles in diameter (0.1 to 0.2 μm) that cannot be sedimented by centrifugation. Organic colloids are derived from the degradation of bacteria, plants, and animals, and have a high affinity for adsorption and binding of nonpolar organic chemicals such as PAHs. High concentrations of dissolved organic carbon, including colloids, from terrestrial and salt marsh origin, can enhance the solubility of PAHs and increase their concentration in the water phase, especially in estuarine regions.

Particle size also has an effect on the adsorption of PAHs. The largest fraction of PAHs (usually high molecular weight PAHs) is associated with the large particulate fraction (>1.2 μm) whereas the fine particulate fraction, and the colloidal fraction present lower concentrations of PAHs. PAHs that are tightly bound to particles are generally inert, have low mobility, bioavailability and toxicity and do not partition into the water phase.[7]

Salinity and temperature also have an effect on PAHs solubility. Solubility tends to decrease with increasing salinity and increase with higher temperatures linearly. In this line, low salinity and high-temperature scenarios as found in tropical estuaries tend to have a high solubility of PAHs. These salinity and temperature relationships with PAHs solubilities have significant consequences on the partitioning between dissolved and sorbed phases in marine and estuarine environments. For instance, adsorption increases as salinity increases and temperature decreases.[7]

2. BIOLOGICAL EFFECTS OF OIL AND PAH CONTAMINATION

Acute effects of oil spills are mainly related to physical interactions between the organisms and oil. Following oil spills, direct contact with oil can smother invertebrate species while physical coating in birds and mammals can affect their insulating qualities, leading to hypothermia and eventual death. Further, oil contamination can directly kill the animals through asphyxiation, or poisoning following the exposure of oil water–soluble components.[9]

Among the toxic compounds associated with oil, PAHs are of greatest concern given their large physical–chemical properties, bioavailability, and toxicity. PAHs can be taken by marine organisms directly from the water column, sediments or through ingestion of food.[7] PAHs are narcotic compounds and are expected to penetrate the lipid bilayer region of membranes and alter lipid proteins. Denaturalization of the membrane structure affects its properties and disturbs the normal function of the cell.[10]

Prolonged exposure to PAHs contamination could reduce the fitness of marine populations, directly threatening species abundance, richness, and survival, and ultimately affecting the ecological structure and functioning of marine ecosystems. However, impacts at the population level usually manifest only after longer periods of time, when the effect has gone beyond remedial and recovery action.[11] Because deleterious effects on populations are often difficult to detect, research aiming at the establishment of early–warning signals, capable of reflecting adverse and sublethal biological responses towards anthropogenic contamination, has been the focus for many years.

Biomarkers were developed as tools to detect sublethal effects of pollutants in exposed organisms. The term biomarker refers to measures of exposure that are expressed at the sub–organism level, such as biochemical, cellular, physiological or behavioral variations that can be measured in tissue or body fluid samples of organisms. [12,13] Biomarkers provide evidence of exposure or effects from one or more contaminants. In this sense, useful biomarkers are sensitive indicators of sublethal ecological effects of pollution and provide warning for the occurrence of deleterious effects at higher levels of biological organization, i.e. at the community and ecosystem level.[12]

Long–term chronic exposure to oil and derivatives is associated with sublethal effects and can be assessed with biomarkers. Biomarkers that have been investigated more extensively include enzymes involved in the detoxication of toxic compounds and

their metabolites —the biotransformation enzymes— and the antioxidant enzymes involved in the oxy–redox system.

Biotransformation of xenobiotic refers to the enzyme–catalyzed process where a xenobiotic compound is converted to a more water–soluble form, which can be excreted more quickly.[11] Biotransformation can be simplified and subdivided into three phases. Phase I biotransformation is characterized by reactions that involve oxidation, reduction or hydrolysis of the xenobiotic. In phase II, conjugation of phase I products occurs, after which catabolization of the conjugated metabolites is performed by phase III enzymes.[11]

2.1. MECHANISMS OF ROS TOXICITY

Reactive oxygen species (ROS) result from the partial reduction of oxygen during its tetravalent reduction to water, coupled with the oxidation of food and the production of energy in aerobic organisms. These partially reduced species comprise both radical and non–radical species. The former include the superoxide anion radical ($O_2 \cdot -$), hydroxyl radical ($OH \cdot$), peroxyl radical ($RO_2 \cdot$), alkoxyl radical ($RO \cdot$), and hydroperoxyl radical ($HO_2 \cdot$). Non–radical species consist of hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), singlet oxygen and peroxynitrite ONOO- a reactive nitrogen species.[14]

Regular ROS production in animal systems accounts for 1-3 % of total O_2 consumed. These unwanted species are continuously produced in animals mainly as by–products of various endogenous processes, like the activity of certain enzymes, auto–oxidation; and membrane electron transport from the mitochondria, the endoplasmatic reticulum, and nuclear membranes. Properties and reactivity of the different ROS differ substantially. The hydroxyl radical $OH \cdot$ has a lifetime of a few nanoseconds and it is the most important free radical of biological and toxicological relevance, capable of reacting instantly and indiscriminately with virtually all organic molecules.[14,15] On the other hand, neither the superoxide anion radical nor hydrogen peroxide are considered highly reactive in aqueous solution, but their production is intimately correlated with the production of more damaging species such as $OH \cdot$. Thus, $O_2 \cdot -$ can dismutase to H_2O_2, with the reaction catalyzed by the superoxide dismutase enzyme (SOD). In the presence of an appropriate redox cycling catalyst (such as iron–chelate), $O_2 \cdot -$ and H_2O_2 can react to yield $OH \cdot$ via the Haber–Weiss reaction.[14] Uncontrolled oxidation by the highly reactive $OH \cdot$ radical would ultimately promote cellular damage through protein oxidation, lipid peroxidation and DNA damage.[16]

2.2. BIOMARKERS OF OXIDATIVE STRESS

Sublethal toxic effects of PAHs are also linked to the production of reactive oxygen species (ROS). ROS are oxygen free–radicals or oxyradicals, mostly generated as a by–product of biotransformation phase I enzyme reactions. ROS are highly reactive chemical elements that will bind to biomolecules causing their oxidation and promoting structural and functional changes at the subcellular and cellular level. ROS reactivity can damage DNA causing strand breakage, and oxidate lipids from cellular and subcellular organelles, which affects membrane permeability and decreases lysosomal stability, which could presumably release damaging hydrolytic enzymes to the cytoplasm.[14] Therefore, aerobic organisms have developed a suite of antioxidant defense mechanisms to neutralize ROS and avoid oxidative damage. Antioxidant defenses include low molecular weight free–radical–scavengers like vitamin A, E, and C, reduced glutathione (GSH) and carotenoids, and specific antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). Altogether, these mechanisms are expected to maintain the redox homeostasis, which is essential for the physiological health of organisms.[15,17]

When ROS production overcomes antioxidant defenses, leading to increased oxidative damage to macromolecules and alterations in critical cellular processes, it is said that the organism is suffering from oxidative stress. To date, several works have demonstrated that a wide range of natural and man–made xenobiotics can induce ROS production.[14] Anthropogenic–related compounds capable of inducing ROS production include organic contaminants such as redox cycling compounds (quinones, nitroaromatics, nitroamines, bipyridyl herbicides), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, dioxins and pentachlorophenol, metal contaminants peroxides, UV–radiation, hypoxia and hyperoxia.[14]

As a proxy for sublethal effects of pollutants on marine organisms, the activity of antioxidant enzymes as biomarkers of oxidative stress has been proposed.[18] In this sense, the induction of antioxidant enzymes is referred to as a counteracting response of exposed organisms, but the same antioxidants can be depleted when overwhelmed.[19] Therefore, both induction and inhibition in the activity of the antioxidant enzymes are referred to as oxidative stress. Conditions like the duration and intensity of the pro–oxidant stressor are the main explanatory drivers for opposite responses in the activity of antioxidant enzymes.[19]

3. ENVIRONMENTAL MONITORING

Environmental monitoring is the repeated observation and study of the environment. An environmental monitoring program describes the activities that are performed in order to assess and monitor the quality of an environment over time and space.[11] It includes the systematic sampling of air, water, soil and biota, which could increase the knowledge about processes occurring in the environment.[20]

Environmental monitoring is regularly performed to assess the effects of anthropogenic activities; and it includes different methods that differ on the object of observation and measurement; as chemicals concentration, chemical bioaccumulation, biological effect, organisms health, and ecosystem monitoring. Biological–effect monitoring assesses exposure and effects by determining early adverse alterations that in case the disturbance is removed, could be partly or entirely reversed to the initial undisturbed scenario.[11] In this sense, biomarkers in selected species have proven to be relevant early–sign indicators of deleterious pollution effects in marine organisms.[18,21–23]

In petroleum related activities, samples are taken on a regular basis to assess change, detect discharges, and record background (natural) variability. Also, monitoring can be conducted following major oil spills, thus providing a quantitative measure of the acute exposure to oil and the recovery of the environment over time. Alternatively, the effects of chronic exposure to oil and derivatives can also be monitored using biomarkers; as performed by the Norwegian Water Column Monitoring Program. In this program, changes in biomarkers from caged fish and blue mussels are used to evaluate the effects of discharges from oil platforms operating in the North Sea.[23]

Before implementing biomarker protocols in environmental monitoring programs, it is necessary to have a sound understanding of the mechanisms underlying their responses. This knowledge includes the identification of factors that can affect the enzymatic system and consequently interfere with the biomarkers signal. In other words, it is necessary to understand the natural variability occurring in the biomarkers response due to confounding variables. These variables can be biotic, such as the organism health, condition, age, reproductive and development status, etc.; or abiotic as temperature, salinity, hydrodynamics and heterogeneity of the environmental pollution.[11,18] Furthermore, the variability related to these confounding factors might change on temporal or spatial scales.[24]

3.1. MULTIVARIATE STATISTICS FOR INTERPRETATION OF BIOMONITORING DATA

No single biomarker can unmistakably measure environmental degradation and only a suite of biomarkers can allow to make a diagnostic about the health of the organisms and the environment. Moreover, biomarkers and environmental parameters (or confounding factors) often have different measuring units. Thus, for interpreting results with multiple variables a technique that can quantitatively and visually present the data is required. When following a multi–biomarker assessment, which is in essence a multidimensional problem, the logical continuation should be to implement multivariate analyses.[25]

Multivariate analyses provide the means to study and interpret the joint relationship of variables in data that contain intercorrelations.[26] Multivariate statistics are routine in ecological research, but they are not common in the ecotoxicology domain. While univariate analysis permits describing associations and relationships between individual variables, multivariate tools are a more powerful statistical mean that combines the effects of, for instance, several response variables (biomarkers) following pollutants exposure, or the combined effect of pollution and environmental variables.[27] A combined view of the biomarkers response ultimately helps with the interpretation and understanding of the data. Biomarker–based biomonitoring studies have traditionally made little use of such approaches, and only a few examples are available in the literature. For instance, multivariate analyses as multi–scaling ordination plots (MDS) and principal component analysis (PCA), have shown to be useful tools for differentiating sites that receive different loads of contamination in British estuaries.[27,28]

Interpretation of biomarker responses using univariate statistical techniques can be very complex. This complexity arises from the fact that pollutant stress triggers a cascade of biological responses, each of which can theoretically serve as a biomarker. Thus, biomarkers responses are often related one to each other, working towards reaching cell homeostasis. Also, for interpretation, comparisons between the biomarkers response and certain threshold, at which the pollutant–responsive biomarker deviates from the reference range of an unstressed scenario, are always performed. The magnitude of the effect is usually determined using univariate statistics, and conclusions about the health of the organisms are drawn based on significance levels. Also, the magnitude of the effect size on biomarkers following pollutants exposure greatly varies. Some, biomarkers increase their activity vs. basal levels in a range between 0-20% while the activity of others may vary by several hundred percents (see Paper II). Also, monitoring programs often include

comparisons of the measured endpoint on either spatial scales, from a reference site versus several contaminated sites, or temporal scales following a contamination event. In this case, many works have listed their major findings based on univariate pairwise comparisons,[29–32] even though these do not provide any indication of how the results can quantitatively discriminate between sites or years. Improper inferences about biomarker responses can lead to false conclusions about pollutant stress and environmental health. In summary, univariate statistics procedures are often inappropriate for visualizing and testing differences in the effect and degree of correlation of multiple biomarkers, or differences among sites exposed to different loads of contaminants.

Multivariate procedures are powerful tools for interpreting complex data, and the ecotoxicology field needs to adopt their use as a standard practice, especially for interpreting field–based data from ecotoxicological experiments that aim to discriminate sites along a contamination gradient.

