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PLASTIC PELLETS: UNDERSTANDING THEIR DYNAMICS AND IMPACTS ON THE COASTAL ENVIRONMENT

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“Eu sou problema de montão, de Carnaval a Carnaval
Eu vim da selva, sou leão, sou demais pro seu quintal”
(Racionais - Negro Drama)

RESUMO

Plásticos são materiais que vêm sendo amplamente utilizados na sociedade moderna. Dentre eles, os microplásticos (plásticos menores que 5 mm de diâmetro) vêm chamando atenção por sua alta capacidade de dispersão e acumulação, sendo amplamente encontrados nas regiões costeiras de todo o globo. Os *pellets* plásticos são a matéria prima de produtos plásticos. De pequenos tamanhos e granulares, estes podem ser perdidos ao longo dos processos industriais e de transporte. Diversos trabalhos reportaram a presença destes materias no ambiente costeiro. Estes também têm focado em sua capacidade de sorção de contaminantes químicos e os impactos da ingestão e exposição de *pellets* em organismos marinhos. Este trabalho tem como objetivo testar os efeitos tóxicos de *pellets* plásticos para organismos da macrofauna bentônica de praias arenosas em condições naturais, além de contribuir com o entendimento de seus padrões de dispersão e acumulação na zona costeira e sua capacidade de sorção de contaminantes químicos hidrofóbicos. Para isso, foram realizados experimentos *in situ* e de laboratório de exposição de *Excirolana armata* a pellets plásticos. As contribuições químicas foram realizadas com um experimento de simulação de derramento de óleo em laboratório seguido de análises químicas e amostragens passivas de pellets em praias com a utilização de imagens aéreas de drone. Em condições ambientais foi observada mortalidade nas menores densidades de *pellets* testadas, e também um aumento nas interações intraespecíficas de canibalismo e agressividade dos organismos testados. Contudo, organismos com ciclo de vida rápido tendem a adquirir resistência a esses estressores. Além disso, os *pellets* plásticos possuem a capacidade de transportar contaminantes hidrofóbicos, especialmente hidrocarbonetos policíclicos aromáticos (HPAs). Naftaleno, fenantreno e criseno foram os principais HPAs adsorvidos e potencialmente carreados pelos *pellets*. Por fim, apresentamos evidências que é possível usar imagens aéreas de drones para monitorar *pellets* plásticos em praias arenosas, sendo um método mil vezes mais eficaz do que os métodos tradicionais ativos de campo.

Palavras-chaves: Microplásticos, Impacto ambiental, Ecotoxicologia, Poluição marinha, Contaminantes químicos hidrofóbicos, Sensoriamento remoto

ABSTRACT

Plastics are materials that have been widely used in modern society. Among them, microplastics (plastics smaller than 5 mm in diameter) have been raising concerns due to their high capacity for dispersion and accumulation, which can be widely found in coastal regions worldwide. Plastic pellets are raw materials for plastic products. Pellets are small in size and have a granular shape. They can be lost during industrial and transportation processes. Several studies have reported the presence of these materials in the coastal environment, their capacity for sorption of chemical contaminants, and the impacts of ingestion and exposure of pellets on marine organisms. This study aimed to test the toxic effects of plastic pellets on benthic macrofauna organisms on sandy beaches under natural conditions. It also contributed to the understanding of their dispersion and accumulation patterns in the coastal zone and their ability of sorbing hydrophobic chemical contaminants. For this, in situ and laboratory experiments were conducted to expose *Excirolana armata* to plastic pellets. Chemical contributions were made through an oil spill simulation experiment in the laboratory, followed by chemical analyses and passive sampling of pellets on beaches using aerial drone images. Under environmental conditions, mortality was observed at the pellet's lowest densities and increased intraspecific interactions of cannibalism and aggressiveness of the organisms tested. However, organisms with fast life cycles tend to acquire resistance to these stressors. Furthermore, plastic pellets can transport hydrophobic contaminants, especially PAHs. Naphthalene, phenanthrene, and chrysene were the main PAHs adsorbed and potentially carried by pellets. Finally, we proved that it is possible to use aerial drone images to monitor plastic pellets on sandy beaches, which is a thousand times more effective than the traditional active field methods.

Keywords: Microplastics, Environmental impact, Ecotoxicology, Marine pollution, Hydrophobic chemical contaminants, Remote sensing

SUMÁRIO

GENERAL INTRODUCTION

Energy and environment. Here, we focused on the intersection of these two areas in an interdisciplinary thesis on microplastics, which is a petroleum-based material, the fossil energy widely used in modern times. Fossil energy is generated from the combustion of fossil fuels. Approximately 82% of the world's energy matrix comes from fossil fuels such as oil, mineral coal, and natural gas (IEA, 2018). The biggest challenge of depending almost exclusively on this type of energy is the constant carbon emissions into the atmosphere, contributing to climate change. In addition, stocks are finite, and this type of energy is nonrenewable.

Among fossil fuels, oil began to be explored in the mid-19th century in an oil well in Pennsylvania, United States. After the Second Industrial Revolution, with the advent of combustion engines, oil began to be used on a large scale. Currently, the world consumes almost 100 million barrels of oil per day (IEA, 2018). Brazil consumes 140 billion liters of oil annually, equivalent to approximately 2 million barrels per day (BEN, 2019).

Co-products from the petrochemical industry, such as most plastic polymers, are widely used in the modern world and in our daily lives. It is estimated that 400 million tons of plastics are produced annually (Geyer *et al.*, 2017), and the improper disposal and persistence of this material in the environment generates impacts that are not yet well described and an emerging concern among scientists. While oil spills have decreased considerably over the last 20 years, the same has not happened for plastic waste discarded into the environment, which has seen an exponential increase at the same time (Galloway *et al*., 2017).

In September 2015, members of the United Nations (UN), such as Brazil, launched an agenda consisting of 17 actions to be implemented by all signatories by 2030, considering the planet's sustainable development. Among these actions, called Sustainable Development Goals, SDG14 has the scope of "Conservation and sustainable use of oceans, seas, and their marine resources for sustainable development.” One of its goals is to prevent and significantly reduce marine pollution of all types by 2025. Another goal is to manage and protect marine and coastal ecosystems and to avoid significant adverse impacts (UN, 2019). Therefore, studies that evaluate the presence of contaminants, such as microplastics, and their impact on marine ecosystems are important for the better management of this ecosystem and to reach the SDG14.

Microplastics are commonly defined as plastics that are smaller than 5 mm in diameter (Hammer *et al*., 2012; Li *et al*, 2020). These particles can be either raw materials for manufactured plastic objects (pellets) or small fragments arising from the breakdown and weathering of larger plastics. Both have heterogeneous characteristics in size, shape, color, chemical composition, and density (Hidalgo-Ruz *et al*., 2012). Plastic pellets are generally cylindrical or rounded in shape, with sizes ranging from nanometers to 5 mm (Mato et al., 2001). Pellets are widely used and sold as raw materials for industrial manufacturing (Karapanagioti and Klontza, 2007; Mato *et al*., 2001, Ogata *et al.*, 2009; Li *et al.*, 2020; Zhang *et al.*, 2020). These microplastics are easy to handle and process because of their small size and granular shape (Wilber, 1987). During their industrial process and, mainly, during their transportation (terrestrial and maritime), it is common unintentional releases into the environment, being carried by rivers and rain, with marine and coastal environments as their final destination (Carpenter and Smith Jr, 1972; Gregory, 1978; Mato *et al.*, 2001; Ogata *et al.*, 2009).

These lost pellets tend to accumulate on sandy beaches and have a series of impacts on local fauna, such as unintentional ingestion (which can causes physical impacts), chemical exposure to plastic industrial additives and hydrophobic contaminants (Milijö, 2001; Endo *et al.*, 2005, Ryan *et al*., 1988; Endo *et al.*, 2005; Teuten *et al*., 2009; Hirai *et al.*, 2011), non-ingested impacts which can induce toxic effects, sublethal effects and ecological impacts (Rochman *et al.,* 2014; Nobre *et al.*, 2015; Gandara e Silva *et* al., 2016; Izar *et al.*, 2019; Nobre *et al*., 2020;) and even facilitating the colonization of alien species (Gregory, 1978; Milijö, 2001; Barnes, 2002; Barnes and Milner, 2005; Majer *et al*., 2012; Lacerda *et al.*, 2020).

Plastic pellets can sorb chemicals from two different ways: industrial additives or sorption of environmental compounds. Industrial additives are chemical substances incorporated into pellets during the industrial process, such as bisphenol A (BPA), alkylphenols, phthalates, flame retardants, fire suppressors, plasticizers, dyes, ultraviolet filters, and metallic catalysts (Koch and Calafat, 2009; Hirai *et al.*, 2011; Engler, 2012; Hammer *et al.*, 2012; Llorca *et al.*, 2014; Taniguchi *et al.*, 2016; Xu *et al.*, 2020). Factory virgin pellets are usually white and have higher concentrations of industrial additives with fast rate of release into the environment (Koch and Calafat, 2009; Hammer *et al.*, 2012; Nobre *et al.*, 2015). The second way is through the sorption of hydrophobic substances from the environment. The nonpolar molecular structure of plastic pellets increases the affinity for

these compounds (Fotopoulou and Karapanagioti, 2012). Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), and dichlorodiphenyltrichloroethane (DDT), and polycyclic aromatic hydrocarbons (PAHs), are the main substances found adsorbed on plastic pellets (Endo *et al.*, 2005; Ogata *et al*, 2009; Teuten *et al*., 2009; Fisner *et al.*, 2013). The concentration of these compounds in pellets is generally higher than in ocean waters (Andrady, 2011), and it can be up to 10^6 times more concentrated in plastic pellets than in the surrounding environment (Mato *et al*., 2001). Yellowish pellets are related to physical degradation caused by the sunlight (photodegradation) (Karapanagioti and Klontza, 2007). However, the yellowish color grade (from yellow to brown) can also represent a concentration gradient of chemicals sorbed in pellets and longer exposure periods in the coastal environment (Endo *et al*., 2005; Yamashita *et al.*, 2018).

In this present work, we address the impacts of plastic pellets on the coastal environment, trying to understand the processes of accumulation of these materials, interactions with chemical contaminants, and the impact induced by the combination of plastics and chemicals on marine organisms. We hypothesize that plastic pellets can cause toxic effects on benthic macrofauna organisms of sandy beaches *in situ* under conditions and factors of the natural environment. Pellets chemical sorption and accumulation patterns on sandy beaches may contribute to this toxicity, and are therefore also studied here. In this thesis, we started with a systematic review of the topic by applying the Drivers-Activities-Pressures-State Changes-Impacts (on Welfare)-Responses (as Measures) (DAPSI[W]R[M]) framework to the plastic pellet production chain, aiming to create an accessible and easy-to-understand product for civil society and public authorities (Chapter 1). Following, we tested the ecotoxicological effects of real plastic pellet densities found on sandy beaches on benthic macrofauna in a field experiment (Chapter 2) and in laboratory experiments with different populations (Chapter 3). Complementing the findings of our main hypothesis, we elaborate a laboratory experiment designed to better understand the dynamics of sorption and equilibrium of hydrophobic chemical contaminants in a pellet-seawater system (Chapter 4). Finally, we developed a methodology for monitoring the input and accumulation of pellets on sandy beaches using Unmanned Aircraft Vehicle aerial drone images (Chapter 5). We also discuss the uniformity of methods and units in microplastic sampling as an additional contribution to this thesis (Appendix 1).

CHAPTER 1

The application of the DAPSI(W)R(M) framework to the plastic pellets chain

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ABSTRACT

Single-use plastic, few global engagement and lack of policies contribute to the global challenge about plastic marine litter. This form of contamination can cause injury and death of marine wildlife. Microplastics (> 5 mm) represent an important fraction of plastic litter. They include the plastic pellets that are used as raw material within plastic industry that can be unintentionally spilled into the environment during the industrial processes. In an initiative in order to facilitate the understanding and communication of plastic pellets as a social and environmental problem, we applied the Drivers-Activities-Pressures-State changes-Impacts (on Welfare)-Responses (as Measures) (DAPSI[W]R[M]) framework to plastic pellets chain. We also analyzed possible mitigating measures and their actions along the plastic pellets chain. This DAPSI(W)R(M) framework aim to show an overview of the plastic pellets chain and solutions for politicians and decision makers to help solve this socio-environmental problem that needs the engagement of all stakeholders involved.

Key words: Plastic, Nibs, Microplastics, Coastal impact, Marine pollution, Plastic debris

1.1. INTRODUCTION

Since 1950, plastic began to establish itself as a common product in our modern society, reaching a current production of about 400 million metric tons annually in the world (Geyer *et al.*, 2017). It is estimated that 12 million metric tons of plastic litter would potentially be released into the environment by 2050 (Geyer *et al.*, 2017). At the moment, plastic residues represent about 90% (or more) of the world's marine litter (Milijö, 2001).

Microplastics are plastic waste with few millimeters of size (> 5 mm). They represent an important fraction of plastic litter present in the environment (Thompson *et al*., 2004; Worm *et al.*, 2017). In 50 years, microplastics spills have exceeded oil spills into the oceans (Galloway *et al*., 2017), and its presence in terrestrial and marine deposits has been proposed as a marker of a distinct stratal component indicating a new geological epoch (Anthropocene) (Zalasiewicz *et al*., 2016). Microplastics are released into the environment from the fragments of larger plastic products, or unintentionally spilled during the industrial process in the form of pellets (Ogata *et al*., 2009). Plastic pellets, microspheres or nibs are granules or cylindrical plastic resins that are used as raw materials for manufacturing plastic objects (Mato *et al*., 2001). During the plastic industrial process, pellets are released directly into the coastal environment or carried out by runoff systems and rivers until reaching the ocean where they drift by the surface currents to the marine and coastal environment (Ogata *et al*., 2009; Karlsson *et al*., 2018). This social-environmental issue is complex and involves many areas of knowledge and different stakeholders. As a complex problem, it needs interdisciplinary solutions.

The inherent complexity of the environmental problem of microplastic pellets raises questions regarding the relative importance of the impacts and priorities for management, for example. For instance, impacts on marine life and human health usually figure as the main issues. However, from the industry point of view, the main problem could be the excessive loss of materials in the industrial processes. Multiple questions and answers show how complex this problem is, as this complex issue can be seen from different perspectives.

In multifaceted issues, with many perspectives and interests, responses may create conflicts among groups involved (Pardini *et al.*, 2021). The Driver-Pressure-State-Impact-Response (DPSIR) framework is a powerful tool used to analyze and describe environmental problems (Gari *et al*, 2015). Based on the previous tool Pressure-State-Response (OECD,

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1993), the DPSIR framework can analyze and describe environmental problems in a clear, transparent, and visual way. This tool can identify the different stakeholders involved and the links of the cause-and-consequence chain of socio-environmental issues and propose possible solutions. However, this tool is not designed to solve the problems analyzed but to better visualize and understand them. DPSIR framework is a communication tool for researchers, interdisciplinary areas, stakeholders, interested parties, and policymakers (Svarstad *et al*., 2008), and is used to develop conceptual models in complex environmental decision-making (Bradley & Yee, 2015). Variations of this tool can be found in the literature reorganizing the DPSIR components in a clearer way to visualize, as the DAPSI(W)R(M) (Elliott *et al*., 2017). This recent derivation of the framework is more accurate and complete, and provides a holistic analysis of cause-effect-response links (Patrício *et al.*, 2016).

Recently, the DPSIR framework was employed to systematize the causal chain related to (micro)plastics, focussing on aquaculture practices (Skirtun *et al*., 2022) or, without focusing on any activity, but on management responses relevant to the problem (Miranda *et al*., (2020). However, there are some types of microplastics, as pellets and fibres, which have their own chain and behavior, and need to be closely understood. In an initiative to facilitate understanding and communication of plastic pellets as a social and environmental problem, this study aims to apply the DAPSI(W)R(M) framework to the plastic pellets chain and analyze possible and existing mitigating measures (responses).

1.2. METHODS

To elaborate the DAPSI(W)R(M) framework for plastic pellets, a list was created for each component (letter in the acronym) of the tool. Items on the list were based on literature. The items were classified following the division proposed by European Environmental Agency (EEA, 2003), reviewed by Gari *et al.* (2015) and reorganized by Elliott *et al*. (2017). Drivers (D) are related to human needs. Activities (A) are human activities or processes that cause Pressures. Pressures (P) are the result of activities/processes that reflect the mechanisms of change and can cause changes in the natural state, introducing or removing matter or energy from the environment. State changes (S) are the environment state description. In other words, it was considered how much and which way the environment is affected by the activities/processes described on Pressures. Impacts (I) are changes in health and in balance of the ecosystem caused by the environmental changes described on State changes that affect the

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society, focosued mainly in human (societal) Welfare and Well-being (W). And Responses (R) are actions taken by society to prevent, mitigate or improve the environmental changes and impacts described on components State changes and Impacts (on Welfare). These responses come as Measures (M) from many stakeholders at different levels and groups in society. Each response is capable to act directly in a component of the framework, or among components, generating different solutions at different levels of the chain.

Information and data to build the DAPSI(W)R(M) framework for plastic pellets was obtained from a literature review using search tools in Google Scholar, Science Direct and Web of Science databases, using plastic pellet as keyword. The analysis considered studies from the main indexed academic journals and published in English, with the exception of the doctoral (PhD) thesis of Pereira (2014), which explains in detail some process of the plastic pellets industry and its failure. The studies were divided by component of the DAPSI(W)R(M) framework and ranked by their degree of relevence, considering number of citations and the published journal. Studies of little relevance or with repeated information were discarded.

1.3. APPLYING DAPSI(W)R(M) FRAMEWORK TO PLASTIC PELLETS

1.3.1. Drivers

Human society is the main plastic consumer since it has some needs in using plastic products due to their practicality for transportation, conservation, and delivery of products. In this way and following the definition propoused by Newton and Elliott (2016), consumers act as stakeholders on Drivers and Activities, because they generate demand for the plastic polymers' production by the industrial sector. With plastic pellets at the beginning of the plastic industrial production chain, the current lifestyle based on disposable (single-use) plastic stuffs and objects is the main driver of the plastic pellets issue.

Another stakeholder for the process is the industry. Generated by human needs (consumers), the manufactured plastic products industry requires raw materials that are easily handled and transported, with low production costs, and consequently, high profit margins. In this point, plastic pellets fit perfect, due their shape and size facilitate the manufacturing and transportation, allowing the use of bulk terminal structures, reducing costs. As raw material of plastic products, it is impossible disassociate plastic pellets from the plastic industry, since the

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last is the demand of the first. Currently, the plastic industry reaches almost 600 billion dollars per year in the global economy (Rochman *et al.*, 2013). In contrast, it generates demand of spending about 520 million dollars to remove plastic litter just in United States West Coast (Rochman *et al.*, 2013). A math that does not close and does not make sense.

1.3.2. Activities

Nowadays, the plastic usage trend accounts for about 40% for packaging and 20% for consumer items (Worm *et al.*, 2017), which raises attention for the excessive use of disposable plastic items in our society. Indeed, since 1950, virgin plastic production has increased over 200 times, with no apparent expectations of reduction (Geyer *et al.*, 2017). Thereat, the industry is an important stakeholder for Activities. Factories, industries, transportation (terrestrial and maritime), and traders respond to the demand of consumers and industry by producing and transporting plastic raw materials (pellets or nurdles) of the most varied polymers. Pellets losses during the industrial and transportation processes result in an important type of pressure on environment. The constantly and unintentionally input of pellets on the environment (Ogata *et al*., 2009; Claessens *et* al, 2011; Fanini and Bozzeda, 2018) together with the long-time persistence of these materials (Endo *et al*., 2005; Wang *et al.,* 2018) are pressures with constant contributions to the plastic impact chain.

According to Pereira (2014), pellets losses occur at all stages of the industrial process. Starting at the industries, pellets are lost during the packaging step (in big bags or packages) by mechanical failures. But it is a small risk, as the process is automated. During the packaging transportation, and storage, there is a chance to damage the packages potentially making pellets leaks. It is estimated a loss of about 90 tons of pellets equivalent approximately to 2.7 million PET nonreturnable bottles (1.5 L) per year by the plastic industry (Lechner and Ramler, 2015).

In the transportation process, made by trucks or trains (terrestrial) and ships (maritime), plastic pellets losses occur by accidents, mishandling of the cargo, or by cleaning the tanks and containers used for storage and transport. In the harbour areas, pellets are commonly accidentally spilled during the loading process due to the limitations of bulk terminal structures and, also, in the cleaning process. Finally, in the factories, plastic pellets are lost by mechanical and manual failures during the handling of packaging/repackaging and

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the manufacturing processes of plastic products (Fig. 1.1). The pellets lost during the industrial processes usually are either carried out by runoff through river waters, eventually reaching the coastal areas or leaked directly into the oceans during ship transportation (Fig. 1.1) (Khordagui and Abu-Hilal, 1994; Ogata *et al*., 2009; Karlsson *et al*., 2018).

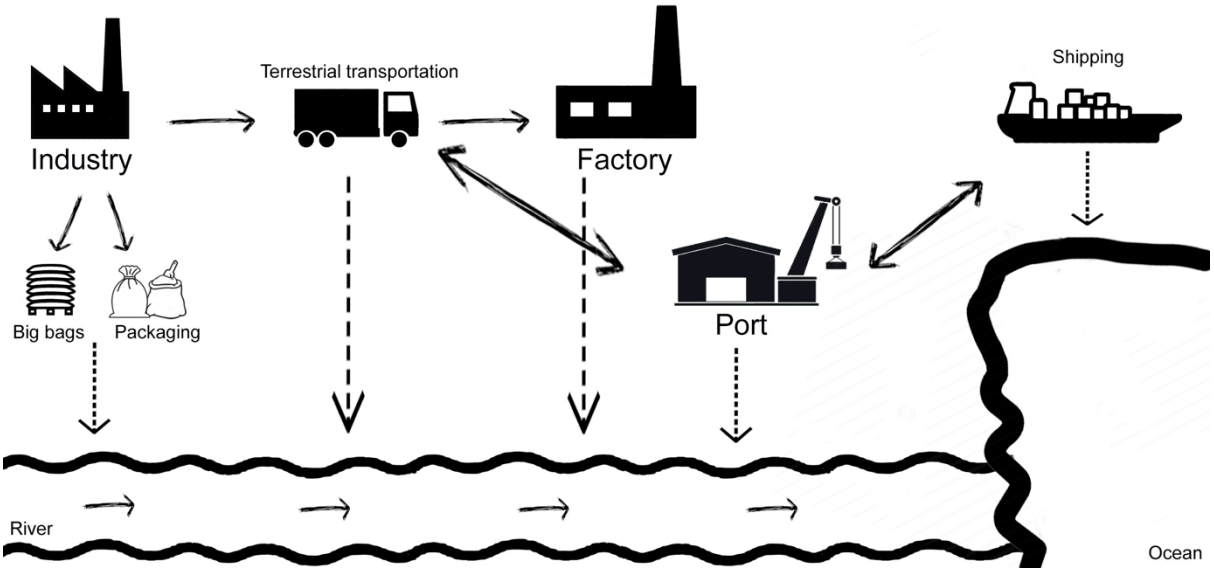

Fig. 1.1. Plastic pellets life cycle. Arrows represent the paths and directions of pellets in the production process and dashed arrows represent the pellets losses along the chain.

1.3.3. Pressures

The pellets that are lost by the industrial sector generate a pressure by the constant input of these materials into the environment. The low density of the plastic pellets facilitates their dispersion within the coastal environment by floating. In addition, the persistence of theses polymers (due to low or very low degradation rate) makes them to remain in the environment for a long time. As explained above (in Introduction), plastic pellets can reach all compartments of marine and coastal environment, accumulating close to port areas (usually pellet source) and dissipating along the coast, reaching remote and contamination-free areas (Izar *et al.*, 2019; Izar *et al.*, 2022). This last point is important, since plastic pellets can carry chemicals sorbed from contaminated areas to contamination-free areas, representing an important pressure both for the environment and for human well-being.

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The chemicals found in the pellets are originated from two different ways: industrial additives used in the plastic production and by the sorption of environmental compounds. Industrial additives are chemical substances incorporated into pellets during the industrial process in order to provide specific properties to the plastic polymers, such as bisphenol A (BPA), which is the raw material for plastic production, alkylphenols used as spermicide, phthalates as plasticizer, some flame retardants (polybrominated diphenyl ethers – PBDEs - and tetrabromobisphenol - TBBPA), or fire suppressors (perfluorooctane sulfonic acid - PFOS - and other perfluoroalkyl substances - PFAS), plasticizers, dyes, ultraviolet filters, and metallic catalysts (Koch and Calafat, 2009; Hirai *et al.*, 2011; Engler, 2012; Hammer *et al.*, 2012; Llorca *et al.*, 2014; Taniguchi *et al.*, 2016; Xu *et al.*, 2020). Virgin pellets (new pellets) are usually white and have higher concentrations of industrial additives with fast rate of release into the environment (Koch and Calafat, 2009; Hammer *et al.*, 2012; Nobre *et al.*, 2015).

The second origin of the chemicals generally found in plastic pellets is through the sorption way, which regards about the pellets' ability to sorving hydrophobic substances from the environment. The nonpolar molecular structure of plastic pellets increases the affinity for these compounds (Fotopoulou and Karapanagioti, 2012). Additionally, some environmental factors, such as salinity and pH, can also influence this sorption process (Holmes *et al.*, 2014; Fred-Ahmadu *et al.*, 2019; Guo and Wang, 2019). Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), and dichlorodiphenyltrichloroethane (DDT), and polycyclic aromatic hydrocarbons (PAHs), are the main substances found adsorbed on plastic pellets (Endo *et al.*, 2005; Ogata *et al*, 2009; Teuten *et al*., 2009; Fisner *et al.*, 2013). Some metallic species, such as major metals and trace metals, may also be sorbed by the pellets (Ashton *et al.*, 2010; Fotopoulou and Karapanagioti, 2012; Holmes *et al.*, 2014; Vedolin *et al.*, 2018; Turner *et al*., 2020), as well as some pharmaceuticals, such as procainamide and doxycycline (Prata *et al.*, 2018). The concentration of these compounds in pellets is generally higher than in ocean waters (Andrady, 2011), and it can be up to 10^6 times more concentrated in plastic pellets than in the surrounding environment (Mato *et al*., 2001). Moreover, plastic pellets can sorb persistent chemicals even those that are banned, for extended periods in the environment (Wang *et al.,* 2018). Almost 80% of the priority pollutants listed by the United State Environmental Protection Agency (US-EPA) are associated with plastic debris (Rochman *et al.*, 2013).

