Marina Ferreira Mourão Santana

Effects of microplastics contamination on marine biota

Dissertação apresentada ao Instituto Oceanográfico da Universidade de São Paulo, como parte dos requisitos para obtenção do título de Mestre em Ciências, Programa de Oceanografia, área de Oceanografia Biológica

Orientador: Prof. Dr. Alexander Turra

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Universidade de São Paulo
Instituto Oceanográfico

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SUMARY

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Agradecimentos

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Resumo

Os microplásticos (< 5mm) são um dos impactos mais difundidos da sociedade moderna. Aqui, eles foram estudados em ensaios experimentais, considerando diferentes composições de exposição de mexilhões à micro-PVCs. O objetivo foi investigar: sinais fisiológicos de estresse sob exposições aguda e crônica; e transferência, assimilação e retenção de microplásticos em cadeias tróficas. Para avaliar seus potenciais riscos na naturaza, a ingestão por mexilhões também foi investigada no Estuário de Santos. As exposições agudas afetaram a fisiologia dos mexilhões, sendo influenciadas pelo tempo e concentração de exposição, e pela presença de aditivos plásticos. Interações entre esses fatores (tempo, concentração e aditivos) foram mais relevantes do que eles individualmente, sugerindo a singularidade dos cenários de poluição. A exposição de longo prazo não afetou os mexilhões, indicando a influência do tempo na aclimatação ao microplástico. O PVC não foi assimilado e retido nas cadeias tróficas, mas biotransferido do tecido das presas para o trato dos predadores, mostrando a influência do estado da presa na efetividade da biotransferência dos microplásticos. Dentre os mexilhões coletados, 75% estavam contaminados, revelando uma importante questão socioambiental. Esse trabalho ilustrou a complexidade dos impactos dos microplásticos para a biota marinha, ressaltando a necessidade de mais estudos sobre seus riscos.

Key-words: lixo marinho; microplásticos; bivalves; ingestão; efetio; transferência de nível trófico; poluição do ambiente; segurança alimentar.

Abstract

Microplastic pollution (particles < 5mm) is one of the most widespread impacts from modern society. Here, microplastic impacts were investigated through experimental assessments considering different exposure scenarios using mussels and micro-PVC as models. These aimed to investigate mussels' physiological signs of stress under acute and chronic exposures and microplastics transference, assimilation and retention along food chains. In acute exposures, PVC intake affected mussels' physiology over time, also influenced by plastics additives and particle concentration. Interactions among exposure factors (time, presence of additives and concentration) were more relevant than their individual effect, indicating the singularity of each contamination scenario. Long-term contact did not affect mussels, indicating the influence of time to acclimation. Microplastics were not assimilated and retained along food chains, but only biotransferred from prey tissues to predators' tract, showing the influence of prey contamination on the effectiveness of microplastics biotransference. To evaluate risks in nature, microplastic ingestion was investigated in mussels from the Santos Estuary. Santos Estuary contained microplastics in 75% of sampled mussels, an issue of environmental and human concern. This study illustrated that microplastics impacts on mussels vary with microplastics characteristics, exposure scenario and species vulnerability, highlighting the need for more toxicological and risk evaluation studies.

Key-words: marine litter; microplastic; bivalve; intake; effect; food-chain transfer; *in situ* contamination; food safety.

Introduction

Coastal and estuarine regions are common fates of urban and industrial wastes, often resulting on environmental and biological contamination (Maia et al., 2006). Among them, the so-called marine litter consists of solid and persistent materials that have been made, used and then lost or abandoned by humans into oceans (Galgani et al., 2010). Since the 1970s, the presence of these solid wastes in marine environments is recognized as one of the major contributors to marine pollution (Santos et al., 2008) and was considered in 1978 as one of the five biggest problems for ocean health by the Protocol of the International Convention for the Prevention of Pollution from Ships (MARPOL – Santos et al., 2008).

Of all materials making up this marine debris, plastics stand out. In 2010, global plastic production reached 265 million tons (ABIPLAST, 2012). As a reflection of such wide production and utilization, along with persistence and floatability, it became the most prevalent pollutant in coastal areas (Graham and Thompson, 2009), reaching remote places with potential to last hundreds of years (Derraik, 2002). Microplastics (plastics < 5mm, Arthur et al, 2009) are a special and worrying component of plastic debris, recently recognized as a threat to marine ecosystems. The source of this pollutant can be either industrially produced already microscopic in size (abrasives beads for cosmetics products, plastic pellets and PVC powder, called "primary microplastics" – Fendall and Sewell, 2009; Andrady et al., 2011) or result from the degradation of larger plastics (named "secondary microplastics" – Browne et al., 2007; Andrady et al., 2011; Zettler et al., 2013). Both reach the ocean due to high consumption and inadequate disposal of plastics products. The quantity of microplastics in oceans corresponds to 92.4% of total marine plastic particles (Eriksen et al., 2014), tending to increase due to the breakdown of macro-particles and recurrent input of new plastic waste (Barnes et al., 2009).

Microplastics are composed of several polymers and plastic additives (EPA, 1992; Teuten et al., 2009). Both, polymers and additives are known to have toxic properties for the environment and to endanger marine biota (EPA, 1992; Ananthaswamy, 2001; Mato et al., 2001; Lithner et al., 2011). Recently, microplastics started to draw the attention of scientists because of their small size, high abundance

and potential biological effects (Moore et al., 2006; Wright et al., 2013a, Turra et al., 2014). As plastics become smaller, larger are their: (i) organic surface areas, prone to adsorption and adherence of other toxic substances and microorganisms (Mato et al., 2001; Browne et al., 2013; Tanaka et al., 2013; Zettler et al., 2013; McCormick et al., 2014); (ii) bioavailability (Von Moos et al., 2008; Santana, 2012); and (iii) interactions with organisms, either by the increased retention in the digestive tract (Santana, 2012), or by the potential increase of cell interactions (Syberbeg et al., 2015). In addition, the problems associated with microplastics marine pollution tend to increase due to high consumption, bad management and the durability of plastics. International organizations (e.g. UNESCO, UNEP and NOAA) have a growing concern with this issue, emphasizing the need to devote more efforts to related research. To illustrate, GESAMP[1] created a working group in 2010 to discuss sources, fates and effects of microplastics in the marine environment. In 2011, they initiated a program of four years of research focusing on this micro-waste, encouraging and prioritizing studies regarding input rates, fragmentation and degradation in the seas, ingestion by marine organisms and impacts on biota.

Microplastics are potentially bioavailable for a wide range of marine animals. Their uptake has been observed in pelagic and benthic species with different feeding strategies and trophic levels (Tourinho et al., 2010; Murray and Cowie, 2011; Lusher et al., 2013; Cole et al., 2013). Once ingested, these particles can be retained and obstruct the digestive tract (Derraik, 2002; Besseling et al., 2013), be assimilated and translocated into tissues (Browne et al., 2008; Von Moos et al., 2012; Farrel and Nelson, 2013) and act as a vector for other toxic substances to the food chain (Mato et al., 2001; Teuten et al., 2009; Browne et al., 2013; Farrel and Nelson, 2013). As a result of such interactions, a series of biological effects may occur, from physical harm (Besseling et al., 2013) to physiological, cellular and molecular stresses (Von Moos et al., 2012; Browne et al., 2013; Cole et al., 2013; Avio et al., 2015).

Microplastics pollution, however, is extremely variable; different exposure

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[1] GESAMP (or the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection) is a group of specialized experts nominated by Sponsoring Agencies (IMO, FAO, UNESCO-IOC, UNIDO, WMO, IAEA, UN, UNEP), whose task is to provide scientific advice concerning prevention, reduction and control of marine environment degradation to the Sponsoring Agencies (GESAMP, 2010).

scenarios may lead to different biological responses. Besides the wide range of polymers and additives that microplastics can be made of, the concentration and residence time of microplastics also vary locally, influencing their interactions with organisms. Differences among plastic types and additives alter microplastics toxicity (Lithner et al., 2011), while concentration and time of exposure influence microplastics' intake, assimilation (Santana, 2012) as well as their possible physiological effects (indicated for other pollutants/stressors – Moreira, 2011). In the environment, microplastics input can be constant (i.e. from sewage disposal, Browne et al., 2011) or episodic (i.e. ship load or industry loss) and can range in magnitude. Natural marine processes such as hydrodynamics and biofouling contribute to microplastic fragmentation, dispersion, and sinking (Andrady, 2011; Cózar et al., 2014; Eriksen et al., 2014), therefore influencing the concentration and period of exposure. This suggests that further studies are still necessary to explore variations and combinations of exposure features (e.g. type of microplastics, concentration and period of exposure), improving risk assessments of microplastics pollution.

Transference through food chains is another risk to marine ecosystems resulting from microplastics intake. Recent studies indicate that organisms with microplastics in their gut can transfer these particles to the next trophic level after being consumed as prey (Farrel and Nelson, 2013; Setäla et al., 2014). From this biotransference, microplastics ingestion may reach organisms that would not ingest plastics directly from the surrounding environment. Moreover, it raises the hypothesis of bioaccumulation through food intake and consequent magnification along food webs, possibly increasing the number of species affected by microplastics impacts. Because field studies found commercially important organisms with ingested microplastics (Lusher et al., 2011; Foekma et al., 2013; Van Cauwenberghe and Jassen, 2014; Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015), further studies could explore microplastics interactions within food webs in which humans participate as an additional issue for risks to fisheries and aquaculture sectors.

Mussels are a good example of organisms with both natural and cultural relevance, susceptible to microplastics intake and further consequences. As with other marine bivalves, these are animals from near the base of the food chain, important as a food source for many organisms including humans. In natural environments, the contamination of blue mussels (*Mytilus edulis*) with microplastics was reported from

polluted and unpolluted coastal regions (Van Cauwenberghe and Jassen, 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015). Even mussels sampled in farm systems and in supermarkets were observed with microplastics (Van Cauwenberghe and Jassen, 2014; Mathalon and Hill, 2014), suggesting the oncoming risks of such interaction. Under laboratory conditions, three species of mussels were shown to ingest microplastics (*M. edulis* – Browne et al., 2008; *Perna perna* – Santana, 2012; and *Mytilus galloprovinciallis* – Avio et al., 2015) and, as a result, several impacts could be observed. Micro-particles of Polyvinylchloride (PVC), for example, persisted in the digestive tract of brown mussels for 12 days after a single exposure (Santana, 2012). Along with PVC, polystyrene (PS) and polyethylene (PE) were assimilated into mussels' tissues (Browne et al., 2008; Von Moos et al. 2012; Santana, 2012), raising the chances of cellular and molecular interactions. Tissue changes, lysosomal instability, inflammatory processes and oxidative stress were already identified in *M. edulis* and *M. galloprovinciallis* that ingested and assimilated PE and PP microparticles (Von Moos et al., 2012; Avio et al., 2015); while microplastics transference from contaminated *M. edulis* to predators were also observed (Farrel and Nelson, 2013; Watts et al., 2014).

The brown mussel *P. perna* is abundant and widely distributed from the Southeastern to the Southern coast of Brazil (Fernandes et al., 2008). As a food resource, this species is commonly collected from natural beds (harvesting) or cultured in systems within the marine environment. Therefore, the microplastics impact on this mussel are relevant not only to the evaluation of ecosystem health but also to human food safety. A previous study indicated that PVC can be ingested, retained and assimilated by *P. perna* (Santana, 2012), so further studies should explore possible related consequences. Polyvinylchloride (PVC) is the third most consumed thermoplastic in the world (ABIPLAST, 2012) and a common example of primary microplastic found in marine environments (Andrady, 2011; Wright et al., 2013a). In Brazil, the consumption of PVC reached 933,240 tons in 2008 (ABIPLAST, 2012), but nothing is known about its presence in nature. The main mode of transport of PVC for further manufacturing within Brazil is maritime shipping along the coastline, making its loss to the marine environment a potential risk.

In this context, this study aimed to further assess biological impacts and environmental risks arising from microplastics' intake by marine biota. Using *P. perna*

and PVC as biological and microplastics models, respectively, we explored (i) possible biological effects under acute (Chapter 1) and chronic (Chapter 2) scenarios of exposure; (ii) biotransference, assimilation and retention along trophic webs (Chapter 3); and (iii) microplastic contamination in nature (Chapter 4). We hypothesized, for short-term exposure, that PVC exposure and intake would induce biological effects in *P. perna* and that the factors related to possible scenarios of contamination (leaching of particles, microplastics' concentration and the period of exposure) would influence mussels' biological responses. In a long-term exposure, we hypothesized that PVC exposure and intake would induce biological effects in *P. perna*. We expected that Polyvinylchloride (PVC) could be transferred, assimilated and retained through food intake under a more realistic experimental contamination scenario. Finally, we supposed that the ingestion of microplastics by brown mussels is already occurring in natural environments potentially subjected to plastics inputs on the Brazilian coast. It is expected that the experimental evidence and discussions presented throughout this document could improve the knowledge of the effects of microplastics on marine biota and be used in risk assessments about microplastics marine pollution, as well as to draw the attention of managers, industry and policy makers on this issue of increasing importance, both locally and globally.

Chapter 1

Contamination features influence the effects of microplastics of the mussel *Perna perna* (Bivalvia, Mytilidae)

Introduction

Despite the benefits that plastic brings to society, its high use and poor management is concerning (Thompson et al., 2009). Plastic debris constitute more than a half of marine litter (Moore et al., 2008; Barnes et al., 2009), and the quantity tends to increase with time, influenced by high consumption, irregular disposal and loss. Their slow degradation (Gregory and Andrady, 2003) and easy dispersal under marine conditions make them widespread (Barnes et al., 2009), increasing the risks for marine environments.

Microplastics (<5mm, Arthur et al., 2009) are a part of the marine contamination caused by plastics that was unknown until recently (Barnes et al., 2009). They originate from the fragmentation of large particles present in the environment, through UV radiation and wave action (*post-consumption microplastics*), or from the loss of industrial raw materials, such as plastic pellets and microbeads for cosmetics and other abrasives (*pre-consumption microplastics*). Microplastics are composed of monomers and plastic additives (EPA, 1992; Teuten et al., 2009), both known to have toxic properties to the environment and endanger marine biota (EPA, 1992; Ananthaswamy, 2001; Mato et al., 2001; Lithner et al., 2011). Microplastic pollution started to draw the attention of scientists because of their small size, high abundance and potential biological effects (Moore et al., 2006; Wright et al., 2013a, Turra et al., 2014). However, such emergent environmental issue still carries uncertainties (GESAMP, 2015).

Indeed, the small size of microplastics make them potentially bioavailable for a wide range of marine species, including invertebrates and other animals with indiscriminate feeding habits that capture anything similar in size to their natural food (Moore et al., 2008; Browne et al., 2008; Graham and Thompson, 2009; Wright et al.,

2013a,b; Cole et al., 2013). Among organisms that possibly ingest microplastics, there are commercially important species already observed to be contaminated in the field (Lusher et al., 2011; Foekma et al., 2013; Van Cauwenberghe and Jassen, 2014; Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015). Therefore, understanding the consequences of microplastics intake by marine organisms can be a good path to understanding how microplastics marine pollution can affect human. Once ingested by marine organisms, these particles can (i) be retained and obstruct the digestive tract (Derraik, 2002; Besseling et al., 2013); (ii) be assimilated and translocated into tissues (Browne et al., 2008; Von Moos et al., 2012; Farrel and Nelson, 2013) and (iii) act as a vector for other toxic substances to the food chain (Mato et al., 2001; Teuten et al., 2009; Browne et al., 2013; Farrel and Nelson, 2013). Thus, microplastic intake can result in a series of biological effects, from physical harm (Besseling et al., 2013) to physiological, cellular and molecular stresses (Von Moos et al., 2012; Browne et al., 2013; Cole et al., 2013; Avio et al., 2015). From these impacts on individual levels, further environmental changes can occur. To illustrate, factors of stress can influence the growth rate and reproduction of an organism (Resgalla Jr. et al., 2007). This may influence the abundance and distribution of its species in a specific locale, which, in turn, can affect the state of this environment.

A range of recent studies showed examples of microplastics intake and biological impacts. High-density polyethylene (HDPE) at 2.5g/L, with an 80µm diameter and irregular shape, was shown to be ingested by the filter-feeding bivalve *Mytilus edulis* and to cause inflammatory responses over time (Von Moos et al., 2012). This species also ingested and assimilated micro-polystyrene (PS) with 3 and 9.6µm diameters, but without any influence on cell viability when exposed to 0.5g/L for 3 h(Browne et al., 2008). The ingestion of polyvinyl chloride (PVC) by the deposit-feeder annelid *Arenicola marina* led to the transfer of chemical additives (Triclosan and PBDE-47) and organic pollutants (nonylphenol and phenanthrene) to their gut tissues, reducing coelomocyte activity and increasing oxidative stress (Browne et al., 2013). *Arenicola marina* exposed to PS microparticles (400-1300µm diameter) for 28 days also showed a positive relationship between plastic concentrations and its intake, reducing feeding activity and weight (Besseling et al., 2013). A reduction in feeding activity was also recorded in copepods *Centropages typicus* exposed to PS particles of about 7.3µm diameter for 24 h (Cole et al., 2013).

The differences among plastic characteristics (e.g. size, type and presence of additives, or their toxic properties) and experimental exposure (as concentration and period) created complex scenarios of pollution that can have led to variation in the biological responses. This suggests that they are important characteristics to consider when addressing microplastics' impacts and risks for marine environments, which can be examined by including different exposure factors and their interactions into experimental studies. To improve risk assessments of microplastics pollution, there should be a better understanding of their basic hazardous properties and more studies on more complex scenarios, closer to real environmental situations (Syberg et al. 2015). Although we logically suppose that higher microplastics' concentration or time of exposure generate greater biological impacts, in a real scenario of marine pollution we do not know whether the interaction of these factors is causing a combined effect different from predicted. Integrating these characteristics and predicting individual responses is not a simple task and should be further explored in laboratory assays.

Here, we investigated microplastics impacts on marine biota under different scenarios of exposure (treatments) through *Perna pena* exposed to micro-PVC. We hypothesized that (*h1*) exposure and intake of microplastics cause biological effects and that (*h2)* factors related to contamination scenarios (leaching of particles, microplastics' concentration and period of exposure) influence the responses. The cultivated brown mussel *P. perna* was exposed to micro-PVC provided by a polymer manufacturer. The effects were investigated using six biomarkers commonly used in ecotoxicological studies: lysosomal stability (i), lipid peroxidation (ii), DNA damage (iii) and the stress proteins Mitogen Activated Protein Kinase (pP38-MAPK) (iv), Allograft Inflammatory Factor-1 (AIF-1) (v) and Heat Shock Protein 70-kDa (HSP70) (vi). These were used to identify different biological receptors (cellular and molecular) possibly affected by microplastics on mussels. That was the first time that stress proteins were used for studying microplastics impacts, helping to expand our current state of knowledge.

Methods

Model organism

Brown mussels *P. perna* (Linnaeus, 1758) are sedentary filter feeders, abundant and well distributed along the Atlantic coast (Fernandes et al., 2008). As other bivalves, this species has commercial value, being widely extracted from the world and cultivated for human consumption (Fernandes, 2008). In Brazil, for example, this is the main mollusc cultivated, accounting for 79.5% of total mollusc production in 2004 (Boscardin, 2008). In addition to the environmental and human importance aspects of *P. perna*, preliminary experiments showed that they can ingest and assimilate micro-PVC particles (unpublished data).

This species is well accepted as a biological model for experimental studies mostly because of its high tolerance to environmental changes, easy access for collecting, and known biology (Ferreira and Magalhães, 2010). It is also a good bioindicator, with their gills, digestive gland and hemolymph as well-known targets for xenobiotics, frequently used for analysis of bioaccumulation and effects of pollutants (Najimi et al., 1997; Ferreira et al., 2004; De Almeida et al., 2007; Pereira et al., 2011).

Mussels (± 6.0 cm) were purchased from a cultivation system at Lagoinha Beach (Ubatuba, São Paulo), cleaned of epifauna and acclimatized for 5 days in a maintenance tank (1000L). They were kept in an open seawater circulation system and under natural abiotic conditions (21˚C, 35, and pH 8.0). Feeding other than that in the circulation was not provided.

Model microplastic

PVC was chosen due to its widespread use, small size and maritime transport while a raw product (Rodolfo et al., 2006). It is the third largest plastic type produced (Lithner et al., 2011) and the one that contains the most associated chemical additives, such as phthalates that can reach up to 50% of PVC weight (Oehlmann et al., 2009). Thus, it is reasonable to assume that the presence of PVC in the marine environment, either by its direct loss as raw material or fragments from larger items, can pose potential risks for marine fauna.

The type of PVC chosen for the assays, Emulsion/Microsuspension PVC, has spherical shape ranging from 0.1 to 1.0µm in diameter, and a tendency to agglomerate in structures of 40 to 50µm (Rodolfo et al., 2006). Thus, the plastic model ranges from nano to micro-sizes, increasing the risks of bioavailability and potential effects (i.e. cellular interactions as observed for nanoparticles, Syberg et al., 2015). These particles are also non-porous, which raises their bulk density (Rodolfo et al., 2006) and facilitate their distribution throughout the water column, influencing on their bioavailability. The PVC was obtained from a polymer manufacturer (Braskem), which did not provide specific details about its chemical composition and additives.

Experimental Setup

Using the mussel *P. perna* and E/M PVC, this study evaluated microplastics' biological effects considering possible variations using the treatments "leaching" (virgin and leached), "concentration" (0.5 and 2.5g/L) and "period of exposure" (6, 12, 24, 48, 96 and 144h).

The factor "leaching" was used in order to understand the influence of microplastics' residence-time in seawater. For this, it included leached (washed) and virgin microplastics (used as they were provided by the industry). Leached ones simulated a scenario in which virgin particles are lost to marine environment and undergo a degradation (washing) process, resulting in the release of constituent material (i.e. plasticizers and other additives) before being ingested (Carmignani and Bennett, 1976; Sajiki and Yonekubo, 2003). This factor presupposes that mussels can contact microplastics recently lost but already transformed by the environment. Thus the concentration of chemical additives (e.g. phthalates) in microplastics could be reduced before they are ingested, decreasing the effects of these compounds, therefore allowing us to observe reactions related to the polymer itself.

To obtain the leached microplastics, particles of virgin PVC were washed in aerated and turbulent seawater for 20 days, a period determined by analysing experimental leached water for phthalate concentration. Because we did not have specific details about the chemical composition of the donated PVC, phthalates were chosen for being the group of additives most widely used by PVC industry (Rodolfo et al., 2006). To stipulate the time of leaching, 2.5g/L of PVC remained in turbulent

seawater for 30 days. Samples from the leaching water were collected every 10 days to be analyzed by gas chromatography/mass spectrometry (GC/MS. 7890/5975, Agilent). *CEIMIC Análises Ambientais Ltda* performed the analysis using the USEPA method 8270 for semivolatile organic compounds. The peak of leaching was identified at the 20th day of rinsing (e.g. 11μg/L of Di-n-butyl Phthalate at the 20th day of rinsing vs. 4.5 and 0.7μg/L at the 10th and 30th days, respectively). This might be related to a decreasing concentration of phthalates within PVC particles along with its rapid degradation in seawater. In marine environments, such degradation is mostly done by microorganisms (Staples et al., 1997). For Di-n-butyl phthalate, for example, degradation reaches 90% in 7 days (Jianlong, 1996). Thus, between the 20th and the 30th day of leaching, PVC particles presumably had low concentrations of phthalates to desorb from their surface, while the phthalates previously identified in seawater were already degraded.

The concentrations of microplastics in tests were chosen based on previous works related to microplastics uptake and effects on bivalves (Browne et al., 2008; Von Moos et al., 2012). Although out of proportion to the current estimates of microplastics present in marine environments (35,540 tons in total in the world's oceans; Eriksen et al., 2014), and almost equivalent to the annual Brazilian input of plastic waste into oceans (70,000 to 190,000 tons; Jambeck et al., 2015), the use of these concentrations allowed us to compare our results with already published data (Browne et al., 2008; Von Moos et al., 2012). Moreover, they inserted our work within a possible context of acute exposures (e.g. accidental loss of a shipping load) or in future scenarios of contamination, since microplastics marine pollution has unavoidable risks of increase. The periods of exposure were chosen to simulate short-term process of acute contamination combined with the time necessary for analysing each biomarker responses as explained later.

For testing the hypothesis *h1*, mussels were arranged in a block design, in which 15 mussels placed in 3 replicates were used in each exposure scenario, each using 12L aquaria with 5 mussels randomly collected from the acclimatizing tank (Figure 1). Apart from controls, all blocks received the "leaching" and the "concentration" treatments in an orthogonal design. This basic setup was repeated for every exposure time. Control groups were also arranged with n = 5 mussels in three aquaria, but their

seawater did not contain microplastics. Thus, a total of 5 blocks per period of exposure were arranged, 4 with scenarios of PVC contamination and one control.

