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# Microplastic contamination in natural mussel beds from a Brazilian urbanized coastal region: Rapid evaluation through bioassessment

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ABSTRACT

Microplastic pollution (particles <5 mm) is a widespread marine threat and a trigger for biological effects, especially if ingested. The mussel *Perna perna*, an important food resource, was used as bioindicator to investigate the presence of microplastic pollution on Santos estuary, the most urbanized area of the coast of São Paulo State, Brazil. A simple and rapid assessment showed that 75% of sampled mussels had ingested microplastics, an issue of human and environmental concern. All sampling points had contaminated mussels and this contamination had no clear pattern of distribution along the estuary. This was the first time that microplastic bioavailability was assessed in nature for the southern hemisphere and that wild *P. perna* was found contaminated with this pollutant. This is an important issue that should be better assessed due to an increase in seafood consumption and culture in Brazil and worldwide.



## 1. Introduction

Over the last few decades, plastic marine pollution has become ubiquitous across the globe (Barnes et al., 2009), with a current estimate of 5.35 trillion particles (~268,940 tons) floating on sea and ocean surfaces (Eriksen et al., 2014). Large-scale consumer use of plastic products and poor management practices (Jambeck et al., 2015) raise the potential risk of being lost to the environment during production, transportation, use and discard; once in the ocean, they are persistent pollutants, lasting hundreds to thousands of years (Moore, 2008; Barnes et al., 2009). Despite this clear potential for accumulation over time, the fate and consequences of plastic marine pollution are just beginning to be understood.

Among global marine plastic debris, 92.4% of the items are microplastics (Eriksen et al., 2014), particles with less than 5 mm diameter (Arthur et al., 2009). These microplastics could either be intentionally produced within this size range (primary microplastics) or originate 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 Janssen, 2014), ascidians (unpublished data), echinoderms (Graham and Thompson, 2009) and sponges (unpublished data). As a consequence, physiological disturbances can occur, already described under laboratory conditions for some marine species (von Moos et al., 2012; Browne et al., 2013; Besseling et al., 2013; Rochman et al., 2014).

Microplastic ingestion by different marine groups and species also made this a plausible pathway for microplastics' transition among marine compartments (e.g. water column and bottom — Eriksen et al., 2014). Microplastic uptake could be responsible for plastic transference from the sea surface to the water column and sea bottom (via plastic 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; Farrell and Nelson, 2013; Setälä et al., 2014; Santana et al., submitted for publication-a), 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

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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' abundances and densely populated areas, suggesting a high relevance of coastal cities to the input of microplastic 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). Coastal areas contain a wide variety of ecosystems (e.g. mangrove forests, estuaries, beaches and coral reef systems), many of them highly diverse and responsible for supporting different goods and services (such as food and the biodiversity itself — Martínez et al., 2007), microplastic input and the resulting impact should be considered an important issue to be assessed.

For humans, the direct risks brought by microplastic marine pollution are associated with their bioavailability to food resources, becoming a matter of food safety. A large proportion of fisheries, shellfisheries and aquaculture systems are concentrated either in or near coastal regions, which makes microplastics another worrying contaminant for human 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., 2013; Foekema et al., 2013; Van Cauwenberghe and Janssen, 2014; Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015), approximating microplastic impacts on humans and thus increasing related concerns.

To investigate microplastic 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, US, 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 the microplastics' re-suspension from bottom sediments and their depth distribution between the bottom and sea surface, increasing the variability of 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 1 μm (Santana et al., submitted for publication-b) up to 80 μm 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 data relevant to the hazard that microplastics pose to marine biota.

The use of biological indicators, in contrast, relies on the relationship between the organism and the polluted environment (EPA, US, 2000), helping improving our understanding of the realistic risks of the potential biotic impacts observed in laboratory studies. Due to the variety of microplastic 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 it calls attention of scientists, 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 et al., 1987 and the Mussel Watch Program), highlighting the need for additional methodological developments. One significant problem of biomonitoring microplastic 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 broaden the scope of 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 estuary of Santos (Southeastern Brazil). Santos estuary is an important Brazilian coastal region, strongly influenced by industrial, port and urban activities and the most urbanized coastal area 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. widespread distribution, sedentary lifestyle, easy sampling and accumulation of chemicals — NOAA. International Mussel Watch Committee, 1995); and (ii) their importance as food resource. In addition, because of the incipient use of bioindicators for microplastic pollution, we also discussed methodological aspects that might be relevant for establishing applicable tools for analyzing biological matrices.