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Yellowish pellets are related to physical degradation caused by the sunlight (photodegradation) (Karapanagioti and Klontza, 2007). However, the yellowish color grade (from yellow to brown) can also represent a concentration gradient of chemicals sorbed in pellets and longer exposure periods in the coastal environment (Endo *et al*., 2005; Yamashita *et al.*, 2018). Contamination processes in plastic pellets commonly occur close to urbanized region and harbor zones (Wang *et al.,* 2018). Pellets can easily carry these chemicals to a contamination-free area by floating transportation though surface ocean currents (Heskett *et al.,* 2012; Taniguchi *et al.*, 2016). According to Yamashita *et al*. (2018), beached pellets from Tokyo Bay had different cocentrations of PCBs among colors: on average white pellets had 71ng g^{-1}, yellowing pellets had 376 ng g^{-1}, and orange and brown pellets had 2052 ng g^{-1}, reaching almost 100000 ng g^{-1} in some samples on the last color.

1.3.4. State changes

1.3.4.1. Physical presence

Once in the ocean, plastic pellets are carried by surface currents to the beach sediments in every continent and remote islands, usually far away from any pellet source (Table 1.1). The first record of plastic pellets in the environment was in 1972 at the Sargasso Sea, in Atlantic North (Carpenter and Smith Jr, 1972). After that, many other records of pellets on seas and beaches were reported, with an exponential increase of papers in the last decade (Akdogan and Guven, 2019; Zhang *et al*., 2020). Pellets can be found in any oceans and seas globally, such as Mediterranean Sea (Morris, 1980), Atlantic Ocean (Colton *et al*., 1974; Wilber, 1987), Pacific Ocean (Day and Shaw, 1990; Moore *et al*., 2001a; Doyle *et al*., 2011; Lebreton *et al.*, 2018), and even in remotes areas as Antarctic Sea (Lacerda *et al*., 2019; Jones-Willians *et al*., 2020), where there are no local sources of these materials.

Pellets tend to accumulate in beaches and ocean gyres, due the influence of wind and surface ocean currents (Browne *et al.*, 2010; Maximenko *et al.*, 2012; van Sebille *et al*., 2020). Indeed, the non-uniformity of units in the studies makes it difficult to compare pellets densities among them, especially in studies that pellets are sampled in different beach zones (high tide line, supralittoral or infralittoral) (Hidalgo-Ruz *et al.*, 2012; Gimiliani and Izar, 2021). In an attempt to compare more effectively different studies, we converted the data that was provided in basic methods information (sample area volume and number of pellets

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sampled) and ranked the top 10 most polluted beaches by pellets. To do that, we use the extrapolation proposed by Turra *et al*. (2014) for pellets per cubic meter (pellets m^{-3}) to 2 meters deep of sediment analyzed by the author (Table 1.2). We use this extrapolation only as a tool of unifying and ranking the measures obtained in different studies.

Plastic pellets are also reported in the digestive tract of some marine animals that unintentionally ingested these particles. Seabirds are the mainly group reported by this ingestion, which about 40 species have already been reported, most Procellariiformes followed by Charadriiformes and Sphenisciformes (Van Franeker, 1985; Fry *et al*., 1987; Ryan, 1987; Ryan and Jackson, 1987; Robards *et al*., 1995; Spear *et al.*, 1995; Blight and Burger, 1997; Vlietstra and Parga, 2002; Ryan, 2008; Barbieri, 2009; Yamashita *et al*, 2011; Hammer *et al.*, 2016). There is some data about plastic pellets in other megafauna species, such as in striped dolphins *Stenella coeruleoalba* (Novillo *et al.*, 2020), Antarctic fur seals *Arctocephalus tropicalis* and *Arctocephalus gazella* (Eriksson and Burton, 2003), loggerhead turtle *Caretta caretta* (Ryan *et al.*, 2016; Pham *et al.*, 2017) and green turtle *Chelonia mydas* (Tourinho *et al.*, 2010). Teleost fishes are another important group affected by pellets ingestion, but there is not much data about it. Pellets were found in species as the Atlantic herring *Clupea harengus* (Carpenter *et al.*, 1972; Hjelmeland *et al*, 1988), the grubby *Myoxocephalus aenus*, the winter flounder *Pseudopleuronectes americanus*, the white perch *Roccus americanus*, the silverside *Menidia menidia*, the pollack *Pollachius virens*, and the sea robin *Prionotds evolans* (Carpenter *et al.*, 1972). Finally, plastic pellets also were found in some invertebrates such as squid (Braid *et al.*, 2012), anemones (Diana *et al*., 2020), and crabs (Costa *et al.*, 2019).

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Table 1.1. Plastic pellets reported from beaches throughout the world.

Continent	Country	Reference
North America	Canada and Bermuda	Gregory, 1983
	United States	Hays and Cormons, 1974
	United States	Moore *et al*., 2001a
	United States	Van *et al*., 2012
South America	Brazil	Costa *et al*., 2010
	Chile	Hidalgo-Ruz and Thiel, 2013
	Brazil	Ivar do Sul *et al*., 2009
	Brazil	Izar *et al*., 2019
	Uruguay	Lozoya *et al.*, 2016
	Brazil	Moreira *et al.*, 2016
	Brazil	Turra *et al.*, 2014
Europe	England	Ashton *et al.*, 2010
	Belgian	Claessens *et* al, 2011
	Germany	Dekiff *et* al., 2014
	Greece	Karkanorachaki *et al.*, 2018
	Portugal	Martins and Sobral, 2011
	Spain	Shiber, 1982
	Sweden	Karlsson *et al*., 2018
	Malta	Turner and Holmes, 2011
Africa	South Africa	Ryan *et al*., 2009
	South Africa	Schumann *et al.*, 2019
Asia	Jordan	Abu-Hilal and Al-Najjar, 2009
	Japan	Endo *et al*., 2005
	South Korea	Heo *et al.*, 2013
	United Arab Emirates	Khordagui and Abu-Hilal, 1994
	Russia and Japan	Kusui and Noda, 2003
	Lebanon	Shiber and Barrales-Rienda, 1991
	Lebanon	Shiber, 1979
Oceania	New Zealand	Gregory, 1978
	Australia and New Zealand	Yeo *et al*., 2015
Remote islands	Maldives	Imhof *et al.*, 2017
	Hawaii	McDermid and McMullen, 2004

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Table 1.2. Top 10 most polluted beaches by plastic pellets in the world. Extrapolations for studies data proposed by Turra *et al*. (2014), in pellets per cubic meter, until 2 meters deep of sediment.

#	Pellets m^{-3}	Beach	Country	Sample Area	Reference
1	1,045,353	Cargo Beach	Hawaii	High Tide Line	McDermid and McMullen, 2004
2	571,181	Itaquitanduva Beach	Brazil	Upper Limit Backshore	Izar *et al*., 2019
3	125,368	Papudo Beach	Chile	High Tide Line	Hidalgo-Ruz and Thiel, 2013
4	115,238	Góes Beach	Brazil	Upper Limit Backshore	Izar *et al*., 2019
5	111,963	Boa Viagem Beach	Brazil	Not Informed	Costa *et al*., 2010
6	68,031	Halawa Valley North Beach	Hawaii	High Tide Line	McDermid and McMullen, 2004
7	43,694	Turtle Beach	Hawaii	High Tide Line	McDermid and McMullen, 2004
8	31,526	Tern Island-South Beach	Hawaii	High Tide Line	McDermid and McMullen, 2004
9	29,775	Santos Beach	Brazil	Upper Backshore	Turra *et al.*, 2014
10	27,557	Mar Casado Beach	Brazil	Upper Limit Backshore	Izar *et al*., 2019

1.3.4.2. Ecological damages

Unintentional ingestion of plastic pellets is the main known impact of this marine litter. The damages on marine fauna are severe. This ingestion causes physical impacts, as obstruction of the intestinal canal, and reduced appetite, which may progress to starvation until the death (Milijö, 2001; Endo *et al.*, 2005).

Chemical exposure in organisms in different routes of transfering (mainly ingestion) may increase desorption of contaminants from the pellets due to the acid gastric from digestive system (Ryan *et al*., 1988; Endo *et al.*, 2005; Teuten *et al*., 2009; Hirai *et al.*, 2011).

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In marine animals, the ingestion of plastic pellets and their transfer along the food web might cause biomagnification and deleterious effects in top chain predators, especially the POPs compounds. The increasing of plastic pellets present in the coastal environment is also related to the ingestion of these particles by seabirds (Robards *et al*., 1995). Also, some marine animals, especially seabirds, have color-selective feeding preferences for bright colors (Sileo *et al*., 1990; Vlietstra and Parga, 2002; Santos *et al*., 2016), which raise a concern about virgin pellets and their industrial chemical additives.

Non-ingested impacts are also identified for marine organisms. Plastic pellets may release into the environment some harzardous compounds that may induce toxicity to the marine biota (Izar *et al.*, 2019). Reported studies done in laboratory showed that direct exposure to plastic pellets may induce acute and chronic toxicity for some marine organisms under controlled conditions (Gandara e Silva *et* al., 2016; Nobre *et al.*, 2015; Izar *et al.*, 2019). In turn, some sublethal effects are possible to be detected by the biomarkers' approach, such as effects on lysosomal membrane stability in oyster (Nobre *et al*., 2020) and down-regulation on genes expression in fishes (Rochman *et al.,* 2014). However, currently there is few information regarding the toxicity and possible sublethal effects.

In regard to ecological impacts, some marine species have the ability to colonize new regions, by drifting across the oceans through surface currents and facilitated by using marine litter as carriers (Milijö, 2001; Barnes, 2002; Barnes and Milner, 2005). Hydroids, diatoms, algae, fungi, and some insects have already been documented on plastic pellets surface, raising concerns regarding the colonization of alien species favored by these particles (Gregory, 1978; Majer *et al*., 2012; Lacerda *et al.*, 2020). Biological invasions have negative consequences to the ecosystems, since ecological imbalance and extinction of native species, and must be considered harmful (Vitousek *et al*., 1996; Minchin and Gollasch, 2003).

1.3.5. Impacts (on Welfare)

For humans, aesthetic (*i.e*. large quantities of plastic pellets on beaches) and economics impacts are the most evident ones. Both bring some interested parties to the framework (Newton and Elliott, 2016): tourists, environmentalists, scientists, and industry - the latter can also act as stakeholder. Scientists and environmentalists identify the social and environmental impacts of plastic pellets and their distribution in the environment and generate

new information regarding pellets (by advancing the frontiers of the knowledge). Beaches with large quantities of plastic pellets and other marine litters usually generate visual discomfort to tourists and recreational goers, resulting in financial losses due to decreased tourism (Botero *et al.*, 2017; Jang *et al*., 2014*;* Leggett *et al.*, 2018). Tourists, scientists, and environmentalists are important actors in the pressure for responses, in addition to the industry due to pressures for decreasing the pellet losses throughout the processes, since it means losses of raw materials, which have an economic impact to the industry profits.

The impacts on human health are still unknown. It is accepted that the principal route which humans consume microplastics probably is through food, especially when consuming fishes and seafood (Smith *et al*., 2018; Naidoo and Rajkaran, 2020). Even though human beings are exposed through food to low concentrations of chemicals and/or microplastics during their lifetime (Frias *et al*., 2010), considering they are the top predators in the food chain, at long-term exposure this may be a significant health problem. Still, for some chemicals there is no safe concentration and even low concentrations can be an issue. Some of those chemicals found in the composition or adsorbed on microplastics are known to cause or suspect, of causing endocrine disruption, cancer, and neurological diseases, among other health-related endpoints (Montagner *et al*., 2017; Pieroni *et al*., 2017). Thus, it is highly recommended to develop more studies in order to tackle possible adverse impacts for human health. Indeed, microplastics in human system are capable of disrupting cellular processes and degrade tissues (Rochman *et al.*, 2013).

As described, impacts by plastic pellets are still poorly understood, both in marine fauna and in humans. However, with the current state of the art about pellets, the DAPSI(W)R(M) framework for this socio-environmental problem can be built (Fig. 1.2). Each topic discussed above is represented in the figure by a blue box of the framework with their related players (stakeholders) subscribed on right.

1.3.6. Responses (as Measures)

Responses are management measures (Borja & Elliott, 2019) and the responsibility for this management lies with the last stakeholders involved in the chain: government, private institutions, scientific academy and non-governmental institutions (ONGs), in addition to civil society and industry, both also acting as interested parties. Government is at the center of the

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responsability in this issue, due to being responsible for creating regulations and laws for their correct application. The others stakeholders involved act suggesting public policies and producing studies and knowledge to support them.

It is important to understand that stakeholders may affect and may be affected in different times and stages along the chain (Fig. 1.2). For instance, the industry is relevant in drivers, activities and impacts components of the framework, while consumers in drivers can be the same person that is impacted in tourism or in human health. This is the main challenge for the implementation of awareness strategies, because there is no direct and immediate impact on the actors who exert the pressure. The impacts affect third parties and are long-term impacts. Tight feedback loops are one of the recognized principles of sustainable societies (Lewis and Conaty, 2012) and, in order to manage plastic pellets issue, the engagement of the whole stakeholders and society is essential. The proposed measures are summarized in the table 1.3 and discussed futher in the following paragraphs.

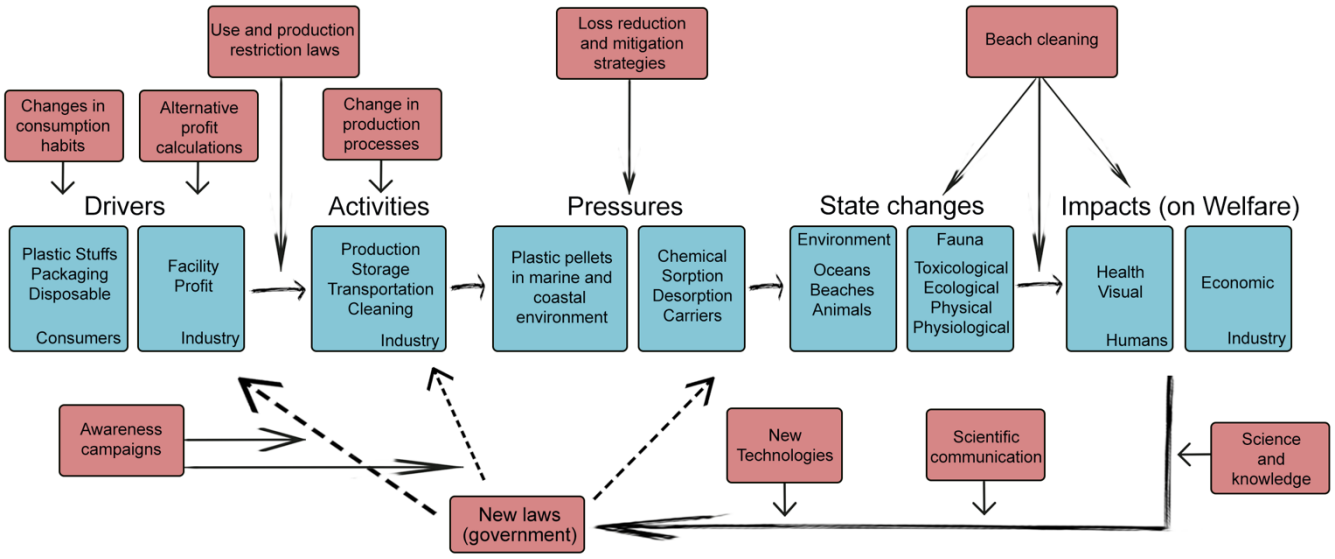

Fig. 1.2. DAPSI(W)R(M) framework applied to plastic pellets chain, focused on Drivers, Activities, Pressures, State changes and Impact (on Welfare) components. Blues boxes represent a group of topics related to the component. Right bottom texts are the stakeholders responsible of each box or the group affected by the box topics. Red boxes represent the Responses (as Measures) applied in each part of the model, which can act directly in the box or between them (arrows).

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Table 1.3. Main Responses (measures) summarized for plastic pellets chain, their effectiveness and the stakeholders involved in each measure, using the DAPSI(W)R(M) framework.

Management Measures	Stakeholders involved	Effectiveness
Change in consumption habits	Consumers	Very high - greatly reduces the need to produce plastics
Alternatives proftis calculations	Industry and Government	High - encourages the industry to look for new ways to produce and profit
A code of conduct for industries	Industry and Government	High - prevents new pellets from entering the environment
Beach cleaning	Non-governmental institutions (ONGs)	Low - local solutuon
Effective comunication	Science, Government and Midia	Medium and long-term
Political awareness and restrictive laws	Government	High - act precisely at the source of pelltes loss
Recycling	Consumers, Industry and Government	Very high - reduces and displace in production of virgins plastic pellets

The change in consumption habits (the culture of disposable items) is an important and essential response that acts at the beginning (drivers) of the plastic pellets chain. Single-use products are the main items generally found in oceans and rivers (Schwarz *et al*., 2019). Reducing the use and dependence of plastic objects, packaging and plastic transport bags would probably decrease the demand for production of these products and plastic raw materials (plastic pellets). However, this change must take place on a large scale, as a social pact. Individual and local initiatives (*i.e.* banning of plastic bags) are important, but have little effect on the overall problem (1% of plastic litter found in the oceans and rivers are packaging

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- Schwarz *et al*., 2019). Changing disposable habits (single-use and hard-to-recycle plastic) is perhaps the biggest challenge proposed in this model, since the industry imposes a pre-determined form of consumption. Once this form of consumption is changed, the demand of production of plastics raw material is reduced. However, rethinking consumption habits should be a worldwide need in a near future. Alternative materials such as wood and glass should also be considered, even the production of these alternatives materials being negative to climate changes (Rochman *et al.*, 2013). In this point, there is a conflicting issue among plastic debris and climate changes that must be considered from both perspectives. As an exemple of this initiative, New Zealand announced this year (2022) the ban of most single-use plastic by 2025, and research funding for alternatives to the use of plastic.

Moreover, still considering the drivers' component, alternatives for guaranteeing the profits can be a proposed solution for the industry players. Within this context, new calculations should be implemented (*i.e.* polluter-pays principle), valuing raw materials and healthy environments. The pellets' losses should be understood as an expense and loss of a significant share of the margin of profit. For this, new technologies should be developed and implemented in order to avoid financial losses to the industry. For a complex issue such as pollution from plastics, both direct regulatory measures (command and control) and economic instruments are needed. In this way, taxation and/or tax exemption are essential as allies for the current profit model to be recalculated and for rethinking alternative models. Laws for restrictions of use and restrictions on the production of plastics should also be used as allies for the reduction of demand, acting among drivers and activities components in the framework. National and/or sub-national reduction targets are needed to determine an agenda to overcome the problem of plastic in the oceans.

Measures of technical improvement should also be considered, especially regarding changes in production and transportation processes. The granular shape of the pellets allows them to be handled and transported in bulk, which generates many points of spills into the environment (Ogata *et al.*, 2009). Effective cleaning, vacuum systems, and installation of sewage screening systems in industries, factories and port terminals can significantly reduce pellets' losses during the industrial and transportation processes (Pereira, 2014; Worm *et al.*, 2017). These suggestions could be used for solving the problems regarding pellets losses in the industrial chain and, consequently, could reflect in increases of the profits of the industry players. It is worth mentioning that plastics are essentially a terrestrial problem, with

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consequences in the marine and coastal environment. Plastic are manufactured and used mostly in the terrestrial environment, and have reached the marine and coastal environment after their inappropriate disposal or loss. Hereupon, a code of conduct for industries producing plastic pellets must be considered, identifying points of loss in the process and implementing remediation and mitigating actions (*i.e.* sweeping and vacuuming of the warehouses, reuse of collected pellets) (Pereira, 2014), such as the change in consumption habits as discussed in the previous paragraphs. Carrot and stick motivation might be an effective approach for this stakeholder (industry), with economic rewards for good conduct pratices and several fines and penalties for industries that do not reach a minimun target.

The cleaning of the beaches is the mitigating measure with greatest prominence and appealing value for the civil society, but it is the least effective one for the issue in the framework. Beach cleaning efforts are important locally and with immediate effectiveness, especially visually and in terms of well-being. However, it is focused on macro marine litter, which might be important to avoid the generation of secondary microplastics. For solid waste of reduced sizes, as plastic pellets and other microplastics, different strategies are needed. Mechanical separation techniques (as sand sieving) might be an alternative that can be effective. However, it requires a huge personal and financial effort, and may have negative effects on the local fauna (Fanini and Bozzeda, 2018). Tow nets, as proposed by Ribic *et al*. (1992), follow the same logic. On the other hand, the afore mentioned response of adopting filtration meshes and filters in sewage systems are essential and effective in mitigating the problem (Karlsson *et al*., 2018) in addition to having the advantage of acting upstream in the chain of causes and consequences.

Science is an important key to managing the issue of plastic pellets in the ocean. Science generates knowledge and identifies possible knowledge gaps, which allow a better understanding of the actors, in order to propose and implement effective measures. The dissemination of the scientific knowledge through scientific communication techniques is a response that goes hand-to-hand with the production of knowledge. It is essential that the knowledge generated is transmitted clearly and easily to all stakeholders involved. Scientific communication can act in any component of the model. It creates the scientific bases and puts pressure on the stakeholders, mainly on decision makers. Such as effective communication (*i.e.* environmental education, public awareness and media) that will make the

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society aware, especially the new generations, of tolerance and morality (ethic), about not leave debris or generate the least amount of debris possible (Borja & Elliott, 2019).

In many countries, the political awareness of the microplastics problem (which include plastic pellets) is inadequate and long overdue (Lechner and Ramler, 2015). Government is a key stakeholder in the chain of causes and consequences of plastic pellets. It is through public policies that measure of education and awareness, economic incentives, and direct regulation are put into practice. The performance of this stakeholder permeates all components of the framework and puts pressure on the other stakeholders for complying with the mitigating measures. This is the main target of dialogue and pressure from civil society. There is in Europe a policy to avoid major pellets spill and leakage events (Karlsson *et al.*, 2018). The first international policy response about marine litter was the International Convention for Protection of Pollution from Ships (MARPOL), which prohibited the disposal of plastic waste in the oceans. MARPOL was approved in 1973, but policies came into effect only in 1988. Few years later, in the United Nation (UN) Conference in Rio de Janeiro, Brazil, in 1992 (Rio-92), it was proposed the reduction of waste emissions, addressed in the Agenda 21. In 2011, the Honolulu Strategy brought orientations for the stakeholders to reduce and manage marine litter. Recently, in 2017, UN Environment launched the Clean Seas Campaign, a global campaign to reduce marine plastic litter, focused on production and consumption of single-use plastics. More than 60 countries have committed to it (United Kingdon, New Zealand, United States of America, Brazil, Colombia, Ecuador, Uruguay, Canadá, India and others). As a global challenge, plastic litter reduction depends on global engagement that reaches all levels of society (Worm *et al*., 2017). All responses would contribute to the UN 2030 Agenda for Sustainable Development, targeting three Sustainable Development Goals (SDGs): SDG11 - Sustainable cities and communities, SDG12 - Responsible consumption and production and SDG14 - Life below water. Strict laws that limit the consumption of single-use and hard-to-recycle plastics, and reduce marine plastic litter act directly on the drivers, forcing the society to change consumption habits and reducing the need to produce more plastic stuff, consequently its raw material - plastic pellets.

Finally, beyond reduce and rethink already addressed in our discussion, recycling must be an option to be considered, completing the 3Rs of waste management. Recycled plastic pellets will displace virgin pellets of the same polymer, in addtional to saving on fossil fuel consuption and helping with greenhouse gas emissions (Basuhi *et al*., 2021). Therefore, the

life cycle of plastics objects have to change, with respect to the mindset of consumers and industry. We must start to understand this life cycle as a cycle that can be reused (circular economy), and not as a line with a beginning (production), middle (use) and end (discard). For this new conception (paradigm), consumers are essential in separating disposable plastics products for recycling. For the industry, to recycle 100% of the plastic used on the world requires a high and pratically unfeasible investment (Basuhi *et al*., 2021). On the other hand, recycled pellets products can be competitive in price in the market, in addional to increasing employment for local communities and distributing income (Borman *et al*., 2019). And finally, government is also a key stakeholder capable of creating laws that force and encourage industries to recycle, and tax incentives for recycled plastic pellets to be competitive in the market. This new paradigm is a long-term response and, as with all the above measures, an integrated effort of all stakeholders involved is needed for a sustainable solution (Agamuthu *et al*., 2019).

1.4. CONCLUSION

DAPSI(W)R(M) framework applied to plastic pellets helps to have an overview of the challenge. Our study organize informations about plastic pellets chain aim to help politicians and decision makers to understand each part of the chain easily and visually. In this framework approach, it is possible to conclude that any solution to solve this problem is interconnected with other possible solutions and needs the engagement of all stakeholders involved. The framework allows us to identify where each solution is acting in the chain and how the interaction between them is necessary to be an effective solution. We hope that this DAPSI(W)R(M) analysis can contribute for future studies that find solutions that minimizing marine plastic litter problem.