The exposure experiments were conducted at a constant water temperature of 21°C and a 12h light-dark photoperiod, but without water exchange and feeding. At the end of the exposure periods, mussels were collected, shell length (average 6.0 ± 0.6cm for all organisms used), height (2.9 ± 1.6cm) and width (2.1 ± 1.5cm) were measured and the soft body (i.e. edible parts) was weighed (7.5 ± 2.4g). Samples of hemolymph were used still fresh, while digestive gland and gills were stored at -80˚C for further analysis.

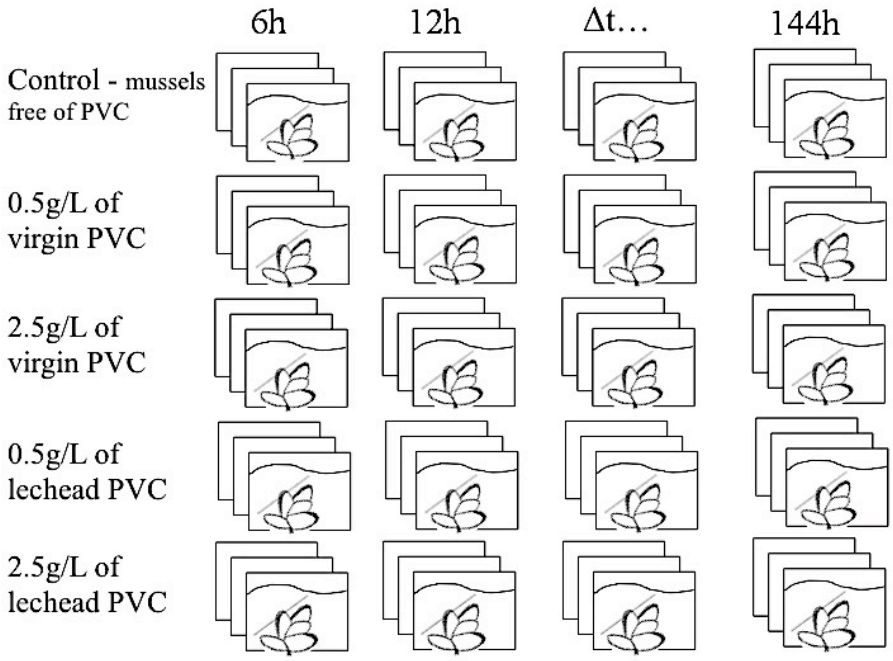

Figure 1.1: Scheme of the experimental design. 15 mussels arranged in three replicates with five mussels per aquaria. Mussels were exposed to four scenarios of PVC contamination, considering concentration (0.5g/L and 2.5g/L of PVC) and leaching (virgin and leached particles). The control for each time of exposure followed the same experimental design but was free of microplastics' exposure.

Assessing microplastics effects – Biomarkers in mussels tissues

Tissues were chosen based on their possible interaction (chemical or physical) with microplastics; and the effects were examined using mussels' gills (von Moos et al., 2012; Avio et al., 2015), digestive gland (von Moos et al., 2012; Avio et al., 2015) and hemolymph (Browne et al., 2008; Avio et al., 2015). Six biomarkers were investigated: lysosomal membrane stability, lipid peroxidation, DNA damage and the stress proteins pP38-MAPK, AIF-1, and HSP70. They can be related to different extracellular stimuli,

to organisms' adaptive responses or to disruption of normal cellular metabolism. Lysosomal integrity (Browne et al., 2008; von Moos et al., 2012; Avio et al., 2015) and DNA damage were previously used to evaluate microplastics' effects on marine biota (Avio et al., 2015), while the others are routinely used in assessments of ecotoxicology to measure biological effects on marine biota (Lewis et al., 1999; Martin et al., 2002; Canesi et al., 2006; Almeida et al., 2007), expanding the diagnosis of effects of microplastics.

The analyzed biomarker varied depending on the exposure period because of their time-dependent responses. We selected the periods analyzed by each biomarker based on previous studies of different types of stress. Lysosomal membrane stability (von Moos et al., 2012; Avio et al., 2015), lipid peroxidation (Variengo et al., 1989; Ribeira et al., 1991) and DNA damage (Almeida et al., 2005; Avio et al., 2015) seemed to need at least 24h to manifest. Stress proteins were expressed within the first 24h of exposure to the stress (Franzellitti and Fabbri, 2005; Gourgou et al., 2010), and potentially continue for several days (Franco et al., 2006; Gust et al., 2013). Stress proteins were therefore analyzed from the 6th hour of PVC exposure, while the other biomarkers were investigated after the first 24h of the experiment. However, quantifying stress proteins is very time-consuming, so we excluded the 96th hour of exposure from these biomarkers' assessments and analyzed six periods of exposure (6, 12, 24, 48 and 144 hours).

Lysosomal integrity – Neutral red retention time assay (NRRT)

Lysosomes are important organelles for cells, with several functions such as accumulating chemical and toxic compounds (i.e. nano and microparticles; Moore et al., 2006a; Canesi et al., 2012 and von Moos et al., 2012; OSPAR, 2013), removing waste substances (Hegaret et al., 2003), and protecting from oxidative stress (Bocchetti and Francesco, 2006). As the concentration of contaminants increase in the lysosome, the structural and functional integrity of its membrane is affected, usually decreasing the cell's viability (Lowe et al., 1994; Lowe et al., 1995).

Lysosomal membrane stability was analyzed following the method of Neutral Red Retention Time (NRRT) described by Lowe et al. (1995). This method relates the hemocyte viability with the ability of lysosomes to retain neutral red dye over time. At

the end of each exposure period, 500µL of hemolymph were collected from the posterior adductor muscle of the mussels with a syringe (2ml volume) containing 0.5 mL saline solution (pH 7.36). After homogenization, 40 μ L of each of these solutions was deposited on microscopy slides pre-treated with agar and incubated in the dark in a humid chamber for 15min. The slides were then exposed to 40 μ L of working solution of neutral red dye and analyzed every 15min under light microscopy. The NRRT was obtained when 50% or more cells showed a leakage of neutral red dye into the cytosol and/or abnormalities in color and size of lysosomes (Lowe et al., 1995; Pereira, 2014).

Oxidative stress – Lipid peroxidation and DNA damage

Oxidative stress is a biological condition caused by an imbalance between the production of reactive oxygen species (ROS) and the ability of an organism to eliminate them and repair the damage (Davies, 1995; Valavanidis et al., 2006). Despite its natural production by cellular metabolism (Valavanidis et al., 2006), pollutants and adverse environmental conditions can increase it, destabilizing the cellular "redox homeostasis" and causing oxidative damage to cellular components (Valavanidis et al., 2006; Almeida et al., 2007), like DNA and other proteins (Meyer and da Silva, 1999).

Lipid Peroxidation (LPO)

Lipid peroxidation was analyzed in samples of gills using the thiobarbituric acid method (TBAR; Wills 1987), which measures lipids' oxidation by the formation of malondialdehyde (MDA, one of LPO chain by products). Weighed samples were homogenized in a 1:4 (weight/volume) buffer solution (NaCl 100mM, HEPES-NaOH 25mM, EDTA 0.1mM, DTT 0.1 mM, pH 7.5). Then, 150µl of that mixture was combined with 300µl of 10% trichloroacetic acid diluted in $FeSO_4$ 1M and 150µl of 0,67% thiobarbituric acid diluted in deionised water. The homogenate was incubated in a 70˚C bath for 10min and duplicates of 200µl were taken from the supernatant, placed in 96 wells plates and cooled on ice. Blanks were represented by the buffer and standards by 0.001% tetramethoxypropano (TMP, diluted in 0.1M HCl). The measures were done via fluorescence (spectrofluorimeter Synergy HT, BioTek) with excitation at 360nm and emission at 450nm. Results were expressed as µg TBARs/mg of total protein.

Total protein was obtained using the Bradford method. For that, samples were diluted two times in TBS extraction buffer (Tris Base 0.5M, 9% NaCl, pH 8.4, dilution of 1:2 and 1:10) and 50µL of each dilution was placed in a 96 wells microplates in duplicate. Thereafter, 50µL of Bradford reagent (Sigma Aldrich) was added to each well. The plate was incubated at room temperature for 10min in the dark. Blank and standards were prepared with TBS and bovine serum albumin (BSA), respectively. Quantification was done by spectrophotometer (spectramax 250) at 595nm of absorbance; the results were expressed as mg of total protein.

DNA damage

DNA is another cellular component susceptible to oxidative stress, with ROS inducing strand breaks and modifications in DNA bases (Valavanidis et al., 2006; Almeida et al., 2007). DNA damage was assessed by an alkaline precipitation assay (Olive, 1998) and quantified through DNA strand breaks by fluorescence (Gagné et al., 1995). For that, samples of gills were weighed and homogenized in a 1:4 (w/v) of buffer solution (same as for LPO). Then, 25µL of homogenized tissue was mixed by inversion with 200µL of 2% SDS buffer (EDTA 10 mM, Tris–base 10 mM and NaOH 40mM) and 200µL of KCl 12 mM. The mixture was incubated in a 60˚C bath for 10min, homogenized by inversion and cooled at 4˚C for 10min. Thereafter, samples were centrifuged at $8x10^3$g for 5min at 4˚C, and replaced into a 96-well plate, where each was mixed with a Hoechst dye working solution (1µg/mL in NaCl 0.4M, Sodium Cholate 4mM and Tris-acetate 0.1M, pH 8.5-9.0, Gagné and Blaise, 1993). After shaking for 300 seconds, the strand breaks were analyzed by fluorescence using 360nm for excitation and 460nm for emission. Salmon sperm DNA (Sigma Aldrich) was used as standard and blanks were prepared with buffer only. The results were expressed as µg of DNA/mg of total protein, with total protein being obtained by the Bradford method, as explained earlier.

Stress proteins

Stress proteins play an important role in cellular homeostasis and repair, being activated early by cellular events derived from toxic exposure (Bierken et al., 1998). Their differences in expression are useful for investigating tissues vulnerable to a

specific stressor (Sanders et al., 1993). pP38-MAPK (Mitogen Activated Protein Kinase), AIF-1 (Allograft Inflammatory Factor-1) and HSP70 (Heat Shock Protein 70-kDa) were analyzed by enzyme-linked immunosorbent assay (ELISA) using methods already established for marine invertebrates (Piza et al., 2007; Zilberberg et al., 2011).

The stress marker pP38–MAPK is part of a superfamily of proteins responsible for initiating a series of reactions related to both cellular survival and apoptosis, depending on the nature of the stress and its duration (Gaitanaki, 2008). The heat-shock Hsp70, in turn, is a chaperone protein. Once activated in stressful scenarios, Hsp70 helps to (i) identify, refold or mark damaged proteins to be eliminated (Meyer and da Silva, 1999) and to (ii) synthesize and mature new proteins to replace the damaged ones (Meyer and da Silva, 1999). In most extreme conditions, Hsp70 also serves as an endogenous modulator of apoptotic cell death (Takayama et al., 2003). Finally, AIF-1 are part of mussels' immune system as a powerful inflammatory cytokine (Martín-Gómez et al., 2014), acting as a modulator of macrophage activation (Gus et al., 2013) and ensuring their efficiency (Tian et al., 2006).

For stress proteins, the number of mussels analyzed was reduced to 5 per treatment. Digestive glands were dissected and homogenized in TBS extraction buffer (Tris Base 0.5M, 9% NaCl, pH 8.4), containing EDTA 1mM and 60µL of protease cocktail inhibitor (Amresco). The homogenate was centrifuged (260x g, 10min at 4˚C) until the supernatant was translucent. Total protein concentration of samples was quantified using a BCA commercial kit (Pierce BCA Protein Assay kit – Thermo Scientific). Samples were diluted three times with TBS extraction buffer (1:2, 1:10 and 1:50), 25µL of the dilutions were placed in duplicates in 96 wells microplates and the BCA reagents mixture were added. After 30min in water bath at 60˚C, total protein was quantified by spectrophotometer (Spectramax 250) at 562nm of absorbance. Based on these results, a volume corresponding to 50µg of proteins was pipetted in triplicates into 96 wells microplates, followed by 100µL of Phosphate Buffered Saline (PBS, NaCl 137mM, KCl 2.7mM, Na_2HPO_4 10mM and KH_2PO_4 2mM). The plates were then washed with PBS containing 0.05% Tween-20 (PBS-T) and incubated with 5% skimmed milk (Molico, Nestlé) solution in PBS-T overnight at 4˚C. The microplates were then washed again in PBS-T and 100µL of primary antibodies were added (rabbit anti-HSP70, rabbit anti-pp38 and goat anti-AIF-1, all Santa-Cruz Biotech), diluted 1:500 in 1% skimmed milk solution. The plates were incubated for 2h30min at 29˚C

and then washed with PBS-T. After this time, the plates received the specific peroxidase-conjugated secondary antibodies (all Santa-Cruz Biotech) diluted 1:1000 in the same buffer; then, the incubation was repeated. The reaction was revealed using 100µl of 3,3,5,5-tetramethylbenzidine solution (TMB revelation kit, Pierce) per well and incubating the plates 10min in the dark at room temperature. The reaction was stopped by adding 50 µl of H_2SO_4 2mM and read in a spectrophotometer (Spectramax 250) at 450nm (optical density or OD 450). The obtained OD450 corresponded to the expression of each protein in each sample.

Statistical Analyses

To test the hypothesis that PVC exposure and intake cause biological effects on *P. perna (h1)*, analyses of variance compared each combination of biomarker and period of exposure. For NRRT, LPO and DNA damage, two-way ANOVAs were done separately for 24, 48, 96 and 144 hof exposure. These analyses had the factors "scenario of contamination" (fixed, with five levels: control, 0.5g/L virgin PVC, 2.5g/L virgin PVC, 0.5g/L leached PVC, 2.5g/L leached PVC) and "aquaria" (fixed and nested using the above factor, with three levels: aquaria 1 to 3, n = 5 mussels). For stress proteins, the factor "aquaria" was excluded as we only analyzed 5 mussels from the 15 exposed per scenario. Thus, one-way ANOVAs only considered the factor "scenario of contamination", for 6, 12, 24, 48 and 144h of exposure independently. When $p < 0.05$, Student–Newman–Keuls (SNK) tests were performed to compare control and exposed scenarios.

To determine the influence of contamination factors and their interactions on biological effects *(h2)*, comparisons were made using four-way ANOVAs for NRRT, LPO and DNA damage, and three-way ANOVAs for stress proteins. As for *h1*, the factor "aquaria" was excluded from stress proteins' statistical analyses. Thus, four-way ANOVAs had the factors "leaching" (fixed, with 2 levels: virgin and leached), "concentration" (fixed and orthogonal, with 2 levels: 0.5 and 2.5g/L), "period" (fixed and orthogonal, with 4 levels: 24, 48, 96 and 144h) and "aquaria" (fixed, nested in the above factors, with 3 levels: aquaria 1 to 3, n = 5 individuals/aquaria); while three-way ANOVAs had all factors apart from "aquaria". Stress proteins also had "period" with 5 levels as for *h1*. These statistical analyses were applied using biomarkers' standardized

responses to controls because the biomarker responses naturally varied over time. Such variation was verified for each biomarker by analyzing control responses during the experiment, using two- or one-way ANOVAs with the factors "period" (controls remained in aquaria) and "aquaria" (for NRRT, LPO and DNA damage). Data were expressed as percentage of control responses (<100% represented a decrease in relation to the control, >100% an increase). For significant factors, SNK tests were done as *a posteriori* comparisons (Underwood, 1997).

All analyses of variance were conducted using WinGMAV 5 (EICC, University of Sydney, Australia). Homogeneity of variance was examined by Shapiro-Wilk and Cochran's tests. When necessary, data was transformed applying the most suitable transformations.

Results

Microplastics intake by *P. perna* induced physiological responses but without a clear pattern among scenarios of exposure and biomarkers (*h1*, $\alpha < 0.05$, Figures 1.2 and 1.3). This finding supports our first hypothesis (*h1*) and corroborates hypothesis 2, by which variations in the features of microplastic contamination ("leaching", "concentration" and "period") can affect the responses of exposed brown mussels. Where the factor "aquaria" was included as factor (NRRT, LPO and DNA damage), significant differences ($p<0.05$) within this factor were found for hypothesis 1 (Figure 1.2.A, E and I) and for hypothesis 2 (Figure 1.4.A-C). This result is handled separately at the end of this section.

Within the first 24h of experiment, all scenarios of exposure affected mussels' lysosomal integrity, but at 48h none of them differed from control. After 96h, only 0.5g/L of virgin PVC showed a response, and by the end of the exposure both concentrations of leached PVC affected lysosomal integrity (Figure 1.2). It is worth noting that in cases where exposed mussels were significantly different from the control, values of NRRT were higher than controls (Figure 1.2.A, C and D). Lipid peroxidation showed evidence of microplastic effects for all exposure periods, at first from the 2.5g/L leached PVC and thereafter from 0.5g/L virgin PVC. In the last period, only mussels exposed to 0.5g/L of leached PVC were significantly affected (Figure

1.2). All significant values of LPO were higher than the control. Regarding DNA damage, mussels from all scenarios analyzed within 24h of exposure had significantly less DNA strand breaks than the control. Within 48h, no differences between exposed and control mussels were observed. 0.5g/L of virgin and leached PVC showed an increase in mussels' strand breaks until the 96th hour. At the 144th hour, no treatment had mussels with significantly higher damage than the control (Figure 1.2.I-L).

The responses of the stress proteins were even more variable, with scenarios of exposure increasing and decreasing their expression without a clear pattern (Figure 1.3). Within 6h, all scenarios of exposure increased pP38-MAPK expressions except for "2.5g/L of virgin PVC", in which mussels had less of this protein than the control. After 12h, 2.5g/L of leached microplastics increased pP38-MAPK production. For 24h, mussels exposed to 2.5g/L of virgin and leached microplastics raised its expression. Within 48h, however, mussels exposed to 2.5g/L of virgin and leached PVC had less pP38-MAPK than control. By the end of the experiment (144h), there were no differences for pP38-MAPK. (Figure 1.3.A-E). For Hsp70, the 6h of exposure only affected mussels exposed to 2.5g/L of leached PVC. In 12h, 0.5g/L of leached microplastics increased mussels' Hsp70 production. In 24h, in turn, 2.5g/L of virgin and leached PVC raised such expression. Thereafter (48h) no differences were observed for Hsp70 expressions until 144h, when mussels exposed to 0.5g/L of virgin and leached PVC decreased it to lower levels than the control (Figure 1.3.F-J). AIF-1 expression of all exposed mussels started high but after 12h, it was similar to the control. Within 24h, 0.5g/L of virgin PVC increased AIF-1 production, while in 48 hours, it was raised by both concentrations of virgin PVC and 2.5g/L of leached particles. After 144h, AIF-1 was negatively affected by both concentrations of virgin PVC and by 0.5g/L of leached microplastics (Figure 1.3.K-O).

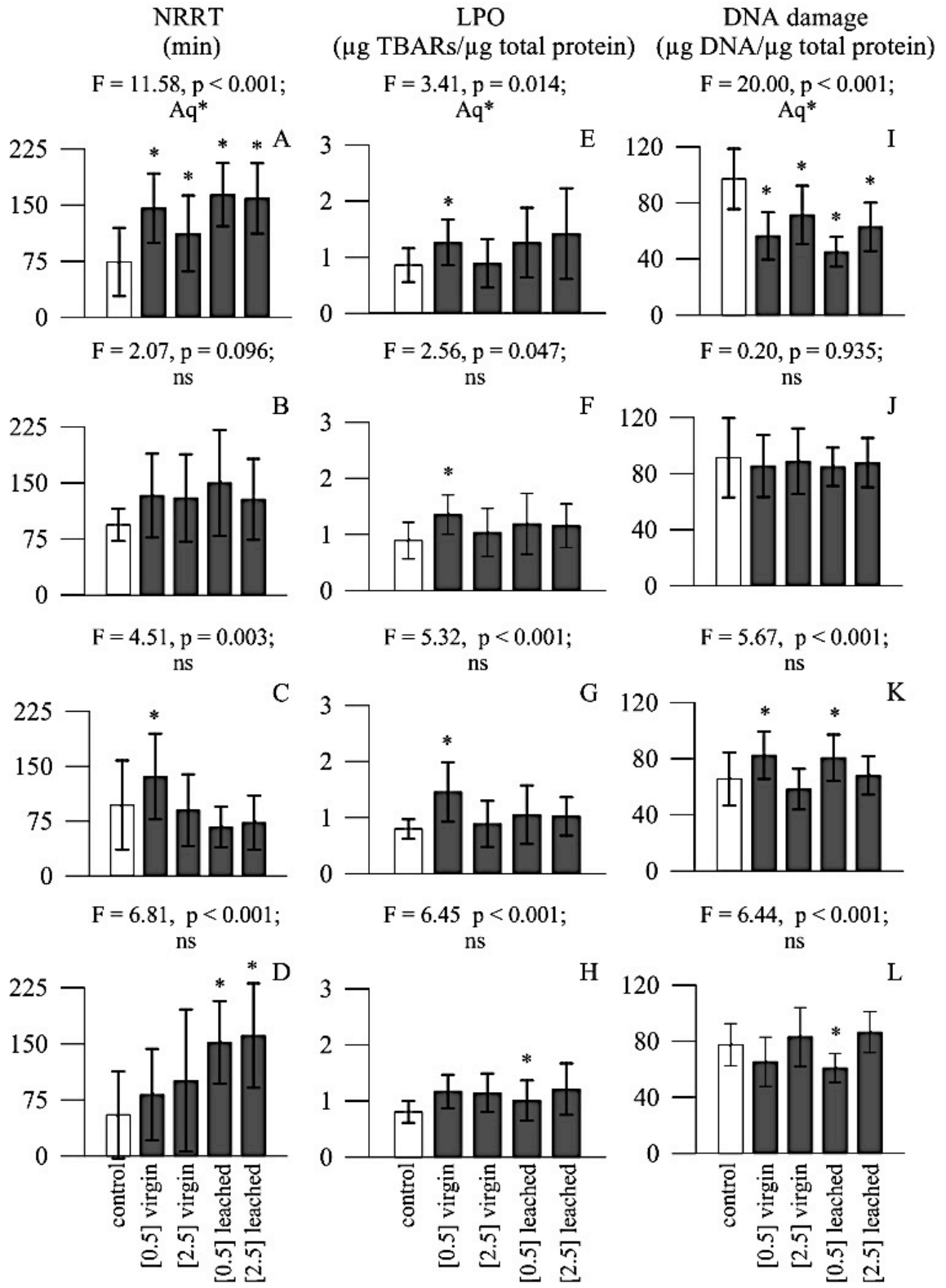

Figure 1.2: Two-way ANOVA evaluation of PVC exposure and intake causing biological effects on *P. perna* (*h1*). Data correspond to neutral red retention time (NRRT, min), lipid peroxidation (LPO, µg TBARs/µg total protein) and DNA damage (µg of DNA/µg total protein) by period of exposure. F and *p* values are related to the factor "scenario of contamination" and graphs represent SNK test for this factor (control, 0.5g/L virgin PVC, 2.5g/L virgin PVC, 0.5g/L leached PVC, 2.5g/L leached PVC). Significant and non-significant differences among aquaria in each scenario are indicated by Aq* or ns, respectively. * indicates that mussels exposed to a given scenario of contamination were significantly different from the control. n = 15.