## 2. Methods

### *2.1. Assessed area: Santos estuary*

The marine environmental health of Santos is of longstanding concern, but not much is known about its level of microplastic pollution. From the beginning of 20th century, this region has been strongly affected by anthropogenic activities (David, 2007); it houses the largest port in South America (Santos Harbor), one of the most important industrial complexes in Brazil (Cubatão industrial complex, Cesar et al., 2007; Fisner et al., 2013a) and has a well-established tourism industry that may attract up to 4.7 million people during the summer (data for 2012; Santos Turism Office, 2014). Considering potential sources of microplastics to coastal regions, all these characteristics can contribute to the microplastic contamination in Santos estuary, as detailed below.

Beside the solid waste produced by vessels that berth in Santos Harbor (including plastic packing ships), virgin plastic pellets (granules with an average diameter of 5 mm, made from different types of polymers, such as polyethylene and polypropylene, EPA, US., 1992), and Emulsion/Microsuspension PVC (small dense microspheres with a size ranging from 0.1 to 1.0 μm diameter, Rodolfo et al., 2006) are among the types of loads handled in this port. Both types of pre-consumption microplastics can potentially be entering 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 microplastic contamination of coastal environments, especially during tourist periods when waste treatment system reach maximum capacity. 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, previous losses of plastic waste from this dumpsite can still serve as a microplastic input for the marine ecosystems of Santos because of slow degradation and the 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 sewage treatment plants are not typically specifically designed to retain microplastic particles (Browne et al., 2011). Large sewage discharges occur clandestinely along the estuary of Santos, without any treatment

(Martins, 2005), raising plastic and microplastic inputs in an undetermined way.

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

### 2.2. Bivalve collection: sampling sites

In September 2014, mussels were collected from 6 randomly selected natural beds near Santos Harbor terminal used for loading microplastics (Fig. 1). The points chosen were downstream of the harbor due to the dominant transport direction of estuarine waters towards the port channel and Santos Bay (Fukumoto, 2007), thus increasing the probability of capturing the effect of several 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 in 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 connects 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 ($4.3 \pm 0.99$ cm in length) for all assessments. Because this was an initial assessment of an area with no previous data regarding the presence of microplastics susceptible to invertebrates' ingestion, it was decided to work with a low sample size to avoid unnecessary use of biological samples. Organisms were removed from substrates by cutting their byssus, then frozen without depuration in clean seawater. That lack of debugging was to ensure the retention of all ingested microplastics, including the particles retained in the digestive tract and not just those translocated to tissues.

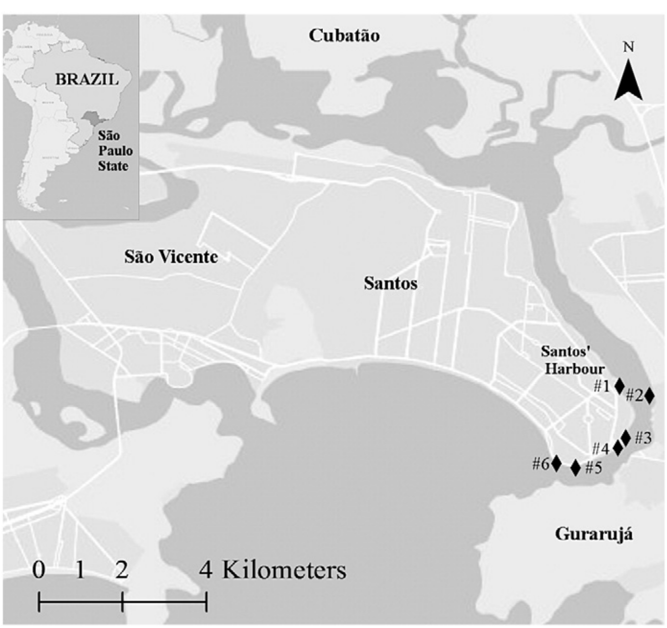


**Fig. 1.** Map of the sampling area – Santos estuary (São Paulo State, Brazil) – indicating the sampling points: #1 (23°58′26.760S, 46°17′35.880W — terminal used to (un)load microplastics at the Santos Harbor); #2 (23°58′34.28S, 46°17′12.47W — irregular occupation with clandestine sewage discharges into the estuary); #3 (23°59′6,75S, 46°17′31.07W — vehicle ferry); #4 (23°59′14.85S, 46°17′36.97 W – 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).