3.2. INDICATOR SPECIES

In most cases, the intensity of the biomarker response, either natural or induced (i.e. following an exposure bioassay), is species–specific (see Paper I). Since monitoring the biomarker response of all the species is not feasible, the use of bioindicator species, taxa that effectively indicate environmental impacts, is widespread.[33]

Indicator taxa are species that respond to a given environmental condition in a manner that is representative for many other taxa in the community. They can be absent (showing low tolerance), or abundant (showing high tolerance) in impacted sites, accumulate contaminants within their tissues, or have a moderate tolerance to environmental variability and present a measurable response to stress.[33,34]

Many indicator species have been proposed for contamination monitoring, whereby the selection often based on the species' socio–economic and ecological attributes. Usually, the selection of indicator species favors those presenting sensitivity to contaminants, high availability along the year, sedentary behavior and habitat–specialism.[33] A good indicator taxon is abundant, can be found in the area subjected to the disturbance of interest, it is moderately tolerant to the contaminant, and has economic or ecologic relevance. Bivalves for instance, have been preferred targets in pollution monitoring studies, primarily because of their sessile lifestyle, high filtration capacity, ability

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to accumulate contaminants and are often commercial species harvested for human consumption.[35,36]

4. ENVIRONMENTAL RISK ASSESSMENT (ERA)

Risk assessment is the process of assessing magnitudes and probabilities of harmful effects of human activities or natural disasters.[11] Environmental risk assessment (ERA) refers to the procedure that estimates the likelihood of adverse effects caused by anthropogenic activities on ecosystems.[11]

The risk assessment process is divided into two sections: risk analysis, a more scientifically oriented process where the potential for a given situation is determined; and risk management, a more politically oriented phase, in which solutions to the problem are examined. Overall, the entire process consists of eight steps: hazard identification, effect assessment, exposure assessment, risk characterization, risk classification, risk–benefit analysis, risk reduction and monitoring.[11]

ERAs for the regulation of chemicals are routinely implemented to ensure the protection of the environment. Within the exposure assessment, the chemical concentration in air, water, soil or biota is measured or modeled. In the effect assessment step, the relationship between dose and length of exposure to a chemical is determined, and the severity of an impact is evaluated. This impact needs to be defined, and usually quantified as an endpoint. Endpoints of pollution of ecosystem relevance (at the population and community level) are meaningful for environmental assessment but are often inappropriate diagnostic tools for pollution impacts because of compensatory processes and adaptive mechanisms. On the other hand, suborganism–level measures, such as biomarkers, are more sensitive diagnostic tools towards pollution exposure, but their direct extrapolation to the population and community level remains poorly understood.[11]

Standard assessment endpoints for chemical risk assessment include effects on survival, growth, and reproduction. These are endpoints at the organism–level, also regarded as an intermediate point between community and sub–organism level endpoints. Based on the effects of chemical exposure on these variables, metrics of risk assessment, or thresholds of exposure like the no–effect levels for ecosystems are generated.[37] Parameters obtained for risk assessment are subsequently used in the risk management part to guide stakeholders and managers to create and implement solutions for environmental chemical pollution.

4.1. RISK ASSESSMENT METRICS

Currently, risk assessments include various metrics of species tolerance, such as the concentration causing lethality in 50% of exposed individuals (LC_{50}), and the no–effect concentration (NEC). These parameters are derived from standardized laboratory exposure protocols, for which the 96 h toxicity test is most commonly used.[38] In acute toxicity tests, biota is exposed to several different concentration of chemicals or chemical mixtures for 96 h.[39]

The NEC, also known as the incipient LC_{50}, represents the concentration of the chemical that does not cause any effects even after prolonged exposure.[40] It is the toxicological threshold below that an organism can be exposed at the infinite time, without an effect on its survival,[41] or as the equivalent to the concentration that does not cause mortality (LC_0) following prolonged exposure.[40]

The NEC is a time independent summary statistic, and as such, it is a more robust risk assessment metric than conventional statistic–based metrics, like the no observed effect concentration (NOEC).[42,43] Similarly, parameters obtained from the interpolation of descriptive regression models of the data, like the LC_{50} and the effect concentration (EC_X), are of limited use mainly because they change with exposure assay duration.[40] Moreover, the time variation observed in LC_{50} metrics is species and chemical specific, offering limited applicability to the metrics.

4.2. TOXICOKINETIC–TOXICODYNAMIC MODELS

Toxicokinetic–toxicodynamic models (TKTD models) simulate the processes that lead to toxicity over time in organisms. Toxicokinetics refers to the chemical uptake, biotransformation and elimination; while toxicodynamics is related to linking the internal concentration of a toxic chemical to its toxic effects which are observed at the individual level over time.[44]

As a first step to model the toxic effects of chemicals over time, parameters for the TK processes such as uptake, biotransformation and elimination rate constants need to be obtained. This can be done by measuring the time–course effect of the chemical of interest in the study species. Thereafter, toxicodynamic parameters, including hazard rate and killing rate are fitted using simulations of the previously estimated TK parameters.[45]

The before mentioned NEC and corresponding confidence intervals, can be estimated as a parameter within the TKTD General Unified Threshold Model of Survival model (GUTS).[44] GUTS is the simplest mechanistic model available that includes toxicokinetics and toxicodynamics.[46] GUTS–SIC–SD, where SIC–SD refers to the use of scaled internal concentration as dose metric, and SD refers to the stochastic death assumption, is also known in the literature as the DEBtox model for survival (i.e. see Klok et al.[47]). Within the model, three parameters that have a physiological meaning co–vary defining the TK–TD of the studied compound.

If one compartment first–order chemical–toxicokinetics is assumed, the model can be described as follows:

(1)
$$\frac{dC_i(t)}{dt} = k_i C_d(t) - k_e C_i(t)$$

where C_i is the internal concentration, C_d external concentration in the exposure media and, k_i and k_e refer to the intake and elimination rates.[44]

When internal concentrations are not available, survival data can provide information about the elimination rate, but not about the intake rate. Dividing both sides of equation 1 by the ratio of the rate constants k_i/k_e or the bioconcentration factor, can solve this problem, resulting in a *scaled* TK model, described by equation 2.

(2)
$$\frac{dC_i^*(t)}{dt} = k_e(C_d(t) - C_i^*(t))$$

When the scaled internal concentration exceeds certain threshold, we can expect to observe effects on survival, which are explained by the hazard rate (h_z). The model assumes that the hazard rate is proportional to the internal scaled concentration once a threshold, the no effect concentration (NEC), is surpassed. Below the NEC, no mortality occurs and under the stochastic death assumption, once the threshold is exceeded, the probability of an individual to die is assumed to increase linearly according to equation 3:

(3)
$$h_z(t) = k_k \max(0, C_i^*(t) - NEC) + h_b$$

where k_k is the killing rate, h_b is the hazard background and the max function selects the maximum between 0 and the difference between the scaled internal concentration and the NEC.[44]

In summary, the model consists of three time–independent parameters; the NEC, the elimination rate that describes when the equilibrium between internal and external concentration is set, and the killing rate, which represents the toxicity of the compound. The higher the killing rate is the more toxic the compound is.[46] Dynamic simulation models quantify toxicity, but because the parameters have a physiological meaning, they can also provide a conceptual framework to better understand differences in species sensitivities to the same chemical.[45,46]

4.3. SPECIES SENSITIVITY DISTRIBUTION (SSD)

Single–species metrics can be combined to predict concentrations affecting a community. Species sensitivity distribution curves (SSD) are routinely used in risk assessment and are generated by plotting risk assessment metrics, like NEC values, in a cumulative distribution function with ranked assigned percentiles.[48]

The SSD approach is based on the premises that at the community/assemblage level, species have different sensitivities to increasing concentrations of physical–chemical toxicants; and also that a range of representative species can adequately represent the whole community sensitivity to a chemical.[43]

By fitting several risk assessment metrics obtained from a variety of species to a statistical distribution, a prediction about the community sensitivity, the hazard concentration (HC$_p$) threshold is derived. The HC$_p$ represents the concentration at which certain percentage (p) of the assemblage of species is assumed to be affected by the chemical of interest.[43,48,49] For example, the HC$_5$ represents the concentration at which 5% of the species are affected; thus 95% of the species are being protected.

5. KNOWLEDGE GAPS AND WORK JUSTIFICATION

The likelihood of adverse effects caused by oil contamination in tropical and subtropical coastal habitats increases with the mounting production and transport of oil and derivatives in these regions. Brazilian marine waters contain significant petroleum resources capable of fuelling economic growth, and, thus, the risk of oil spills on the Brazilian coast is increasing. Also, Brazilian energy demands suggest that oil combustion and pollution from diffuse sources will continue to grow in the coming years. Despite this, there are still many unanswered questions regarding the impacts that such rapid development would have on the important marine ecosystems the Brazilian territory holds. Therefore, environmental managers need to put in motion mechanisms for oil–spill monitoring and risk assessment that could better protect the environmental health of tropical and subtropical coastal habitats.

Marine oil spills are often major environmental disasters where high amounts of liquid petroleum hydrocarbon enter the ecosystem. The effects of such accidents are associated with the physical interaction between marine organisms and oil and are notoriously detrimental. Following oil spills, physical coating by oil on animals has deleterious effects on animal health either by directly killing the animals through asphyxiation, or poisoning following exposure of water soluble oil components. However, a significant share of the total amount of hydrocarbons that enter the marine environment from human activities is from diffuse sources (i.e. oil that leak from marine vessels, runoff from paved roads, sewage, etc.). The impact from these sources is often disregarded and is hardly considered as an oil spill. Among the different sources of diffuse contamination, the diesel oil that leaks from marine vessels poses a real risk to the species inhabiting the Paranaguá Estuarine System (PES), which host the third largest harbor of the country. Such inputs of oil are often not considered as oil spills under the premise that the quantity spilled is very low and the concentration of toxic components of oil will rapidly decrease due to weathering process. However, leaks from marine vessels is an untraceable chronic source of contamination that can occur anywhere a vessel travels and may have different effects that depend on the physical–chemical characteristics of the environment into which is released. Therefore, tools for biomonitoring the effects of acute and chronic exposure to diffuse oil contamination are much needed.

Regardless their characteristics and their wide use in biomonitoring programs in European waters, there are several shortcomings that need to be addressed before

incorporating the use of antioxidant biomarkers into routine environmental monitoring at subtropical and tropical regions. These shortcomings are related to the effect that environmental variables have in their responses (which adds "noise" to their response); the uncertainties encountered when extrapolating from the suborganism–level to higher levels of biological organization; and finally, the challenges involved in hypothesis testing for causality between biomarkers response and pollutants exposure and results interpretation. In addition, the large number of species potentially affected by oil exposure makes risk assessment a real challenge. Current research indicates that differences in species sensitivities to narcotic compounds could be predicted based on the ecological traits of the affected species, and linked directly with the toxicokinetics of the chemical.[46]

In order to efficiently incorporate the use of biomarkers into biomonitoring practices in subtropical and tropical ecosystems, it is necessary to set the ground for their appropriate implementation. Establishing biomarkers as tools for monitoring in Brazilian coastal habitats means that their background levels, natural variation, and responsiveness to oil contamination, should be validated using representative species. Moreover, methods for interpreting the combined responses of multiple biomarkers are much needed. For risk assessment purposes, the factors underpinning species sensitivities, and thus affecting risk assessment metrics, need to be identified and understood.

6. OBJECTIVES

Recent offshore petroleum exploration in Brazil has increased the risks of oil spills in tropical habitats highlighting the need to implement monitoring and risk assessment practices adapted to subtropical and tropical coastal habitats of Brazil. The general objectives of this Ph.D. thesis are to validate the use of antioxidant biomarkers as tools for biomonitoring programs for coastal estuarine habitats in Brazil, and to determine if risk assessment metrics generated from temperate and Arctic species are applicable to the subtropical region. To accomplish these objectives, a baseline of biomarker values in different tropical species were established; the antioxidant biomarker response in two common species characterized; and the biomarker response after chronic exposure to diesel oil *in situ* evaluated. Also, differences in sensitivity related to exposure to 2–Methylnaphthalene, a toxic PAH present in oil, were identified as a way to distinguish the modifications needed to adapt risk–assessments strategies developed for temperate areas for use in subtropical and tropical regions.

ASSESSING THE EFFICACY OF ANTIOXIDANT ENZYMES AS POST–SPILL MONITORING TOOLS

Baseline of antioxidant enzymes activity (Paper I)

- What are baseline values for five antioxidant biomarkers in 5 common estuarine species?

- How do these values compare with levels identified in other areas?

- Are there significant differences in the antioxidant response between seasons?

- Do background levels of organic contamination influence biomarker values?

- Are there significant differences in the antioxidant response among species?

- Do the results identify appropriate sentinel species?

Antioxidant biomarkers after exposure to diesel oil spiked sediments under laboratory controlled conditions (Paper II)

- Are the studied species potential bioindicators of organic contamination?

- Are biomarker responding to variations in diesel oil concentration and time of exposure?

In situ *assessment of antioxidant biomarkers (Paper III)*

- Are biomarkers suitable indicators of oil exposure under field scenarios?

- Do their responses vary over time after repeated and accumulative exposures to diesel?

- Is the antioxidant–short–exposure response (48 h post–exposure) different from the long–term response (1 and 2 weeks)?

RISK ASSESSMENT STRATEGIES FOR SUBTROPICAL AND TROPICAL COASTAL HABITATS

Species sensitivity to 2–methylnaphthalene (Paper IV)

- Does species sensitivities, expressed in terms of their threshold concentration for survival, the no effect concentrations (NEC), to 2–Methylnaphthalene (2MN) vary across regions?

- Are Species Sensitivity Distribution (SSD) curves identifying the differences in sensitivities for species with different ecological traits (taxonomy groups, feeding guilds and trophic levels) or geographical distribution?

- What are the recommendations related to the effect assessment of 2MN exposure for oil risk assessment practices in coastal environments of Brazil?

7. Materials and Methods

Field experiments and sampling were conducted at the Paranaguá Estuarine System, southern Brazil. Laboratory tests were carried out in conjunction between the Federal University of Paraná (UFPR) and the Center for Marine Studies (CEM, UFPR) in Brazil, and Akvaplan–niva AS in Norway.