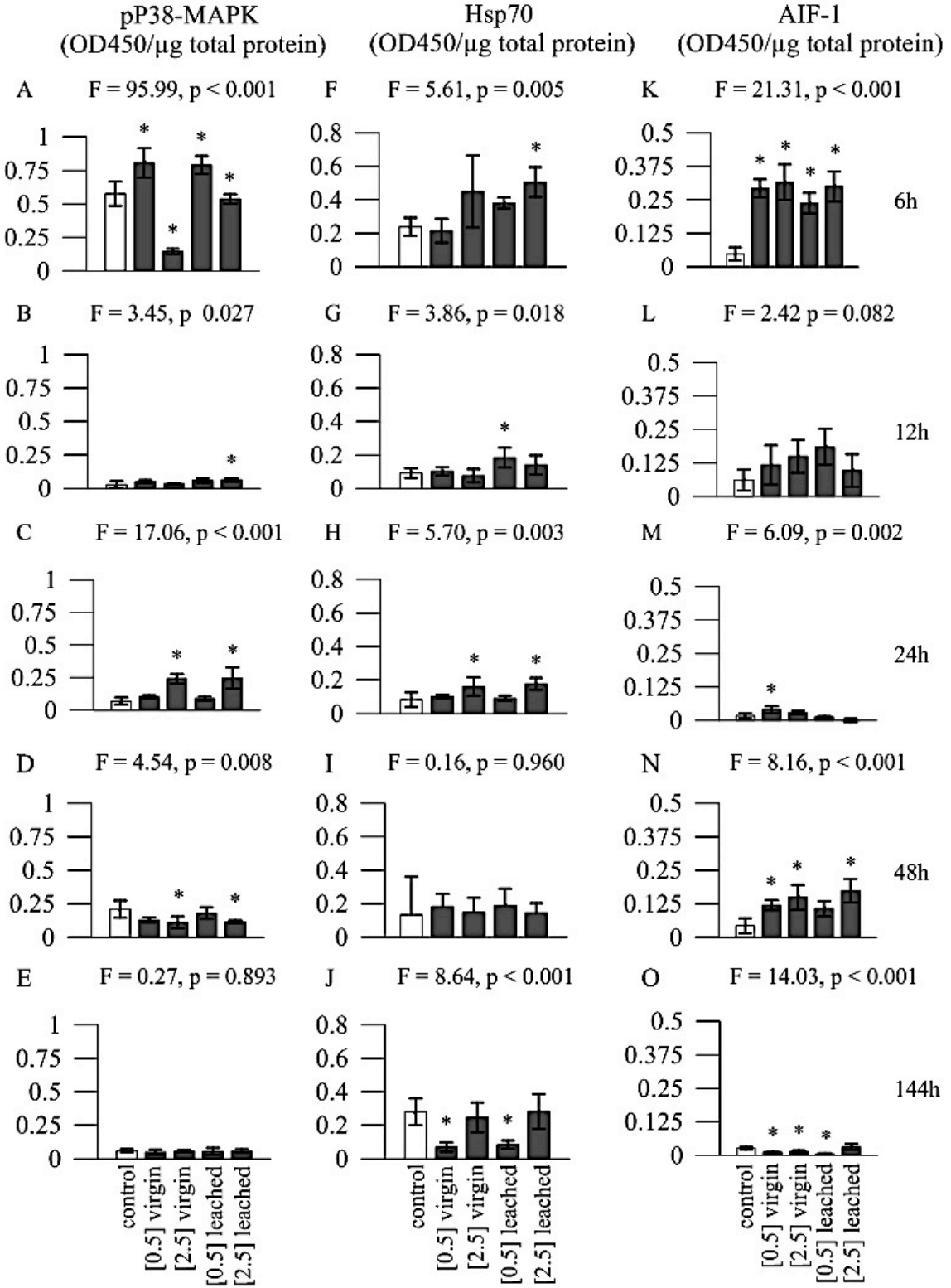

Figure 1.3: One-way ANOVA evaluation of PVC exposure and intake causing biological effects on *P. perna* (*h1*). Data correspond to pP83-MAPK (OD450), Hsp70 (OD450) and AIF-1 (OD450) by period of exposure. F and *p* values are related to the factor "scenario of contamination" and graphs represent SNK test for this factor (control, 0.5g/L of virgin PVC, 2.5g/L of virgin PVC, 0.5g/L of leached PVC, 2.5g/L of leached PVC). * indicates that mussels exposed to a given scenario of contamination were significantly different from the control. n = 5.

The three and four-way ANOVAs showed significant interactions among contamination factors for all biomarkers. Individual and combined (interaction) effects were also not consistent among biomarkers (Figure 1.4, Table 1).

For NRRT, there was a significant interaction between the factors “leaching” and “period”, without influence from microplastics concentrations (Figure 1.4.A, Table 1). The leached PVC caused a clear tendency of decreasing lysosomal integrity until 96h of exposure, when the mean NRRT achieved its minimum, similar to the control levels (Figure 1.4.A). Thereafter, it increased sharply, reaching its maximum retention time after 144h of exposure, with leached PVC presenting higher values than virgin ones. A similar but not significant tendency was observed for virgin plastics (Figure 1.4.A).

Lipid peroxidation had a significant interaction between “leaching” and “concentration” (Figure 1.4.B, Table 1), in which all exposed mussels had more LPO than the control. Within “leaching” levels, mussels exposed to 0.5g/L of virgin PVC had more LPO than those the exposed to 2.5g/L, while leached PVC did not vary between concentrations (Figure 1.4.B). Within concentrations, 0.5g/L of virgin PVC had a stronger effect than leached ones, with no difference between leached and virgin particles at 2.5g/L.

The DNA damage had a significant interaction between “concentration” and “period” (Figure 1.4.C, Table 1). Mussels exposed to both concentrations increased the quantity of DNA strand breaks from 24 to 48 hours, but without surpassing the control (Figure 1.4.C). For *P. perna* exposed to 0.5g/L of PVC, however, this increase continued until 96h, exceeding the control and decreasing thereafter (144h, Figure 1.4.C). The exposure to 2.5g/L of PVC did not have significant variations over time from 48h of assay, but showed higher values than mussels exposed to 0.5g/L at 144h (Figure 1.4.C).

Table 1.1: Summary of 4-way ANOVAs for the influence of contamination factors (leaching, Le; Concentration, Co; Period, Pe; Aquaria, Aq) and interactions (*hypothesis 2*, $\alpha < 0.05$) on lysosomal integrity (NRRT), lipid peroxidation (LPO) and DNA damage biomarkers. Cochran's tests (C) and type of transformation utilized (Logarithmic, Ln(X+1); Square-Root, $\sqrt{(X + 0.5)}$; and Arcsine, $\arcsin\sqrt{X}$, transformations; or ns in case of absence).

		NRRT C= 0.106 ns			**LPO** C= 0.055 Ln(X+1)			**DNA damage** C= 0.217 ns		
Source	*df*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Leaching Le	1	6.82	5.18	**0.029**	0.02	0.13	0.726	0.0	0.82	0.371
Concentration Co	1	0.72	0.55	0.464	0.17	1.07	0.309	0.0	0.85	0.364
Period Pe	3	19.08	14.50	**<0.001**	0.14	0.89	0.458	0.0	23.68	**<0.001**
Aquaria Aq(Le*Co*Pe)	32	1.32	2.45	**<0.001**	0.16	1.62	**0.025**	0.0	2.01	**0.002**
Le*Co	1	0.49	0.38	0.544	1.26	7.93	**0.008**	0.0	1.19	0.283
Le*Pe	3	7.81	5.93	**0.002**	0.27	1.70	0.185	0.0	0.77	0.517
Co*Pe	3	0.54	0.41	0.746	0.12	0.77	0.518	0.0	9.72	**<0.001**
Le*Co*Pe	3	0.44	0.33	0.801	0.07	0.45	0.720	0.0	1.24	0.311
Residual	192	0.54			1.00			0.0		

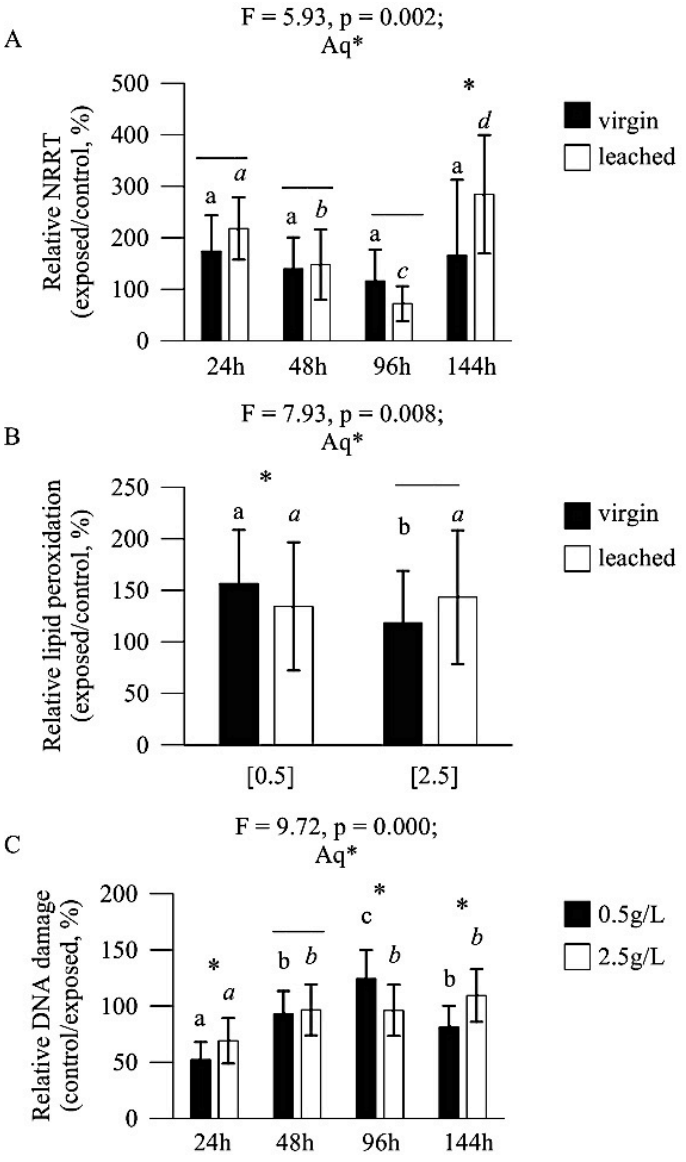

Figure 1.4: Representation of the 4-way ANOVAs for the influence of contamination factors and their interactions (*hypothesis 2*) on (A) NRRT ("leaching" * "period"), (B) LPO ("leaching" * "concentration"), and (C) DNA damage ("concentration" * "period"). Graphs show SNK test for significant interactions between factors (discriminated F and p). Significant and non-significant differences among aquaria in each scenario are indicated by Aq* or ns, respectively. Data are expressed as percentage responses in relation to the control (<100% = decrease in relation to the control, >100% = increase). Regular letters represent significant differences between 1st columns (see specific legends above). Italic letters represent significant differences between 2nd columns (see specific legends above). – represents non significant differences between 1st and 2nd columns within levels from horizontal axis. * represents significant differences between 1st and 2nd columns within levels from horizontal axis. n = 15.

The responses of the three stress proteins generally declined with time but not with the same influence of factors (Table 2; Figures 1.5 and 1.6). The pP38-MAPK levels were influenced by two interactions: "leaching" and "period", and "concentration" and "period" (Figure 1.5.A and B, Table 2). Mussels exposed to 2.5g/L virgin and leached PVC began with low expression of pP38, increasing thereafter until

24h, when we observed a peak in mussels exposed to 2.5g/L and virgin PVC. From 48 to 144h, however, all exposed mussels decreased their pP38 expression, with values near or below the control. Bivalves exposed to 0.5g/L of particles started with higher expressions than the control, reaching a peak within 12h and decreasing thereafter (Figure 1.5.A and B). Within 48h of exposure, these mussels had lower expression of pP38, similar to the control, presenting no changes thereafter.

For Hsp70 expression there was significant interaction between "concentration" and "period" (Figure 1.5.C, Table 2). For the first hours of assay, *P. perna* exposed to 0.5g/L had similar expression of Hsp70 to the control. They did not show differences over time until the 144th hour, when levels declined sharply to below control values. Mussels exposed to 2.5g/L had two times more Hsp70 than the control at the 6th hour of assay, followed by a decrease at 12h and an increase in 24h, with lower values at 48 and 144h (Figure 1.5.C). The lowest level of Hsp70 in this concentration was never lower than the control (See Figure 1.3).

The AIF-1 production had significant interactions among "leaching", "concentration" and "period" (Table 2, Figure 1.6). This mainly represents the expression over time for mussels exposed to virgin and leached PVC, and their differences at 6 and 24h of exposure. For all concentrations and leaching levels, the highest expression was from mussels exposed for 6 hours. This peak was followed by a decline to values generally similar to the control, and by an increase or stability until 48h. Thereafter, the quantity of AIF-1 reduced again and reached similar or smaller concentrations to those found in uncontaminated mussels (Figure 1.6). No pattern of responses among "concentration" and "leaching" factors over time was observed.

Table 1.2: Summary of 3-way ANOVAs for the influence of contamination factors (leaching, Le; Concentration, Co; Period, Pe) and interactions (*hypothesis 2*, $\alpha < 0.05$) on pP38-MAPK, HSP70 and AIF-1 stress proteins. Cochran's tests (C) and type of transformation utilized (Logarithmic, Ln(X+1); Square-Root, $\sqrt{}(X + 0.5)$; and Arcsine, $\arcsin\sqrt{}X$, transformations; or ns in case of absence).

		pP38-MAPK C= 0.219 ns			**Hsp70** C= 0.196 ns			**AIF-1** C= 0.151 ns		
Source	*df*	MS	F	*p*	MS	F	*p*	MS	F	*p*
Leaching Le	1	0.12	1.20	0.276	0.00	7.74	**0.006**	2.54	3.11	0.082
Concentration Co	1	0.06	0.53	0.469	0.01	10.10	**0.002**	2.89	3.55	0.063
Period Pe	4	9.98	96.34	**<0.001**	0.01	15.50	**<0.001**	4.00	81.28	**0.000**
Le*Co	1	0.02	0.16	0.686	0.00	0.00	0.988	0.24	0.29	0.592
Le*Pe	4	0.87	8.36	**<0.001**	0.00	2.01	0.101	2.18	2.67	**0.038**
Co*Pe	4	5.33	51.46	**<0.001**	0.00	7.16	**<0.001**	1.73	2.12	0.086
Le*Co*Pe	4	0.24	2.36	0.060	0.00	0.32	0.867	4.00	2.68	**0.037**
Residual	80	0.10			0.00			0.82		

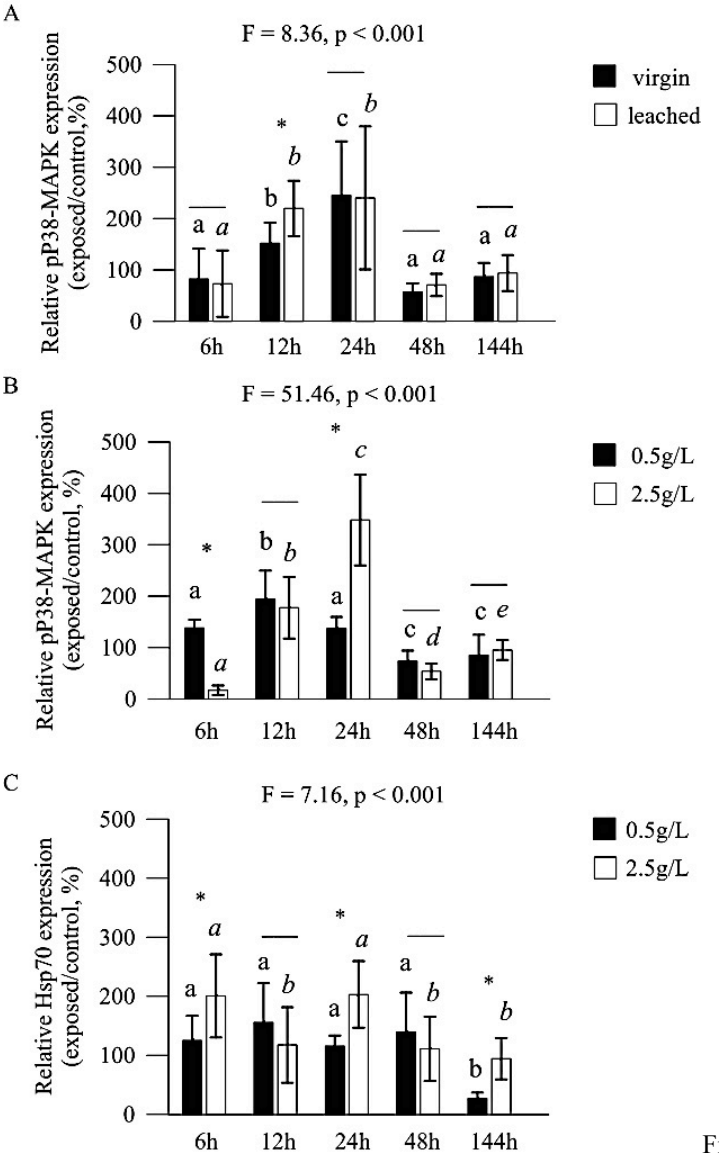

Figure 1.5: Representation of the 3-way ANOVAs for the influence of contamination factors and their interactions (*hypothesis 2*) on (A and B) pP38-MAPK ("leaching" * "period" and "concentration" * "period", respectively) and (C) Hsp70 ("concentration" * "period"). Graphs show SNK analysis for significant interactions between factors (discriminated F and p). Data are expressed as percentage responses in relation to the control (<100% =

decrease in relation to the control, >100% = increase). Regular letters represent significant differences between 1st columns (see specific legends above). Italic letters represent significant differences between 2nd columns (see specific legends above). – represents non significant differences between 1st and 2nd columns within levels from horizontal axis. * represents significant differences between 1st and 2nd columns within levels from horizontal axis. n = 5.

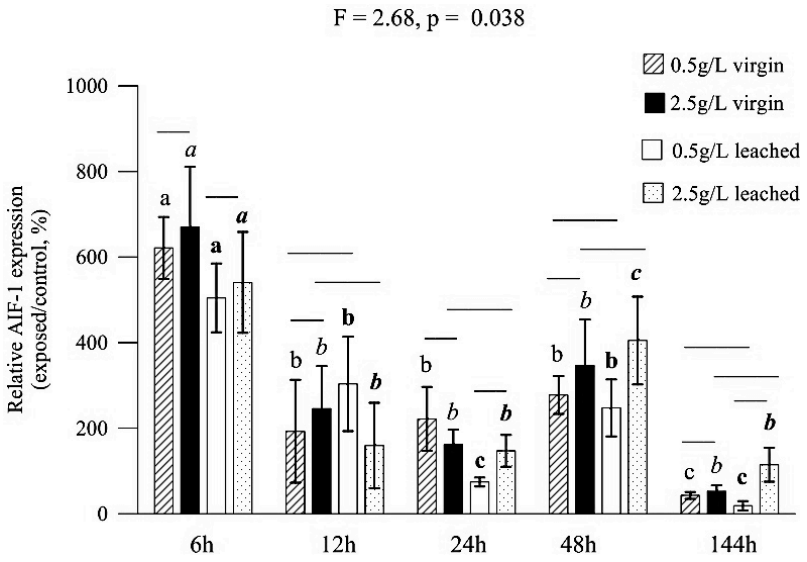

Figure 1.6: Representation of the 3-way ANOVAs for the influence of contamination factors and their interactions (*hypothesis 2*) on AIF-1 stress proteins ("leaching" * "concentration" * "period"). Graphs show SNK analysis for significant interactions between factors (discriminated F and p). Data are expressed as percentage responses in relation to the control (<100% = decrease in relation to control, >100% = increase). Regular letters represent significant differences between 1st columns (see specific legends above). Italic letters represent significant differences between 2nd columns (see specific legends above). Regular and bold letters represent significant differences between 3rd columns (see specific legends above). Italic and bold letters represent significant differences between 4th columns (see specific legends above). (–) represents non significant differences between extreme columns within time. Columns not connected by (–) means that they are significantly different. n = 5.

For the NRRT, LPO and DNA damage biomarkers, the ANOVAs included 'aquaria" as a factor. These were significantly different ($p<0.05$) for all biomarkers after 24h of exposure (h1) (Figure 1.2.A, E and I) and also for hypothesis 2 after 24 and 144h of exposure to 2.5g/L of PVC (Figure 1.4.A-C; Figure 1.7). Nevertheless, this factor was not remarkable for all scenarios of exposure as a generalized effect. Responses for this factor also occurred without a pattern among scenarios and biomarkers (see examples on Figure 1.7).

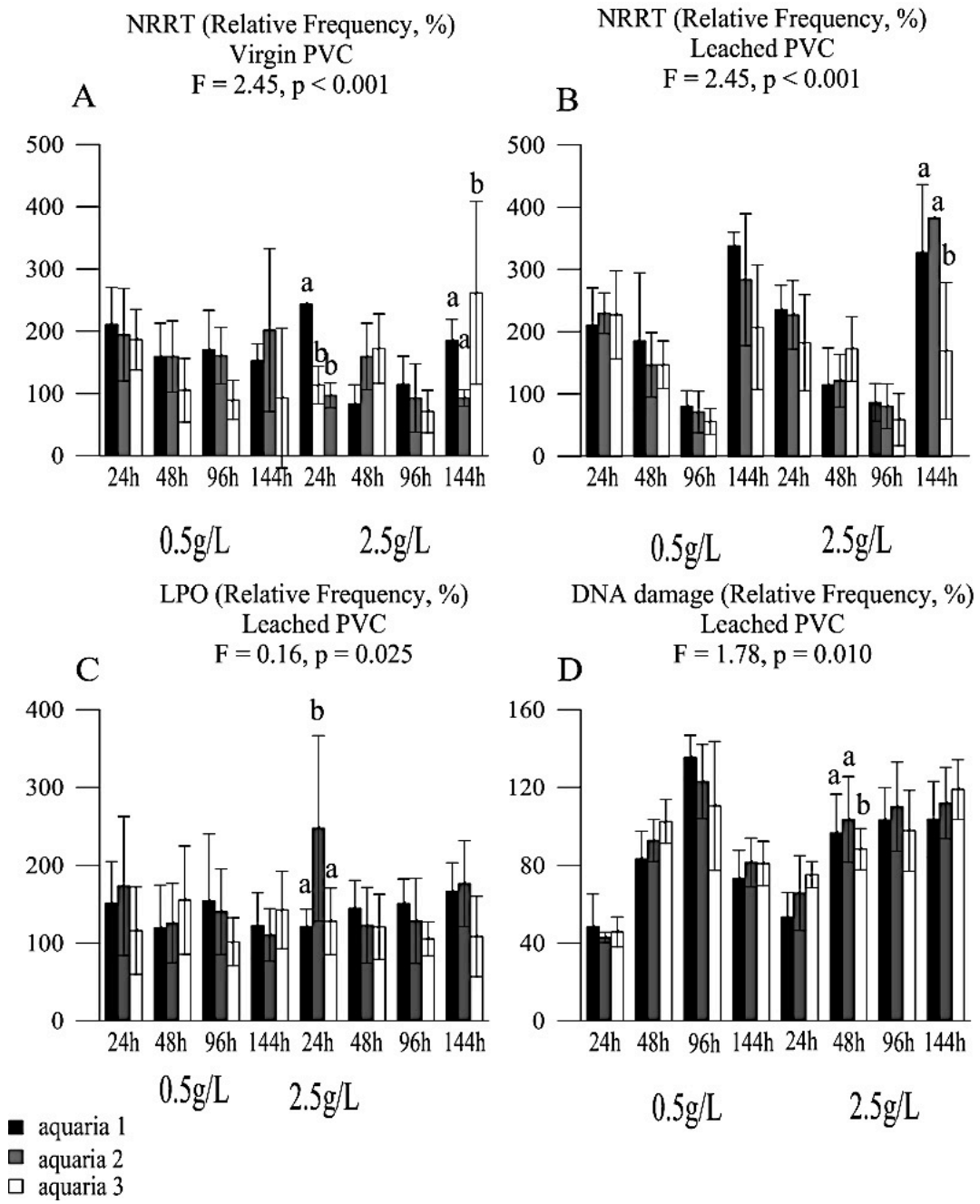

Figure 1.7: Representation of ANOVAs for the influence of the nested factor "aquaria" on (A) NRRT of mussels exposed to virgin PVC; (B) NRRT of mussels exposed to leached PVC; (C) LPO of mussels exposed to leached PVC; and (D) DNA damage of mussels exposed to leached PVC. Graphs show SNK analysis for the influence of the nested factor "aquaria" (discriminated F and p), by "period" of exposure (hours). Data are expressed as percentage responses in relation to the control (<100% = decrease in relation to the control, >100% = increase). Letters indicate the significant differences between levels of "aquaria" in each scenario of contamination to PVC. n = 15.

Discussion

Exposure and intake of E/M PVC microparticles induced biological effects on mussels, as all six biomarkers were influenced. Biomarkers share the advantage of

indicating scenarios of contamination that realistically threaten organisms, giving an idea of how environmental health might change according to exposure features (Bierkens et al., 1998). Our results indicated that exposure features can influence microplastics' impacts on marine bivalves, a potential approach for evaluating the risks in natural systems (Domouhtsidou et al., 2004). Generally, the period of exposure was a decisive factor for triggering responses, but it was also influenced by the other experimental factors. Apart from LPO, all biomarkers responded to microplastic contamination in a time-dependent manner. LPO showed a dose-dependence influenced by "leaching". Moreover, the nested factor "aquaria" indicated a significant but random influence on the effects on mussels.