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CHAPTER 2

Plastic pellets make *Excirolana armata* more aggressive: intraspecific interactions and mortality in field and laboratory ecotoxicological assays

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ABSTRACT

Microplastics, including plastic pellets, get stranded on sandy beaches. They persist in the oceans for long periods and frequently carry contaminants. Acute and chronic toxicity has been observed when marine organisms are exposed to high densities of plastic pellets in laboratory assays. We investigated the toxicity of beach-stranded plastic pellets on macrobenthic populations (*Excirolana armata;* Crustacea; Isopoda) under natural conditions (*in situ)*. We simulated different pellets densities on a beach not contaminated by pellets, exposing isopods for 6 hours and testing possible behavioral responses (i.e., vertical displacement) and mortality effects. No effect was observed on vertical displacement, but higher mortality was reported for organisms exposed to plastic pellets. The lowest pellet density tested commonly found in coastal areas was sufficient to trigger mortality. We also observed that lethargic individuals (near-death) were preyed on by the healthy individuals remaining in the test chambers.

Keywords: Microplastics, Marine pollution, Ecotoxicology, Ecological impacts, Field experiments, Sandy beaches

2.1. INTRODUCTION

In a world that produces over 400 million metric tons of plastic annually (Geyer et al., 2017), nine out of ten items of marine litter are plastic (Milijö, 2001). In this scenario, microplastic waste (diameter < 5 mm) becomes a problem, especially when the main route of transport for the plastic raw material (plastic pellets) is maritime. Unintentional plastic pellets losses during manufacture and transportation cause their release and spread across the marine environment (Ogata et al., 2009).

The most described impact of microplastics on marine life is the unintentional ingestion by vertebrates (Ryan, 2008; Tourinho et al., 2010; Carpenter et al., 1972; Novillo et al., 2020) and some invertebrates (Costa et al., 2019; Diana et al., 2020). Unintentional ingestion can obstruct the digestive tube reducing appetite and absorption of nutrients, as well as causing internal lesions. Moreover, during their time floating in the ocean, plastic pellets can adsorb and concentrate hydrophobic chemical compounds (Mato et al., 2001). Consequently, industrial additives can also be desorbed into the environment from plastic pellets (Hammer et al., 2012). Such debris are hazardous, as they strand and accumulate along coastal areas globally, which may promote the persistence of these contaminants in the environment (Teuten et al., 2009), especially on sandy beaches where these particles tend to accumulate (Moreira et al., 2016; Corcaran et al., 2020).

Non-ingestion impacts have also been reported in some marine organisms tested in laboratory assays (Nobre et al., 2015; Izar et al., 2019). In those cases, the release of chemicals from the plastic pellets induced acute and chronic toxicity (Silva et al., 2022). However, these results show the reality of laboratory settings with controlled variables and use of extremely high plastic densities to induce toxicity. There is a gap in information about realistic plastic pellet densities and field studies (Moreira et al., 2016; Ma et al., 2020; Pinheiro et al, 2020) that could provide information about the real impacts of this debris on coastal areas, where the natural conditions may vary largely It thus becomes necessary to test if the ecotoxicological effects of exposure to plastic pellets under natural conditions remains the same in the presence of other environmental factors.

Sandy beaches are especially important for human populations, and are heavily threatened by microplastic pollution (Turra et al., 2014). Macrobenthic populations are responsible for sediment bioturbation and secondary production in these beaches (Machado et

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al., 2004; Petracco et al., 2012a). They contribute to the energy-nutrient cycle on coastal food webs. Nevertheless, macrobenthic species are sensitive to human impacts (Schlacher et al., 2008; Amaral et al., 2016. In long term, microplastic pollution could jeopardize the delicate ecological balance and functions of beach ecosystems.

Cirolanid isopods are an important and abundant macrobenthic taxa in sandy beaches, especially in supralittoral and intertidal zones around the world (Wendt and McLachlan, 1985). *Excirolana armata* has high mobility and abundance on beaches with fine sands from Rio de Janeiro to northern Patagonia (Defeo et al., 1997; Lozoya et al., 2010). This is an important feature regarding model organisms for plastic pellets toxicity since there is a tendency for such particles to accumulate in sandy beaches with fine sands (Corcoran et al., 2020; Vermeiren et al., 2021). *E. armata* is also resistant to environmental stress (Laurino and Turra, 2021), and ecologically dominant, in terms of abundance throughout many sandy beaches of South America (Lercari and Defeo, 2003). The population is distributed in patches and leaves trails on the sand surface in the washout zone until the intertidal upper limit (Gandara-Martins et al., 2015) and has also been used as a model organism for the ecological effects of climate change (Laurino et al., 2020).

We aimed to test the toxicity of beach-stranded plastic pellets on macrobenthic populations of isopods (*E. armata* - Crustacea) in field-natural conditions. We expected that high pellet densities would induce vertical displacement as a behavioral mechanism to escape from the stressor or cause mortality since toxic effects were already observed in laboratory studies for high densities of plastic pellets (Izar et al., 2019). We conducted three experiments (one *in situ* and two in a controlled environment): 1) Beach simulations: which consisted of an assay with real densities of beach-stranded plastic pellets in field-natural conditions to test our main hypothesis; 2) Closed system exposure: which compared the exposure to pellets for the same population of *E. armata* in a small scale and laboratory-controlled assay; and 3) Survival Analysis: a laboratory-controlled assay testing the organisms' survival after being exposed to plastic pellets in a seawater matrix. In addition, a biomarkers analysis was performed to generate information on the sublethal impacts of experiments 1 and 2.

2.2. METHODS

2.2.1. Beach simulations: field-natural experiment

This simulation consists of an assay with real densities of beach-stranded plastic pellets in natural conditions to test if high pellet densities induce vertical displacement. Experiments were conducted in Dura beach (Ubatuba – Brazil), located on the coast of São Paulo (Southeast Brazil). This beach is far from the Santos port (140 km), the main regional source of plastic pellets. Due to the distance, geographical and oceanographic characteristics, this beach has naturally low pellet densities (Moreira et al., 2016; Izar et al., 2019). Thus, Dura beach may be considered as the negative control condition, allowing experiments that simulate exposures to different pellets' densities.

We used beach-stranded plastic pellets (2.5 - 4.5 mm size) collected on 3 beaches (Itaquitanduva, Góes, and Itaguaré) in June 2020 and March 2021 for all the experiments in this study. All sampled beaches are under the influence of Santos Port - the largest port in Latin America, and close to the major industrial complex of Cubatão. Itaquitanduva and Góes beaches are located inside Santos Bay (where the Santos Port is located), and Itaguaré beach is located outside Santos Bay close to the mouth of the Bertioga Channel, which also contributes with pellets to the region (Izar et al., 2022) and is located inside a state park. Pellets from those beaches presented high concentrations of hydrophobic adsorbed contaminants, especially PCBs, DDTs, and PBDEs (Taniguchi et al., 2016; Ohgaki et al., 2021). For this investigation, plastic pellets were sampled by active search, in which the pellets were manually collected (~5 thousand in total) from the sand surface along the high tide line after visual identification. To avoid biological contamination or loss of adsorbed contaminants during storage time, sampled plastic pellets were mixed, stored in glass containers, and kept refrigerated (5 to 10^{o} C) until use. In all experiments of this study, beach-stranded plastic pellets were chosen at random, regardless of color, polymer type (not analyzed in this study), or beach that they were sampled. This was done to simulate the heterogeneous conditions of plastic pellets in the natural environment (Izar et al., 2022).

PVC cylindrical corers (15 cm diameter and 25 cm high) were inserted (20 cm) into the sediment (leaving 5 cm of corer above the sand surface) in a rectangular limit experimental area (150 m in length and 10 m in width). Corers were aligned in the upper intertidal zone parallel to the drift line. Pellets densities were calculated for the corer volume and placed on the surface layer (0, 3, 18, 100, and 200 pellets corer^{-1}). We made three replicates for each treatment and control, totaling 15 corers. The order of each treatment inside the sampling area was randomly sorted to the buried corers. In each corer, we exposed

10 juvenile individuals (~3 mm size) of the semiterrestrial crustacean *E. armata* (Isopoda: Cirolanidae) for 6 h (Fig 2.1. - #1). We buried the corers in the transition between the intertidal and supralittoral zones. This minimized the presence of previous organisms in the sediment layer. After the individuals were added to the surface of each corer, we covered the top exit using a 0.5 mm mesh net to prevent escaping, as described by Laurino et al. (2020), and added seawater (~100 ml) every 30 minutes to avoid desiccation and to facilitate the organisms' mobility. At the end of the exposure time, corers were dug up and each sediment layer was sifted according to the depth: surface (0 - 3.5 cm) and deep (3.5 - 20 cm). *E. armata* individuals were accounted for within each layer in each treatment. The experiment was repeated for eight days in different sites on the same beach, totaling 120 replicates divided into the five treatments described above (n = 24 per treatment).

The number of plastic pellets used in each corer (pellet densities) was based on information reported for other sites around the world: A - control (absence of pellets); B - the most frequent density found on beaches along the São Paulo coast, Brazil (Izar et al., 2019), and on some Hawaii beaches (Mcdermid and Mcmullen, 2004) (20,000 pellets m^{-3}); C – pellet density in a beach located inside the Santos Bay, close to the Santos port (100,000 pellets m^{-3}); and D – the highest density of plastic pellets found in Brazil (Izar et al., 2019) (600,000 pellets m^{-3}), and E - the highest pellet densities found in the world (Mcdermid and Mcmullen, 2004) (1,000,000 pellets m^{-3}). The pellet densities were estimated following the extrapolation method suggested by Turra et al. (2014) for cubic meters, in an attempt to unify units as proposed by Gimiliani and Izar (2022). These densities were chosen to try to represent realistic plastic pellets exposure conditions, and possible simulation of future exposures. Thus, these densities do not necessarily represent those found on the beaches where the tested isopods occur.

Vertical displacement and mortality of *E. armata* were measured in each treatment as dependent variables. Vertical displacement was determined as the total number of organisms found in the deep layer (3.5 - 20 cm). Mortality was the total number of dead organisms found in both layers. Missing individuals were considered dead because of the scavenger behavior of this species (Bergamino et al., 2011). Thus, we assumed that the missing individuals were eaten after death by the surviving individuals since only about 10% of non-living (missing + dead) organisms were accounted for in the field experiment. For both dependent variables, we used a Generalized Mixed Model (GLMM) with a Poisson distribution, as it is the most

appropriate distribution for counting data and it was the model with the best fit for our data (smaller Akaike Information Criterion - AIC). We built the model with two independents variables: Date (Factor 1, random, 8 levels: regarding the temporal replications) and Treatment (Factor 2, fixed, 5 levels: regarding the pellets concentrations), adopting a significance level at 95% ($p \leq 0.05$ for all analyses in this study). The ratio rate (RR) was calculated for each significant treatment compared with the control. All statistical analyses performed in this study were built using the open statistical software Jamovi (2021).

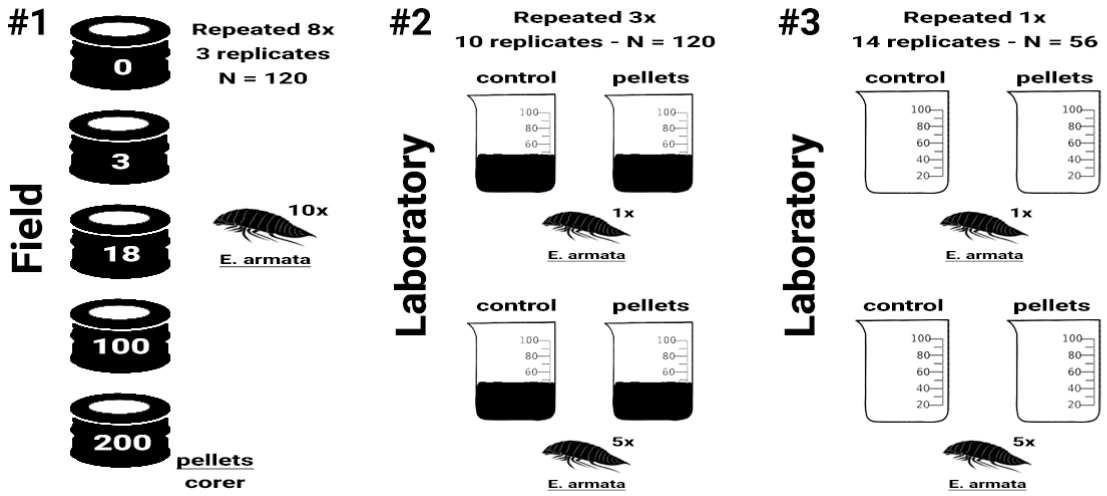

Fig. 2.1. Experimental designs performed in this study. #1 - Beach simulations: field-natural experiment. #2 - Closed system exposure: laboratory-controlled assay. #3 - Survival Analysis: laboratory-controlled assay

2.2.2. Closed system exposure: laboratory-controlled assay

The field experiment described in the previous section has some advantages such as observing toxicity effects under natural conditions, but it was also an open-environment approach with risks such as losing organisms and where the natural variables were not controlled. As previously mentioned, considering that *E. armata* is a scavenger animal, we assumed that the missing individuals at the field experiment were eaten after death by the surviving individuals. So, to validate this assumption, we elaborated a simple controlled experiment in a closed system. These experiments were conducted using the *E. armata* population from Dura beach (the same beach chosen for the previous experiment).

The experiment had two treatments: control (no pellets exposure), and a very high pellets density that would ensure a toxic effect (~40 pellets per beaker = 2,000,000 pellets m^{-3} - twice as much as found in Hawaii by Mcdermid and Mcmullen [2004]). Beach sediments

were sampled and previously sieved to avoid isopods in the samples. 20 mL of this sieved sediment was placed into a glass beaker (50 mL). Plastic pellets were then added to the surface layer. In each treatment, we exposed 1 and 5 organisms of *E. armata* per beaker for 6 h (the same time as the beach simulations). Natural populations of *E. armata* are commonly observed in high densities, reaching 500 - 2000 individuals per square meter (Lozoya et al., 2010; Petracco et al., 2012b), thus the number of individuals used in this experiment should not be an additional stressor. Moreover, we compare the pellet treatment results with a control experiment in which the same number of individuals are present in the beaker without pellets, further eliminating the possibility of a purely density dependent effect. We replicate the exposure ten times for each group of organisms (1 and 5). This number of individuals was chosen to confirm that dead individuals are eaten by surviving individuals (scavenger habit) since this behavior could occur in the chambers containing 5 individuals but not in those with a single individual. Moreover, the container (beaker) was closed and did not allow the organisms to escape, unlike our field experiment where there is a very low possibility of escaping, which needs to be considered. At the end of the exposure time, sediment was sifted, and the organisms were accounted for in each treatment. Dead and missing organisms were individually counted per beaker and summed to obtain total mortality since missing individuals were considered dead (consumed by the surviving individuals). The experiment with ten replications per group of organisms (1 and 5) was repeated three times, totaling 120 replicates (n = 30 per treatment) (Fig. 2.1 - #2). A Generalized Mixed Model (GLMM) with a Poisson distribution was built for each group of organisms (1 and 5 individuals) for mortality (dependent variable) with two factors: Date (Factor 1, random, 6 levels), and Treatment (Factor 2, fixed, 2 levels).

2.2.3. Survival Analysis: laboratory-controlled assay

Also aiming to validate our assumption regarding the scavenger behavior on the *in-situ* results, we elaborated a third experiment. We exposed 1 and 5 organisms of *E. armata* from the Dura beach population to two treatments (control and pellets), following the same pellets density from the closed system exposure. However, this experiment was performed without sediments, allowing us to visualize the whole process including the scavenger behavior of surviving individuals. The organisms were exposed in a transparent glass beaker with 40 mL of seawater from Dura beach for 6 h (the same exposure time as the field experiment). We

then visually evaluated the first mortality and the event of after-death consumption in each beaker (through the glass), hourly for 96 h. In this experiment we had 14 replicates for each treatment and group, totaling 56 replicates divided into two treatments for two groups of organisms exposed (n = 14 per treatment) (Fig 2.1 - #3). Associations between both events (mortality and after-death consumption) were tested using a Cox regression model based on Kaplan-Meier estimates for each group of organisms (1 and 5 individuals), with treatment as an explanatory variable and time to each event as the dependent variable.

2.2.4. Sublethal effects: biomarkers analysis

To address the sublethal effects of exposure of *E. armata* to plastic pellets, we analyzed biochemical biomarkers in the surviving organisms from the beach simulations and the closed system exposure (experiments 1 and 2). After exposure, the surviving organisms were immediately frozen in ice and transported to the laboratory, where they were stored in an ultrafreezer (-80°C). Contamination biomarkers were then analyzed, namely oxidative stress (Lipid peroxidation - LPO) and DNA damage. LPO analysis was performed following the thiobarbituric acid method proposed by Wills (1987), using fluorescence with 516 nm of excitation wavelengths and 600 nm of emission wavelengths. Blanks and tetramethoxypropane standards were prepared in homogenizing solution. Results were expressed in μM TBARs mg^{-1} of total protein. DNA damage was evaluated by the alkaline precipitation proposed by Olive (1998), using salmon sperm DNA standard curve for damage normalization and Hoechst solution to give luminescence to the sample. The damage was quantified by fluorescence, applying 360 nm of excitation wavelengths and 450 nm of emission wavelengths. Results were expressed in μg DNA mg^{-1} of total protein.

In both analyses (beach simulations and close system exposure), for LPO, a Generalized Linear Model (GLzM) with a Gamma (link function = Identity) distribution was built, for the factor: Treatment (Factor 1, fixed, 5 and 2 levels, respectively). For DNA damage, a General Linear Model was built for the same factor.

2.3. RESULTS

2.3.1. Beach simulations: field-natural experiment

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For vertical displacement, on average, 0.75 ± 1.19 organisms sunk to deeper layers in the control experiment, while the highest sinking rate was 0.88 ± 1.80 in treatment E (higher pellets density). In 65% of replicates, no organisms left the superficial layer. No significant difference was observed for vertical displacement between treatments (AIC = 278.5; X^2 = 0.48; p = 0.95) and random factor (Date, p = 0.46).

The average mortality in the control treatment was 0.17 ± 0.38, whereas it reached 1.33 ±1.61 in the treatment with higher mortality (D). In the control, 16.5% of replicates showed at least one dead individual and in the presence of plastic pellets these proportions were: B = 54.0%, C and D = 62.5%, and E = 58.0%. Mortality was significant higher for all treatments with plastic pellets (B, C, D and E) compared to the control (AIC = 326.6; X^2 = 16.4; p = 0.003) (Fig. 2.2), reaching a maximum of eight times more deaths in presence of pellets (RR = 6 - 8) (Table 2.1). The random factor was not significant (Date, p = 0.17).

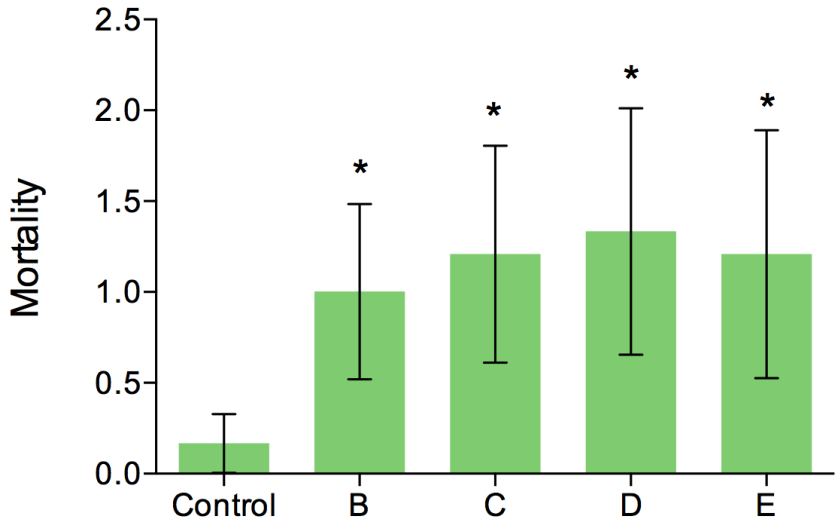

Fig. 2.2. Mortality of *Excirolana armata* per treatment: Control = 0 pellets, B = 3 pellets corer^{-1}, C = 18 pellets corer^{-1}, D = 100 pellets corer^{-1} and E = 200 pellets corer^{-1}. Error bars represent a 95% confidence interval and asterisks represent toxic effects compared to the control.

Table 2.1. Fixed effects parameter estimates in GLMM (Poisson) for mortality of *Excirolana armata* in presence of plastic pellets. Control = 0 pellets, B = 3 pellets corer^{-1}, C = 18 pellets corer^{-1}, D = 100 pellets corer^{-1} and E = 200 pellets corer^{-1}. Effect = Treatment compared to Control, exp(B) = Ratio Rate, 95% CI = Confidence interval, and p = p-value

Effect	exp (B)	95% CI	p

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B - Control	6.00	2.09 - 17.2	< 0.001
C - Control	7.25	2.57 - 20.5	< 0.001
D - Control	8.00	2.85 - 22.4	< 0.001
E - Control	7.25	2.57 - 20.5	< 0.001

2.3.2. Closed System Exposure: laboratory-controlled assay

No mortality was observed in treatments with 1 organism exposed. For the group with 5 organisms exposed, the average mortality in the control was 0.03 ± 0.18, while in the pellets treatment it was 0.57 ± 0.73. Mortality was significantly higher for plastic pellet treatments compared to the control (AIC = 73.66; X^2 = 7.58; p = 0.006), with 17 times more isopods deaths in presence of pellets (RR = 17, % CI = 2.26 - 127.74) (Fig. 2.3). Random factor was also significant (Date, p < 0.001). At the end of the assays, five organisms (8.3% of the total and 27.8% of the total mortality) and 13 organisms were missing (21.7% of the total and 72.2% of the total mortality).

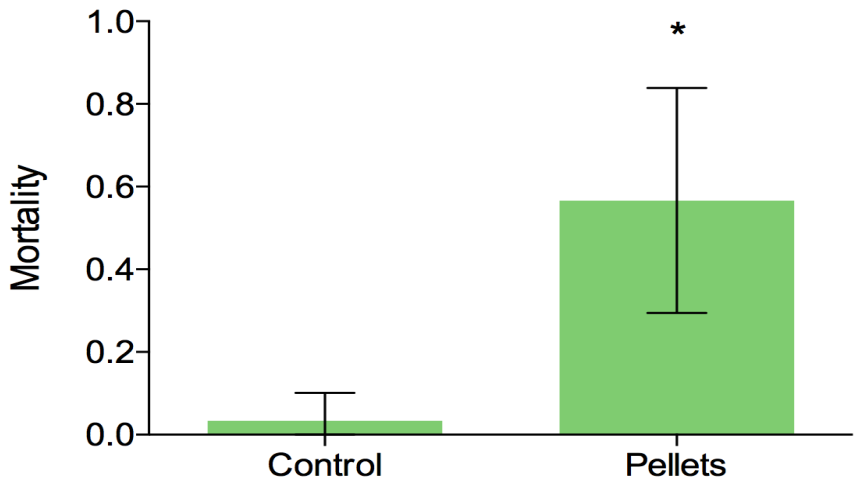

Fig. 2.3. Mortality of *Excirolana armata* per treatment: Control and Pellets. Error bars represent a 95% confidence interval and the asterisk represents the toxic effect compared to the control of the same beach population.

2.3.3. Survival Analysis: laboratory-controlled assay

After the 6 h exposure, no mortality was observed in both group of organisms. At the end of 96 h, no mortality was observed in treatments with 1 organism exposed. For the 5 individuals group, the first death was observed after 24 h of exposure to the pellet treatment, and after 30 h in the control. At the end of the experiment, 50% of replicates had at least one event of mortality and scavenger behavior events (after-death consumption) in the pellet treatment and 21.5% in the control treatment.

For the 5 individuals group (the only one with mortality), there was no difference between mortality and scavenger behavior events ($p = 1$), since both were observed at the same time in both treatments. We observed the surviving individuals feeding on dead individuals. We also observed a cannibalism behavior, with lethargic individuals (in a near-death state) being preyed on by healthy individuals. As soon as an individual died or showed decreased activity (lethargy), it was immediately cannibalized regardless of pellet exposure. We thus considered both events as one in the analysis. Organisms in pellet treatments tended to have a 2.77 times higher risk of mortality and/or cannibalism (CI95%: 0.71 - 10.73, $p = 0.13$) than those placed in the control treatment (Fig. 2.4).