7.1. Area of Study

The Paranaguá Estuarine Complex, located in southern Brazil, is an extensive estuarine system (612 km^2) which includes a high diversity of habitats, such as islands, coastal dunes, mangroves, salt marshes, rivers, tidal creeks, rocky shores, seagrass meadows and sandy beaches.[50] It divides into two main sections, the northern and the western section. The northern part is composed by Laranjeiras, Guaraqueçaba, and Pinheiros Bays, while the West axis, also known as Paranaguá Estuarine System (PES), includes the bays of Paranaguá and Antonina. The Paranaguá Bay is a relatively shallow (average depth 5.4 m), semi–closed estuarine system, with a surface of about 250 km^2.[51] Intertidal mudflats are a predominant habitat at PES, with extensions of up to 2 km wide, colonized by mangroves and marshes.[51] Tidal currents and seasonal freshwater input regulate the estuarine hydrodynamics.[52] Climate at PES is classified as subtropical humid mesothermic with two main seasons during the year: (i) a dry season from April to September and (ii) a rainy season between October and March.[51]

The PES sustains artisanal fisheries, urban and touristic activities, industries, fuel terminals, and the principal grain shipping port in South America.[2] PES is susceptible to multiple sources of anthropogenic disturbance, which includes domestic discharges and sewage from the harbour and industries, inappropriate disposal of solids, and pollution from fertilizer manufacturing industries. Additionally, Paranaguá Harbor hosts the Transportation Terminal of Paranaguá (TEPAR), which operates refining, storing and transporting of oil and its derivatives.[3] The wide range of human activities that take place at PES, highlight its economic importance, but also the multiple potential sources of disturbance. For instance, growing oil production and intense ship traffic, carrying greater amounts of petroleum products, increase the risk of oil spills to occur on the PES, as evidenced by the explosion of the Vicuña oil tanker in 2004.[2,3] The Vicuña oil tanker was loaded with approximately 1,265,000 L of bunker oil, 173,000 L of diesel oil and around 4,079 tons of methanol[53], and released around 9 million litters of methanol into the bay.[54]

7.2. STUDIED SPECIES

Nine species showing characteristics suitable for monitoring of pollution impact were studied. Thus, benthic, sessile or limited mobility and numerically dominant species were selected. In addition to these criteria, we selected species with diverse life strategies, with different trophic levels, and belonging to various taxonomic groups.

Table 1. Overview of species included in this thesis. For identification of the site of collection the reader is referred to figure 1 on page 17.

Species	Class	Paper	Main characteristics	Habitat	Collection Site
Anomalocardia flexuosa	Bivalvia	I-IV	Clam. Abundant infaunal suspension feeder, harvested for human consumption.	Unvegetated tidal mudflat	I: 6C, 4P II-IV: 6C
Crassostrea rhizophorae	Bivalvia	I	Oyster. Euryhaline, sessile, filter–feeding, harvested for human consumption.	Mangrove roots	I: 7C, 2P
Neritina virginea	Gastropoda	I, IV	Snail. Grazer with limited mobility, found in high abundance.	Salt marshes	I: 7C, 3P
Laeonereis culveri	Polychaeta	I, II	Polychaete. Infaunal detritivorous, dominantly abundant.	Unvegetated tidal mudflat	II-IV: 10C_2
Uca maracoani	Malacostraca	I	Crab. Omnivorous, relatively abundant, lives in shallow burrows in mudflats.	Unvegetated tidal mudflat	8C, 5P
Genidens genidens	Actinopterygii	I	Fish. Detritivorous–carnivorous, benthic behavior, harvested for human consumption.	Subtidal benthos	9C, 1P
Monokalliapseudes schubarti	Malacostraca	IV	Tanaid. Deposit–feeder	Unvegetated tidal mudflat	10C_2
Clibanarius vittatus	Malacostraca	IV	Hermit crab. Scavenger	Mangrove roots	6C
Phrontis vibex	Gastropoda	IV	Snail. Carnivorous–scavenger	Unvegetated tidal mudflat	6C

7.3. ENDPOINTS

Different biomarkers of effect and exposure, mostly of antioxidant stress, were studied. Chemical endpoints as total polycyclic aromatic hydrocarbons (PAH) concentration in sediment and in animals soft tissue were also determined.

Table 2. Overview of measured endpoints included in this thesis.

Endpoint	Paper	Description	Protocol Reference
SOD	I, II, III	Dismutase the superoxide anion radical ($O_2^{\cdot-}$) into hydrogen peroxide (H_2O_2).	Gao et al. 1998 [55]
CAT	I, II, III	Degrades H_2O_2 to form water.	Aebi, 1984 [56]
GPx	I, II, III	Degrades H_2O_2 and lipid hydroperoxides to form water using reduced glutathione (GSH) as a electron donor.	Hafeman et al. 1974 [57]
GST	I, II, III	Adds an endogenous polar compound to hydrophobic xenobiotics or products from phase–I biotransformation reactions. Reduces lipid hydroperoxides to alcohol, with the concomitant oxidation of GSH to GSSG	Keen et al. 1976 [58]
LPO	II	Lipid hydroperoxides (lipid radicals) produced by hydroxyl radicals ($\cdot$OH).	Jiang et al. 1991 [59]
MDA	I, III	MDA, a byproduct from lipid peroxidation, is measured.	Shaw et al. 2004 [60]
PHAs in sediments	II, III	Quantification of chemical concentration of main polyaromatic hydrocarbons (PAH) in experimental sediments.	Dauner et al. 2016 [61]
PHAs in biota	III	Quantification of chemical concentration PAH in animal tissue.	
Mortality	IV	Quantification of number of dead organisms following exposure.	

PAPER I

Baseline levels of oxidative stress biomarkers in species from a subtropical estuarine system (Paranaguá Bay, southern Brazil)

Baseline levels for four major antioxidant enzymes and a biomarker of oxidative stress were studied in five tropical and subtropical species from PES. Five numerically dominant species (Table 1) were sampled during austral winter 2014 and austral summer 2015 at two different locations with varying levels of contamination (Fig. 1). Reference and polluted locations were not the same for all species since they live in different habitats.

Polluted locations were near the Paranaguá City, which usually presents the highest values of total PAH in sediments of the Cotinga sub–estuary (28.7–232.74 ng g^{-1}) [62–64] After collection, the collected species were transported to the lab, immediately dissected and stored at -80 ºC until further analysis. Enzyme activity determination was performed in the laboratory facilities of Akvaplan–niva (Norway) following standard spectrophotometer protocols (Table 2). Species–specific variations, seasonal and spatial variation in the biomarker response were studied and described for the selected species following univariate and multivariate assessments. In addition, a comparison with literature data summarized from other estuaries along the Brazilian coast was made.
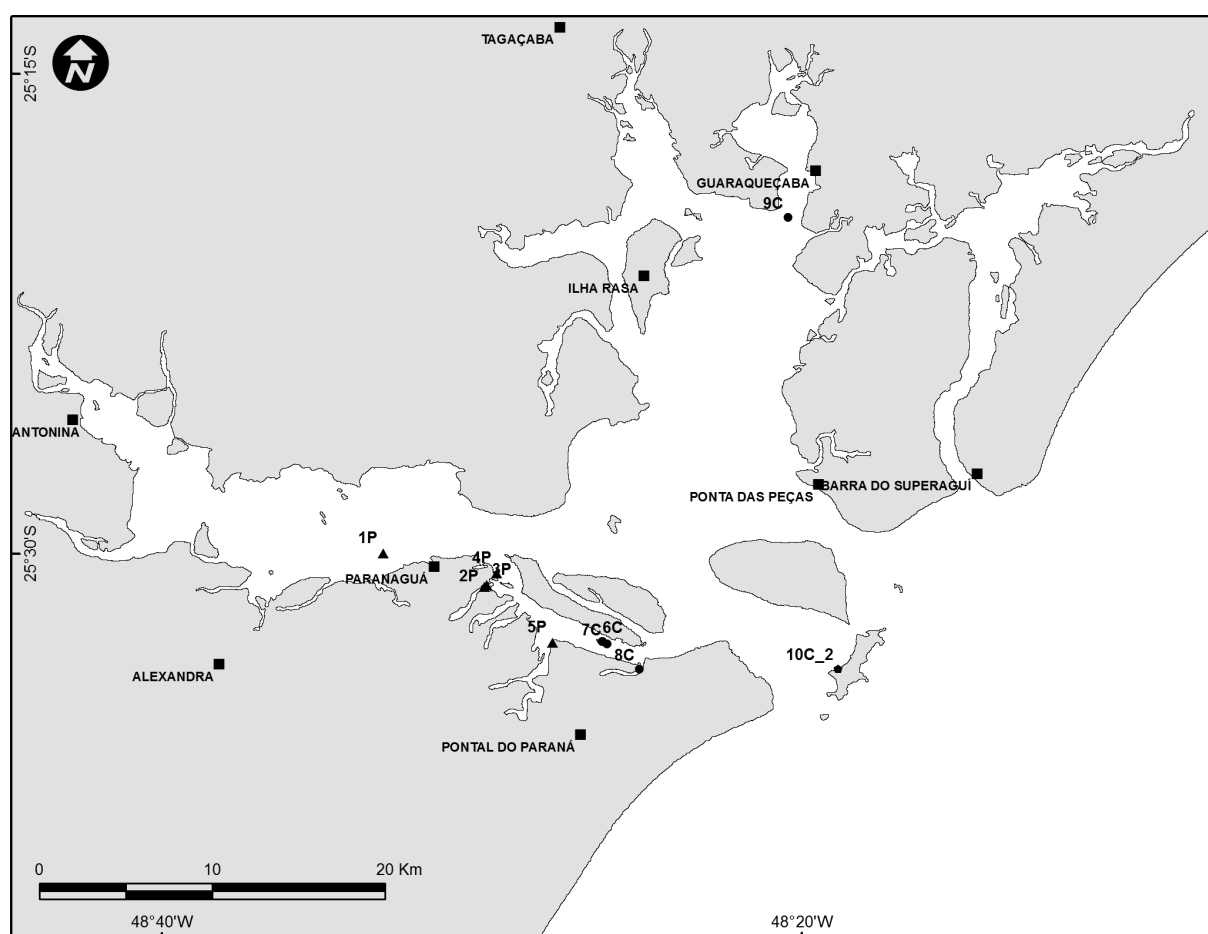
Figure 1. Sampling locations at PES southern Brazil. Points labelled with C indicate locations considered as control or reference sites; while points labelled with P refer to contaminated locations.

Oxidative stress in two tropical species after exposure to diesel oil

The activity of antioxidant enzymes in the polychaete *Laeonereis culveri* and the bivalve *Anomalocardia flexuosa* were studied after exposure to oil–spiked sediments under laboratory conditions. Clams and polychaetes were collected from reference sites (AF: Papagaios Island; LC: Saco do Limoeiro, Table 1) and transported to the lab. After 96 h of acclimation, an acute bioassay was conducted with fixed temperature and photoperiod (20° C and 12 light-12 dark regime). A 2–factor experimental design was conducted to assess biomarker responses to diesel oil, with oil concentration and time of exposure as fixed factors. Three concentrations and two times of exposure were tested, making a total of 6 treatments. Sediment samples were collected at the end of the experiment and levels of PAHs were measured according to United Nations Environment Program method.[65] Differences among treatments were tested using PERMANOVA and if significant, further compared using pairwise *t*–test with the Bonferroni correction.
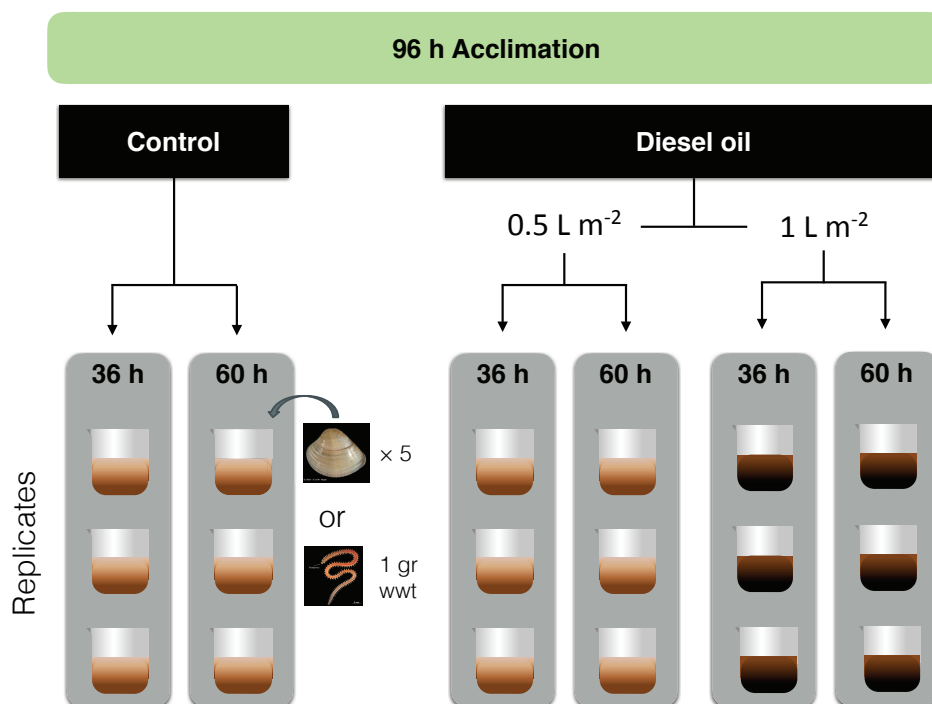
Figure 2. Exposure design employed for the laboratory exposure experiment. Each treatment included 3 replicates sampled at two different times of exposure. In exposed treatments, sediments were spiked with 500 or 1000 mL of diesel oil at day 0.