The fact that biomarkers were not equally affected by the analyzed factors indicates that those factors may interfere differently with biological process, tissues and structures. Interactions among them surpassed their main effects and made microplastics' impacts directly related to the exposure scenario and its conditions (different combinations of factors' levels), illustrating the complexity of microplastics' risks to marine environment. This study showed that E/M PVC could also impact all tissues previously associated with the pathway of bivalves' ingested microplastics (see Browne et al., 2008 and von Moos et al., 2012), but the use of different biomarker for each tissue made it difficult to integrate the observed threads. However, the variable influence of factors among biomarkers should not be attributed to the tissues. LPO and DNA damage, as well as the three stress proteins, were analyzed in the same tissues (gills and digestive glands, respectively) and had different results. Further works could explore this issue, seeking to understand why those differences occurred, focusing on the mode of action of each biomarker and in how their expressions were triggered by microplastics exposure. Thereby we would be able to select the most appropriate biomarker to evaluate the effects of microplastics contamination.

Lysosomal stability measured in mussels' hemolymph had a time-dependent response influenced by the factor "leaching", clearly observed in mussels exposed to leached PVC for 96h. These mussels had a decreasing NRRT with time until its minimum at 96h, increasing again thereafter. Only in this critical time did these mussels have, on average, lower NRRT than others. Despite having the same tendency, bivalves exposed to virgin microplastics did not exhibit a significant effect of time. Therefore, period of exposure had stronger effect on mussels exposed to leached PVC, especially

within 96 hours, indicating a particular scenario of exposure to note when considering leached microplastics present in marine environments. Previous studies have already demonstrated lysosomal membrane destabilization on hemolymph and digestive gland of mussels as a cellular response to microplastics intake (Von Moos et al., 2012; Avio et al., 2015). In our study, however, control mussels presented similar or lower NRRT than exposed ones (regardless of "leaching"), and were generally considered as "stressed" organisms by the indices of Moore et al. (2006b). This contradicts the principle of the assay, by which healthy cells from unexposed mussels would have higher NRRT than contaminated ones (Lowe et al., 1995).

Focusing on the control data, other research on the Brazilian coast had similar results for *P. perna* (Abessa et al., 2005; Francioni et al., 2007; Pereira et al., 2011), suggesting that healthy tropical mussels may have lower NRRT than those from temperate waters (Pereira et al., 2011). This, however, does not justify the higher NRRT of exposed mussels. Along with other studies, ours suggests that pollutants do not always compromise the lysosomal membrane stability (Matozzo et al., 2002; Matozzo and Marin, 2005; Dissanayake et al., 2008), which can be related to "lysosome autophagy". This process corresponds to the degradation of intracellular components by lysosomes, recycling malfunctioning components and producing energy to sustain the cell itself (Cuervo, 2004). Lysosome autophagy and its capacity of being up regulated is notable in times of stress (Cuervo, 2004) and its presence in marine mussels may be a significant factor for tolerance to stressful aquatic environments (Moore et al., 2006b; OSPAR, 2013). During mussels' exposure to PVC, lysosome autophagy may have been up regulated, increasing NRRT in comparison to the control. Nevertheless, further studies should investigate the effecs of microplastics on lysosome autophagy to better infer this suggestion.

Comparison between mussels' NRRT from virgin and leached treatments indicated a lower stability of lysosomes associated to virgin microplastics. This can be related to the plastic additives present on them, which might have increased PVC toxicity, affecting lysosomal organelles and decreasing NRRT. Virgin microplastics were already showed to be more toxic for *Lytechinus variegatus* than beach-collected ones (Nobre et al., 2015), corroborating our expectations that virgin PVC should be more hazardous than leached ones.

This indicates that mussels are sensitive to plastic additives and that the ingestion of virgin E/M PVC can act as a vector of these toxic substances that may reach circulatory fluids in marine biota. During plastic manufacturing, chemicals such as flame-retardant and antimicrobial compounds are commonly added to polymers to give them specific features (EPA, 1992; Teuten et al., 2009). Many of these compounds are toxic and cause adverse effects in organisms (Ananthaswamy, 2001). Plastic additives moved from ingested microplastics to the tissues of the polychaete *A. marina* and the associated disrupted feeding led to the death of some individuals (Browne et al., 2013). Our results corroborate such findings, adding cellular responses of mussels to the physiological assessment of lugworms. The use of particles from industry production, however, prevented us from knowing the quality and quantity of additives present on the PVC available for organisms. Yet, this is a clear demonstration that these microplastics (produced in large quantities, used and lost in marine environments) are raising the risks of biological impacts related to plastic additives. As PVC is one of the plastics with more chemical additives (Oehlmann et al., 2009), the potential risks of virgin particles should be better investigated.

Microplastics exposures also caused oxidative stress in mussels' gills, but the responses of associated biomarkers differed from each other. Lipid peroxidation had a significant interaction between "leaching" and "concentration", characterized by a decrease in LPO of mussels exposed to virgin PVC with increased concentration of plastics. When exposed to 0.5g/L, mussels had higher concentrations of LPO compared to those exposed to leached PVC, but when submitted to 2.5g/L, both PVCs caused similar levels of LPO. The decreasing impact of virgin particles can be related to the tendency of E/M PVC to agglomerate. The 2.5g/L of PVC can have increased clustering, decreasing the surface to volume ratio and desorption of additives. However, our understanding of factors influencing desorption of chemical compounds on microplastics particles is still poor (Bakir et al., 2014), increasing the difficulty of further hypothesis formulation and results interpretation. Nevertheless, concentration of virgin microplastics can be an important issue for assessing the risks of microplastics' pollution in marine systems. Not less important, leached PVC also caused lipid peroxidation but without influence of concentrations. This suggests that, for mussels, microplastics present in seawater for longer periods (leached) pose the same threat for LPO under either acute or more chronic exposures. DNA strand breaks, in turn, were

slightly influenced by microplastic exposure. In this case the interaction was between "concentration" and "period" of exposure, but only mussels exposed to 0.5g/L of PVC for 96h had more strand breaks than the control. This interaction was observed due to the damaged DNA of mussels exposed to 0.5g/L. These mussels had a DNA damage increased with time, surpassing the control and mussels exposed to 2.5g/L within 96h. Thereafter, the amount of DNA damage of these mussels decreased to similar or lower quantities than control. Mussels exposed to 2.5g/L were only slightly impacted, increasing and stabilizing their strand breaks until 48h, with values close to the control. The quantity of chemical additives in PVC ("leaching" factor) did not influence the DNA damage, suggesting that such impact is not remarkably affected by their toxicity.

Oxidative stress caused by microplastics' intake was already observed in marine biota regardless of plastics additives. Ingested PVC without additives and adsorbed pollutants (corresponding to our leached particles) increased oxidative stress in lugworms *A. marina* by 30% (Browne et al., 2013), while PS and PE microparticles increased DNA strand breaks in mussels *Mytilus galloprovinciallis* (Avio et al., 2015). It is worth noting that for *M. galloprovinciallis*, DNA strand breaks seemed to be the first sign of damage caused by ROS after microplastics exposure (Avio et al., 2015), while *P. perna* showed little influence of PVC on DNA damage. This difference reinforces that biological responses can vary greatly according to scenarios of exposure and its combination of factors. Avio et al. (2015) exposed mussels to microplastics for 144h and in a concentration of 20g/L in seawater. Based on our results, the interaction between "concentration" and "period" of microplastics' exposure influence the amount of DNA damage to bivalves, explaining the difference between studies. None the less, the chosen model species, plastic types and analyzed tissue could also have contributed to these differences.

Besides oxidative stress, impacts of leached PVC were observed for the digestive gland and hemolymph of mussels by the expression of stress proteins and lysosomal integrity. Depending on the scenario of exposure and biomarker, they were equally or more damaging to mussels than virgin PVC (e.g. LPO significant responses and levels of pP38 within 12h). Thus, even being washed and retaining fewer chemical additives, E/M PVC still affected mussels, supporting the argument that the microplastic particles themselves (apart from the chemical compounds carried by them) are also harmful, as already indicated by previous studies (Von Moos et al. 2012;

Besseling et al. 2013). Increasing concentrations of PVC on sediment caused weight loss in *A. marina* (Besseling et al. 2013), while HDPE exposure increased granulocytoma formation and decreased lysosomal stability on *M. edulis* (Von Moos et al. 2012). The Marine Strategy Framework Directive Task Group 10 also suggested effects such as decrease in feeding stimulus, reduction of growth rates, and reproductive failure (Galgani et al., 2010). However, a great challenge regarding microplastics' effects is still evaluation of whether the impacts measured are caused by the chemicals adsorbed or by a physical process (Von Moos et al., 2012).

If microplastics impacts are physical, we could correlate them with non-toxic particles suspended in seawater. Suspended solids in water can have a direct effect on filter feeders by increasing physical harm due to abrasion, choking and changes on feeding mechanisms (Hughes, 1976; Hellawell, 1986). However, filter-feeding bivalves have adaptive mechanisms for high concentrations of particulate matter that reduce their intake (Foster-Smith, 1975; Lucas et al., 1987; Barillé et al., 1993), avoiding higher impacts. As an example, concentrations up to 2 g/L of suspended and uncontaminated sediments did not change Hsp70 levels of oysters (Cruz-Rodriguéz and Chu, 2002). This makes us suppose that mussels should not have had different effects between 2.5g/L and 0.5g/L of leached PVC as observed on AIF-1 expression.

Based on literature, we suggest that these results are related to chemical impacts brought on by monomers' toxicity (Mato et al., 2001). Although plastics are considered inert due to their general large molecular size, reactions are frequently not finished during polymerization and monomers may remain within the polymeric materials, being released during use and after disposal (Lithner et al., 2011). Once released, they can interact with cellular and molecular structures, leading to toxic effects. Based on the health and environmental impacts of monomers, PVC is one of the most hazardous plastics (Lithner et al., 2011), which might have contributed to our results. However, considering the utilized biomarkers and their possible relationship with diverse causes of tissue damage, we cannot discard physical impacts. The small size of E/M PVC ($\leq$ 1μm of diameter) could also contribute to physical stresses by raising the chances of the particles being transported across cells membranes as observed for engineered nano-particles (Syberg et al., 2015). To clarify this subject, however, studies using different shapes of microplastics (e.g. spherical E/M PVC and irregular PEAD abrasives) and a control composed of uncontaminated (inert) particles

should be performed. Immunohistochemical methods could also be explored so that we could actually see the damage microplastics are causing to tissues. However, our results suggest that microplastic itself can pose more than physical threats and should not be treated as a typical biochemically inert particle.

Interestingly, while the expression of pP38 was affected by all factors, only interactions between pairs were significant ("leaching"*"period" and "concentration"*"period"). Combinations among all three factors ("leaching"*"period"*"concentration") did not affect its production. This suggests that all analyzed factors were relevant for pP38 activation but in different ways according to their combination. Combinations between concentrations and leaching states of microplastics, for example, had no effect. Generally, microplastics progressively increased pP38 expression until 24h, when exposed mussels had almost 5 times more signs than control. Thereafter it decreased with time, getting levels similar to the control by 144h. Still, interactions were present; some scenarios were more variable and influenced by time than others, becoming more or less of an impact from exposure. By 12h, for instance, mussels exposed to leached PVC had more signs of pP38 than those exposed to virgin ones. Before and after that time, however, the mussels' expression was similar between exposures. In the case of "concentration" and "period", 2.5g/L of PVC caused an interaction within 24h, when they induced more Pp38 expressions than 0.5g/L. Despite this time, both had similar time-influence. Expressions of Hsp70, in turn, were only influenced by the combination of "concentration" and "period" and its levels in mussels exposed to 2.5g/L. Their expression oscillated with time, from higher to similar levels to mussels exposed to 0.5g/L, generally close to the control. At 6 and 24h, mussels exposed to 2.5g/L of PVC had levels of Hsp70 two times greater than others. By the end of the assay, however, both concentrations caused similar or lower levels than the control. Such a response indicates that this biomarker can be extremely variable and not useful to furnish clear information regarding microplastics' risk assessment. Finally, AIF-1 was the only biomarker significantly affected by the combination among all analyzed factors, suggesting that this protein can be more sensitive to differences in scenarios of exposure, considering the factors alone or in combination. Although all exposed mussels had the same tendency of increasing AIF-1 expression within 6 and 48h of assay, interaction was remarkable and the significance of each factor and its levels were highly variable. For each exposure period, the

sequence of great impacting scenarios changed, making it difficult to establish a standard risk, like Hsp70. Overall, however, there was a tendency for AIF-1 be down-regulated with time, just as for pP38 and Hsp70.

This study evaluated, for the first time, microplastics impacts on marine biota using stress proteins. Based on their time-dependent response, we suggest that pP38 and Hsp70 expressions were antagonist to AIF-1, because both proteins can have roles of protection and may act according to signs of inflammation (Jacquier-Sarlin et al., 1994; Herlaar and Brown, 1999). AIF-1 can have been expressed first, as a cytokine signaling inflammation, and pP38 can have activated Hsp70 thereafter, in an anti-apoptotic condition (Kefaloyianni et al., 2005; Banerjee et al., 2009; Gourgou et al., 2010), initiating one of the mussels' reactions to microplastic exposure. With time, all proteins tended towards control levels of expression, indicating a decrease of the effects of microplastics for digestive glands or an adaptation to them. Differences among scenarios of exposure can influence such a response, highlighting their singularity and hindering general statements.

Considering the analyzed significant factors in this study, the most frequent one was "period". Apart from DNA damage, biomarkers exhibited a fluctuating response with time, influenced differently by plastic concentration and leaching. From 24 to 96h of exposure mussels generally had a stress peak, and by 144h most biomarkers had returned to the control levels. For *M. edulis*, time also influenced biological responses and the highest signs were observed at the end of their exposure, in 96h of HDPE contamination (Von Moos et al., 2012). The results corroborate these findings, but also showed a tendency to decreased effects after this period. With time, oxidative and inflammatory conditions were observed together with positive biological responses by the rise of AIF-1 and Hsp70 levels, besides possible lysosomal autophagy. Thus, microplastics' contamination might have activated different defensive systems, leading to further acclimation. According to Evans and Hofmann (2012), the capacity of an organism to surpass ocean changes throughout physiological acclimatization can be an indicative of phenotypic plasticity. In this context, high levels of biomarker responses are temporary, and they return to regular quantities when the acclimatization period restores cellular homeostasis (Kültz, 2003; Kültz, 2005). Added to the high resistance of *P. perna* and its capacity to adapt to adverse conditions (Ferreira and Magalhães, 2010), our results suggest that this species may have phenotypic plasticity

to deal with the microplastics' exposure. Yet, further studies are needed in order to understand *P. perna* responses to microplastic contamination under chronic exposure and, from that, conclude if acclimatization can occur.

Although not related to our hypothesis and objective, but to the experimental design, the factor "aquaria" showed a relevant source of variability for mussels' responses. Experimental assays are suggested to have organisms housed individually to ensure interdependence between samples. Here, significant differences among "aquaria" suggested dependence between samples. Mussels from the same aquaria might have created a group and not an individual response. This is interesting to note because mussels normally live in dense beds and not as solitary individuals (Nicastro et al., 2012); and an aggregation commonly behaves as a whole, having different responses from single organisms (Parrish and Edelstein-Keshet, 1999). Such a response shows the importance of properly reproducing an experimental design, taking into account the ecology of the model organism in statistical analyses to develop more realistic data.

Marine pollution caused by microplastics has different sources, fates and effects, creating complex anthropogenic influences on ocean health, difficult to monitor and manage. Even if not produced intentionally, microplastic pollution tends to increase in the marine environment through the fragmentation of larger plastics already lost in the seas (Wright et al., 2013a), highlighting how important is to better understand their consequences. This study aimed to approach the risks of microplastics for marine bivalves using an integrated perspective, considering the influence of different scenarios of contamination on a range of biomarkers and biological tissues possibly impacted by plastic ingestion. Our results suggest that microplastic pollution lead to biological stress, generally in a time-dependent manner, but also to defense responses, perhaps allowing organisms to overcome their effects or acclimate over time. Factors such as leaching and concentration can also affect the responses, and their interactions were more relevant than individual factors. Combination of contamination's characteristics is important to consider when addressing microplastics' environmental risks, which is also a function of hazard and effects (GESAMP, 2015). Further studies are still necessary to provide detailed data about the mechanisms involved in the evolution of the identified stress to understand their causes. Despite that observed responses may also vary according to microplastic type, PVC should be more focused on due to its large-volume and widespread use, size, presence of toxic monomers and high quantities of additives.

Chapter 2

Chronic low-concentration exposure to microplastic does not cause physiological effects on cultivated mussel *Perna perna*

Introduction

Microplastic pollution is an emerging and worldwide threat for marine ecosystems (Boerger et al., 2010). Originated by land-base and maritime activities (Barnes et al., 2009), these small particles (<5mm, Arthur et al., 2009) can be produced by industries for direct applications (i.e. medicine and cosmetics, named as primary microplastics) or be a consequence of large plastics degradation (secondary microplastics; Ye and Andrady, 1991; Erikson and Burton, 2010). The presence of microplastics in marine environments has already been reported in urban and remote locations (Cole et al., 2011), as well as their contamination with other chemical pollutants, and their biological impacts such as uptake by wild and cultured species (Hirai et al., 2011; Murray and Cowie, 2011; Fisner et al., 2013; Foekma et al., 2013; Van Cauwenberghe and Janssen, 2014). Because plastic degradation in marine environment tends to be slow (Gregory and Andrady, 2003), their persistence and impacts tend towards an unquestionable rise.

The greatest concerns involving this contaminant are related to its size (Moore et al., 2006; Wright et al., 2013a,b). Being small, microplastics present a relatively large hydrophobic surface area, facilitating the adsorption and adherence of other toxic substances and microorganisms liable to leach to organisms after uptake (Mato et al., 2001; Browne et al., 2013; Tanaka et al., 2013; Zettler et al., 2013; McCormick et al., 2014). Their small sizes, especially for those in micrometer scale, also make them broadly bioavailable, including for groups from lower trophic levels and sizes, which are important for energy flux in the food chain (see as examples: Cole, 2011; Cole et al., 2013).

Microplastics exposure and intake were already observed to cause physiological impacts, from stress signs and tissue damage to alteration of physiological

rates (Von Moos et al., 2012; Wegner et al., 2012; Browne et al., 2013; Besseling et al., 2013; Cole et al., 2013; Avio et al., 2015). However, published data mostly investigated microplastics' responses due to acute exposures, while information about chronic contamination are still limited (Van Cauwenberghe et al. 2013). In scenarios of chronic exposure, toxic agents are released periodically, in sublethal concentrations and for long periods (Schvartsman, 1991). The effects can take longer to be manifested, and also differ from those observed for acute exposure (Moreira, 2011). When chronically exposed to a stresses, marine organisms were already reported to have (i) changes in ecologically important sublethal endpoints (e.g. health, growth, and reproduction, Peterson et al., 2003); (ii) increasing susceptibility to disease and other pressures (Blakley, 1985; Di Giulio & Scanlon, 1985) but also; (iii) recoveries and adaptations (Hamdoun et al., 2003).

Coastal environments are examples of regions commonly impacted by chronic inputs of xenobiotics (Morales-Caselles et al., 2008) and for microplastics it is probably not different. About 80% of marine plastics originate from land-based activities (Andrady, 2011). Apart from the slow breakdown of larger plastics (Jambeck et al., 2015), coastal urbanized areas are susceptible to constant inputs of primary and secondary microplastics from domestic and industrial activities (Fendall and Sewell, 2009; Browne et al., 2011; Fisner et al., 2013; Mathalon and Hill, 2014; Gallagher et al., 2015). As a consequence, coastal polychaetes and mussels may commonly ingest microplastics in nature (Mathalon and Hill, 2014), setting up a long-term exposure to microplastics as expected for chronic contaminations. Thus, it is important to test the consistency of biological responses to microplastics observed under acute exposures, in chronic scenarios. This will support a more complete risk assessment for microplastics marine pollution by adding different scales of exposure (Underwood and Peterson, 1988). Coastal mussels are good biological models due to their susceptibility to ingest, retain and assimilate microplastics after acute exposures in laboratory experiments, suffering physiological effects as a consequence (Browne et al., 2008; Von Moos et al., 2012; Avio et al., 2015; Santana et al., submitted). With that, ingestion is already noted in nature (Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015).

The relevance of studying the effects of chronic exposures to microplastics on mussels goes beyond marine ecosystems conservation, becoming an issue of human interest. Humans worldwide eat mussels as seafood; and part of this supply originates

from farm systems made mainly by plastic materials (i.e. plastic sock nets and polypropylene long lines, Mathalon and Hill, 2014). In 2008, mussels comprised 12.4% of the total cultured molluscs in the world (FAO, 2012), reaching almost 2 million tons in 2010 (FAO, 2011). Even though both wild and farmed mussels are susceptible to microplastics contamination (Van Cauwenberghe and Jassen, 2014; Mathalon and Hill, 2014), a previous study showed that cultured mussels were more contaminated than wild ones (Mathalon and Hill, 2014). This suggests that farm systems can be other potential and constant source of microplastics to mussels, highlighting the importance of knowing the associated risks. Thus, understanding the biological effects of chronic exposures to microplastics allow the evaluation of possible consequences of microplastics marine pollution to the environment and how that might affect the seafood industry and its management.

In this context, we investigated the effect from chronically exposing the mussels *Perna perna* to polyvinyl chloride (PVC) particles to their (i) physiological rates; and (ii) signals of biochemical stress and health condition.

Methods

Microplastic and Organism

Biological and microplastic models were chosen to better illustrate a possible contamination scenario in nature, approximating laboratory responses to *in situ* events. PVC is the third most used thermoplastic in the world (ABIPLAST, 2012) and its intake by marine benthic invertebrates, including mussels, was already observed (Graham and Thompson, 2009; Browne et al., 2013). The industrial grade Emulsion/Microsuspension PVC (E/M PVC) is composed of spherical granules ranging from 0.1 to 1.0μm in diameter (Rodolfo et al., 2006). Their lack of porosity increases their bulk density (Rodolfo et al., 2006), facilitating the sinking and dispersion in the marine environment, raising their bioavailability.

The sessile nature and filter-feeding habits of mussels constantly subject them to different types of environmental stress (Goldberg, 1975), including microplastic pollution. *P. perna* is a coastal mussel of commercial importance (Fernandes et al., 2008), widely distributed in the Mediterranean and in tropical and subtropical regions of

the Atlantic and Indian Oceans (Henriques, 2004). As with other bivalves, it is easily cultivated and still extracted from natural banks for human consumption (Fernandes et al., 2008). Preliminary experiments showed that *P. perna* is susceptible to the intake of E/M PVC, to its assimilation in the hemolymph and retention in the gut for over 12 days (unpublished data). These data suggest that both this polymer and animal would be good models for testing biological effects of ingested microplastics.

Experimental Setup and analysis of effects

One hundred mussels (2.5cm average shell length) were purchased from a farm located at Lagoinha Beach, an unpolluted site in Ubatuba (São Paulo state, Brazil – 23˚31'S 45˚12'O). Epifauna was removed and mussels were left together for five days to acclimatize under salinity and temperature-controlled conditions (35‰; 21˚C). During this period, the organisms were fed only by the organic particulates present in the natural seawater.

The experimental setup consisted of 80 mussels (2.5 ± 0.43cm) randomly assigned to individual aquaria (2L) for 90 days. Forty mussels (half) were exposed to 0.125g/L ($1.115x10^{10}$ particles/L) of E/M PVC powder and the others were maintained as a control, under the same environmental conditions but without contact with microplastics. Ideally, the experimental design should have a concentration of microplastics based on field data for long-term exposures (e.g. amount of microplastics found in urbanized coastal environments). However, (i) there is a lack of field information on the quantity of plastics within the size range of our model (0.1 – 1.0μm of diameter); and, (ii) the breakdown of larger plastics, together with the use of primary microplastics of this size, increase the possibility that small microplastics (<100μm) might exist in marine environments in higher concentrations than has been measured for larger sizes. Thereby, we based the concentration on previous experimental research of microplastics impacts on mussels. The concentration of E/M PVC had to guarantee the intake of microplastics, but be lower than those tested before, and with a longer period of exposure. Thus, selection of the E/M PVC level used the lowest concentration tested for microplastics intake by mussels (0.125g/L, unpublished data). Although it corresponds to a much higher number of microplastics particles than field data, it is also 4 to 20 times lower than what was experimentally tested with mussels until now. The 90 days of constant exposure to the PVC was 10 to 720 times longer than the periods of

contamination already tested with mussels. This allowed us to study growth rate; which, in optimal conditions, represents 2cm mussels growing to 9cm in about 8 months (Ferreira and Magalhães, 2010).