### 2.3. Tracking ingested microplastics in mussels: procedures for organic matter digestion and microplastic 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 matter to be analyzed. For that, mussels were individually submitted to an overnight digestion with $HNO_3$ (22.5 M) at room temperature, followed by: 15 min of boiling, dilution with distilled water, and filtering. The period of boiling was shorter than the validated protocol, aiming to minimize the risks of microplastic degradation during the acid digestion (Claessens et al., 2013). In contrast, the final solution was filtered through 0.7 μm GF/F filters (Whatman, 25 mm 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 microplastic 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).

To prevent sample contamination with airborne microplastic 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 flasks and other apparatus, whenever possible, 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.

### 2.4. Establishing the relative frequency of microplastic contamination

As soon as one particle of microplastic was found in a mussel, the sentinel was considered contaminated. Using the ratio between contaminated mussels and total number 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 microplastic pollution of Santos estuary. Therefore, the quantity of microplastics found in the organism was not counted nor will be discussed here. Nevertheless, the relevance of these and other types of data (e.g. polymer type) is discussed later.

## 3. 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 (Fig. 2). The least contaminated mussel bed was #6, with only one *P. perna* contaminated. This site 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 $\#6 < \#4 < \#2 < \#1 = \#3 = \#5$.

The procedures used for mitigating possible contamination of samples during lab activities were efficient. The main sources of


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

microplastic contamination seemed to be avoided as only two of the microplastics identified were fibers. The majority of microplastics found were white and had irregular shapes (Fig. 3A and B). However, the organic matter digestion and the ethanol dilution of the filtered remains (before mounting on glass slides) were not fully effective. In some slides, we could still identify organic matter and clumps of samples that could have hindered the identification of ingested plastic particles (Fig. 3C).

## 4. Discussion

Seventy-five percent (75%) of the mussels sampled in Santos estuary had ingested microplastics. For 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 collecting 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, already being ingested by the marine biota. Other studies have reported microplastic uptake by invertebrates in natural habitats (Van Cauwenberghe and Janssen, 2014; Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015), but this is the first assessment for the species *P. perna*, an organism found in several countries and directly 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 the 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. It is worth noting that as a food resource for humans, this species is commonly collected from natural beds or cultured in systems imposed into marine environments, allowing their food supply to come from natural seawater (due to filter-feeding). Therefore, even cultured mussels are not free from the risks of microplastic ingestion, as observed by Mathalon and Hill (2014) and Van Cauwenberghe and Janssen (2014)). Although mussel farms are not common in the study area, the shores in the Santos region are among the greatest natural beds of *P. perna* in São Paulo State (Henriques et al., 2001), and harvesting in the estuary for human consumption and sale is a common practice (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, respectively, after a single exposure (Browne et al., 2008, unpublished data). Unfortunately, organic matter digestions do not allow us 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, this residence time of microplastics within mussels suggests that environments with potential frequent inputs, such as Santos estuary, have high risks of mussels being constantly contaminated. We therefore suggest the adoption of debugging procedures as a sanitary precaution for shellfisheries. Further research could be dedicated to better understand possible variations in retention periods by marine species (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 foreign 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 microplastic input in the Santos estuary.

Despite the lack of data about further consequences of microplastic 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 for publication-b). Persistent organic pollutants (POPs) and other

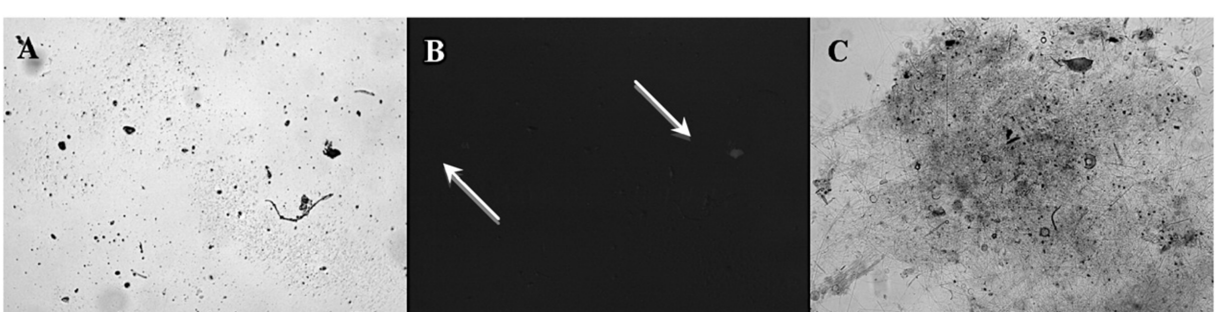


**Fig. 3.** (A) Illustrative figure of sample slides seen under microscopy (B) and polarized light microscopy, PLM, with arrows indicating polarized particles of plastic. (C) Example of organic matter remains in clogged samples.

