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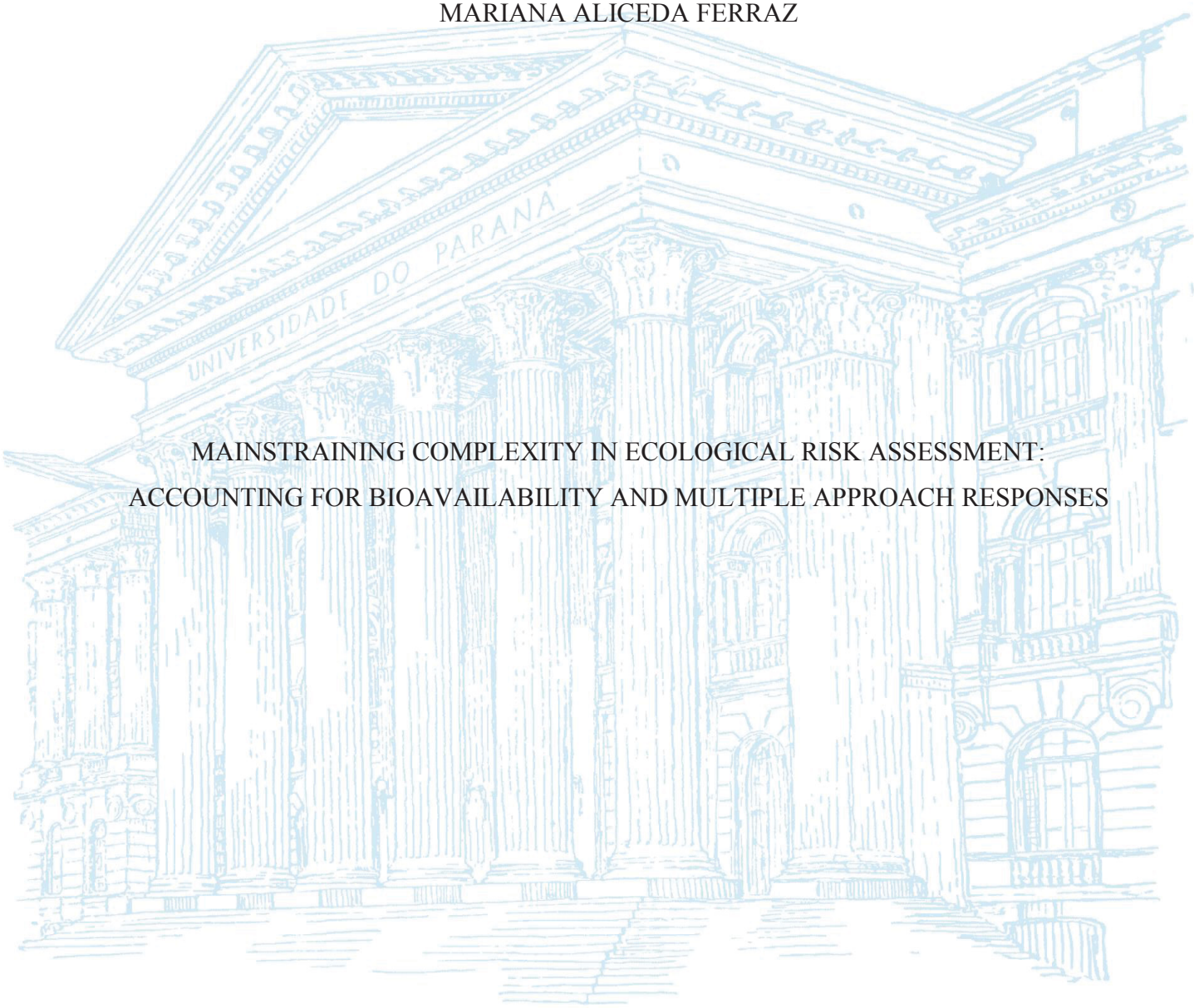

MAINSTRAINING COMPLEXITY IN ECOLOGICAL RISK ASSESSMENT: ACCOUNTING FOR BIOAVAILABILITY AND MULTIPLE APPROACH RESPONSES

PONTAL DO PARANÁ

2020

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Tese apresentada ao curso de Pós-Graduação em Sistemas Costeiros e Oceânicos, Centro de Estudos do Mar, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutor em Sistemas Oceânicos e Costeiros.

Orientador(a): Profª. Drª. Fabiane Gallucci

Coorientador: Prof. Dr. Rodrigo Brasil Choueri

PONTAL DO PARANÁ

2020

FICHA CATALOGRÁFICA

CATALOGAÇÃO NA FONTE:
UFPR / SiBi - Biblioteca do Centro de Estudos do Mar
Fernanda Pigozzi – CRB 9/1151

Ferraz, Mariana Aliceda
F413m Mainstraining complexity in ecological risk assessment: accounting for bioavailability and multiple approach responses. / Mariana Aliceda Ferraz. – Pontal do Paraná, 2020.
120 f.: il.; 29 cm.

Orientadora: Profª. Drª. Fabiane Gallucci.
Coorientador: Prof. Dr. Rodrigo Brasil Choueri.

Tese (Doutorado) – Programa de Pós-Graduação em Sistemas Costeiros e Oceânicos, Centro de Estudos do Mar, Setor Reitoria, Universidade Federal do Paraná.

1. ARE – Avaliação de Risco Ecológico. 2. Parâmetros ambientais. 3. Biomarcadores. 4. Microcosmo. 5. Copépodes. 6. Assembleia de nemátoda. I. Título. II. Gallucci, Fabiane. III. Choueri, Rodrigo Brasil. IV. Universidade Federal do Paraná.

CDD 577.2

MINISTÉRIO DA EDUCAÇÃO
REITORIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO SISTEMAS COSTEIROS E OCEÂNICOS - 40001016054P6

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em SISTEMAS COSTEIROS E OCEÂNICOS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MARIANA ALICEDA FERRAZ** intitulada: **Mainstreaming complexity in ecological risk assessment: accounting for bioavailability and multiple approach responses**, sob orientação da Profa. Dra. FABIANE GALLUCCI, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

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AGRADECIMENTOS

Agradeço primeiramente a Deus.

Agradeço aos meus pais por todo apoio nesta incansável jornada.

Agradeço a minha orientadora Drª Fabiane Gallucci por ter me aturado todos esses anos! 😊 E de ter divido toda a sua sabedoria em me orientar e compartilhado de momentos de risadas, confraternizações, coletas, aulas, reuniões e desesperos!!

Agradeço ao meu orientador Drº Rodrigo Brasil Choueri por todo apoio desde as coletas, ensaios e desenvolvimento da tese.

Agradeço por todo o suporte da Drª Paloma Gusso-Choueri nas análises de biomarcadores.

Agradeço ao Drº Denis Moledo de Souza Abessa por ter permitido que as análises de biomarcadores fossem desenvolvidas em seu laboratório.

Agradeço ao Drº Ítalo Braga Castro e a sua aluna Fiamma Eugênia Lemos Abreu pela realização das análises químicas.

Agradeço a Cecilia Simon por ter realizado o primeiro ensaio, bem como a identificação dos nemátodas.

Agradeço a Simone por ter auxiliado na identificação dos nemátodas.

Agradeço a Aline Vecchio Alves que desde o meu mestrado me ajuda em com todos os ensaios laboratoriais.

Agradeço aos alunos de iniciação cientifica, Vagner, Ana Carolina, Amanda e Guilherme por terem encarado as incansáveis horas de coletas e laboratório (quem nunca montou um ensaio em plena Copa do Mundo né?!)

Agradeço a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – código 001) por ter me concedido a bolsa de doutorado.

RESUMO

A Avaliação de Risco Ecológico (ARE) é um processo usado para avaliar o impacto das atividades humanas no meio ambiente e é uma parte importante do portfólio de informações sobre a política ambiental. Em uma ARE amplamente satisfatória, a análise precisa indispensavelmente abranger uma seleção de ensaios, incluindo uma variedade de respostas relevantes provenientes de diferentes níveis de organização biológica, bem como diferentes espécies, não apenas com base na cadeia alimentar, mas na variabilidade em outras características ecológicas. Portanto, a fim de entender os mecanismos de ação tóxica e os efeitos ecológicos em sistemas complexos, há a necessidade de considerar múltiplas abordagens que incorporem processos moleculares, fisiológicos e ecológicos. Esta tese tem como objetivo compreender como a variabilidade ambiental, as características de espécies e o uso de múltiplas abordagens influenciam a caracterização de efeitos ecológicos de acordo com ARE. Para isso, uma série de experimentos foi realizada para analisar os efeitos ambientais de um contaminante orgânico emergente e frequentemente encontrado no meio ambiente (o biocida anti-incrustante Irgarol),, sob diferentes condições ambientais, utilizando diferentes espécies (isoladamente ou em ensaios de comunidade) e abordagens ecotoxicológicas. Os principais resultados da tese foram: (1) o carbono orgânico total do sedimento influencia os efeitos do Irgarol sobre assembléias de nemátoda; (2) O pH da água intersticial não apresenta influência na toxicidade de Irgarol sobre assembléias ne nemátodas; (3) Os efeitos do Irgarol dependem das características ecológicas dos organismos; (4) Os ensaios de nível individual de uma única espécie podem ser menos sensíveis à contaminação do que os parâmetros nos níveis mais baixo e mais alto de organização biológica; e (4) Diferentes abordagens exibem diferentes caracterizações de efeitos ecológicos. Os resultados dos diferentes estudos que fazem parte desta tese fornecem evidências substanciais de que a interação entre "disponibilidade ambiental", "biodisponibilidade ambiental" e "biodisponibilidade toxicológica" acabará por determinar os efeitos ecológicos dos contaminantes nos organismos vivos. Como tal, as principais conclusões desta tese podem dar suporte ao projeto de futuras estruturas de ERA relativas à contaminação de sedimentos estuarinos.

Palavras-chave: Avaliação de Risco Ecológico, microcosmo, assembleia de nemátoda, parâmetros ambientais, biomarcadores, copépodes.

ABSTRACT

Ecological Risk Assessment (ERA) is a process used to evaluate the impact of human activities on the environment and it is an important part of the information portfolio that improves the environmental policy. A widely satisfactory ecological risk assessment, must necessarily cover a selection of assays, including responses at different levels of biological organization, and different species considering not only the food chain but other ecological traits of model organisms. As such, there is a need to consider multiple approaches that incorporate molecular, physiological as well as ecological processes in order to understand the mechanisms of the toxic action as well as the ecological effects on complex systems. This thesis aims at understanding how the environmental variability, species traits and the use of multiple approaches influence the characterization of ecological effects in ecological risk assessment. For that, a series of experiments were conducted to analyze the environmental effects of an emerging organic contaminant (antifouling biocide Irgarol), under different environmental conditions, using different species (in isolation or in community assays) and ecotoxicological approaches. Main findings from the thesis were that (1) Sediment total organic carbon influence the effects of Irgarol on nematode assemblages, (2) Pore-water pH does not affect Irgarol toxicity over nematode assemblages; (3**)** Effects of Irgarol depends on organisms' ecological traits; (3) Single-species individual level assays can be less sensitive to contamination than endpoints at lower and higher levels of biological organization; (4) Different approaches display different characterizations of ecological effects. The results from the different studies that make part of this thesis provide substantial evidence that the interplay between ''environmental availability'', ''environmental bioavailability'' and ''toxicological bioavailability'' will ultimately determine ecological effects of contaminants in living organisms. As such, the main findings from this thesis may give support to the design of future ERA frameworks concerning contamination of estuarine sediments.

Keywords: Ecological Risk Assessment, microcosm, nematode assemblages, environmental parameters, biomarkers, copepod.

SUMMARY

GENERAL INTRODUCTION

Ecological Risk Assessment

Ecological risk assessment (ERA) is a process used to evaluate the impact of human activities on the environment and is an important part of the information portfolio that improves environmental policy (Finizio and Villa, 2002; Power and Keegan, 2001; Sekizawa et al., 2003; Suter, 2008). According to the USEPA, the ERA "evaluate the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors" (USEPA, 1992, 2012). Although ERA is applicable to any type of anthropogenic stressor, it is generally used to the regulation of manufactured chemicals.

The essential elements of an ERA include three steps: (1) Problem formulation, (2) Analysis and (3) Risk characterization (Figure 1; USEPA, 1998). In the first step, risk assessors develop a strategy of analysis selecting goals and endpoints for assessments. In the second step, the procedures are implemented to evaluate exposure to stressors and the relationship between stressor levels and ecological effects (hazard). In the third step, the risk is estimated and described by lines of evidence which produce information to be reported to risk managers.

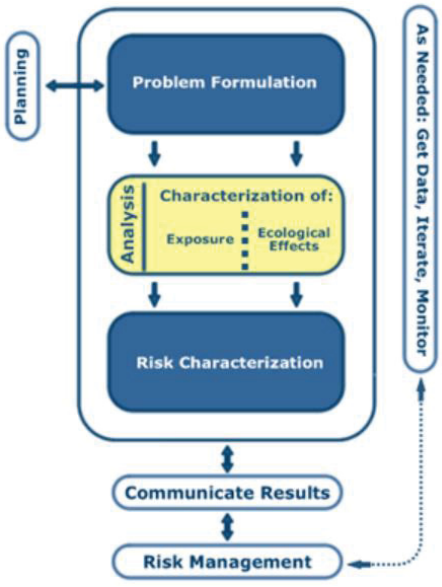


Figure 1. Planning the Ecological Risk Assessment. Figure from EPA (https://www.epa.gov/risk/conducting-ecological-risk-assessment).

The ERA process was developed by the scientific and regulatory communities (ERA, 2008), with the main objective to develop simple tools which could provide answers to growing and pressing problems (USEPA, 1998). Risk characterization is usually represented by a simple

number (PEC/PNEC or TER, Toxicity Exposure Ratio), which represents a threshold that must not be exceeded to avoid adverse effects to ecosystems. Within this context, it is important to discern between the terms "hazard" and "risk", which have different meanings. Whereas "hazard" implicates in exposure and ecotoxicological effects (step 2. Analysis), the term "risk" describes the likelihood of a chemical compound to cause a negative effect in the environment (step 3. Risk characterization). In simplistic terms, a potential for adverse effects (risk) may occur when the exposure concentration exceeds (by some predetermined margin) the concentration at which effects have been observed from toxicity studies (USEPA, 1992; Cortez et al., 2012; Cortez et al., 2018, Pusceddu et al., 2018). As such, risk characterization is estimated from ecotoxicological data which is generally produced with a small number of selected test organisms. The choice of organisms and endpoints, which should take place at the first step (i.e. Problem formulation), are based on standard laboratory toxicity test using a model organism and measure of toxicity (e.g., LC50, NOAEC, etc.) (Rohr et al., 2016; EPA, 1998). The main drawback here is that the standardized protocols do not determine whether an assessment endpoint is appropriate to achieve the ultimate goal of estimating how ecosystem functioning and biodiversity are affected by chemical substances (EPA, 1998). In addition, the standard conditions of laboratory tests do not account for the high the variability of environmental scenarios that may strongly affect both exposure (environmental fate, bioavailability, etc.) and effects of toxic chemicals (Vighi and Villa, 2013).

Currently used approaches for ERA represented the basis for the development of international regulations and have led to an increased level of chemical control and improvement of environmental quality (Vighi and Villa, 2013). However, their oversimplification limits the prediction of real consequences to the structure and functions of the natural ecosystems, leading to a high level of uncertainty in the characterization of ecological risks. As such, there is a pressing need for the development of tools that can increase the ecological realism of exposure and effect assessment, considering the properties and the complexity of potentially exposed ecosystems. In that respect, main challenges of ERA include the evaluation of the availability of the compounds to organisms and the improvement of approaches combining ecotoxicological responses at different levels of biological organization (e.g. biochemical, cellular, histological, individual, population, community), which may show both early and ecologically relevant biological responses to assess the potential risk of chemical compounds in ecosystems (ECHA, 2013).

Bioavailability

Conceptually, bioavailability has a wide variety of meanings. According to Di Toro et al. (1991), "bioavailability is the fraction of a substance that is free or available for uptake by an organism and subsequently transported to a site of action/receptor, or target organ". This means that total concentrations of contaminants are not always directly related to effects since exposure of biota may be limited by processes that render them unavailable for uptake. As such, bioavailability is the key to understanding toxicity.

Peijnenburg et al. (1997) proposed a very interesting and wide view for the term bioavailability, that bioavailability should be understood as a dynamic process that involves two distinct phases: a physicochemically driven desorption process (also referred to as ''environmental availability'') and a physiologically driven uptake process also referred to as ''environmental bioavailability''). In addition, since the internal concentration of the organism (also referred to as ''toxicological bioavailability'') is related with organ–effect levels, it is the latter that is determinant for the actual bioavailability (Peijnenburg et al., 1997). Considering this dynamic process, bioavailability of contaminants for aquatic fauna can be determined by at least three main factors: 1- physical and chemical properties of the molecules which in turn determine preferential partitioning in different environmental compartments (Di Toro et al., 1991; Alexander, 2000), 2- Environmental parameters of exposure media (pH, salinity, oxygen availability, carbon amounts etc.) (Bangkedphol et al., 2009; Basallote et al., 2015; Sandrin and Maier, 2003) and 3- several different aspects related to organism biology, including differences in uptake routes (Chapman et al., 2002a; Forbes et al., 1998; Lu et al., 2004), levels of interaction with environmental matrices (Forbes et al., 1998; Ortega-Calvo et al., 2015), organisms behavior (Peijnenburg et al., 1997; Ortega-Calvo et al., 2015), etc.

Regarding the influence of physical and chemical properties of the molecules, N-octanol water (Kow), defined as the ratio of the concentration of a chemical in n-octanol and water at equilibrium, is an important characteristic of organic molecules often used to predict partitioning in the environment (Niemi et al., 1992). Usually expressed as logKow, it is a relative indicator of the tendency of an organic compound to adsorb to the organic phase of sediments and living organisms (ECHA, 2017). LogKow are generally inversely related to water solubility, so that substances with high logKow values tend to adsorb more readily to organic matter in sediments because of their low affinity for water. As such, higher Know will

imply in lower bioavailability of the compound in the water but greater bioavailability in particulate organic matter (Eggleton and Thomas, 2004). The Kow property also defines the affinity of a compound for biotic tissue or fat since cell walls are composed of lipids (phospholipids, lipoproteins and free fatty acids), the polarity of which is similar to octanol (Coquillé et al., 2018).

Environmental parameters of a given media, such as redox potential, salinity, pH, organic matter content, can influence the partitioning of chemical substances between different environmental compartments and also interfere with the stability of contaminants in the sediment matrix, promoting more or less availability of chemical substances to the biota (Bangkedphol et al., 2009; Basallote et al., 2014). Particularly for apolar organic compounds, an important class of environmental pollutants, the organic matter content of the sediment combined with the partition coefficient (Kow) of the chemical strongly influence bioavailability for aquatic organisms (Landrum et al., 1987; Peijnenburg et al., 1997). This is so because this class of contaminants are generally hydrophobic, so that they tend to partition with the organic phase of the sediment (Fuchsman and Barber, 2000). As such, the amount of organic matter in the sediment is expected to influence the bioavailability and therefore the toxicity of organic contaminants to benthic organisms (Quang et al., 2017). Other environmental variables like pH, for example, potentially influences water solubility as well as lipophilicity (therefore cell permeability) of substances by ionization of the molecules (Pagliarani et al., 2013; Reijonen et al., 2016). The acid dissociation constant (pKa) is a measure of ionization constant, i.e. it infers on the amount of H^+ of a given substance. Acid pHs can affect substances with low pKa making them more lipid soluble as well as substances with high pKa which turn more water soluble (Schwarzenbach et al., 2016). In this way, ionized molecules are more water soluble and less lipophilic (therefore less permeable to cellular membranes) (Larini, 2009).

Finally, organisms' life history strategy such as feeding behavior and direct contact with the sediment, have potential relevance for bioavailability (ECHA, 2012). For instance, deposit-feeding organisms can concentrate particle-bound contaminants by selective ingesting the organic-rich fraction of the available sediment (Lopez & Levinton, 1987). The degree to which they can concentrate ingested organic matter varies from species to species, but concentration factors are typically on the order of two or more (Lopez & Cheng, 1982; Lopez & Cheng, 1983; Lee et al., 1990). In this case, the concentration of the contaminant within an animals' gut will often be two or more times that of the bulk sediment. Such evidences point to the potential role

of organism's biology and feeding behavior in controlling contaminants uptake. Another example is contamination via the water phase in nematodes, through their permeable cuticle. The cuticle of a nematode is a physiologically active structure that exhibits selective permeability to molecules including water, certain ions as well as organic compounds (Bird & Bird, 2012). The permeation of these compounds through the cuticle is a dynamic process which is actively controlled by the living nematode. Substances such as nematicides, for instance, can enter the body at different rates independent of other chemical compounds, and permeation rates differ strongly among different nematode species (Castro and Thomason, 1971). As such, cuticle permeability can actively influence the amount of chemicals that enter the nematode body, and therefore, has the potential to substantially influence bioavailability.

The way in which the factors mentioned above may affect bioavailability has been considered in scientific studies (e.g., Peijnenburg et al., 1997). However, more than each factor in isolation, it is the interplay between them that will ultimately determine toxicity (Figure 2). For instance, the relevance of environmental variables to bioavailability (and therefore toxicity) will depend on physical and chemical properties of the contaminants. Molecules which are highly hydrophobic, for instance, will be more influenced by organic content of the media than those that are not. In this case, the influence of organic content will also depend on organisms' exposure route (Peijnenburg et al., 1996; Rudel, 2003). A higher organic content offers more binding sites for organic contaminants (i.e. lower contaminant levels in the interstitial water), which means a potential lower toxicity for organisms whose exposure route is mainly through contaminated interstitial water (Hoss et al., 2011; Chapman, 1998; Di Toro et al., 1991). On the other hand, a higher POM level could mean a higher toxicity for deposit-feeding organisms, which can assimilate the contaminant by ingestion of contaminated particles (Hoss et al., 2011; Höss et al., 2001; Schlekat et al., 1999).

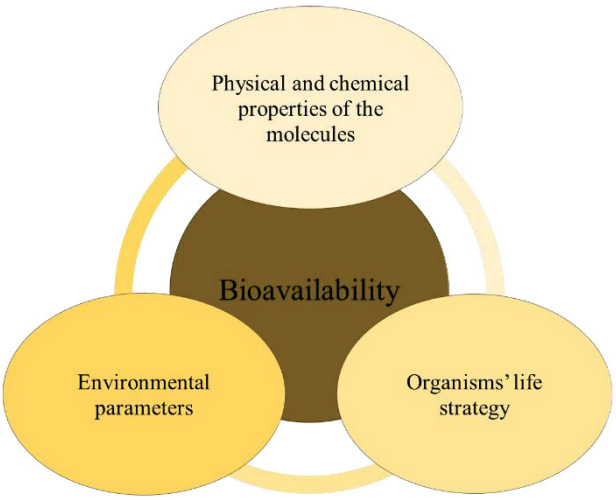


Figure 2. Factors that affect bioavailability of chemical substances and their interactions.

Multiple Approach Responses for ERA

Characterization of ecological effects in ERA (Analysis, Figure 1) are commonly based on a set of single-species, short-term, toxicity tests. The choice of species and endpoints to be measured take place at the problem formulation stage of an ERA (Figure 1). Although guidelines that recommend the use of three to four different species representative of the food chain exist (OECD, 2007), endpoints are usually restricted to individual life-cycle traits such as growth, reproduction and survival (Jager et al., 2014; Kammenga et al., 1996; Rohr et al., 2007). The use of biological endpoints at the individual level raises questionings regarding the extrapolation of these responses (Franco et al., 2017; SCENIHR, 2012), and the lack of understanding of the mechanisms of the stressor action (Hagger et al., 2006). Besides, using biological endpoints at the individual level prevents the management, as the observations are limited to more drastic biological responses (i.e. mortality, reduced growth and/or reproductive performance). However, it has been recognized that studies that integrates different levels of biological organization are better suited to predict toxicity in a complex natural environment since they provide different types of information necessary for ecological risk assessment (Clements, 2000).

Effects of contaminants at lower levels of organization (e.g. molecular and biochemical responses) occur faster than other levels and may provide early warnings of toxicological effects on individuals and populations (Hagger et al., 2006). In addition, some biochemical and physiologic biomarkers are already commonly used (including metallothionein induction, acetylcholinesterase inhibition, cytochrome P450 induction, persoxisome proliferation and

various indicators of oxidative stress; Cajaraville et al., 2000) and they can provide information on possible mechanisms of toxicity or exposure to potential toxic substance (Forbes et al., 2006; Hagger et al., 2006). Despite the ability of the endpoints to directly link responses to exposure, the ecological significance of these responses is generally unknown and remains to be established (Rohr et al., 2016). As such, the characterization of molecular, biochemical and physiological responses within ERA framework may not be enough.

Single-species assays are also effective in assessing cause-effect relationships. They provide straightforward results such as lethal concentration in 50% of the organisms (LC50 - acute), Concentration of Not Observed Effect (NOEC) (Chronic), or the Concentration of Lesser Observed Effect (LOEC) (chronic), which allows very objective information for risk managers. However, observed effects are obviously species-specific, excluding any emergent responses that arise from interactions and indirect effects that regulate biological communities and ecosystems. Thus, community bioassays in microcosm or mesocosm setups offer a more real alternative between the impossible complexity of the ecosystem and the artificiality of single-species tests (Brink et al., 2000; Passarelli et al., 2018; Rohr et al., 2016; Van den Brink et al., 2013).

The community level assays are clearly a more representative approach, as they consider the response of many species with different life traits and sensitivities, and preserve species interactions and interactions with the physicochemical environment, which mediate the results of the tests (Rohr et al., 2016). As such, community level assays are ecologically more relevant than single-species tests, and potentially represent a powerful tool for improving ecological realism of risk assessment (Cairns and Pratt, 1993; Chapman, 2002; Höss and Kühn-institut, 2014; SCENIHR, 2012; Schratzberger et al., 2002).

In addition to consider the information held by the different levels of biological organization of bioassays, biological traits and ecological function of the species within the community should be considered. For instance, the trophic behavior and burrowing capacity in the sediment may affect the direct routes of exposure to the contaminants. On the same way, biological characteristics such as cuticle permeability and life-history traits may affect sensibility, or the way species respond to stress (Bongers, 1990; Egres et al., 2019; Santos et al., 2018; Schratzberger and Ingels, 2018). Even ecologically similar species have shown distinct, sometimes even divergent, responses to stress (Monteiro et al., 2018). This was also observed for taxonomically close species (Monteiro et al., 2018). In that context, it has been

shown that integrating the results of assays with different organisms (e.g., amphipod, copepod, tanaid and bivalve) promote a more precise view of negative effects, because they contemplate different ecological strategies and sensitivities (Campos et al., 2019).

Each level of biological organization is responsible for promoting mutually exclusive responses, as for example, the mode of action against ecological relevance, and potentially relevant to risk characterization. Besides, the use of model species with variable biological and ecological characteristics may produce a heterogeneous set of responses that it can complement each other. In this sense, one chemical stressor may affect particularly one endpoint, e.g. reproduction whereas other compound may affect another, e.g. mortality. As such, absence of an effect in one attribute does not mean protection of all attributes. Intrinsically, the ERA needs an interdisciplinary approach that incorporates molecular, physiological as well as ecological processes in order to understand mechanisms of toxic action as well as ecological effects on complex systems.