During the assay, all aquaria were aerated and maintained with a 12h light-dark illumination regime, 23˚C, pH 7.5 - 8.0, and 35‰. Concentrations of ammonia, nitrite, and nitrate were also monitored. For logistical reasons (e.g. the distance between the source of seawater and the experimental site) the water in each aquarium was partially replaced three times per week. The organisms were fed every two days with 3 x 10^4 cells/mL of a 7:3 mixture of *Chaetoceros muelleri* to *Isochrysis* sp. (Microalgae Bank of Oceanography Institute - University of São Paulo, IO-USP). With each seawater renewal, PVC was replaced and microplastics intake was inferred by the presence of PVC in the feces of mussels. All experimental procedures were performed at IO-USP (São Paulo, SP).

After exposure, mussels were further analyzed to understand the effects of such microplastic contamination. The existence of physiological effects was evaluated through physiological rates (clearance rate, absorption efficiency, and growth rate) and signals of biochemical stress and health condition (biomarkers – lysosomal integrity, lipid peroxidation and DNA damage – condition index and mortality). To optimize the use of these indicators, single mussels were used for more than one type of analysis. The number of mussels analyzed for each physiological parameter is defined below.

Clearance Rate (CR)

The CR (volume of filtered water per hour) was estimated measuring the decrease in algal cell density of the experimental aquaria in relation to time, using the equation proposed by Omory and Ikeda (1984):

$$CR\,(L/h) = \frac{V}{N}\left\{\frac{Ln\,(Ci\,) - Ln\,(Cf\,)}{\Delta t} - \mathrm{f}\right\}$$

V = Volume of test flask (L)
N = Number of organisms per test flask
Ci = Initial concentration of phytoplankton
Cf = Final concentration of phytoplankton
Δt = Time of incubation (h)

f = Factor of correction (obtained by the same equation applied for control flasks)

After 24h of starvation in filtered seawater (0.7µm), 30 individuals exposed to microplastics and 30 from the control group were individually arranged in aquaria with filtered seawater and 140cells.mL^{-1} of *C. muelleri* for 1h. The quantities of phytoplankton present in the water at the beginning and end of the assay were measured by the Welschmeyer fluorometric method (excitation 665nm and emission at 665 and 673nm). The assay was conducted in the dark in order to avoid planktonic growth, under controlled conditions of salinity and temperature (35‰ and 23˚C).

Absorption Efficiency (AE)

The AE was determined using the relative content of organic matter (OM) present in the food (seston) given to mussels (I) compared to the OM of their feces collected at the end of the assay (F) (Conover, 1966):

$$EA(\%) = \left\{ \left(\frac{I - F}{1 - F} \right) I \right\} \times 100$$

The test was carried out with the same 60 mussels and under the same conditions as the CR assay, but run for 24h. The OM related to the phytoplankton and to the feces of the mussels was obtained by combustion in a muffle furnace for 2h, following the method described in Resgalla Jr. et al. (2007).

Growth Rate (GR)

The growth for each of the 80 mussels used in the experiment was estimated as proposed by Resgalla Jr. et al. (1999), considering the variation of their weight during the 3 month experiment (Wi and Wf):

$$GR\ (g/month) = \frac{[Ln\ (Wf) - Ln\ (Wi)]}{\Delta t}$$

Biomarkers

As early signs of biological impacts, cellular and molecular biomarkers of stress were analyzed in 30 randomly selected mussels, 15 from each treatment. Biomarkers were analyzed in samples of both gills and hemolymph due to their possible interaction with microplastics (Browne et al., 2008; von Moos et al., 2012; Avio et al., 2015), with interaction differences expected for the two tissues. The contact with the gills is an intense process because all microplastics in the aquaria are susceptible of it (due to mussels' filter-feeding habit). The contact between microplastics and hemolymph is more persistent, but requires a longer time period to occur as it depends on the translocation process within organisms. In addition, it also involves a smaller quantity of particles (Browne et al., 2008; unpublished data).

Lysosomal integrity – Neutral red retention time assay (NRRT)

The lysosomal integrity was evaluated in mussels' hemolymph using the neutral red technique, which relates hemocyte viability to the capacity of lysosomes to retain neutral red dye over time (Lowe et al., 1995). For that, 500µL of hemolymph were collected from the posterior adductor muscle of the mussels with a 2ml syringe containing 0.5mL of saline solution (pH 7.36). After homogenization, 40 μ L of each sample were placed on pre-treated slides with AGAR and incubated in a dark humid chamber for 15min. Thereafter, the slides were exposed to 40 μ L of working solution of neutral red dye (Moore, 1990, reagent from Invitrogen) and analyzed every 15min under light microscopy (400x) until 50% or more cells showed a leakage of neutral red dye into the cytosol and/or abnormalities in the color and size of lysosomes (Lowe et al., 1995; Pereira, 2008).

Lipid Peroxidation (LPO)

LPO was analyzed by the thiobarbituric acid method (TBAR) described by Wills (1987) whereby lipid oxidation is correlated to production of malondialdehyde (MDA, one of LPO chain byproducts). Gill samples were weighed and homogenized with a 4-time volume buffer (NaCl 100mM, HEPES-NaOH 25mM, EDTA 0.1mM, DTT 0.1 mM, pH 7.5). Thereafter, 150µL of the solution was mixed with 300µl of 10%

tricholoacetic acid (TCA, diluted in 1M $FeSO_4$) and 150µl of 0,67% thiobarbituric acid (TBA, diluted in deionized water). The mixture was incubated at 70˚C for 10min, and 200µL duplicates from supernatant were placed in a 96 well fluorescence microplate reader to be cooled on ice. The MDA produced was analyzed via fluorescence (spectrofluorimeter Synergy HT, BioTek) with excitation at 360nm and emission at 450nm. Quantification used a standard curve prepared with 0.001% tetramethoxypropane (TMP, diluted with 0.1M HCl). Blank wells were prepared with buffer used for homogenization. The results were expressed as µTBARs/mg of total protein. The total protein was obtained by Bradford method (reagent from Sigma Aldrich), following the manufacturer's instructions (quantification done by spectrophotometer (Spectramax 250) at 595nm absorbance, with blank and standards prepared using TBS).

DNA damage

DNA damage was quantified in gill samples by alkaline precipitation assay (Olive, 1998) and DNA strand breaks via fluorescence (Gagné et al., 1995). After weighing and homogenization with a 4-time volume buffer (same as for LPO), 25µL of tissue was mixed by inversion with 200µL of 2% SDS (with EDTA 10mM, Tris–base 10mM and NaOH 40mM) and 200µL of KCl. Thereafter, samples were incubated at 60˚C for 10min, homogenized by inversion, cooled at 4˚C for 10min and centrifuged at $8x10^3$g for 5min at 4˚C. In a 96 well microplate, they were mixed with a Hoechst dye working solution (1µg.mL^{-1} in NaCl 0.4M, Sodium Cholate 4mM and Tris-acetate 0.1M, pH 8.5-9.0, Gagné and Blaise, 1993). After shaking for 300 seconds, the strand breaks were analyzed by fluorescence (spectrofluorimeter Synergy HT, BioTek) at 360nm excitation and 460nm emission. The standard curve was prepared with salmon sperm DNA (Sigma Aldrich) and blank wells were prepared with buffer. The results were expressed as µg of damage DNA/µg of total protein, with total protein again obtained by the Bradford method.

Condition Index (CI)

The CI was calculated by each of the 80 mussels cultivated, half in each treatment, using the method suggested by Baird (1958), expressed by the ratio between soft body wet weight and the total weigh of living individuals:

$$CI = \frac{Wet\ meat\ weight\ (g)}{Total\ wet\ weigh\ (g)}$$

Data analysis

The results of biochemical, cellular and physiological parameters were compared between control and exposed mussels using Student-t tests. The significance level was $\alpha = 0.05$ for all tests. When necessary, data was transformed applying the most suitable transformations.

Results

At the beginning of the experiment (t = 0), all mussels were placed at the bottom of the aquaria. Over time, however, some migrated and got fixed on the surface of the water column, partially emerged (exposed to air). At the end of the experiment, 70% of the mussels were fixed at the surface. Filter-feeding activities were observed in both control and exposed groups through the presence of feces and pseudofeces in the aquaria. Animals partially emerged also continued to filter-feed. Microplastics intake by exposed mussels was confirmed by the presence of E/M PVC in their feces. Pseudofeces was found in much higher quantities in the aquaria of organisms exposed to PVC (Figure 2.1).

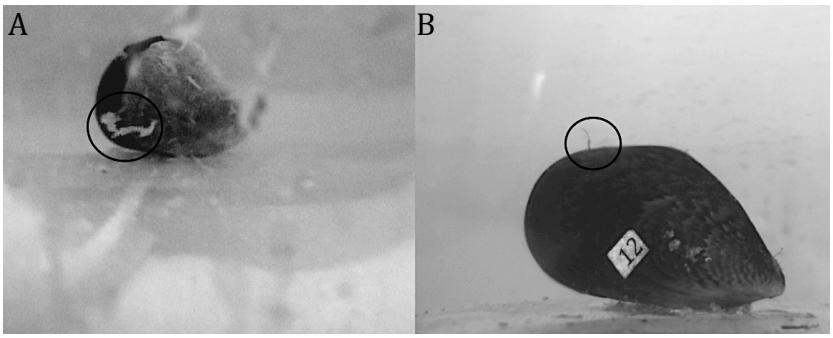

Figure 2.1: Production of pseudofeces by *Perna perna* exposed to PVC (A) and of feces by control (B).

No significant effects in physiological rates, biomarkers and health condition were evident for mussels chronically exposed to E/M PVC ($p > 0.05$, Figure 2.2). All responses varied widely among individuals, which may have influenced the significance of statistical analyses. Nevertheless, slightly trends between control and exposed mussels were observed.

The constant presence of microplastics in the water of exposed mussels led to 15.9% of an elevation in their CR (non-significant), rising from 1.57L/h filtered water by control mussels to 1.82L/h by exposed ones (Figure 2.2.A). The effect on the AE had opposite and even less remarkable results: 2.6% lower in mussels in contact with PVC. For both treatments, AE did not exceed 50% on average, with a response variability of ± 17.7% and 10.6% for control and exposed mussels, respectively (Figure 2.2.B).

Despite the absence of significant difference in AE, exposed mussels tended to have average higher growth rates than controls. The difference among treatments was 8.5% on average, with exposed mussels tending to grow from 1.85 ± 0.75 to 2.35 ± 1.01g over (0.20 ± the three months and control growing from 2.16 ± 0.70 to 2.65 ± 0.8g. Their growth rates were therefore 0.08 ± 0.07 and 0.07 ± 0.06g/month, respectively (Figure 2.2.C). Although exposed and control mussels did not have the same average size at t=0, growth rates were similar between the treatments, indicating that such differences did not affect our results.

Regarding stress biomarkers, the most remarkable trend was seen for NRRT. Neutral red dye leaked from lysosomes earlier for cells of exposed mussels than the control (48.0 ± 33.4min and 64.5 ± 32.9min, respectively). That corresponds to an average difference of 25.6%, but also with high variability among organisms from both treatments (Figure 2.2.D). Quantities of LPO were more similar between exposed and control *P. perna*, with a difference of only an average 4.8% (Figure 2.2.E). Contrary to

the other two markers, DNA damage was 7.4% lower in exposed mussels, corresponding to 78.5 ± 36.4µg damaged DNA/µg total proteins in comparison to 84.8 ± 32.5 µg damaged DNA/µg total proteins for control mussels (Figure 2.2.F). The average condition index (CI) of exposed mussels was 0.19 ± 0.04, 5.6% lower than unexposed mussels 0.05, Figure 2.2.G). During the 90 days of assay, no mortality was registered.

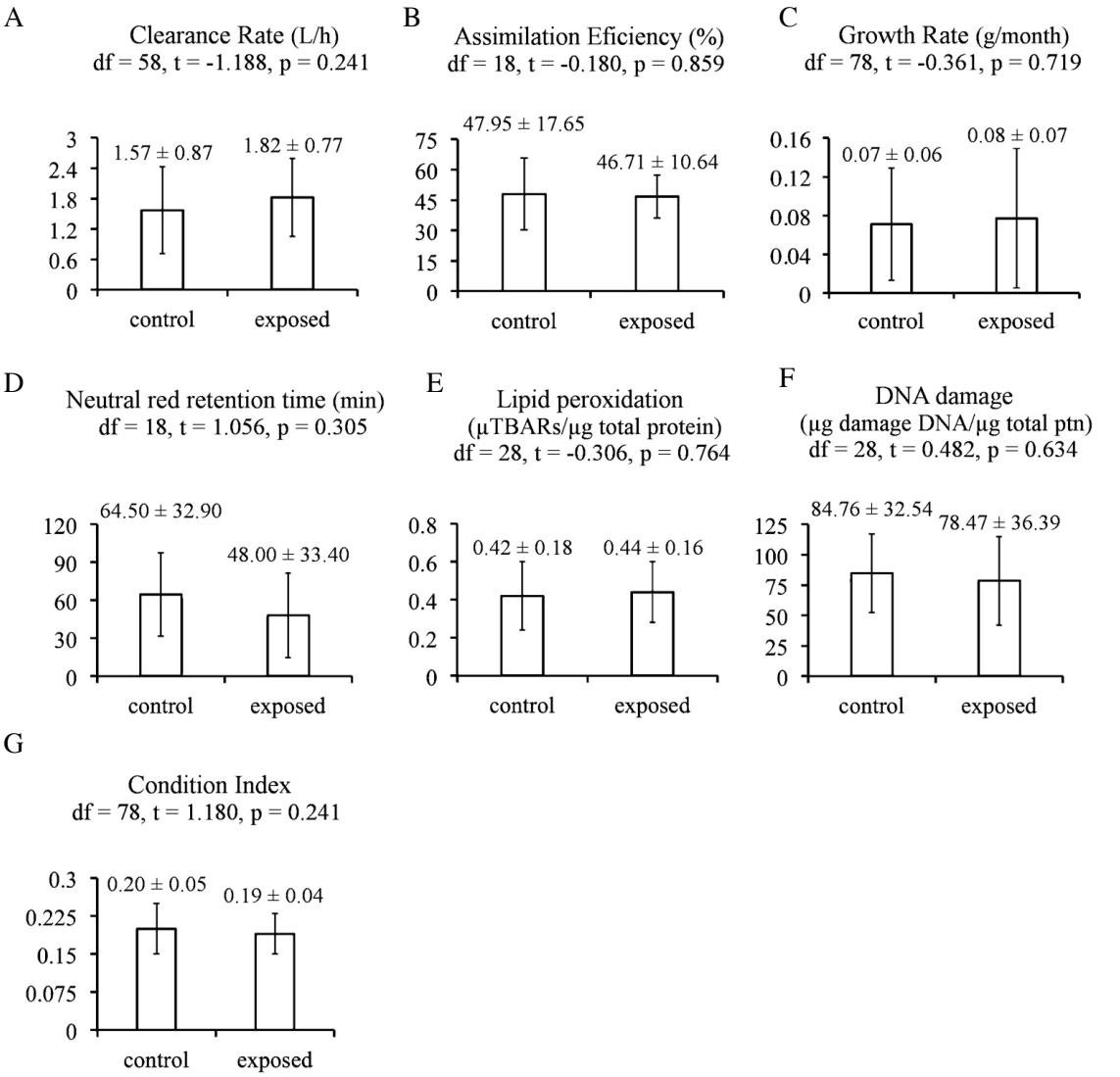

Figure 2.2: Physiological rates, biomarker and health condition results (mean ± SD and Student-t test. df = degree of freedom, $\alpha < 0.05$) of control and exposed mussels to Emulsion/Microsuspension PVC (E/M PVC) under chronic conditions (0.125g/L for 90 days). (A) Clearance Rate (L/h); (B) Absorption Efficiency (%); (C) Growth Rate (g/month); (D) Lysosomal Integrity measured by Neutral Red Retention Time Assay (min); (E) Lipid peroxidation (µTBARs/µg of total protein); (F) DNA damage (µg damage DNA/µg of total protein) and (G) Condition Index.

Discussion

Our results show that under the conditions tested, chronic exposures to microplastics did not trigger physiological impacts in mussels, a positive perspective considering chronic scenarios of contamination in marine environments. This, however, goes against what current research has been indicating, making necessary a critical understanding of our experimental features and limitations, including their possible effects on results.

To illustrate, a drop in the filtering activity of *Mytilus edulis* was observed due to nanoplastics (Wegner et al., 2012). Because the chosen nanoplastic model tended to aggregate to diameters of up to 1µm, differences in particulate size is not a plausible reason for our divergent results. The feeding activity of the lugworm *Arenicola marina* and the copepod *Centropages typicus* are also negatively affected by acute microplastics contamination (Besseling et al., 2013; Cole et al., 2013), while molluscs bivalves express oxidative stress, inflammation and apoptotic responses to microplastics intake (Von Moos et al., 2012; Avio et al., 2015; Santana et al., submitted). These examples were based on exposures that lasted from hours to few days (maximum of 28 days – Besseling et al., 2013), thus simulating short-term exposure and not chronic scenarios of contamination. A reasonable source of differences could be the long-term exposure evaluated in the present study. However, considering our model species, its biological features, and some of our experimental features, this lack of responses could equally be related to (i) mussels' low retention/capture efficiency for particles smaller than 1µm of diameter (i.e. leading to a low impact of E/M PVC intake); (ii) the low concentration of microplastics per aquaria (compared to most acute exposure studies); (iii) the high variability of responses among organisms; and (iv) mussels' phenotypic plasticity and their abilities for long-term recovery and acclimation to natural or anthropogenic stresses.

Some studies suggest that mussels have limited efficiency for capturing particles with very small sizes (Ward and Shumway, 2004; Ward and Kasch, 2009; Cranford et al., 2009). This could influence the impacts associated with the intake of small microplastics, leading to results similar to those presented here. However, not only did we observe PVC within mussel feces, but previous work also showed E/M PVC ingestion and assimilation by *P. perna* (unpublished data; Santana et al.,

submitted). Considering that even nanoplastics can be ingested by *M. edulis* (Wegner et al., 2012), we strongly agree that particle features other than size may contribute to the process of uptake selection by mussels (see Ward and Shumway, 2004), making argument (i) above inconsistent. Regarding the concentration of microplastics chosen for this study and those of preceding research, it is also reasonable to suggest that the effects previously observed were influenced by the enormous (and unrealistic) quantity of microplastics added to the aquaria. The concentration chosen for this assay was still high in comparison to field data and even sufficient to promote the rejection of pseudofeces by exposed mussels. During the three-month exposure, without exception, all mussels exposed to PVC (n = 40 in each treatment) frequently produced pseudofeces with microplastics, while control mussels rarely did so. Considering functions related to pseudofeces (rejection of non nutritive particles before intake and defense against high quantities of particulate matter suspended in the water) and the presence of PVC in the feces of exposed mussels (an indication of microplastics intake), we suggest that exposed mussels produced more pseudofeces to prevent an overload of particulate matter (Jorgensen, 1981) due to microplastics presence in the water. In this case, despite our consideration that 0.125g/L of PVC was a low concentration appropriate for chronic simulation, it induced defense mechanisms in the mussels, making assumption (ii) also inconsistent.

It seems reasonable now to suggest a reduction in the concentrations chosen for laboratory research for the investigation of contamination scenarios to simulate *in situ* conditions. However, current methodologies for sampling microplastics in the field are limited, underestimating the environmental abundance of microplastics of smaller sizes (e.g. < 100μm; GESAMP, 2015). These small particles are the most susceptible to marine bioavailability and the most used in experimental studies of microplastics' biological impacts. Thus, studies with microplastics smaller than 100μm remain restricted to hypothetical concentrations of microplastics in the environment, despite the advances in field data.

The variability of responses among organisms can also have influence on results (assumption iii). Some distinctions between samples are less likely to occur with highly variable samples (Underwood, 1997), so we could suggest that "the variability" influenced the power of Student-t test. This type of limitation could be avoided by increasing sample sizes to decrease estimate errors (Underwood, 1997). However, other

studies on microplastics' impacts on marine biota have been using sample sizes smaller than or similar to this study (e.g. Browne et al., 2008; von Moos et al., 2012; Wegner et al., 2012), and most found significant differences between control and exposed samples. Several intrinsic and extrinsic properties can naturally influence biological variability, from genetic aspects to environmental conditions (Underwood, 1997). Thermal stress (Helmuth and Hofmann, 2001), wave beating (Hunt and Scheibling, 2001), food availability (Foster-Smith, 1975), size (Zilberberg et al., 2011), and reproductive cycle (Bocchetti and Regoli, 2006) are examples of factors influencing organisms' responses. In our assay, some of these natural influences can have acted, leaving only slight trends of differences between control and exposed mussels. Seventy percent (70%) of mussels, for example, spent part or all experiment in the air-water interface of aquaria. This can have increased their variability on filtering and ingesting the food offered, and their stress related to desiccation, resulting in variable clearance rate and lysosomal integrity. Interferences like this can also occur in natural habitats and cannot be controlled in experimental studies. Considering our sample size and those used in previous studies, the variability of responses should not invalidate our results and their trends. Nevertheless, further studies could be done increasing the sample size to better understand this issue.

After evaluating all possibilities, we suggest that the most likely reason for the absence of physiological impacts is assumption (iv). It involves mussels' long-term acclimation to stresses, which seem to be a well-accepted process, but at the same time rarely investigated in laboratory studies of environmental pollution. Phenotypic plasticity is the ability of some genotypes to respond to environmental changes by modifying their phenotypic expression through morphological adjustments and/or physiological conditions (Schlichting, 1986). It is also related to biological responses to stress and their capacity of being up-regulated. For sessile and sedentary organisms in particular, survival can depend on phenotypic plasticity (Halpin et al., 2004).

We intended to put our research in the context of studies of physiological rate acclimations to marine pollution, but just few studies were found. Broadly considering abiotic factors, Bohle (1970) showed that mussels exposed to seawater with reduced salinity (75% of seawater salinity) required 4 weeks to adapt and regulate their filtration rates and reach similar clearance rates to the control. Juveniles of *Ostrea edulis*, if submitted to decreasing or increasing water temperatures (14 and 26˚C, respectively)

have acute physiological rate reactions followed by a process of compensation that rendered an overall improvement in their energy available for growth (Beiras et al., 1995). Thus, compensation mechanisms might also have contributed to our results, causing no significant differences among control and exposed mussels, but only slight variations between physiological rate treatments.

The E/M PVC led to a small but non-significant increase of the mean of CR. This slightly high mean for the CR of exposed mussels could be a consequence of microplastics chronic input and the elevation of non-nutritive matter present in the water. In this case, mussels could have acclimated to filtering more to efficiently ingest and assimilate nutritive particles (Foster-Smith, 1975; Bayne, 1993; Navarro, et al., 1996). Winter (1973) found a similar tendency for *M. edulis* exposed to low concentrations of algae, reinforcing this argument. The increase of CR on mussels exposed to PVC may also be the reason why AE (and GR) remained similar between groups. With the increased volume of filtered water, exposed mussels were able to maintain ingested and assimilated organic content as if under regular conditions.

According Resgalla and Brasil (2007), AE is the second most important parameter for energy gain in bivalves, responsible for enhancing the development of these organisms under optimal conditions. In this study, the AE of both exposed and control mussels was lower than 50% and did not have significant differences between treatments. This low AE can have influenced mussels' GR, which was notably small, especially for lab conditions. In optimal conditions, mussels (~2cm) can reach up 9cm in about 8 months (Ferreira and Magalhães, 2010), which did not happen in this work. This is probably related to the majority of mussels being partially emerged in aquaria, and to the lack of daily feeding of animals. Emerged mussels may have reduced the ability to capture the food offered in the seawater, reducing their growth. Moreover, logistics make it impossible to offer food to mussels every day or in a continual flux. Although we tried to compensate by offering a food density that exceeded nutritive values found in natural environment (see Anderson et al., 2002), the lack of constant food supply might have contributed to the low growth recorded.

Although long-term exposure did not cause significant effects on *P. perna* physiological rates, it could have caused adverse effects on lower levels of organization in exposed mussels, such as cellular and molecular structures. According to Helmuth (2009), very different patterns can exist through scales of biological signs, especially

when analyzed at the organismal level. Recently, several studies, including those related to microplastics pollution, evaluate cellular and biochemical signs of stress responses, relating them to preceding effects at higher levels of biological organization (Lam, 2009, e.g. Browne et al., 2008; Von Moos et al., 2012; Besseling et al., 2013). Regarding a mussel's susceptibility to ingest, retain and assimilate micro and nano particles with potential toxicity (Browne et al., 2008; Ward and Kach, 2009; Von Moos et al., 2012; Wegner et al., 2012; Avio et al., 2015; Santana et al., submitted), small microplastics are likely to have cellular and biochemical interactions and effects.