Effects on an experimental in situ oil spill in oxidative stress biomarkers in the clam Anomalocardia flexuosa *of tidal flats from subtropical estuary, Brazil.*

A 5 week *in situ* experiment was conducted during austral summer 2016 where the effects of 3 successive diesel oil spills, with two weeks of recovery time between exposures, were tested. The experimental design consisted of an undisturbed control treatment and an experimental treatment. Each treatment had two blocks, each including four quadrats replicates of 0.25 m^2 disposed 10 m distance apart from each other. Quadrats within treatment blocks were separated by a 40 m distance while between–treatment blocks had a minimum distance of 80 m (Fig. 3). We selected the clam species *Anomalocardia flexuosa* as the study organism. At each exposed replicate the concentration of diesel oil spilled was equivalent to 2 L m^{-2} spill (500 mL) per exposure event, and performed during low tide when the sediments were completely exposed. *Anomalocardia flexuosa* specimens were always collected before the spilling. A zinc frame of 0.25 m^2 area was placed and buried in the sediments to keep diesel from spreading around. These frames were in place for at least 40 min, to allow diesel to percolate in the sediment. Animal collection was done in both exposed and control replicates every 48 h, 7 and 14 days after each exposure event (Fig. 4). Antioxidant enzyme activity was determined as denoted in Table 2. Levels of PAHs in sediment and in animal tissue were also quantified.
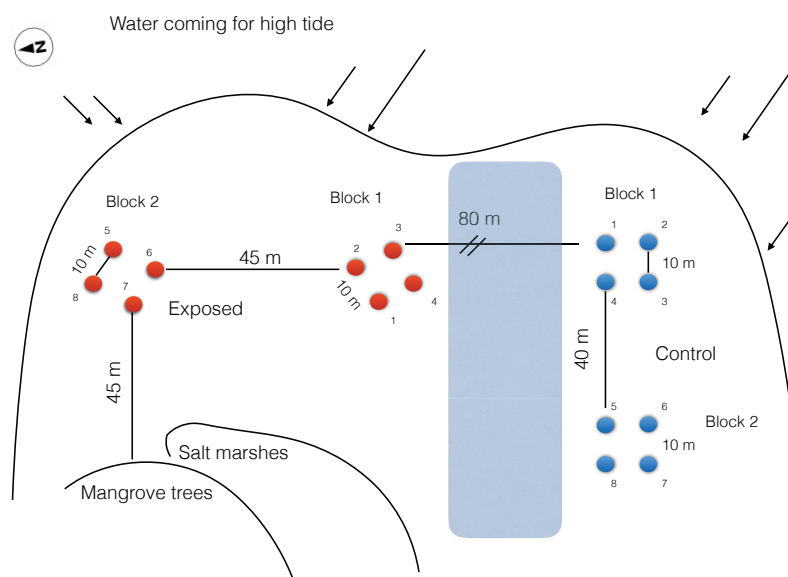
Figure 3. Experimental design scheme representation from a mudflat in Papagaios Island PES. Red points represent experimental quadrats and blue points indicate control quadrats.
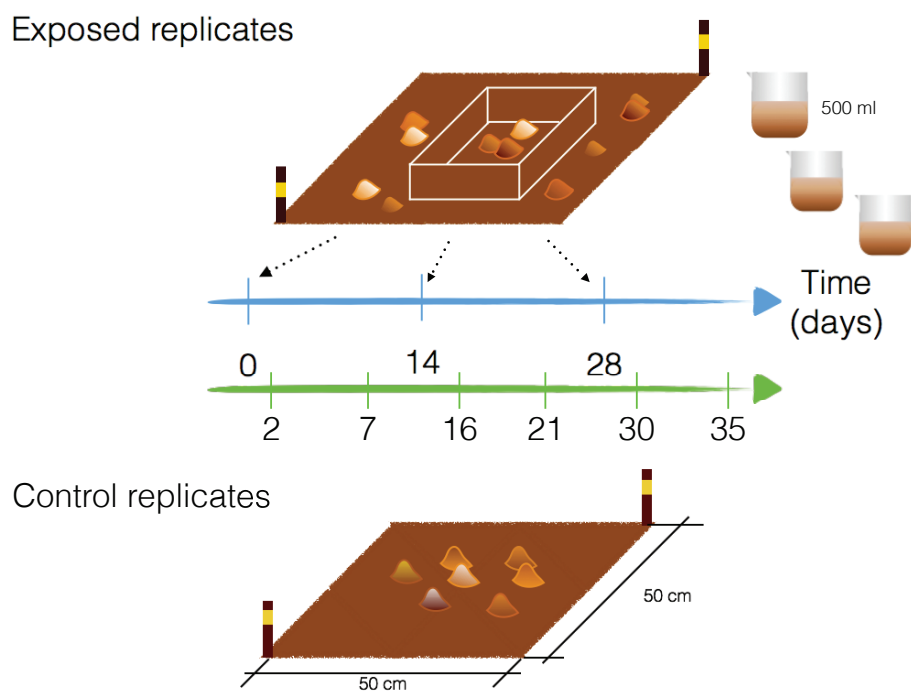
Figure 4. Exposure regime employed for the *in situ* exposure experiment. Animal collection was made at every sampling day in exposed and control quadrats (*n*=8 per treatment). In exposed quadrats, 500 mL of diesel oil was spilled on days 0, 14 and 28.

PAPER IV

Exploring inter–species sensitivity to a model hydrocarbon, 2–Methylnaphthalene, using a process–based model

Six different species (Table 1) were collected from reference sites at PES (Fig. 1) and transported to the laboratory. After 72 h of acclimation, an exposure bioassay to six treatment concentrations of 2–Methylnaphthalene (2MN) was performed as described by Olsen et al.[38] Each treatment included 4 replicated aquaria; making a total of 28 aquaria per species–experiment. Mortality and unusual behavior were monitored for each replicate at 2, 4, 6, 8 and 24 h, and thereafter every 24 h until the end of each experiment. No effect concentrations (NEC) were derived fitting a GUTS–SIC–SD model using time–concentration–response relationships derived for each species exposed to 2MN. Acute toxicity data for 2MN exposure to 11 Arctic and 6 temperate species exposed to 2MN were taken from Olsen et al.[38] Also, a search was carried out in the U.S. Environmental Protection Agency (U.S. EPA) ECOTOX database (http://cfpub.epa.gov/ecotox/, free access, data retrieved on 07-2016). To explore patterns in species sensitivity to 2MN, species sensitivity distributions (SSDs) with respect to biological traits as phylogeny, trophic mode and geographic distribution were constructed. SSDs resulted from fitting

NECs values to a log–normal distribution. By coupling GUTS model parameters with fate and transport models, it is possible to forecast toxicity in time of other untested narcotic compounds, which ultimately allows for extrapolation at the ecosystem level or for other untested species (Fig. 5).
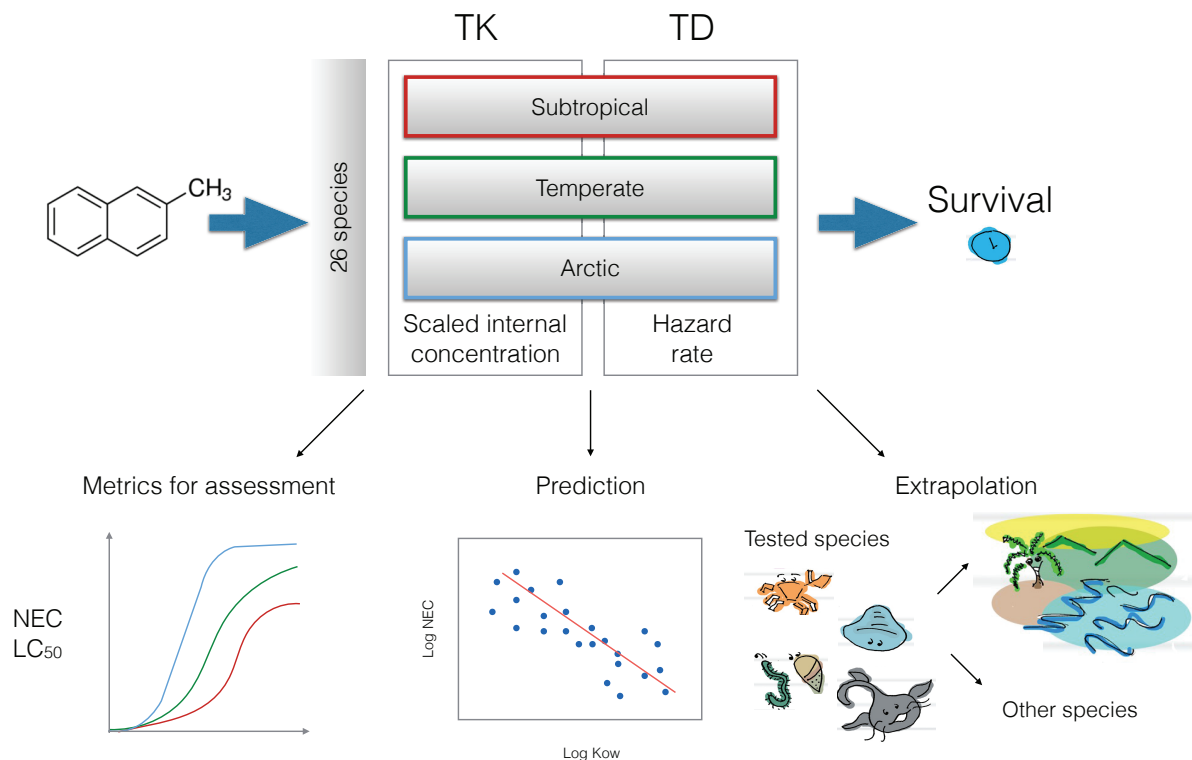
Figure 5. Framework used for exploring inter–species sensitivity to 2MN. Model parameters for 26 species were estimated based on survival data over time. The species sensitivity was assessed using the SSD approach. Toxicity towards other narcotic compounds was predicted using theoretical relationships between the NEC and Log K_{ow} of chemicals.

8. SUMMARY OF RESULTS AND DISCUSSION

Before a robust well–designed biomonitoring program can be implemented, it is necessary to establish causality between the measured variables and the impacts the program is monitoring.[33] Often, measuring environmental stressors is difficult, costly and time–consuming. Moreover, direct chemical quantification of chronic and low–concentration contamination, such as petroleum–related pollution from diffuse sources, can also prove difficult to measure because chemical analyses often rank in the no detectable range.

In this work, we propose and test the use of biomarkers of oxidative stress as pollution indicators. For that, we have guided our experimental work following the framework of Goodsell et al.,[33] which suggests testing several null hypotheses (H_0) for the establishment of causality relationships between a proposed indicator taxon and an environmental stressor (Fig. 6). Instead of testing causality between an indicator taxon and an environmental stressor, in this Ph.D. thesis we aim to test the causal relationship between the oxidative stress biomarkers and diesel oil contamination.

8.1. ANTIOXIDANT ENZYMES AS POST–SPILL MONITORING TOOLS

Baseline of antioxidant enzymes activity and its natural variation

"H_0: There is no consistent correlation between the response of the biomarker and levels of the contaminant"

Causality relationships can be tested through the comparison of contaminated versus reference sites over varying time scales.[33] In this context, the current knowledge gap on natural variation in basal biomarker levels in subtropical species justified a baseline study.

Our results depicted a seasonal signal in the activity of antioxidant enzymes for all studied species (Table 3). Although this seasonal variation is most likely attributed to changes in temperature, salinity, food availability, and reproductive cycles, our experiment did not aim to establish a direct relationship between the before mentioned drivers, and the variation in the biomarker response for each species. Our objective was to understand the effect of temporal and spatial variation as for their interaction, on the biomarkers response of representative species of the PES. Temporal variation was defined here by two seasons (dry and rainy seasons), while spatial variation is associated with different levels of contamination.

Although the seasonal signal was far more important for total variation, the antioxidant activity also varied significantly between reference and contaminated locations for most target species (Table 3). Significant univariate variations of average values from reference vs. polluted sites were more frequent for endpoints measured in the mangrove oyster *C. rhizophorae*, the clam *A. flexuosa* and catfish *G. genidens* species (Fig. 7).

No overall pattern was observed in a given biomarker for all the species (Fig. 7). An extension of our alternative hypothesis of a correlation between biomarker responses and contamination, was to verify if such association was common and consistently observed among the studied species, finding a shared biomarker response among very diverse organisms. A biomarker of exposure that continually responds to contamination in several species represents a better indicator than a biomarker that answers in certain species only. However, significant species–specific variation in levels of biomarkers activities among diverse species was always observed, regardless of univariate or multivariate approaches. The activity of SOD was the only biomarker that showed a consistent pattern in several species, being significantly higher in locations labeled as polluted than in reference sites in 3 out of the 5 studied species. Yet, it is important to highlight that given the complexity of biochemical reactions during redox homeostasis, the choice of a single biomarker that works for several species is correspondingly complex. Duration and intensity of the pro–oxidant stressor can drive opposite responses, and both increases or decreases in the enzymatic activity are referred to as oxidative stress.[18] In this sense, in order to achieve redox homeostasis many enzymes are expected to respond as part of an integrated system, and a decrease in one antioxidant enzyme is often coupled with an increase in another antioxidant enzyme, highlighting the importance of following a multi–biomarker and multivariate approach.[27]

CONTAMINANT STRESS

- What is the putative impact?
- Is it difficult, costly or time-consuming to measure it directly?

PROPOSED BIOMARKER

- Are biomarkers likely to respond to contaminant stress?
- What is the measurable response? is it simpler to quantify? is it reproducible?

Establish correlation
H_0: There is no consistent correlation between the response of the biomarker and levels of the contaminant

TEST: sample the response of the biomarker in several sites with known levels of contaminant (combine biomarkers response with chemical analysis) over time scales relative to natural fluctuations in the biomarker

Retain   Reject H_0

Establish cause
H_0: There is no causal relationship between the contaminant and the observed response of the biomarker

TEST: compare biomarker response after experimental application of the stress (experimental manipulations) over time scales relative to natural fluctuations in the biomarker

Retain   Reject H_0

Establish a direct response
H_0: the response of the biomarker is not directly associated with doses of the contaminant

TEST: experimental application of the contaminant at a range of doses, i.e. bioassay and ecotoxicology experiments

Retain   Reject H_0

Continued verification of relationship in stressed system. Repeat steps to verify the response

BIOMARKER is a pollution indicator

Figure 6. Steps involved for testing causal relationship between proposed biomarkers and contaminants. Modified from Goodsell et al.[33] Used with permission.

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Table 3. Analysis of variance using permutation test (PERMANOVA) for enzymatic activities in tropical species collected at different seasons and locations with different levels of contamination. Statistically significant differences are highlighted in bold. Abbreviations stand for *F:* pseudo-F-ratio; R2: coefficient of determination, *P:* probability of *F*.