Aims and Outline of the Thesis

This thesis aims at understanding how environmental variability, species traits and the use of multiple approaches influence the characterization of ecological effects in ecological risk assessment (i.e. Analysis, Figure 1). For that, a series of experiments were conducted to analyze the environmental effects of an organic contaminant frequently encountered in the environment under different environmental conditions, using different species (in isolation or in community assays) and ecotoxicological approaches. The contaminant used was the antifouling biocide Irgarol, one of the most commonly biocide used in antifouling paints. This herbicide is highly hydrophobic, precipitating as soon as it gets in contact with the aquatic environment, where it become associated with organic matter (Konstantinou and Albanis, 2004). Irgarol belongs to the s-triazine group (2-methythiol-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) and acts as a photosystem-II (PSII) inhibitor, causing interruption of electron transport in chloroplasts during photosynthesis (Dahl and Blanck, 1996; Moreland, 1980) showing high toxicity to autotrophic aquatic species such as macrophytes, symbiotic dinoflagellates in corals, cyanobacteria and algae (Bao et al., 2011). Despite being an herbicide, ecotoxicological studies have also shown negative effects of Irgarol on the development and metabolism of invertebrates (Kobayashi and Okamura, 2002; Manzo et al., 2006; Perina et al., 2011), fish (Key et al., 2008;

Van Wezel and Van Vlaardingen, 2004), and on meiobenthic communities (Gallucci et al., 2015; Hannachi et al., 2016). Specifically, the following questions were addressed:

(1) Does total organic carbon concentration in the sediments influence the ecological effects of Irgarol on meiobenthic communities?

(2) Does pore water pH influence the ecological effects of Irgarol on meiobenthic communities?

(3) Does the combination of approaches using organisms with distinct ecological traits and endpoints encompassing different levels of biological organization complement each other to improve ecological risk assessment?

To answer these questions the thesis is divided in two main chapters. The Chapter 1 focuses on the influence of environmental variability and species traits in the characterization of ecological effects of Irgarol. This Chapter is divided in two parts. The first part is devoted to the understanding of the role of sediment organic content and organisms' trophic ecology on Irgarol toxicity. Specifically, it tests the hypothesis that toxicity of Irgarol on meiobenthic communities differ between sediments with the same community composition but different organic carbon contents and that species with different ecological traits are differently affected. The second part investigates the role of sediment pH on Irgarol toxicity. Because variations in pH can affect both hydrophilicity and lipophilicity of organic compounds and therefore potentially influence their bioavailability, I test the hypothesis that Irgarol will exhibit different toxicity at different pH. However, since the potential effects of pH in Irgarol water solubility and lipophilicity are counteracting, it is hard to hypothesize on the direction of pH influence on Irgarol toxicity. In both chapters, I have tested the toxicity of Irgarol in microcosm assays using meiobenthic communities. Within sedimentary environments, nematodes comprise the most abundant and species-rich metazoans (Coomans, 2000; Lambshead and Boucher, 2003), which contribute to important ecosystem functions such as decomposition of organic matter, nutrient recycling (Bonaglia et al., 2014; Hubas et al., 2010; Freckman, 1988;), and energy transfer to higher trophic levels (Woodward, 2010). Due to the general lack of planktonic larvae, the manipulation of natural assemblages with several species belonging to different trophic levels (e.g. bacterivores, microvores, herbivores, omnivores, and predators) in laboratory microcosms is relatively simple.

The second chapter of the thesis was devoted to the comparison of different ecotoxicological tools which provide information of different responses, of organisms with different ecologies, and contemplating different levels of biological organization (biomarkers, individual, and community levels) in order to assess potential convergences, divergences, redundancies or complementarities of the different approaches and therefore understand their influence in the final outcome of the Analysis step in ERA. For that, I have compared Irgarol toxicity over a series of bioassays with different organisms and endpoints. Three different ecotoxicological tools for testing sediment toxicity were used: (1) biomarkers assessment with bivalves, (2) reproduction assessment with harpacticoid copepods, and (3) meiobenthic community structure assessment with *ex situ* microcosms. To account for species-sensitivity variability, two different species were used in each of the single-species tools.

At the end of the thesis, I make a synthesis of what were the main finding from these studies and how these may contribute to increase ecological realism of exposure and effect assessment and therefore reduce uncertainty in risk characterization in ERA.

Chapter 1 – Part I

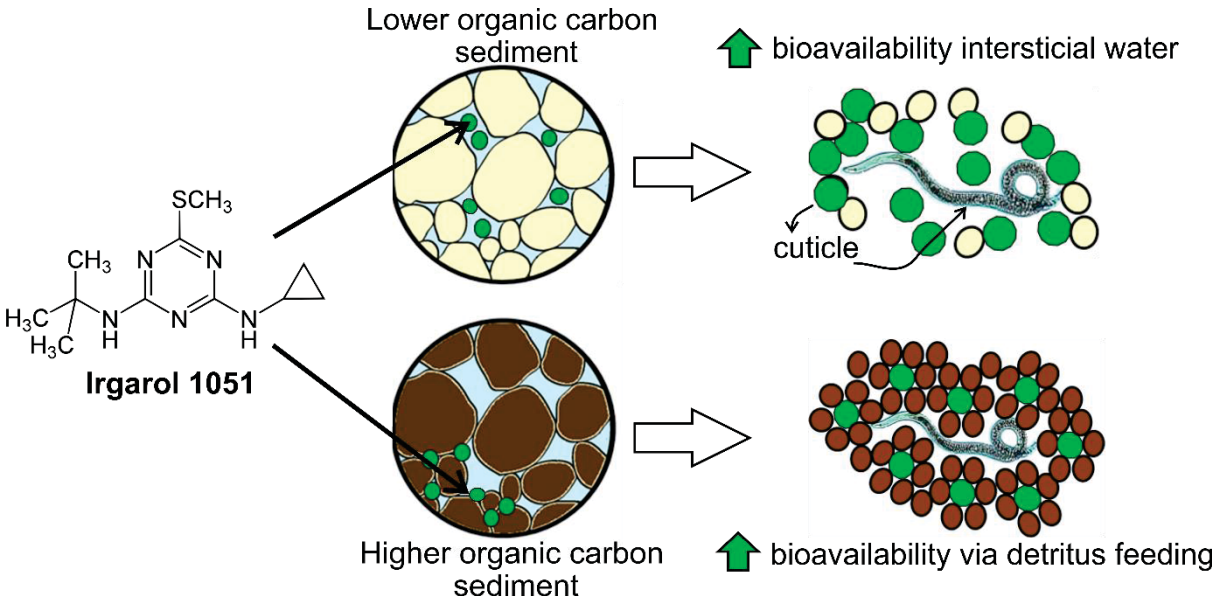
Title: Influence of sediment organic carbon on toxicity depends on organism's trophic ecology

Authors: Mariana Aliceda Ferraz, Rodrigo Brasil Choueri, Ítalo Braga Castro, Cecília Simon da Silva and Fabiane Gallucci

Published in *Environmental Pollution – Journal Elsevier*.

DOI: https://doi.org/10.1016/j.envpol.2020.114134

GRAPHICAL ABSTRACT



ABSTRACT

Studies which showed the influence of organic carbon on the toxicity of sediment-associated contaminants on benthic invertebrates suggest this was primarily due to its influence on the interstitial water concentrations of the contaminant. A higher organic content offers more binding sites for organic contaminants, which means lower toxicity for organisms whose exposure route is mainly through contaminated interstitial water. However, a higher organic content in the sediment could mean a higher toxicity for deposit-feeding organisms, which can assimilate the contaminant by ingestion of contaminated particles. To investigate the influence of sedimentary organic carbon content on the toxicity of an organic contaminant on a benthic community, a microcosm experiment was carried out where natural nematode assemblages were exposed to three concentrations of Irgarol in sediments with two different levels of organic carbon for 7 and 35 days. The response of the nematode assemblage to sediment contamination by Irgarol differed between organically "Lower organic carbon" and "Higher organic carbon" sediments. Responses were genus specific and although community composition was the same in both sediments in the beginning of the assay, contamination by Irgarol affected different genera at each sediment type. Also, the differential amount of organic carbon promoted responses of different functional groups. In Lower organic carbon sediments, contaminated treatments showed lower abundances of the genus *Viscosia* and the group of predacious nematodes, which were probably affected by an increased availability of Irgarol in the interstitial water in this treatment. In Higher organic carbon sediments, the group of deposit-feeders were mainly affected, suggesting the ingestion of contaminated food as the main route of contamination in this condition. These results indicate that the bioavailability of toxic substances in sediments is not only determined by their partitioning between the different phases of the sediment but also by the organism's trophic ecology.

Keywords: benthic community; antifouling; multispecies toxicity test; sediment toxicity

Capsule: Influence of sediment organic carbon on toxicity depends on organism's trophic ecology

1. Introduction

Most of the contaminants, once introduced into the aquatic environment, tend to settle and accumulate in the sediments where they interact with dissolved ions, particulate (POM) and dissolved (DOM) organic matter, chemical compounds, and the sedimentary matrix (Eggleton and Thomas, 2004; Chapman and Wang, 2001). The organic content of the sediment (POM and DOM) interferes on stability of the apolar organic compounds and the sedimentary matrix, influencing their bioavailability to the benthic (De Orte et al., 2014; Bangkedphol et al., 2009; Landrum et al., 1987). A higher organic content offers more binding sites for organic contaminants (i.e. lower contaminant levels in the interstitial water), which means a potential lower toxicity for organisms whose exposure route is mainly through contaminated interstitial water (Hoss et al., 2011; Chapman, 1998; Di Toro et al., 1991). On the other hand, a higher POM level could mean a higher toxicity for deposit-feeding organisms, which can assimilate the contaminant by ingestion of contaminated particles (Hoss et al., 2011; Höss et al., 2001; Schlekat et al., 1999). As such, the bioavailability of toxic substances in sediments is not only governed by their partitioning between the different phases of the sediment but it may also be determined by the organism's trophic ecology.

Studies which showed the influence of organic carbon on the toxicity of sediment-associated contaminants on benthic invertebrates suggest this was primarily due to its influence on the interstitial water concentrations of the contaminant. In general, increased toxicity has been linked to a decreasing content of total organic carbon in the sediment suggesting the dissolved form in interstitial water as the primary route of contamination when compared to accumulation from contaminated food (Boufahja et al., 2011; Austen and McEvoy, 1997; Meador et al., 1997; Gunkel and Streit, 1980; Streit, 1979). However, Armenteros et al. (2010) and Louati et al. (2013) have shown the ingestion of contaminated organic carbon, in the mode of bacterial cells or bacterial mucus, as an important route of contamination for benthic deposit feeders. Whereas contrasting results might have several reasons, it might be possible that the different species studied, with different lifestyle and feeding behavior might also differ in their ecology and therefore their main route of contamination.

The present study aims at investigating the influence of sediment organic carbon content on the toxicity of an organic contaminant on a diverse benthic assemblage represented by many species with different life traits and feeding strategies. This will be achieved by means of a

microcosm experiment with a natural meiobenthic community, with emphasis on the nematode assemblages. Within sedimentary environments, nematodes comprise the most abundant and species-rich metazoans (Coomans, 2000; Lambshead and Boucher, 2003), which contribute to important ecosystem functions such as decomposition of organic matter, nutrient recycling (Bonaglia et al., 2014; Hubas et al., 2010; Freckman, 1988;), and energy transfer to higher trophic levels (Woodward, 2010). Due to the general lack of planktonic larvae, the manipulation of natural assemblages with several species belonging to different trophic levels (e.g. bacterivores, microvores, herbivores, omnivores, and predators) in laboratory microcosms is relatively simple. As organic contaminant, we used Irgarol 1051, one of the biocides most commonly used in antifouling paints. This herbicide is highly hydrophobic, precipitating to sediment as soon as it is in contact with the aquatic environment, where it is associated with organic matter (Konstantinou and Albanis, 2004). It belongs to the s-triazine group (2-methythiol-4-*tert*-butylamino-6-cyclopropylamino-*s*-triazine) and acts as a photosystem-II (PSII) inhibitor, causing interruption of electron transport in chloroplasts during photosynthesis (Dahl and Blanck, 1996; Moreland, 1980) showing high toxicity to autotrophic aquatic species such as macrophytes, symbiotic dinoflagellates in corals, cyanobacteria and algae (Bao et al., 2011). Despite being an herbicide, ecotoxicological studies have also shown negative effects of Irgarol on the development and metabolism of invertebrates (Kobayashi and Okamura, 2002; Manzo et al., 2006; Perina et al., 2011), fish (Key et al., 2008; Van Wezel and Van Vlaardingen, 2004), and on meiobenthic communities (Gallucci et al., 2015; Hannachi et al., 2016). Irgarol 1051, also known as cybutryne, was recently disapproved as an active substance for use in antifouling products by the European Union due to environmental concerns (EC, 2016).

Specifically, we aimed to test the hypotheses that (i) toxicity of Irgarol 1051 on meiobenthic communities differ between sediments with the same community composition but different organic carbon contents and (ii) species with different feeding traits are differently affected. With this respect we expected that with the increase in sediment organic carbon content, the group of deposit feeders would be preferentially affected.

2. Material and methods

2.1 Experimental design

A natural meiobenthic community was exposed to three concentrations of Irgarol in sediments with two different levels of natural organic carbon for 7 and 35 days. The experiment

included three factors: (1) Total organic carbon (TOC) treatment: sediment with lower content of total organic carbon ("Lower organic carbon sediment") and sediment with higher content of total organic carbon ("Higher organic carbon sediment"), (2) Contamination treatment: two controls- Negative control and Acetone control and three nominal concentrations of Irgarol corresponding to “Low”, “Moderate” and “High” Irgarol concentrations, and (3) Exposure time: 7 and 35 days (Figure 1). The two levels of total organic carbon used represent intertidal sandy estuarine sediments which were spatially adjacent and had similar grain size but showed natural differences in organic matter content. This organic matter was constituted of material from terrestrial origin, particularly derived from mangrove forests present nearby as well as autotrophic organisms such as benthic diatom, bacterial and protist populations, which formed biofilms typical of estuarine intertidal flats. Irgarol nominal concentrations were chosen to represent those found in natural sediments in the field. The low concentration of Irgarol is analogous to values found in sites with higher volume of water exchange (Biselli et al., 2000), while the moderate concentration of Irgarol consider the levels found in areas of marinas and estuaries where water exchange is restricted (Biselli et al., 2000). Finally, the high concentration of Irgarol was based on values detected in regions of marinas and ports, where boat dockings are carried out (Boxall et al., 2000). The equilibrium concentration of Irgarol in porewater was within a range able to cause a wide range of effects to many marine invertebrates. The exposure time were selected to observe short-term effects related mainly to mortality (7 days) and long-term changes resulting from lethal plus sublethal effects as well as effects apparent toxicity due to environmental changes and species interactions (35 days). Four replicates were sampled for each contamination treatment from each TOC treatment at each time interval, totaling 80 microcosms.

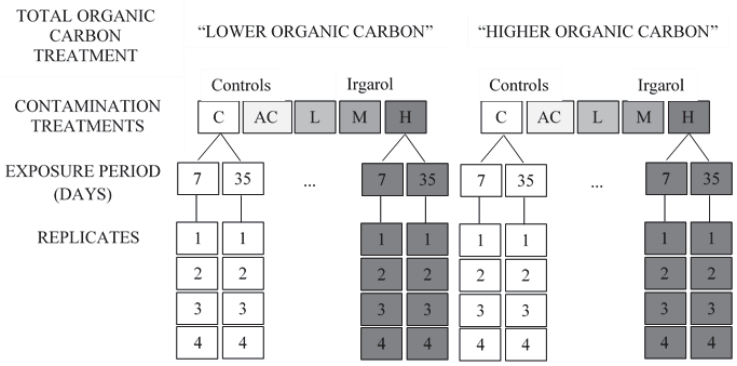


Figure 1. Experimental design showing each level of each tested factor.

2.2 Sediment sampling and defaunation

Sediments were sampled from the intertidal zone of two adjacent sites in the Itaguaré Estuary: the sediment with higher TOC from an inner part of the river (site 1) and the sediment with lower TOC from a site located slightly closer to the beach (site 2). The Itaguaré Estuary is a pristine area located in the municipality of Bertioga - São Paulo (Brazil) (23 ° 46'45.60 "S, 45 ° 58'9.31" W) and was chosen since the river Itaguaré along with its mangrove were decreed *Marine Environmental Protection Area of the Coastal Center* by the state government of São Paulo on October 8, 2008 on decree number 53.526. Sediment sampling and defaunation followed Schratzberger et al. (2002). Briefly, defaunation was performed by alternately freezing homogenized sediments for 12 hours and thawing it at room temperature for 48 hours. This process was repeated at least three times or until no organisms were found in the sediment.

2.3 Sediment characterization

Total Organic Carbon (TOC) content were analyzed according to methodology described by Luczak et al. (1997). The TOC% were determined in CHNS elemental analyzer Perkin Elmer 2400 Series II may, after decarbonation in a desiccator with hydrochloric acid (HCl, 37%). For grain size analysis, sieves with mesh of 1.0, 0.50, 0.250, 0.125 and 0.063mm were used to dry-sieve sediment samples and classify them according to Wentworth (1922).

2.4. Sediment spike

An aliquot of 10% of total sediment was dried at 80ºC for 48 hours. The moisture content of the sediment was checked in order to calculate the concentrations of stock solutions of Irgarol and the contamination based on the sediments dry weight. Following ASTM (2008) procedures, dried sediments were spiked with volumes of the stock solutions to obtain the target concentrations (25 ng g-1, 500 ng g-1 and 2000 ng g-1). Irgarol presents low solubility in seawater, therefore, acetone (0.05%) was used as co-solvent to promote a maximum solubilization during sediment spikes. The acetone was chosen due to its high volatility and low toxicity (Canty et al., 2007; Tao et al., 2013). Dried contaminated sediments were homogenized with the remaining moist defaunated sediment of each treatment by manual shaking 3 times a day for 5 minutes for 3 days. At this stage of contamination concentrations of Irgarol were double the desired, considering its subsequent mixing with the uncontaminated sediments containing meiofauna. Sediments were stored at controlled temperature (22ºC), in the dark to

avoid photodegradation of the biocide, for a period of 24 hours to volatilize the co-solvent. Negative control treatment consisted in meiobenthic community exposed to clean sediments and filtered seawater only, whereas the acetone control treatment was prepared with clean sediments, filtered seawater and acetone. Except for the negative control, all contamination treatments received 1,15 mL of the co-solvent acetone.

2.5 Microcosm set-up

A sampling was performed at the shallow sublittoral (ca. 1m depth) adjacent to site 1 to obtain the meiofauna for the set-up of the experiment. The sediments containing meiofauna were transferred to boxes and gently mixed to ensure an even distribution of the meiofauna among microcosms. Microcosms were set up with 4 cm of sediment layer placed in 500 mL beckers (14 x 8cm) by gentle mixing of spiked sediments with samples containing meiofauna. The sediment layer was then carefully covered with brackish water (salinity 8,25) from the field. The same homogenized sediments containing the meiofauna seeded both the "Lower organic carbon" and "Higher organic carbon" sediments. The microcosms were constantly aerated and covered by parafilm to prevent evaporation and salinity increase. Microcosms were randomly assigned on the bench and maintained under constant temperature (22 °C) and dark conditions to reduce microalgal growth (Schratzberger et al., 2002) and photodegradation of Irgarol. Four sediment samples of each treatment were preserved in formaldehyde 4% for later analysis of meiofaunal community structure at the beginning of the experiment (T0).

2.5.1 Microcosm sampling and samples processing

Four replicates of each treatment (Negative control, Acetone control, Low, Moderate and High concentration of Irgarol) were randomly sampled after exposure time of 7 and 35 days (T7 and T35). Firstly, the overlying water was removed by siphoning and passed over a 45µM mesh sieve to retain any organism in suspension. After, the redox potential was measured at the sediment surface (ca. 1 cm depth) using a *Hanna Instruments HI 991003*. Then $3cm^3$ of sediment were sampled with a syringe with cut-off ends to analysis of chlorophyll-*a* and phaeopigments concentrations as a proxy of the microphytobenthos biomass. The remaining sediment was fixed with formaldehyde 4% to analysis of the meiofauna (nematode genera). A fifth replicate from each contamination treatment was separated at the beginning of the

experiment (T0) and after 7 (T7) and 35 (T35) days of exposure to the determination of real Irgarol concentrations.

Meiofauna samples were washed through a 45µM mesh sieve and extracted by flotation with Ludox TM 50 (specific density 1.18) (Heip et al., 1985). All meiofaunal organisms were counted and identified under a stereomicroscope. Fifty percent from the total nematodes per microcosm were randomly picked, evaporated in anhydrous glycerol and mounted on permanent slides for identification at genus level. A lowest and highest limit of 100 and 250 nematodes were established.

Microphytobenthic samples were immediately frozen (-20 ºC) and evaluated indirectly through the analysis of concentrations of the photosynthetic pigments. The pigments were extracted from the sediment with 10mL of acetone 90% for 24 hours at a temperature of 4 °C and analyzed from absorbance spectra at 665nm and 750nm before and after acidification with HCl with a digital spectrophotometer. Lorenzen (1967) equations were used to estimate the concentrations of the photosynthetic pigments.

2.6 Determination of measured Irgarol concentrations and estimation of porewater concentrations

Analytical confirmation of the spiked concentrations of Irgarol was done according to Batista-andrade et al. (2018). After the extraction procedure using solid phase extraction cartridges (SPE / C18), the extracts were injected to the Alliance Separations liquid phase chromatography system, model 2695, Waters (Milford, MA, USA). The equipment with automatic sampler, quaternary pump, degassing system and mass spectrometry detector - EM (Micromass® Quatro MicroTM API Waters with API source), used “electrospray” ionization mode, data acquisition system through software Masslynx 4.0 Waters and analytical column XTerra® C18 3.5 µm 144 Å (50 x 3 mm di) (Waters, Millford, MA, USA). The limit of quantification (LQ) for this method is 0.1 ng g^{-1} for Irgarol. The quantification was performed by external standardization and the quality control performed through the analysis of interspersed blanks with the samples, as well as fortified samples for the analyte.

Irgarol porewater concentrations were estimated following the equation suggested by Simpson et al (2016):

Equation: $C_{pw} = C_{sed}/f_{oc}*K_{oc}$

Where C_{pw} is the concentration of Irgarol in the porewater (ng/mL), C_{sed} is the measured concentration of Irgarol in the sediments (ng/g), f_{OC} is the fraction of organic carbon in the same sediment, and K_{OC} is the organic carbon: water partition for Irgarol (log K_{OC} = 3.15; K_{OC} = 1413) (EU, 2011).

2.7 Data analysis

Data from the microcosm assays were analyzed using multifactorial permutational analysis of variance (PERMANOVA) (Anderson, 2001) for univariate and multivariate data considering "TOC treatment" (two levels: "Lower organic carbon" sediment and "Higher organic carbon" sediment), "Contamination treatment" (five levels: Negative control, Acetone control, and Low, Moderate and High Irgarol concentrations) and "Exposure time" (two levels: 7 and 35 days) as fixed factors. Pairwise a posteriori multiple comparisons tests were performed when significant differences were detected ($p<0.05$). PERMANOVA tests were conducted on Euclidean-distance and Bray-Curtis similarity matrices for univariate and multivariate data, respectively. The residuals were permutated using unrestricted permutation of raw data. Monte Carlo *p*-values were used when the number of permutations were lower than 50 (in the context of 999 permutations asked to the program to be performed, see Anderson et al. (2008).

Univariate indices included nematode density, number of nematode genera, sediment chlorophyll-*a* and phaeopigment concentrations, and sediment redox potential. To test hypothesis ii (i.e. species with different ecological traits are differently affected), nematode genera were assigned to feeding types based on the morphology of the buccal cavity according to Wieser's (1953) classification: selective deposit feeders (1A), non-selective deposit feeders (1B), epigrowth feeders (2A) and predator/omnivores (2B). The relative abundance of each feeding type was calculated for each sample and differences tested as described above.

To visualize the multivariate structure of nematode assemblages, non-metric multi-dimensional scaling ordination (nMDS) (Clarke and Ainsworth, 1993) was performed based on the same Bray-Curtis similarity matrix used for the PERMANOVA. The contribution of different genera to the differences between groups of samples indicated by the PERMANOVA were investigated through the similarity percentage analysis (SIMPER). Finally, to assess the contribution of chlorophyll-*a*, phaeopigments and redox potential to variation in nematode assemblage structure, we carried out multivariate regressions using distance-based redundancy

analysis (db-RDA; Mcardle and Anderson, 2001). A distance-based multivariate linear model (DistLM routine), using forward selection, was used to test the significance of these contributions by fitting a linear model based on Bray-Curtis dissimilarity. Given the significant interactions between "TOC treatment" vs. "Contamination treatments" vs. "Exposure time" observed in the PERMANOVA analysis for nematode genera abundances, multiple regressions were carried out independently for each sediment type at each time interval.

In order to assess possible "microcosm effects", two-way PERMANOVA was applied to test for differences in environmental and fauna univariate measures and multivariate structure of nematode assemblages, only from the negative control, of each sediment type (fixed, 2 levels: "Lower organic carbon" sediment and "Higher organic carbon" sediment) at the different exposure time (fixed, 3 levels: 0, 7 and 35 days). Pairwise a posteriori multiple comparisons tests were performed when significant differences were detected ($p < 0.05$).

3. Results

3.1 Sediment characteristics

Both Lower organic carbon and Higher organic carbon sediments were composed predominantly by fine sand, followed by very fine sand (Table S1). Sediment TOC varied from 0.11% to 0.37% and were significantly different between "Lower organic carbon" and "Higher organic carbon" sediments (ANOVA, $p < 0.05$) (Figure S1).

3.2 Irgarol measured concentrations and estimated concentrations in porewater

At the start of the experiment, measured concentrations of Irgarol were lower than nominal concentrations for both sediment types (Table S2). Nonetheless, measured concentrations still could be considered as different discrete treatments of increasing Irgarol concentrations (Low, Moderate and High Irgarol concentrations) as initially proposed in the experimental design. As expected, sediments with lower levels of organic carbon showed higher estimated porewater concentrations of Irgarol and during the experimental period, Irgarol concentrations in both sediments decreased progressively (Table S2).

3.3 Microcosm effects on nematode assemblages

Nematode densities at the start of the experiment (T0) ranged between 464 and 887 individuals per microcosm and the number of genera from 22 to 30 per microcosm considering both sediments. After 7 and 35 days of exposure, both genus richness and nematode density in the negative control treatment were significantly lower when compared to T0, independently of sediment type (Table S3, Figure S2). However, no differences on these parameters were observed between 7 and 35 days, as well as between Lower organic carbon and Higher organic carbon sediments (Table S3).

The negative control treatment presented a total of 62 nematode genera. *Desmodora* was the dominant genus (26,82%) followed by *Viscosia* (8,02%), *Sabatieria* (7,53%), *Sphaerotheristus* (4,65%) and *Oxystomina* (3,70%). Other genera represented each less than 3% of the total nematode assemblage per treatment. The multivariate structure of nematode assemblages in the negative control treatment differed marginally between 'lower organic matter' and 'higher organic matter' sediments (p-value 0,056) and did not differ at any exposure time (Table S3). For both sediment types, nematode assemblages from the start of the experiment (T0) were significantly different from nematode assemblages after 7 and 35 days (Table S3, Figure S2). However, differently from the univariate data, at 35 days assemblages significantly differed from those at 7 days (PERMANOVA post-hoc, $p<0.05$). As shown by the MDS, there was an increase in variability between replicate microcosm after 35 days of experiment (Figure S3).

3.4 Influence of organic carbon content on Irgarol toxicity to nematode assemblages

3.4.1 Nematode taxonomic structure

Permanova analysis for nematode density and genus richness showed no significant interaction between the factors "TOC treatment" and "Contamination treatment" (Table S4). Indeed, nematode diversity and number of genera did not differ between controls and concentrations of Irgarol (Table S4, Figure S4), indicating no effect of the biocide on these univariate parameters.