Plastic polymers can act as vector of toxic chemical compounds such as plastic additives (EPA, 1992; Teuten et al., 2009; Ananthaswamy, 2001; Tanaka et al., 2013) along with lesser known plastic monomers (Lithner et al., 2011). These and other types of pollutants generally have low molecular sizes, enabling them to interact with cells and other molecular components, generating physiological effects (Von Moos et al., 2012; Browne et al., 2013; Avio et al., 2015; Santana et al., submitted). Rationally, those responses could increase if the plastic exposure was chronic, indicating a time-response of microplastics impacts on *P. perna*. However, our results for lysosomal integrity, lipid peroxidation and DNA damage did not support this hypothesis.

None of the three biomarkers showed significant signs of stress responses; helping to invalidate our primary rationale that chronic exposure to microplastics would lead to great biological impacts. In contrast, the similarity of biomarkers' responses among exposed and control bivalves once again suggests that under long-term contamination from microplastics, *P. perna* may activate mechanisms of acclimatization (phenotype plasticity), enabling them to live regularly under these circumstances. Unfortunately no works relating phenotype plasticity to the chosen biomarkers exist to better illustrate this reasoning, and caution should be used due to the high variability of individual responses for all parameters tested, as demonstrated earlier. Nevertheless, adaptive responses were already observed for other molecular biomarkers as heat-shock proteins. Studies indicate that heat-shock proteins have different expressions related to adaptive and acclimatization responses to stresses (Hamdoun et al., 2003; Evans and Hofmann, 2012). In oysters, for example, the effects of suspended sediment on the expression of heat-shock proteins are related to acute and not chronic exposures (Cruz-Rodriguéz and Chu, 2002), highlighting the role of acclimatization in an analogous situation.

Considering previous studies with short-term exposures and our results, we could say that during the first days of PVC exposure, lysosomal integrity, lipid peroxidation and DNA damage can have indicated signs of stress (as observed by Santana et al. (submitted) under simulated acute exposures). However, over time and due to constant contamination their responses can have been up-regulated and their threshold induction (Morimoto, 1998) modified for the worst exposure conditions (e.g. higher concentration), an indication of acclimation. If culture farms can also be a source of microplastics due to the materials utilized in the structures (Mathalon and Hill, 2014), the model mussels could have been in prior contact with microplastics. This contact could have already induced adaptive responses on control and exposed mussels before the assay, leading to our results. Further studies could explore whether there is a time within 90 days when the responses of mussels become similar between treatments. This is a key information for investigations into risks from microplastics that should be investigated. Because it is suggested that farmed mussels can bet more contaminated by microplastics than wild ones (Mathalon and Hill, 2014), potential difference on in the responses/acclimation of these mussels to microplastic should also be explored.

The CI of exposed and control mussels were also not significantly different. Moreover, they were within the range considered as "good" by Marques (1988), suggesting that animals were in good health and that exposed mussels were not under stress (Lucas and Beninger, 1985), despite microplastic exposure. Together with the results of GR and mortality, the CI of mussels reinforce that "chronic exposures to microplastics do not affect brown mussels". Considering this, our results can be an indicative that chronic exposures to microplastics might not affect the productivity of commercialized *P. perna.* PVC exposure did not result in mortality and did not affect mussel health or weight gain over time. Therefore, the abundance and quality of brown mussels in a farm or natural bank would not be impacted by such exposure. However, while *P. perna* showed adaptability, other cultured and fished organisms may not have this ability. As suggested before, coastal environments and culturing systems can be chronic sources of microplastics (Browne et al., 2011; Mathalon and Hill, 2014; Gallagher et al., 2015), highlighting that effects of this type of scenario (and their complex variations) should be explored for multiple coastal and commercial species susceptible to microplastics intake. In addition, microplastics could chronically affect the health and development of marine organisms in other ways, which should also be

explored in the context of aquaculture and fishing systems. Microplastics can, for instance, act as a vector for pollutants and microorganisms adsorbed/attached on their surface (e.g. Lithner et al., 2009; Chua et al., 2014; Zettler et al., 2013; McCormick et al., 2014), both important issues commonly controlled by health surveillance.

Assessing a scenario of chronic contamination by microplastics not only allowed to study issues of environmental relevance but also some of social importance. After an exposure to 0.125g/L of PVC during 90 days, our results suggest that brown mussels *P. perna* may be able to adapt to chronic exposure to microplastics, which has important implications for environmental health and human food resources. In this scenario, time and potential acclimation were important features for mussels' physiological responses to microplastics contamination. However, this might not apply to other marine organisms susceptible to microplastics intake, especially non-filter feeding organisms, which may not have adaptive mechanisms to deal with particulate matter. Given the importance of this result for risk assessments of microplastics pollution, further works should explore similar scenarios of chronic exposures using different biological models.

Chapter 3

From biotransference to biomagnification of microplastics: what really threatens marine predators from microplastics pollution?

Introduction

Microplastics particles (plastics < 5mm, Arthur et al, 2009) have become a widespread threat to marine ecosystems, a material found from coastal regions to open ocean and deep sea (Claessens et al., 2011; van Cauwenberghe et al., 2013; Cózar et al., 2014). Their total quantity in oceans corresponds to 92.4% of the total plastic particle count (Eriksen et al., 2014), and their tendency is towards increase due to the breakdown of macro-particles and the recurrent input of new plastic waste (Barnes et al., 2009). This pollutant can be industrially produced on the microscopic scale (as abrasives beads for cosmetics products, plastic pellets and PVC powder, called "primary microplastics" – Fendall and Sewell, 2009; Andrady et al., 2011), or be a result of a larger plastic's degradation ("secondary microplastics"; Browne et al., 2007; Andrady et al., 2011; Zettler et al., 2013). Nevertheless, both reach ocean compartments (e.g. water column and bottom) due to large-volume consumption and inadequate disposal of plastic products. Along with increasing quantities, natural marine processes such as hydrodynamics and biofouling contribute to microplastics' fragmentation, dispersion, and sinking (Andrady, 2011; Cózar et al., 2014; Eriksen et al., 2014), widening their distribution within environments.

Beyond abundance, microplastics are of concern because of their small size and potential to affect wildlife. As plastics get smaller, higher are their chances of being ingested and wider is the range of organisms susceptible to it (Cózar et al., 2014). The ingestion of microplastics was reported in different marine species, from pelagic and benthic habitats, with different feeding strategies and from different trophic levels (Tourinho et al., 2010; Murray and Cowie, 2011; Lusher et al., 2013; Cole et al., 2013). Most studies, however, only associate it with a direct uptake from the environment (sediment or water), usually related to organisms' feeding habits (e.g. filter- and

deposit-feeders), or when animals confound plastics with their prey (e.g., fish, sea birds and turtles). The intake of microplastics through contaminated food was poorly understood as a route of contact of marine biota and until recent years, being just speculative, even for organisms from higher trophic levels (e.g. fish).

Given this knowledge gap, experimental works were developed aiming to investigate the biotransference of microplastics (transfer of compounds from a food source to its consumer – Barwick and Maher, 2003). Using different types of polymers, along with benthic and pelagic species, the few studies conducted supported this hypothesis, but most merely observed microplastics' transference to the gut of predators (Murray and Cowie, 2011; Farrel and Nelson, 2013; Watts et al., 2014; Setäla et al., 2014), suggesting a temporary presence in the organism with no cellular interactions. One study, however, also observed microplastics' assimilation by predators (Farrel and Nelson, 2013), thereby increasing the effectiveness of microplastics' biotransference and its associated risks (e.g. persistence and cellular stress). Microplastics biotransference to the tissue of predators also raise the hypothesis of bioaccumulation through food intake and, as a consequence, magnification along the food web. If microplastics accumulate along the food web, the chances of higher trophic levels be exposed to the impacts related to microplastics intake could increase (e.g. oxidative stress, inflammatory reactions, endocrine disruptions, fitness disturbances and transference of other toxic substances – von Moos et al., 2012; Browne et al., 2013; Wright et al., 2013a,b; Besseling et al., 2013, Rochman et al., 2014; Santana et al., submitted), highlighting the need for works related to these processes. Although these processes are better studied for dissolved compounds, bioaccumulation has been observed for nano-particulate matter in marine organisms (e.g. Pan et al., 2012; Hanna et al., 2013), which may be an indication that such risks exist for microparticles.

Bioaccumulation and biomagnification correspond to biological processes by which organisms take up and accumulate assimilated chemicals and/or particles (i) from abiotic and biotic (food) environments (bioaccumulation) or (ii) exclusively from contaminated food (biomagnification). Both, therefore, promote a higher concentration of the pollutant in these organisms in comparison to what was found in the previous environment or trophic level (Connell, 1990; Rand et al., 1995 – Figure 3.1.C). This higher concentration is the main issue for the risks of a pollutant for high trophic levels. Both processes presuppose that predators go through more than one episode of exposure

to the contaminant (i.e. uptake of microplastics from the water more than once or intake from more than one contaminated prey). For dietary bioaccumulation to occur, for example, predators should feed on more than one contaminated prey, allowing the concentration of the pollutant to rise despite predators' mechanisms of regulating storage within their bodies (Gray, 2002 – Figure 3.1.C). Furthermore, biomagnification also requires that at least two trophic levels (prey and predator) accumulate the compound (Barwick and Maher, 2003 – Figure 3.1.C), which has not been confirmed for microplastics.

It is also worth mentioning that the potential occurrence of these processes depends on microplastics passing the first barrier of organisms' digestive system, translocating to the tissues of predators and being retained there (Setälä et al., 2014 – Figure 3.1.A, B and C). Therefore, prior to bioaccumulation and magnification, assessments should look for microplastics' assimilation and retention in higher trophic organisms, so far only observed in one species of blue crab (Farrel and Nelson, 2013).

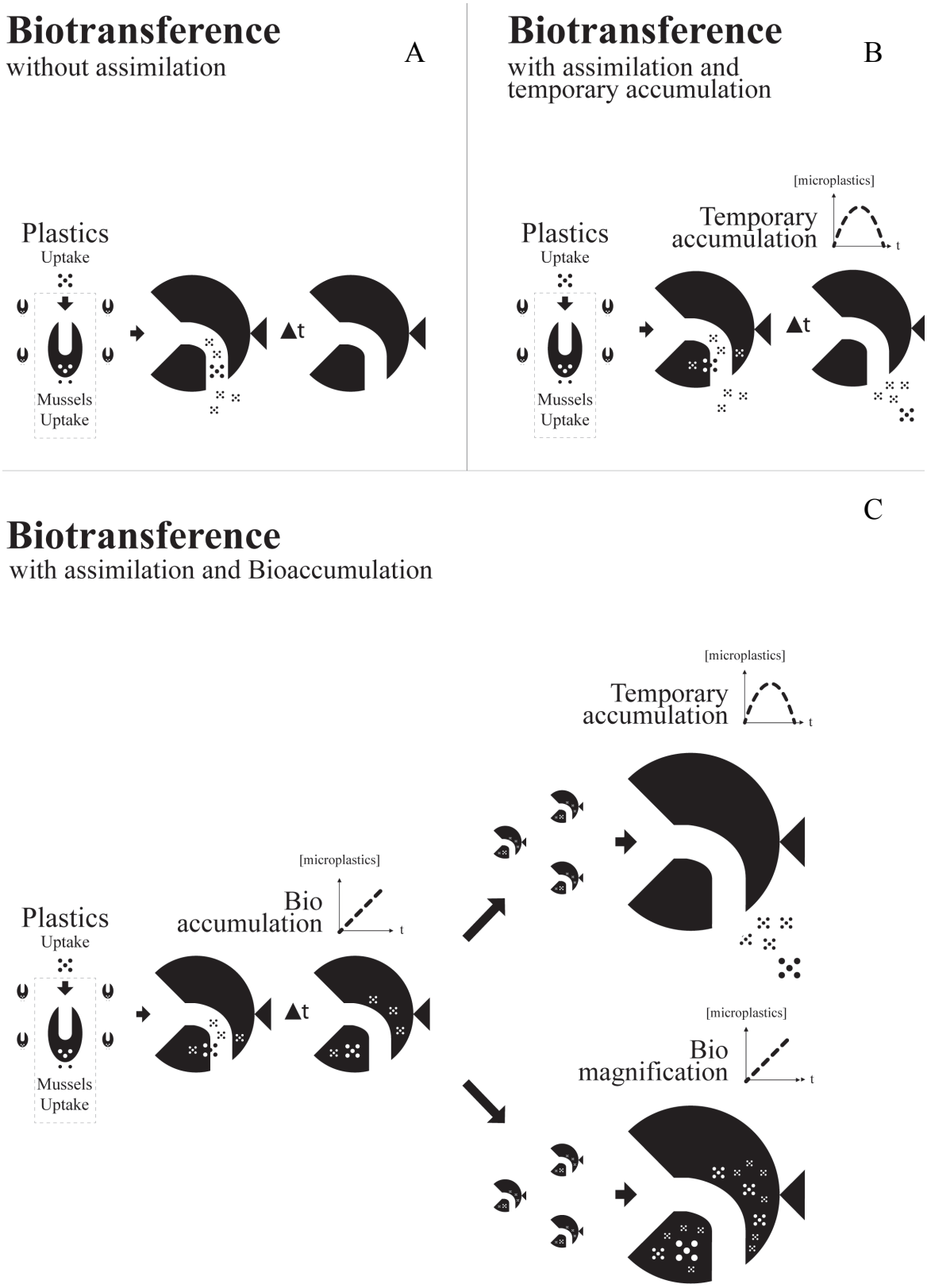

Figure 3.1: Theorical scheme of microplastics uptake leading to (A) biotransference of microplastics without assimilation of particles; (B) biotransference of microplastics with assimilation and temporary accumulation or particles; (C) biotransference of microplastics with assimilation and increasing accumulation of particles, causing a greater concentration of microplastics in the organism than in previous environment (bioaccumulation). As a consequence of bioaccumulation, following predators could biomagnify or reject the ingested microplastics with time (C). Microplastics accumulation on organisms depends on their capacity of rejecting the ingested and assimilated particles within tissues.

As some top predators are not directly exposed to microplastics uptake from the environment, biotransference is hypothesized to be an important route for establishing this contamination, and microplastics' assimilation and accumulation along food web would be the main trigger of their potential effects. Humans are good examples for this situation and, in this case, such environmental threat also becomes an issue for fisheries and aquaculture sectors. To illustrate this risk, microplastics were found in the digestive tract of commercial marine organisms such as fish (cod, herring and mackerel – Lusher et al., 2011; Foekma et al., 2013), mussels (Van Cauwenberghe and Janssen, 2014; Mathalon and Hill, 2014) and lobster (Murray and Cowie, 2011). Moreover, microplastics' contamination of bivalves has been observed under cultured conditions (Van Cauwenberghe and Janssen, 2014; Mathalon and Hill), highlighting how close the risk of microplastics' biotransference can be to humans. Although most commercial species do not have their digestive tract included among the edible parts, the presence of microplastics in their gut suggests that microplastics assimilation can be occurring in these organisms, increasing the susceptibility of this animal to accumulate this pollutant. The presence of microplastics in some of these commercial species is also an indication that microplastics' biotransference could already be occurring in the natural environment.

This study assessed microplastics' transference, assimilation and retention within the food chain using an experimental approach that simulated a more realistic scenario of prey contamination and predation, and that also allowed us to have some indication of the risks of bioaccumulation. Contaminated but depurated (i.e., without microplastics in the digestive tract) brown mussels (*Perna perna*) were used as prey, and as predators a swimming crab (*Callinectes ornatus*) and a fish (*Spheoeroides greeleyi*). We assumed that microplastics' pollution is dynamic and occurs in pulses (patches) in coastal systems; thus not constantly contaminating organisms susceptible to direct uptake from environment (such as mussels), and reducing the average chance of predators to eat contaminated prey immediately after their exposure to microplastics (i.e. full of microplastics in their digestive tract). To infer indications of bioaccumulation, predators were fed for 10 days with contaminated prey. We hypothesized that microplastics could be transferred, assimilated and retained along the food webs, but in different ways between predator species due to their potentially distinct mechanisms of regulating the concentration of xenobiotics within the body.

Beyond being the first assessment of microplastics' biotransference with features that allow dietary bioaccumulation, this is the first investigation of microplastics' assimilation in fish, an important issue considering its relevance for the environment and food industry. Since mussels are already known to assimilate micro and nanoplastics (Browne et al., 2008; Ward and Kach, 2009), their chances of becoming prey while contaminated increase, making direct predators more susceptible to biotransference and further impacts. While the confirmation of these hypotheses increases the risks of bioaccumulation and expands the number of species susceptible to microplastics impacts, opposite results may minimize potential microplastics impacts, suggesting that food intake cannot be the major route of concern for microplastic uptake by and accumulation in organisms from higher trophic levels.

Methods

Due to the variety of microplastics types, sizes and shapes, there are numerous exposure scenarios to explore for any associated biological impacts. In the present study, we aimed to include a reliable scenario of microplastic contamination that could maximize the chances of assimilation and retention occurrence through food intake. Therefore, part of experimental design is in accordance to what can be found in the environment (e.g. type of microplastic) but parts are not (e.g. quantity of microplastics to which mussels were exposed). We prefer to overestimate the chances of accumulation in mussel tissues to evaluate if the biotransference processes between trophic levels is feasible.

Microplastic Model

Polyvinyl chloride (PVC) is one of the polymers most produced worldwide and, not surprisingly, is commonly encountered in marine environments (Andrady, 2011). Its versatility makes it present everywhere, from tubes to civil industry, children's toys and hospital supplies (Rodolfo et al., 2006). Among the types of PVC produced, we worked with Emulsion/Microsuspension (E/M) resins. They are spherical particles with a size range of 0.1 to 1.0μm in diameter, but reaching sizes of 100 μm (50 μm on average) due to their tendency to agglomerate (Rodolfo et al., 2006). The

particles of E/M PVC have high densities and tend to sink in seawater, spreading easily throughout the water column and sea bottom, so reach a wide range of organisms. E/M PVC can be found in marine environments due to their loss during marine translocations, particularly when vessels are being loaded and unload (Pereira, 2014). In addition, the size of this microplastic approximates the nano-scale, possibly increasing its similarity with nanoparticles, already observed to be bioaccumulated in marine organisms.

Biological Models

Mussels were chosen as prey model due to their susceptibility to ingestion and assimilation of microplastics (Browne et al., 2008; von Moos et al., 2012). More specifically, the brown mussel *Perna perna* was chosen for susceptibility to ingestion and assimilation of E/M PVC (unpublished data). The filter-feeding nature of mussels facilitates the uptake of inorganic particles (such as plastics) along with food, perhaps allowing the temporary accumulation of microplastics (Setälä et al., 2014). In Brazil, this species can be found in abundance from the Espírito Santo to Rio Grande do Sul states (Fernandes et al., 2008), and is an important food source for organisms from higher trophic levels, including humans. In 2006, 90 tons of mussels were cultured for human consumption in the State of São Paulo alone (IBAMA, 2008), the macro region from which mussels were collected for the assays.

Blue crabs and pufferfish were chosen as natural predators of molluscan bivalves, enabling a realistic simulation of a trophic chain. *Callinectes ornatus* is a portunidae swimming crab, common in tropical marine and estuarine environments (Guerra-Castro et al., 2007). This species is abundant in the region (Mantelatto and Fransozo, 2000) and has a diverse omnivorous diet, with a clear preference for crustaceans, molluscs (including bivalves) and fish, regardless of their size and sex (Mantelatto and Christofoletti, 2001). *Spheoeroides greeleyi* is a tetraodontidae pufferfish that is abundant along the Brazilian coast (Figueiredo and Menezes, 2000), typically found in bays and estuaries (Schultz et al., 2002). They feed mostly on gastropods, bivalves and crustaceans (Targett, 1978) and are an important component of trophic chains in their natural habitats (Schultz et al., 2002). Both species were collected at the Ubatuba research field station of the Oceanographic Institute of the University of

São Paulo, located on the north coast of São Paulo State, where the assays were conducted.

Contaminated Mussels

Two hundred mussels (4.98 ± 0.32cm in shell length) were purchased from an aquaculture system (Lagoinha Beach, Ubatuba, São Paulo) and acclimatized for three days. They were maintained in a tank (1000L) with open circulation system of natural seawater, where abiotic factors (temperature, pH and salinity) were monitored. Mussels fed natural organic matter from seawater in the tanks.

After acclimatization, three mussels were collected to verify if prey were free of microplastics prior to E/M PVC exposure. These mussels had their hemolymph and soft tissues sampled for subsequent analyses of background microplastic contamination. The other mussels were placed in 20 aquaria (12L each) (10 mussels per aquarium) and exposed to 0.5g/L of E/M PVC for 3h, following Browne et al. (2008). During PVC exposure, all mussels appeared to be feeding (e.g. opened valves and production of feces and pseudofeces). Following exposure, mussels were returned to the previous tank (1000L) with clean seawater, and remained there for 12 days to depurate their digestive tract but also to ensure the maximum assimilation of microplastics in their hemolymph, as observed by Browne et al. (2008). The idea behind the depuration period was that predators are not always going to eat prey while microplastics are retained in their tract. This step of the experimental design aimed to simulate a more realistic scenario of microplastics biotransference, assuming that prey do not ingest large amounts of microplastics continuously, raising their chances of being consumed with only assimilates microplastics (clean gut but contaminated tissues). That is because (i) microplastics keep moving in a marine environment (Eriksen et al., 2014), which makes an organism's contamination something dynamic (they are not constantly ingesting microplastics); and (ii) organisms will, eventually, defecate the particles present in their tract. Previous experiments already showed that *P. perna* eliminate ingested E/M PVC from their gut within 12 days after a single exposure, while assimilated particles can be found in their hemolymph until 20 days in clean seawater (unpublished data). For depurating, mussels were kept under the same conditions as in acclimatization period. By the end of 12 days, all were collected, opened and had their soft tissues separated

from their valves. The soft tissues were then frozen to be further used as prey (food) for blue crabs and pufferfish.

Assuming that some natural variation could act on mussels' uptake and assimilation of microplastics, three *P. perna* were randomly selected to quantify the E/M PVC present on their hemolymph and soft tissue after the 12 days of depuration. For each mussel, 500µl of hemolymph was collected from the abductor muscle and smeared directly onto glass slides. Thereafter, slides were fixed in methanol (15min) and stained with H&E (hematoxylin and eosin) for microplastics quantification (see below). The soft tissue was cut into 5mm diameter pieces, placed into glass bottles and fixed with 70% ethanol for further microplastic quantification. The quantity of microplastics assimilated by mussels (important information for assessing bioaccumulation through the food web) was established afterward as the mean quantity of PVC found assimilated within these three mussels. This method was also applied to the mussels collected in the field and used as a control.

Feeding Assay

The experimental design of the feeding assay consisted on predators being fed *ad libitum* (i.e., animals ate as much as they wanted, with no restriction) for 10 days with contaminated mussels and during 10 subsequent days with uncontaminated prey. Nine blue crabs (5.39 ± 0.45cm in length) and nine pufferfish (10.27 ± 0.42cm in total length) were individually placed in seawater aquaria (12L) for acclimation and assay. During the whole period pre- and post-exposure, seawater was constantly aerated, partially changed (30%) every 24 hours, and had abiotic factors monitored (i.e. temperature, salinity and pH). The light and dark conditions followed cycles of 12h.

The 10 days of feeding with contaminated mussels guarantee that predators ate enough mussels to allow the development of possible scenarios of bioaccumulation through food intake. Feeding occurred once per day and the quantity (in grams) of contaminated mussels eaten by each crab and pufferfish noted after every meal. Over the following 10 days, however, both species of predators remained in aquaria and were fed with fish, mussels and shrimps without assimilated PVC. The days of depuration allowed *C. ornatus* and *S. greeleyi* to pass through intake, assimilation and rejection stages, enabling the evaluation of microplastics' retention within organisms.

Theoretically, if predators still contained assimilated microplastics after this last period, there would be a potential risk of bioaccumulation through food intake, which could be better investigated in further works.

After the 10 days of depuration, *C. ornatus* and *S. greeleyi* were collected and parts of their tissues were sampled to investigate the transference, assimilation and retention of microplastics along trophic levels. For sampling, organisms were anesthetized at 4º C. Thereafter, blue crabs and pufferfish were measured in length (cm), weight (g) and had their digestive tract, hepatopancreas (blue crabs), liver (pufferfish), gonads, hemolymph (blue crabs) and blood (pufferfish) sampled. The hemolymph and the blood were collected from the base of the first pereopod (Nelson and Farrel, 2013) and from the vessels of caudal region (Ishikawa et al., 2010), respectively. Just as for mussels, both solutions were smeared onto glass slides, fixed in methanol (15min) and stained with H&E for further analysis. The rest of the tissues were cut in 5mm pieces, placed into glass bottles, fixed with Bouin for 24h and preserved in ethanol 70% for further microplastics quantification.