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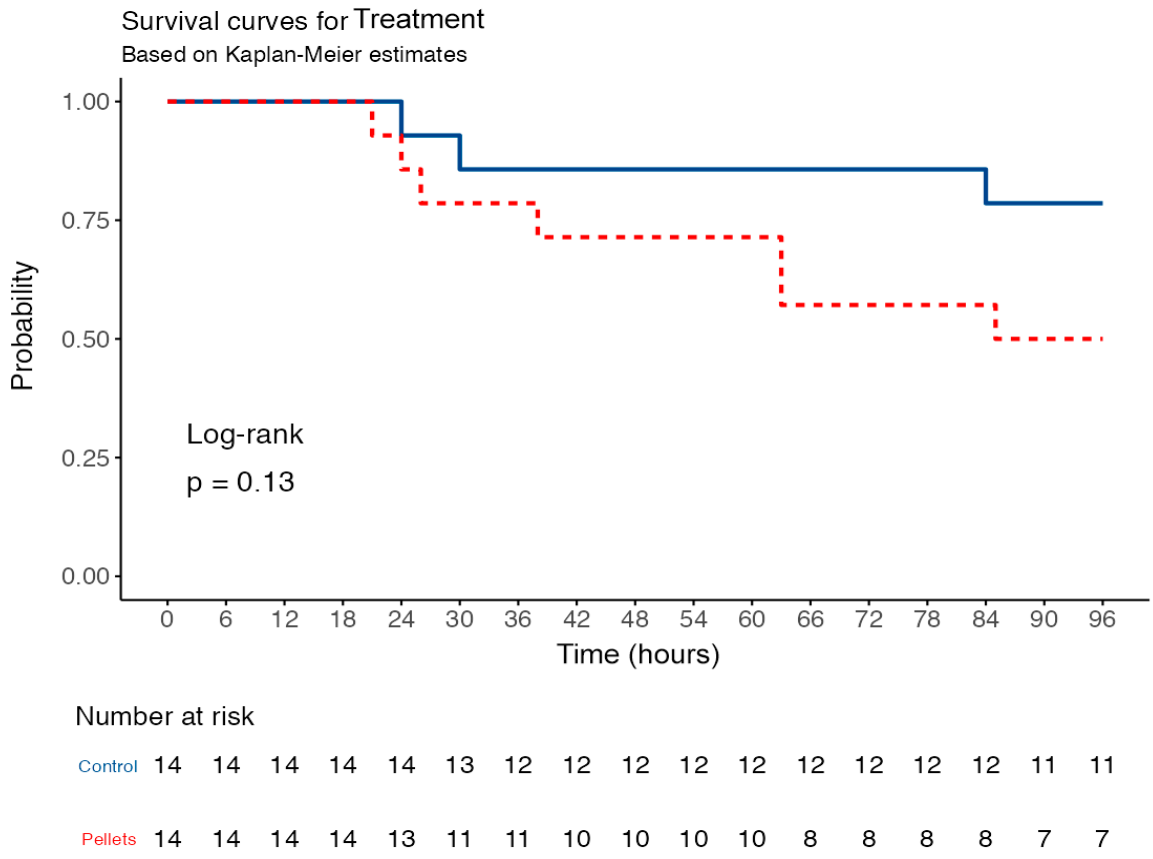

Fig. 2.4. Survival curves for Treatment based on Kaplan-Meier estimates. Probability of mortality and/or cannibalism events for 96 hours in 5 individuals of the *Excirolana armata* group exposed to pellets - 14 replicates per treatment (n = 28). The continuous blue line represents control treatment and the dashed red line represents pellets treatment. The number at risk is the number of replicates without mortality until the observation.

2.3.4. Sublethal effects: biomarkers Analysis

For beach simulations surviving organisms, lipid peroxidation (LPO) was, on average, 0.483 ± 0.176 µM of TBARS mg^{-1} of total protein in control, and 0.694 ± 0.346 µM of TBARS mg^{-1} of total protein in the treatment with higher oxidative stress (D). For DNA damage, the average was 443 ± 123 µg of DNA mg^{-1} of total protein in control and 587 ± 242 µg of DNA mg^{-1} of total protein in the treatment with the higher oxidative stress (D) (Fig. 2.5). Both biomarkers at different plastic pellet densities tested showed no significant difference compared to the control. However, there is a trend of increase (not significant) in

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the level of both biomarkers, until treatment D (100 pellets corer^{-1}), the same treatment that was found the highest mortality.

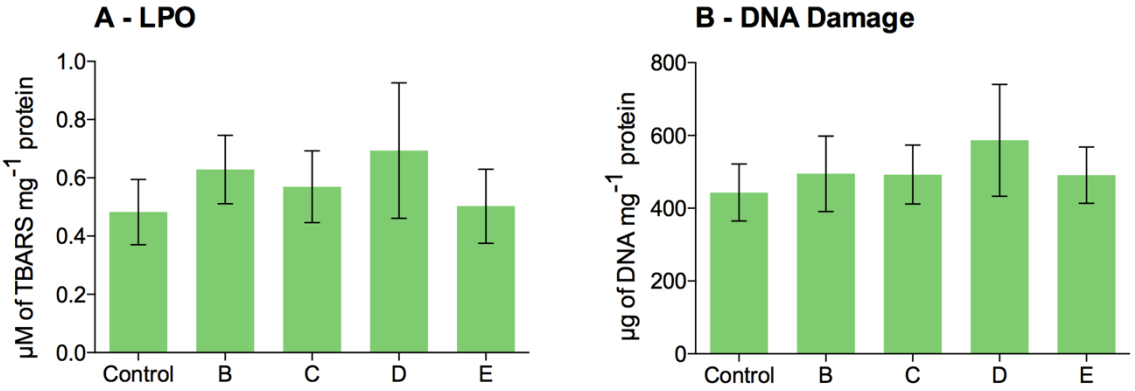

Fig. 2.5. A - Lipid peroxidation analysis (LPO) expressed in µM TBARs mg^{-1} of total protein for *Excirolana armata* per treatment. **B -** DNA damage analysis expressed in µg DNA mg^{-1} of total protein for *Excirolana armata* per treatment. Error bars represent a 95% confidence interval. Control = 0 pellets, B = 3 pellets corer^{-1}, C = 17 pellets corer^{-1}, D = 100 pellets corer^{-1}, and E = 200 pellets corer^{-1}

For organisms from the closed system exposure, the average values for LPO in the control experiment were 0.582 ± 0.076 µM of TBARS mg^{-1} of total protein and significantly higher (AIC = 15.26; X^2 = 5.98; p = 0.014) than those found in pellets treatment, which were 0.432 ± 0.103 µM of TBARS mg^{-1} of total protein (Fig. 2.6A). For DNA damage, average values were 574 ± 99 µg of DNA mg^{-1} of total protein and 598 ± 100 µg of DNA mg^{-1} of total protein in the control and pellets treatment, respectively. No significant differences were found between treatments (Fig. 2.6B).

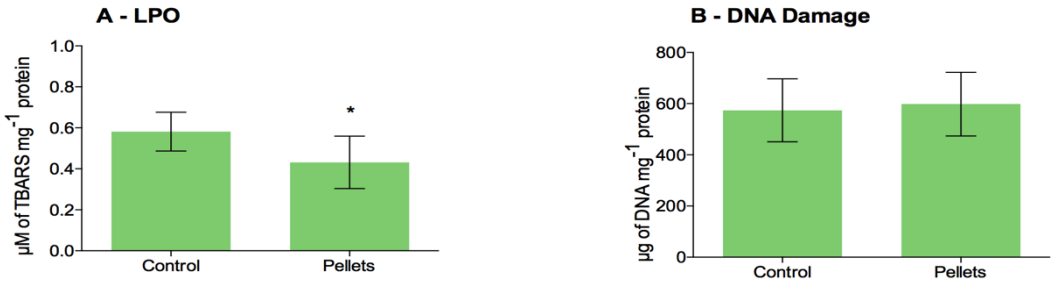

Fig. 2.6. A - Lipid peroxidation analysis (LPO) expressed in µM TBARs mg^{-1} total protein for *Excirolana armata* per treatment: Control and Pellets. **B -** DNA damage analysis expressed in µg DNA mg^{-1} of total protein for *Excirolana armata* per treatment: Control and Pellets. Error bars represent a 95% confidence interval.

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2.4. DISCUSSION

Here we found significant acute toxicity in the isopod *E. armata* when exposed to plastic pellets. In our three experiments, the mortality of *E. armata* was significantly higher in the pellet treatments than in the controls. However, there was a difference in toxicity effects between substrates (beach sediments), depending on the number of animals in the replicates (i.e. single exposure or pooled). Plastic pellets did not induce mortality in the organisms when tested individually but induced significant acute toxicity (mortality) when 5 or 10 organisms were exposed together, suggesting the potential importance of the intraspecific interactions for the ecotoxicological effects. Moreover, pellets in beach sediment induced faster and higher acute toxicity than those in seawater, as was demonstrated in the differences in our two laboratory experiments.

Mortality and the high effect size (6 to 8 times higher than the control) in all pellet treatments from the beach simulation experiments indicate that beach-stranded pellets are capable of inducing toxicity regardless of their density since a range of plastic pellets densities were simulated from lowest to the highest density ever found. Concentrations of hydrophobic contaminants in pellets are not homogeneous, but pellet colors can be good indicators of the adsorbed chemicals, thus different pellet colors may indicate contamination gradients (Endo et al., 2005; Izar et al., 2022). White and clear colors have high concentrations of industrial additives, while yellowish pellets have a gradient of hydrophobic contaminants from light yellow to dark brown (Endo et al., 2005; Yamashita et al., 2018). Because plastic pellets were chosen at random (regardless of color) in our experiments, this could explain at least part of the variability between treatments, replicates, and the significance of the random effect (Date - replication day) in the closed system exposure. Although this could represent a possible source of variation in our experiments, it is still a better representation of the real conditions found in the natural environment, since plastic pellets do not accumulate in equal color densities along the entire beach. In general, negative effects were observed in all densities in the field experiment. The presence of only a few pellets was enough to induce toxicity, regardless of density, which is possibly due to the release of pollutants (as observed by Silva et al., 2022). Biological variability is ruled out since there was a control treatment for each day replication and all plastic pellet treatments were compared with their respective daily control. Even considering the biological variability

between all tested organisms, the difference between the control and plastic pellets treatments remained standard on each day.

A similar laboratory study was conducted with the amphipod *Tiburunella viscana*, using the same pellet density proportions and no acute toxicity was reported (Izar et al., 2019), despite *T. viscana* species being a sensitive organism (Melo and Abessa, 2002). Causes for such differences could be the distinct feeding habit of these two organisms, differences in their activity when in the test chambers, or the way they interact with individuals of the same species. *E. armata* is more active than *T. viscana*, which could increase its exposure. It is possible that, in addition, or previous to lethality, pellet exposure may also interfere with *E. armata* physiology and behavior. Thus, for some individuals such exposure could increase or stimulate the activity while some individuals could become lethargic. As a consequence, healthy individuals would feed on lethargic ones, as expected due to their food habits and natural behavior. Competitive behavior for food was reported in *E. armata* (Petracco et al., 2010), and despite the short-term exposures used in our study, stressed animals could demand more energy when exposed to plastic pellets, which could explain, at least partially, the results obtained. This hypothesis is supported by data resulting from our experiments where single individuals were exposed (closed system exposure and survival analysis) and there was no mortality. This result increases the importance to consider intraspecific interactions in ecotoxicological tests since the difference between both studies (Izar et al., 2019 and this study) might be the intraspecific interactions (cannibalism or competition) of the tested species, and not mortality caused by toxicity. In our study, plastic pellets made isopods lethargic and these weaker organisms were attacked and eaten by the remaining individuals who were likely stressed due to the pellets exposure. The scavenger habit of the species would explain this cannibalism event observed, as an expected and common behavior, which occurred with greater intensity in the groups of organisms exposed to plastic pellets. Moreover, considering that individually exposed organisms did not present toxic effects caused by the plastic pellets (no mortality), intraspecific interactions become the most likely explanation for mortality. This hypothesis does not invalidate other possible explanations, such as intraspecific competition, neurotoxicity, and behavioral changes.

Vertical displacement was not significant, unlike results for salinity differences from a previous study performed in the same location (Laurino et al., 2020), which showed significant vertical displacement for these organisms. *E. armata* did not bury themselves in

the presence of plastic pellets. Mortality occurs before the organisms have the chance to flee (digging into the deeper layers), considering the behavior of the species to escape from environmental stressors, based on Laurino et al. (2020) results. Thus, plastic pellets represent an important threat to these organisms, even though the species proved to be resistant to other natural and anthropogenic stressors (Lercari and Defeo, 2003; Laurino et al., 2020). Sublethal effects (LPO and DNA damage) were non-significant, unlike results found for microplastics in other studies with mussels *Mytilus galloprovincialis* (Avio et al., 2015), freshwater fish *Oreochromis niloticus* (Hamed et al., 2020), and sea bass *Dicentrarchus labrax* (Barboza et al., 2018).

In the closed system exposure, mortality (cannibalism) of *E. armata* was higher in the presence of plastic pellets, following the pattern found in the *in situ* beach simulations experiment conducted in sediment from the same location. According to our biomarker data, the organisms from Dura beach had low levels of LPO and DNA damage. In this case, mortality occurs before oxidative stress, which can indicate that organisms are in their stress limit or that mortality occurs by other metabolic pathways, not due to oxidative stress. Unlike our study, Prokic et al. (2019) reported increasing LPO levels and DNA damage caused by microplastics. This raises a hypothesis about the influence of another factor (the quality of the sediments) on toxicity. Although there are not many plastic pellets on Dura beach, the sediment of this beach is moderately contaminated, especially by polycyclic aromatic hydrocarbons (PAHs) from biomass combustion sources (Moreira et al., 2021) and receives considerable sewage inputs (CETESB, 2019), especially during summer and/or after thunderstorms. Muddy sediments (sublittoral zone) from Dura beach were toxic to amphipods (Moreira et al., 2021). Despite the fact that beach sand tends to concentrate fewer contaminants than muddy sediments, such toxicity found for amphipods should be considered and may suggest a stressful environment for the local biota. This chemical contamination in Dura beach sediment might constitute a stressor to the isopods, which can increase the species' sensitivity. Thus, in the presence of an additional stressor represented by the plastic pellets, the effect is the isopod's mortality. This phenomenon (stress upon stress) could explain the lack of toxicity in a study with copepods (*Nitokra* sp.) exposed to reference sediments containing plastic pellets under laboratory ecotoxicological assays (Nobre et al., 2022). In this subject, more studies are needed to better understand and explain the obtained

results. Our next step would be performing laboratory assays with different *E. armata* populations to be able to unequivocally define this new hypothesis.

The survival analysis experiment made it was possible to visualize the surviving organisms feeding on the dead isopods (necrophagy event) immediately after the death of the organism. This confirms missing individuals as dead in the other experiments. Cannibalism also occurred on a dying individual, after multiple attacks from others, supporting the scavenger and opportunistic behavior of the species. Marginal significance in *p*-value shows the sensitivity of the statistical test, since Cox regression was designed for medical analysis with a large number of replicates, especially when is based on Kaplan-Meier estimates Rich et al., 2010). This methodological limitation should be considered when Cox regression is used in environmental studies. *E. armata* tended to exhibit higher mortality in the presence of plastic pellets, triggering the scavenging behavior on dead or dying individuals, which was more evident on Dura beach. Results corroborate those previously reported by Nobre et al. (2015) and Izar et al. (2019), in which beach-stranded pellets induced toxicity for marine organisms in seawater after long exposures. Also, our survival results corroborate with a similar study (McCormick et al., 2020) that found higher mortality of fish exposed to microplastic than to degraded habitats.

All our results, along with those from the aforementioned (Nobre et al., 2015; Izar et al., 2019), bring some information on the effects of plastic pellets at a population level and reinforce the idea that plastic pellets are capable to transfer adsorbed contaminants to the water column and organisms, as already observed by Silva et al. (2022). Biofilm can also contribute to this transfer of hydrophobic contaminants, since it colonizes the surface of plastic pellets, modifying their physical properties (Rummel et al., 2017), and should be considered in future studies. Our findings raise an ecological concern in the coastal environment since *E. armata* has a high ecological importance as a primary consumer subject to high predation pressures (Bergamino et al., 2011). Furthermore, our results represent real plastic pellet densities and were tested in the field using a very resistant macrobenthic species (Lercari and Defeo, 2003; Laurino et al., 2020).

Regarding the field experiments, we could observe that some natural factors can influence, interact or confuse the results. Low plastic pellet densities, including those commonly found on some beaches around the world, induced toxic effects in isopods in field simulations, and such toxic effects were confirmed in laboratory assays. Assuming that the

sorption equilibrium of hydrophobic contaminants is similar for the majority of microplastics present in the coastal environment (Koelmans et al., 2016), our results could be extrapolated to possibly represent the effects caused by microplastics in general. Such potential toxic effects associated with microplastic pollution, as a result of simple exposure (non-ingested), are particularly concerning to coastal and marine protected areas, where microplastic pollution sources are normally inexistent but microplastics can deposit as a result of oceanographic processes. In such cases, microplastics would consist of new stressors for the local marine biota, being capable to cause acute toxicity, and sublethal physiological effects and/or behavioral changes, as shown in our study.

2.5. CONCLUSION

Though challenging to pinpoint the exact process leading to the observed results, using real-world environmental conditions in ecotoxicological experiments provides key insights on how biodiversity reacts to anthropogenic disturbances. Mortality observed using pellet densities as low as 3 pellets highlight the importance of taking intraspecific interactions into account. These interactions might be confounding or masking toxicity results, or may lead to worse toxic effects, as observed in this study. In addition to lethal effects, chronic toxicities of plastic pellets can be associated with behavioral changes caused by plastic pellets, in which pellets do not induce direct toxicity, but rather generate a physiological imbalance, reflected in behavioral changes, that can lead to population imbalances. We urge the need to better understand the effects of microplastic contamination on biodiversity in natural settings so information can be provided for more effective management strategies.

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CHAPTER 3

Plastic pellets make *Excirolana armata* more aggressive: intraspecific interactions and isopod mortality differences between populations

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ABSTRACT

Plastic pellets represent a significant component of microplastic (< 5 mm) pollution. Impacts caused by plastic pellets involve physical harm and toxicity related to ingestion and non-ingestion (such as the release of chemicals in leachates). The latter is the main route of exposure for invertebrate macrobenthic populations. This study aimed to compare the toxicity of plastic pellets in distinct marine macrobenthic populations, considering the influence of sediment characteristics (organic matter and grain size) and quality (contamination by hydrophobic chemicals) on ecotoxicological effects, as well as the influence of color on the toxicity of beach-stranded plastic pellets. We performed three experiments on plastic pellet exposure using *Excirolana armata* from beaches with high and low pellet density. When exposed to pellets, populations that inhabit beaches without pellets demonstrate higher mortality than those inhabiting beaches with high pellet densities. The mortality of *E. armata* to pellets was higher when the exposure occurred in sediment with high organic matter (OM), suggesting that chemicals were transferred from pellets to OM. Yellowish beach-stranded pellets induced higher mortality of *E. armata* than the white tones did. We also observed lethargic (near-dead) and dead individuals being preyed upon by healthy individuals, a

cannibalistic behavior that raises an ecological concern regarding the negative effects of this exposure on intraspecific interactions in marine macrobenthic populations.

Keywords: Microplastics, Marine pollution, Sandy beaches, Ecotoxicology, Ecological impacts, Macrobenthos.

3.1. INTRODUCTION

Microplastics (plastic pieces with a diameter smaller than 5 mm) are potentially toxic to marine organisms, although their effects on the environment are still not well understood. Plastic pellets (or nurdles) consist of primary microplastics, which are used as raw materials for manufacturing plastic objects. These particles are commonly and constantly released into the environment because of unintentional losses. About 90 tons of plastic pellets (the equivalent of ~2.7 million 1.5 L nonreturnable PET bottles) are estimated to be lost annually by the plastic industry (Lechner and Ramler, 2015), unintentionally spilled into the environment during manufacturing and transportation (Ogata et al., 2009; Corcaran et al., 2020). These spills often fall into runoff systems and rivers, which carry pellets toward the coastal environment (Ogata et al., 2009; Karlsson et al., 2018), where they tend to accumulate mainly on sandy beaches (Moreira et al., 2016; Corcaran et al., 2020).

Newly manufactured plastic pellets are usually white or translucent and can be called virgin pellets (Hammer et al., 2012). These pellets frequently contain high concentrations of chemical substances that are incorporated into polymers during industrial processes (Koch and Calafat, 2009; Hammer et al., 2012; Andrady and Rajapakse, 2016; Yamashita et al., 2021). Once plastic pellets are lost, these chemical additives can quickly leach into the environment (Teuten et al., 2009; Nobre et al., 2015). Unlike virgin pellets, beach-stranded plastic pellets are usually yellowish or brownish owing to degradation (Karapanagioti and Klontza, 2007). Long periods of exposure to the environment darken the yellowish tone of the plastic pellets to shades of orange and brown. This color gradient is derived from hydrophobic chemicals present in the pellets or their degradation products, which are generally high present in dark tones (Endo et al., 2005; Fotopoulou and Karapanagioti, 2012; Yamashita et al., 2018). These plastic pellets hydrophobic chemicals can reach concentrations higher than

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those found in ocean waters (Andrady, 2011; Mato et al., 2001). Thus, the pellet color grade can be used as an indicator of chemical contamination (Endo et al., 2005; Izar et al., 2022a).

Marine invertebrates from infauna constantly interact with microplastics in contaminated coastal environments. Benthic organisms can bury microplastics into deeper layers of sediment during bioturbation and burrowing (Näkki et al., 2017; Gebhardt and Foster, 2018; Capparelli et al., 2022). Laboratory assays have shown acute and/or chronic toxicity of plastic pellet leachates to mussels (Gandara e Silva et al., 2016), sea urchins (Nobre et al., 2015; Izar et al., 2019), and sand dollar embryos (Albanit et al., 2022), copepods and amphipods adult organisms (Izar et al., 2019). The sublethal effects of microplastic exposure have been observed using biomarkers in oysters (Nobre et al., 2020) and crabs (Silva et al., 2022; Nobre et al., 2022). A first attempt at *in-situ* ecotoxicological tests for macrobenthic invertebrate populations was performed on a pristine sandy beach using real-world plastic pellet densities exposed to *Excirolana armata* (Isopoda) individuals, which caused toxic effects at all tested densities (Izar et al., 2022b). Isopods are ecologically important because of its ability to transfer plastic particles to higher trophic levels in food chains (Anbumani and Kakkar, 2018). As a shortcoming of our previous work (Izar et al., 2022b), *in-situ* experiments can introduce numerous environmental confounding factors, mainly with regard to the bioavailability of hydrophobic contaminants in the sediment. The properties of the local sediment can influence or mask toxic effects on organisms, placing the sediment as a factor and a natural stressor that can act in a multiple stressor toxic chain (Maulvault et al., 2019; Wieringa et al., 2022). The bioavailability of chemical compounds associated with the leachates of plastic pellets may vary according to the matrix and its properties to which the organisms are exposed, i.e.: sediment organic matter content (OM), among others (Koelmans *et al*., 2016).

The cirolanid isopod *E. armata* is abundant and ecologically dominant on sandy beaches with fine granulometry throughout South America, ranging from Rio de Janeiro to Northern Patagonia (Defeo et al., 1997; Lercari and Defeo, 2003; Lozoya et al., 2010). The preference for fine sand is an important factor in determining the exposure of this species to microplastics, as such particles (including plastic pellets) tend to accumulate in fine grain-sized areas (Enders et al., 2019; Corcoran et al., 2020; Vermeiren et al., 2021). Furthermore, it has been reported that micrometer-sized microplastics are ingested by *E. armata* (Vermeiren et al., 2021). However, this species is highly resistant to environmental stress and human

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activities (Lozoya and Defeo, 2006; Thompson and Sánchez de Bock, 2007; Lozoya et al., 2010; Gandara-Martins et al., 2015; Fanini et al., 2017; Laurino and Turra, 2021) and fits perfectly as a model organism. This has been highlighted in previous studies testing the responses of species to environmental stress (Laurino et al., 2020; Laurino and Turra, 2021) and microplastic pollution (Izar et al., 2022b). Once *E. armata* is affected, the entire trophic chain may be compromised because of its importance as a primary consumer (Lercari et al., 2010; Bergamino et al., 2011; Costa and Zalmon, 2017). This high resistance of the species leads us to a possible adaptation facility to stressors or natural selection. In mosquitoes, selection resistance has already been observed for insecticides, with some adaptive mechanisms, such as cuticle modification, increased detoxification enzymes, and target-site mutations (Nkya et al., 2013). As isopods are arthropods, they may experience acquired resistance. These observations led us to believe that populations on beaches that are more contaminated by plastic pellets present different responses to toxicity. Acute toxicity (mortality), as described above, is one of these responses and another one are some adaptive biological responses, or sublethal effects. These sublethal effects are early biochemical warnings of toxic effects that it has been studied by biomarkers neurotoxicity and oxidative stress to microplastics (Barbosa et al., 2020; Nobre et al., 2020).

In the present study, we aimed to understand why different populations of the macrobenthic cirolanid isopod *E. armata* respond differently to plastic pellet stress depending on the environment in which they inhabit. To do this, (1) we exposed two *E. armata* populations from different sites to plastic pellets under equal conditions to verify differences in toxicity between populations. Here, we tested the hypothesis of acquired resistance (natural selection) of this species to plastic pellets when inhabiting beaches with high densities of these particles. The sublethal effects of this exposure in both populations were measured by analyzing biomarkers for neurotoxicity (cholinesterase-like activity) and oxidative stress (lipid peroxidation and DNA damage). (2) We also exposed a single population of *E. armata* to different beach sediments, in order to test the influence of sediment characteristics (OM and grain size) and quality (organic and metal contamination) in the ecotoxicological bioassays. Here, we expected that higher OM, fine granulometry and/or contamination would induce more toxicity when associated with plastic pellets, a multiple-stressor phenomenon, owing to the high affinity of hydrophobic contaminants to organic matter, making them more bioavailable. Finally, (3) we tested the toxic effects of the different beach-stranded plastic

pellet colors under the hypothesis that darker pellets are more toxic than white tones. Results are critically discussed.

3.2. METHODS

In this study, we performed three experiments exposing individuals of two distinct populations of *E. armata* to plastic pellets. The first population inhabits the Dura Beach in Ubatuba city (23°29'41" S, 45°10'21" O - Fig. 3.1), on the Northern coast of São Paulo state (Southeast Brazil). Dura Beach is a microtidal (tidal range < 2 m) dissipative beach with about 1700 m in length, sheltered at the deeper portion of a large bay, with a higher concentration of OM due to the presence of an estuary on one of its sides (Laurino et al., 2020; Izar et al., 2022b). It is located 200 km away from Santos Port, the largest port in Latin America, and the main source of plastic pellets for the entire coast of São Paulo (Izar et al., 2019). Once the beach is far from pellet sources, it is considered to be free from plastic pellet contamination (Moreira et al., 2016; Izar et al., 2019). In the last decade, Dura Beach was rated as regular/good for bathing (CETESB, 2020a) and is considered a moderately impacted area, likely due to the presence of marinas and boat traffic in the region (CETESB, 2020b). Even with its proximity to state park (*Parque Estadual da Serra do Mar*), this protected area is restricted to mountainous areas on the coast, allowing construction and real estate speculation on the sandbanks of the region's beaches. The sediment of the region is moderately contaminated by polycyclic aromatic hydrocarbons (PAHs) from biomass combustion sources (Moreira et al., 2021) and contains a considerable amount of sewage, especially in the summer during the high tourism season (CETESB, 2019).