However, differently from the univariate data, the multivariate structure of nematode assemblages showed a significant interaction between "TOC treatment", "Contamination

treatment" and "Exposure time", i.e. differences in nematode assemblage structure between the Irgarol contaminated treatments depended on the type of sediment as well as the time of exposure (Table 1). nMDS ordination for each exposure time illustrate the differential effect of Irgarol on nematode assemblages at each sediment type (Figure 2). After 7 days of exposure, only Lower organic carbon sediments showed differences between Irgarol concentrations. In this type of sediment, nematode assemblages were significantly different between acetone control and all Irgarol contaminated treatments. Simper analysis showed that these differences were mainly due to lower mean densities of the genus *Desmodora* and *Viscosia* in contaminated treatments. Whereas *Desmodora* showed higher abundances in the acetone control compared to all other treatments including the negative control, *Viscosia* abundance was significantly lower at high concentration of Irgarol when compared to acetone but also the negative control treatment ($p<0.05$, Figure 3a).

After 35 days of exposure, Lower organic carbon sediments showed no significant differences between contamination treatments. On the other hand, in Higher organic carbon sediments nematode assemblages exposed to low and moderate Irgarol concentrations were significantly different from negative control, acetone control and the high concentration of Irgarol (Figure 3b). According to SIMPER analysis, these differences were mainly due to lower mean abundances of *Daptonema* in sediments exposed to low and moderate concentrations of Irgarol and higher mean abundances of *Desmodora* and *Sabatieria* in the same treatments (Figure 3b).

Table 1: Results of PERMANOVA and Monte Carlo post hoc tests for assessing differences in the multivariate structure of nematode assemblages from "Lower organic carbon" or "Higher organic carbon" sediments, contamination treatments (Negative control, Acetone control, Low, Moderate, or High concentrations of Irgarol), and exposure period (7 and 35 days). TOC: TOC treatment; Treat: Contamination treatment; Time: Exposure time; C: Negative Control; AC: Acetone Control; L: Low concentration; M: Moderate concentration; H: High concentration. Values in bold indicated $p< 0.05$.

	df	***MS***	**Pseudo-F**	**P(perm)**
TOC	1	7386,1	11,027	0,001
Treat.	4	1348,4	2,013	0,016
Time	1	48422	72,29	0,001
TOC x Treat.	4	1358,5	2,0281	0,006
TOC x Time	1	3259,8	4,8666	0,001
Treat. x Time	4	1277,8	1,9076	0,019
TOC x Treat. x Time	4	1179,6	1,761	**0,029**
Residual	60	669,84		

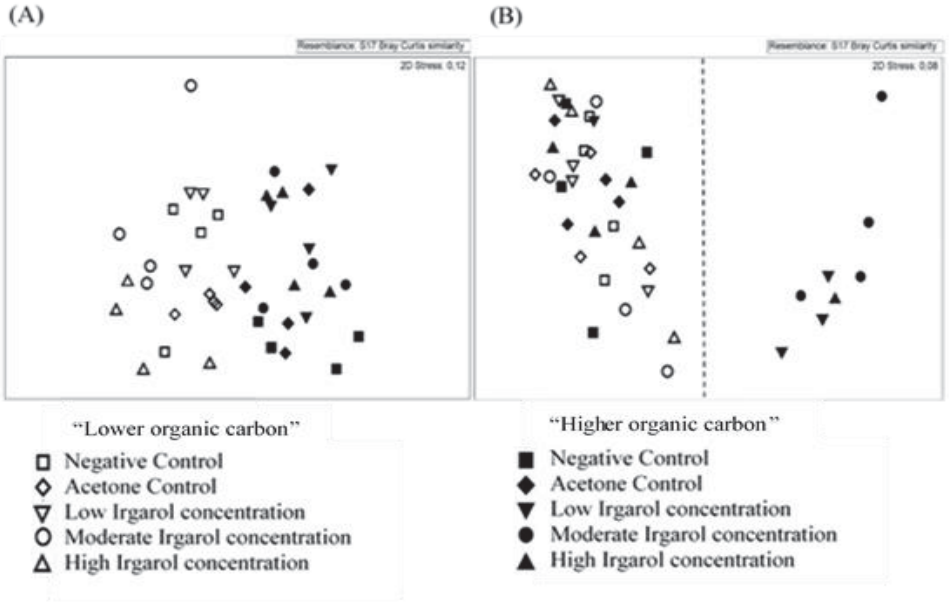


Figure 2. Ordination of nMDS of nematode genera in "Lower organic carbon" and "Higher organic carbon" sediments with different concentrations of Irgarol after 7 days (A), and 35 days of exposure time(B).

3.4.2 Nematode functional structure

All feeding types of nematodes (1A: selective deposit feeders, 1B: non-selective deposit feeders, 2A: epigrowth feeders and 2B: predators/omnivorous), although at different proportions, were represented in all treatments. The group of selective deposit feeders (1A) showed significant interactions between the factors "TOC treatment", "Contamination treatment" and "Exposure time" (Table S5). Monte Carlo post-hoc analysis showed significantly higher relative abundance of this group in Higher organic carbon sediments exposed to moderate Irgarol concentration after 35 days of exposure time when compared to the other contamination treatments (Figure 4). Likewise, the group of epigrowth feeders (2A) was significantly more abundant in sediments exposed to low and moderate concentration of Irgarol after 35 days of exposure in the Higher organic carbon treatment (Figure 4). Conversely, non-selective deposit feeders (1B), showed significantly lower abundances in low and moderate concentrations of Irgarol in both sediment types after 35 days of exposure, with lower mean relative abundance particularly in Higher organic carbon sediments. Finally, the group of predacious nematodes (2B) had significantly lower relative abundance in sediments with high

concentration of Irgarol when compared to negative control and acetone control treatments, exclusively in Lower organic carbon sediment (Table S5, Figure 4).

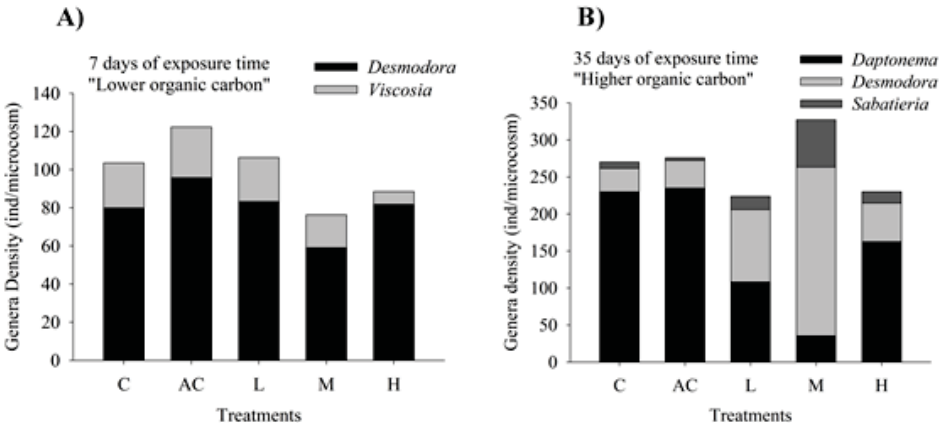


Figure 3. Mean abundance of the nematode genera that mostly explained the differences observed in PERMANOVA, as revealed by SIMPER analysis. (A) "Lower organic carbon" sediment after 7 days of exposure time and (B) "Higher organic carbon" sediment after 35 days of exposure time. C: Negative Control; AC: Acetone Control; L: Low concentration; M: Moderate concentration; H: High concentration.

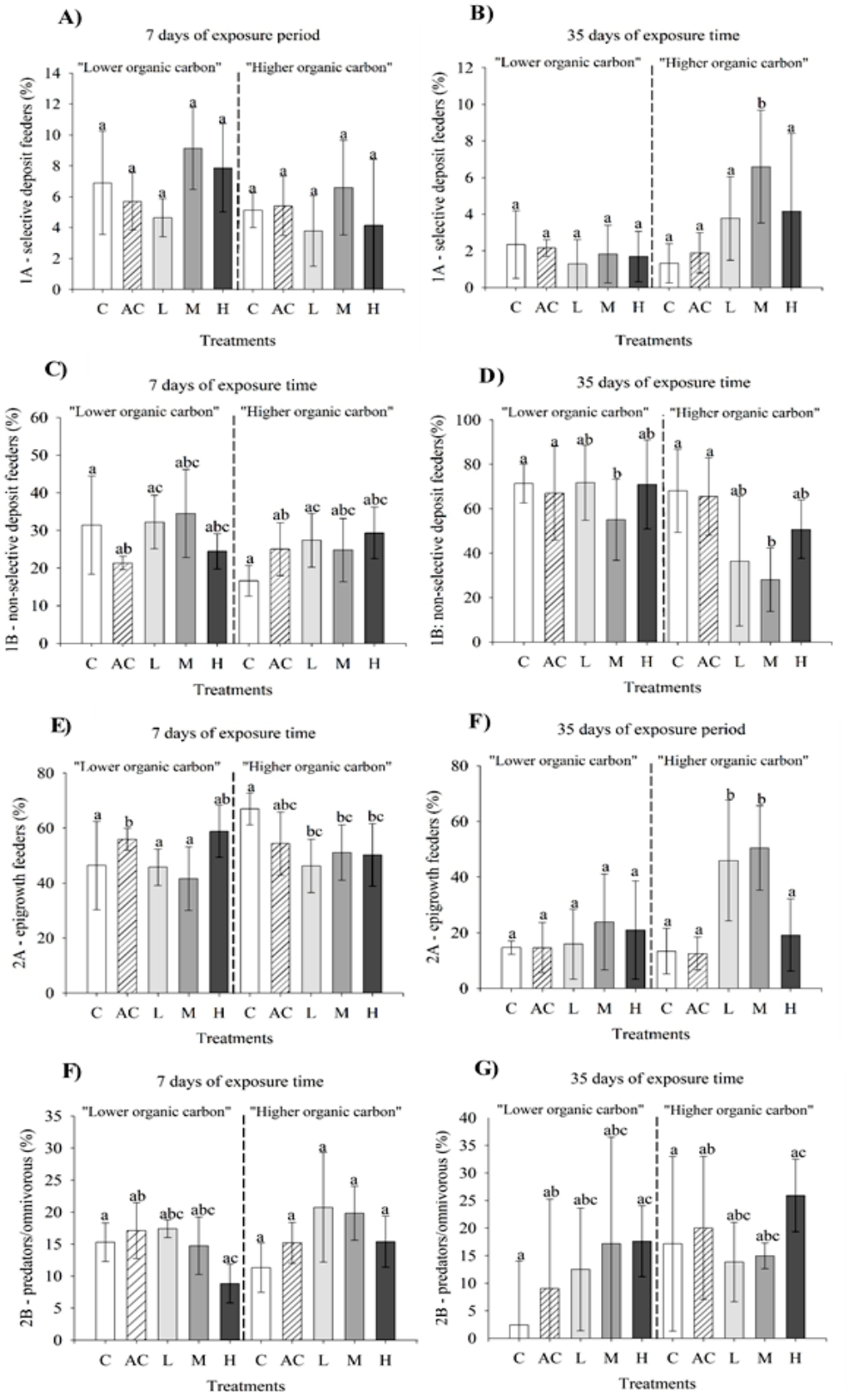


Figure 4. Mean and standard deviation of relative abundances of feeding types (1A, 1B, 2A and 2B) of nematode assemblages exposed to the different concentrations of Irgarol in "Lower organic carbon" and

"Higher organic carbon" sediments, for 7 and 35 days. C: Negative Control; AC: Acetone Control; L: Low concentration; M: Moderate concentration; H: High concentration.

3.5 Environmental variables and their relationship with nematode assemblages

Sediment redox potential ranged from 24 to 294 mV in Lower organic carbon sediments and -110 to 185 mV in Higher organic carbon sediments. Permanova analysis showed significant interactions between "TOC treatment" and "Exposure time". The redox values showed significant difference between sediment types in both exposure time. There was also significant interaction between "Contamination treatment" and "Exposure time" (Table S6). After 7 days of experiment, redox potential values were significantly lower at the moderate concentration of Irgarol when compared with both controls (Figure S5a). In 35 days of exposure time there were no significant differences (Figure S5b). The concentrations of chlorophyll-*a* ranged from 0.11 to 4.49 μg/cm^3 and showed significant differences between Irgarol treatments which depended on the exposure time (Table S6). After 7 days of exposure, in both sediment types, chlorophyll-*a* concentrations were significantly higher at contaminated treatments (low, moderate and high concentrations of Irgarol) when compared to control treatments (Figure S5c and Figure S5d). Mean concentrations were particularly higher at low concentrations of Irgarol. After 35 days, sediment chlorophyll-*a* concentration from low and moderate concentrations of Irgarol remained significantly higher whereas sediments with high concentrations of Irgarol did not differ from controls. Phaeopigments showed values between 0.00 and 7.59 μg/cm^3 in Lower organic carbon sediments and 0.00 to 3.85 μg/cm^3 in Higher organic carbon sediment and showed significant interaction between all factors ("TOC treatment", "Contamination treatment" and "Exposure time") (Table S6). High concentrations of Irgarol showed significantly lower concentrations of phaeopigments after 7 days of exposure in Lower organic carbon sediment (Figure S5e). In Higher organic carbon sediments, phaeopigments concentrations were significantly lower in low and moderate concentrations of Irgarol when compared to the negative control and acetone control treatments after 35 days of exposure (Figure S5f).

The DistLM analysis for each TOC treatment and Exposure time independently, did not show significant relationships between environmental variables and nematode assemblage structure (Table S7). Exception was for the "Higher organic carbon" treatment after 35 days of exposure, where chlorophyll-*a* significantly explained 22% of the variability in the multivariate structure of nematode assemblages ($p<0.05$) (Table S7, Figure S6).

4. Discussion

The differences in sediment organic carbon content showed a clear influence on the toxicity of Irgarol to meiobenthic communities. In the short exposure time, high levels of Irgarol significantly lowered absolute abundances of the genus *Viscosia* in sediments with lower organic carbon. Similar results had been observed in previous experiments performed with nematode assemblages from a different study site with different genus composition. Gallucci et al. (2015) and Santos et al. (2018) observed lower absolute abundances of the genus *Oncholaimellus*, which belongs to the same family (Oncholaimidae) and is morphologically and ecologically similar to *Viscosia*, in sandy sediments, low in organic carbon content, which were exposed to Irgarol and Diuron (Gallucci et al., 2015) and to interstitial water from sediments influenced by a sewage outfall (Santos et al., 2018). Likewise, both studies observed a decrease in the relative abundance of predacious nematodes in contaminated treatments, as was the case in the present study. Nematodes are known to uptake pollutants from the sediment interstitial water through their permeable cuticle (Fichet et al., 1999;Kammenga et al., 1994; Howell, 1983). As such, they suggested that the observed response directed to this functional group might have resulted from a higher permeability of their cuticle when compared to more ornamented cuticles (Fonseca and Fehlauer-Ale, 2012). The same holds true for *Oncholaimellus* and *Viscosia* which also have smooth cuticles. If this is the case, then higher or lower bioavailability of a contaminant will depend not only on the amount of organic carbon in the sediment but also on the primary route of exposure of organisms (Forbes et al, 1998).

The estimated porewater Irgarol concentrations corroborate the affirmation that some genera could have been affected via interstitial water, especially in the lower organic carbon sediment. The equilibrium concentration of Irgarol in porewater (305.87 to 584.70ng/mL), in the lower organic carbon sediment spiked with higher Irgarol concentrations, was within a range able to cause a wide range of sublethal and even lethal effects to many marine invertebrates (USEPA, 1992; Khandeparker et al., 2005; Manzo et al., 2006; Downs and Downs, 2007; Finnegan et al., 2009; Bao et al. 2011). The moderate Irgarol porewater concentrations for the lower organic carbon sediment (51.85 to 195.91 ng/mL), and the higher estimated porewater concentrations (37.58 to 307.2470ng/mL) for the higher organic carbon treatment, is able to cause biological effects as well, although most of them are only sublethal responses (biochemical, physiological, behavioral, developmental, and on growth or reproduction) (USEPA, 1992; Khandeparker et al., 2005; Manzo et al., 2006; Downs and Downs, 2007;

Finnegan et al., 2009; Bao et al. 2011). The lowest porewater concentrations (1.03 to 7.39ng/mL), estimated for the "Low Irgarol" treatment in higher organic carbon sediment, are reported to cause only few, subtler effects, such as inhibition of photosynthesis in the coral *M. mirabilis* (Owen et al., 2002) and impairment of the ability of serpulid larvae to detach from sediment (Khandeparker et al., 2005).

Whereas several studies have suggested the interstitial water as the primary route of contamination for benthic organisms, the ingestion of contaminated particles with food should also be a relevant pathway, since other organic contaminants like Irgarol have high affinity to organic particles (Castro et al., 2011; Thomas and Brooks, 2010). If this is the case, then higher or lower bioavailability of a contaminant will depend not only on the amount of organic carbon in the sediment but also on the primary route of exposure of organisms. Differently from the short-term response to contamination observed at the Lower organic carbon sediments treatment, in Higher organic carbon sediments differences between the treatments were observed only after 35 days of exposure. This long-term response was characterized by different nematode assemblages in sediments contaminated with low and moderate concentrations of Irgarol when compared to controls and the high concentration treatment. According to Simper analysis, differences were mainly due to lower absolute abundances of the genus *Daptonema* and higher densities of the genus *Desmodora* and *Sabatieria*. This was reflected in the trophic structure which showed lower relative densities of non-selective deposit feeders (1B) and higher relative densities of selective deposit feeders (1A) and epigrowth feeders (2A). Interestingly, Hannachi et al. (2016) also reported a significant decrease in absolute densities of one species of *Daptonema* (*D. normandicum*) as well as the lower relative abundance of group of deposit feeders, with a concomitant higher relative abundance in the group of epigrowth feeders, in sediments contaminated with Irgarol at concentrations within the range of the "Moderate Irgarol" concentration used here.

Lower absolute abundance of *Daptonema* (1B) and relative abundance of the group of non-selective deposit feeders (1B) corroborate the idea of the ingestion of contaminated particles with food as a relevant pathway for contamination. In this case, the higher organic carbon content of sediments, the higher the bioavailability of contaminants for these organisms. In addition, it is possible that with increasing food ingestion nematodes form more lipid reserves, which due to high sorption capacity for hydrophobic organic chemicals, would result in higher internal contaminant concentrations in the organisms and therefore a greater apparent toxicity with higher food densities (Fisher et al. 2016). However, this response was observed

only at low and principally moderate levels of contamination. Why did *Daptonema* densities and relative abundances of 1B did not differ between sediments with high concentrations of Irgarol and control treatments? Although any tentative to answer this question will be speculative, if we consider the ingestion of food as the main pathway for contamination, it might be that this contaminated food resource was not available at high levels of contamination, eventually due to toxic effects of Irgarol to such living resources. Deposit-feeder nematodes, including *Daptonema*, are known to feed highly on protozoa (Moens and Vincx, 1997), particularly ciliates, which might have been affected at high levels of Irgarol contamination.

In opposition to the decrease of *Daptonema* absolute densities, a concomitant increase in absolute densities of *Desmodora* and *Sabatieria*, and in the higher relative density of the group of selective deposit feeders and epigrowth feeders (2A) at low and moderate levels of contamination in Higher organic carbon sediments suggests a diverse and complex response to contamination in these particular treatments. In one hand, increased densities might be an apparent toxicity response to contamination since species can respond to low and moderate levels of contamination by investing in reproduction (Gusso-Choueri et al., 2012) and therefore contribute to population density increase. Such a response is in concordance with the relatively slower response at population level of these species (registered only at the 35 days-exposure observation) when compared to the short-term (7 days of exposure time) toxicity response exhibited by absolute abundance of *Viscosia* and the relative abundance of group of predators in the Lower organic carbon sediment treatment.

On the other hand, increased densities might be an apparently toxicity effect of contamination which has caused the death of abundant species, leaving resources (e.g. space, food) available for opportunistic species to flourish. *Sabatieria*, which is allocated in the same trophic group as *Daptonema*, is known as having an apparent toxicity positive relationship with anthropogenic disturbance (Schratzberger et al., 2009), being highly adapted to fit into new habitats, conditions or resources and as being an indicator of a poor ecological quality status due to its tolerance to pollution (Soetaert et al., 1995; Austen and Somerfield, 1997; Moreno et al., 2011; Santos et al., 2018). It is also possible that environmental changes induced by Irgarol have favored the higher relative densities of a particular functional group or increase absolute densities of species. *Desmodora* and the group of epigrowth feeders, which are expected to feed upon microalgae, might have responded to increased concentrations of chlorophyll-*a* in contaminated treatments, observed particularly at low and moderate concentrations of Irgarol. This possible causal mechanism is supported by the relationship between chorophyll-*a* and

nematode assemblage structure in Higher organic carbon sediments, as pointed by DistLim analysis. However, an increase in chlorophyll-a is not necessarily related to an increase in algal biomass, but it can reflect an increase in the number of chloroplasts as a compensatory response to the decrease in photosynthetic activity caused by Irgarol (Ricart et al., 2009; Chesworth et al., 2004). The above possibilities are not mutually exclusive, and a combination of the processes might have occurred.

The current study showed that subtle time-dependent differences on organic carbon content in sediments lead to different responses of the meiofauna community structure to contamination by Irgarol. The responses may be a consequence of the complex relationships among sediment-contaminant partitioning, organisms' morphology (type of cuticle), feeding habits, life history, and ecological interactions. By using a microcosm assay, the current study provided empirical data which reinforces the concept that the risk assessment to higher environmental levels may not be supported by reductionist data. For example, it has long been debated in the field of ecotoxicology the ability of single-species (or monospecific) tests to assessing prospective or retrospective ecological or environmental risks (Cairns and Niederlehner, 1987; Chapman, 1995; Vlaming and Norberg-king, 1999). Although they probably represent the most common approach employed for regulatory ecological/environmental risk assessment (ERA) and chemicals registration frameworks (Hernando et al., 2006; Rohr et al., 2016 and references therein), limitations of monospecific tests in predicting effects to more complex biological levels, or under field conditions, are extensively argued.

The results of the current study suggest that the complexity of ecotoxicological responses of contaminants in sediments must be approached by superseding the reductionist, linear paradigm in ecotoxicology, by testing different sediment types and enabling complex sediments-to-species and species-to-species interactions at different times of exposure. In this way, laboratory microcosm tests contribute to fill the gap, for both prospective or retrospective and ERA, between single-species experiments and predictions of field responses at community level since their setup is affordable, have easily manipulable variables, and provide complex responses at community level.

5. Conclusions

The response of the nematode assemblage to sediment contamination by Irgarol differed between Lower organic carbon and Higher organic carbon sediments, corroborating the

hypothesis that toxicity of Irgarol on meiobenthic communities is influenced by the organic carbon content in the sediment. Responses were genus specific and although community composition was initially the same in both sediments, contamination by Irgarol affected different genera at each sediment type. Also, the differential amount of organic carbon promoted responses of different functional groups, corroborating the hypothesis that the influence of sediment organic carbon on toxicity depends on organism's trophic ecology.

Acknowledgment

We are grateful to Fiamma Eugênia Lemos Abreu by Chemical analyses of Irgarol concentrations. This article resulted partially from a PhD thesis from the 'Programa de Pós-Graduação em Sistemas Costeiros e Oceânicos (PGSISCO) offered by Universidade Federal do Paraná (UFPR). M.A.F and C.S.S. was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, finance code 001). I.B.C thanks for a research grant from CNPq (PQ 306486/2015-6). The study was supported by Financiadora de Estudos e Projetos (FINEP / CT-Hidro 1111/13 − AIBRASIL2).

Supplementary material

Table S1: Percentual of grain size fractions from two replicates (R1 and R2) in "Lower organic carbon" and "Higher organic carbon" sediments.

Grain size	**"Lower organic carbon" (%)**		**"Higher organic carbon" (%)**	
	R1	R2	R1	R2
Very Coarse Sand	0,618	0,345	0,239	0,123
Coarse Sand	2,482	1,098	1,375	1,415
Medium Sand	9,430	5,053	4,164	4,687
Fine Sand	41,176	78,633	76,366	66,053
Very Fine Sand	45,998	14,860	16,870	27,036
Silt/Mud	0,297	0,011	0,986	0,686

Table S2: Sediment nominal and measured concentrations, and estimated porewater concentrations of Irgarol in "Lower organic carbon" or "Higher organic carbon" sediments during 7 and 35 days of exposure time. C: Negative Control; AC: Acetone Control; L: Low concentration; M: Moderate concentration; H.: High concentration. N.d. = not detected. N.c. = not calculated.

"Lower organic carbon"				**"Higher organic carbon"**			
Start of experiment							
Treatmen	Nominal (ng/g)	Measure (ng/g)	Porewat (ng/mL)	Treatmen	Nominal (ng/g)	Measure (ng/g)	Porewat (ng/mL)
C	0	n.d	n.c.	C	0	n.d	n.c.
AC	0	n.d	n.c.	AC	0	n.d	n.c.
L	25	11,48	7,39	L	25	19,85	3,80
M	500	304,50	195,91	M	500	229,71	43,94
H	2000	908,80	584,70	H	2000	1606,28	307,24
7 days of exposure time							
C	0	n.d	n.c.	C	0	n.d	n.c.
AC	0	n.d	n.c.	AC	0	n.d	n.c.
L	25	5,13	3,30	L	25	11,61	2,22
M	500	108,46	69,78	M	500	217,82	41,66
H	2000	818,04	526,31	H	2000	901,82	172,49
35 days of exposure time							
C	0	n.d	n.c.	C	0	n.d	n.c.
AC	0	n.d	n.c.	AC	0	n.d	n.c.
L	25	2,85	1,83	L	25	5,36	1,03
M	500	80,59	51,85	M	500	201,00	38,45
H	2000	475,41	305,87	H	2000	196,49	37,58

Table S3: Results of the PERMANOVA multivariate analysis with the data from the negative control treatments at the start of the experiment (T0), and at 7 and 35 days of exposure time. TOC: TOC treatment; Time: Exposure time. Bold values indicate $p < 0.05$.

Nematode Density	***df***	***SS***	**MS**	**Pseudo-F**	**P(perm)**
TOC	1	1,0417	1,0417	0,13915	0,733
Time	2	599,08	299,54	40,013	**0,001**
TOC x Time	2	14,083	7,0417	0,94063	0,387
Residual	18	134,75	7,4861		
Genus Richness					
TOC	1	6061,7	6061,7	0,44549	0,51
Time	2	7,7591E5	3,8796E5	28,512	**0,001**
TOC x Time	2	7089,6	3544,8	0,26052	0,761
Residual	18	2,4493E5	13607		
Multivariate Structure					
TOC	1	962,34	962,34	2,0673	0,056
Time	2	31799	15900	34,155	**0,001**
TOC x Time	2	1493,1	746,55	1,6037	0,086
Residual	18	8379,2	465,51		

Table S4: Results of the univariate PERMANOVA and Monte Carlo post-hoc test for total density and genus richness (S') of nematode assemblages exposed to different Irgarol concentrations in "Lower organic carbon" and "Higher organic carbon" sediments for 7 and 35 days. TOC: TOC treatment; Treat: Contamination treatment; Time: Exposure time. Bold values indicate $p < 0.05$.