Species	Source of variation	*F*	R2	*P*
Anomalocardia flexuosa	Season (Se)	20.14	0.34	**<0.0001**
	Condition (Cond)	0.84	0.01	0.47
	Se:Cond	3.14	0.05	**0.024**
Crassostrea rhizophorae	Season (Se)	10.26	0.18	**<0.0001**
	Condition (Cond)	9.55	0.17	**<0.0001**
	Se:Cond	1.29	0.02	0.26
Neritina virginea	Season (Se)	40.12	0.50	**<0.0001**
	Condition (Cond)	2.43	0.03	0.078
	Se:Cond	2.77	0.03	0.057
Uca maracoani	Season (Se)	16.55	0.28	**<0.0001**
	Condition (Cond)	2.93	0.05	**0.026**
	Se:Cond	1.68	0.02	0.15
Genidens genidens	Season (Se)	53.66	0.52	**<0.0001**
	Condition (Cond)	4.61	0.04	**0.012**
	Se:Cond	2.80	0.03	0.058

Figure 7. Confidence plots derived from log-transformed enzymatic activities in studied species. Plots represent the mean (red points), 50% confidence intervals (boxes) and 95% confidence intervals (dispersion lines). Effects of significant interaction are given as estimated changes between polluted (P) and control (C) samples for winter (W) and summer (S). When significative, the marginal effect of season and condition are also denoted. Enzyme activity units are, CAT: mMol.min^{-1}.mg^{-1} of protein; GPx and GST: µMol.min^{-1}.mg^{-1}; of protein; SOD: U mg.ml^{-1} of protein; MDA: nM g^{-1} wet weight.

Anomalocardia flexuosa **Crassostrea rhizophorae** **Neritina virginea** **Uca maracoani** **Genidens genidens**

No significant effects

season (p=0.02)   effect -28%
condition (p=0.001) effect +61%

season (p<0.0001)
effect +682%

season (p<0.0001)
effect +287%

season (p<0.0001)   polln.eff(W) +31%
condition (p<0.0001) polln eff.(S) -10%
interaction (p<0.0001)

No significant effects

season (p=0.0001) seas.effect +73%
interaction(p=0.04) polln eff.(S) +59%

season (p<0.0001) polln eff.(W) -35%

season(p=0.0003)
effect +123%

season (p<0.0001)
effect -78%

season (p<0.0001) polln eff.(W) -56%
condition(p=0.04) polln.eff. (S) +50%
interaction(p=0.03)

season (p=0.02) seas.effect -53%

season (p<0.0001)
effect -73%

No significant effects

No significant effects

season (p=0.007) polln eff.(W) -49%
condition(p=0.01) polln.eff. (S) +5%
interaction(p=0.06)

condition (p<0.0001) eff.+242%

season (p<0.0001)
effect -57%

season (p<0.0001)
effect -78%

season (p<0.0001) effect +160%
condition (p=0.03) effect +32%

season (p=0.001)   season eff. -77%

season (p<0.0001) effect +166%

interaction (p<0.0001)
polln eff. (W) +28%
polln eff. (S) +263%

season((p<0.0001)
effect -51%
condition (p<0.0001)
effect +97%

condition (p=0.04)
effect -24%

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Results from multivariate analyses revealed that baseline levels of the antioxidant biomarkers for *G. genidens*, *U. maracoani*, and *C. rhizophorae* species varied significantly between reference and polluted locations (Table 3 and Fig. S3, Paper I). However, the observed seasonal effect in antioxidant biomarkers of *G. genidens* and *U. maracoani* was stronger. Interestingly, significant differences between reference and contaminated sites were not observed when biomarkers were analysed individually following univariate approaches. A similar result was obtained by Gagnon and Rawson,[66] who observed deterioration on fish health only when integrating the biomarker responses with multivariate analysis. For the mangrove oyster, *C. rhizophorae*, differences in the levels of contamination between locations were stronger, as highlighted by the percentage of variance explained by RDA2 (Fig. 8). Contamination had an effect on the activities of GST and SOD, which were higher in polluted areas. Our results are in accordance with previous studies, that highlight the response of biotransformation and antioxidant enzymes from the mangrove oyster as suitable biomarkers for contamination.[29,67–69] Results from multivariate analyses in the clam *A. flexuosa*, indicated the interaction between season and contamination as significant, with the variation in the biomarker response between locations being more evident in winter season than summer.
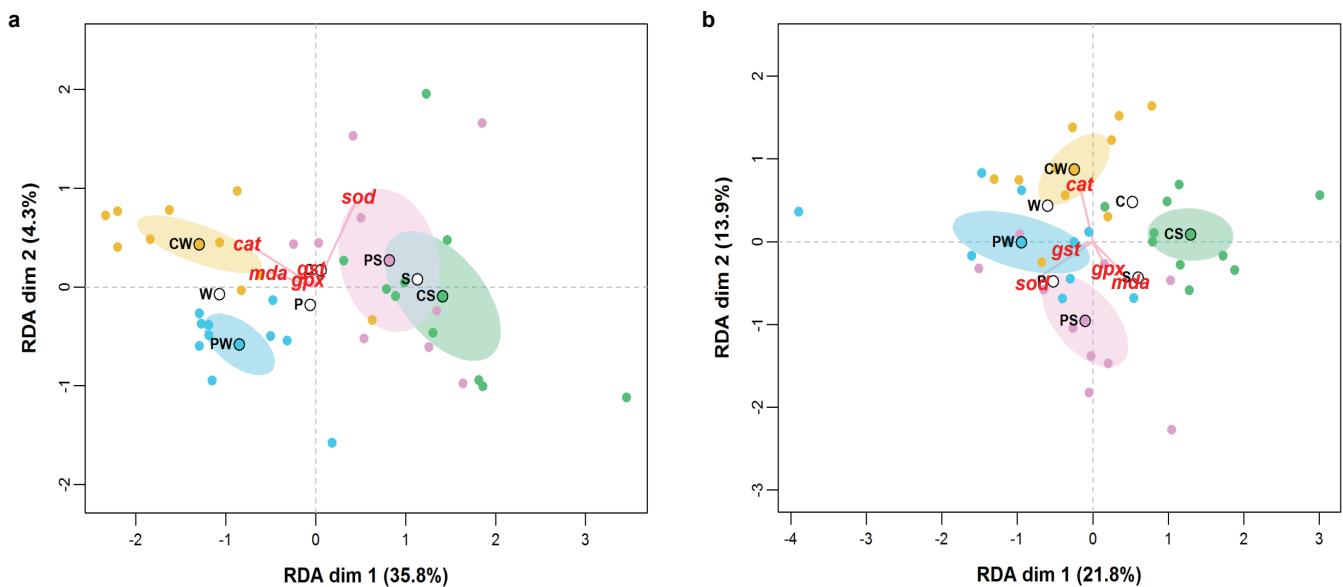
Figure 8. RDA biplots derived from log transformed enzymatic activities in a) *Anomalocardia flexuosa* and b) *Crassostrea rhizophorae* sampled during winter and summer seasons in locations with different levels of contamination. Ellipses represent 95% confidence intervals from centroids of the interaction between season and condition. Abbreviations stand for: C: control; P: polluted; CW: control winter (yellow); PW: polluted winter (blue); CS: control summer (green); PS: polluted summer (purple).

Similar comparisons of multi–biomarker responses in a set of diverse organisms are still scarce in the literature, and consistent biomarker validation has been done for only a few species, mainly bivalves. As a result, biomarker responses in selected indicator species or sentinels may not express or reflect the sensitivity of other species or functional groups within a community. This obviously may hinder the development of consistent strategies for species selection in monitoring programs. Based on the results obtained within this study the bivalve species *A. flexuosa*, *C. rhizophorae,* and the catfish *G. genidens* are proposed as sentinels of contamination, since the integrated response of their antioxidant enzymes allowed to discriminate locations with different levels of contamination. Such surveys of background or natural variability are a necessary step for the development of consistent and cost–effective tools for the detection of ecotoxicological effects *in situ.*

Antioxidant biomarkers after exposure to diesel oil spiked sediments under laboratory controlled conditions (Paper II)

"H$_0$: The response of the biomarker is not directly associated with doses of the contaminant"

In this study, the response of antioxidant enzymes was evaluated following an acute exposure to experimentally contaminated sediments. Our results indicate that the activity of the studied antioxidant enzymes is significantly different from control conditions and that these differences vary over time (Fig. 1, 2, and 3 Paper II).

The experimental design aimed to identify the combined effect between the concentration of diesel oil spiked to the sediment, and the time following the exposure. Nevertheless, for most of the measured endpoints, the interaction between exposure concentration and time was not significant.

The observed variability was primarily explained by the time of exposure (Table 4) and none of the endpoints varied significantly among concentration treatments. The last result is most likely related to the small differences in measured PAHs obtained among concentration treatments, which showed little correspondence with nominal concentrations (Table 1, Paper II). Also, the activity of the enzymatic endpoints was different between species, which is in accordance with results from the baseline study that showed that the activity of antioxidant enzymes is species–specific.

Effects of pulsed diesel oil exposure on oxidative stress biomarkers in the clam **Anomalocardia flexuosa***. An* **in situ** *approach (Paper III).*

"H$_0$: There is no causal relationship between the contaminant and the observed response of the biomarker "

In this study, we departed from the null hypothesis of not finding significant differences in the biomarker response in clams from the control treatment vs. those from the exposed treatment, which received three sequential low–dose diesel oil spills under field scenarios.

Table 4. Permutational ANOVA for mean percentage change in activity from control of enzymatic activities and lipid peroxide levels after exposure to diesel oil. Abbreviations stand for df: degree of freedom; MS: mean squares; *F:* F-ratio; *P:* probability of *F*.

		(i) *Anomalocardia flexuosa*			(ii) *Laeonereis culveri*		
	df	MS	*F*	*P*	MS	*F*	*P*
(a) SOD							
Concentration	1	370.79	0.35	0.72	619.23	2.26	0.17
Time	1	45.78	0.04	0.94	104.66	0.38	0.55
C x T	1	879.29	0.82	0.49	298.71	1.09	0.32
Residual	8	1072.34			258.81		
(b) CAT							
Concentration	1	2002.60	2.56	0.16	98.886	0.38207	0.56
Time	1	9994.70	12.77	**<0.05**	2.932	0.01133	0.92
C x T	1	36.80	0.05	0.80	196.322	0.75855	0.41
Residual	8	782.70			273.92		
(c) GPx							
Concentration	1	23.91	0.28	0.65	46.58	1.3058	0.26
Time	1	33.80	0.40	0.58	912.48	25.5813	**<0.01**
C x T	1	14.48	0.17	0.73	30.33	0.8504	0.37
Residual	8	85.04			35.67		
(d) GST							
Concentration	1	168.70	0.91	0.37	400.13	1.3358	0.27
Time	1	4010	21.72	<0.01	2147.59	7.1697	**<0.05**
C x T	1	1229.60	6.66	**<0.05**	9.57	0.0319	0.88
Residual	8	184.70			299.54		
(e) LPO							
Concentration	1	220.33	0.13	0.79	80	0.07	0.79
Time	1	1676.35	0.98	0.39	31899	27.59	**<0.01**
C x T	1	21.37	0.01	0.94	116	0.10	0.79
Residual	8	1715.59			1156		

Moreover, the design aimed to test if the biomarker response varies over time following repeated pulsed exposure to diesel oil, which resumes as a second null hypothesis: the biomarker response from exposed animals will not change regardless of the number of spills (pulses) to which animals were subjected. Also, differences between short (48 h post–spills) and long–term (1 and 2 weeks) response of the spills were expected, so a third null hypothesis was formed: departures from control levels in the biomarkers will not vary (or be more evident) over 1 – 2 weeks following exposure.

Based on our results, we reject the null hypothesis of no difference in the biomarker response associated with experimental spills. The results indicate that the activities of GST and SOD in both measured tissues, responded significantly to experimental treatments. The activity of these enzymes also varied over time, with marked increases or decreases in their activities 48 h post–spills, that following 7 and 14 days post–spills recovered to basal levels, as seem by the not significant differences with their respective controls (Fig. 9). Moreover, the activity of none of the assessed biomarkers suggested a continuous or cumulative effect and most of the differences were observed 48 h following of the spill. Thus, we accepted the second null hypothesis, whereas our third null hypothesis, that presumed that departures from control levels in the biomarkers will not vary over 1 – 2 weeks following exposure, was also rejected (Fig. 9).

Because abiotic variables, such as temperature, dissolved oxygen, pH, and salinity have an effect on redox reactions,[70] and these abiotic variables also vary over time in the field, we expected to obtain high variability in the biomarker response in clams from the control treatment. PAHs levels in tissues and sediments from the control quadrats, allowed us to confirm that the observed variability in the control treatment was due to natural variation. No significant differences were obtained in total levels of PAHs bioaccumulated in animal tissues, but the levels of PAHs showed to be higher in exposed sediments than in control ones, except that they were always within a range considered as uncontaminated.[61,64]

Changes in the biomarker response due to the experimental spills would not have been identified if an independent control treatment was not included in the experimental design. Without a control treatment, natural fluctuations of the enzymes would have confounded the experimental treatment, and the magnitude of the response attributed to the experimental treatment would have been much higher. The effect of natural variation in the biomarker response was evident within the RDA biplot, that shows a steady

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progression in the activity of biomarkers over time, split on 3 different trajectories, the first trajectory observed from day 0 to day 7, the second from day 14 to day 28 and the last one from days 30 to 35 (Fig. 2, Paper III).