The second population inhabits the Boracéia Beach, in Bertioga city (23°45'25" S, 45°49'8" O - Fig. 3.1), on the middle coast of São Paulo state. It is close to the Santos port (~ 50 km) and has a moderate pellets density (Izar et al., 2019; Izar et al., 2022a). Boracéia Beach is also a microtidal (tidal range < 2 m) dissipative beach with a gentle slope and fine granulometry (Laurino and Turra, 2021). However, it is longer than Dura Beach, with about 8,000 m in length. Its sediment is relatively uncontaminated by inorganic and organic elements/compounds, owing to the low input of sewage (CETESB, 2019), the proximity of a State Park (*Parque Estadual Restinga de Bertioga*) and a Marine Protected Area (Área de Proteção Ambiental Marinha Litoral Centro - APAMLC) and for the characteristics of an exposed beach. Boracéia Beach was rated as good for bathing in most of the years of the last

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decade (CETESB, 2020a). Furthermore, Boracéia Beach is located in the same bay as Itaguaré Beach, whose sediment is considered a control site for contamination in ecotoxicological studies (Ferraz, 2013).

The beach-stranded plastic pellets used in all experiments were sampled at Itaquitanduva Beach in September 2021. This beach is located on the west side of Santos Bay (23°59'50" S, 46°23'28" O - Fig. 3.1), close to the Santos Port and to the major industrial complex of Cubatão (8 km far from the mouth of the Santos Channel, where Santos Port is located, in the east side of the Santos Bay). This beach is affected by the discharge of plastic pellets and chemical contaminants (Moreira et al., 2016; Taniguchi et al., 2016; Izar et al., 2019; Izar et al., 2022a). Itaquitanduva Beach has a high pellet density that is comparable to the highest in the world (Izar et al., 2022c). The plastic pellets from Itaquitanduva Beach presented high concentrations of adsorbed hydrophobic contaminants, such as dichlorodiphenyl trichloroethanes (DDTs), polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs), and polychlorinated biphenyls (PCBs) (Taniguchi et al., 2016; Ohgaki et al., 2021), which were found in extremely high concentrations (> 500 ng g^{-1} of pellets) (Ohgaki et al., 2021).

Plastic pellets were picked from the sandy beach surface sediment along the high tide line by active searching, visually identified, and collected. The beach-stranded pellets sampled (~ 8 thousand) were stored in glass containers, regardless of color or polymer type, and kept refrigerated (5 to 10 ^{o}C) until the exposure in experiments. To simulate the distribution of plastic pellets in the natural environment, collected pellets were randomly selected for each exposure.

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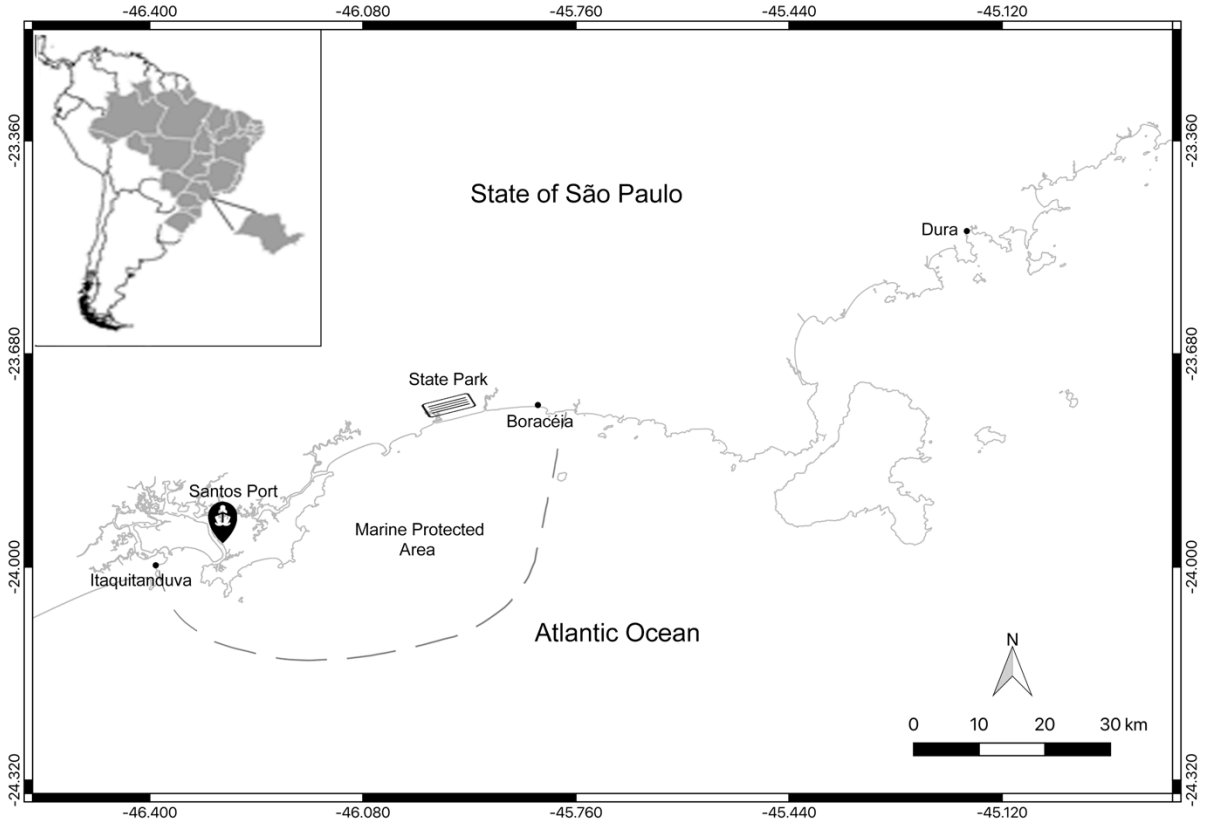

Fig. 3.1. Map of the São Paulo north and center coast (Brazil) showing the locations of the three beaches used in this study and their distance to the Santos port. Plastic pellets were collected from Itaquitanduva Beach. The beach sediment and individuals of *Excirolana armata* used in the experiments were sampled at the Boracéia and Dura beaches. Dashed line represents the Marine Protected Area and the rectangle the State Park.

3.2.1. Different populations under the same conditions

To ensure the same conditions for both *E. armata* populations tested in the bioassays, in September 2021, we exposed them to the same sediment from Dura Beach. The sediment of this beach was chosen because it has a higher level of PAHs contamination and sewage (CETESB, 2019 and 2020a), allowing us to test the effect of multiple stressors (plastic pellets and contaminated sediment).

At Boracéia Beach, we collected about a thousand individuals of the macrobenthic isopod, finding the tracks left on the sand surface, digging those tracks, and sifting the sand to collect the organisms. We visually selected only adult individuals (> 3 mm in length; Petracco et al., 2010) and stored them in a plastic bucket with sifted local sediments and seawater for transportation. In the laboratory, the organisms collected at Boracéia Beach had one day of

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acclimatization with constant oxygenation due to the transport stress. The population of Dura Beach was sampled following the same methods described for the population of Boracéia Beach. After sieving, individuals had one hour of acclimatization due the stress of collection management and placed in beakers to be exposed to the assay.

Dura Beach sediment was sieved to remove organisms from the samples used in the bioassays. A total of 20 mL of sieved sediment was added to a 50 mL glass beaker and local seawater was added until a thin layer of water covered the sediment. We exposed both populations of *E. armata* to two treatments: (1) control – no pellets, and (2) pellets – a very high pellet density (40 pellets) was added to the sediment surface layer per beaker, which was equivalent to twice that found in Hawaii by Mcdermid and Mcmullen (2004). This pellet density was chosen to ensure that the toxic effect would occur and to simulate a future catastrophic plastic pellet density.

In each treatment, five individuals of the same *E. armata* population were added to each beaker and exposed for 6 h. At the end of the exposure period, the sediment from each beaker was sifted, and the surviving individuals accounted for in each treatment and population. Missing individuals were considered dead because the cannibalistic behavior of the species had previously been observed in similar experiments (Izar et al., 2022b), and it is highly unlikely that the tested organisms would escape the beakers. The experiment had ten replicates for each group and was repeated thrice, totaling 120 replicates (n = 30 per treatment).

A Generalized Mixed Model (GLMM) with a Negative Binomial distribution was built for mortality, considering the number of dead or missing individuals (the dependent variable). Three factors were tested in the model: Date (Factor 1, random, 3 levels), Beach population (Factor 2, fixed, 2 levels), and Treatment (Factor 3, fixed, 2 levels). For all statistical analyses, we adopted a confidence level of $p \leq 0.05$, and the distribution used in the models was determined by the best fit to the model (a smaller Akaike information criterion - AIC, and the normality of residuals). The rate ratio (RR) indicates the effect size with a 95% Confidence Interval (CI95%). All statistical analyses were performed using the open-source statistical software Jamovi (2021).

The sublethal effects of exposure were determined by biochemical biomarker analyses. At the end of the experiment, surviving *E. armata* were immediately frozen on ice, transported for 4 h to the laboratory, and subsequently stored in an ultrafreezer (-80 °C). We

analyzed the neurotoxicity biomarker cholinesterase-like (ChE) and two biomarkers of oxidative stress, lipid peroxidation (LPO), and DNA strand break (single break) - DNA damage.

Neurotoxic effects were evaluated based on ChE-like activity, using the method proposed by Ellman et al. (1961) and adapted by Herbert et al. (1995). We added a solution of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and acetylcholine iodide to each sample, and the absorbance was measured at a wavelength of 415 nm. The results were expressed as nmol min^{-1} mg^{-1} of total protein.

LPO was evaluated based on the protocol described by Wills (1987), in which thiobarbituric acid reactive substances (TBARS) and tetramethoxypropane standards were added and diluted in a homogenizing solution. The measurement was performed using fluorescence, in which an excitation wavelength of 516 nm and an emission wavelength of 600 nm were applied. The results were expressed in μM TBARs mg^{-1} of total protein.

DNA damage was evaluated using the alkaline precipitation method proposed by Olive (1988). We applied a salmon sperm DNA standard curve for damage standardization and used Hoechst 33342 solution to provide luminescence to the sample. Damage was quantified by fluorescence, using a wavelength of 360 nm for excitation and 450 nm for emission. The results were expressed as μg DNA mg^{-1} of total protein.

The total protein content used to normalize the biomarkers data was evaluated using the Bradford method (1976). Each replicate of the biomarker analyses (10 organisms) was composed of 5 organisms from each of the two replicates of the previous experiment. Thus, biomarker analyses had five replicates for each group (beach and treatment) and were performed for the last two days of the experiment (days 2 and 3), totaling 40 replicates (n = 10 per treatment).

For ChE and LPO, a Generalized Mixed Model (GLMM) with Gamma distribution (link function = Identity) was built with three factors: Date (Factor 1, random, 2 levels), Beach population (Factor 2, fixed, 2 levels), and Treatment (Factor 3, fixed, 2 levels). For DNA damage, a General Mixed Model (GMM - Linear distribution) was built using the same statistical software with the same previous three factors.

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3.2.2. Different sediments for the same population

To test the influence of sediment characteristics and quality as a factor for toxicity, we repeated the previous assay, following the same methods and experimental design, with a standard population exposed to the same pellet density. This test was performed using the Boracéia Beach population of *E. armata* in sediments from Boracéia and Dura beaches. The population of Boracéia Beach was chosen because it is resistant to the stress of plastic pellets in their natural environment (sediment), according to the results of our first experiment. The experiment had ten replicates for each group, and was repeated three times per beach sediment, totaling 120 replicates (n = 30 per treatment).

A Generalized Mixed Model (GLMM) with a Negative Binomial distribution was built for the dependent variable mortality with three factors tested: Date (Factor 1, random, 6 levels), Sediment (Factor 2, fixed, 2 levels), and Treatment (Factor 3, fixed, 2 levels).

As the organisms were tested in different sediments, it was considered that sediment properties could be a confounding factor. Therefore, to address the possible influence of OM during pellets exposure, sediment was collected from both beaches in 10 random replicates. In the laboratory, OM was measured in the dried sediment (60 ^{o}C for 48 h) by weight loss after incineration (550 ^{o}C for 6 h) for each sample. To determine the grain size (Φ), samples were sifted following the procedure proposed by McCave and Syvitski (1991), and classified as parameters following Folk and Ward (1957). We sifted the dry sediment into six different granulometric fractions (2, 1, 0.5, 0.212, 0.125, and 0.063 mm) for 15 min at a speed of 3,600 bpm and individually weighed each fraction. Sediment properties from both sites were statistically compared using the Student's t-test and Welch's correction, if necessary.

Beach sediments were also analyzed for organic compounds and mercury (Hg), to address the possibility that sediment contamination influences isopod mortality when associated with pellet exposure. Three sediment samples from each beach (Dura and Boracéia) were collected, stored in calcined aluminum containers, refrigerated in ice, and transported to the laboratory, where they were kept frozen (-14°C to -25°C). The sediments were lyophilized in the laboratory. For chemical analyses, we prepared samples using a miniaturized solid–liquid extraction method (Santos et al., 2016, 2018), in which 25 mg of sediment from each beach sample was added to a microextraction device (Whatmann Mini ™

UniPrep Filters without syringe, Whatmann, USA) with 500 µL of a mixture of solvents (18% acetonitrile + 82% dichloromethane) and sonicated for 23 min. The final filtered extract was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS QP-2010 ULTRA, Shimadzu, Japan) to analyze PAHs and their derivatives (oxy- and nitro-PAHs). The condition of the GC-MS is described in Santos et al. (2016). Quantification was performed using the calibration curve for PAHs. The limit of detection (LOD) and limit of quantification (LOQ) of each analyte were calculated following the International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al., 2002): LOD = (3SE/α) and LOQ = (10SE/α), where SE is the standard error of the linear regression and α is the slope of the linear regression of the calibration curve (all these information are in the supplementary data, Table 3.S1). LOD was applied for each analyte. Analytical quality control parameters (evaluating, precision and accuracy) followed the method developed for sediments in Santos et al. (2018), in which reference certificated marine sediment deuterated standards (fluorene-D10 and pyrene-D10) were added to determinate control parameters and repeated ten consecutive times. Relative recovery for PAHs ranged from 73% to 118% and from 104% to 106% for deuterated PAHs. In order to avoid contamination, all laboratory instruments and non-volumetric glassware used for extraction were cleaned with highly polar organic solvents and muffled at 500 °C for 4 h, following the Method 610 for PAHs analysis from the United States Environmental Protection Agency (US EPA, 1986). The analyzed compounds are in the supplementary data (Table 4.S1).

Hg determination in the sediment samples was performed using the Direct Mercury Analyzer DMA-80 Tri Cell (Milestone, Sorisolev (BG), Italy). During the analysis, the lyophilized sediment samples were weighed (approximately 100 mg) and placed in nickel boats. Mercury determination using a direct mercury analyzer (DMA-80) allowed for three calibration curves, which showed the following results: 1) linear range (cell 0 = 0.010–3 ng; cell 1 = 3–10 ng; cell 2 = 10 –100 ng); 2) linear regression (cell 0 = 0.1410 mHg + 0.0004; cell 1 = 0.0510 mHg + 0.0064; cell 2 = 0.00085 mHg + 0.00019); and 3) coefficient of determination (R^2) = 0.9994, 0.9991, and 0.9990, respectively. The LOD and LOQ were determined using the standard deviation (SD) values of the blank replicates following the same PAH equation. The LOD and LOQ values were 0.004 and 0.012 ng g^{-1}, respectively, and the LOD was applied. Method accuracy was realized and confirmed using certified reference materials of marine sediment and fish protein (MESS-3 and DORM-4) from the

National Research Council, Canada (NRCC). The certified reference values were 0.091 ± 0.009 mg kg^{-1} and 0.410 ± 0.055 mg kg^{-1}, respectively. The results obtained by the DMA method were 0.086 ± 0.003 for sediment marine and 0.414 ± 0.003 mg kg^{-1} for fish protein. All chemical results were analyzed for differences using Student's t-test for the factor Beach (Dura and Boracéia) and Welch's correction, if necessary.

3.2.3. Different plastic pellet colors

To test the toxicity of plastic pellets of different colors, *E. armata* from the Dura Beach population was exposed to two color groups of plastic pellets (white and yellowish) and a control treatment (without pellets) using the Dura Beach sediment, following the method described in the previous sections of this manuscript. We chose the Dura Beach population because it presented the best sensitivity to the toxic effects of plastic pellets. Pellets were visually separated into two groups: the white (white, translucent, and gray tones) and the yellowish (yellow, orange, and brown tones). A total of 40 pellets of each color group were added to the surface layer of the sediment within a beaker and exposed for 6 h, as in the previous assays. Each treatment had nine replicates, and the experiment was repeated four times (108 replicates total, 36 per treatment).

A Generalized Mixed Model (GLMM) with a Poisson distribution was built and the dependent variable (mortality) was tested using two factors: Date (Factor 1, random, 4 levels), and Treatment (Factor 2, fixed, 3 levels).

3.3. RESULTS

3.3.1. Different populations under the same exposure conditions

Fig. 3.2 shows the mortality of individuals of *E. armata* sampled in Dura and Boracéia beaches, exposed to plastic pellets. Treatment effect on *E. armata* populations was dependent on the beach assessed (Beach x Treatment: AIC = 203.9, X^2 = 3.9, p = 0.04), since a difference in mortality between control and pellet treatments was noted only for the Dura Beach population ($p_{bonferroni}$ = 0.03, Fig. 3.2). Individuals in the population from Dura Beach exposed to plastic pellets had 3.5 times higher mortality values than those in the control treatment (RR = 3.56, CI95%: 1.51 - 8.40). The mortality in Dura Beach was also 3.1 times

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higher than the Boracéia Beach population, when exposed to plastic pellets (RR = 3.12, CI95%: 1.41 - 6.98). There were no significant differences in mortality of control and pellets treatment to individuals from Boracéia Beach ($p_{bonferroni} = 1$, Fig. 3.2). The random factor Date was also significant for both groups (p = 0.03).

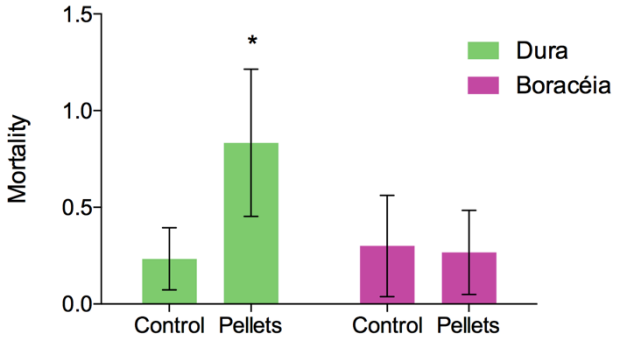

Fig. 3.2. Average mortality of *Excirolana armata* in raw count numbers in each treatment (control and pellets) per beach population (Dura Beach and Boracéia Beach) tested under the same conditions: exposed in Dura Beach sediment at the same time. Error bars represent a 95% confidence interval and asterisks represent toxic effects compared to the control of the same beach population.

Fig. 3.3 shows the biomarkers results for *E. aramta* from Dura Beach and Boracéia Beach populations when exposed to plastic pellets. None of the analyzed biomarkers were statistically significant for any factor, including random factor (Fig. 3.3).

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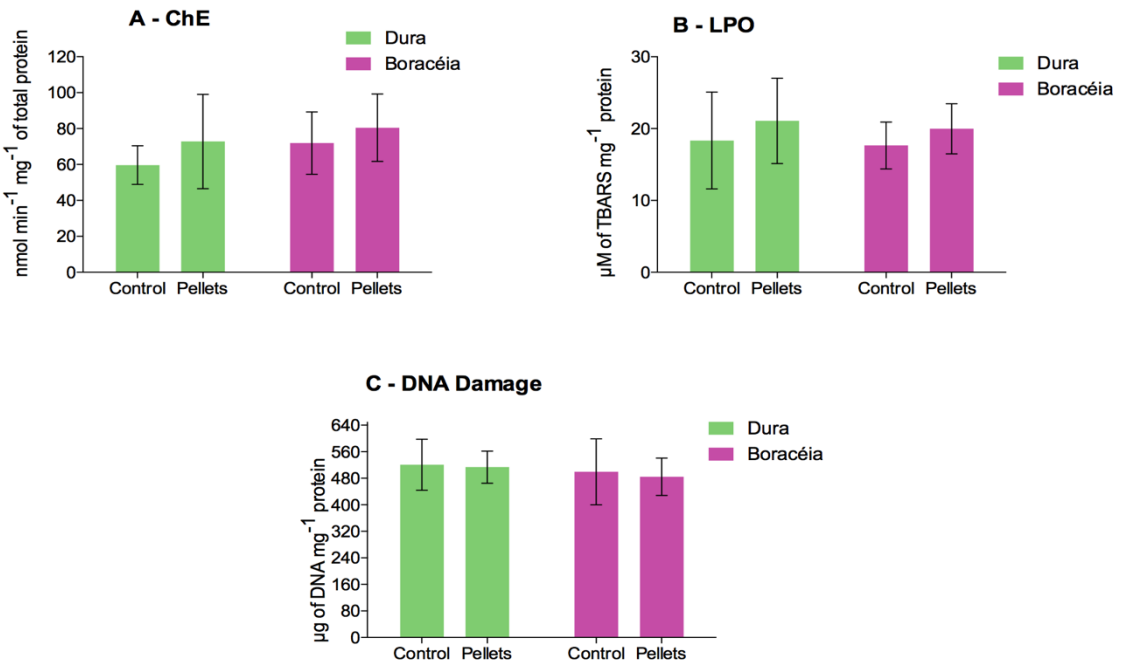

Fig 3.3 Average values of cholinesterase (ChE) expressed in nmol min^{-1} mg^{-1} of total protein (A), lipid peroxidation analysis (LPO) expressed in µM TBARs mg^{-1} total protein (B) and DNA damage analysis expressed in µg DNA mg^{-1} of total protein (C) for *Excirolana armata* per treatment (Control and Pellets) and per beach population (Dura Beach and Boracéia Beach). Error bars represent 95% confidence intervals.

3.3.2. Different sediments for the same population

Conversely, we did not find any effect of pellet treatment on *E. armata* mortality when comparing the control group to the pellets exposed group ($p = 0.92$). We found 4 times higher mortality (RR = 4.24, CI95%: 1.16 - 15.18) for treatments (control and pellets) exposed to Dura Beach sediment than those exposed to Boracéia Beach sediment (AIC = 123.3, $X^2 = 4.8$, $p = 0.02$) (Fig 3.4). The random factor Date was not significant ($p = 0.07$).

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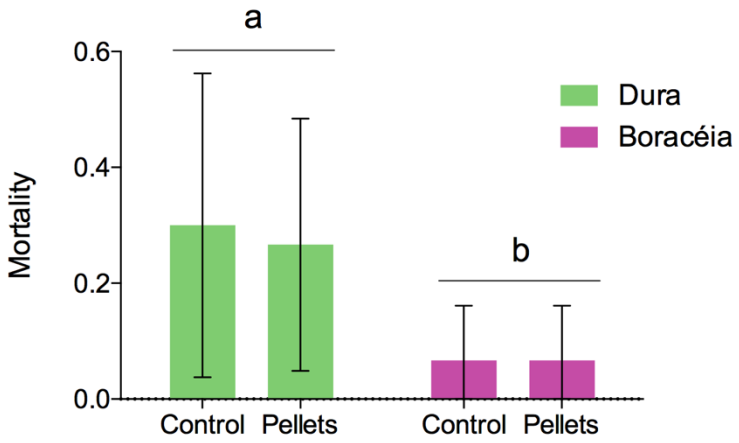

Fig. 3.4. Average mortality of *Excirolana armata* in raw count numbers from the Boracéia Beach population was tested for sediment from Dura Beach and Boracéia Beach. Error bars represent 95% confidence intervals. Different letters indicate significant differences between beach sediments in terms of toxicity.

The average organic content of the sediment in Dura Beach was 0.68% (CI95%: 53-83), while on Boracéia Beach it was 0.47% (CI95%: 32-63). The average grain size (Φ) was 3.42 (CI95%: 3.34-3.51) at Dura beach and 2.81 ± (CI95%: 2.73-2.90) at Boracéia Beach. Dura Beach had higher OM concentration and smaller grain size (Φ) than Boracéia Beach ($t = 2.02$, $df = 18$, $p = 0.059$ [marginal]; $t_{welch} = 10.7$, $df\ 9.2$, $p < 0.001$; respectively). The concentrations of PAHs, N-PAHs, and O-PAHs in the sampled sediment in both beaches were below LOD for all analyzed compounds (Supplementary Material, Table 3.S1). The average concentrations of Hg at Dura and Boracéia beaches were 2.6 ng g^{-1} (CI95%: 1.5-3.8) and 2.4 ng g^{-1} (CI95%: 1.2-3.5), respectively, with no significant difference between beaches ($t = 0.45$, $df = 4$, $p = 0.67$).

3.3.3. Different plastic pellet colors

We found a mortality difference between pellet treatments ($AIC = 237.8$, $X^2 = 15.9$, $p < 0.001$), with 2.6 times more mortality in white pellet exposure compared to the control ($RR = 2.6$, CI95%: 1.25 - 5.38), and 4 times more mortality in yellowish pellet exposure than in the control ($RR = 4$, CI95%: 2 - 7.98) (Fig. 3.5). There is no significant difference between color groups ($p_{bonferroni} = 0.25$). The random factor Date was significant ($p = 0.04$).