Nematode Density	***df***	***SS***	**MS**	**Pseudo-F**	**P (perm)**
TOC	1	1,3894E5	1,3894E5	9,4088	**0,001**
Treat.	4	14530	3632,6	0,24599	0,922
Time	1	1,8107E5	1,8107E5	12,261	**0,001**
TOC x Treat.	4	47701	11925	0,80754	0,517
TOC x Time	1	16589	16589	1,1233	0,321
Treat. x Time	4	26701	6675,2	0,45202	0,79
TOC x Treat. x Time	4	32532	8133	0,55074	0,729
Residual	60	8,8605E5	14767		
Genus Richness (S')					
TOC	1	56,113	56,113	9,4905	**0,002**
Treat.	4	14,45	3,6125	0,61099	0,667
Time	1	427,81	427,81	72,357	**0,001**
TOC x Treat.	4	29,7	7,425	1,2558	0,298
TOC x Time	1	46,512	46,512	7,8668	**0,009**
Treat. x Time	4	50,75	12,687	2,1459	0,108
TOC x Treat. x Time	4	44,3	11,075	1,8732	0,108
Residual	60	354,75	5,9125		

Table S5: Results of PERMANOVA for assessing the effects of sediment type, Irgarol treatments and exposure time on the relative abundance of feeding types of nematode assemblages. TOC: TOC treatment; Treat: Contamination treatment; Time: Exposure time. Values in bold indicated p< 0.05.

1A	**df**	**MS**	**Pseudo-F**	**P(perm)**
TOC	1	0,010	0,002	0,962
Treat.	4	8,445	1,815	0,117
Time	1	214,790	46,151	0,001
TOC x Treat.	4	5,124	1,101	0,379
TOC x Time	1	58,874	12,650	0,001
Treat. x Time	4	1,959	0,421	0,800
TOC x Treat. x Time	4	15,955	3,428	**0,019**
Residual	60	4,654		
1B				
TOC	1	2329,2	2329,2	0,001
Treat.	4	1185,2	296,3	0,228
Time	1	20200,0	20200,0	0,001
TOC x Treat.	4	1190,1	297,5	0,202
TOC x Time	1	881,5	881,5	0,047
Treat. x Time	4	3121,7	780,4	**0,009**
TOC x Treat. x Time	4	1131,2	282,8	0,274
Residual	60	12153	202,56	
2A				
TOC	1	1031,4	7,2	0,01
Treat.	4	134,2	0,9	0,458
Time	1	16330,0	114,5	0,001
TOC x Treat.	4	422,86	2,96	0,022
TOC x Time	1	193,40	1,36	0,246
Treat. x Time	4	957,83	6,72	0,001
TOC x Treat. x Time	4	374,54	2,63	**0,048**
Residual	60	142,62		
2B				
TOC	1	263,98	5,41	**0,021**
Treat.	4	40,56	0,83	0,502
Time	1	0,10	0,00	0,966
TOC x Treat.	4	114,27	2,34	**0,052**
TOC x Time	1	65,79	1,35	0,25
Treat. x Time	4	64,92	1,33	0,259
TOC x Treat. x Time	4	78,65	1,61	0,179
Residual	60	48,836		

Table S6: Results of PERMANOVA for environmental variables (redox potential, concentrations of chlorophyll-*a* and phaeopigments) TOC: TOC treatment; Treat: Contamination treatment; Time: Exposure time. Values in bold indicated p< 0.05.

	df	SS	MS	Pseudo-F	P(perm)
Redox					
TOC	1	123010	123010	94,407	**0,001**
Treat.	4	7449	1862	1,429	0,239
Time	1	219140	219140	168	**0,001**
TOC x Treat.	4	3595	899	0,690	0,596
TOC x Time	1	6975	6975	5,353	**0,019**
Treat. x Time	4	13955	3489	2,678	**0,032**
TOC x Treat. x Time	4	3257	814	0,625	0,653
Residual	60	78178	1303		
Chlorophyll- a					
TOC	1	13,83	13,83	47,16	**0,001**
Treat.	4	59,11	14,78	50,38	**0,001**
Time	1	1,19	1,19	4,06	0,067
TOC x Treat.	4	0,84	0,21	0,71	0,616
TOC x Time	1	0,65	0,65	2,21	0,157
Treat. x Time	4	6,01	1,50	5,12	**0,003**
TOC x Treat. x Time	4	2,25	0,56	1,91	0,107
Residual	60	17,598	0,2933		
Phaeopigments					
TOC	1	0,030	0,030	0,023	0,863
Treat.	4	6,835	1,709	1,284	0,300
Time	1	12,257	12,257	9,210	**0,005**
TOC x Treat.	4	31,215	7,804	5,864	**0,002**
TOC x Time	1	15,700	15,700	11,797	**0,001**
Treat. x Time	4	16,056	4,014	3,016	**0,025**
TOC x Treat. x Time	4	15,097	3,774	2,836	**0,028**
Residual	60	79,851	1,3308		

Table S7: Results of Distance based Linear Model of each TOC treatment at each exposure time. Values in bold indicated p< 0,05.

7 days of exposure time				
"Lower organic carbon"	**SS (trace)**	**Pseudo-F**	**P**	**Prop.**
Redox (mV)	484,12	1,27	0,24	0,065
chlorophyll-*a*	245,28	0,62	0,70	0,033
phaeopigments	433,33	1,13	0,35	0,058
"Higher organic carbon"				
Redox (mV)	268,90	0,72	0,59	0,038
chlorophyll-*a*	795,66	2,31	0,06	0,113
phaeopigments	659,97	1,88	0,09	0,094
35 days of exposure time				
"Lower organic carbon"	**SS (trace)**	**Pseudo-F**	**P**	**Prop.**
Redox (mV)	380,85	0,44	0,73	0,023
chlorophyll-*a*	178,25	0,20	0,95	0,111
phaeopigments	156,58	0,18	0,95	0,097
"Higher organic carbon"				
Redox (mV)	1022,10	0,62	0,58	0,335
chlorophyll-*a*	6931,80	5,30	**0,004**	0,227
phaeopigments	2594,70	1,68	0,18	0,085

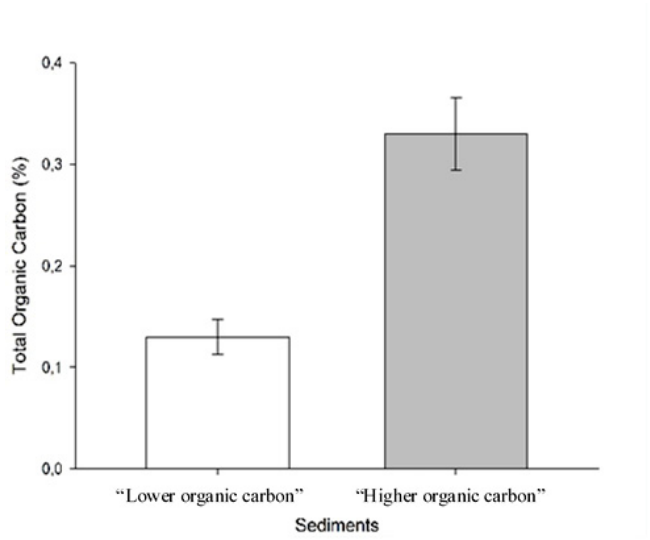


Figure S1. Mean and standard deviation for Total Organic Carbon percentages (TOC) in "Lower organic carbon" and "Higher organic carbon" sediments.

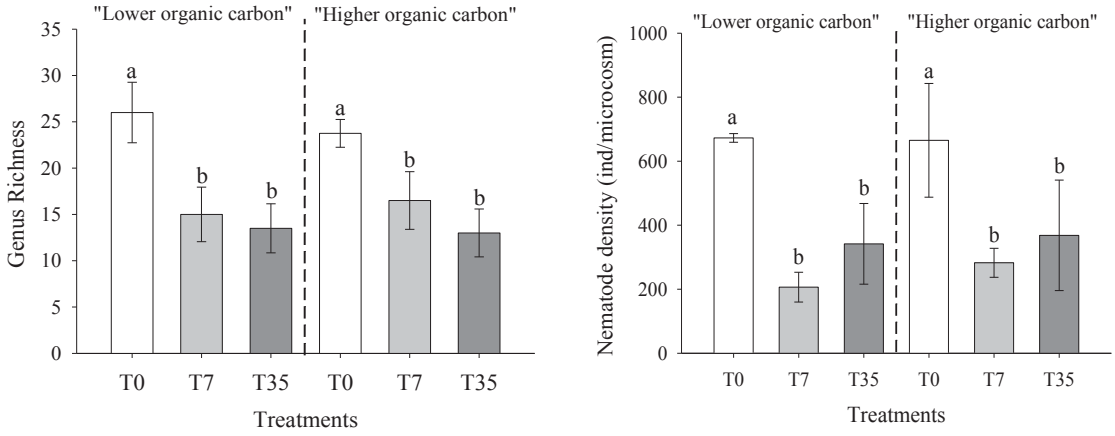


Figure S2. Mean and standard deviation for genus richness and nematode density in the negative control treatments from microcosms with "Lower organic carbon" or "Higher organic carbon" sediments at the start of the experiment (T0), and after 7 (T7) and 35 days (T35) of exposure time. Different letters above the bars mean significant differences after the PERMANOVA post-hoc tests.

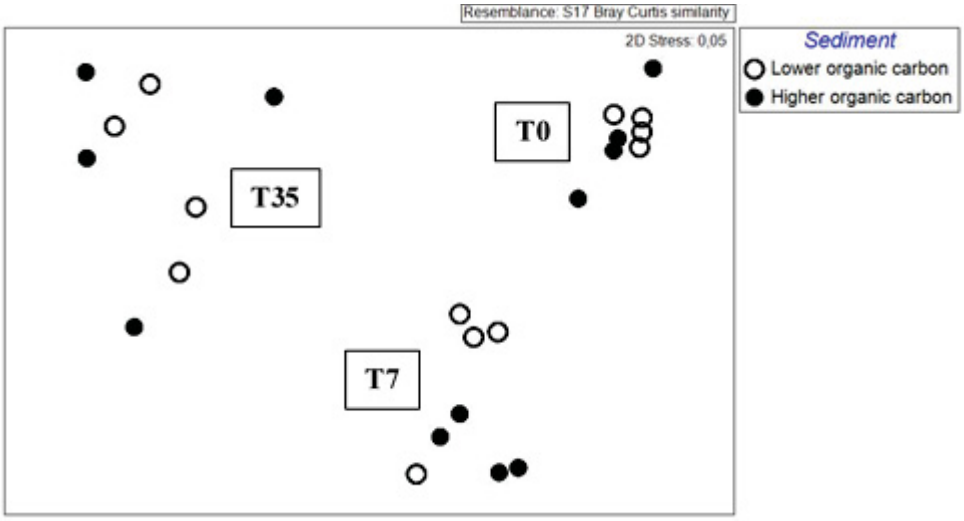


Figure S3. Ordination of nMDS of nematode genera densities in samples at the start of the experiment (T0) and in the negative control after 7 (T7) and 35 (T35) days of exposure time.

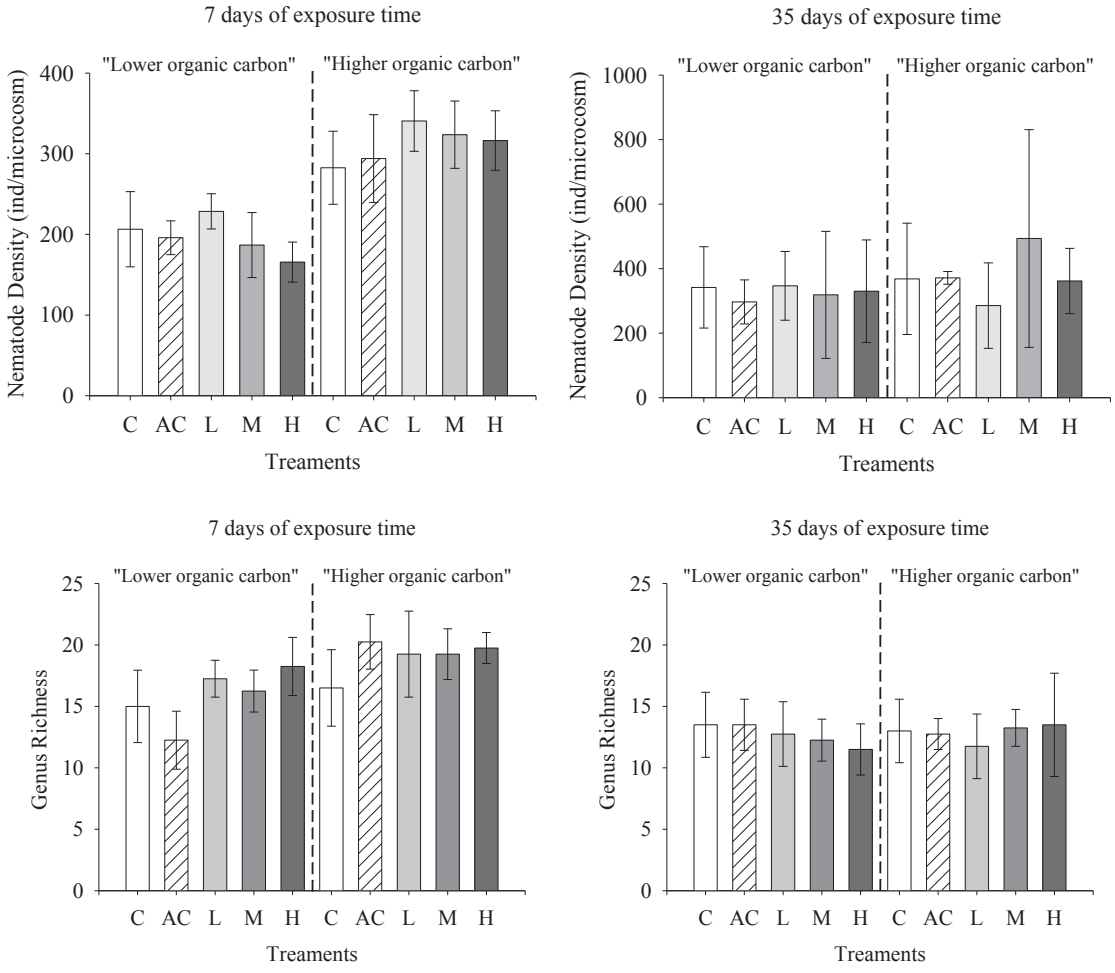


Figure S4. Mean and standard deviation for density (individual/microcosm) and genus richness (number of genus/microcosm) of nematode assemblages exposed to different Irgarol concentrations in "Lower organic carbon" and "Higher organic carbon" sediments after 7 and 35 days of exposure time. C: Negative control; AC: Acetone control; L: Low concentration; M: Moderate concentration; H: High concentration.

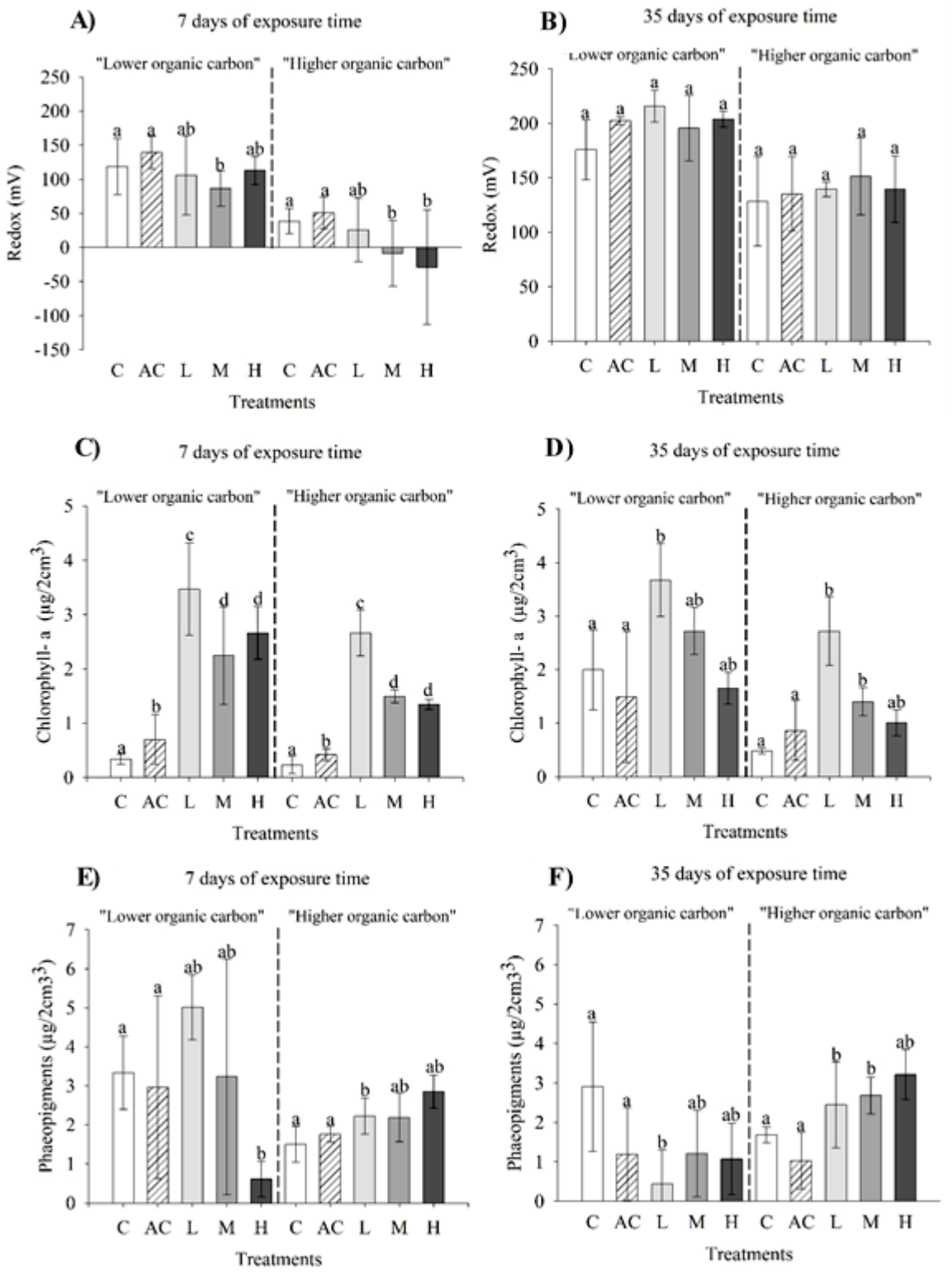


Figure S5. Mean values of redox potential (mV), concentrations of chlorophyll-*a* and phaeopigments (µg/2cm^3) in "Lower organic carbon" or "Higher organic carbon" sediments, Contamination treatments, at 7 or 35 days of exposure time. Error bars represent standard deviation. C: Negative Control; AC: Acetone Control; L: Low concentration; M: Moderate concentration; H: High concentration.

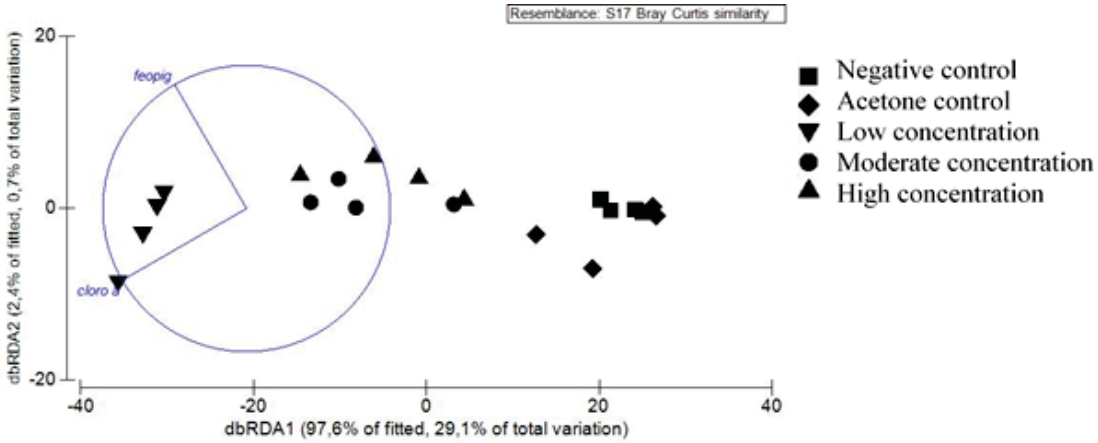


Figure S6. Distance-based multivariate linear model plot of contaminated treatments of "Higher organic carbon" sediment at 35 days of exposure time.

Chapter 1 – Part II

Title: Does environmental pH variation affect the estuarine organisms and influence the bioavailability of organic contaminants? The case of Irgarol toxicity on nematode assemblages

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Intention to submit in *Ecotoxicology and Environmental safety – Journal - Elsevier*

ABSTRACT

Although pH values vary largely from locations, ecosystems and time periods, tests for ecological risk assessment tend to use fixed pH values from protocols. Changes in water pH may change the organism's physiology and the characteristics of compounds found in the environment. More acidic pH values can cause acidosis internally of the organisms, and the bioavailability of organic contaminants such as Irgarol to organisms. In order to investigate the effect of two different pH conditions on the toxicity of a hydrophobic organic compound on a benthic community, a microcosm experiment was conducted to evaluate the response of nematode assemblages to the exposure to environmentally relevant concentrations of Irgarol at two naturally occurring pHs conditions. Estuarine nematode assemblages were exposed to two concentrations of Irgarol at pH 7.0 and 8.0 for periods of 7 and 35 days. While there was no significant effect of pH over the contaminant toxicity, the results showed that a continuous exposure of lower pH to some nematodes may be stressful, affecting density. Our results showed that higher concentration of Irgarol affected nematodes (i.e. lower diversity, different nematode assemblage structure), independently of the pH value. Although estuarine nematodes are known to cope with pH variability, our results suggest that the longer exposure (7 days) to low pH values can impact these communities. Toxic substances under acidic conditions may be available in interstitial water due to an increase in their ionization which makes them more water soluble, but less lipophilic to cell membranes, counter acting effects.

Keywords: bioavailability, nematode assemblages, estuarine pH, Irgarol

1. Introduction

Natural seawater pH may change drastically between marine habitats, locations and periods of time (Fischer et al., 2013; Hofmann et al., 2011; Heugens et al., 2001). In coastal ecosystems like estuaries, high pH variability can be observed in the scale of hours (Hofmann et al., 2011), and may reach values under 7.0 and over 8.0 in days (Burnett, 1997; Ringwood and Keppler, 2002). In the open ocean, in turn, pH tends to present stable patterns between locations and around annual cycles. However, due to the increase of pCO2 in the atmosphere in the long term, projections predict a strong decrease in open ocean pH of the to the end of the current century (Caldeira and Wickett, 2005), which can also influence in estuarine pH (Feely et al., 2010). However, the Ecological Risk Assessments are based on standardized testing protocols (e.g. OECD, USEPA guidelines) which do not consider variations on environmental parameters, including pH.

Organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), organohalogen compounds, pharmaceuticals and personal care products, antifouling biocides and others, are widespread in marine and estuarine environments (Abessa et al., 2005; Harino et al., 2006; Viana et al., 2019). Acidification can affect hydrophilicity or lipophilicity of such substances by protonation, making organic acids (substances with low pKa) more lipid soluble, and organic bases (high pKa) more water soluble (Schwarzenbach et al., 2016). Therefore, varying environmental pH potentially changes the solubility and toxicity of organic contaminants, as it modifies both sediment:water partitioning and may modify biological uptake and absorption of these molecules. Recently, the European Chemicals Agency (EACH) oriented that a chemical should be tested “at a pH consistent with the more toxic form of the substance” (ECHA, 2017) as part of the information requirements under the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation.

The biocide Irgarol is a triazine widely used in antifouling paints and is commonly found in marine environments (Ali et al., 2013; Fernandez and Gardinali, 2016; Viana et al., 2019). When this compound is released from paint layers in the marine environment, it can adsorb to suspended particulate matter or it can be introduced as part of paint particles, accumulating in marine sediments from 100 to 200 days (Dafforn et al., 2011). In seawater with pH close to 8.3, Irgarol is presented almost entirely in its neutral form. However, as pH diminishes, the

protonated forms of the compound become more representative, increasing water solubility of the molecules (Homepage: chemicalize.com/2019) and potentially modifying both the route of exposure to the biota and the absorption mechanism by the organisms. Therefore, understanding the effect of pH variation on the toxicity of contaminants is important to a relevant ecological risk assessment.

To investigate the effect of different pH conditions on the toxicity of Irgarol, we set up a microcosm experiment using nematode assemblages. Although less used for management purposes due to lack of standardization (Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems), analysis at the community level have more ecological relevance because it considers the interaction between species and consequently includes indirect effects to the analysis (Rohr et al., 2016), such as, for example, predator-prey relationships and competition for resources. Additionally, since exposure multispecies, with different ecological traits and sensitivities to contaminants are tested simultaneously. For instance, Ferraz et al. (2020) showed that the bioavailability of toxic compounds depends on their partitioning between water and sediment phases but also on the trophic ecology of the species. Therefore, the simultaneous investigation of the responses of different species with diverse ecological traits allows the better elucidation of the influence of an environmental variable on toxicity.

In the present study we focus on nematode assemblages which are highly abundant and diverse in marine and estuarine sediments (Heip et al., 1985) and are relatively easy to maintain in microcosms in the laboratory (Armenteros et al., 2010; Haegerbaeumer et al., 2018; Schratzberger et al., 2002). Wexpect that Irgarol will exhibit different toxicity at different pH, because variations in pH can affect both hydrophilicity and lipophilicity of organic compounds and therefore potentially influence their bioavailability (Peijnenburg et al., 1996; Rudel, 2003). However, since potential effects of pH in Irgarol are counteracting, and we do not know the magnitude of this interactions, it is hard to hypothesize on the direction of pH influence on Irgarol toxicity. For instance, at lower pH, Irgarol is expected to be less lipophilic, and therefore less permeable to cell membranes, but at the same time it is also expected to be more water soluble, therefore increasing its bioavailability in the water.

2. Material and Methods

2.1 Experimental Design

In order to evaluate the influence of pH over the toxicity of Irgarol in meiobenthic communities, three factors were manipulated: (i) 'pH': pH 8.0 and pH 7.0, (ii) 'Contamination': Control (environmental water + sediment + co-solvent acetone), Low, and High Irgarol concentrations (environmental water + co-solvent acetone + Irgarol) and (iii) 'Exposure period': 7 and 35 days. Each treatment had 3 replicates, totaling 24 microcosms (Figure 1). The two pH levels chosen represent values found in the estuarine environment and Irgarol nominal concentrations were chosen to represent those found in natural sediments in the field according to Ferraz et al. (2020). The exposure periods were selected to observe short-term effects related mainly to mortality (7 days) and long-term changes resulting from lethal plus sublethal effects as well as toxicity due to environmental changes and species interactions (35 days). To analyze any possible effect of the co-solvent acetone, a negative control treatment (environmental water + sediment) was included at each pH for each exposure period.

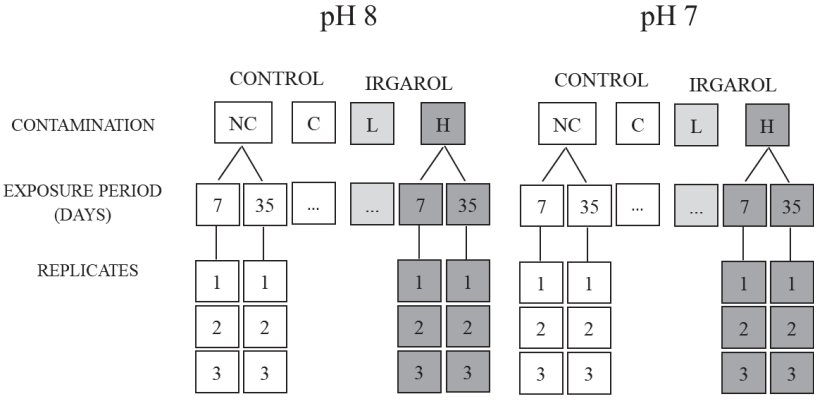

Figure 1. Schematic representation of the experimental design.; C: Control; L: Low Irgarol concentration; H: High Irgarol concentration.