On the first trajectory, SOD in gills decreases and levels of SOD in digestive glands increases accompanied by lower levels of lipid peroxides in the digestive gland. The second trajectory includes a moderate increase of GPx activity in the digestive gland, a moderate decrease in GST activity and levels of lipid peroxides in the gills; and a mixed (contradictory) response in the activity of SOD in gills for days 16 and 21. The last trajectory is mainly expressed by a steady increase in SOD activity in both tissues and lower levels of MDA in digestive glands. This result suggest that the activity of SOD in digestive glands is the first biomarker to respond to the diesel oil spill stress and that such induction in the antioxidant machinery presumably leads to a chain of antioxidant reactions that reverse lipid peroxidation, converting lipid hydroperoxides back to lipids and alcohol by the activity of GPx.[18] Further, the contradictory response in the second trajectory suggest that the response of the measured biomarkers varies over short periods of time, as observed in the univariate approach, where most of the enzymes recover to control levels following 7 or 14 days post–spills. The last trajectory suggest the re–induction of the activities of SOD in gills and digestive glands, which is in line with the fast responsiveness of this enzyme towards all experimental spills.
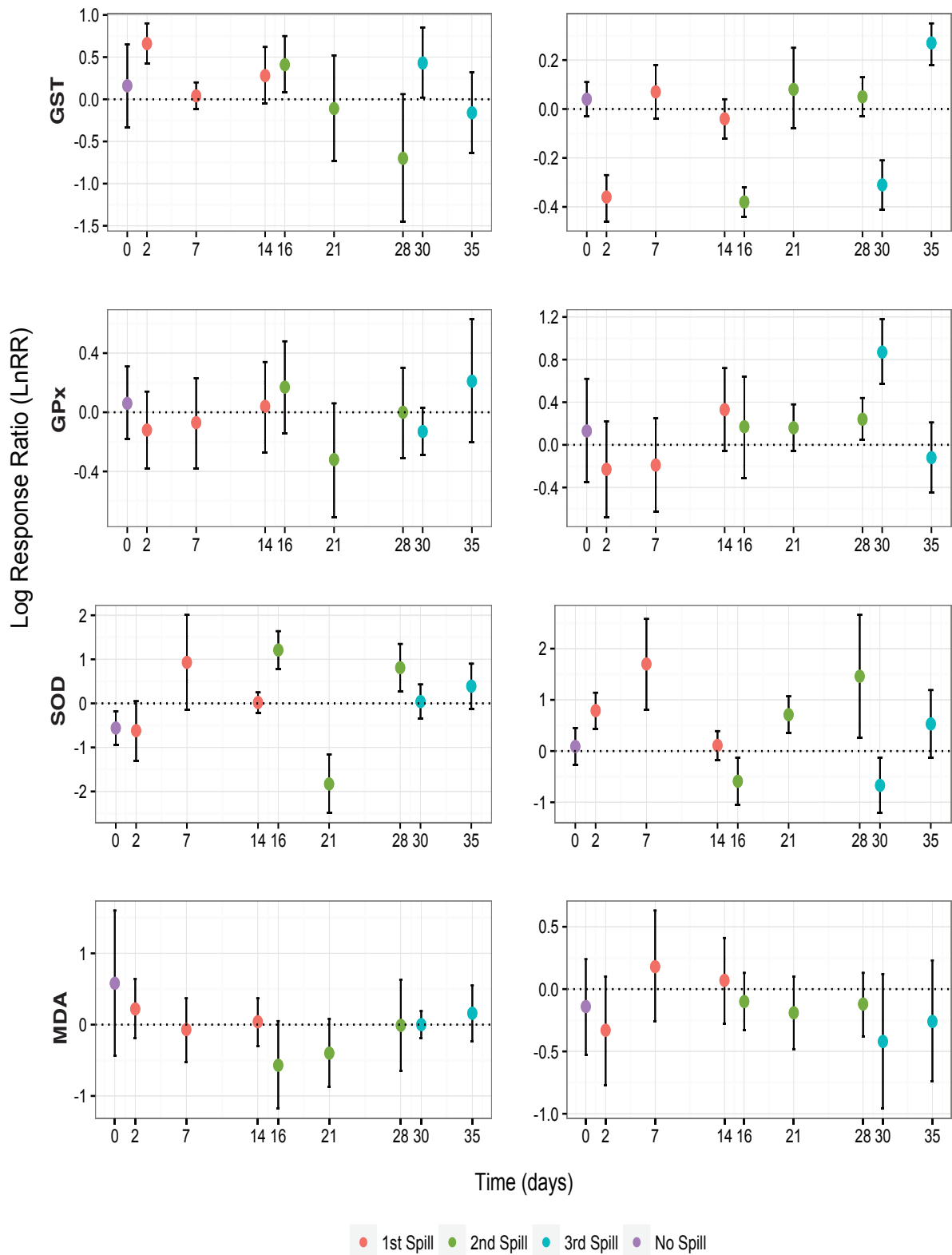
Figure 9. Relative effects of experimental diesel oil spills (denoted as log ratios) in *Anomalocardia flexuosa* antioxidant enzymes in gills (left panel) and digestive glands (right panel). Error bars represent 95% confidence intervals (CIs) for relative effects. If a CI does not cross the horizontal line at 0, the effect is significantly different from 0 at 95% confidence–level.

8.2. RISK ASSESSMENT STRATEGIES FOR SUBTROPICAL AND TROPICAL COASTAL HABITATS

Exploring inter–species sensitivity to a model hydrocarbon, 2–Methylnaphthalene, using a process–based model

"H$_0$: There are no differences in the sensitivity to 2–Methylnaphthalene in species distributed in the Arctic, temperate or subtropical region "

Model parameters for 26 species were estimated (Table 1, Paper IV). The goodness of fit was in all cases very good ranging from 0 to 0.07 with an overall mean of 0.02. Many data sets suggest that kinetics are either "fast" or "slow" — and this means that in practice there is no exact value for the elimination rate. In fast kinetics the equilibrium between external and internal concentrations is rapidly reached, which is followed by high mortality. In "slow kinetics" the time to reach the equilibrium between the internal and the external concentration is not within the experimental time frame, which means that the elimination rate is not significantly different from zero and equilibrium is only achieved after very long exposure.[71] Slow kinetics cases are problematic because it is not possible to estimate all model parameters precisely, and the only information we have is that the time to reach equilibrium exceeds the assay duration (4-5 d).

Species sensitivity to 2MN was compared by fitting accumulative lognormal distributions based on NEC values. We did not observe significant differences in the SSD curves made for any of the proposed comparison criteria (Fig. 10). In general, the SSD approach provided little insight into understanding the drivers accounting for the differences in the species sensitivity to 2MN. In fact, differences in species sensitivities were better explained by the elimination rate. As the NEC is a threshold for toxicity, effects on survival will be observed once the NEC is surpassed, but once the concentration exceeds the NEC, death is not immediate.[41] By integrating the information provided by the NEC and the k_e parameters, the time to observe an effect or the time to reach the NEC can be calculated. The results suggest that the time to observe an effect on survival is different for species inhabiting the Arctic, the temperate or the subtropical region (Fig. 4, Table IV).
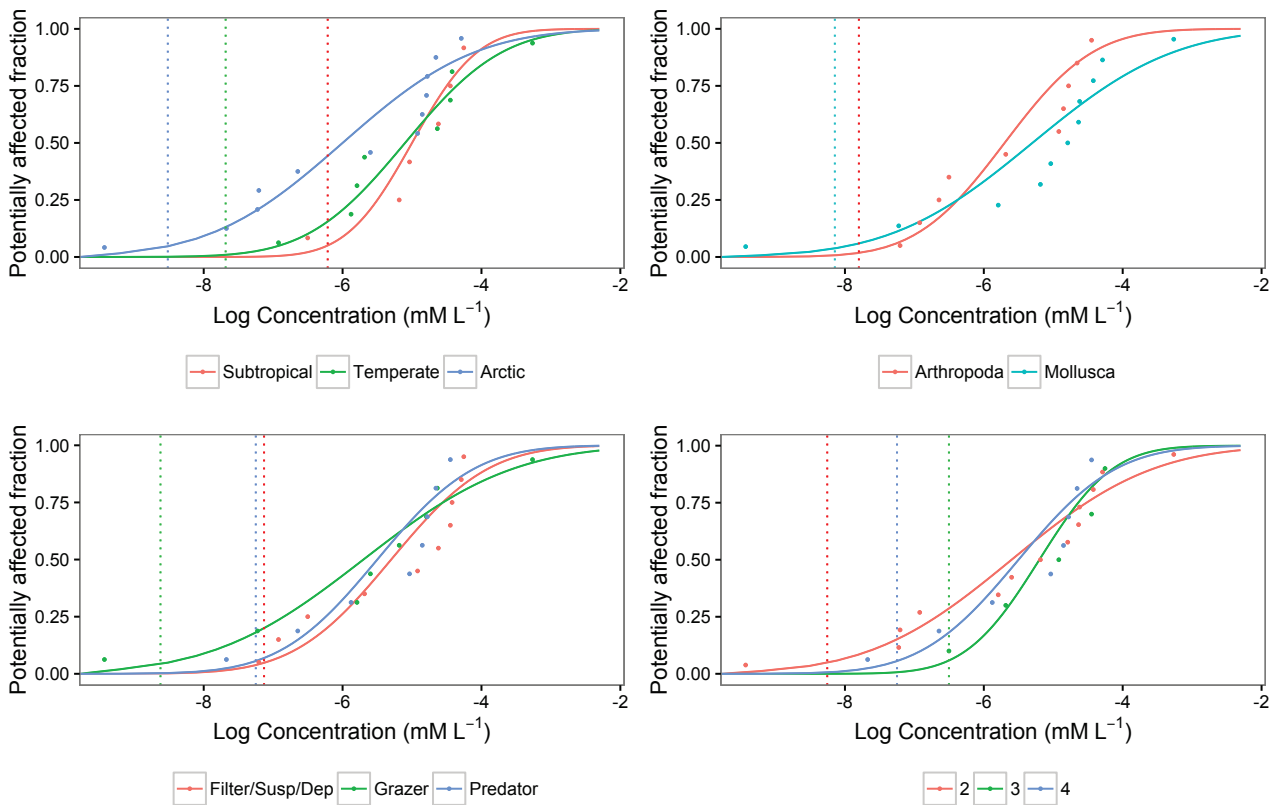
Figure 10. Cumulative lognormal distribution fits of the NECs for species according to A) geographical distribution, B) Taxonomic classes, C) Feeding guilds and D) Trophic level. Only groups with 6 or more species were considered. Colored dashed lines indicate HC_5 thresholds.

This comparison was done for concentrations that exceeded by 5%, 10% and every 10% increases until 100% the NEC obtained from the GUTS model for each species (Fig. 4, Paper IV). For risk assessment purposes it might be more interesting to compare the sensitivities among species for a common concentration value. Thus, we have applied a temperature correction to the rate parameters and estimated the time to effect at an arbitrary external concentration. We selected 0.014 mM, which is a value higher than the entire NEC range of values reported within this work. On average, Arctic species start showing effects on survival after 6 hours of exposure, while temperate and subtropical species require roughly 2 days of exposure (Table 5).

It is interesting to highlight that among the temperate species, the clam *M. edulis* presents the highest tolerance and pushes up the group average from 14 h to 50 h. There is a good amount of literature that highlights the capacity of this species to accumulate significant amount of contaminants within their tissues without threatening their survival. [72,73]

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Table 5. Time to observe an effect in survival at a external concentration of 0.014 mM of 2MN and with k_e corrected to a reference temperature of 20 °C.

Region	Species	Hours	Days
Subtropical	*Phrontis vibex*	14.3	
	Clibanarius vittatus	19.0	
	Monokalliapseudes schubarti	56.9	2.4
	Neritina virginea	85.9	3.6
	Laeonereis culveri	94.0	3.9
	Anomalocardia flexuosa	118.2	4.9
	Mean	**64.7**	**2.7**
Temperate	*Gammarus sp.*	0.3	
	Patella depressa	0.9	
	Oncorhynchus mykiss	1.4	
	Dicentrarchus labrax	4.9	
	Semibalanus balanoides	10.5	
	Gibbula umbilicalis	38.5	1.6
	Mytilus edulis	293.8	12.2
	Mean without *M. edulis*	13.8	
	Mean	**50.0**	**2.1**
Arctic	*Nymphon gracile*	0.4	
	Testudinalia testudinalis	0.9	
	Margarites helicinus	1.5	
	Sclerocrangon boreas	2.6	
	Strongylocentrotus droebachiensis	2.6	
	Gammarus sp.	2.7	
	Anonyx nugax	4.0	
	Pandalus borealis	6.6	
	Boreogadus saida	7.7	
	Litorina littorea	11.0	
	Chlamys islandica	17.2	
	Balanus balanus	18.2	
	Mean	**6.3**	

When assessing the results using several of the model–based parameters, differences among the species sensitivity to 2MN distributed in different regions are distinguished. This result allows us to reject the null hypothesis of no differences in species sensitivities across different regions.

9. SYNTHESIS

Coastal marine areas as estuaries are among the most ecologically sensitive and economically important ecosystems.[74] They are subjected to a variety of stressors, both natural and anthropogenic, that can impair the health and fitness of biota. Among the stressors, nutrients load, hypoxia, suspended sediments, turbidity, hydrologic regimes and pollutants releases can impact marine resources through single, cumulative or synergistic processes.[13] Because estuaries are at risk of environmental impact associated with anthropogenic activities, establishing causal relationships between stressors and effects on marine resources is fundamental. Currently, there is not a widely accepted and proven approach for establishing such causal relationships.[13] Proof of causality would reduce uncertainty on management decisions and cut down the cost involved in implementing and complying environmental policies.[13]

The three first scientific works conducted within this Ph.D. thesis aimed to provide the necessary scientific evidence for establishing causality between the activity of antioxidant biomarkers and diesel oil contamination. Their ultimate goal was to develop a framework for biomonitoring diesel oil–related impact at subtropical estuaries that incorporates the use of biomarkers as biomonitoring tools. Based on the results of these experiments it is possible to conclude:

- The response of antioxidant biomarkers correlates with diesel oil contamination and varies at different temporal scales.

- The direct response of antioxidant biomarkers following experimental manipulations (both laboratory and field conditions), suggests a causal relationship between activities of these biomarkers of exposure and diesel oil contamination.