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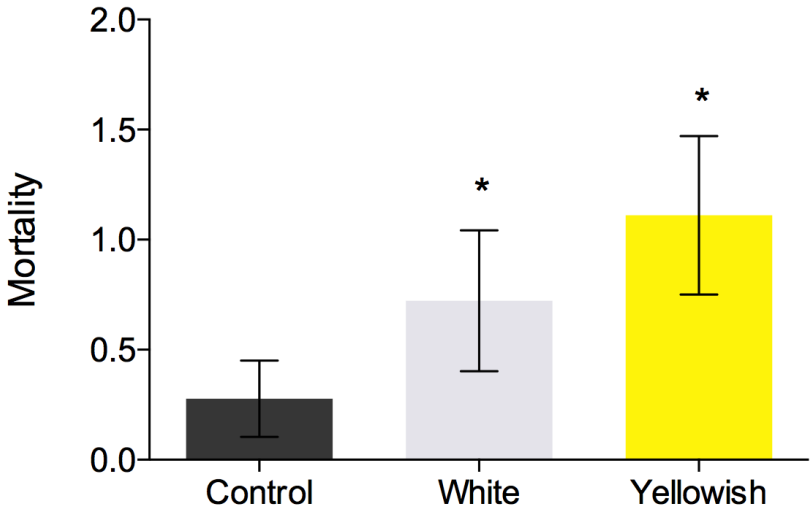

Fig. 3.5. Average mortality of *Excirolana armata* in raw count numbers from the Dura Beach population in Dura Beach sediment exposed to different pellet color groups (white and yellowish) and control without pellets. Error bars represent 95% confidence intervals and asterisks represent toxic effects compared to the control.

3.4. DISCUSSION

Different *E. armata* populations exposed to plastic pellets exhibited distinct responses. When exposed to the same sediment under the same conditions, only the population of Dura Beach showed signs of toxicity, in which pellets induced 3.1 times higher mortality (or cannibalism behavior; see Izar et al., 2022b). The Boracéia's population seemed to be resistant to plastic pellet stressors, which can be caused by the fact that this beach is closer to Santos Port (a plastic pellet source) and has a higher number of beached plastic pellets per area than Dura beach (Izar et al., 2019). This population showed very low mortality (similar to the control values) regardless of the sediment tested (Dura Beach or Boracéia Beach sediments).

E. armata has proven to be highly tolerant to environmental and anthropogenic stressors because of its resistance to plastic pellets, as observed in the Boracéia Beach population. This isopod has already demonstrated some degree of resistance to abrupt salinity variations (Lozoya and Defeo, 2006; Lozoya et al., 2010; Fanini et al., 2017; Laurino and Turra, 2021), anthropogenic erosion (Thompson and Sánchez de Bock, 2007), and to non-ingested plastic pellets exposure. High mortality in the Dura Beach population exposed to plastic pellets and their changes in vertical distribution to avoid floods with different salinities (Laurino et al., 2020) suggest that this resistance selection is possibly acquired over generations when exposed to a new (unfamiliar) stressor. This resistance can be behavioral,

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avoiding the stressor, as is the case with changes in salinity (Laurino et al., 2020), or physiological. The resistance observed in this study seems to be caused by this last case. Physiological adaptations have already been described for mosquitoes (Nkya et al., 2013), and are related to their fast life cycle. Similar to mosquitoes, isopods are arthropods with a fast life cycle, suggesting that this resistance can be acquired in a short time (Nkya et al., 2013). Nonetheless, we did not test this possible resistance heritability here, a hypothesis that must be explored in future studies.

Interestingly, no sublethal effects were found in either isopod population when exposed to plastic pellets. This is unlike the pattern of increasing LPO and DNA damage levels caused by microplastic exposure found by Prokić et al. (2019), however follows the pattern found in our previous study (Izar et al., 2022b). The results of our studies may be associated with behavioral changes when this species is exposed to plastic pellets and does not reach physiological stress. The same values for all biomarkers could represent a sublethal effect at the same intensity with or without pellets exposure, corroborating with the idea of behavioral changes rather than lethal effects such as mortality.

The population of Boracéia Beach responded differently when tested in different sediments. This population showed approximately 4 times more intense response when exposed to Dura Beach sediment in both treatments (control and pellets). Therefore, the characteristics of the sediment should be considered. Dura Beach sediment grains are finer than those of Boracéia Beach sediment. These characteristics are favorable for *E. armata*, once this species shows a preference for and increased abundance in fine sands (Defeo et al., 1997; Lozoya et al., 2010; Fanini et al., 2017). In addition, OM might also be considered when evaluating toxicity. It can be an important route of contamination, facilitating the bioavailability of some contaminants from plastic pellets (Ferraz et al., 2020). For *E armata*, this is an important factor due to its higher abundance and preference for sediments with low levels of OM (Lozoya et al., 2010), as those from Boracéia Beach. Even with no levels of PAHs contamination and similar and very low Hg contamination on both beaches, other contaminants not tested may be present in the beach sediment. Pharmaceuticals are emerging pollutants that have proven to be toxic when associated with plastics (Nobre et al., 2020 and 2022). Ammonia may also be considered because high toxicity can be associated with its presence in the sediment (Araujo et al., 2013; Campos et al., 2016). Both may be present in high concentrations at Dura Beach because of the elevated sewage input (CETESB, 2019;

Nobre et al., 2020). The proximity of mangroves to the beach can also be a natural source of ammonia in the region. However, for the isopod population from Boracéia Beach, Dura Beach sediment induced four times more toxicity with or without pellet exposure than when exposed to Boracéia Beach sediment. The non-toxic effect of plastic pellets on this population reinforces the importance of considering the characteristics of the sediment as a factor for toxicity. On the other hand, there was no difference in PAHs or mercury contamination in beach sediments between the two beaches, contrary to what was found for marine sediments and sewage, in which sediments from the Dura Beach area were more contaminated than those from Boracéia Beach (CETESB, 2019; CETESB, 2020b; Moreira et al., 2021).

All chemicals analyzed were below Level 1 (low probability of adverse effects to biota) of the Brazilian Environmental Council (CONAMA 454/2012), on both beaches. The same chemical contamination level (PAHs and Hg) in the sediments of the studied beaches does not completely refute our hypothesis of multiple stressors. However, OM may act as a route for the bioavailability of other contaminants not tested carried by plastic pellets to sandy beaches. In our first experimental results (different populations in the same exposure conditions), indicate the toxicity found for the Dura Beach population when exposed to plastic pellets, unlike the Boracéia Beach population (both tested in Dura Beach sediment), suggests toxicity for multiple stressors. Toxicity in the Dura Beach sediment to the *E. armata* population from Boracéia Beach, with or without exposure to plastic pellets, reinforces this hypothesis. The higher level of OM in the Dura Beach sediment can also increase the bioavailability of contaminants to the benthic macroinvertebrate community and might be a persistent and natural stressor in the local isopod population. Ammonia can also present as a natural stressor, especially in Dura beach due the proximity of a mangrove. When these isopods are exposed to an additional stressor (plastic pellets carrying more contaminants that are bioavailable by OM), they reach a stress threshold, resulting in mortality (cannibalism behavior). Similar results were found by Maulvault et al. (2019), in which stress on juvenile fish was more severe when the three stressors acted simultaneously.

In all experiments, mortality of *E. armata* was observed as a result of intraspecific interactions such as necrophagy and cannibalism, corroborating Izar et al. (2022b). In both studies, only living individuals were found inside beakers after the exposure time, with no signs of dead individuals. The presence of plastic pellets intensifies the agonistic interactions of this species (Bergamino et al., 2011; Bergamino et al., 2012), in which living individuals

attack dead or dying individuals. Mortality (cannibalism) was more evident in the population of Dura Beach, although it was also observed in the population of Boracéia Beach.

Beach-stranded plastic pellets were toxic in all exposed combinations: mixed, regardless of color, and separated by color (white and yellowish). When separated, pellets of both colors were toxic, with yellowish tones tending to cause higher toxicity than white tones. Beach-stranded plastic pellets tend to concentrate more contaminants in the darkest tones of the yellow (Endo et al., 2005; Fotopoulou and Karapanagioti, 2012). For example, Yamashita et al. (2018) observed polychlorinated biphenyl (PCB) concentrations up to 29 times higher in orange and brown than in white plastic pellets. The toxicity of white pellets is related to industrial additives that tend to be volatile (fast biodegradation) and they are easily released when a virgin pellet reaches the environment (Teuten et al., 2009). Therefore, according to our data, corroborating with Gandara e Silva et al. (2016), industrial additives seem to be less concentrated and therefore less toxic than hydrophobic contaminants adsorbed, which are present in higher concentrations in beach-stranded plastic pellets. Unlike the results of Nobre et al. (2015) and Izar et al. (2019), which showed higher toxicity of virgin plastic pellets to sea urchin larvae and no difference in toxicity between white and colored beach-stranded plastic pellets, we found toxicity in both color groups. This difference in toxicity might be related to the exposure method, since in Nobre et al. (2015), sea urchin larvae were exposed to leachate from plastic pellets, in which virgin pellets tend to desorb more volatile chemical compounds (industrial additives). In contrast, in the Izar et al. (2019) assays, the density of the pellets tested was extremely high, probably reaching the stress limit of the organisms tested in both pellet colors (white and colored). In addition, both studies used embryos in their exposures, which tend to be a sensitive stage in the life cycle of organisms, prone to toxicity, and masking small differences in toxicity. Our study was performed using adults of a very resistant species, and this natural resistance might have highlighted the subtle difference in toxicity between pellet colors because resistant species take longer to reach their toxicological stress limit.

3.5. CONCLUSION

Organisms with a fast life cycle can acquire resistance to stressors and potentially mask their toxic effects. We encourage further studies in this regard. Plastic pellets can act as carriers of hydrophobic contaminants to sandy beaches, and these contaminants can become

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bioavailable to sandy beach macrobenthic populations through organic matter. Plastic pellets should also be considered a multi-stressor toxicity matrix. We observed an increase in *E. armata* necrophagy and cannibalism when exposed to a new stressor (plastic pellets). To avoid misunderstanding or masking toxicity results, we encourage the consideration of intraspecific and interspecific interactions in ecotoxicological assays. Finally, pellet color is relevant as a factor for toxicity, with yellowish colors inducing higher toxic effects than white ones. Hydrophobic contaminants adsorbed in plastic pellets are more toxic to macrobenthic populations because of their high concentrations in beach-stranded plastic pellets.

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3.6. SUPPLEMENTARY DATA

Table 3.S1. Chemical compounds analyzed by GC-MS, with their respective mass-to-charge ratio (*m/z*), retention time (RT), R-squared of the regression (R^2) of the calibration curve, limit of detection (LOD) and limit of quantitation (LOQ) of each chemical compound expressed in µg L^{-1} and ng g^{-1} for 25 mg of sediment.

Compounds	m/z	RT	R^2	LOD (µg L^{-1})	LOQ (µg L^{-1})	LOD (ng g^{-1})	LOQ (ng g^{-1})
PAHs							
Naphthalene	128	5.48	0.9988	0.66	2.21	13.2	44.2
Acenaphthylene	152	6.91	0.9991	0.58	1.93	11.6	38.7
Acenaphthene	153	7.18	0.9988	0.66	2.20	13.2	44.0
Fluorene	166	7.73	0.9986	0.70	2.36	14.1	47.2
Phenanthrene	178	9.54	0.9986	0.72	2.40	14.4	48.0
Anthracene	178	9.68	0.9993	0.49	1.64	9.85	32.8
Fluoranthene	202	13.98	0.9991	0.56	1.88	11.3	37.7
Pyrene	202	14.95	0.9993	0.50	1.68	10.1	33.7
Benz[a]anthracene	228	20.70	0.9996	0.39	1.30	7.85	26.1
Chrysene	228	20.85	0.9995	0.44	1.49	8.95	29.8
Benzo[b]fluoranthene	252	25.53	0.9997	0.33	1.13	6.78	22.6
Benzo[k]fluoranthene	252	25.64	0.9995	0.42	1.42	8.54	28.4
Benzo[a]pyrene	252	26.64	0.9995	0.41	1.37	8.25	27.5
Benzo[e]pyrene	252	26.83	0.9998	0.27	0.91	5.49	18.3
Perylene	252	27.17	0.9997	0.30	1.02	6.16	20.5
Indene[1,2,3-cd]pyrene	276	31.01	0.9998	0.29	0.96	5.81	19.3
Benzo[ghi]perylene	276	31.86	0.9999	0.14	0.47	2.85	9.52
Dibenz[a,h]anthracene	278	31.13	0.9990	0.60	2.02	12.1	40.5
Coronene	300	36.50	0.9992	0.54	1.82	10.9	36.4
Oxy-PAHs							
1,4-naphthoquinone	158	6.64	0.9992	0.55	1.85	11.1	37.1
9,10-anthraquinone	208	12.26	0.9991	0.57	1.91	11.5	38.3
Benzanthrone	230	21.37	0.9997	0.33	1.11	6.66	22.2
Nitro-PAHs							
2-nitrofluorene	165	14.65	0.9978	1.95	6.53	39.1	130
2-nitrobiphenyl	199	8.43	0.9978	1.95	6.52	39.1	130
4-nitrobiphenyl	199	10.27	0.9987	1.47	4.92	29.5	98.4
5-nitroacenaphthene	199	12.91	0.9980	1.83	6.12	36.7	122
9-nitroanthracene	223	15.16	0.9996	0.87	2.91	17.5	58.3
3-nitrophenanthrene	223	16.49	0.9998	0.65	2.17	13.0	43.5
9-nitrophenanthrene	223	17.34	0.9986	1.70	5.67	34.0	113
3-nitrofluoranthene	247	22.19	0.9991	1.37	4.58	27.5	91.7
2-nitropyrene	247	23.07	0.9960	2.91	9.73	58.3	194
6-nitrobenz[a]pyrene	267	31.61	0.9939	4.58	15.2	91.6	305
6-nitrochrysene	273	27.27	0.9994	2.24	7.47	44.8	149

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CHAPTER 4

Adsorption rates of polycyclic aromatic hydrocarbons in plastic pellets from marine water

UNPUBLISHED PAPER 1

ABSTRACT

Factory virgin plastic pellets have a high chemical affinity for hydrophobic substances (nonpolar molecules) as organic contaminants. Polycyclic aromatic hydrocarbons (PAHs) are of particular concern because they are highly toxic and represent the combustion of fossil fuels and petrogenic sources of contamination. Our goal was to determine the PAH adsorption rates in polypropylene (PP) and polyethylene (PE) plastic pellets at three different exposure times (24h, 7, and 15 days) and two pellet densities (20 and 100 pellets) per 100 mL^{-1} of seawater (S = 35). In seawater, pellets were exposed to crude oil (oil from the Reconcavo Basin in Bahia State - 1 g L^{-1}). The PAHs in seawater and plastic pellets were determined using gas chromatography coupled with mass spectrometry (GC-MS). We observed increased PAH adsorption with longer exposure times, reaching approximately 90% removal under almost all conditions after 15 days. Accordingly, the quantity of pellets also influences the adsorption rate by increasing the adsorption surface area of the polymer in the environmental system. In addition, PAH removal using PE pellets was more efficient than PP. In summary, plastic pellets efficiently removed PAHs from seawater, acting as traps and carriers of these contaminants in the environment.

Keywords: Microplastics, PAHs, Oil contamination, Sorption, Oil spillage, Remediation and mitigation.

4.1. INTRODUCTION

Plastic pellets (or nurdles) are microplastics (< 5 mm) of primary origin because they are raw materials for manufacturing plastic objects in the plastic industry. They raise special concerns in coastal areas because of their unintentional environmental leakage during industrial processes (*e.g.,* manufacturing and transportation) (Ogata et al., 2009; Corcoran et al., 2020). Spilled plastic pellets often fall into runoff systems and rivers, commonly reaching the coastal environment (Ogata et al., 2009; Karlsson et al., 2018), or are spilled directly in harbor areas, where such losses are common during the transportation process (Izar et al., 2019).

Factory virgin plastic pellets are usually white or colorless and contain high concentrations of industrial additives (Hammer et al., 2012; Andrady and Rajapakse, 2016; Yamashita et al., 2021). Once in the environment, plastic pellets quickly release their chemical additives to water and organic matter (Teuten et al., 2009; Nobre et al., 2015); consequently, new active sites in their structure become available for interaction and adsorption of hydrophobic substances (Teuten et al., 2007; Fotopoulou and Karapanagioti, 2012). Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants that can be adsorbed by pellets (Endo et al., 2005; Ogata et al., 2009; Teuten et al., 2009; Fisner et al., 2013; Taniguchi et al., 2016), and usually represent or are markers of combustion processes (pyrogenic origin) or the presence of fossil fuels (petrogenic origin) (Yunker et al., 2002; González-Fuenzalida et al., 2019).

In scenarios of large oil spills, such as those that have already occurred and are well documented, the high adsorption capacity of hydrophobic contaminants in plastic pellets can turn them into carriers, and this idea can be interesting from the point of view of environmental remediation. Plastic pellets can adsorb hydrophobic contaminants, reaching concentrations from 85 thousand to 1 million times higher than those found in the surrounding ocean waters (Mato et al., 2001; Andrady, 2011; De Frond et al., 2019). The contaminants adsorbed onto the pellets during their period of floating and drifting in the ocean end-up being removed from the seawater and incorporated into the plastic polymer matrices. Considering good public policies to mitigate losses and highly efficient removal protocols for these particles from the marine environment, as proposed by Pereira (2014), the removal of plastic pellets could cause the withdrawal of contaminants adsorbed on their polymeric matrices and a possible decrease in their concentration in coastal environments. In this sense, plastic pellets can be used as *"allies*" in environmental disasters such as oil spills and/or industrial effluents.

To consider this cutting-edge technology, it is necessary to first establish the adsorption rates of these contaminants in plastic pellets at different exposure times, plastic polymers, and pellet densities. Therefore, this study aimed to determine the PAH adsorption rate in polypropylene (PP) and polyethylene (PE) plastic pellets at three different exposure times and two pellet densities in seawater.

4.2. METHODS

4.2.1. Experimental design

Three experiments were performed in the laboratory to simulate the environmental conditions. For all experiments, artificial seawater (distilled water and artificial sea salt, Red Sea Salt - Coral Pro model, which simulates the Red Sea conditions, at a salinity of 35) was contaminated by crude oil from the Reconcavo Basin in the Bahia State, Northeastern Brazil (71.5% saturated, 18.4% resins, and 10.1% aromatics), in a proportion of 1 g L^{-1} of artificial seawater. The seawater and oil mix was stirred for 30 min for homogenization and then placed in glass beakers for exposure to plastics experiments.

Factory virgin plastic pellets were obtained from the manufacturer Braskem located in the industrial complex of Cubatão city (São Paulo-Brazil) and used in all experiments in this study. The polypropylene (PP) and polyethylene (PE) pellets were exposed to oil in seawater. We tested two pellet densities: the most commonly found in port areas - 200 pellets L^{-1}, approximately 60 g L^{-1} (Izar et al., 2019), and the highest density ever found in the world to date - 1000 pellets L^{-1}, approximately 300 g L^{-1} (Mcdermid and Mcmullen, 2005). Exposure assays were carried out for 24 h, 7 days, and 15 days, with each endpoint independent of a new exposure. The matrix control (only artificial seawater) and matrix oil-contaminated control (artificial seawater and oil) were performed independently for all exposure times, and each treatment had three replicates (n = 3) (Fig. 4.1). All the experiments were performed at a controlled temperature (16 ^{o}C) throughout the exposure time.

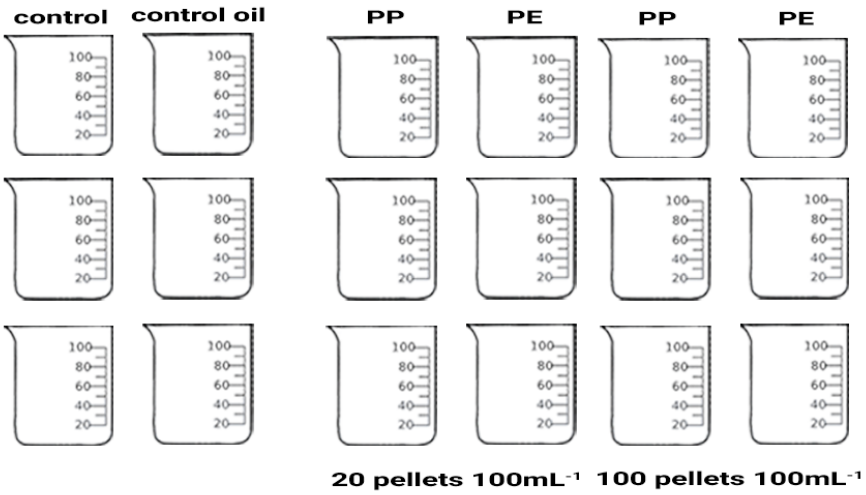

Fig. 4.1. The experimental design of the laboratory assay was based on exposing seawater mixed with oil to plastic pellets of different polymers and densities. Polypropylene (PP) and polyethylene (PE) plastic pellets were exposed to two different densities: 20 and 100 pellets per 100 mL of seawater. Three different exposure times were used: 24 h, 7 days, and 15 days. There were three replicates (N = 3) for each treatment, and the experiment was repeated three times for different exposure times: 24 h, 7 days, and 15 days.

At the end of each exposure period, plastic pellets were removed from seawater using metal tweezers, stored in aluminum packaging, and immediately frozen in a freezer (- 14 ^{o}C to - 20 ^{o}C). Seawater was stored in the beakers used in the experiment, covered with aluminum, and refrigerated at 5 to 10 ^{o}C. Seawater chemical analyzes were performed immediately after the end of each exposure time, and plastic pellet analyzes were performed 5 months after the end of the experiment.

4.2.2. Extraction and quantification procedures

Seawater and plastic pellets were analyzed for the 16 priority PAHs according to the United States Environmental Protection Agency (USEPA) - naphthalene, acenaphthylene, acenaphthene, phenanthrene, anthracene, fluorene, fluoranthene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, pyrene, benzo[a]pyrene, indeno[1,2,3-cd]pyrene, dibenz[ah]anthracene and benzo[ghi]perylene.

The extraction procedure for seawater followed the method proposed by Nascimento et al. (2019), which was adapted for 50 mL of seawater. This volume was placed in a cylindrical glass tube (length: 150 mm, top opening: 20 mm, and bottom opening: 9.0 mm) with 100 mg of octadecylsilyl silica C_{18} sorbent (particle size: 55–105 μm) from Waters

(Milford, Massachusetts), and 1 mL of 2-propanol (99.7%) from JT Baker (Center Valley, USA). The bottom of the tube was closed using polytetrafluoroethylene tape and aluminum foil to avoid loss during the process. The top layer was then closed using a glass cap. The system was stirred for 32 min in an IKA MS3 (Staufen, Germany) vortex mixer at 2000 rpm. The solution was filtered under vacuum through a 5 mL micropipette tip attached to the glass tube bottom opening, containing 20 mg of glass wool used to retain the octadecylsilyl silica C_{18} sorbent. The filtered solution was transferred to a microextraction device (Whatmann Mini ™ UniPrep Filters without syringe, Whatmann, USA) with 500 µL of *n*-hexane (98%) from Merck (Darmstadt, Germany) and sonicated for 20 min before being filtered through the microextraction device.

For the plastic pellets, we adapted the extraction method proposed by Santos et al. (2018) for sediments. After the pellets were lyophilized, five pellets (~ 0.10 - 0.17 g - Table 4.S1 in Supplementary data) were weighed and transferred to a glass tube with 1000 µL of a solvent mixture (18% acetonitrile into 82% dichloromethane) and sonicated for 23 min. The solvent was removed using a 1 mL micropipette and transferred to a microextraction device to filter the final solution.

The final filtered extract of seawater and plastic pellets was injected (1 µL) into a gas chromatograph coupled to a mass spectrometer (GC-MS QP-2010 ULTRA, Shimadzu, Japan). GC-MS conditions followed the method developed by Santos et al. (2016) and improved by Nascimento et al. (2019). Briefly, the temperature ramp set was adjusted as follows: starting at 70 ^{o}C for 2 min, increasing at 30 ^{o}C min^{-1} to 200^{o}C for 5 min, and ending at 5 ^{o}C min^{-1} to 330 ^{o}C for 0.67 min. The injector and the transfer line were set at 310 ^{o}C and 280 ^{o}C, respectively. The detector voltage was set to 1.0 kV. Chromatograms were analyzed using GC-MS/SIM, in which each analyte was determined by the mass-to-charge ratio (*m/z*) and retention time in the temperature ramp. For quantification, a calibration curve was constructed for each analyte using a 16 priority PAHs standard solution from Supelco (St. Louis, USA). For all analyses, the minimum value adopted for the calibration curve's linear regression coefficient (R^2) was 0.99 (Tables 4.S2 and 4.S3 in the supplementary data).

For seawater and plastic pellet extractions, deuterated PAH standards (fluorene-D10, phenanthrene D-10, and pyrene-D10) were used as internal standards. The relative recoveries for deuterated standards in the samples ranged from 79% to 120% in PP and from 76% to 126% in PE. The limit of detection (LOD) and limit of quantification (LOQ) of each analyte were calculated using LOD = 3SE/α and LOQ = 10SE/α, where SE is the standard error of the

linear regression and α is the slope of the linear regression of the calibration curve. All LOD and LOQ information is provided in the supplementary data in Table 4.S2 for seawater and Table 4.S3 for plastic pellets.

The methods used for seawater and plastic pellets were validated according to the criteria established by the International Union of Pure and Applied Chemistry (IUPAC) (Thompson et al., 2002). Nascimento et al. (2019) have already validated the seawater extraction method. For the extraction of plastic pellets, a standard solution of 16 priority PAHs and deuterated PAHs standards (fluorene-D10, phenanthrene D-10, and pyrene-D10) was added to virgin plastic pellets (10 µL - 1mg L^{-1}), with two replicates for each polymer, to determine the accuracy and precision of the method. The relative recoveries ranged from 60% to 98% for all PAHs analyzed in PP and from 80% to 120% in PE. Precision was calculated by dividing the replicates' standard deviation by their average, multiplied by 100. The precision ranged from 0.4% to 18% for PP and from 2% to 28% for PE, with two analytes over 20% (ideno[1,2,3,c,d]pyrene and benzo[ghi]perylene). For deuterated standards, relative the recoveries ranged from 78% to 120% in PP and from 74% to 108% in PE. The precision ranged from 1% to 5% for PP, and from 14% to 18% for PE. All materials used in the chemical analysis were decontaminated to evaluate organic compounds at trace levels following Method 610 from the United States Environmental Protection Agency (US EPA, 1986).