2.2 Sediment collection and defaunation

Sediments used in the experiment were collected in the Itaguaré river mouth, located in Bertioga, São Paulo, Brazil (coordinates: 23.7807715, - .9684255), a widely used location for this purpose as it is protected by the Restinga de Bertioga State Park along the Itaguaré River. The sediment for Irgarol spiking was collected and immediately transported to the laboratory,

where it was homogenized and stored in a freezer. Defaunation consisted in freezing the sediment for 12 hours, thawing for 48 hours and repeating the same process at least three times, until complete defaunation (Schratzberger et al., 2002). Defaunated sediments were stored at -20ºC. Meiofauna was collected on the same day the experiment began, at the same location where sediments for defaunation and contamination were previously sampled. This sediment was then transferred to the laboratory, gently homogenized to ensure even distribution of organisms among all experimental units, and immediately transferred to the microcosm beakers.

Total Organic Carbon (TOC) content was analyzed according to the methodology described by Luczak et al. (1997). TOC percentage was determined in CHNS elemental analyzer Perkin Elmer 2400 Series II, after decarbonation with hydrochloric acid (HCl 37%) in a desiccator. Sediment grain size was analyzed by Mudroch & MacKnight (1994) and classified based on the scale proposed by Wentworth (1922).

2.3 Sediment spike

Acetone was chosen as co-solvent due to its rapid dissipation in sediment, which facilitates its volatilization and removal (Northcott and Jones, 2000). Irgarol stock solution was prepared with the co-solvent and added to sediments to final nominal concentrations of 100ng kg^{-1} and 2000ng kg^{-1} for low and high Irgarol concentrations, respectively. It was used 100g of defaunated sediments per replicate, which were individually spiked with the appropriate amount of Irgarol stock solution plus 50mL of environmental water from Itaguaré River (salinity 21) to facilitate homogenization. The final concentration of acetone in the control, low Irgarol and high Irgarol treatments was 0,05% of the total volume. Environmental water was added to the negative control at the same volume of the other treatments. All replicates were shaken vigorously for 15 minutes and kept refrigerated at 4ºC in the dark for 48 hours to achieve compound equilibration time and co-solvent volatilization.

2.4 Microcosm set-up

Initially, 500mL (14 x 8cm) beakers containing 100g of sediment with meiofauna and 350mL of environmental water (salinity 21) were kept in the closed test-systems under CO_2 injection to acclimatize the organisms for 3 days. After acclimatization, the overlaying water was removed and 100g of the treated defaunated sediment (spiked with Irgarol, pure acetone or environmental water) were added. Sediments of each beaker were carefully homogenized and

topped with 350mL of environmental water. An aeration system kept the microcosms individually aerated, at constant 25 ºC ±1 temperature and protected from light to prevent excessive microphytobenthos development and/or Irgarol photodegradation.

2.5 CO_2 injection

An indirect CO_2 injection system was used to keep the pH constant in the test-systems throughout the exposure periods. The beakers containing microcosms were accommodated in closed polypropylene boxes (adapted from Kita et al., 2013). In addition to the beakers with treatments, two more beakers were added to each box, one containing only water and receiving direct CO_2 input by bubbling, and the other one, an identical replicate of the control microcosms, was placed in the far opposite side of the box with a pH measuring electrode in the water, with the aim of measuring pH changes due to the CO_2 diffusion throughout the whole system's atmosphere (Figure 2). pH was continuously monitored through the ApexFusion online software, programmed to detect 0.1 variations in pH from the set value (7,0). When a variation was detected, the solenoid valve was automatically opened to release a CO_2 flux until the set values were reestablished, which immediately halted CO_2 injection. The pH in the experimental units in the boxes that received the injection of CO_2 presented the pH average of 6,98 (equipment data), while the pH in the microcosms in the boxes that did not receive the CO_2 showed an average of 8,2 (equipment data).

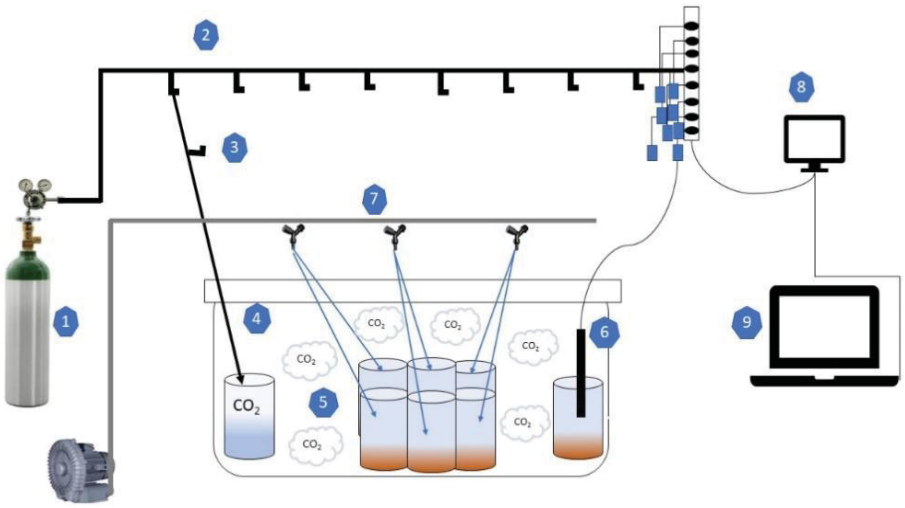

Figure 2. Representation of the CO_2 injection system in polypropylene box. Adapted from Kita et al. (2013).(1) CO_2 cylinder; (2) CO_2 circulation system; (3) solenoid valve; (4) CO_2 injection; (5) CO_2 rich atmosphere; (6) pH detection electrode; (6) pH measuring electrode; (7) individual aeration; (8) Control display; (9) Software in computer.

2.6 Sampling

At the end of the experiment the water was carefully removed and the sediment in each beaker was fixed with 4% formaldehyde for analysis of the meiofauna.

2.7 Sample processing

Meiofauna was extracted by flotation using Ludox ™ 50 (1.18 specific density) according to the procedure described by Heip et al. (1985). Samples were washed through a 45µm mesh and the retained material was stored in 4% formaldehyde and stained with Bengal Rose for subsequent total meiofauna quantification and nematode *genera* identification.

All meiofaunal organisms were counted and identified under a stereomicroscope. Ten percent from the total nematodes per microcosm were randomly picked, evaporated in anhydrous glycerol and mounted on permanent slides for identification at genus level (minimum of 100 and maximum of 200 nematodes).

2.8 Statistical analysis

Possible effects of the co-solvent acetone were investigated by permutational multivariate analysis of variance (PERMANOVA) with 'Control treatments' (2 levels: Negative Control and Control), 'pH' (2 levels: pH 8 and pH 7) and 'Exposure period' (2 levels: 7 and 35 days) as fixed factors.

The influence of pH on Irgarol toxicity on univariate metrics (nematode density, richness and diversity) and multivariate data (matrices of densities of nematodes genera) was analyzed by PERMANOVA with 'pH' (2 levels: pH 8 and pH 7), 'Contamination' (3 levels: Control, Low Irgarol and High Irgarol) and 'Exposure Period' (2 levels: 7 and 35 days) as fixed factors. According to the experimental design, the influence of pH on toxicity is identified as an interaction between the factors 'Contamination' and 'pH' (Cont. vs. pH), or between the factors 'Contamination', 'pH' and 'Exposure Period' treatments (Cont. vs. pH vs. Exp.). For univariate metrics, PERMANOVA analysis was done based on Euclidian distance resemblance matrix (Anderson, 2017). For the multivariate structure of nematode assemblages, Bray-Curtis similarity index was used to construct the resemblance matrix. Data were log (x+1) transformed to reduce the influence of the few dominant *genera*. In addition, because there were large differences in densities between the two exposure periods, we have also performed a

PERMANOVA analysis on relative abundance data, which precludes the influence of total densities of samples. In the case of significant interactions of interest (as described above: Cont. vs. pH and Cont vs. pH vs. Exp.), pairwise a posteriori comparisons were performed and a Percentage Similarity Analysis (SIMPER) was applied (Clarke, 1993) to identify the taxa that contributed the most to the differences detected. Alpha level was set at 5%.

3. Results

3.1 Sediment analyzes

The mean percentage of total organic carbon of sediments was 0,28 % and the sediment particle size was classified by fine sediment (higher percentage retains in the size of 0,125mm).

3.2 Effects of the co-solvent acetone, pH and exposure period on nematode assemblages

Nematode densities in the controls (negative and acetone control) varied from 320 to 2543 individuals per microcosm and did not differ significantly between treatments (negative control vs. acetone control), indicating no effect of the co-solvent acetone.

Although acetone did not affect nematode densities, the analysis of the controls alone showed a significant interaction between ‘pH’ and ‘Exposure period’ (Table S1). Pairwise analyzes showed significantly lower densities at pH 7.0 compared to pH 8.0 after 7 days of exposure period (Figure S1). Also, at both pH 7.0 and 8.0, nematode densities were significantly lower after 35 days of exposure compared to densities at 7 days (Figure S1).

Nematode *genus* richness varied from 7 to 17 per microcosm and showed a significant interaction between the factors ‘Control treatments’ and ‘Exposure period’ (Table S1). Pairwise analysis showed no differences between acetone control and negative control treatments but showed significant increase in richness after 35 days, compared to 7 days, only at the acetone control treatment. (Figure S1).

A total of 40 nematode *genera* were identified in the control microcosms (negative and acetone controls). *Desmodora* was the dominant genus, accounting from 47 to 87% of total nematode densities per microcosm. *Anoplostoma* (0 to 22%), *Oncholaimus* (0 to 16%) and *Viscosia* (0 to 9,5%) were the next most abundance *genera*. Multivariate PERMANOVA analysis showed significant differences in nematode assemblage structure between pHs and between exposure periods (Table S1). Simper analysis showed that differences between pH 7.0 and pH 8.0 were mainly due to lower abundance of *Desmodora* and *Anoplostoma,* the most abundant *genera*, at pH 7.0.

The multivariate analysis with relative abundances of nematode assemblages presented the interaction between factors ‘Controls’ and ‘pH’, and between ‘Controls’ and ‘Exposure period’ (Table S2). The posthoc showed the difference between negative control and acetone control only at pH 8.0 and 35 days of exposure period (Table S2). The Simper analysis results presented a decrease in the relative abundance of the genus *Desmodora* followed by an increase in the genus *Oncholaimus* in the acetone control compared with the negative control in 35 days of exposure period (Figure S2).

3.2 Influence of pH on Irgarol toxicity

3.2.1 Univariate analysis

Nematode densities varied from 234 to 3053 individuals per microcosm and did not show any significant results for the factor ‘Contaminant’ either in isolation or in interaction with ‘pH’ and/or ‘exposure period’ (Table 1). There was, however, significant differences in nematode densities between exposure periods, with significantly lower values after 35 days of exposure (Table 1, Figure S2).

The number of nematode *genera* per microcosm varied from 6 to 18 and showed no significant effect of any factor (Table 1). Nematode genus diversity, on the other hand, showed significant differences between levels of the factors ‘Contamination’ and ‘Exposure period’ (Table 1). Posthoc analysis revealed significantly lower diversity at high concentrations of Irgarol when compared to the acetone control treatment (Figure 3). It also showed significantly higher diversity after 35 days of exposure period independently of contamination and pHs.

Table 1. Results of the Permanova analysis for nematodes density, genus richness and genus diversity for the fixed factors 'Contamination'(Cont.), 'pH' and 'Exposure Period' (Exp.). Values in bold indicate p<0,05.

Density	df	SS	MS	Pseudo-F	P(perm)
Cont.	2	177630	88816	0,980	0,388
pH	1	104980	104980	1,158	0,320
Exp.	**1**	22686000	22686000	250,340	**0,001**
Cont. x pH	2	326050	163030	1,799	0,194
Cont. x Exp.	2	292540	146270	1,614	0,212
pH x Exp.	1	126260	126260	1,393	0,266
Cont. x pH x Exp.	2	303990	152000	1,677	0,206
Residual	24	2174900	90623		
Richness					
Cont.	2	29,556	14,778	2,264	0,118
pH	1	4,694	4,694	0,719	0,449
Exp.	1	0,250	0,250	0,038	0,858
Cont. x pH	2	5,556	2,778	0,426	0,660
Cont. x Exp.	2	18,667	9,333	1,430	0,269
pH x Exp.	1	1,361	1,361	0,209	0,660
Cont. x pH x Exp.	2	1,556	0,778	0,119	0,898
Residual	24	156,670	6,528		
Diversity					
Cont.	2	0,591	0,296	4,758	**0,019**
pH	1	0,000	0,000	0,003	0,961
Exp.	1	0,336	0,336	5,405	**0,025**
Comt. .x pH	2	0,006	0,003	0,046	0,958
Cont. .x Exp.	2	0,354	0,177	2,850	0,086
pH x Exp.	1	0,003	0,003	0,048	0,815
Cont. x pH x Exp.	2	0,042	0,021	0,335	0,724
Residual	24	1,492	0,062		

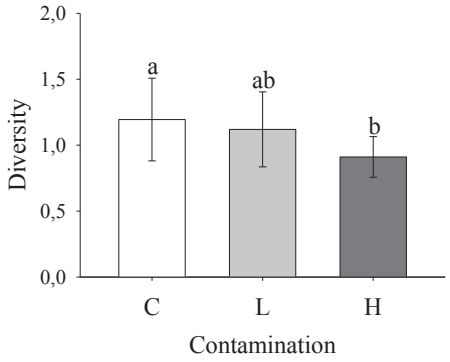

Figure 3. Mean and standard deviation of nematode diversity for levels of contamination independently of pH and exposure period. C: Control; L: Low Irgarol concentration; H: High Irgarol concentration. Small letters indicate differences.

3.2.2. Multivariate data

A total of 44 nematode *genera* was identified in the microcosms. *Desmodora* was the dominant genus in all microcosms, accounting from 46 to 87% of total nematode densities per microcosm. *Anoplostoma* (0 to 22%), *Oncholaimus* (0 to 16%) and *Viscosia* (0 to 9,5%) were

the next most abundance *genera*. As for nematode densities and genus richness, multivariate PERMANOVA analysis on nematode genus densities showed significant differences in nematode assemblage structure between exposure periods (Table 2). Given the large differences between exposure periods, a PERMANOVA analysis was performed with genus relative abundances data. Results from this analysis revealed significant differences between levels of 'Contamination' and 'Exposure period' factors (Table 2). Pairwise comparisons showed significant differences in nematode assemblages between high Irgarol concentration and acetone control treatments (Table 2). Simper analysis indicated that differences were mainly due to higher relative abundances of the dominant genus *Desmodora*, and lower relative abundances of *Anoplostoma* and *Oncholaimus* at the Irgarol contaminated treatment.

Table 2. Multivariate Permanova analysis for total and relative abundance of nematode genera for the fixed factors 'Contamination'(Cont.), 'pH' and 'Exposure period' (Exp.). Values in bold indicate p< 0,05.

Total abundance	*df*	*SS*	*MS*	*Pseudo-F*	*P(perm)*
Cont.	2	246890	123440	1,924	0,135
pH	1	47188	47188	0,736	0,413
Exp.	1	13533000	13533000	210,970	**0,001**
Cont. x pH	2	210940	105470	1,644	0,199
Cont. x Exp.	2	216320	108160	1,686	0,207
pH x Exp.	1	65852	65852	1,027	0,318
Cont. x pH x Exp.	2	194460	97230	1,516	0,227
Residual	24	1539500	64146		
Relative abundance					
Cont.	2	770,930	385,460	2,420	**0,024**
pH	1	85,528	85,528	0,537	0,777
Exp.	1	1936,300	1936,300	12,155	**0,001**
Cont. x pH	2	476,870	238,440	1,497	0,122
Cont. x Exp.	2	525,960	262,980	1,651	0,104
pH x Exp.	1	81,290	81,290	0,510	0,812
Cont. x pH x Exp.	2	216,340	108,170	0,679	0,791
Residual	24	3823,000	159,290		

4 Discussion

Microcosm efficiency and quality of controls

Along the present study, nematode densities in the microcosms showed a considerable decrease from 7 to 35 days, evidencing a "microcosm effect". Other microcosm experiments have already reported decreases in nematode densities in similar time intervals (Gallucci et al., 2015; Ingels et al., 2018; Santos et al., 2018; Schratzberger et al., 2002). However, whereas an initial decrease might result from damage caused by the preparation of microcosms and/or adaptation of the organisms to the microcosm's conditions, a delayed reduction as observed

here might have resulted from the high density of the assemblage at day 7 (mean of 1838,33 ind./microcosm) which might have surpassed the microcosm carrying capacity causing species populations to decline. Similar results were observed by Vieira et al. (submitted) in a laboratorial microcosm experiment where treatments with high immigration rates (mimicked by artificial addition of organisms) have resulted in a decrease in nematode abundance and changes in community structure, as observed here. Despite the reduction in population sizes as a response to the microcosm incubation, it was still possible to observe the effects of and/or interaction between the other factors in univariate and multivariate parameters.

Regarding the co-solvent acetone, there were no significant differences in univariate parameters and multivariate assemblage structure between the negative control treatment and the acetone control, indicating no major effects of the co-solvent used to prepare the stock solutions on the fauna. This agrees with previous meiofauna microcosm experiments which have used this co-solvent (Gallucci et al., 2015; Haegerbaeumer et al., 2018 and Ferraz et al., 2020) and is probably due to the high volatilization of this compound during the period that contaminated sediments are homogenized and left to reach equilibrium.

pH effects on nematode assemblages

Nematode densities were significantly lower at pH 7.0 compared to pH 8.0, suggesting that more acidic conditions were less suitable for those nematode populations. These results agree with previous studies, which have reported negative responses of invertebrates physiology (Miles et al., 2007; Widdicombe et al., 2009), populations (Hofmann et al., 2011) and community structure and diversity (Christen et al., 2013) to acidified conditions, including meiofaunal assemblages (Barry et al., 2004; Hale et al., 2011; Widdicombe et al., 2009). However, these results are also somehow surprising, given that estuarine nematodes have naturally cope with daily variability and relatively low pHs (Burnett, 1997). Particularly for meiofauna organisms, studies that have reported negative responses of organisms to acidification were mainly conducted in the deep-sea, and infer that such effects of pH variability on the fauna are probably due to the constant environmental conditions typical of the deep-sea, which confers higher sensitivity to these organisms (Seibel and Walsh, 2003, 2001; Thistle et al., 2007). We expected that daily exposure to ambient fluctuations in pH as well as lower pH values typical of estuarine systems (Ringwood and Keppler, 2002) would translate into populations that were acclimatized to variable pH or lower pH, and therefore resistant to our pH experimental exposure. However, our results showed that this range of change, (which is

small compared to those manipulated at deep-sea experiments, but is typical of coastal ecosystems), can significantly affect these communities.

Elevated carbon dioxide pressures in the water (hypercapnia) produce an acidosis in the blood of organisms (Pörtner et al., 2004). Studies of hypercapnia in some estuarine organisms (Burnett, 1997; Cameron, 1978) showed that some of them partially compensate the acidosis by ionic exchanges between the blood and the aquatic environment. In some cases, compensation may not complete, i.e. hemolymph or blood pH is not completely restored to the values present before the hypercapnic challenge. The compensation that does occur takes several hours and the degree of compensation depends upon the magnitude of the challenge. In estuarine systems, a combination of oceanography information, resident biological processes, and residence time of the water are likely driving the daily variability in water pH (Ringwood and Keppler, 2002), contributing to the pH minimum value, pH maximum value, diurnal and seasonal variability. It is not clear what aspect of this variability is most biologically significant (e.g. minimum pH, maximum pH, hours spent below the yearly mean low pH). It is possible that the nematode assemblages from this study would be able to cope with pH 7.0 for hours given compensation mechanisms, but they were not in a 7 days continuous exposure. Indeed, other laboratory and field studies have reported adverse effects on estuarine organisms when pH levels are below 7.5 (Ringwood and Keppler, 2002). As pointed by Hale et al. (2011), extreme range of environmental variability does not necessarily translate to extreme resistance to acidification. Instead, such a range of variation may mean that the organisms resident in estuarine regions are already operating at the limits of their physiological tolerances so that future acidification may drive the physiology of these organisms closer to the edges of their tolerance windows (Hofmann et al., 2011).

pH influence on Irgarol toxicity

The acid dissociation constant (pKa) is a measure of ionization, i.e. it infers about the amount of H^+ of a given substance. When environmental pH is at the same value as the constant pKa, then ionized and non-ionized molecules are in similar proportion (50% each). Ionized molecules are more water soluble and less lipophilic (therefore less permeable to cellular membranes) (Larini, 2008). At pH 7.0 about 67.5% of the Irgarol molecules are at its non-ionized form (13.8% at the ionized form), whereas at pH 8.0 about 97% of the Irgarol molecules are expected to be at its non-ionized form (only 1.8% would be ionized). Thus, at the same total Irgarol concentration, it is reasonable to assume that there will be more Irgarol dissolved in the

interstitial water at pH 7.0 than at pH 8.0. Ferraz et al. (2020) observed that nematode *genera* with more permeable cuticles were affected mainly via interstitial water (i.e. waterborne exposure), while nematodes with less permeable cuticle were affected when Irgarol was adsorbed to organic matter, probably via ingestion of contaminated food (i.e. dietborne exposure). In the current study, we should expect that at pH 7.0 nematodes with more permeable cuticles would be preferentially affected when compared to those at pH 8. However, at pH 7.0 Irgarol is expected to be less lipophilic, and therefore less permeable to cell membranes, counteracting the possible effects of higher solubility in water. This counteract action of pH in Irgarol water solubility and lipophilicity may explain why there is no net effect of this variation of pH on Irgarol toxicity.

Despite the lack of pH influence, effects of Irgarol contamination on nematode assemblages were observed at high Irgarol concentrations. Effects were mainly in the proportion of relative abundances of particular genera, with an increase in dominance of the most abundant genus *Desmodora* and a decrease in the relative abundances of *Anoplostoma* and *Oncholaimus*. The decrease in abundance of the genus *Oncholaimus* in response to contamination by organic compounds have already been observed (Gallucci et al., 2015; Mahmoudi et al., 2005; Moreno et al., 2011). Given the widespread observation of this phenomena (i.e. lower densities of *Oncholaimus* in contaminated sites when compared to pristine locations), this genus has been considered as sensitive to contaminants and its absence where this genus is expected to naturally occur can be an indication of sediment pollution (Moreno et al., 2011). In addition, the lower abundances of this genus and other from the same family in face of an environmental stress, as well as the lower abundances of the genus *Anoplostoma* observed at this study, all *genera* with smooth cuticle, may be related to a higher permeability of this cuticle type (Gallucci et al., 2015; Schratzberger et al., 2002; Ferraz et al. 2020), indicating the interstitial water, in this case, as a relevant route of contamination at the present study regardless of environmental pH.

While some *genera* were adversely affected, *Desmodora* relative abundance increased. This genus has already shown a slight tolerance to TBT contamination (Austen and McEvoy, 1997). Somerfield et al. (1994) observed an increase in *Desmodora* abundances as a response to contamination by heavy metal and have suggested the development of resistant strains which are tolerant to toxic stress. *Desmodora* also increased in abundance at similar Irgarol concentrations in a previous experiment testing the same community. Ferraz et al. (2020)

suggests that *Desmodora* increase in abundance as an indirect response to an observed increase in its feeding resources (microphytobenthos). On the other hand, this genus tolerance to pollution is not widespread. Indeed, it is believed that it gets contaminated mainly via the ingestion of contaminated food such as microphytobenthos and ciliates (Danovaro and Gambi, 2002; Losi et al., 2013; Semprucci et al., 2015). Morphological characteristics of species of the genus *Desmodora*, such as body strength and the presence of a thick cuticle, may represent a possible adaptation to the habitat (Cesaroni et al, 2017; Samprucci et al, 2010). These cuticular characteristics can mean greater ecological amplitude due to the relatively impermeable cuticle and consequently greater tolerance to pollutants found in estuarine habitats (Bongers, 1999).

Conclusion

The present study indicates that there is no acidification influence over the toxicity of Irgarol for benthic meiofauna, as it might become more soluble in interstitial water and less permeable in biological membranes, presenting counteracting effects. The results show that estuarine nematode assemblages are impacted by long exposure to low but naturally occurring pHs. This indicates that estuarine organisms are under naturally high physiological pressure and that permanent changes in the ecosystem's environmental factors, such as future acidification, may drive organisms closer to the edges of their tolerance windows.

Supplementary data

Table S1. Permanova analysis for nematode density, genus richness, genus diversity and total nematode abundance for fixed factors 'Controls', 'pH' and 'Exposure period' (Exp.). Values in bold indicate p<0,05.

Density	*df*	*SS*	*MS*	*Pseudo-F*	*P(perm)*
Controls	1	172890	172890	2,569	0,127
pH	**1**	338200	338200	5,025	**0,044**
Exp	**1**	11007000	11007000	163,540	**0,001**
Controls x pH	1	173910	173910	2,584	0,130
Controls x Exp.	1	1650	1650	0,025	0,875
pH x Exp.	**1**	322710	322710	4,795	**0,043**
Controls x pH x Exp.	1	149940	149940	2,228	0,163
Residual	16	1076800	67303		
Richness					
Controls	1	0,008	0,008	0,056	0,815
pH	1	0,024	0,024	0,176	0,690
Exp.	1	0,221	0,221	1,595	0,208
Controls x pH	1	0,348	0,348	2,511	0,121
Controls x Exp.	**1**	0,771	0,771	5,571	**0,031**
pH x Exp.	1	0,004	0,004	0,031	0,865
Controls x pH x Exp.	1	0,014	0,014	0,099	0,727
Residual	16	2,215	0,138		
Multivariate structure					
Controls	1	925,230	925,230	2,023	0,061
pH	1	984,260	984,260	2,152	**0,032**
Exp.	1	4933,500	4933,500	10,788	**0,001**
Controls x pH	1	934,800	934,800	2,044	0,061
Controls x Exp.	1	653,160	653,160	1,428	0,177
pH x Exp.	1	756,040	756,040	1,653	0,118
Controls x pH x Exp.	1	625,170	625,170	1,367	0,249
Residual	16	7317,000	457,310		

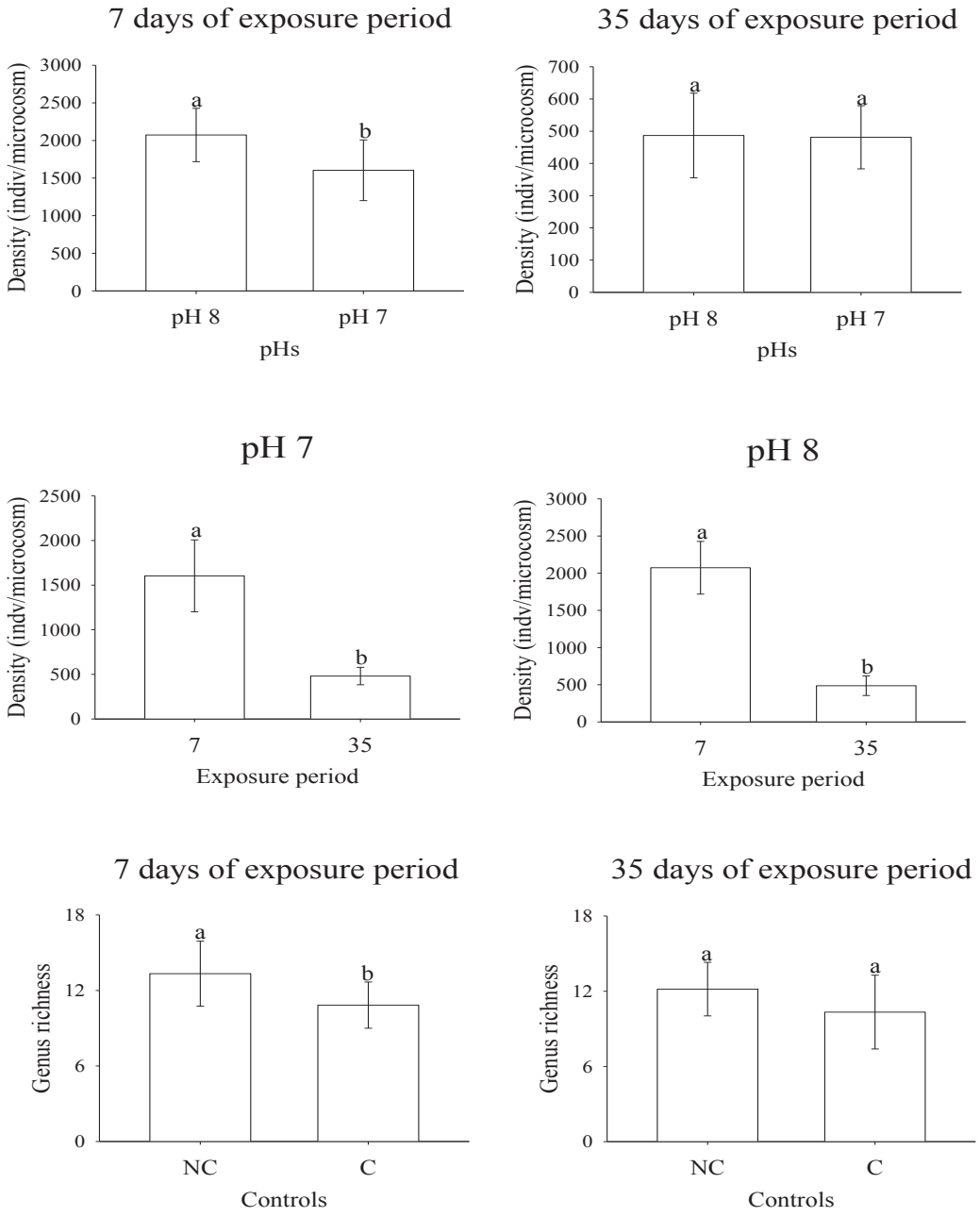

Figure S1. Mean and standard deviation of nematode density at pH 7 and 8 after 7 and 35 days of exposure period, and for nematode genus richness at the control and negative controls after7 and 35 days of exposure period. Small letters indicate significant differences.