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 than ingested sediment for lugworms (Browne et al., 2013). Thus, it is feasible to suggest that the higher the contamination of a certain area (with microplastics, POPs and others), the higher the chances are of microplastic adsorption and vectorization of chemical toxic substances to organisms. In the study area, plastic pellets (~5 mm diameter) sampled in Santos Bay were highly contaminated by PAHs (Fisner, 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 an organic pollutant is a consequence of adsorption mechanisms during their residence in the highly polluted Santos estuary (Manzano, 2009; Fisner et al., 2013a). Given that decreasing the size of a particle increases the surface/volume ratio (Mato et al., 2001), we fear that greater quantities of dissolved compounds are being adsorbed on these micrometric plastics ingested by mussels in the Santos estuary. Considering local harvesting of *P. perna* for human consumption, it is important to better understand the risks of ingested microplastics as a vector for other pollutants. This is a matter of global concern, so the impacts of microplastics on commercial species should be further explored. As important, few studies identified the role of microplastics as a substrate for microorganisms' development (Zettler et al., 2013; Reisser et al., 2014; McCormick et al., 2014). Although incipient, this indicates that microplastics can also act as a transport for diseases (McCormick et al., 2014). Whenever this impact reaches commercial species, both 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 pattern can vary over a short scale (Ryan et al., 2009). Santos estuary illustrates that well, presenting contaminated mussels at all sampling sites but without a predicted distribution among sampling points. The three mussels beds fully contaminated with microplastics were not consecutive along the Santos 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-flow rivers, 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 microplastic bioavailability along the Santos estuary has a stochastic pattern, varying temporally according to environmental conditions and type of microplastic input, which should be further investigated. This highlights the importance of characterizing microplastic 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 long-term 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 microplastic input to bioavailability in Santos estuary. Identifying microplastic sources is extremely relevant for public policies. Those policies should be more incentivized to reduce the input of microplastics into marine ecosystems. For further studies, we strongly suggest this type of analysis, more specifically for ingested microplastics since bioavailability does not always directly reflect the environmental state (EPA, US, 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, we performed a simple and rapid assessment that reflected the coverage of microplastic contamination in Santos estuary (75% of mussels contaminated and 5/6 of sampling sites with occurrence of microplastic 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 pinpoint sampling sites for further evaluations of the quantity and quality of microplastics ingested 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 (<1 mm are difficult to identify, with difficulty increasing with decreasing size). Based on the present results, further quantification and monitoring of Santos estuary should be considered at 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 microplastic pollution in the region (e.g. quantifying and qualifying ingested microplastics, tracking changes according to environmental conditions, etc).

Obtaining the relative frequency of contamination also presented difficulties through 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 underestimations. Moreover, using concentrated nitric acid may have discolored the ingested microplastics, as observed in a previous test (unpublished data). Although this did not lead to any hindrance to our research, these limitations can disturb further discussions regarding characterization of ingested plastics and their associated impacts. Especially for small organisms, tissue sections analyzed with microscopy techniques (e.g. polarized light microscope, von Moos et al., 2012) could be an accurate method for assessing microplastic intake and assimilation. Besides allowing the separation of what is in the digestive tract from what was assimilated, histology does not prejudice the quality of microplastics (as acid digestions might do), providing a more detailed and accurate analysis. However, histological procedures are time-consuming, making them infeasible for a large 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 an appropriate simple strategy for seeking 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 need the image for processing data (e.g. determining size of ingested particles). This did not seem to occur for previous studies that used PLM for microplastic identification. Such limitation can be related to the content of the glass slides and may not occur with stained tissue sections. For studies with organic matter digestion, however, this should be considered.

## 5. Conclusion

Apart from obstacles, the oncoming rise in microplastic pollution in marine environments drives 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 scope of 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 throughout the study area, posing potential risks to environmental and human health. This was the first assessment for the southern hemisphere of microplastic bioavailability in nature using benthic invertebrates as a sentinel. In addition, this was the first time that *P. perna* was found field-contaminated with microplastics. Similar data should be collected in other places where this or other species have importance as a food resource. Finally, we strongly suggest long-term and more complex assessments of microplastics' bioavailability for monitoring purposes around the world.

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