4.2.3. Data treatment

To allow comparisons of the same magnitude and some units with the literature, the results for seawater were converted and expressed in ng L^{-1}, and the results for plastic pellets in ng g^{-1}. A significance level of 95% ($p \leq 0.05$) was adopted for all statistical analyses. They were performed using in the open statistical software Jamovi Version 2.3 (2022).

We built a General Linear Model (GLM) with the total concentration of PAHs as the dependent variable. The comparison was made between treatments in the respective exposure times in seawater (Factor 1, fixed, 6 levels) and plastic pellet (Factor 1, fixed, 4 levels) matrices. Another GLM model was built for the adsorption rate (in percentage) of PAHs as the dependent variable in the seawater matrix. The adsorption rate was calculated as the total concentration found in each treatment with pellets added, multiplied by 100, and divided by the average of the chemical contaminants found in the respective oil control treatment. The

results were subtracted from 100 to obtain the adsorbed concentration from the seawater matrix. The adsorption rate was compared between treatments with pellets (Factor 1, fixed, 4 levels). Tukey's post-hoc test was used for the all described models. We also built another GLM model for the adsorption rate grouping factors: polymer type (Factor 1, fixed, 2 levels), pellet density (Factor 2, fixed, 2 levels), and exposure time (Factor 3, fixed, 3 levels).

4.3. RESULTS

Overall, the lowest concentrations of all PAHs analyzed in the seawater matrix were found in treatments containing 100 PE pellets at all exposure times. All PAHs analyzed were found at levels below the LOD in seawater controls at all exposure times. Fluoranthene, benz[a]anthracene, indene[1,2,3-cd]pyrene, benzo[ghi]perylene, and dibenz[a,h]anthracene were found at levels below the LOD in all treatments of the seawater matrix, including the treatment of seawater with oil without plastic pellets, at all exposure times (Table 4.1). After 7 days of exposure, acenaphthylene, anthracene, pyrene, benzo[b]fluoranthene, and benzo[k]fluoranthene were added to this group, totaling 10 analytes found at levels below the LOD (Table 4.2). After 15 days, naphthalene and benzo[a]pyrene were found at levels below the LOD in all treatments, totaling 12 analytes found at levels below the LOD (Table 4.3, Fig. 4.2). The oil PAHs composition was determined in the oil treatment without plastic pellets, in which naphthalene (49.86%), phenanthrene (24.68%), fluorene (9.04%), and chrysene (6.50%) were the most abundant PAHs in oil after 24h; naphthalene (74.69%), phenanthrene (13.25%), and fluorene (7.05%) after 7 days; and phenanthrene (68.57%) and fluorene (23.50%) after 15 days of exposure.

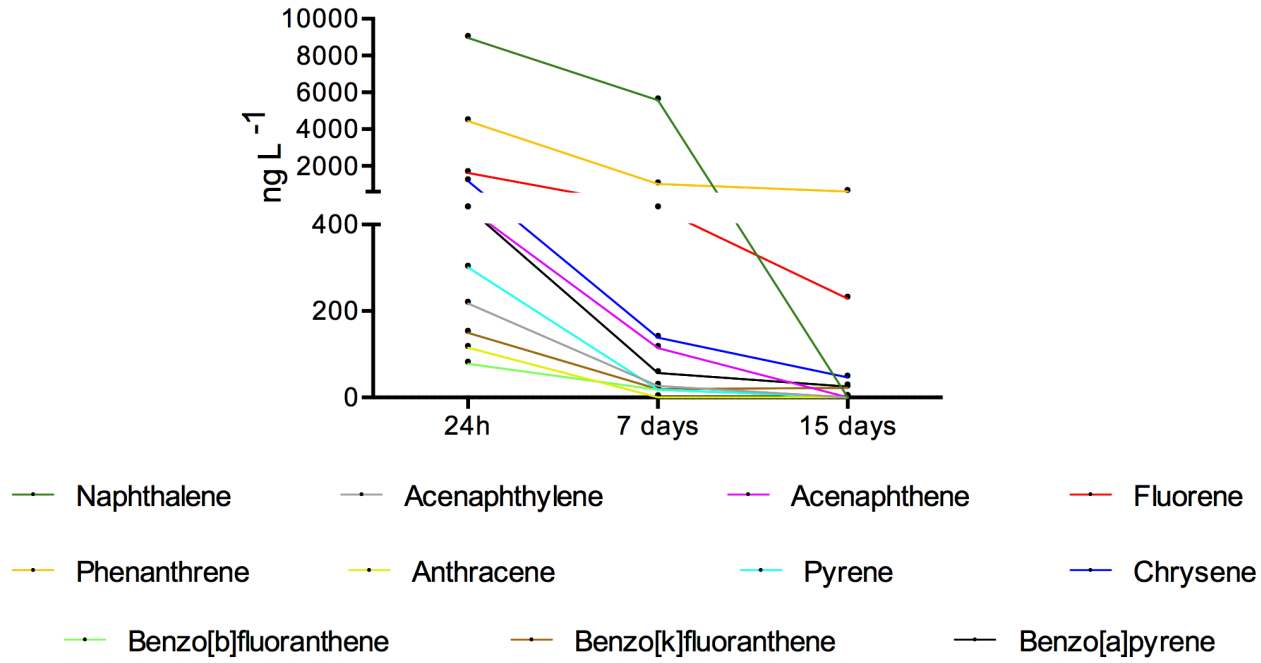

Fig. 4.2. Concentrations of each priority PAH analyzed in seawater with oil without plastic pellet treatment at 24 h, 7, and 15 days. Each line represents the average concentration of each PAH in the seawater matrix. Fluoranthene, benz[a]anthracene, indene[1,2,3-cd]pyrene, benzo[ghi]perylene, and dibenz[a,h]anthracene were not included because all presented values below the LOD at all exposure times.

About the analytes that remained in the seawater matrix, at 24 h of exposure time, acenaphthylene, anthracene, pyrene, benzo[b]fluoranthene, and benzo[k]fluoranthene reached levels below the LOD just in PE treatment with 100 pellets, while anthracene and benzo[k]fluoranthene were also found at < LOD in PE treatment with 20 pellets. Only anthracene was found at low levels in three treatments, including in PP treatment with 100 pellets (Table 4.1). After 7 days of exposure, benzo[a]pyrene was the only analyte removed from the water, reaching levels close to < LOD in all pellet treatments. Acenaphthene, fluorene, chrysene, and phenanthrene reached < LOD levels just in the PE treatment with 100 pellets, while acenaphthene and chrysene also reached low levels in the PP treatment with 100 pellets. Only acenaphthene was found at < LOD levels in the PE treatment with 20 pellets, in addition to the two treatments already mentioned (Table 4.2). Finally, at 15 days, pyrene was the only analyte removed from the seawater, reaching levels close to < LOD in all pellet treatments. Fluorene and phenanthrene reached < LOD levels just in the PE treatment with 100 pellets, while chrysene also reached low levels in the PP treatment with 20 and 100 pellets (Table 4.3).

Table 4.1. Average concentrations of each priority PAH in seawater (ng L^{-1}) and plastic pellet (ng g^{-1}) matrices analyzed by GC-MS after exposure for 24 h for different plastic pellet densities. **Sw** = seawater control, **Oil** = Seawater control + Oil, **PP20** = Seawater control + Oil + 20 PP pellets, **PP100** = Seawater control + Oil + 100 PP pellets, **PE20** = Seawater control + Oil + 20 PE pellets, and **PE100** = Seawater control + Oil + 100 PE pellets. The average PAH concentrations below the limit of detection (LOD) are represented by <, followed by the respective LOD values.

	Seawater (ng L^{-1})						Plastic Pellets (ng g^{-1})			
$_{16}$PAHs	**Sw**	**Oil**	**PP20**	**PP100**	**PE20**	**PE100**	**PP20**	**PP100**	**PE20**	**PE100**
Naphthalene	< 66.3	8964	9205	4857	3471	1327	1051	5005	3802	11819
Acenaphthylene	< 58.1	218	176	119	107	< 58.1	135	709	195	704
Acenaphthene	< 66.0	412	309	205	171	83.8	209	1010	392	1240
Fluorene	< 70.8	1626	1308	1041	883	487	1260	3832	1687	4361
Phenanthrene	< 72.0	4438	3364	2376	2070	1341	11871	24097	6546	14065
Anthracene	< 49.2	115	92.7	< 49.2	< 49.2	< 49.2	270	1089	175	651
Fluoranthene	< 56.6	< 56.6	< 56.6	< 56.6	< 56.6	< 56.6	191	949	133	590
Pyrene	< 50.6	301	205	115	116	< 50.6	987	2423	529	1242
Benz[a]anthracene	< 39.2	< 39.2	< 39.2	< 39.2	< 39.2	< 39.2	287	1198	223	687
Chrysene	< 44.8	1169	831	470	456	282	8514	17411	4677	8221
Benzo[b]fluoranthene	< 33.9	78.0	56.1	39.8	42.9	< 33.9	1253	2701	886	1562
Benzo[k]fluoranthene	< 42.7	150	109	50.0	< 42.7	< 42.7	866	1848	492	984
Benzo[a]pyrene	< 41.2	462	329	189	184	132	208	917	134	526
Indene[1,2,3-cd]pyrene	< 29.1	< 29.1	< 29.1	< 29.1	< 29.1	< 29.1	66.7	590	77.6	371
Benzo[ghi]perylene	< 14.2	< 14.2	< 14.2	< 14.2	< 14.2	< 14.2	157	798	113	467
Dibenz[a,h]anthracene	< 60.9	< 60.9	< 60.9	< 60.9	< 60.9	< 60.9	160	821	117	529
Σ_{16}PAHs	**< LOD**	**17982**	**16014**	**9500**	**7574**	**3803**	**28275**	**66929**	**21424**	**47307**

Table 4.2. Average concentrations of each priority PAH in seawater (ng L^{-1}) and plastic pellet (ng g^{-1}) matrices analyzed by GC-MS after exposure for 7 days for different plastic pellets densities. **Sw** = seawater control, **Oil** = Seawater control + Oil, **PP20** = Seawater control + Oil + 20 PP pellets, **PP100** = Seawater control + Oil + 100 PP pellets, **PE20** = Seawater control + Oil + 20 PE pellets, and **PE100** = Seawater control + Oil + 100 PE pellets. The average PAH concentrations below the limit of detection (LOD) are represented by <, followed by the respective LOD values.

	Seawater (ng L^{-1})						Plastic Pellets (ng g^{-1})			
$_{16}$PAHs	**Sw**	**Oil**	**PP20**	**PP100**	**PE20**	**PE100**	**PP20**	**PP100**	**PE20**	**PE100**
Naphthalene	< 66.3	5579	3148	1597	2768	512	969	3685	2730	3857
Acenaphthylene	< 58.1	< 58.1	< 58.1	< 58.1	< 58.1	< 58.1	125	683	146	467
Acenaphthene	< 66.0	115	77.3	< 66.0	< 66.0	< 66.0	149	883	255	608
Fluorene	< 70.8	533	462	326	343	< 70.8	371	1702	948	1692
Phenanthrene	< 72.0	1014	949	553	859	< 72.0	2221	9933	3669	6297
Anthracene	< 49.2	< 49.2	< 49.2	< 49.2	< 49.2	< 49.2	172	845	134	568
Fluoranthene	< 56.6	< 56.6	< 56.6	< 56.6	< 56.6	< 56.6	168	853	124	530
Pyrene	< 50.6	< 50.6	< 50.6	< 50.6	< 50.6	< 50.6	490	1709	282	740
Benz[a]anthracene	< 39.2	< 39.2	< 39.2	< 39.2	< 39.2	< 39.2	157	810	104	410
Chrysene	< 44.8	138	74.8	< 44.8	112	< 44.8	1682	4928	759	1469
Benzo[b]fluoranthene	< 33.9	< 33.9	< 33.9	< 33.9	< 33.9	< 33.9	346	1287	231	633
Benzo[k]fluoranthene	< 42.7	< 42.7	< 42.7	< 42.7	< 42.7	< 42.7	631	1911	289	651
Benzo[a]pyrene	< 41.2	57.0	< 41.2	< 41.2	< 41.2	< 41.2	174	888	109	500
Indene[1,2,3-cd]pyrene	< 29.1	< 29.1	< 29.1	< 29.1	< 29.1	< 29.1	108	622	77.9	395
Benzo[ghi]perylene	< 14.2	< 14.2	< 14.2	< 14.2	< 14.2	< 14.2	175	869	120	456
Dibenz[a,h]anthracene	< 60.9	< 60.9	< 60.9	< 60.9	< 60.9	< 60.9	196	982	135	587
Σ_{16}PAHs	**< LOD**	**7522**	**4761**	**2476**	**4157**	**512**	**8387**	**33460**	**10274**	**20253**

Table 4.3. Average concentrations of each priority PAH in seawater (ng L^{-1}) and plastic pellet (ng g^{-1}) matrices analyzed by GC-MS after exposure for 15 days for different plastic pellets densities. **Sw** = seawater control, **Oil** = Seawater control + Oil, **PP20** = Seawater control + Oil + 20 PP pellets, **PP100** = Seawater control + Oil + 100 PP pellets, **PE20** = Seawater control + Oil + 20 PE pellets, and **PE100** = Seawater control + Oil + 100 PE pellets. The average PAH concentrations below the limit of detection (LOD) are represented by <, followed by the respective LOD values.

	Seawater (ng L^{-1})						Plastic Pellets (ng g^{-1})			
$_{16}$PAHs	**Sw**	**Oil**	**PP20**	**PP100**	**PE20**	**PE100**	**PP20**	**PP100**	**PE20**	**PE100**
Naphthalene	< 66.3	< 66.3	< 66.3	< 66.3	< 66.3	< 66.3	543	2774	660	2625
Acenaphthylene	< 58.1	< 58.1	< 58.1	< 58.1	< 58.1	< 58.1	229	1235	186	767
Acenaphthene	< 66.0	< 66.0	< 66.0	< 66.0	< 66.0	< 66.0	847	3474	719	2443
Fluorene	< 70.8	228	187	137	119	< 70.8	256	2167	1021	1499
Phenanthrene	< 72.0	612	457	387	398	< 72.0	1032	6576	5018	5992
Anthracene	< 49.2	< 49.2	< 49.2	< 49.2	< 49.2	< 49.2	169	1051	204	653
Fluoranthene	< 56.6	< 56.6	< 56.6	< 56.6	< 56.6	< 56.6	193	997	198	540
Pyrene	< 50.6	140	< 50.6	< 50.6	< 50.6	< 50.6	389	1656	541	844
Benz[a]anthracene	< 39.2	< 39.2	< 39.2	< 39.2	< 39.2	< 39.2	395	628	137	336
Chrysene	< 44.8	46.7	< 44.8	< 44.8	85.1	< 44.8	1581	3364	2004	1330
Benzo[b]fluoranthene	< 33.9	< 33.9	< 33.9	< 33.9	< 33.9	< 33.9	288	722	256	429
Benzo[k]fluoranthene	< 42.7	< 42.7	< 42.7	< 42.7	< 42.7	< 42.7	740	1132	726	585
Benzo[a]pyrene	< 41.2	< 41.2	< 41.2	< 41.2	< 41.2	< 41.2	240	704	136	418
Indene[1,2,3-cd]pyrene	< 29.1	< 29.1	< 29.1	< 29.1	< 29.1	< 29.1	249	632	88.7	356
Benzo[ghi]perylene	< 14.2	< 14.2	< 14.2	< 14.2	< 14.2	< 14.2	472	1744	515	1324
Dibenz[a,h]anthracene	< 60.9	< 60.9	< 60.9	< 60.9	< 60.9	< 60.9	884	1242	217	576
Σ_{16}PAHs	**< LOD**	**935**	**691**	**591**	**720**	**22.7**	**8598**	**30518**	**12809**	**20976**

PAHs concentrations in the seawater matrix were significantly different among treatments at all exposure times (24 h: $F = 19.0$, $df = 5$, $p < 0.001$; 7 days: $F = 11.4$, $df = 5$, $p < 0.001$; 15 days: $F = 4.05$, $df = 5$, $p = 0.022$). In the comparison between the seawater control and oil treatment without pellets (Oil mean - Seawater mean), the average PAH concentration in the oil treatment was 17,982 ng L^{-1} higher than in the seawater control after 24 h of exposure, 7,522 ng L^{-1} higher after 7 days, and 935 ng L^{-1} higher after 15 days (Fig. 4.3A). Regarding pellet treatments compared to the oil control treatment, treatments with 20 PP pellets did not differ from oil control treatments after any time of exposure, whereas the other pellet treatments decreased PAHs levels ($p_{tukey} < 0.05$) at 24 h and 7 days. After 15 days of exposure, only the PE treatment with 100 pellets differed from the oil control treatment ($p_{tukey} < 0.001$). PE treatment with 100 pellets had the lowest PAH levels, close to the seawater control after 7 days of exposure, and close to < LOD after 15 days (Fig. 4.3A). In the plastic pellet matrix, treatments with 100 plastic pellets adsorbed more PAHs, with a slight difference in the adsorption of the PP polymers over PE. This pattern was observed for all exposure times (Fig. 4.3B).

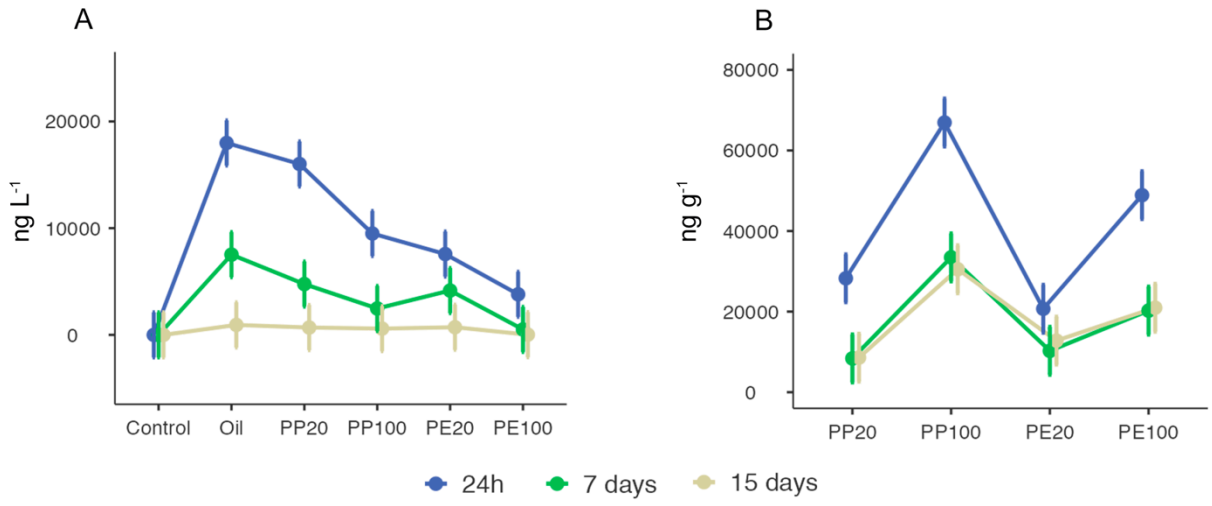

Fig. 4.3. The sums of the concentrations of the 16 priority PAHs found in seawater (A - ng L^{-1}) and plastic pellet (B - ng g^{-1}) matrices mixed with oil exposed to plastic pellets for 24 h (blue line), 7 (green line), and 15 days (beige line). Control = seawater control, Oil = seawater mixed with oil control, PP20 = seawater mixed with oil control and 20 PP pellets, PP100 = seawater mixed with oil control and 100 PP pellets, PE20 = seawater mixed with oil control and 20 PE pellets, and PE100 = seawater mixed with oil control and 100 PE pellets. The dots represent the treatment mean and the error bars represent the 95% confidence interval. Confidence intervals that do not intersect represent a significant difference.

In terms of PAHs adsorption by plastic pellets, there was a significant difference in the average adsorption rate of the interaction between the polymers and exposure time (F = 5.16, df = 2, p = 0.014). The differences in the average adsorption rate between polymers after just 24 h of exposure, with the adsorption rate being 21% higher in PE than in PP (p_{tukey} = 0.002). Treatments with 100 PE pellets removed almost all PAHs (nearly 100%) from seawater after 7 days of exposure, except for naphthalene (Table 4.2) and all PAHs after 7 days of exposure (Table 4.3, Fig. 4.4 and 4.5). Fluorene and Phenanthrene persisted in seawater for 15 days, with plastic pellet adsorption rates below 50% (Fig. 4.5A, B, and C), except in PE treatments with 100 pellets, in which both were completely removed (Fig. 4.5D).

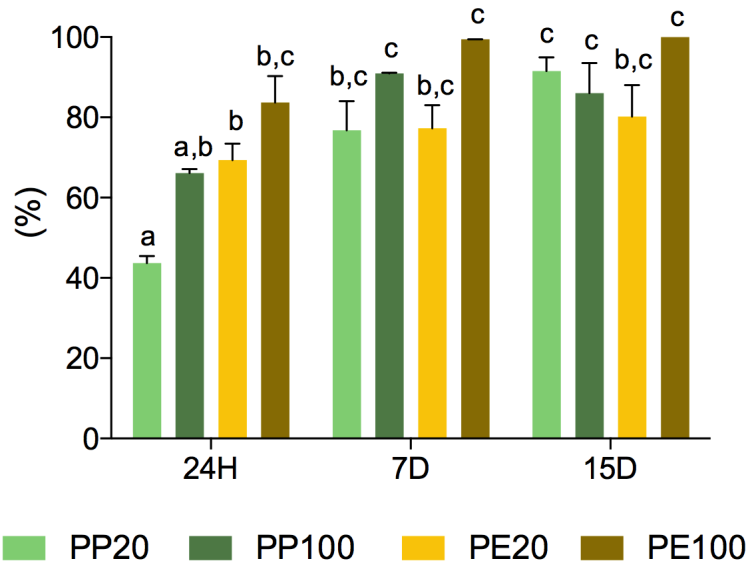

Fig. 4.4. The adsorption rate (%) of 16 priority PAHs was determined by exposing plastic pellets to the seawater matrix with oil for 24 hours, 7, and 15 days. Different colors represent treatments: light green (PP20) = PP with 20 pellets, dark green (PP100) = PP with 100 pellets, orange (PE20) = PE with 20 pellets, and brown (PE100) = PE with 100 pellets. Error bars represent 95% confidence intervals; different letters indicate significant differences between the treatments.

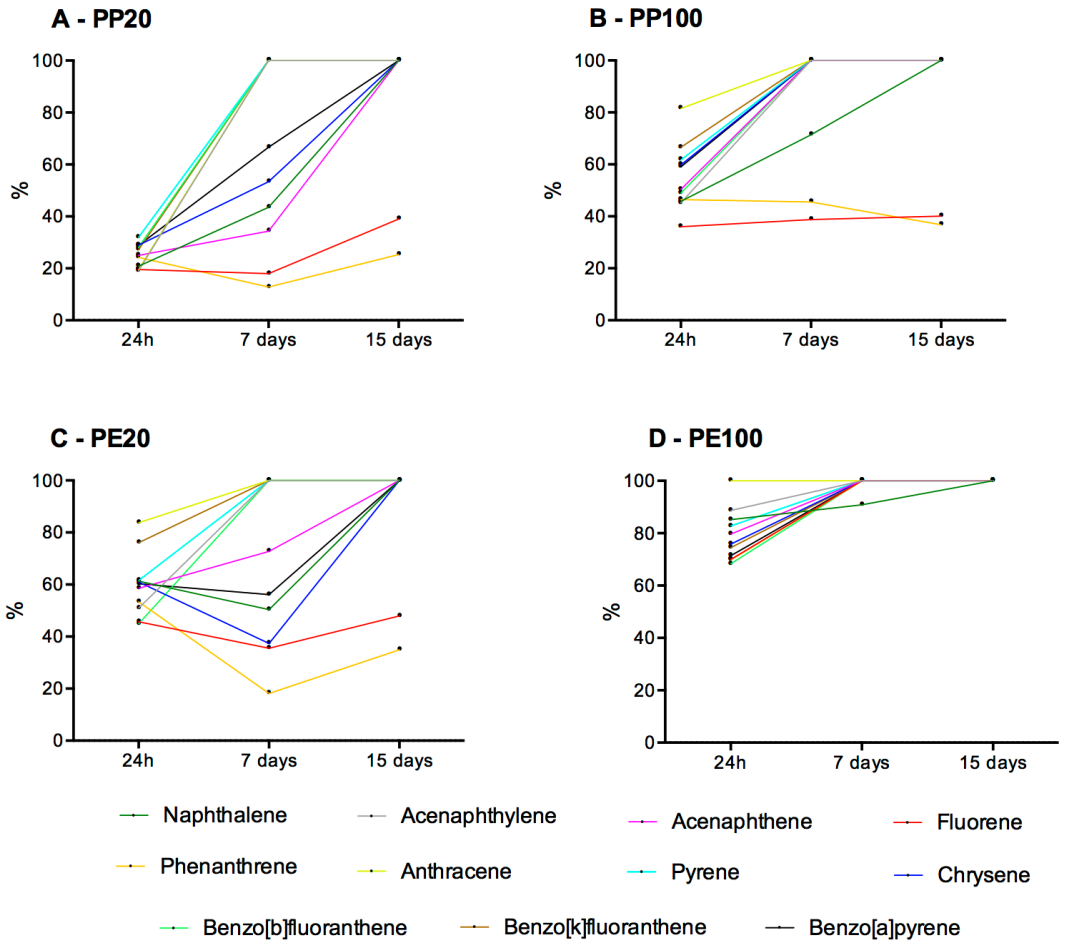

Fig. 4.5. The adsorption rate (%) of 16 priority PAHs by plastic pellets exposed to the seawater matrix with oil for 24 h, 7 and 15 days. A - PP20 = PP with 20 pellets, B - PP100 = PP with 100 pellets, C - PE20 = PE with 20 pellets and D - PE100 = PE with 100 pellets. Each line represents the adsorption rate of each PAH by the plastic pellets for each treatment. Fluoranthene, benz[a]anthracene, indene[1,2,3-cd]pyrene, benzo[ghi]perylene, and dibenz[a,h]anthracene were not included because all presented values < LOD at all exposure times.