Table S2. Multivariate Permanova analysis for relative abundances of nematode *genera* for fixed factors 'Controls', 'pH' and 'Exposure period' (Exp.). Values in bold p<0,05.

	df	SS	MS	Pseudo-F	P(perm)
Controls	1	317,990	317,990	1,517	0,195
pH	1	359,940	359,940	1,717	0,151
Exp	1	1203,400	1203,400	5,739	**0,002**
Controls x pH	1	578,160	578,160	2,757	**0,036**
Controls x Exp	1	666,060	666,060	3,176	**0,026**
pH x Exp	1	137,040	137,040	0,654	0,617
Controls x pH x Exp	1	120,510	120,510	0,575	0,704
Residual	16	317,990	317,990	1,517	

	Posthoc	
Negative control	t	P(perm)
pH 8 x pH 7	**1,868**	**0,046**
Control		
pH 8 x pH 7	1,211	0,213
pH 8		
NC x C	2,039	**0,012**
pH 7		
NC x C	1,321	0,11
7 days of exposure period		
NC x C	1,011	0,387
35 days of exposure period		
NC x C	**1,8144**	**0,035**

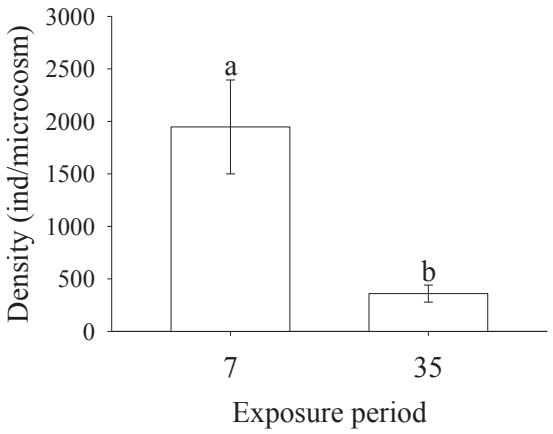

Figure S2. Mean and standard deviation of nematode density after 7 and 35 days of exposure period independently of factor 'Contamination' and 'pH'. Small letters indicate significant differences.

Chapter 2

The need for multiple approaches in ecological risk assessment

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Intention to submit in *Science of the Total Environmental – Journal – Elsevier*

ABSTRACT

Organisms have distinct biological/ecological characteristics and different levels of biological organization are responsible for promoting mutually exclusive responses, as for example, the mode of action against ecological relevance. As such, multiple response studies which use model organisms with distinct ecological traits and endpoints encompassing different levels of biological organization should be ideal to predict toxicity in complex natural environments. In this study we compare different ecotoxicological tools which provide information of different responses, of organisms with different ecologies, and contemplating different levels of biological organization (biomarkers, individual, and community levels) in order to assess potential convergences, divergences, redundancies or complementarities. We have performed a series of bioassays with different organisms and endpoints: (1) biomarkers assessment with bivalves (*Crassostrea brasiliana* and *Mytella charruana*) in 48 and 96 hours of exposure time , (2) reproduction assessment with harpacticoid copepods (*Nitokra* sp. and *Tisbe biminiensis*) in 10 days of exposure time, and (3) meiobenthic community structure assessment with *ex situ* microcosms in 7 and 35 days of exposure time. The "Contamination" factor had 3 levels: "Control" (water + sediment + acetone) plus two environmentally relevant concentrations of the biocide Irgarol ("Low" 100 ng/g and "High" 2000ng/g). Negative controls (water + sediment) were also performed to assess possible effects of the solvent acetone used. The results showed significant effects of Irgarol in the biomarker endpoints, but sensibility was different between the two bivalve species. Also, Irgarol affected different biomarkers in each species and exposure time. responded were different between the two bivalve species. Individual level endpoints of both harpacticoid copepod species were not affected by contamination. Finally, the community endpoint showed effect of Irgarol by changes in the structure of nematode assemblages. According to the results, Irgarol can cause biological effects at a sub-individual level and changes in the structure of meiobenthic communities showing different sensitivities and complemented each other. This relationship between different tools generates important subsidies for ERA's step 1, contributing to determine the goals and endpoints in a less arbitrary way by the protocols.

Keywords: multiple approaches, biomarkers, copepods harpacticoid, nematode assemblages, ecological risk assessment.

1 Introduction

Ecotoxicology is focused in identifying effects of natural or synthetic substances on the biota, including individual organisms, populations (animal, vegetable and microbial), communities, and human health within ecosystems (Gallucci et al, 2015; Walker et al, 2012; Moriarty, 1988; Truhaut, 1969). The challenge is to directly relate the concentrations of chemical substances with effects caused in different levels of biological organization, from subcellular processes to ecosystems functions. Ecological risk assessments (ERA) use information held by ecotoxicology to predict the likelihood of future effects (prospective) on ecosystems or evaluate the likelihood that observed changes are caused by past exposure to stressors (retrospective)" (see on https://www.epa.gov/risk/ecological-risk-assessment). In this way, risk assessors should analyze the risks of substances already introduced or to be introduced in the environment and provide the information to decision makers (e.g. managers, public agencies, among others) (ERA, 2008).

An important step in any ERA framework is the characterization of ecological effects, in which the relationship between the stressor and measurement endpoints (i.e. effects) is quantified and cause-and-effect interactions are evaluated (USEPA, 1992). However, the estimation of ecological effects is usually the result of extrapolations based on mortality, growth, or reproduction data obtained from single-species short-term toxicity tests (ECHA, 2002; Hagger et al., 2006; Franco et al., 2016). The use of biological endpoints at the individual level raises questionings on at least 3 fronts: 1. these tests and their responses do not have ecological realism (Franco et al., 2016; SCENIHR, 2012); 2. there is a lack of understanding of the mechanisms of stressor action (Hagger et al., 2006); 3. it is impossible to manage preventively as the results are limited to more drastic biological responses (i.e. mortality, reduced growth and/or reproductive performance). It is recognized that studies that integrates different levels of biological organization are better suited to predict toxicity in complex natural environments (Clements, 2000), although this is not embraced by ERA frameworks.

Many ecotoxicological tools are available to aid the establishment of the relationship between chemical stressors and biological and/or ecological effects, with potential to promote a more informative ERA. Biomarkers, for example, are known to be sensitive tools that show early warning effects of pollutants (Hagger et al., 2006; Whitacre, 2012). However, biomarkers

alone are usefulness to extrapolate the effects in other levels of biological organization (Forbes et al., 2006). For higher levels of organization, community assays performed in microcosm assays are now a relatively common method (Ferraz et al., 2020; Nasri et al., 2020; Gallucci et al, 2015; Hedfi et al, 2007; Gyedu-Ababio & Baird, 2006) in which multiple species exposures have been shown to be very similar to the natural environment (Tolle et al., 1985; Niederlehner et al, 1990, Hägerbäumer et al, 2015). These experiments provide highly relevant ecological information on the toxicity since different species are exposed at the same time in the same experimental medium, enabling the identification of direct toxicity effects to several different species, with different sensitivities and subjected to exposure routes, as well as indirect ecological effects related to interactions between species (Beyers and Odum, 1993; Chapman, 2002).

An acceptable ERA must be informative, protective, and able to use multiple ecotoxicological tools, encompassing different responses, with ecologically distinct organisms, and contemplating different levels of biological organization. Thus, provide complementary information for a suitable characterization of ecotoxicological effects. For example, integrating the results of assays with different organisms (amphipod, copepod, tanaid and bivalve) promote a more precise view of negative effects, because such approach contemplate different ecological strategies and sensitivities (Campos et al, 2019), even though a battery of single-species toxicity tests do not allow for species to interact. Pereira et al (2014) performed a multi-level environmental assessment of marine and estuarine zones, including a biomarker and community structure parameters, and showed associations between cellular stress (lysosomal membrane stability), impaired embryo-larval development, and changes in the structure of the benthic macrofauna. In a critical review Rohr et al (2016) discussed the pros e cons of using different levels of biological in ERA and argue that future approach to ERA should include low and high levels of endpoints (molecular, organismal, community and ecosystem).

In this study we use multiple approaches in ecological risk assessment, providing information of different responses on ecologically distinct organisms, and encompassing different levels of biological organization (biomarkers, individual, and community levels) in order to assess potential convergences, divergences, redundancies, or complementarities. Because bioavailability and toxicity responses depend on organisms' ecological traits and the investigated endpoint, we expected responses to differ between the approaches.

2 Material and Methods

2.1 Study design

In order to compare different ecotoxicological tools which provide information from organisms with different ecologies, and contemplate different levels of biological organization, we have performed a series of bioassays with different organisms and endpoints. Three different ecotoxicological tools for testing sediment toxicity were used: (1) biomarkers assessment with bivalves (*Crassostrea brasiliana* and *Mytella charruana*), (2) reproduction assessment with harpacticoid copepods (*Nitokra* sp. and *Tisbe biminiensis*), and (3) meiobenthic community structure assessment with *ex situ* microcosms. To account for species-sensitivity variability, two different species were used in each of the single-species tools. The contamination factor was common for all ecotoxicological tests, with 3 levels: "control" (water + sediment + acetone) plus two concentrations of the Irgarol biocide as a model contaminant ("Low" 100 ng/g and "High" 2000ng/g), which is an antifouling biocide commonly encountered in marine and estuarine sediments (Biselli et al., 2000; Boxall et al., 2000) (Figure 1). Negative controls (water + sediment) were also performed to assess possible effects of the solvent acetone used. For the bivalve's biomarker and nematode assemblages' assays, two exposure times were investigated, which were 48 and 96 hours for biomarkers (two tissues were analyzed: gills and digestive glands) and 7 and 35 days for assemblages. For the copepods fertility rate, only a single exposure period was analyzed (10 days). Exposure periods were chosen based in protocols and/or procedures most adopted in the literature (Cortez et al, 2018; Galluci et al, 2015; Lotufo and Abessa, 2002). These are expected to best suit the time scale of processes involved in the responses of the different endpoints. All experiments was kept under controlled temperature (25 °C) and photoperiod (16:8h light:dark).

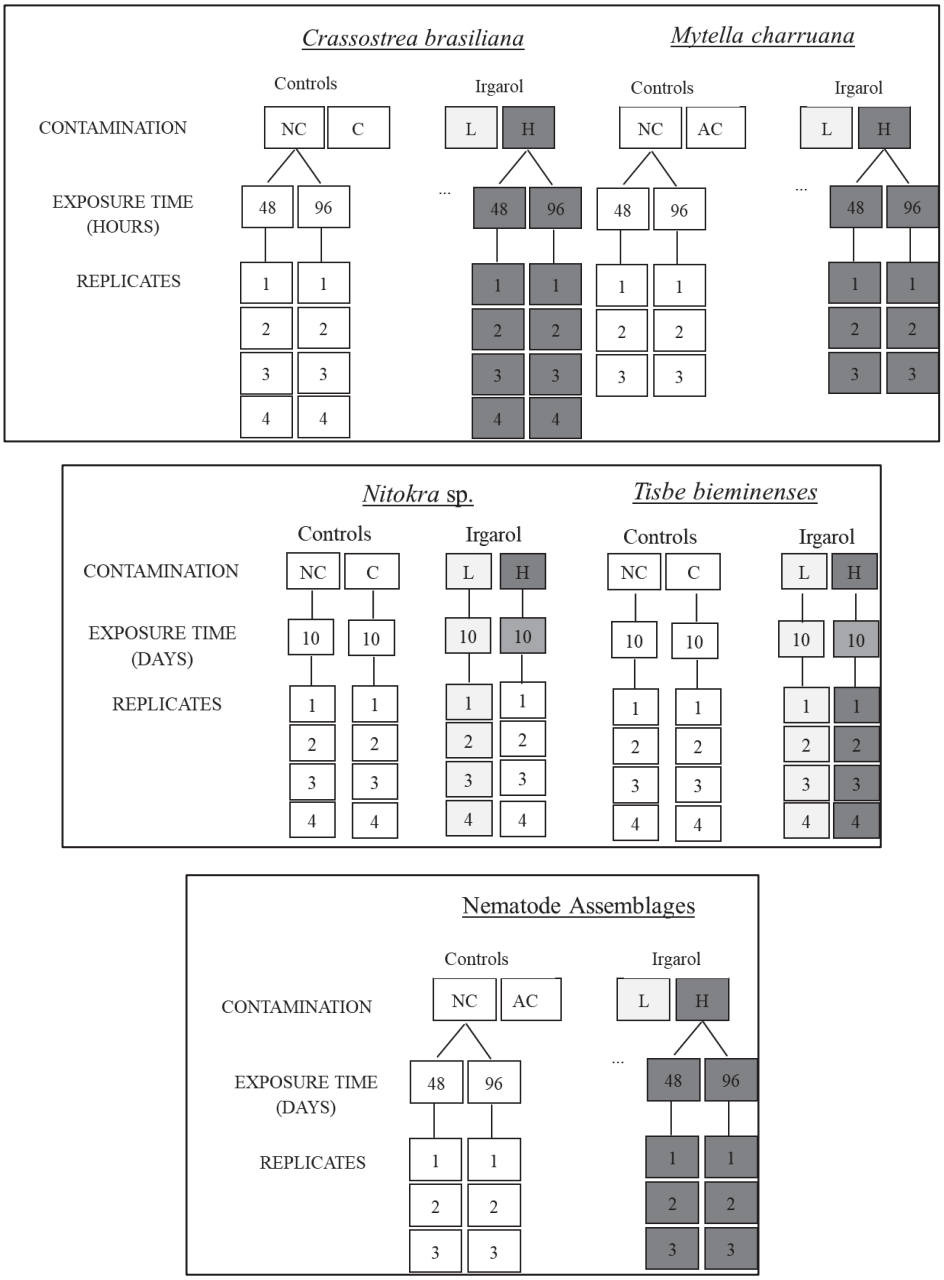

Figure 1. Experimental design of the bioassays with *Crassostrea brasiliana, Mytella charruana, Nitokra sp., Tisbe bieminenses* and meiofauna community.

2.2 Collection and defaunation of sediment

The sediment used in all experiment was collected in the Itaguaré river mouth, located in Bertioga, São Paulo, Brazil (coordinates: 23 ° 46'45.60 "S, 45 ° 58'9.31" W). This is a widely used location for this purpose because it presents low levels of contaminant interference, since

it is protected by the Bertioga Restinga State Park along the Itaguaré river. Sediments were sampled using corer, storage in tanks and were immediately transported to the laboratory, where they were homogenized and stored in a -20ºC. Before contamination of the sediments for the experiments, these were defaunated by 12 hours freezing followed by 48 hours thawing at room temperature, for at least three times or until there were no more organisms in the sediment (Schratzberger et al., 2002). After this procedure, sediments were stored in a freezer.

2.3 Sediment spike

Irgarol stock solution was prepared with the co-solvent acetone and diluted to final nominal concentrations of 100ng g^{-1} and 2000ng g^{-1} for Low and High Irgarol concentrations, respectively. Acetone was chosen as co-solvent due its rapid dissipation in sediment, which facilitates it's volatilization and removal (Northcott and Jones, 2000). The defaunated sediments were added to replicate experimental units (300g for bivalves, 8g for copepods and 100g for nematode assemblages' experiments) which were each individually spiked with Irgarol solution corresponding to the estimated nominal concentrations of the different treatments. Estuarine natural water from the field (salinity 23 for bivalves and nematode assemblages) or reconstituted water (salinity 17 and 33, used for *Nitokra* sp. and *T. biminiensis*, respectively) were added until sediment saturation to facilitate homogenization. The final concentration of the co-solvent acetone in the 'acetone control', 'low Irgarol' and 'high Irgarol' treatments was 0,05% of the total solution volume. Natural or reconstituted water was added to the 'negative control' at the same volume of the other treatments. All replicate units were shaken vigorously for 15 minutes and kept refrigerated at 4ºC in the dark for 48 hours to achieve compound equilibration time and co-solvent volatilization.

2.4 Ecotoxicological tests

2.4.1 Biochemical biomarkers with organisms-test *C. brasiliana* and *M. charruana*

C. brasiliana (BIVALVIA: Ostreidae) and *M. charruana* (BIVALVIA: Mytilidae) are bivalve organisms commonly found in estuarine environments along the Brazilian coast. *C. brasiliana* are sessile organisms found in the intertidal and infralittoral regions, fixed both in rocky substrates and in mangrove roots filter phytoplankton and sediment particles (Absher, Vergara and Christo, 2000). Despite being an euryhaline species, the best range of salinity to cultivate is between 15 to 25 (Pereira et al, 2003). *M. charruana* have similar ecological traits

to other bivalves (*Modiolus demissus* and *Modilus metealfei*), that found from the surface up to 10 cm depth, they gather in groups of 3 to 4 individuals, with the bissus entangled and attached to the mud particles, sand and pieces of shells (Stanley, 1972; Morton, 1977). Both are commonly used in the ecotoxicology biomarkers assays. Individuals of *C. brasiliana* were purchased from Cananéia - SP mariculture and *M. charruana* were obtained from the field (Bertigoa-SP). The organisms were placed in tank with 500 L of diluted sea water (salinity 25), acclimated for 7 days for their recovery. During this period organisms were kept in constant aeration and fed with phytoplankton solution.

Experimental units consisted in 3L glass flasks containing 300g of sediment and 1.2L of marine water with salinity 29. Two individuals of *C. brasiliana* (4 replicates for each level of "Contamination") and four individuals of *M. charruana* were added to each experimental unit (3 replicates for each level of "Contamination") and were kept for 48 and 96 hours with constant aeration. The tanks had the externally lower part coated with aluminum foil to avoid the minimum degradation of the compound during the exposure time. After all exposure times (T-48 and T-96), euthanasia of individuals was performed and tissues (gills and digestive glands) were collected and kept in the ultra-freezer (-80°C) until further analyzes. The following biomarkers were analyzed: GST, GSH, GPx, LPO, AChE, DNA damage.

First step it was homogenize the tissues with buffer solution and the activity of the glutathione-S-transferase (GST) were quantified since the enzyme take part in the phase II of the metabolism of xenobiotic organic compounds. Antioxidant system responses, which may also be related to exposure of organic xenobiotics, were assessed by the quantification of non-protein thiols (reduced glutathione, GSH) and the activity of glutathione peroxidase (GPx). Potential toxic effects on exposed organisms were observed through of the levels of lipoperoxidation (LPO) (oxidative damage on lipids), acetylcholinesterase (AChE) (neurotoxicity) and quantification of levels of DNA damage (genotoxicity) (Figure 2).

Activity of GST (Keen et al., 1976) and GPx (Sies et al., 1979) were determined by spectrophotometry at 340 nm. Levels of GSH were measured by spectrophotometry at 415nm (Sedlak and Lindsay, 1968). Analysis of AChE activity were quantified at 415nm using the colorimetric method proposed by Ellman et al. (1961). The levels of lipid peroxidation (LPO) were quantified by the method of thiobarbituric acid reactive substances (TBAR) proposed by Wills (1987), by fluorescence (λex 532nm and λem at 556nm). The method used to evaluate

DNA damage were the alkaline precipitation (Gagné and Blaise, 1995). The assay is based on the precipitation of the protein-bound genomic DNA through SDS (sodium dodecyl sulfate), leaving the single-strand breaks in the DNA-protein chain free in the supernatant. These DNA strands were quantified using fluorescence (λex 360nm and λem at 450nm) after staining with Hoescht dye. Standard solutions of salmon sperm DNA were used for calibration. All analyzes were normalized using BSA (bovine serum albumin protein) as a standard and read by spectrophotometry at 595nm (Bradford, 1976). The reading of absorbance and fluorescence were performed on a microplate reader.

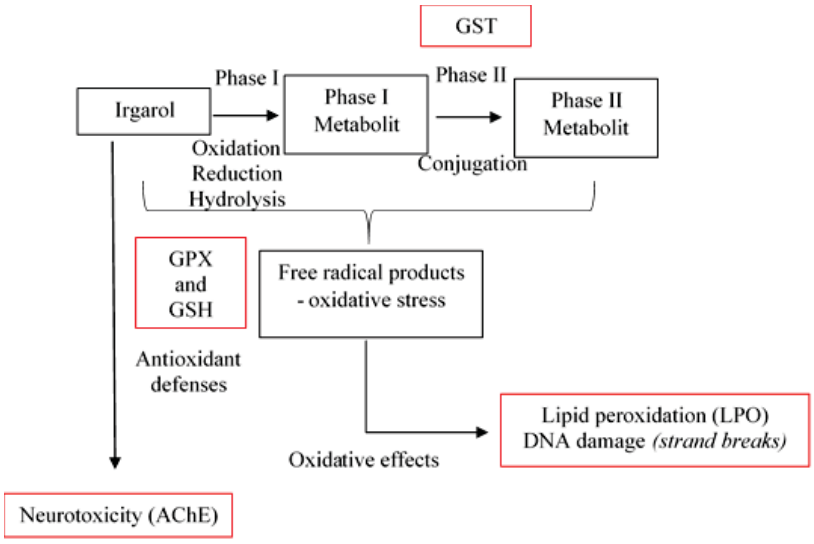

Figure 2. Flowchart on the exposure of organisms to xenobiotics and the responses of enzymatic activities in phases I and II and the oxidative effects.

2.4.2 Fertility rate of ovate females of the harpacticoid copepods Nitokra sp. and Tisbe biminiensis

Nitokra sp. and *T. biminiensis* are benthic free-living harpacticoid copepods with benthic habit and generally live in estuarine areas. In a natural environment it feeds on microorganisms and debris trapped in the sediment grains, in addition to seaweed. Adult copepods integrate an important part of the food chain (it is part of the diet of marine organisms such as fish and even other crustaceans), with a length varying between 1 or more than 5 mm (Ruppert and Barnes, 1996). The complete life cycle of this organism consists of six naupliar stages, five stages of copepodites and an adult stage, which lasts about 20 days according to Lotufo and Abessa (2002) to reach the adult stage. They are considered as suitable for toxicity

testing due to their ease of cultivation and handling, relatively short life cycle (3 to 4 weeks) and good laboratory reproduction rates, survival in water and sediment, and sensibility to several contaminants (Lotufo and Abessa, 2002).

The experiments were performed according to guidelines recommended by Lotufo & Abessa (2002), with adaptations. *Nitokra* sp. and *T. biminiensis* individuals for the experiments were taken from cultures. The cultures were stored in 1000mL Erlenmeyers, containing 800mL of reconstituted seawater, prepared in the laboratory using Pro Coral RedSea® sea salt, with salinity 17 for *Nitokra* sp. and 33 for *T. biminiensis*, maintained in the germination chamber, with controlled temperature and photoperiod (25 ± 2ºC and 16 / 8h light/dark). They were fed twice a week with compost based on fish feed and the water changed carried out monthly. The organisms were exposed in beckers (35 x 56 mm) containing 8 g of sediments and 40 mL of reconstituted water with salinity 17 for *Nitokra* sp. and 33 for *T. biminiensis*. Each replicate (4 replicates for each level of "Contamination") contained 10 ovate females and were exposed to the whole sediment for 10 days. The experiment received 10ul of food, which was the same used to feed the cultures, and it were maintained without aeration. The beckers had the externally lower part coated with aluminum foil to avoid the degradation of Irgarol during the exposure time. At the end of the exposure time, a solution of 10% formalin was added to each becker to fix the organisms and Bengal Rose to facilitate the sorting. After 48h the sediment were sifted and the total number of offspring (nauplii + copepodite) and adult females was subsequently analyzed with the support of a stereomicroscope.

2.4.3 Univariate and multivariate analysis of nematode assemblages

Sediment containing nematode assemblages was collected at the day of the start of the experiment, at the same location of the previously defaunated sediment. Sediment was transferred to the laboratory, carefully homogenized to ensure uniformity of the community and immediately added to each experimental unit. Microcosms consisted of 500 ml beackers (14 x 8cm) filled with 100g of sediment with meiofauna and 100g of defaunated contaminated sediment ('low Irgarol' and 'high Irgarol') or non-contaminated defaunated sediment ('acetone control' and 'negative control'). Sediment with meiofauna and the defaunated sediment (contaminated or not) were carefully homogeneized and natural estuarine waters from the field with salinity 21 was gently added to each experimental unit. Microcosms were constantly aerated and kept in the dark to reduce microalgal growth (Schratzberger et al., 2002) and

photodegradation of Irgarol. At the end of the exposure times (7 days and 35 days) the water was carefully removed with the assistance of a siphon and the sediment was fixed with 4% formaldehyde for analysis of the nematode assemblages.

The extraction of all meiofauna organisms was done by flotation using Ludox ™ 50 (1.18 specific density) according to the procedure described by Heip et al. (1985). After extraction from the sediment, sediments were washed through a 45μm mesh and the retained material was stored in 4% formaldehyde and stained with Bengal Rose. All nematodes were counted under a stereomicroscope and 10 %, from each replicate was randomly picked, evaporated in anhydrous glycerol for 48 hours and mounted on permanent slides for identification to genus level (Warwick et al., 1998). A lower and higher limit of 100 and 200 organisms, respectively, were established for identification.