The presence of PVC was also investigated in the feces of predators, helping to confirm microplastics' intake (biotransference). Feces were sampled daily, after 24h of predator feeding. Samples of feces were separated into two groups through time (corresponding to the periods when predators were fed with contaminated or uncontaminated prey) and preserved in 70% ethanol for further analysis. As a control group, three extra blue crabs and three extra pufferfish (5.57 ± 0.45cm and 10.27 ± 0.42cm in length, respectively) were collected at the same site as the others and directly dissected, fixed and preserved. The absence of microplastics in their tissues was an indicator that exposed organisms were free of microplastics before the present study.

Sample Analysis

The soft tissues of mussels and the fecal material of predators were processed following the optimized alkaline digestion protocol for organic matter proposed by Cole et al. (2013) (digestion efficacy of 91.3 ± 0.4%). Samples were maintained in glass tubes with 10M NaOH at 60º C for 24h to reduce the biological material, leaving mostly the inorganic matter. Post-digestion, samples were vacuum-filtered onto GF/F filters (Whatman, 25mm diameter, 0.7µm mesh) and washed with distilled water. Filter

contents were then removed and placed on glass slides to be analyzed under optical microscopy with polarized light (PLM), a method suggested by von Moos et al. (2012) for investigating microplastic particles. Each mussel's hemolymph was also analyzed by PLM.

The preserved tissues of *C. ornatus* and *S. greeleyi* were dehydrated with an ethanol series, included in Paraplast, cut in 4µm thick sections with a semi-manual microtome, and stained with H&E. The histological procedure applied followed Behemer et al. (1976) and the slides were also analyzed by PLM under stereomicroscope (Leica DM750). Despite being time-consuming, histological sections allow us to identify the specific location where microplastics are allocated in organisms, unlike the method of organic matter digestion. Thus, it allowed identification of the assimilated particles and the elimination of potential samples with particles still present in the digestive tract of the analyzed tissues, which would not be related to assimilation and retention.

Results

The exposure to 0.5g/L of E/M PVC followed by the 12 days of depuration made all three analyzed mussels assimilate an average of 12.67 ± 2.49 microplastics/µL within their hemolymph. Their soft tissues were free of microplastics.

Table 1 discriminates the quantity (in grams) of contaminated mussels preyed on by *C. ornatus* and *S. greeleyi* during the first 10 days of feeding. During this first phase, 4 pufferfish did not to eat mussels for a few days, but not during the whole contaminated feeding period (Table 1). Since the average weight of mussels offered was 2.58 ± 0.51g, all predators had eaten more than one bivalve after 10 days of predation, ensuring a condition of microplastics contamination that could reflect the risks of bioaccumulation through food web (Table 1).

Table 3.1: Quantity (in grams and units of mussels) of contaminated mussels preyed on by *C. ornatus* and by *S. greeleyi* during the first 10 days of feeding. For total quantity, (A) and (SD) represent average and standard deviation. (--) represents the days on which pufferfish did not eat.

Quantity of contaminated mussels eaten by predators														
	Quantity (in grams) per day and in total											Total quantity (in units)		
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day	8th day	9th day	10th day	Total	A	A - SD	A + SD
Callinectes ornatus														
1	1.48	0.96	1.1	2.38	1.3	1.5	1.4	1.00	0.56	1.56	13.24	5.14	4.29	6.41
2	1.63	0.97	1.1	2.76	0.94	1.25	1.01	1.7	0.61	0.78	12.75	4.95	4.13	6.17
3	0.71	1.25	0.6	3.12	0.79	1.54	1.82	1.41	0.99	1.29	13.52	5.25	4.38	6.55
4	1.05	0.77	1.9	2.87	2.38	1.71	0.83	1.69	1.10	0.9	15.2	5.90	4.93	7.36
5	0.99	0.83	2.4	3.24	1.84	1.6	2.02	1.45	1.16	1.0	16.53	6.42	5.36	8.00
6	1.33	1.06	2.4	3.18	2.35	2.25	1.80	2.10	1.87	1.25	19.59	7.61	6.35	9.49
7	0.83	1.01	1.8	2.50	1.05	1.81	0.75	0.92	0.86	0.84	12.37	4.80	4.01	5.99
8	1.26	0.95	1.5	4.03	0.7	1.92	1.01	1.00	0.72	0.92	14.01	5.44	4.54	6.78
9	1.11	0.97	2.8	3.03	2.06	1.5	2.20	1.65	0.89	1.8	18.01	6.99	5.84	8.72
Spheoeroides greeleyi														
1	1.43	0.64	--	--	--	1.15	1.50	1.30	1.44	1.14	8.6	3.33	2.78	4.15
2	1.87	0.95	2.20	1.22	1.45	1.70	1.50	1.36	1.89	1.8	15.94	6.18	5.16	7.70
3	1.19	1.3	1.20	1.35	1.05	1.09	1.69	1.04	0.82	1.2	11.93	4.62	3.86	5.76
4	1.07	--	0.85	1.27	1.35	1.08	0.90	0.82	0.74	1.00	9.08	3.52	2.94	4.39
5	--	--	--	0.69	0.60	0.83	1.91	0.65	0.55	0.83	6.06	2.35	1.96	2.93
6	1.06	1.00	0.50	0.66	1.44	0.69	1.30	1.15	1.78	1.02	10.6	4.11	3.43	5.12
7	--	--	0.70	2.36	1.04	1.54	0.62	1.20	0.94	0.98	9.38	3.64	3.04	4.53
8	1.07	1.10	0.80	1.06	1.95	1.57	0.65	1.56	1.95	1.4	13.11	5.08	4.24	6.33
9	--	--	0.90	1.69	0.80	0.78	0.99	0.99	1.09	0.90	8.14	3.16	2.63	3.93

At the end of the assay, after the predators' contamination and depuration period, microplastics were only identified in their feces, without being observed assimilated in their tissues. This suggests that although microplastics were biotransferred from contaminated prey (where the particles of PVC were assimilated in the hemolymph) to the digestive tract of predators, it was either (i) followed by a temporary and rapid assimilation, or (ii) directly rejected as feces, both preventing

microplastics' retention in organisms. Nevertheless, all *C. ornatus* and *S. greeleyi* had PVC in their feces, only while they were fed with contaminated mussels. During their feeding with uncontaminated prey, feces were free of microplastics.

The quantity of mussels eaten by predators (Table 1) showed that the methodological precaution of 10-day feeding with contaminated prey ensured that *C. ornatus* and *S. greeleyi* ate several mussels, enabling discussion of dietary accumulation risks. Under the present experimental conditions, the lack of microplastics in tissues of predators reduce the possibility of this pollutant bioaccumulating through food intake.

Discussion

The marine environment is continuously loaded with microplastic pollution and not much is known about the consequences. Although some organisms are susceptible to direct uptake from seawater and, the risks for higher trophic level organisms (e.g. humans) still needs to be understood. Microplastics' transference, assimilation and retention through food ingestion are key processes to better understand the real impacts of these pollutants along marine food webs (e.g. Pan et al., 2012; Buffet, et al., 2012; Hanna et al., 2013).

This study explored the potential of these processes to occur by simulating a scenario where the offered prey (source of microplastics to the food web) had only contaminated tissues (and no particles in the digestive tract). When preyed, mussels had already rejected the microplastics previously retained in their gut and not assimilated in their tissues. The rationale of this approach is that this situation is closest to realistic conditions for predators becoming contaminated through their prey. Plastic pollution keeps moving in the ocean by the action of winds and surface currents (Eriksen et al., 2014). Therefore, apart from input regions and sink environments, such dynamic exposure probably promotes microplastics' short-term contact with organisms, raising their chances of having a gut clearance between uptake events. This would reduce the probability of predators feeding on prey immediately after their exposure to microplastics, while they still have a digestive tract full of these particles.

At the same time, we extrapolated some experimental components to promote an environment fully supportive of testing the hypothesis of assimilation and retention,

and to discuss possible indications of bioaccumulation. Mussels were (i) exposed to 0.5g/L of PVC, a concentration a thousand times greater than what is currently estimated to the total ocean surfaces (relation based on published data by Eriksen et al., 2014); and (ii) left depurating for 12 days to be eaten by predators with the maximum quantity of assimilated microplastics in the hemolymph (Browne et al., 2008; unpublished data). Predators also fed *ad libidum* on these maximum contaminated prey for 10 days, which may also not easily occur in the environment. Results showed that E/M PVC was not retained in predators. Thus, we suggest that bioaccumulation of microplastic particles through food intake is unlikely given the current ocean contamination levels and these tested organisms.

Contaminants can be metabolized or excreted by organisms (Gray, 2002), making their concentration a balance between their uptake, assimilation and elimination. Such balance can preclude bioaccumulation and biomagnification if animals from higher trophic levels (predators) do not assimilate the pollutant or eliminate it in quantities that end up less concentrated than in their prey. Here, all *C. ornatus* and *S. greeleyi* ingested the E/M PVC particles but also eliminated them within their feces. Microplastics were not found in the hemolymph and blood, nor in the other tissues of these blue crabs and pufferfish, suggesting that they rejected PVC particles without accumulating or even assimilating them. Such rejection created, therefore, a balance that prevents the risks of microplastic bioaccumulation through food chain.

Similarly to our experiment, Watts et al. (2014) fed *C. maenas* with blue mussels contaminated with 10µm polystyrene (PS) microspheres (0.5g/L) and also did not identify microplastics assimilation. However, 0.5µm PS microspheres were observed for 21 days within the hemolymph of *C. maenas* fed with only one contaminated mussel (with a peak after 24h, Farrel and Nelson, 2013). Since both studies used the same polymer and species as models, we hypothesize that the different result is related to the size of the plastic models. It would be reasonable to hypothesize that E/M PVC could translocate from the midgut of *C. ornatus* and be assimilated within their tissues as observed by Farrel and Nelson (2013). Moreover, since PS beads were retained in *C. maenas* up to 21 days after they fed on contaminated mussels, the 10 days of microplastics-free feeding of our assay is not a plausible reason for the lack of PVC assimilation and retention. The most notable difference between our experimental designs could perfectly explain such apparent incongruence. Farrel and Nelson (2013)

fed their crabs with fresh contaminated mussels without depuration. Thus, the amount of microplastics ingested by *C. maenas* was much greater than *C. ornatus* and *S. greeleyi* were susceptible of eating in the present study (*P. perna* had only 12.67 ± 2.49 PVC microparticles/µL of hemolymph), increasing the chances that *C. maenas* assimilated 0.5µm PS particles. This suggests that the concentration of microplastics in the prey has a relevant effect for a hazardous level of biotransference, in which microplastics are not just eaten by predators but also assimilated and retained.

The period of exposure is also important for such biotransference. Mussels (and probably other prey) depurate microplastic with time, clearing their gut and decreasing the quantity of particles within their bodies. In addition, both the quantity of microplastics in the environment (seawater) and their period of exposure were observed to influence their intake and assimilation by mussels (unpublished data). Thus, these factors could have a wide influence on microplastics assimilation by marine organisms, regardless of if it occurs directly from the environment (as for filter feeders) or through the food web (from the tissues of prey to the tissues of predators). The variety of experimental designs brought us several clues to evaluate the risks of an efficient transfer of microplastics along marine food webs (i.e. with microplastics' assimilation by predators). It highlights how dynamic and unpredictable such risks can be, apparently changing depending on (i) species, (ii) microplastics type, and (iii) scenario of prey contamination and predation.

Because our experimental design aimed to simulate a realistic scenario, the lack of retention of PVC in *C. ornatus* and *S. greeleyi* suggests that the chances of microplastics reaching the next trophic level (i.e. predators of crabs and pufferfish) are low and associated with the period that PVC were present in their digestive tracts (estimated as 24h in the present study). Nevertheless, microplastic particles were transferred from mussels to the digestive tract of predators, which is concerning by itself. It is reasonable to suppose that if biotransference occurs in a less effective manner (i.e. without microplastics assimilation, as observed in this study), the risks of interactions among microplastic particles and cellular components (Syberg et al., 2015), for example, will decrease and some physiological effects may not exist. However, the simple presence of microplastics in the digestive tract can also bring consequences, as the vectorization of toxic compounds adsorbed on their surface (e.g., organic and inorganic pollutants, and plastic additives, Teuten et al., 2009; Bakir et al; 2014;

Koelmans et al., 2014; Mizukawa et al., 2009). After its ingestion, these substances can leach from the surface of the polymer by digestive mechanisms and be assimilated within organisms' tissues (e.g.: Bakir et al., 2014). The transfer of microplastics along food chains may, therefore, create a new route for contaminants to reach animals from high trophic levels, ones that usually take up pollutants from food and, seldon, from the environment. Microplastic intake and the leaching of adsorbed chemicals were already shown to cause accumulation of PCB and disruption of feeding in lugworms *Arenicola marina* (Besseling et al., 2013; Browne et al., 2014, respectively), signs of stress in brown mussels *Perna perna* (Santana et al., submitted), and endocrine disruption in the fish *Oryzias latipes* (Rochman et al., 2014). However, these pieces of evidence of physiological effects were identified in organisms whose microplastics uptake happened directly from the environment and not through their food, demonstrating a further lack of information that should be explored to assess microplastics' risks.

Different types of polymers, with different sizes, were shown to be translocated from zooplankton, bivalves and fish to crabs, lobsters and other fish (Murray and Cowie, 2011; Farrel and Nelson, 2013; Watts et al., 2014; Setälä et al., 2014), demonstrating that both benthic and pelagic food webs may be susceptible to microplastics transfer (Setälä et al., 2014). Because marine predators have a wide range of prey (Gray, 2002), this risk is not restricted to a few pathways and could therefore reach different food chains. Organisms that uptake microplastics directly from the environment, for instance, can also be directly preyed on by top predators, a threat still little explored in current research. Humans (i.e.) consume organisms susceptible to microplastics intake from different trophic levels, including those that uptake the plastics directly from the environment. Considering this, which risks of microplastics contamination have potential to impact human life? Our results suggest that commercial organisms eaten as a whole (including the digestive tract) are the most worrying. In this case, biotransference does not depend on microplastics assimilation and accumulation, but only on their intake by the prey (food) and the residence time of the particles, particulary in their digestive tract.

To illustrate, humans eat mussels and other shellfish as a whole organism, which can become a potential vector of microplastics for us. Wild and cultured mussels were already observed as contaminated with microplastics particles (Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe et al., 2015) and a recent study

observed that farmed mussels could be more contaminated than wild ones (Methalon and Hill, 2014). This might be related to mussels' constant contact with fragments of plastic materials used for farming structures (Mathalon and Hill, 2014), which means that the risks of contamination and biotransference can be higher in scenarios of continuous microplastic input (another indication of the relevance of the period of exposure for the impacts of microplastics pollution). Once studies showed that microplastics do not bioaccumulate on mussels (Browne et al., 2008; unpublished data), depuration procedures could be a requirement for the sanitary certification of shellfish sellers to prevent microplastics' biotransference to humans.

As observed in this study, dietary accumulation of xenobiotics is not a rule in marine ecosystems because pollutants can be eliminated by organisms (Gray, 2002). In a more realistic scenario of food web transference, microplastics were not assimilated and retained in predators of mussels. Microplastics' transference only occurred from prey tissues to predators' tract, reducing the likelihood of the existence of bioaccumulation through food intake in current reality. Some factors were suggested to influence the magnitude of microplastics' biotransference, enabling predators to assimilate these particles, and increasing intake-associated impacts. Such discussion highlighted complex and dynamic processes that should be considered to assess ecological and feeding risks of microplastics.

Chapter 4

Microplastics contamination in mussels' natural beds from a Brazilian urbanized coastal region: an initial evaluation for further bioassessments

Introduction

In the last decades, plastic marine pollution became ubiquitous across the globe (Barnes et al., 2009), recently estimated at 5.35 trillion particles (~268,940 tons) floating along sea and ocean surfaces (Eriksen et al., 2014). The large-scale consumption of plastic products and poor management practices (Jambeck et al 2015) raises their potential risks of being lost to the environment during production, transportation, use and discard; once in the ocean, they act as persistent pollutants by lasting hundreds to thousands of years (Moore, 2008; Barnes et al., 2009). Despite this clear accumulation trend, the fate and consequences of plastics marine pollution are just beginning to be understood.

Among global marine plastic debris, 92.4% of the items are microplastics (Eriksen et al., 2014). These comprise plastic particles with less than 5mm diameter (Arthur et al., 2009), intentionally produced within this size range (primary microplastics) or originating from the fragmentation of larger plastic products (secondary microplastics) (Andrady, 2011; GESAMP, 2015). Microplastics are suggested to pose a special threat to marine ecosystems due to their high bioavailability, persistence, and capacity to adsorb and to be a vector of toxic substances to marine biota (Mato et al., 2001; Moore, 2008; Turra et al., 2014). Their small size makes them available for ingestion by a large number of organisms, including a variety of small invertebrates such as zooplankton (Cole et al., 2013), polychaetes (Besseling et al., 2013), bivalves (Browne et al., 2008; Van Cauwenberghe and Jassen, 2014), ascidians (unpublished data), equinoderms (Graham and Thompson, 2009) and sponges (unpublished data). As a consequence, physiological disturbances can in theory occur; some have already been described under laboratory conditions (von Moos et al., 2012;

Browne et al., 2013; Besseling et al., 2013; Rochman et al., 2014).

Microplastics ingestion by different marine groups and species also made it a plausible pathway for microplastics' exportation among marine compartments (e.g. watter column and bottom – Eriksen et al., 2014). In this case, microplastics' uptake could be responsible for plastics transference from the sea surface to the water column and sea bottom (via plastics rejection as feces and marine snow, Wright et al., 2013a), or to the trophic chains (via ingestion of contaminated prey by higher trophic levels (Murray and Cowie, 2011; Farrel and Nelson, 2013; Setäla et al., 2014, Santana et al., submitted), both broadening the risks of microplastic pollution to a wide range of marine organisms and ecosystems.

About eighty percent of plastics present in marine systems originate from land-based activities (Andrady, 2011). Therefore, densely urbanized coastal areas are both great sources and sinks of microplastics. Worldwide coastal populations contribute marine debris (including plastics) either through litter or inadequate disposal of wastes that eventually enter the ocean via rivers, wastewater outflows, etc. (Jambeck et al., 2015). Fifty percent of primary microplastics produced in the USA and used in cosmetics products, for instance, were estimated to pass through sewage treatment and reach marine environments (Gouin et al., 2011). Browne et al. (2011) reported eighteen shorelines along six different continents as contaminated with microplastics, and found a positive relationship among these particles' abundance and densely populated areas, suggesting a high relevance of coastal cities to the input of microplastics marine pollution. Microplastics have also been reported in estuaries and sandy beaches all over the world (e.g. Cole et al., 2011; Lima et al., 2014; Lee et al., 2013; Turra et al., 2014; Vedolin, 2014; Gallagher et al., 2015). When coastal areas hold a large variety of ecosystems (e.g. mangrove forests, estuaries, beaches and coral reef systems), many of them considered the most diverse of the world and responsible for supporting different goods and services (such as food and the biodiversity itself – Martinéz et al., 2007), microplastics input and impacts from them should be considered an important issue to be assessed.

For humans, the direct risks brought by microplastics marine pollution are associated with their bioavailability to food resources, becoming a matter of food safety. A great part of fisheries, shellfisheries and aquaculture systems are concentrated in coastal regions or nearby, which makes microplastics another worrying contaminant for

humans' health beyond those already well known, such as persistent organic pollutants (POPs) and metals. Recent studies addressed the contamination of commercial organisms in nature (Lusher et al., 2011; Foekma et al., 2013; Van Cauwenberghe and Jassen, 2014; Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015), approximating microplastic impacts to humans and thus increasing related concerns.

To investigate microplastics' contamination in nature, three marine compartments could be used: water column, sediment and biota. However, the abundance (concentration) in water or sediment does not always reflect the quality of the living resources (EPA, 2000), which should be considered the major concern for environmental health. The presence of microplastics in seawater and on the sea bottom seem to have a stochastic pattern, influenced by oceanographic biotic and abiotic forces, such as the development of biofilms, bioturbation, flood tide, winds, currents and wave fronts (Turra et al., 2014; Eriksen et al., 2014; GESAMP, 2015; Gallagher et al., 2015). All these factors can temporally influence either microplastics' re-suspension from bottom sediments and distribution in depth or along sea surface, raising the variability of estimates on microplastics' abundance in these compartments. The composition of microplastics in an environment can vary according to the sampling materials, and the ability to identify them varies with plastic size (GESAMP, 2015). To illustrate that, most studies assessing water column have used plankton nets for collecting samples (Gallagher et al., 2015), which underestimates the abundance of microplastics smaller than their mesh size. Experimental studies on microplastics intake and effects on marine biota use particles with less than 1µm (Santana et al., submitted) up to 80µm diameter (von Moos et al., 2012) as plastic models, sizes that are not retained by plankton nets. This methodological bias suggests that the current evaluation of abiotic compartments may not be fully supportive of risk assessments, leaving out relevant data to the hazard of microplastics to marine biota.

The use of biological indicators, in contrast, relies on the relationship between the organism and the polluted environment (EPA, 2000), helping to understand the realistic risks of the potential biotic impacts observed in laboratory studies. Due to the variety of microplastics types, sizes and shapes, bioassessments allow the understanding of the most threatening plastics for marine biota, for example. Initiatives of evaluating microplastic pollution in marine environments using sedentary invertebrates as

bioindicators are just beginning but already drew attention, especially when bivalves for human consumption were reported contaminated (Van Cauwenberghe and Janssen, 2014). Nevertheless, there are no standardized protocols for assessment of microplastics in organisms as there are for persistent organic pollutants (e.g. Tanabe and Tatsukawa, 1987 and the Mussel Watch Program), highlighting the need for additional methodological developments. One significant problem of biomonitoring microplastics pollution is the lack of efficient and standardized methodologies for extracting and identifying the particles, making it difficult to compare studies and discuss the results.

The goal of this study was to widen the estimates of microplastic contamination in nature using marine biota as sentinels. We analyzed the presence of microplastics on the filter-feeding mussel *Perna perna* around Santos Estuary (Southeastern Brazil). Santos Estuary is an important Brazilian coastal region, strongly influenced by industrial, port and urban activities, being the most urbanized area on the coast of São Paulo State, Brazil. As a first and rapid method to assess the state of microplastic contamination of the region, we identified the frequency of occurrence of such contamination on six natural mussel beds in the area. The use of this species of bivalve was based on (i) their features commonly appreciated for the purpose of bioassessments (e.g. wide distribution, sedentary lifestyle, easy sampling and accumulation of chemicals – NOAA, 1995); and (ii) their importance as food resource. In addition, because of the incipient use of bioindicators for microplastics pollution, we also discussed methodological aspects that might be relevant for establishing applicable tools for analyzing biological matrices.

Methods

Assessed area: Santos Estuary

The marine environmental health of Santos is a longstanding cause of concern but not much is known about its state regarding microplastic pollution. From the beginning of 20th century, this region has been strongly affected by anthropogenic activities (David, 2007), housing the largest port in South America (Santos Harbour); one of the most important industrial complexes in Brazil (Cubatão industrial complex, Cesar et al., 2007; Fisner et al., 2013a) and a well-established tourism flux that may reached up to a 4.7million people in the summer season (data for 2012; Santos Turism

Office, 2014). Considering potential sources of microplastics to coastal regions, all these characteristics can contribute to the microplastics contamination in Santos Estuary, as detailed below.

Besides the solid waste produced by vessels that berth in Santos Harbor (including plastic packing ships), virgin plastic pellets (granules of 5mm, average diameter, made from different types of polymers, such as polyethylene and polypropylene, EPA, 1992), and Emultion/Microsuspension PVC (small dense microspheres with a size ranging from 0.1 to 1.0µm diameter, Rodolfo et al., 2006) are among the types of loads handled in this port. Both types of pre-consumption microplastics can potentially be impacting the estuary after accidental losses (Pereira, 2014), putting marine biota at risk from their associated impacts. Probably as a consequence of these losses, Santos Bay was already observed to have high quantities of pellets, with a standing stock calculated at 762 million particles (Turra et al., 2014).

Other pollution sources such as landfills and sewage also contribute to the degradation of this estuary; these are important sources for microplastics' contamination of coastal environments, especially during tourist periods when waste treatments reach maximum capacities. For over 30 years, all solid waste from Santos' city was destined for a dumpsite in the neighborhood of *Alemoa*, an area close to the estuarine system. Although currently inactive, old losses of plastic waste from this dumpsite can still serve as a microplastics input for the marine ecosystems of Santos, because of slow degradation and persistence of plastics in marine environments (David, 2007). Sewage discharges may also be introducing both microplastics used in cosmetic industries (Fendall and Sewell, 2009) and those derived from washing synthetic clothes (e.g. polyester fibers, Browne et al., 2011) because, generally speaking, sewage treatment plants are not specifically designed to retain microplastics particles (Browne et al., 2011). Large volumes of sewage discharges along the estuary of Santos occur clandestinely and without any treatment (Martins, 2005), raising plastic and microplastics input in an immeasurable way.