- Considering the assumptions and conditions for the experiments, all tested null hypothesis were, rejected. Certain biomarkers were more responsive to the contamination than others (i.e. GST and SOD), highlighting the necessity of following a multi–biomarker approach and developing tools for their visualization and interpretation (such as multivariate statistics).

- Biomarker natural variability is an important confounding source of variation. Thus, implementation of an adequate biomonitoring program demands a thoughtful design. To efficiently discriminate the biomarker response caused by contaminant exposure

from natural background variability, experiments that include adequate control treatments are much needed.

Based on our conclusions and the knowledge obtained from our experimental work, the following practical framework is proposed to establish causality between biomarkers and contamination (Fig. 11).
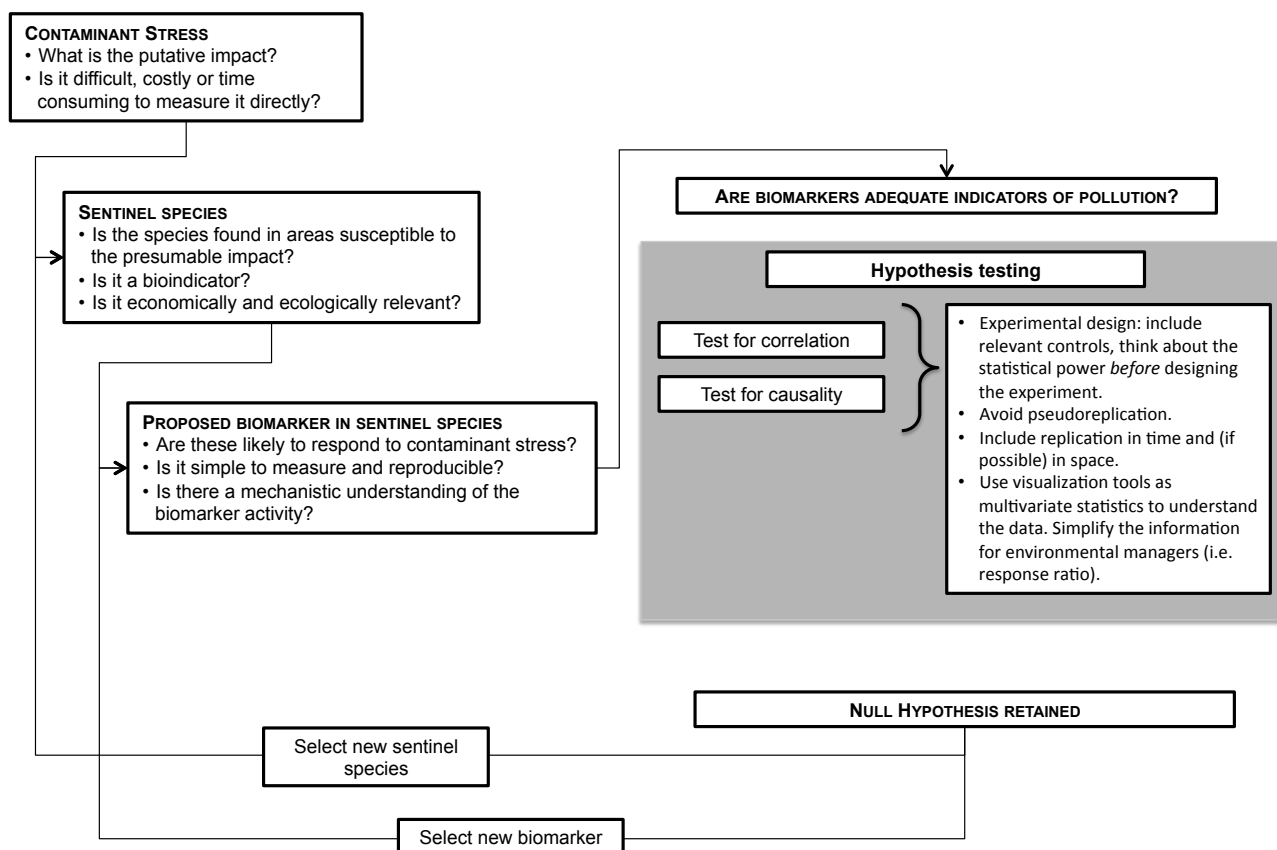
Figure 11. Proposed framework for implementing biomarkers as contamination biomonitoring tools.

First, it is necessary to select a sentinel species, which ideally should serve as a bioindicator of contamination at the community level. Therefore, when selecting the species at which biomarker analyses are to be conducted, the species should meet at least one of the following criteria:

- The species occurs in the area that is at risk and in a reference area.

- Variations in the species abundance or density correlates with a gradient of contamination, suggesting either sensitivity or tolerance to the contaminant.

- The species is ecologically or economically relevant.

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Second, it is necessary to select a group biomarkers for hypothesis testing and for proving a correlation between the measured biomarkers and the impact (See Fig. 6). Before testing correlation between biomarkers and the impact, consider the potential of the proposed biomarker as an effective indicator by simply asking the following questions:

- Is the biomarker likely to respond to the contaminant impact?

- Is the measurement of the biomarker reproducible, simple and cost–efficient?

- Is there enough mechanistic understanding of the activity of the proposed biomarker?

Once a set of biomarkers is selected, proceed with hypothesis testing. If correlation is obtained, proceed with testing more rigorous hypothesis that allows for establishing causality (Fig. 6). However, as highlighted by Adams et al.[13] "Establishing causal relationships between stressors and effects on marine resources is difficult because of the physiochemical and biological complexity of these systems, the variety of biotic and abiotic factors that can modify responses of biota to stressors, compensatory mechanisms that operate in populations, the orders of magnitude involved in extrapolation over temporal and spatial scales, and the many pathways by which stressors can disrupt the normal functioning of ecosystems."

The challenges associated with establishing causality between environmental impacts and the endpoint that presumably indicates the magnitude of their effects can be tackled if statistical power in planning and interpreting the impact is considered.[75] In this regard, particular attention should be given to the experimental design. A good example to convey this point are the results from Paper III, for which interpretation of the impact would not have been possible if an independent control plot was not included and followed over time to be compared with the experimental treatment.

Third, interpretation and visualization of data need to be improved. The use of multivariate tools in ecotoxicology is not widespread, and it is necessary to find ways to present the results in a simpler and more approachable way. Methods that opt for classifying the impact are not recommended. These are susceptible to be biased towards the experimentalist perception of impact and also create confusion because such classes vary depending on the scale or biological organization level. The recommendation is to conduct robust experiments explicitly designed for testing specific hypothesis for which inferential statistics can be applied.

Remarks about laboratory bioassays vs. in situ *manipulations*

Establishing causality between biomarkers and contamination, usually conducted under laboratory conditions, is a necessary pre–requisite before using biomarkers as tools to diagnose stress or as indicators within biomonitoring programs.[33] However, experiments conducted under laboratory conditions do not account for natural variability of the measured markers[76] and are often performed using high and constant concentrations. In the real–world, however, chemicals might only be released in small pulses at a release point, scenarios for which laboratory tests as formally described are nor appropriate.[76] Most importantly, laboratory assays fail to reproduce biotic and abiotic variables (and their associated variability) occurring in natural conditions and omits the effect that biological and ecological process might have in the chemical toxicity, as regarded by changes in bioaccumulation, incorporation and detoxification mechanisms. Under laboratory conditions, all the variables are held constant except for the variable for which the hypothesis is tested; while in field scenarios, only the variable being tested is held constant and all variables are allowed to vary.[76] In field assessment scenarios the goal of pollutant management then becomes understanding the ecological and biological circumstances where pollutants are influential, as a substitute of demonstrating and understanding 'damage' from pollutants.[77]

In this regard, a significant amount of effort is spent standardizing individual biomarkers and characterizing the range for which their response can be considered 'normal' and not affected by detection procedures, differences between experimentalist and laboratory conditions.[78,79] This practice is particularly important for data quality assessment when comparing data between laboratories is the objective. Biomarker responses are known to vary considerably with environmental factors which drastically differ among regions. Thus, to consolidate the use of biomarkers into routine environmental monitoring, such standardizations and quality control comparisons with results available in the literature are insufficient, and baseline or appropriate reference data is needed.[25,80] Also, regarding the literature comparison approach, comparing values from *in situ* biomonitoring studies, where exposure to complex contaminant mixtures unequivocally occurs, with results obtained under laboratory conditions, is not appropriate.[77]

Remarks about experimental designs

One of the principal challenges within field–based assessments of environmental impacts is to deal with the natural spatial and temporal variability in the biomarker responses. A way to prevent confusion between the noise associated with natural variability and the effect of the impact the environmental assessment wishes to measure, is to consider the statistical power of the experiment before conducting it. Statistical power is the probability of rejecting the null hypothesis of "no effect" when it is false. Therefore, to implement well–designed experiments, it is necessary to have a clear hypothesis to test, for which the measurement of environmental parameters at an adequate sampling intensity is feasible, given financial, and logistic constraints.[75] Because calculation of statistical power requires specification of the number of replicates and the ratio between the size of an effect and the variability among the replicates,[75] it is also important to clearly specify what is considered as the effect, variability and replicate. Also, is common that environmental assessment designs lack of spatial replication which is in some cases, justified by the fact that there is only one disturbed location (e.g. a source of disturbance, such as the presence of nuclear power plants are not replicated and randomly located along the coast).[24,75] However, chronic hydrocarbon contamination in estuarine habitats is *a priori* a replicable scenario if the chemical load is sufficiently similar among sites. Temporal replication is also necessary to determine whether an apparent difference from one time to another is part of the effect of the disturbance or a consequence of variation due to intrinsic ecological processes such as predation, competition and recruitment, or to natural disturbances such as storms, extreme temperatures, and floods.[24]

Because biomarker responses vary depending on environmental factors, it is often predicted that major differences in the activity of antioxidant markers relate to seasons, where changes in temperature, precipitation and food availability are anticipated. Thus, the temporal scale at which basal levels are studied is often framed according to seasonal changes. The problem with this approach is that the selection of time for sampling of baseline studies, if available, is often arbitrarily chosen and once trends over time at the selected scales are detected, it is rare to find a follow–up study that examines variability on a different (higher resolution/smaller) time scale. Even if by doing so sounds like it is an overwhelming amount of work, this is in essence the type of work that would serve as a proper base/ground for appropriate biomonitoring design. Within this Ph.D. thesis we restricted the temporal basal variation to only 2 samplings along the year (Paper I). Yet,

natural temporal variation was observed in the activity of clams collected within a 35 day period (Paper III), highlighting that further research on natural temporal variation of these measures is required.

Remarks about interpretation of multi–biomarker results

Multi–biomarker studies represent a useful tool for physiological impairment caused by contaminants. Within multi–biomarker studies the interpretation of how combinations of different biomarkers reflect the integrated toxic effect of a pollutant is more relevant than standardizing individual biomarker responses towards contamination.[25] As proposed by Luoma (1996),[77] a more coherent framework should include pollutants as another of the many variables influencing organisms in the field. So understanding the ecological and biological circumstances, for which pollutants effects are significant as a replacement for identifying the best tool to demonstrate 'damage' from pollutants, becomes the objective.[77] In this sense, multivariate procedures are powerful tools for visualizing and interpreting complex data such as multi–biomarker responses. Also, for management purposes, data presentation tools that easily answer the questions asked and the hypotheses tested are needed. Plots that efficiently convey the size of the effect following contamination disturbance could significantly improve the dialogue between scientist and environmental managers. In this regard, the adoption of log response ratio and percentage change from control are hereby proposed as tools for biomonitoring purposes.

Risk assessment tools

Risk assessment metrics derived from ecotoxicity bioassays are still the primary scientific output used for the regulation of chemicals substances discharged to the environment.[81] To date, the vast majority of the risk assessment data available are from temperate species, generated by developed countries based on temperate regions. As a consequence, water quality criteria for tropical and subtropical regions often rely on extrapolations from temperate species, and is conducted following a surrogate approach, which assumes that the sensitivity of tropical and temperate species are sufficiently similar.[81]

The last manuscript of this thesis was designed to investigate the extent of which the geographical distribution of the studied species can explain their sensitivity. We did this by comparing temperate and Arctic species sensitivity, as denoted by mortality following acute exposure to 2–Methylnaphthalene, a main component of petroleum.

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Our results indicate that, in its simplest form, there are not differences in sensitivity among studied regions. The simplest form refers to comparisons among concentration thresholds, as the No–Effect Concentration (NEC), obtained for the different studied species. This result might be related to the toxic mode of action (MoA) of PAH. PAHs are classified as narcotic compounds and are expected to denature cell membranes, being their physical–chemical characteristics the ones driving the velocity of such degeneration. Thus, the internal concentration threshold at which the chemical compound start causing deaths should, theoretically, be the same regardless the species,[46] and in line with this assumption, differences among region were not depicted. However, when defining sensitivity as the time to observe an effect, a metric that includes the concentration threshold (NEC) and a toxicokinetic parameter like the elimination rate, differences in sensitivity among regions are detected. Our results allowed us to conclude that species from Arctic to subtropical regions might have similar NEC thresholds, but the time they need to reach that threshold varies, and this variation relates to each species' biological traits, with phylogeny and trophic level best–explaining differences among the studied species. Arctic species had in average shorter times for starting to show an effect, followed by subtropical and finally temperate species. Our results highlight the need to conduct further research on this topic and revisit the surrogate approach, which could be underestimating the effects of oil and oil components for Arctic and subtropical regions.

10. Recommendations and concluding remarks

Within this thesis, it has been discussed that for the validation and implementation of biomarkers as tools for biomonitoring programs, it is necessary to detect causal relationships between their activity and the contamination event. Also, the comparison of biomarkers responsiveness without a proper control treatment would lead to either overestimation of the effect, in which case protective measures will be more conservative, or underestimation of the impact for which the monitoring will not accomplish its very basic objectives of prevention and protection from chemical effects. In order to detect such causal relationships and avoid the interaction of confounding factors, the general recommendation is to improve the experimental design of ecotoxicological tests. This implies conducting thoroughly experimental designs and the establishment of clear hypothesis to be tested. Also, considering the statistical power as a key variable for the experimental design is advised. Having clearly stated hypothesis and having identified the methods for refuting them (i.e. inferential statistics and visualization tools as multivariate

ordinations) would significantly improve the interpretation of the results, which can be affected by natural background variation, and therefore, be confusing and hard to interpret.