2.4 Data Analysis

Firstly, possible effects of the co-solvent acetone were assessed. For the experiment with the organisms-test *Crassotrea brasiliana* and *Mytella charruana,* a two-way permutational multivariate analysis of variance (PERMANOVA) was performed considering all the six biomarkers as variables and the factors 'Control treatments' (2 levels: Negative Control and Acetone Control), and 'Exposure Time' (2 levels: 48 and 96 hours) as fixed factors. For the experiments with the copepods *Nitokra* sp. and *Tisbe bieminensis,* a one-way univariate PERMANOVA was conducted to test for differences in fertility rates (number of offspring divided by the number of adult females) between the Negative control and Acetone control. For nematode assemblages, two-way PERMANOVA analysis was conducted considering 'Control treatments' (2 levels: Negative Control and Control), and 'Exposure Time' (2 levels: 7 and 35 days) as fixed factors.

To test for Irgarol toxicity, a three way PERMANOVA considering all biomarker data was performed separately for each bivalve species considering the factors "Contamination" (3 levels: Control, Low and High Irgarol contamination), "Tissue" (2 levels: gills and digestive gland) and "Exposure Time" (2 levels: 48 and 96 hours) as fixed factors. For the levels of the factor "Contamination", the control with acetone was considered as the "Control" since all Irgarol contaminated treatments also have acetone. Data was normalized prior to the construction of the Euclidian distance resemblance matrix. In the case of significant results, the pairwise a posteriori comparison was performed. Percentage Similarity Analysis (SIMPER)

was applied (Clarke, 1993) to identify the biomarker that contributed the most to the differences detected. Alpha level was set at 5%.

Differences in fertility rate between contamination treatments was analyzed by one-way PERMANOVA with “Contamination” (3 levels: Control, Low and High Irgarol contamination) as fixed factor. The analysis was done separately for each copepod species. In case of significant differences, *a posteriori* pairwise comparisons were performed.

For nematode assemblages, effects on univariate metrics(i.e. nematode density and richness), and on multivariate data, (i.e. densities of nematodes *genera*) were analyzed by two-way PERMANOVA with ‘Contamination’ (3 levels: Control, Low Irgarol and High Irgarol contamination) and ‘Exposure Time’ (2 levels: 7 and 35 days) as fixed factors. According to the experimental design, the toxicity is identified as an interaction between the factors ‘Contamination’ and ‘Exposure Period’ treatments (Cont. vs. Exp.) or levels of “Contamination”. For univariate metrics, PERMANOVA analysis was done based on Euclidian distance resemblance matrix (Anderson, 2017). For the multivariate structure of nematode assemblages, Bray-Curtis similarity index was used to construct the resemblance matrix. In addition, because there were large differences in densities between the two exposure times, we have performed the PERMANOVA multivariate analysis on relative abundance data, which excludes the influence of total densities of samples. In the case of significant results, pairwise *a posteriori* comparisonS were performed. Percentage Similarity Analysis (SIMPER) was applied (Clarke, 1993) to identify the genera that contributed the most to the differences detected. Alpha level was set at 5%.

3 Results

3.4.1 Biochemical biomarkers with organisms-test *C. brasiliana* and *M. charruana*

For both bivalve assays, results from multivariate PERMANOVA analysis integrating the six biomarkers testing for possible effects of the cosolvent acetone showed no significant differences between the negative control and acetone control (Table S1).

The PERMANOVA multivariate analysis for the factors “Contamination” (3 levels: acetone control, low Irgarol and High Irgarol), “Exposure Time” (2 levels: 48 and 96 hours) and “Tissue” (2 levels: gills and digestive gland) showed significant interaction between the three factors for both bivalve species. For *C. brasiliana,* posthoc analysis showed significant

differences only for the digestive gland. Integrated structure of biomarkers was significantly different at low Irgarol concentrations compared to the control at both time of exposure whereas high Irgarol concentrations did not differ from the controls (Table 4).

Table 1. Results of multivariate PERMANOVA analyses for all respective factors of experiments with biochemical biomarkers with tissues of *Crassostrea brasiliana and Mytella charruana*. Cont.: Controls; Exp.: Exposure Time. Values in bold p<0,05.

Crassostrea brasiliana				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	11,674	2,454	0,01
Exposure time	1	17,444	3,668	0,005
Tissue	1	43,215	9,086	0,001
Cont. x Exp.	2	9,5973	2,018	0,034
Cont. x Tissue	2	9,6124	2,021	0,038
Exp. x Tissue	1	17,533	3,686	0,009
Cont. x Exp. x Tissue	2	9,3539	1,967	**0,044**
Residual	67	4,7563		
Mytella charruana				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	10,255	2,4393	0,004
Exposure time	1	24,316	5,7836	0,001
Tissue	1	71,523	17,012	0,001
Cont. x Exp.	2	5,67	1,3486	0,226
Cont. x Tissue	2	6,871	1,6343	0,095
Exp. x Tissue	1	15,050	3,580	0,002
Cont. x Exp. x Tissue	2	9,1047	2,1656	**0,013**
Residual	56	4,2043		

Although differences were observed for both exposure times, SIMPER analysis showed that at 48 hours, increased activity of AChE and GST were the main responsible for the significant differences observed (Figure 3) whilst at 96 hours, differences were mainly due to increased DNA damage at low Irgarol concentration when compared to the control (Figure 3) . Data for all the six biomarkers analyzed for each tissue, at each contaminated treatment and exposure time are shown on the supplementary Figures S1, S2, S3 and S4.

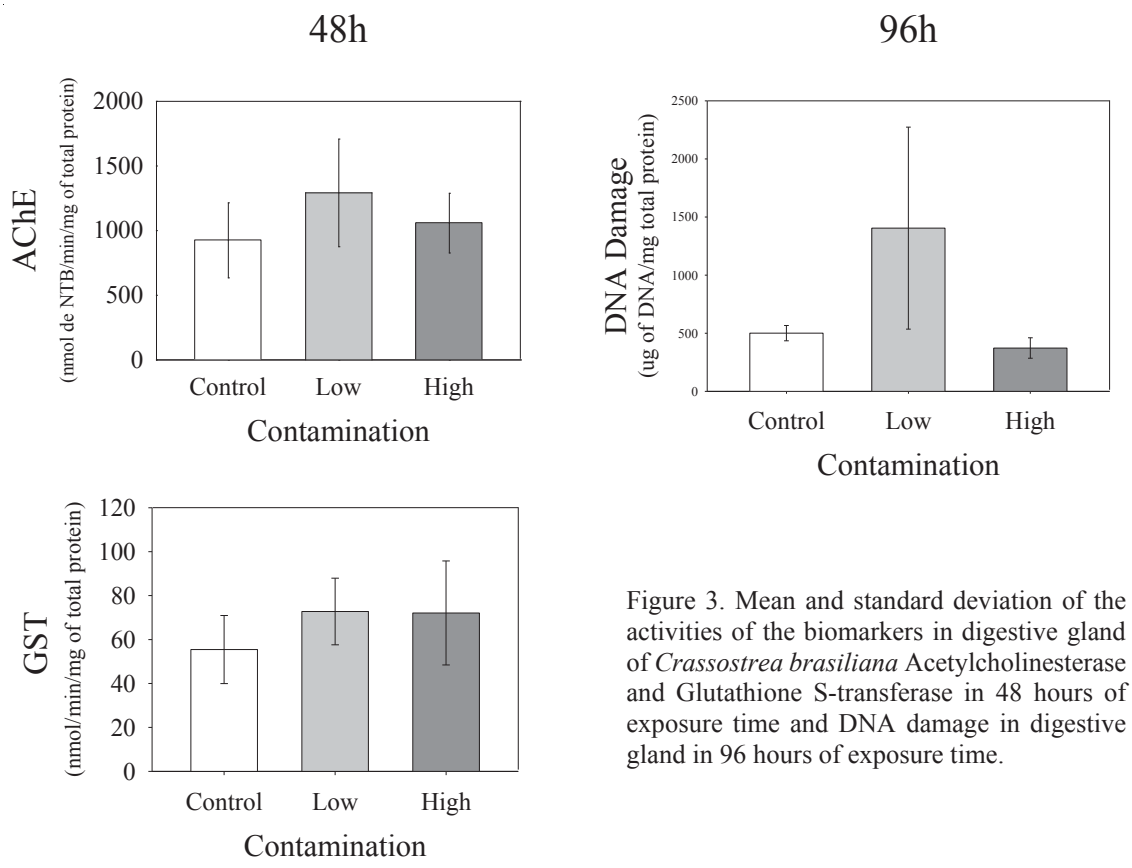

Figure 3. Mean and standard deviation of the activities of the biomarkers in digestive gland of *Crassostrea brasiliana* Acetylcholinesterase and Glutathione S-transferase in 48 hours of exposure time and DNA damage in digestive gland in 96 hours of exposure time.

For *M. charruana,* posthoc results from biomarker analysis of the gills showed significant differences between the low Irgarol concentration and the control at the 48 hours exposure treatment (Table 4). According to SIMPER this difference was mainly explained by lower GPX activity at the low Irgarol treatment (Figure 4). For the digestive gland, significant differences were observed only for the 96h exposure period (Table 4). At this exposure time, both low and high Irgarol treatments were significantly different from the control. Differences were mostly due to higher GST activity at both concentrations of Irgarol compared to the control (Figure 4). Data for all the six biomarkers analyzed for each tissue, at each contaminated treatment and exposure time are shown on the supplementary Figures S5, S6, S7 and S8.

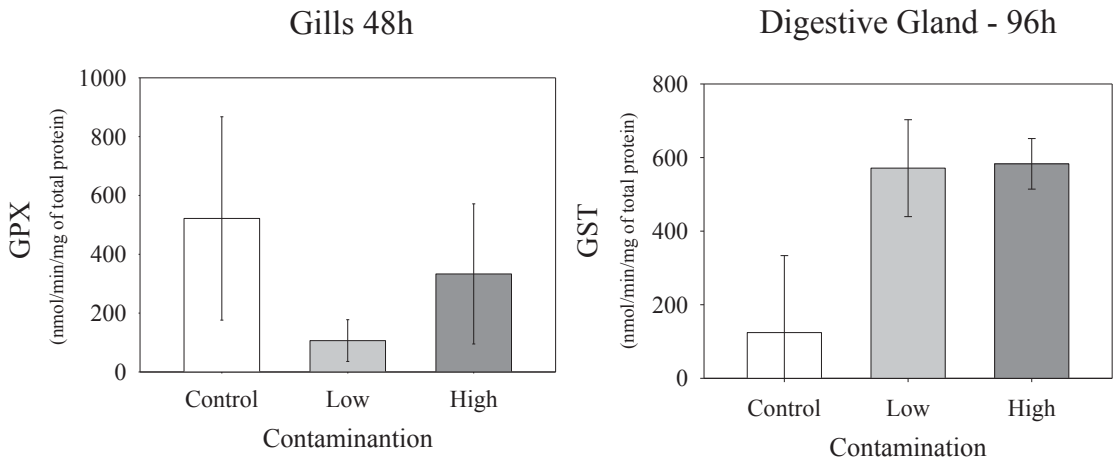

Figure 4. Mean and standard deviation of the activities of the biomarkers Glutathione peroxidase in the gill of *Mytella charruana* in 48 hours of exposure time and Glutathione S-transferase in the digestive gland of *Mytella charruana* in 96 hours of exposure time.

3.4.2 Fertility rate of ovate females of the harpacticoid copepods Nitokra sp. and Tisbe biminiensis

Fertility rates varied between 4 and 16 proles (nauplii+copepodit) per female for *Nitokra* sp and between 21 and 45 prole per female for *Tisbe biminiensis* (Figure S9). As for the integrated biomarker analysis, fertility rates for both copepod species did not differ between negative and acetone controls (Table S1). Univariate PERMANOVA analysis for the factor "contamination" showed no significant differences in fertility rates between levels of the "contamination" treatments (Table 2) for both species.

Table 2. Results of multivariate PERMANOVA analyses for all respective factors of experiments with fertility rate of ovate females of the harpacticoid copepods *Nitokra* sp. and *Tisbe biminienses*. Cont.: Controls; Exp.: Exposure Time. Values in bold p<0,05.

***Nitokra* sp.**				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	0,000	0,353	0,709
Residual	12	0,000		
Tisbe biminienses				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	0,000	1,258	0,332
Residual	6	0,000		

3.4.3 Univariate and multivariate analysis of nematode assemblages

Nematode densities in microcosm ranged from 247 to 2667 and did not differ between contamination levels (Table 3). However, nematode densities were significantly lower after 35 days when compared to 7 days of exposure time.

Microcosm nematode assemblages showed a total of 15 nematode genera. Genus richness varied from 7 to 15 genera per microcosm and did not differ between "Contamination" and "Exposure time" treatments (Table 3). *Desmodora* was the dominant genus representing between 45.6 and 86.7% of the total nematodes per microcosm. The following genera with the greatest relative abundance was *Anoplostoma* (0 to 20.6%), *Oncholaimus* (0 to 16.3%) and Viscosia (0 to 8.3%). The PERMANOVA multivariate analysis performed with the absolute abundances of nematode genera showed no significant difference in the structure of the nematode assemblages (Table 3). The PERMANOVA multivariate analysis performed with the relative abundances of nematode genera showed a significant difference in the structure of the nematode assemblages between the levels of the "Contamination" and "Exposure Time" factor (Table 3). Posthoc demonstrated that the significant difference in the "Contamination" factor was between the control and high Irgarol concentration treatments (Table 4). The results of Simper analysis showed that differences were mainly due to higher relative abundances of *Desmodora* and lower relative abundances of *Oncholaimus* in the high concentration of Irgarol compared to the control treatment (Figure 5).

Table 3. Results of univariate and multivariate PERMANOVA analyses for all respective factors of experiments with nematode assemblages' structure. Cont.: Controls; Exp.: Exposure Time. Values in bold p<0,05.

Univariate				
Density				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	65153,000	1,152	0,306
Exposure time	1	13099000,000	231,550	**0,001**
Cont. x Exp.	2	144880,000	2,561	0,114
Residual	12	56570,000		
Richness				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	7,389	1,357	0,298
Exposure time	1	0,222	0,041	0,830
Cont. x Exp.	2	7,389	1,357	0,303
Residual	12	5,444		
Multivariate of absolute abundance				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	435,430	1,858	0,069
Exposure Time	1	22282,000	95,054	**0,001**
Cont. x Exp.	2	421,170	1,797	0,064
Residual	12	234,410		
Multivariate of relative abundance				
	df	***MS***	***Pseudo-F***	***P(perm)***
Contamination	2	422,77	2,5249	**0,043**
Exposure time	1	1104	6,5934	**0,003**
Cont. x Exp.	2	260,28	1,5545	0,182
Residual	12	167,44		

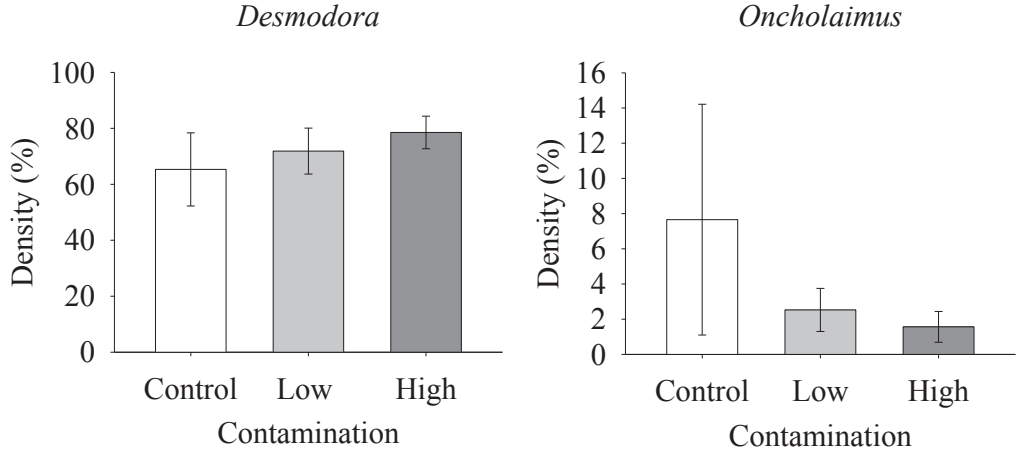

Figure 5. Mean and standard deviation of nematode relative density of genera *Desmodora* and *Oncholaimus* by levels of "Contamination" factor.

Table 4: Summary table integrating the posthoc results of all bioassays. YES: Significant difference between the levels of the contamination factor (p<0,05); NO: No significant difference between the levels of the contamination factor (p>0,05).

Biochemical biomarkers				
Crassotrea brasiliana				
Gills		CONTAMINATION		**Detailed diagnosis**
		Low	High	
	48h	NO	NO	No effect of Irgarol on gills after 48h and 96h of exposure time
	96h	NO	NO	
Digestive gland				
	48h	YES	NO	Increased AChE and GST at the lowest Irgarol level in 48h of exposure time. Increased DNA damage only at the lowest Irgarol level in 96h of exposure time
	96h	YES	YES	
Mytella charruana				
Gills		Low	High	
	48h	YES	NO	GPx inhibition at the lowest Irgarol level in 48h of exposure time
	96h	NO	NO	
Digestive gland				
	48h	NO	NO	Increased GST activity in organisms exposed to Irgarol in 96h of exposure time
	96h	YES	YES	
Fertility rate of ovate females of the harpacticoid copepods				
		Low	High	No effect of Irgarol on *Nitokra*'s and *Tisbe*'s fecundity
Nitokra sp.		NO	-	
Tisbe biminienses		NO	NO	
Multivariate structure of nematode assemblages				
		Low	High	Highest Irgarol level induced increase relative abundance *Desmodora* and decrease of *Oncholaimus*
Change in the structure of nematode assemblages		NO	YES	

4 Discussion

Although Irgarol is a herbicide, there is evidence that this contaminant causes toxicity in other groups of organisms, such as nematode assemblages, sea urchin and oyster embryo development, larva and adult grass shrimp (Ferraz et al., 2020; Galluci et al., 2015; Mai et al., 2013; Key et al., 2008). In the present study, ecotoxicological tools were used at 3 levels of

biological organization, involving sub-individual (analysis of biomarkers), individuals (copepod fertility rate) and community (nematode assemblages' structure) which showed varied results.

The first line of evidence was observed in the changes in the biomarkers of the oyster and mussel test organisms. First, within 48 hours of exposure, the increased activity of the GST enzyme in the digestive gland of the oyster indicates an induction caused by the low concentration of Irgarol probably as part of the process of metabolizing Irgarol. The effect of Irgarol on AChE activity is nuclear. Park et al. (2016) showed no effect of Irgarol on the AChE activity in oysters's gills (although AChE mRNA expression was downregulated); on the other hand, Key et al. (2006) reported an increase in the activity of AChE in grass shrimp larvae, which was attributed to a compensatory mechanism. Nowadays it is known that, apart from its classical role of degrading acetylcholine in the termination of nervous signals at cholinergic synapses, other roles have been attributed to AChE related to non-catalytic roles, such as the regulation of cell death or proliferation in apoptosis (depending on the AChE isoform involved) (Jiang and Zhang, 2008).

It is noticeable that Irgarol exerted effects in oysters (i.e. induced activity of GST and AChE) mostly at the low concentration treatment. This can be a result of a biphasic pattern of response, characterized by a stimulus at low concentrations of the stressor (Calabrese & Baldwin, 2003) followed by inhibition as concentration increases, as observed for biomarkers responses to Chlorotalonil, another antifouling biocides (Morais, 2016; Utsumi, 2019). These effects are called hormesis and are characteristic of enzymatic changes in order to see if the repair was carried out properly without major subsequent effects over time (Clabrese, 2002). On the other hand, after 96 hours of exposure there is evidence of DNA damage, however this genotoxic effect of Irgarol is still unknown and, therefore, inconclusive result (Mai et al, 2012).

Indications of biotransformation of Irgarol were observed in the digestive gland of *M. charruana* after 96h of exposure for both concentrations of Irgarol. The increase of the activity of GST suggests that the organisms were biotransforming Irgarol since the enzyme catalyze the conjugation of GSH on carbon, sulfur, or nitrogen atoms of xenobiotic substrates (Hayes et al., 2005).

The two species used to investigate effects at lower level of biological organization showed different responses. While the oyster showed some effect of exposure to Irgarol in 48h,

effects in 96h were inconclusive. Yet *M. charruana* showed clear responses to exposure to the contaminant and these were more congruent at 48 and 96 h of exposure time. This observed differential response may be due to the different ecologies of the organisms. Ryan (2002) reported that the contamination of molluscs occurs mainly from the capture of food resources present in the interstitial water than from the ingestion of sedimentary particles. So, probably, the main route of exposure is by the capture of food resources through the filtration of small particles present in the water (Lees, 2000). Unlike *C. brasiliana*, *M. charruana* has a burial habit and, in fact, during the exposure the organisms buried. As the contamination of Irgarol came from the sediment, probably *M. charruana* may have been more exposed to particulate matter and contaminated water than the oyster, which throughout the exposure remained on the sediment layer.

Our results of ecotoxicological tests at the individual level (i.e. copepod fecundity) were less sensitive than biomarker and community-level approaches, since the environmental concentrations of Irgarol had no effect on individual level endpoints. Normally, ERA decision-making is based on laboratory tests that contemplate only the level of individual organization and these results show that the use of tools only at this level underestimate the effects both at the sub-individual level and at the community level.

The observed changes in the structure of the nematode assemblage caused by Irgarol had already been reported elsewhere (Ferraz et al., 2020; Galluci et al., 2015). In the same way, increase of the genera *Desmodora* and decrease of *Oncholaimus* was also detected. According to Santos et al. (2018), the *Desmodora* genus can be considered tolerant to polluted environment. The decrease in the relative abundance of the genus *Oncholaimus*, on the other hand, may be related to its smooth cuticular morphology, which suggests a higher permeability to contaminants (Santos et al., 2018; Gallucci et al., 2015; Fonseca and Fehlauer-Ale, 2012). Apart from morphological aspect, changes in communities could also be a result of indirect effects of the stressor that interfere on the interactions between species (eg. consumer-resource relationships, competition, positive interactions) (Ferraz et al., 2020). As such, the responses to changes in the community structure showed ecological effects of Irgarol, which this fact is reinforced with the biomarker responses that showed the availability of Irgarol not only at higher, but at lower Irgarol concentration. In this sense, the approaches not only promoted different information, but complemented and enriched the ecological effects characterization in ERA.

The multiple approaches in ecological risk assessment used in this study included different endpoints, exposure times, with organisms that have different ecologies (and, therefore, different routes of exposure to contaminants), and different levels of biological organization. Our results showed that Irgarol, in the tested concentrations (environmentally relevant), cause biological effects at a sub-individual level and change the structure of meiobenthic communities. As also revealed by Pereira et al (2014), that xenobiotic compounds can induce physiological stress, and alter benthic structures, degrading the ecological condition of the environment over time. These conclusions could only be draw with the use of multiple ecotoxicological tools. The use of only a classic ecotoxicological tool, the copepod fecundity test, tested with two different species, was not sensitive to the effects of Irgarol at these environmental concentrations. Integrated multilevel approaches produce complementary information and improve the decision-making process on ecological risk.

Correlating heterogeneous data from different lines of evidence, with different levels of biological organization, are key steps for determining the risks associated with polluted sediments in complex systems, and thus providing a coherent and acceptable tool for the assessment of ecological risks and management decisions. The multiple approaches in ecological risk assessment used generates important subsidies for ERA's step 1, contributing to determine the goals and endpoints in a less arbitrary way as usually done by the protocols. The multiple approaches showed different sensitivities and were clearly complementary. While biochemical responses were mechanistic, showing direct effect relationships caused by exposure to the stressor, the responses of the microcosm assay include different species subjected to indirect relationships with each other, produce ecologically relevant information, such as structural changes in the community. This relationship between different tools generates important subsidies for ERA's step 1, contributing to determine the goals and endpoints in a less arbitrary way as usually done by the protocols.

Supplementary material

Table S1. Results of multivariate PERMANOVA analyses between Negative control and Acetone Control for experiments with *Crassostrea brasiliana and Mytella charruana* biomarkers, *Nitokra* sp. and *Tisbe biminienses* fecundity and multivariate structure nematode assemblages. Cont.: Controls; Exp.: Exposure Time. Values in bold indicate p<0,05.

Crassostrea brasiliana				
	df	**MS**	**Pseudo-F**	**P(perm)**
Controls	1	5,406	0,986	0,439
Exposure time	1	2,452	0,448	0,837
Tissue	1	22,722	4,147	**0,001**
Cont. x Exp.	1	4,286	0,782	0,566
Cont. x Tissue	1	1,331	0,243	0,972
Exp. x Tissue	1	3,434	0,627	0,739
Cont. x Exp. x Tissue	0	No test		
Residual	49	268,510	5,480	
Mytella charruana				
	df	***MS***	***Pseudo-F***	***P(perm)***
Controls	1	7,369	1,472	0,186
Exposure time	1	6,146	1,227	0,304
Tissue	1	50,948	10,174	**0,001**
Cont. x Exp.	1	4,367	0,872	0,488
Cont. x Tissue	1	2,008	0,401	0,839
Exp. x Tissue	1	6,620	1,322	0,273
Cont. x Exp. x Tissue	1	2,855	0,570	0,698
Residual	38	5,008		
Nitokra* sp. *fecundity				
	df	***MS***	***Pseudo-F***	***P(perm)***
Controls	1	0,001	0,471	0,487
Residual	8	0,002		
Tisbe biminienses fecundity				
	df	***MS***	***Pseudo-F***	***P(perm)***
Controls	1	0,000	3,739	0,197
Residual	4	0,000		
Nematode assemblages				
	df	***MS***	***Pseudo-F***	***P(perm)***
Controls	1	574,75	2,3987	0,115
Exposure Time	1	714,06	2,9802	**0,042**
Cont. x Exp.	1	553,79	2,3113	0,107
Residual	8	239,61		

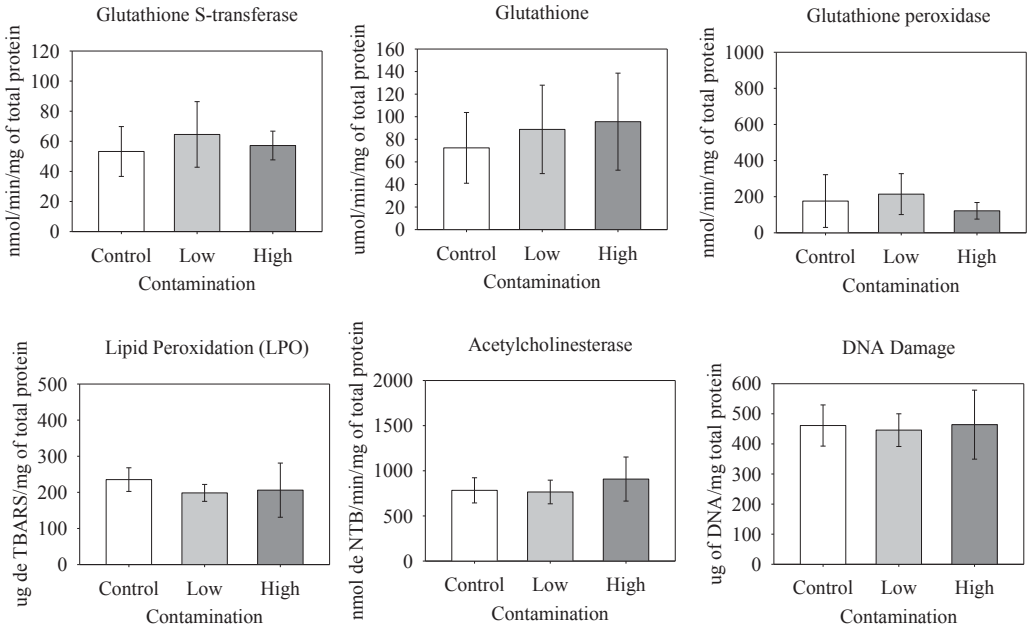

Figure S1. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the gill of *Crassostrea brasiliana* in 48 hours of exposure time.