The multiple uses of the region also resulted in the introduction of several other contaminants in the estuary. Some of them were adsorbed on the surface of pellets sampled in Santos Bay (Fisner et al., 2013a and b), bringing toxic effects to marine organisms whenever leached (Nobre et al., 2015), increasing the risks to local biota.

Bivalve collection: sampling sites

In September 2014, mussels were collected in 6 random natural beds downstream from the Santos Harbor terminal used for loading microplastics (Figure 1). The chosen points were downstream of the harbor due to the preferable transport of the estuarine waters towards the port channel and Santos Bay (Fukumoto, 2007), thus increasing the chances of covering any of the possible sources of microplastics mentioned above. The sampled area of the estuary covered: an area close to the terminal used to (un)load microplastics at the Santos Harbor (sampling point #1); a substrate close to an irregular occupation that clandestinely discharges sewage into the estuary (sampling point #2); a point close to the vehicle ferry that bridges the cities of Santos and Guarujá (sampling point #3); fishing warehouses (sampling point #4); ferries (sampling point #5); a pier commonly used for fishing (sampling point #6) and other anthropogenic activities.

At each sampling point, five *P. perna* were randomly collected during the low tide, totaling 30 mussels (4.3 ± 0.99cm in length) for all assessments. As a first assessment of an area where no previous data exists on the presence of microplastics susceptible to invertebrates' ingestion, it was decided to work with a low sample size, avoiding unnecessary uses of biological samples. Organisms were removed from substrates by cutting their byssus, then frozen, without depuration in clean seawater to ensure preservation of all ingested microplastics, including the particles retained in the digestive tract and not just those translocated to tissues.

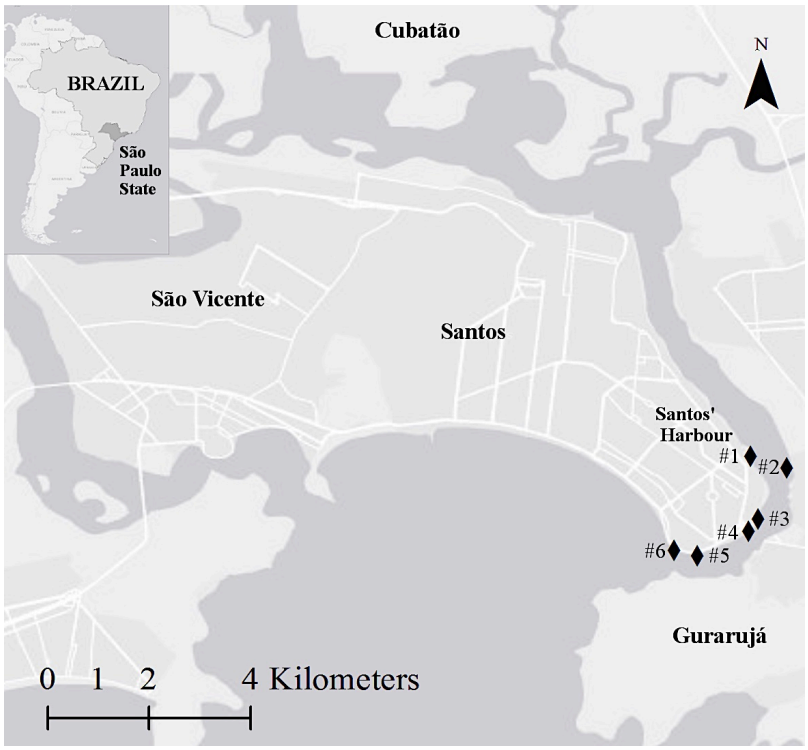

Figure 4.1: Map of the sampling area – Santos Estuary (São Paulo State, Brazil) – indicating the sampling points: #1 (23˚58'26.760S, 46˚17'35.880W – terminal used to (un)load microplastics at the Santos Harbor); #2 (23˚58'34.28S, 46˚17'12.47W – irregular occupation with clandestine sewage discharges into the estuary); #3 (23˚59'6,75S, 46˚17'31.07W – vehicle ferry); #4 (23˚59'14.85S, 46˚17'36.97W – fishing warehouses); #5 (23˚59'30.62S, 46˚18'9.88W – ferries); #6 (23˚59'27.08S, 46˚18'24.79W – pier used for fishing).

Tracking ingested microplastics in mussels: procedures for organic matter digestion and microplastics identification

The evaluation of microplastics within collected mussels was performed by adapting the microplastics' extraction method proposed by Claessens et al. (2013) and analyzing the samples under a polarized light microscope (PLM). This extraction method is a procedure of organic matter digestion that removes biological materials from samples, leaving mostly dissolved and particulate inorganic matters to be analyzed. For that, mussels were individually submitted to an overnight digestion with HNO_3 (22.5M) at room temperature, followed by: 15 minutes of boiling, dilution with distilled water, and filtering. The period of boiling was less than the validated protocol, aiming to minimize the risks of microplastics degradation during the acid digestion (Claessens et al., 2013). In contrast, the final solution was filtered in 0.7µm GF/F filters (Whatman, 25mm of diameter) to optimize the size range of microplastics retained by the filter mesh. The material trapped on filters was carefully scraped, slightly diluted

with absolute ethanol and placed on glass slides, thereby avoiding formation of clusters and accelerating the drying. Thereafter, glass slides were observed by PLM for microplastics identification. PLM is a contrast-enhancing technique used for anisotropic materials (natural or not), and suggested by von Moos et al. (2012) for microplastics' investigation in stained tissue sections (biological samples). In this study, it was adapted to analyze the glass slides prepared with the remains of organic matter digestion.

To prevent sample contamination with airborne microplastics fibers, all material and equipment used for the organic matter digestion was cleaned with distilled water prior to use, and the procedures were performed in a fume hood. The lab coat used during the assay was 100% cotton and, whenever possible, flasks and other apparatus were made of glass. As we did not use a blank sample to normalize our results with airborne fibers (Witte et al., 2014; Van Cauwenberghe and Janssen, 2014), we decided to disregard fibers found in samples (only two records). Hypothetically, another possible error could come from the contamination of glass slides by GF/F fibers scraped with the sample after the digestion procedure. To verify if GF/F zests could interfere with the assessment, glass slides were prepared solely with these fibers and analyzed by PLM to check the polarization of such material. The results showed that GF/F do not polarize.

Establishing the relative frequency of microplastics contamination

If one particle of microplastic was found in a mussel, it was considered contaminated. Based on the relation between contaminated mussels and total of organisms collected per bed, we established the relative frequency of contamination of each sampling point. It is worth remembering that the purpose of this study was to perform a simple and rapid survey of the status of microplastics pollution of Santos Estuary. Therefore, the quantity of microplastics found in the organism was not counted. Nevertheless, the relevance of these and other types of data (e.g. polymer type) are discussed later.

Results

Microplastics were detected in 75% of mussels sampled in Santos Estuary. All sampling sites had at least one contaminated *P. perna*, and 3 sites had all mussels contaminated by polymer particles (Figure 2). The less contaminated mussel bed was #6, with only one *P. perna* contaminated. This point was the farthest point of the study area and the closest to Santos Bay. Sampling sites #1, #3 and #5 had 100% of analyzed mussels contaminated with microplastics. Sampling site #4 showed 60% contaminated. The ascending order of sampling points in relation to the frequency of contaminated mussels was *#6 < #4 < #2 < #1 = #3 = #5*.

Figure 4.2: Illustrative figure showing the relative amount of contaminated mussels per sampling point. Circle sizes illustrate how contaminated each mussel bed were (in percentage of mussels sampled containing microplastics – see legend). Ascending order of sampling points contamination: *#6 < #4 < #2 < #1 = #3 = #5*.

The procedures used for mitigating possible contamination of samples during lab activities were efficient. The main sources of microplastics contamination seemed to be avoided because only two of the microplastics identified were fibers. The majority of microplastics found were white and had irregular shapes (Figure 3A and B). However, the organic matter digestion and the ethanol dilution of the filtered remains (before mounting the glass slides) were not fully effective. In some slides, we could still identify organic matter and some areas had clogged samples that could have hindered the identification of ingested plastic particles (Figure 3C).

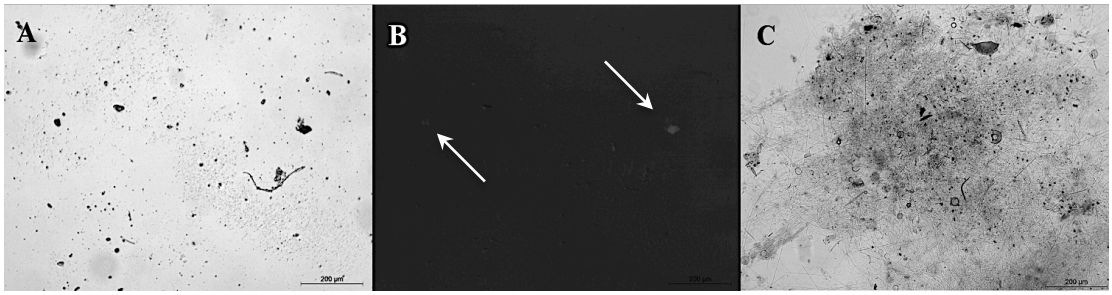

Figure 4.3: (A) Illustrative figure of sample slides seen under microscopy (B) and polarized light microscopy, PLM, with arrows indicating polarized particles of plastic. (C) Example of organic matter remains in clogged samples.

Discussion

Seventy-five percent (75%) of the mussels sampled in Santos Estuary had ingested microplastics, and at five out of the six sampling sites more than 50% of the mussels were contaminated. This was a preliminary assessment, with a small sample size (n = 5) and just a few sampling points (n = 6), with no quantitative data regarding the microplastics found in mussels. Still, our results suggest that Santos Estuary is highly polluted by microplastics, as clearly shown by sentinels organisms. Other studies have reported microplastics uptake by invertebrates in natural habitats (Van Cauwenberghe and Jassen, 2014; Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015), but this is the first assessment for *P. perna* species, an organism found along different coasts and frequently consumed by humans.

P. perna is an abundant organism, widely distributed across tropical and subtropical coastal environments of the Atlantic and Indian Oceans (Henriques, 2004), and found from Southeastern to Southern Brazilian coasts (Fernandes et al., 2008). As other marine bivalves, it is an animal from near the base of the food chain, important as a food resource for higher trophic level organisms, including humans. As a food resource for humans, this species is commonly collected from natural beds or cultured in systems deployed directly in the marine environments, allowing their food supply to come from natural seawater. Therefore, even cultured mussels are not free from the risks of microplastics ingestion, as observed by Mathalon and Hill (2014) and Van Cauwenberghe and Jassen (2014). Although mussel farms are not common in the study

area, the shores of Santos region are among the largest natural beds of *P. perna* in São Paulo State (Henriques et al., 2001), and harvesting is a common practice in the estuary for human consumption and sale (David, 2007). Therefore, the presence of microplastic particles in mussels in this area is a relevant issue to not only the environmental health of the estuary but also human food safety. Previous studies indicate that microplastics can be ingested and assimilated by mussels, persisting in the digestive tract and hemolymph for over 12 and 48 days after a single exposure, respectively (Browne et al., 2008). Unfortunately, organic matter digestions do not allow to infer if particles are assimilated in tissues or retained in the mussels' digestive tracts, important if estimating the magnitude of mussels' risks in nature. Nevertheless, these residence time of microplastics within mussels suggest that environments with potential frequent inputs, such as estuaries, have high risks of mussels being permanently contaminated. Thereby, we suggest the adoption of debugging procedures as a sanitary precaution for shellfisheries. Further research could be dedicated to better understand possible variations in these periods of retention according to organisms (focusing on commercial species, including other bivalves such as oysters), type of plastic pollution and contamination scenario (i.e. concentration and time of exposure).

About 60% of the largest cities in the world are located around estuaries, an important ecosystem for both marine biota (known as marine nurseries) and human activities (Martins, 2005). As a connection between land and ocean, estuaries serve as receptacles of natural and anthropogenic products; the entry of odd substances into them can harm living resources, including humans (Miranda et al., 2002). Plastic debris can enter an estuarine system through both land-based activities and oceanic waters. The former source, however, is responsible for nearly 80% of plastics found in the marine environment (Andrady, 2011) and we suggest it as the main contributor to microplastics input in the Santos Estuary.

Despite the lack of data about further consequences of microplastics ingestion in the field, laboratory studies have already shown cellular damage, feeding disruption and signs of related stress (von Moos et al., 2012; Browne et al., 2013; Besseling et al., 2013; Wright et al., 2013b; Rochman et al., 2014; Santana et al., submitted). Persistent organic pollutants (POPs) and other xenobiotics can also be transported and released by microplastics (Browne et al., 2013; Besseling et al., 2013), increasing, therefore, the health risks of an environment contaminated by such particles. As an example, ingested

microparticles of PVC with nonylphenol and triclosan were observed to be a better vector of these chemicals for lugworms than ingested sediment (Browne et al., 2013). Thus, it is feasible to suggest that the higher the contaminations of a certain area (with microplastics, POPs and others), the higher are the chances of microplastics adsorption and vectorization of chemical toxic substances to organisms. In the study area, plastic pellets (~5mm diameter) sampled in Santos Bay were highly contaminated by PAHs (Fisner et al., 2012). Although these pellets were found in sedimentary compartments of Santos Bay beaches, the authors suggest that their main source is Santos Harbor and their contamination with organic pollutant is a consequence of adsorption mechanisms during their residence in the highly polluted Santos Estuary (Manzano, 2009; Fisner et al., 2013a). Knowing that decreasing in size raises the surface/volume ratio of a particle (Mato et al., 2001), we fear that greater quantities of dissolved compounds are being adsorbed on these micrometric plastics ingested by mussels at Santos Estuary. Considering local harvesting of *P. perna* for human consumption, it is important to better understand the risks of ingested microplastics act as a vector for other pollutants. This is a matter of global concern and the impacts of microplastics on commercial species should be further explored. As important, few studies identified the role of microplastics as a substrate for pathogenic and non-pathogenic microorganisms' development (Zetler et al., 2013; Reisser et al., 2014; McCormick et al., 2014). Although incipient, this indicates that microplastics can also act as a vector for diseases (McCormick et al., 2014). Whenever this impact reaches commercial species, organisms susceptible to plastics ingestion and humans could be vulnerable.

According to Browne et al. (2010), plastic particles can be found in almost any habitat of an estuary, and their spatial distribution patterns can vary on a short scale (Ryan et al., 2009). Santos Estuary illustrates that well, presenting contaminated mussels at all sampling sites but without a predictable spatial distribution. The three mussel beds fully contaminated with microplastics were not consecutive along the channel, but intercalated with less contaminated ones; from sampling site #5 to sampling site #6, the relative abundance of contaminated mussels fell 80%. The specific type, size and density of microplastics may be the reason for that due to their influence on particle distribution (Browne et al., 2010; Cole et al., 2011; Lima et al., 2014). Low-density polymers, for instance, tend to float in the water column, and in an estuary their sinking will depend on processes such as water fronts (Cole et al., 2011). High-density

polymers are temporally suspended in the water as a function of turbulence, tidal fronts and high rivers flow, but their tendency is to deposit faster than others (Browne et al., 2010; Cole et al., 2011), which decreases their ability to disperse in the environment. Finally, small plastic particles are easily transported by the water flow and tend to sink where the hydrodynamics are less intense (Browne et al., 2010), which varies according to meteorological and oceanographic conditions. With all these variables, we suggest that microplastics bioavailability along the Santos Estuary have a stochastic pattern, varying temporally according to environmental conditions and type of microplastics input, which should be further investigated. This highlights the importance of characterizing microplastics types and environmental conditions of a contaminated area to develop a full risk assessment. Moreover, because environmental conditions can change with time, it is also essential to consider having a follow-up/monitoring of these features to better identify the most vulnerable situations for organisms.

Due to methodological issues, we did not classify the ingested microplastics according to type or origin. Thus we were not able to establish the relative importance of each microplastics input to bioavailability in Santos Estuary. Identifying microplastics sources is extremely relevant for public policies and should be more incentivized to reduce the input of microplastics into marine ecosystems. For further studies, we suggest this type of analysis, more specifically for ingested microplastics since bioavailability does not always directly reflect environmental contamination (EPA, 2000); the major type of microplastic present in the water column may not be the most ingested and harmful microplastic to marine biota.

Although *in-situ* evaluations and monitoring of microplastic ingestion by marine organisms are important, they are not trivial tasks. Sampling and assessing methodologies are still in development, and many limitations need to be remedied to enrich data and support more discussions. In our study, a simple and rapid assessment reflected the range of microplastics contamination in Santos Estuary (75% of mussels contaminated and 5/6 of sampling sites with occurrence of microplastics intake), an important factor to be considered in evaluations of microplastic pollution in a region. Thus, this seems to be a suitable method and good indicator that could also be used for management strategies and to further research planning in this and other regions. An initial assessment, such as presented here, is essential to identify the exactly sampling points for further evaluations of the quantity and quality of microplastics by organisms.

Quantifying microplastics and identifying the type of field-collected polymers can be complex, time consuming and too expensive for large number of samples with micrometric plastics (<1mm so difficult to identify, with difficulty increasing with decreasing size). Based on the present results, further quantification and monitoring of Santos Estuary could happen in sampling sites with 75% or more mussels contaminated. Considering the relatively simple methodology presented here, we also suggest an increase in the number of mussels sampled per site to better illustrate real scenarios of contamination. Moreover, we highlight the importance of further studies that deepen the issue of microplastics pollution in the region (e.g. quantifying and qualifying ingested microplastics, tracking changes according to environmental conditions, etc).

Getting the relative frequency of contamination also presented its difficulties in dealing with biological samples as sentinels. The acid digestion protocol proposed by Claessens et al. (2013) and adapted in the present study was not fully efficient in digesting mussels' soft tissue. Some tissue fractions remained after digestion, hampering microscopic analysis. Despite adaptation, this method can also damage pH-sensitive polymers, while the high temperature during the digestive process can melt particles depending on the glass transition temperature of the polymer (Claessens et al., 2013). All these issues can complicate the identification and count of ingested plastics, creating conservative estimates. Moreover, using concentrated nitric acid may have discolored the ingested microplastics. Although this did not lead to any hinderance for our research, these limitations can disturb further discussions regarding ingested plastics characterization and associated impacts. Especially for small organisms, tissue sections analyzed under microscopy techniques (e.g. polarized light microscope, von Moos et al., 2012) could be an accurate method for assessing microplastics intake and assimilation. Besides allowing the separation of what is in the digestive tract from what was assimilated, histology does not compromise the quality of microplastics (as acid digestions might do), providing a more detailed and accurate analysis. However, histological procedures are too time-consuming, making them unfeasible for a great number of samples or rapid assessments. Thus, this method could be used to further specific investigations after the contamination status of area has been recognized and more sophisticated objectives have been outlined.

The use of PLM was appropriate for finding microplastics in a sample with more than one matrix (i.e. samples with inorganic matter such as sediment and

microplastics mixed with organic remains from acid digestion) because polarization of microplastics facilitated its identification among other materials. However, it was difficult to capture images of samples when the polarized filter was applied, and this should be considered for more detailed studies that needs the image for processing data (e.g. size of ingested particles). This did not seem to occur for previous studies that used PLM for microplastics identification. Such limitation can be related to the content of the glass slides and may not happen with stained tissue sections. For studies with organic matter digestion, however, this should be considered.

The oncoming rise in microplastics pollution in marine environments demands the development of efficient and viable methods for field studies. This study investigated the relative frequency of contamination of sampling sites as a simple and rapid assessment to widen the estimates of microplastic contamination in nature. For that we analyzed the presence of microplastics in biological samples, using the sedentary filter-feeder *P. perna* as a sentinel (since bioavailability does not always reflect the environmental contamination). Moreover, this study revised, tested and adapted an acid digestion protocol for destroying organic matter to seek microplastic presence in mussels. As a result, we found that microplastics can be largely bioavailable along the study area, posing potential risks to environmental and human health. This was the first assessment for the southern hemisphere of microplastics bioavailability in nature using benthic invertebrates as a sentinel. In addition, this was the first time that *P. perna* was found contaminated with microplastics in the wild. Similar data should be collected in other places where this or other species have importance as food resource. Finally, we suggest long-term and more complete assessments of microplastics' bioavailability for monitoring purposes around the world.

Final Considerations

It has been more than 30 years that plastics were recognized as one of the major contributors to marine pollution (Santos et al., 2008). Now, attention has been given to the micro-particles of plastics present in marine environments, which corresponds to 92.4% of the total plastic particles in oceans (Eriksen et al., 2014) and has an unavoidable tendency towards increase (Barnes et al., 2009). Given the growing interest of international organizations but little scientific data, this study aimed to contribute to new discussions on biological impacts and environmental risks arising from microplastics' intake by marine biota. Using *P. perna* and micro-PVC as biological and microplastics models, we assessed: (i) the influence of different sets of exposure on mussels' physiological responses (under acute and chronic scenarios of contamination); and (ii) the potential risk of microplastics be transferred, assimilated, and retained along food chains. Also, to better understand the potential risks of these hypotheses occurring in nature, we investigated the prevalence of microplastics intake in field collected mussels.

Combining the results from acute and chronic assays (chapters one and two), we observed that despite microplastics pollution leading to biological stress, it can also activate organisms' defense responses, allowing them to overcome effects and acclimate with time. The period of exposure was, therefore, a decisive factor in triggering mussels' responses to microplastics' pollution. This indicates that microplastics' risks for marine biota can be positively influenced by time, and that microplastics' chronic exposures (e.g. urbanized coastal areas, with sewage disposal and other sources) may not lead to relevant hazards for organisms, as also suggested by our results. In a short-term exposure, however, all six biomarkers analyzed were influenced by the intake of E/M PVC. In this assay, the factors "leaching of additives" and "concentration of microplastics" could also affect mussels' responses, and the interactions among factors surpassed their main effects. Such interaction made microplastics' impacts directly related to the scenario of exposure (i.e. different combinations of factor levels) highlighting the singularity of each combination of contamination's characteristics. The fact that biomarkers were not equally affected by all analyzed factors also indicates that, for microplastics pollution, those factors may interfere differently within biological process, tissues and structures.

Using a more realistic approach (in which prey have only contaminated tissues and no microplastics in the digestive tract), we observed microplastics' transference from prey tissues to predator tracts and not to tissues. Thus, microplastic bioaccumulation through food intake seems unlikely to occur. However, concentration (of microplastics within prey) and time (between prey contamination and predation) were suggested to influence the magnitude of microplastics' biotransference. Scenarios where predation occurs immediately following ingestion of microplastics by prey may enable predators to assimilate plastic, possibly increasing intake-associated impacts. The complex variability of microplastics' risks to the marine environment once more stood out.

In addition to the ecological relevance, mussels are important organisms for humans as food resource. Different species of mussels are widespread along coastal regions, being harvested or farmed for human consumption. Even around urban areas, where microplastics input (and thus intake) can be frequent, local people harvest mussels for selling and eating. Santos Estuary illustrates that well, as it comprises great natural beds of *P. perna* and harvesting is a common practice there. Seventy-five percent (75%) of sampled mussels had ingested microplastics and all mussels' beds had at least one mussel with this pollutant. By conducting a rapid and simple assessment, we identified the high bioavailability of microplastics within the study area, which could be related to a significant input from urban activities of the region. The bioavailability of microplastics in Santos is, therefore, a relevant issue to environmental health and human food safety that should be better explored by the scientific community and policy makers. This assessment also revealed a random distribution (in quantity) of contaminated mussels among sampling points, which can be related to the large variety of microplastics types available and to hydrodynamic conditions. These results reaffirm that microplastics' risks to the marine environment seem complex and highly variable. The applied method also proved to be an efficient tool for primary evaluations of the contamination state of coastal environments using mussels as sentinels.

Along with field data, the different experimental designs and results of this study brought a few considerations for evaluating the risks of microplastics pollution to marine biota. A great variety of factors modulate microplastics' scenarios of exposure, while interactions among these factors influence their effects on marine biota. Based on a critical analysis of our results and of previous studies, the dynamic and unpredictable

risks of microplastics debris stood out, and their impacts seemed to change depending on interactions among (i) marine species, (ii) microplastics' type and characteristics, (iii) and environmental conditions. Thus, it is important to consider factors with multiple levels and interactions when addressing microplastics' environmental risks, which should be further widened and deepened.

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