The antioxidant machinery of the clam *Anomalocardia flexuosa* was identified as a tool of diesel oil chronic an acute exposure. In specific, the activity of SOD and GST were the more sensitive and more indicative biomarkers. Their response was at its 'maximum' following 48 h of exposure. Characterizing the time frame in which the studied biomarkers reach their maximum is relevant for the design of biomarker–based biomonitoring programs. Moreover, regarding assessing the risk of subtropical and tropical ecosystems exposed to oil and derivatives, it was shown that the implementation of risk assessment metrics generated from temperate and Arctic species is not advisable. Also, it was suggested that in the search for metrics for safeguarding the marine ecosystem, attention should not be given only to concentration thresholds, like the no–effect–concentration (NEC), which was similar among the 26 studied species regardless of their geographical distribution. The NEC did not fully explain the complexity of the species sensitivities. Such differences in sensitivity were better reflected by the time to reach and effect, a measure which combines the information from the NEC and the rate of incorporation of the chemical in the animal body. Thus, for biomonitoring coastal estuarine habitats in Brazil, the use of biomarkers in the clam *Anomalocardia flexuosa* is advised, and risk assessment metrics generated from temperate and Arctic species are not applicable to the subtropical ecosystems.

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ABBREVIATIONS

2MN	2–Methylnaphthalene
AChE	Acetylcholinesterase
CAT	Catalase
CEM	Centro de Estudios do Mar
EIA	U.S. Energy Information Agency
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutathione S–transferase
GUTS	General Unified Threshold Model of Survival
H_2O_2	Hydrogen peroxide
$HO_2\cdot$	Hydroperoxyl radical
HOCl	Hypochlorous acid
IAP	Instituto Ambiental do Paraná
$K_{OC}S$	Partition coefficient octanol/colloids in sediments
K_{OW}	Partition coefficient octanol/water
LC_0	Lethal concentration 0%
LC_{50}	Lethal concentration 50%
MDS	Multidimensional scaling
MoA	Mode of action
NEC	No effect concentration
NOEC	No observed effect concentration
$O_2\cdot-$	Anion radical
$OH\cdot$	Hydroxyl radical
$ONOO-$	Peroxynitrite
PAH	Polycyclic aromatic hydrocarbon
PCA	Principal component analysis
RDA	Redundancy analysis
$RO\cdot$	Alkoxyl radical

RO2·	Peroxyl radical
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TEPAR	Terminal Aquaviário Paranaguá - Petrobras
TKTD	Toxicokinetics/Toxicodynamic
UFPR	Universidade Federal do Paraná

GLOSSARY

ABSORPTION

In chemistry, the phenomenon in which atoms, molecules or ions enter some bulk phase. Molecules absorbed are taken up by the volume and not by the surface (adsorption). In biology, the process by which a substance is assimilated by a living organism.

ACCLIMATION

The reversible process whereby an individual organism adjusts to short–term changes in the environment. Adjustment can refer to physiology, morphology or behavior changes. Can occur in natural habitats but here refers to experimental conditions.

ADSORPTION

The process by which a substance becomes attached to another.

BIOACCUMULATION

Uptake and retention of a bioavailable chemical from any, or all, possible external sources (water, food, substrate, air). To occur, the rate of chemical uptake must be higher than the rate of loss of the chemical from the tissues of the organism.

BIOAVAILABILITY

The extent to which a chemical can be absorbed or adsorbed by a living organism by active (biological) or passive (physical or chemical) processes. It may also refer to the presence of the chemical in organisms tissues in the form that can react with cellular biochemicals, eliciting biological responses (Neff, 2004).

BIOINDICATOR

Structural entities, such as sentinel species or communities, that provide indication of exposure to contaminants or biological effects at higher levels of biological organisation (Adams et al., 2005).

BIOMARKER

At the sub–individual level, changes in a biological response, ranging from molecular, cellular, and physiological measures, that can be related to environmental chemicals and can indicate deviation from the normal status of the organism (van der Oost et al., 2003).

FITNESS

The capacity of an individual organism to survive and reproduce in a particular environment.

HOMEOSTASIS

Property of a system, particularly the physiological system of higher animals that allows maintaining internal stability by actively regulating any variable (for instance, body temperature) to remain nearly constant. Also, the ability of the evil of biological organization, be it at the individual, community or ecosystem, to withstand/tolerate/adjust/ adapt to stressors (Elliot and Quintino, 2007).

KINETICS

the study of rates of chemical processes.

PYROGENIC

Produced under conditions involving intense heat.

PYROGENIC PAHS

Hydrocarbons produced from the rapid and incomplete combustion of organic materials (petroleum, wood, coal, etc.) occurring a very high temperatures (~700 ºC).

PETROGENIC

Related to hydrocarbons formed by decomposition of organic matter, such as petroleum, at elevated pressure and temperatures.

PETROLEUM PSEUDOCOMPONENTS

Groups of chemicals described by their chemical and physical properties that are assumed to have a similar distribution and fate in the environment.

SENTINEL SPECIES (BIOINDICATOR TAXON)

Species that because of their sensitivity are often employed to detect potential risks pose to humans. Organism that can provide information on the environmental conditions of its habitat by its presence, absence or its behavior (van der Oost et al., 2003). Also, organisms that can be used to infer conditions or population responses in a particular habitat.

SORPTION

Physical and chemical process by which a substance becomes incorporated (absorption), adhered or bonded (adsorption), or exchanged between an electrolyte solution and a complex.

TOXICOKINETICS

Refers to the collective processes of toxicants intake, elimination, transformation and transportation (to target sites) into the body and their variation within time.

TOXICODYNAMICS

Link between the internal concentration of a toxicant and the observed effects in organisms over time. Usual studied endpoints include mortality, growth and reproduction.

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PAPERS

APPENDIX

Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 52859-1	Data da Emissão: 11/03/2016 08:03	Data para Revalidação*: 10/04/2017

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Paulo da Cunha Lana	CPF: 370.764.697-15

Título do Projeto: Avaliação dos níveis de estresse oxidativo em espécies bênticas expostas a derrame experimental de óleo diesel em estuário subtropical

Nome da Instituição : UNIVERSIDADE FEDERAL DO PARANÁ	CNPJ: 75.095.679/0001-49

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Levantamento bibliográfico	02/2016	12/2016
2	Ajustes metodológicos	02/2016	03/2016
3	Experimento de derrames de óleo diesel em campo	03/2016	03/2016
4	Processamento das amostras biológicas em laboratório	03/2016	03/2016
5	Analises dos níveis de hidrocarbonetos policíclicos aromáticos em laboratório	04/2016	05/2016
6	Analises da atividade anti-oxidante	04/2016	05/2016
7	Redação de relatórios técnicos e artigos científicos	05/2016	12/2016
8	Analise dos dados	06/2016	09/2016

Observações e ressalvas

1	As atividades de campo exercidas por pessoa natural ou jurídica estrangeira, em todo o território nacional, que impliquem o deslocamento de recursos humanos e materiais, tendo por objeto coletar dados, materiais, espécimes biológicos e minerais, peças integrantes da cultura nativa e cultura popular, presente e passada, obtidos por meio de recursos e técnicas que se destinem ao estudo, à difusão ou à pesquisa, estão sujeitas a autorização do Ministério de Ciência e Tecnologia.
2	Esta autorização NÃO exime o pesquisador titular e os membros de sua equipe da necessidade de obter as anuências previstas em outros instrumentos legais, bem como do consentimento do responsável pela área, pública ou privada, onde será realizada a atividade, inclusive do órgão gestor de terra indígena (FUNAI), da unidade de conservação estadual, distrital ou municipal, ou do proprietário, arrendatário, posseiro ou morador de área dentro dos limites de unidade de conservação federal cujo processo de regularização fundiária encontra-se em curso.
3	Este documento somente poderá ser utilizado para os fins previstos na Instrução Normativa ICMBio n° 03/2014 ou na Instrução Normativa ICMBio n° 10/2010, no que especifica esta Autorização, não podendo ser utilizado para fins comerciais, industriais ou esportivos. O material biológico coletado deverá ser utilizado para atividades científicas ou didáticas no âmbito do ensino superior.
4	A autorização para envio ao exterior de material biológico não consignado deverá ser requerida por meio do endereço eletrônico www.ibama.gov.br (Serviços on-line - Licença para importação ou exportação de flora e fauna - CITES e não CITES).
5	O titular de licença ou autorização e os membros da sua equipe deverão optar por métodos de coleta e instrumentos de captura direcionados, sempre que possível, ao grupo taxonômico de interesse, evitando a morte ou dano significativo a outros grupos; e empregar esforço de coleta ou captura que não comprometa a viabilidade de populações do grupo taxonômico de interesse em condição in situ.
6	O titular de autorização ou de licença permanente, assim como os membros de sua equipe, quando da violação da legislação vigente, ou quando da inadequação, omissão ou falsa descrição de informações relevantes que subsidiaram a expedição do ato, poderá, mediante decisão motivada, ter a autorização ou licença suspensa ou revogada pelo ICMBio, nos termos da legislação brasileira em vigor.
7	Este documento não dispensa o cumprimento da legislação que dispõe sobre acesso a componente do patrimônio genético existente no território nacional, na plataforma continental e na zona econômica exclusiva, ou ao conhecimento tradicional associado ao patrimônio genético, para fins de pesquisa científica, bioprospecção e desenvolvimento tecnológico. Veja maiores informações em www.mma.gov.br/cgen.
8	Em caso de pesquisa em UNIDADE DE CONSERVAÇÃO, o pesquisador titular desta autorização deverá contactar a administração da unidade a fim de CONFIRMAR AS DATAS das expedições, as condições para realização das coletas e de uso da infra-estrutura da unidade.

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Gisele Cavalcante Morais	Pesquisadora	931.275.023-20	82738697-4 SSP-MA	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1		PR	Baía de Paranaguá	Fora de UC Federal

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Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 52859-1	Data da Emissão: 11/03/2016 08:03	Data para Revalidação*: 10/04/2017

* De acordo com o art. 28 da IN 03/2014, esta autorização tem prazo de validade equivalente ao previsto no cronograma de atividades do projeto, mas deverá ser revalidada anualmente mediante a apresentação do relatório de atividades a ser enviado por meio do Sisbio no prazo de até 30 dias a contar da data do aniversário de sua emissão.

Dados do titular

Nome: Paulo da Cunha Lana	CPF: 370.764.697-15

Título do Projeto: Avaliação dos níveis de estresse oxidativo em espécies bênticas expostas a derrame experimental de óleo diesel em estuário subtropical

Nome da Instituição : UNIVERSIDADE FEDERAL DO PARANÁ	CNPJ: 75.095.679/0001-49

Atividades X Táxons

#	Atividade	Táxons
1	Coleta/transporte de espécimes da fauna silvestre in situ	Anomalocardia brasiliana (*Qtde: 1000)

* Quantidade de indivíduos por espécie, por localidade ou unidade de conservação, a serem coletados durante um ano.

Material e métodos

1	Método de captura/coleta (Invertebrados Aquáticos)	Peneira, Coleta manual, Draga, pegador (Van veen, Box corer, Holme, Petersen, etc.)

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	UNIVERSIDADE FEDERAL DO PARANÁ	
2	Akvaplan-Niva	

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Registro de coleta imprevista de material biológico

De acordo com a Instrução Normativa nº 03/2014, a coleta imprevista de material biológico ou de substrato não contemplado na autorização ou na licença permanente deverá ser anotada na mesma, em campo específico, por ocasião da coleta, devendo esta coleta imprevista ser comunicada por meio do relatório de atividades. O transporte do material biológico ou do substrato deverá ser acompanhado da autorização ou da licença permanente com a devida anotação. O material biológico coletado de forma imprevista, deverá ser destinado à instituição científica e, depositado, preferencialmente, em coleção biológica científica registrada no Cadastro Nacional de Coleções Biológicas (CCBIO).

Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

Este documento (Autorização para atividades com finalidade científica) foi expedido com base na Instrução Normativa nº 03/2014. Através do código de autenticação abaixo, qualquer cidadão poderá verificar a autenticidade ou regularidade deste documento, por meio da página do Sisbio/ICMBio na Internet (www.icmbio.gov.br/sisbio).

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* Identificar o espécime no nível taxonômico possível.

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THE SURVEYORS: WATCHING FOR OUR PARADISE HEALTH

BRAZIL PARADISE

FOUND OIL

+

= SAD PARADISE CONTAMINATED SEA

IDEA   IDEA

MEASURE BIOMARKERS HELP CLEAN THE SEA

PLEASE SIGN IN

LAB GEEKS

OUR HEROES !

+

LAB

AFTER TIME AND ORGANIZATION

SUPER CRABY

CULVERI

LITTLE VIRGINEA

EVERY ONE HAVE THEIR POWER
ALL TOGETHER WILL FIND THE BIOMARKER

WILL THE DO IT???

SHEELLY

BIG GENIDENS

THEY GOT ALL TOGETHER AND TRAINED VERY HARD

POW

LABORATORY TESTS

HEAT

WATER   SEDIMENTS   HAZARD ELEMENTS

+

DIFFERENT MIXES AND CONCENTRATIONS

=

LAB CHAMPIONS!

#1

#1

AND THEN ...

FINAL TEST
HOW THEY WORK IN PLACE
OUR CHAMPIONS WILL
GO BACK HOME AND PROVE
THE THEORY

THE CHAMPION !
BEST HERO AND BIOMARKER DUO

FINALLY HAVING
PAM PAM PAAAAAAMMMM...