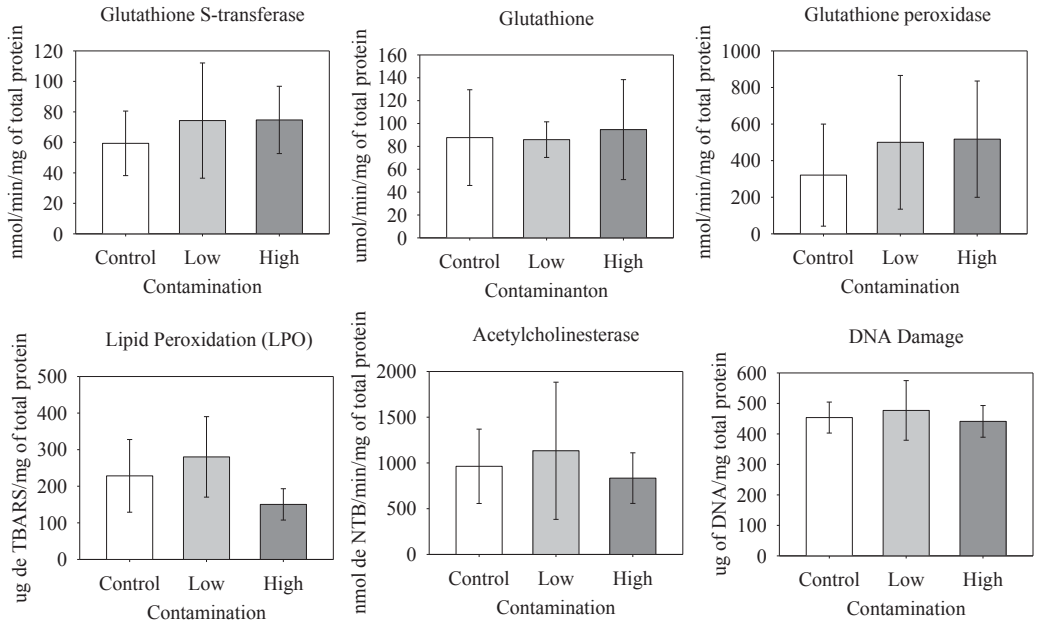

Figure S2. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the gill of *Crassostrea brasiliana* in 96 hours of exposure time.

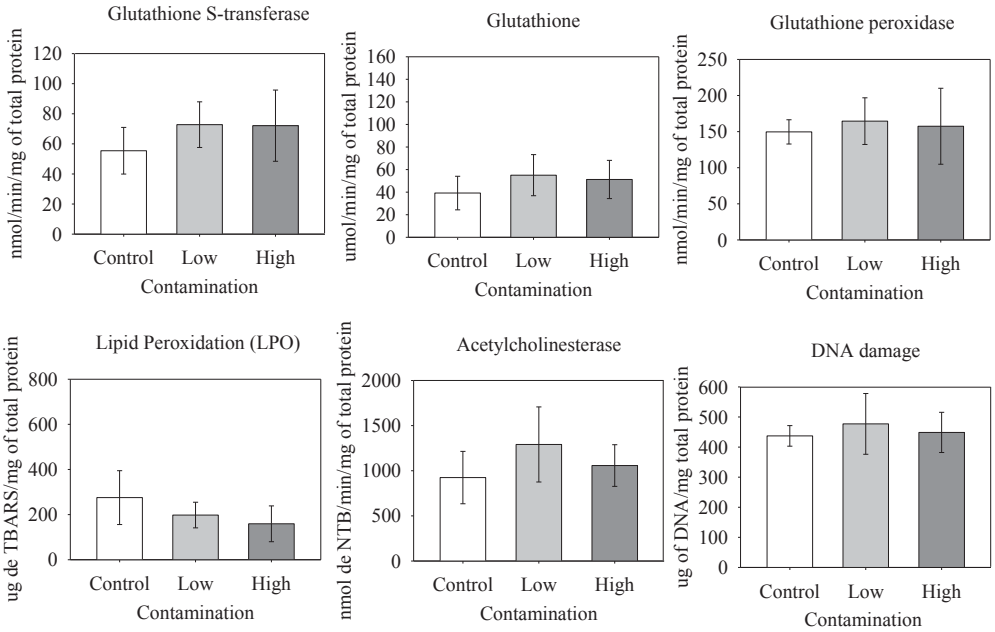

Figure S3. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the digestive gland of *Crassostrea brasiliana* in 48 hours of exposure time.

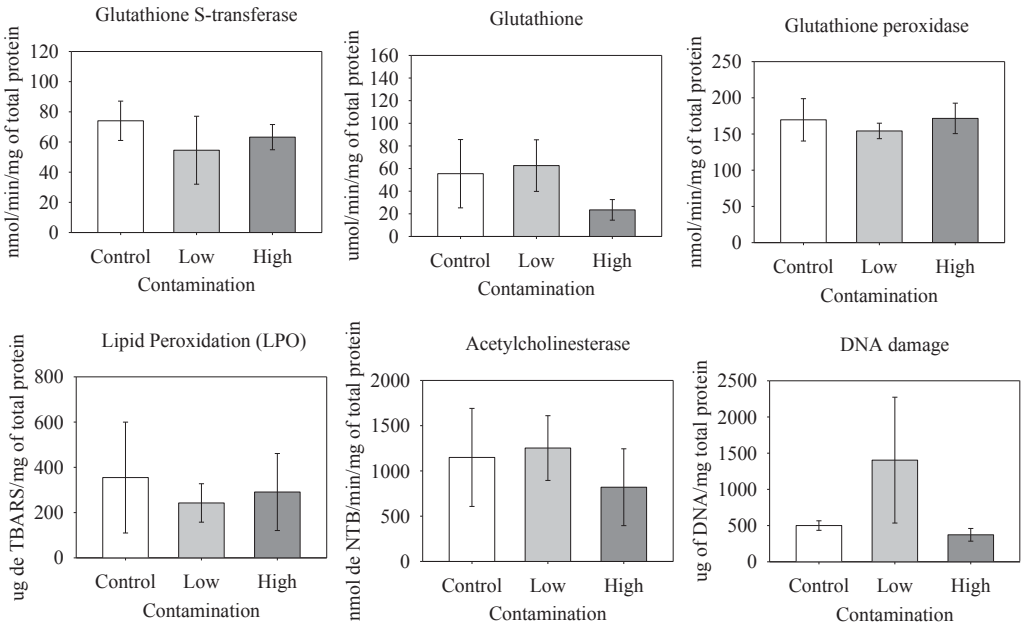

Figure S4. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the digestive gland of *Crassostrea brasiliana* in 96 hours of exposure time.

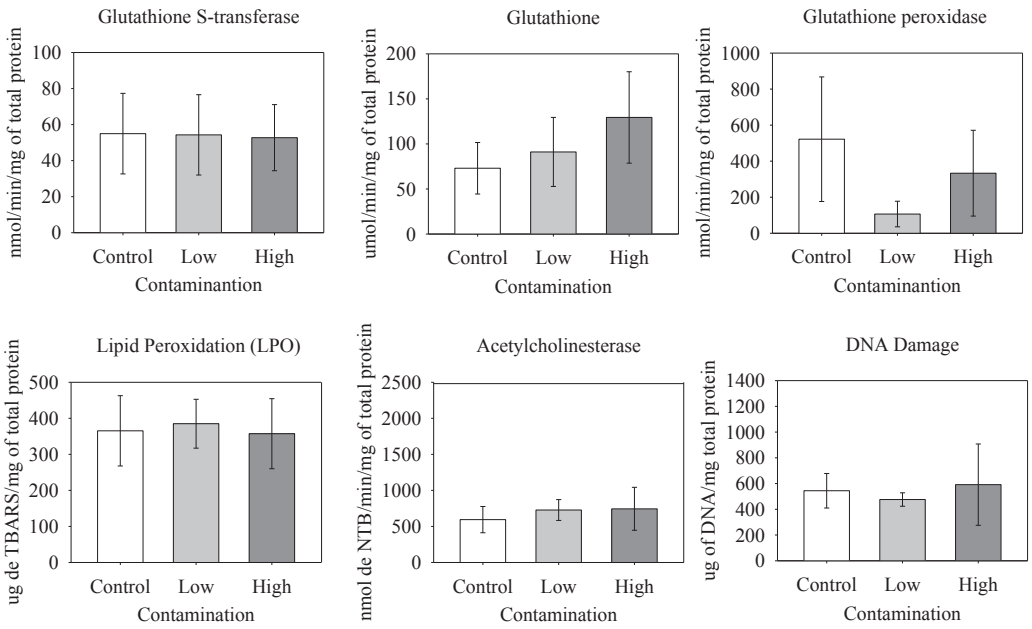

Figure S5. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the gill of *Mytella charruana* in 48 hours of exposure time.

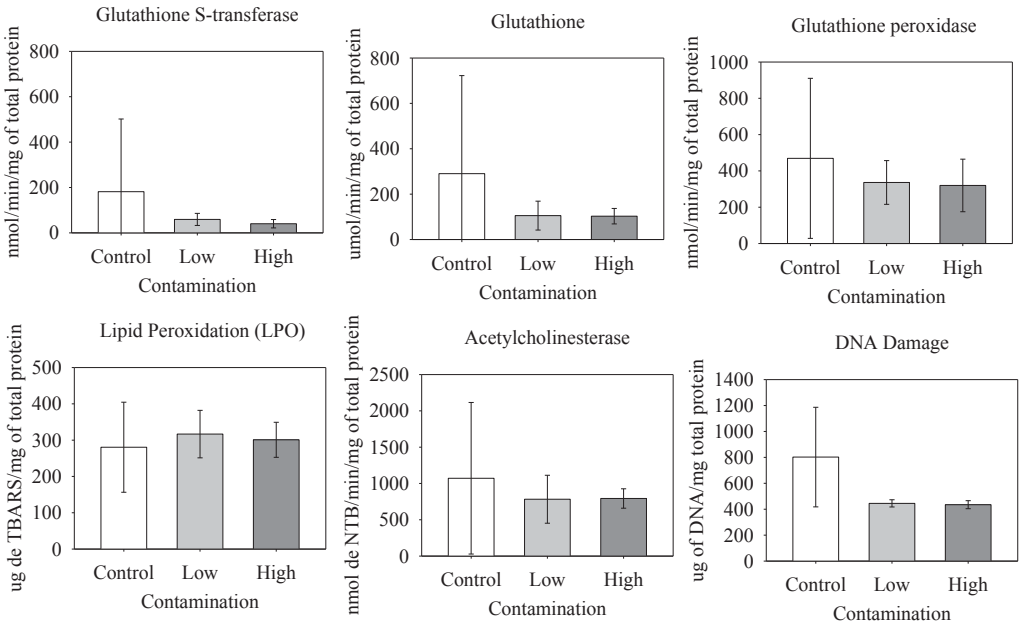

Figure S6. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the gill of *Mytella charruana* in 96hours of exposure time.

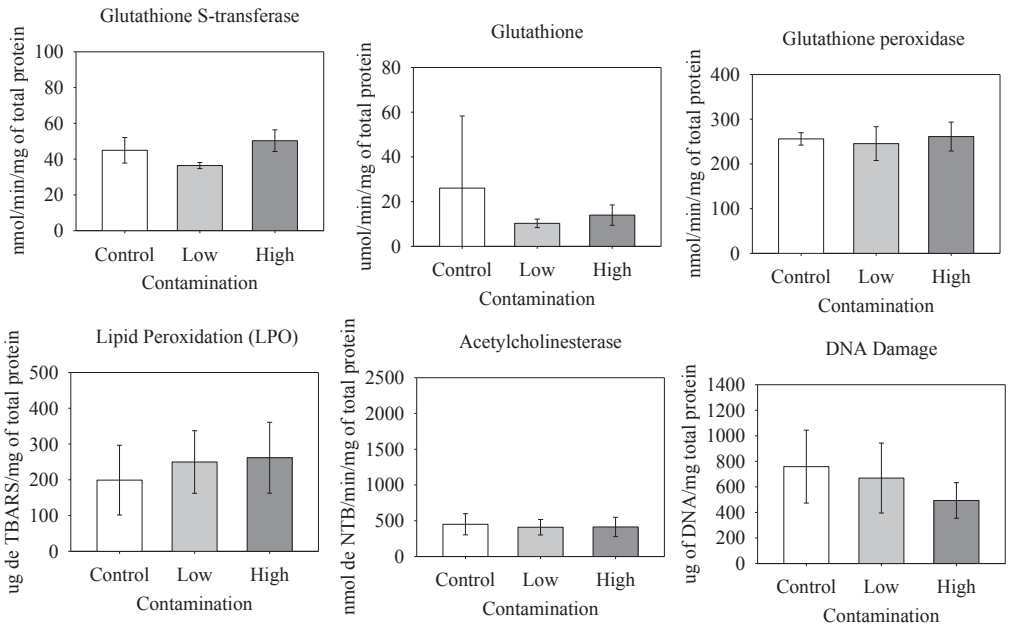

Figure S7. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the digestive gland of *Mytella charruana* in 48 hours of exposure time.

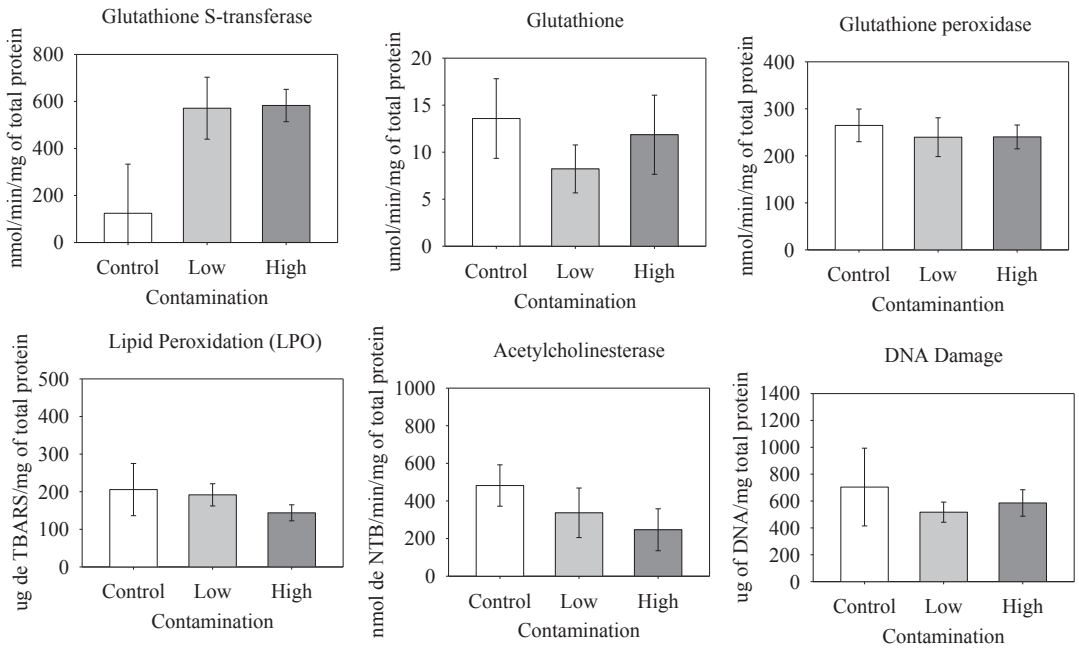

Figure S8. Mean and standard deviation of the activities of the biomarkers Glutathione S-transferase (GST), Glutathione (GSH), Glutathione peroxidase (GPX), Acetylcholinesterase (AChE), Lipid Peroxidation (LPO) and DNA damage in the digestive gland of *Mytella charruana* in 96 hours of exposure time.

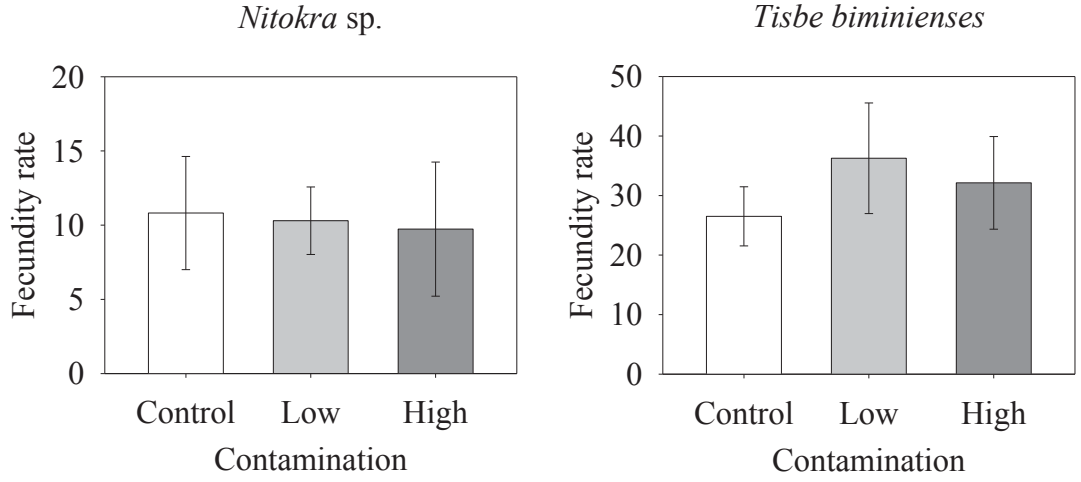

Figure S9. Mean and standard deviation of copepod *Nitokra* sp. and *Tisbe biminienses*

SYNTHESIS

The aim of this thesis was to understand how changes in the environmental settings and the use of multiple approach (using organisms with distinct ecological traits and endpoints encompassing different levels of biological organization) affect the ecological effects of an emergent contaminant, the biocide Irgarol, in estuarine sediments. Our results showed that oversimplification of the approaches used in the ecological effects of contaminants in ERA (step 2 in ERA) limits the prediction of real consequences to natural ecosystems and increases the uncertainty of ecological risks. As such, we call for an urgent update of ERA, given the lack of ecological realism of exposure and effect assessments, and considering the biological complexity of potentially exposed ecosystems as well.

The main findings of this thesis are as follows:

(1) Sedimentary organic carbon influences the effects of Irgarol on nematode assemblages, but pH does not

Environmental parameters, by affecting physical and chemical properties of the molecules, can interfere with the stability of contaminants in the matrix influencing the partitioning of chemical substances between different environmental compartments (Basallote et al., 2014; Bangkedphol et al., 2009). In the first part of Chapter 1, the differential amounts of organic carbon content of the sediments promoted clear differential responses of the fauna to Irgarol contamination. The responses from the different trophic groups supported the idea that this differential effect may have been mediated by the exposure route of organisms, and therefore was probably mediated by differential partitioning (and therefore bioavailability) of Irgarol in the water and sedimentary compartments. In the second part of this chapter, however, sediment pH had no significant influence on the effects of the same contaminant, Irgarol, on the nematode assemblage.

The observed influence of sediment organic carbon on Irgarol toxicity corroborates the expected idea that for apolar organic compounds (high Kow), the organic matter content of the sediment strongly influence bioavailability for aquatic organisms (Landrum et al., 1987; Peijnenburg et al., 1997). This is so because this class of contaminants are generally hydrophobic, so that they tend to partition with the organic phase of the sediment (Fuchsman and Barber, 2000). The parameter pH, on the other hand, is expected to influence bioavailability

by altering the characteristics of chemical compounds through ionization of the molecules (Pagliarani et al., 2013; Reijonen et al., 2016) but how these modifications alter partitioning and bioavailability of organic compounds is less clear. Ionized molecules are generally more water soluble but at the same time less lipophilic (therefore less permeable to cellular membranes) (Larini, 2009). Because variations in pH can affect both hydrophilicity and lipophilicity of organic compounds it potentially influences their bioavailability, so that I expected that Irgarol would alter bioavailability and therefore exhibit different effects on nematode species at the different pH tested. However, in contrast to the well-known influence of organic carbon content on organic hydrophobic compounds, the interaction of pH with this class of contaminant is less understood so that it was hard to hypothesize on the direction of pH influence on Irgarol bioavailability. The counteract action of pH in Irgarol water solubility and lipophilicity might explain why there was no net effect of this variation of pH on Irgarol toxicity, but it is also possible that the variation of pH studied here exerts no influence on Irgarol bioavailability. The contrasting results from parts I and II of Chapter 1, indicate that influence of environmental variables on bioavailability of contaminants will depend on the potential interactions between the particular environmental variable and the contaminant. As such, the understanding of the behavior of compounds and their interactions with the biota under different environmental settings is of ultimate importance in order to drive hypothesis oriented decisions in the first step of ERA ("Problem Formulation" *sensu* USEPA, 1998) where decisions about the tests to be conducted to characterize ecological effects are taken.

(2) Effects of Irgarol depends on organisms' ecological traits

In the first part of Chapter 1, the differential amount of organic carbon promoted responses of different functional groups. The group of deposit-feeders were mainly affected in sediments with higher organic carbon, suggesting the ingestion of contaminated food as the main route of contamination in this condition. At the same time, contaminated treatments in sediments with lower organic carbon content, showed lower abundances of the genus *Viscosia* and the group of predacious nematodes, which are characterized by a smooth and therefore more permeable cuticle, what suggests they might have been affected by an increased availability of Irgarol in the interstitial water in this treatment. These results indicate that the bioavailability of toxic substances in sediments is not only determined by their partitioning between the different phases of the sediment but also by the organism's trophic ecology,

challenging generally accepted notions, such as that equilibrium partitioning alone, can predict toxicity (Di Toro et al., 1991; Peijnenburg et al., 1997). Interestingly, in the experiment from part II of chapter 1, a decrease in the relative abundance of the nematode genera *Anoplostoma* and *Oncholaimus,* both with smooth cuticle, in treatments contaminated with Irgarol, corroborate the idea that a higher permeability of smooth cuticle when compared to more ornamented cuticles (Fonseca and Fehlauer-Ale, 2012), may support the uptake of pollutants from the sediment interstitial water. Similar results had been observed in previous experiments performed with nematode assemblages from a different study site (sandy sediments with low organic matter content), with different genus composition and exposed to different contaminants (Gallucci et al. 2015, Santos et al., 2018).

In Chapter 2, the bivalve species studied also differed in their biomarker responses to Irgarol exposure. Such differential responses to the same Irgarol concentrations, under the same environmental conditions, might also be explained by the different ecologies of the organisms. The mussel *M. charruana* showed clearer responses to the exposure to Irgarol when compared to the oyster *C. brasiliana*. Ryan (2002) reported that the contamination of molluscs occurs mainly from the capture of food resources present in the interstitial water than from the ingestion of sedimentary particles. So, probably, the main route of exposure is through the capture of food resources through the filtration of small particles present in the water (Lees, 2000). Unlike *C. brasiliana*, *M. charruana* has a burial habit and, in fact, during the exposure the organisms buried themselves. As the contamination of Irgarol came from the sediment, *M. charruana* may have been more exposed to particulate matter and contaminated interstitial water than the oyster, which throughout the exposure remained on the sediment layer.

(3) Single-species individual level assays are less sensitive to contamination than endpoints at lower and higher level of biological organization

In the present study, the results of ecotoxicological tests at the individual level (i.e. copepod fecundity) were less sensitive than biomarker and community-level approaches, since Irgarol had no effect on this endpoint. Fecundity rate tests with both copepod species used here, *Nitokra sp*. and *Tisbe biminiensis*, have already shown to be sensitive to various contaminants (Araújo-Castro et al., 2009; Camargo et al., 2015; Campos et al., 2019; Ihara et al., 2010). However, Santos et al. (2018) showed that *Nitokra* sp fecundity assay can be less sensitive to subtle pollution gradients, when compared to meiofauna community exposure in microcosms. One cannot discern whereas the lack of sensitivity in this case was due to the organism-test

used or the particular endpoint investigated, but it clearly demonstrates that standard single-species tests that contemplate only the level of individual underestimate the effects both at the sub-individual level and at the community level for environment concentrations.

(4) Different approaches display different characterizations of ecological effects

Ecotoxicology traditionally relies on the reductionist paradigm of science for knowledge production, which can be inferred from studies being mostly based on a single species exposed to ideal laboratory conditions, where only one condition is manipulated at time. Such approach is important to understand mechanistically a phenomenon but lacks on the realism of the complex nature of the pollution phenomena. As stated by the Science Advisory Board of USEPA's Environmental Futures Committee (USEPA, 1995), "(US)EPA will be unable to respond quickly and effectively to what are likely to be complex, synergistic problems if it continues to use a one-at-time, single-stressor, single-species, single-medium, single-endpoint approach". In Chapter 2, Irgarol exposure had significant effects in the biomarker endpoints, but sensibility was different between the two bivalve species. Also, Irgarol affected different biomarkers in each species and exposure time. Individual level endpoints of both harpacticoid copepod species were not affected by contamination. Finally, it caused changes in the structure of nematode assemblages that included both decrease in abundances of one genus (*Oncholaimus*), which suggests mortality or other sublethal effects, as well as increases in abundances of other species (*Desmodora*), which may not be a direct effect of Irgarol but rather may results from indirect effects. The different ecotoxicological tools used here different sensitivities and complemented each other, since the biochemical responses are mechanistic, showing direct effect relationships caused by exposure to the stressor, but have less relation with ecological endpoints. The responses of the microcosm assay include different species subjected to indirect relationships with each other, not allowing to discriminate what would be direct or indirect effects of the stressor, but which produce ecologically relevant information, such as structural changes in the community. These results illustrate the need for a multiple approach ecological risk assessment in order to reduce the uncertainties and inaccuracies in the determination of risks associated with polluted sediments in complex systems.

FINAL CONSIDERATIONS

The results presented in this thesis provide substantial evidence that the interplay between ‘‘environmental availability’’, ‘‘environmental bioavailability’’ and ‘‘toxicological bioavailability’’ (*sensu* Peijnenburg et al. 1997) will ultimately determine ecological effects of

contaminants in living organisms. For instance, the relevance of environmental variables to bioavailability depends on how they affect the physical and chemical properties of the organic contaminant (‘‘environmental availability’’). Molecules which are highly hydrophobic, for instance, will be more influenced by organic content than by pH, as observed in Chapter 1 (parts I and II). In this case, the influence of organic content will also depend on organisms’ exposure route (‘‘environmental bioavailability”), as suggested by results from Chapters 1 (Part I) and 2. Finally, the ‘‘toxicological bioavailability’’ is related with biological effects. That refers to paths taken by the chemical following the uptake across a physiological membrane, (which can occur externally or internally to an organisms as, for example, in the lumen of the gut in the case of ingestion of contaminated food), for example, metabolic processing or exerting a toxic effect within a particular tissue.

One way of improving complexity and ecological realism in ERA, is by increasing our understanding of these processes and the interplay between them. For instance, by understanding the behavior of a particular contaminant under different environmental scenarios (“environmental availability”) and how its “environmental bioavailability” may be mediated by organisms ecological traits as well as its possible modes of action once inside a living organism (“toxicological bioavailability”), it is possible to build up full conceptual models including all these steps in order to predict its toxic effects to different organisms under different circumstances. Such conceptual models may therefore generate important subsidies for ERA's step 1, supporting the development of well-founded risk hypothesis to guide the determination of goals and endpoints in a less arbitrary way as usually done by the protocols. As such, the main findings from this thesis may give support to the design of future ERA frameworks concerning contamination of estuarine sediments. By developing such conceptual models, one can eventually predict even emergent responses of the systems, which results from interactions between the components of the systems and the processes involved and are usually highly unpredictable.

The use of similar approaches to the ones employed here may be further adopted in order to quantify the relationships involved in the bioavailability processes described above, offering subsidizes to the development and future calibration of models that, based on hypothesis-driven ecotoxicological data, take into consideration how changes in important parameters (e.g. organic carbon content, main exposure route, species interactions) may affect the outcome of the tests, therefore including complexity and ecological realism in the step of risk characterization, decreasing uncertainty in ERA.

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