4.4. DISCUSSION

In general, priority PAHs showed high depletion in seawater after 15 days in the presence of plastics, and 7 days were sufficient to deplete most PAHs to levels below the LOD, demonstrating an increasing relationship with exposure time. These results corroborate those found by Mato et al. (2001), who found a constant increase in the concentrations of PCBs and DDT in virgin pellets exposed to seawater in a field experiment for 6 days. Both results demonstrate that the exposure time is important for depleting hydrophobic plastic contaminants in seawater. For comparison purposes, the metal Pb's absorption rates were approximately 70% (Turner et al., 2020). We reached adsorption rates for PAHs of 88% for PP and 90% for PE after 15 days. In line with other studies (Teuten et al., 2007; Fisner et al.,

2017; Léon et al., 2018), our data proved that PE is more efficient than PP for scavenging PAHs from seawater. PE is a polymer with a large surface area and a large volume of internal cavities, facilitating the adsorption of hydrophobic compounds (Teuten et al., 2007). These characteristics could be the key factors in our experiment: the number of plastic pellets exposed. A higher number of pellets provides a greater surface area and internal polymer cavities, enabling greater adsorption of PAHs.

As previously reported, the exposure time is also important because all analytes were completely depleted from seawater after just 15 days of exposure, the maximum period tested in this study. Desorption rate of PAHs compounds from plastics to seawater is approximately 12% (Léon et al., 2018), proving the ability of plastic pellets to serve as traps for PAHs in marine environments. This conclusion can also be extrapolated to aquatic and estuarine environments, as salinity has already been shown to have no significant effect on the sorption of persistent organic pollutants on plastic polymers (Bakir et al., 2014). Future experiments under natural conditions are recommended, since desorption and equilibrium of chemicals seem to be faster in natural environments (Endo et al., 2013).

In this study, the average concentrations of the 16 priority PAHs varied between 8500 and 66000 ng g^{-1} in PP pellets and 10000 and 47000 ng g^{-1} in PE pellets, approaching the environmental concentrations found in beached pellets collected in harbor areas. Previous studies found that the average PAH concentrations in environmental beached pellets ranged from 500 to 3000 ng g^{-1} in beaches near cities and reached 9000 to 27000 ng g^{-1} in beaches located in harbor areas (Karapanagioti et al., 2011; Van et al., 2012; Fisner et al., 2013; Taniguchi et al., 2016), highlighting the interaction between PAHs and petroleum fractions, as harbor areas have high vessel and ship traffic. Moreover, in plastic pellets from seawater, the average PAHs' concentrations range from 39 to 1200 ng g^{-1} (Rios et al., 2007), demonstrating the ability of these marine litter particles to adsorb greater amounts of hydrophobic contaminants during their drift period in the ocean, as beached pellets have higher PAH concentrations.

Among the 16 priority PAHs analyzed, phenanthrene and fluorene were among the highest PAH concentrations adsorbed by plastic pellets, according to previous environmental studies (Frias et al., 2010; Fisner et al., 2013; Mizukawa et al., 2013; Léon et al., 2018; Arias et al., 2023). Naphthalene and chrysene have completed this list. In seawater, PAHs with low molecular weight (2-3 rings) tend to be more abundant and easier to absorb into particles in the water column because of their less hydrophobic properties (Luo et al., 2006).

Phenanthrene and fluorene are commonly found in high concentrations in petroleum and its derivatives, representing petrogenic sources (Budzinski et al., 1997; Tam et al., 2001; Latimer and Zheng, 2003), and they have a high affinity for PE polymers (Bakir et al., 2014). This might explain the presence of both compounds in the water after 15 days of exposure to plastic pellets, except in the treatment with 100 PE pellets. Even so, the high concentration of both compounds in the oil possibly prevented them from being completely removed from seawater after the maximum exposure time tested. It was necessary 100 PE pellets to achieve maximum depletion of these compounds from seawater, even though the half-life of phenanthrene is approximately 15 days in seawater (Brakstad et al., 2018), indicating that the quantity of plastics used in the exposure is an important factor in increasing the available adsorption surface of the polymer. For PP and PE20, both analytes remained in seawater even after 15 days of exposure, probably due to the short exposure time, or the finite number of active sites in the polymeric matrix available for adsorption. However, for naphthalene, plastic pellets at any density or polymer tested were not able to remove this PAH from seawater before 7 days of exposure. This behavior of naphthalene can be explained by its higher solubility in water (Latimer and Zheng, 2003), which makes this chemical more liquefiable, especially in seawater (Jing et al., 2014). Moreover, naphthalene is the priority PAH with the lowest affinity for plastics, with a desorption rate of approximately 20% (Léon et al., 2018). However, this analyte represents a large component of PAHs in crude petroleum (Sporstol et al., 1983; Truscott et al., 1992), and its high volatility may have been an ally for low concentrations at 15 days, close to its half-life (Lofthus et al., 2018). The same idea can be extended to acenaphthene and acenaphthylene (Table 4.S4).

Our results support the idea that plastic pellets act as carriers of organic contaminants in coastal environments, especially in the seawater-to-coastal area route (Mato et al., 2001; Gorman et al., 2019; Izar et al., 2024), in which PAHs tend to accumulate in sediments and biota due to their high affinity for organic matter (Kim et al., 1999; Teuten et al., 2007; Teuten et al., 2009). In coastal areas with PAH sources, besides plastic pellets and sediment, PAHs are also capable of accumulating in tissues of organisms, such as on the Brazilian coast affected by the 2019 oil spill, in which PAHs concentrations ranged from 8 to 418 ng g^{-1} wet weight were found in finfish and shellfish tissues (Magalhães et al., 2022), or in the region of the largest harbor in Latin America, Santos Port, where PAHs were found from 23 to 495 ng g^{-1} wet weight in guitarfishes and angelsharks (Martins et al., 2023), highlighting the high lipophilicity of these contaminants. Moreover, the sorption properties of sediments are

affected by the presence of plastics, which can lead to toxicological effects and the bioaccumulation of these organic contaminants in the local biota (Teuten et al., 2007). Finally, industrial additives incorporated into pellets should be considered, as they can be released into the environment during the plastic pellets presence in the system (Nobre et al., 2015; Tanaka et al., 2023), which may generate a new environmental impact while trying to solve another. Hence, we suggest that the findings of this study should be used very carefully, as we may not only deplete hydrophobic contaminants from seawater but also switch these contaminants to industrial additives, especially PE, which leaches more additives than PP (Tanaka et al., 2023).

4.5. CONCLUSIONS

Plastic pellets are efficient for removing PAHs from seawater. However, it is important to consider the polymer to be used. PE is more effective than PP, and the greater the quantity of pellets added, the greater the adsorption surface available in the environment. The exposure time is an important factor, with 15 days seeming to be the ideal time for the total removal of all priority PAHs by PE pellets. The volatilization of the compounds to be adsorbed is also a factor to be considered, as this can act as an ally for this removal. However, the volatilization of compounds not only removes them from seawater but also deposits these contaminants in another environmental compartment (atmospheric air), which can be harmful in terms of toxicity and environmental risks. Finally, it is important to consider the desorption rates of each contaminant (industrial additives and organic contaminants) to better understand the real and safe effectiveness of using plastic pellets to remove hydrophobic contaminants from seawater and their potential risks.

4.6. SUPPLEMENTARY DATA

Table 4.S1. Total weight of 5 plastic pellets (g) used in the chemical analysis at the 3 different exposure times (24 h, 7 days and 15 days).

Treatment	24 h	7 days	15 days
PP1_20	0.1144	0.1090	0.1218
PP2_20	0.1168	0.1015	0.1134
PP3_20	0.1156	0.1052	0.1059
PP1_100	0.1140	0.1044	0.1083
PP2_100	0.1100	0.1054	0.0946
PP3_100	0.1058	0.1051	0.1141
PE1_20	0.1421	0.1138	0.1158
PE2_20	0.1757	0.1431	0.1474
PE3_20	0.1592	0.1383	0.1308
PE1_100	0.1774	0.1236	0.1952
PE2_100	0.1096	0.1752	0.1520
PE3_100	0.1655	0.1731	0.1390

Table 4.S2. Chemical compounds in seawater analyzed by GC-MS, with their respective mass-to-charge ratio (*m/z*), retention time (RT), R-squared of the regression (R^2) of the calibration curve, limit of detection (LOD) and limit of quantitation (LOQ) of each chemical compound expressed in µg L^{-1}, and the equation of the linear regression curve.

Compounds	m/z	RT	R^2	LOD (µg L^{-1})	LOQ (µg L^{-1})	Equation of the linear regression curve
PAHs						
Naphthalene	128	5.48	0.9988	0.66	2.21	y = 9174.4x + 2531.0
Acenaphthylene	152	6.91	0.9991	0.58	1.93	y = 7335.9x + 697.1
Acenaphthene	153	7.18	0.9988	0.66	2.20	y = 5181.3x + 286.4
Fluorene	166	7.73	0.9986	0.70	2.36	y= 5918.8x + 487.7
Phenanthrene	178	9.54	0.9986	0.72	2.40	y = 8352.0x + 2519.7
Anthracene	178	9.68	0.9993	0.49	1.64	y = 7770.9x + 831.4
Fluoranthene	202	13.98	0.9991	0.56	1.88	y = 8730.1x + 4060.6
Pyrene	202	14.95	0.9993	0.50	1.68	y = 8351.2x + 3564.0
Benz[a]anthracene	228	20.70	0.9996	0.39	1.30	y = 5896.0x + 610.3
Chrysene	228	20.85	0.9995	0.44	1.49	y = 5837.8x + 1236.0
Benzo[b]fluoranthene	252	25.53	0.9997	0.33	1.13	y = 6187.8x - 1219.1
Benzo[k]fluoranthene	252	25.64	0.9995	0.42	1.42	y = 6205.4x - 1450.8
Benzo[a]pyrene	252	26.64	0.9995	0.41	1.37	y = 5873.1x - 392.2
Indene[1,2,3-cd]pyrene	276	31.01	0.9998	0.29	0.96	y = 5514.6x - 1063.1
Benzo[ghi]perylene	276	31.86	0.9999	0.14	0.47	y = 6246.0x - 430.0
Dibenz[a,h]anthracene	278	31.13	0.9990	0.60	2.02	y = 5557.9x - 1575.6

Table 4.S3. Chemical compounds in plastic pellets analyzed by GC-MS, with their respective mass-to-charge ratio (*m/z*), retention time (RT), R-squared of the regression (R^2) of the calibration curve, limit of detection (LOD) and limit of quantitation (LOQ) of each chemical compound expressed in µg L^{-1}, and the equation of the linear regression curve.

Compounds	m/z	RT	R^2	LOD (µg L^{-1})	LOQ (µg L^{-1})	Equation of the linear regression curve
PAHs						
Naphthalene	128	5.61	0.9994	1.33	4.44	y = 4433.6x - 1158.1
Acenaphthylene	152	7.06	0.9992	1.57	5.22	y = 3762.4x - 3522.6
Acenaphthene	153	7.26	0.9981	1.60	5.35	y = 2579.3x - 2600.2
Fluorene	166	7.88	0.9991	1.60	5.33	y = 2429.3x - 1772.1
Phenanthrene	178	9.69	0.9991	1.60	5.35	y = 3507.2x - 2134.3
Anthracene	178	9.80	0.9987	2.15	7.17	y = 3046.6x - 5004.9
Fluoranthene	202	14.02	0.9984	2.23	7.43	y = 3083.8x - 3482.6
Pyrene	202	14.97	0.9988	1.91	6.38	y = 2924.8x - 2639.4
Benz[a]anthracene	228	20.66	0.9990	1.71	5.71	y = 1768.6x - 1566.7
Chrysene	228	20.85	0.9993	1.48	4.94	y = 2206.6x - 1661.0
Benzo[b]fluoranthene	252	25.53	0.9981	2.45	8.16	y = 1747.1x - 2102.7
Benzo[k]fluoranthene	252	25.63	0.9996	1.06	3.52	y = 2816.5x - 1617.6
Benzo[a]pyrene	252	26.81	0.9991	2.45	8.16	y = 1188.9x - 991.2
Indene[1,2,3-cd]pyrene	276	31.02	0.9984	2.23	7.42	y = 694.7x - 668.7
Benzo[ghi]perylene	276	31.86	0.9975	2.79	9.29	y = 572.5x - 581.3
Dibenz[a,h]anthracene	278	31.16	0.9968	3.17	10.5	y = 1247.0x - 1691.4

Table 4.S4. Physicochemical properties of the 16 PAHs.

Compounds	Number of rings	Point of melting (°C)	Point of boiling (°C)	Solubility (mg L^{-1})	Log K_{ow}
PAHs					
Naphthalene	2	81	218	31.7	3.40
Acenaphthylene	3	92	265	16.1	4.07
Acenaphthene	3	95	279	3.47	3.93
Fluorene	3	116	295	1.98	4.18
Phenanthrene	3	101	340	1.29	4.50
Anthracene	3	216	342	$7.3x10^{-2}$	4.60
Fluoranthene	4	111	375	$2.6x10^{-1}$	5.22
Pyrene	4	149	360	$1.4x10^{-1}$	5.18
Benz[a]anthracene	4	158	400	$1.4x10^{-2}$	5.61
Chrysene	4	255	448	$2.0x10^{-3}$	5.91
Benzo[b]fluoranthene	5	167	481	$1.2x10^{-3}$	6.12
Benzo[k]fluoranthene	5	217	480	$5.5x10^{-4}$	6.84
Benzo[a]pyrene	5	179	496	$3.8x10^{-3}$	6.50
Indene[1,2,3-cd]pyrene	6	163	536	$6.2x10^{-2}$	6.58
Benzo[ghi]perylene	6	222	545	$2.6x10^{-4}$	7.10
Dibenz[a,h]anthracene	5	266	524	$5.0x10^{-3}$	6.50

CHAPTER 5

Is it possible to estimate beach-stranded plastic pellets using Unmanned Aerial Vehicles (UAV) drone images?

UNPUBLISHED PAPER 2

ABSTRACT

We establish a relationship between natural wrack and plastic pellet density on the sandy beach using Unmanned Aerial Vehicles (UAV). Floating marine debris (natural or anthropogenic), including plastic pellets, tend to coalesce, drift, and strand together on sandy beaches. Using high-resolution images, UAV drones are cheap and widely available technology that can identify microplastics with a few millimeters. Therefore, this work verified if it is plausible to indirectly estimate plastic pellet densities using UAV drone images by identifying the local coverage of natural wrack. Pellets and natural debris were manually sampled *in situ* on 3 beaches on the coast of São Paulo-Brazil to verify the relationship between the pellets and debris. Once a model was established, UAV drone aerial images were taken on the largest beach (Itaguaré - 2 km length), and heat maps of the distribution of natural debris from both methods were compared. Plastic pellets tend to accumulate along with natural debris on sandy beaches, especially in the high tide line. The regression models from the field method predicted pellets with 40% accuracy. Other factors not considered in this study (as wave energy and sediment composition) may have influenced this accuracy. However, when comparing the pellet dispersion heat maps obtained from both methodologies (field and UAV drone), it is possible to conclude that the method was effective, especially in identifying hotspots for pellet entries onto beaches. The UAV drone methodology allows a single person to sample plastic pellets in 35 thousand times more beach area than the conventional field methodology, in just over 1 hour flight. This methodology can be fast, easy, and scalable.

Key words: Microplastics, Marine Pollution, Marine Debris, Sandy Beaches, Unmanned Aerial Vehicles, Natural Wrack

5.1. INTRODUCTION

Plastic pollution is a global problem that has been increasingly studied over the past decades. Plastics can directly give rise to microplastics (< 5 mm) through the fragmentation and degradation of these larger plastic debris or in the manufacturing process of larger plastic objects since the plastic raw material is made in a granular form of a few sizes (2.5 - 4.5 mm of diameter) (Mato et al., 2001; Ogata et al., 2009). These factory granular microplastics are called plastic pellets or nibs and may be either intentionally or unintentionally spilled into the environment during the industrial and transportation processes (Ogata et al., 2009; Karlsson et al., 2018). Once in the sea, these plastic particles tend to float and drift with oceanic surface currents (Browne et al., 2010; Maximenko et al., 2012; Iwasaki et al., 2017), reaching coastal areas, and especially sandy beaches (Van Cauwenberghe et al., 2015; Moreira et al., 2016; Corcoran et al., 2020).

Plastic pellets have been reported on beaches around the world, highlighting the beaches from Hawaii (McDermid and McMullen, 2004), Chile (Hidalgo-Ruz and Thiel, 2013) and Brazil (Izar et al., 2019; Turra et al., 2014), which are locations with the highest disclosed densities for these particles. Gregory (1983) reported a relationship between high plastic pellet densities and the wrack close to the high tide zone. Pellet accumulation patterns on beaches are random, even on nearby beaches, with winds and surface currents acting as the main forces in the distribution of these floating particles (Kim et al., 2015). This suggests that floating marine debris tends to drift in the surface sea currents together with the natural floating debris (algae and land-plant material) and accumulate together on beaches. If this relationship is true, there is an important ecological implication for benthic macrofauna, which tends to be more abundant in natural wracks than in the presence of plastic debris (Laurino et al., 2023).

Methods to estimate plastic pellet density on beaches are based on direct sampling efforts from a limited area of beach sediment (square, cylindrical corer, or transect) and the separation of pellets from the sampled sediment by density, filtration, visually/manually or sieving (Hidalgo-Ruz et al., 2012). These techniques are accurate and reliable but require an

enormous physical effort and it is time-consuming to sample at a small scale on the beach. Other techniques are necessary to estimate pellet densities on beaches without high human efforts. Therefore, the relationship between floating debris in sea surface currents might be a factor to be considered. The hydrodynamical modeling of particles by ocean currents and their drift trajectories have been well documented and calculated for marine debris (Kubota, 1994; Martinez et al., 2009), microplastics (Maximenko et al., 2012; Iwasaki et al., 2017; Alosairi et al., 2020) and recently for plastic pellets (Gordon et al., 2020; Izar et al., 2022). Numerical models are powerful tools for understanding the distribution, points of source, and endpoint of plastics and microplastics (Hardesty et al., 2017).

Remote sensing is a novel and more accurate approach to estimate and identify marine debris in the environment. Satellites, manned aircrafts, and unmanned aerial vehicles (UAV), popularly known as drones, are thus used. Satellites are the most frequent platform used because they can provide a large amount of data, with repetitions in time and various scales. This gives the researcher a global view with low cost and relatively low human effort (Salgado-Hernanz et al., 2021). Optical satellite data has been used to identify marine debris in coastal environments with 85% of accuracy in the ocean (Biermann et al., 2020) and 88% of accuracy in beaches (Acuña-Ruz et al., 2018). Nevertheless, it cannot identify microplastics due to its resolution and is very sensitive to the sunglint phenomenon, cloud cover, atmospheric aerosol, sensor saturation, and zenith angle (Salgado-Hernanz et al., 2021). Manned aircrafts are an expensive platform that solves those limitations, targeting small plastic items. On the other hand, UAV drones are now widely available and cheaper than manned aircraft flights, even though they have low flight autonomy. This platform can fly at lower altitudes, providing high-resolution images, which are a perfect fit for sampling marine debris on sandy beaches. However, to identify microplastics within a few millimeters, the UAV drone needs to fly at low altitudes (< 10 m). The lower the UAV drone's flight, the smaller its field of view, diminishing its ability to sample large-scale areas. An alternative is to indirectly sample small fragments of marine debris, since there is a correlation between the natural beach stranded materials and microplastics.

To develop a novel technique to sample marine debris, we aimed to estimate plastic pellet densities on beaches using UAV drone images. Firstly, we tested the difference in pellet densities between beach regions with and without natural waste. In addition, we established the relationship between plastic pellets and natural wrack densities to build a statistical model under the hypothesis that floating marine debris (natural or anthropogenic) tends to coalesce,

drift, and strand together. Then, we indirectly estimated pellet densities using UAV drones images to identify natural wrack.

5.2. METHODS

5.2.1. Natural debris vs. no natural debris

This study was divided into three steps, with different samples and analyses for each one. The first step addressed differences in plastic pellet densities in different beach areas with varied coverage of natural wrack in a standardized beach region (high tide line). This step was performed on three beaches in the Baixada Santista metropolitan area, close to the Port of Santos, which is the region's main source of plastic pellets (Izar et al., 2019; Izar et al., 2022). The beaches of Góes, Praia Preta, and Itaguaré were sampled in June 2021 (Fig. 5.1). All beaches had difficult access points and no public cleaning services or sand removal. Góes and Praia Preta beaches are smaller than 500 m in length, and Itaguaré is about 2 km.

At each beach, sediment samples were collected from a square superficial area (50 cm side x 5 cm deep), totaling 0.0125 m^3 of sand. Sample squares were divided into two treatments: (i) Debris - which had at least 5% of natural debris inside the square sample area, and (ii) No Debris - which had no debris inside the square sample area (less than 5% of natural debris in the square sample area). All squares were sampled every 10 m inside a rectangular sample area of 50 m x 2 m in the high tide line visible on the day, covering the whole sandy beach length. In Itaguaré Beach, 3 rectangular sample areas (right, center, and left) were made due to its large extension.

Natural wrack coverage was determined by photo analysis. Each square area was photographed parallel to the ground from a height of 1.50 m and analyzed by pixels in the Image J software (Schneider et al., 2012). To determine the percentage of natural debris coverage, sand area (white pixels) was separated (subtracted) from the total pixels of the square, using color and contrast threshold and mixing the balance of brightness, saturation, and hue. To determinate the pellet density on each square sampled, beach sediment was dug and placed in a bucket with local seawater to separate beach-stranded plastic pellets from the sediment by density difference, using the flotation method (Turra et al., 2014). Pellets were sifted out of the bucket using a 0.5 mm mesh net and counted the total of each sample square at the sampling site.

Fig. 5.1. Map of the Baixada Santista Metropolitan coast, showing the Port of Santos in Santos Bay and the three beaches selected for pellet sampling.

In Góes Beach, we sampled 25 replicates for each treatment (n = 50); in Praia Preta Beach, we sampled 15 replicates for each treatment (n = 30); and in Itaguaré Beach, we sampled 15 replicates for each treatment (5 per beach area, n = 30). Differences in replicates between beaches are due to their size. For each sampled beach, a Generalized Linear Model (GLzM) with a Poisson distribution (link function Log) was built for the total of plastic pellets (dependent variable) with the factor Treatment (Factor 1, fixed, 2 levels), co-varying by the percentage of natural debris coverage of each square. For all analyses, we adopted a significance threshold of 95 % ($p \leq 0.05$). The rate ratio (RR) was calculated by comparing the statistically significant treatments among their levels. All statistical analyses performed in this study were built using the open statistical software Jamovi Project (2022).

5.2.2. Plastic pellets vs. Natural debris - Is there a relationship?

In October 2022, another sample was made in Itaguaré Beach, collecting beach-stranded plastic pellets using the same square method described in the previous section. To establish the relationship between natural debris and plastic pellets, squares were sampled

along the beach in the high tide line, in which three sample squares were made every 100 m. Natural wrack coverage was chosen at random, and it was analyzed using the same previous photo method. Starting on the Southwestern side of the beach, a total of 57 squares were sampled in Itaguaré beach, covering 2 km of the beach length. In Góes Beach (300 m length), the squares of the previous section were used for this estimative, totaling 50 squares sampled on this beach. Praia Preta Beach was not considered in this section due to its small size (less than 100 m).

A Generalized Linear Model (GLzM) with a Negative Binomial distribution with Log link function (smaller Akaike Information Criterion - AIC) was built for the total amount of plastic pellets (dependent variable) co-varying by the percentage of natural debris coverage of each square for both beaches sampled. For each sample point, the model calculated predicted pellet densities using the model equation for negative binomial regression: **log (predicted pellets) = intercept + percentage of natural debris coverage**. The model was considered assertive when the residuals (in z-score) were lower than ± 0.5 for each sample point. Residual values between ± 0.5 and ± 0.7 were considered marginal.

The geographic coordinate of each sampling point was recorded using GPS (Garmin - Etrex10) with an accuracy of 15 meters. Heat maps were created in the open software QGIS (3.36 version), using the Kernel density estimation algorithm for pellet density and natural debris coverage. The radius was set at 100 m for Itaguaré Beach and 20 m for Góes Beach, with the variables mentioned (pellet density and natural debris coverage) as the weight factor of each sample respective point and pixel size 0.1 quartic. The color spectrum was divided into 5 colors, using the spectral color gradient, in which the color values were defined as: blue = 0 to 15, green = 15 to 35, yellow = 35 to 50, orange = 50 to 70 and red = over 70 for plastic pellets density (pellets square^{-1}), and blue = 0 to 15, green = 15 to 30, yellow = 30 to 45, orange = 45 to 60 and red = over 60 for natural debris coverage (%), values set based on the maximum value found for each variable. The coordinate reference systems used in all cartographic projections of this study was the European Petroleum Survey Group (EPSG) - Spatial Reference Identifier number 4674 - SIRGAS 2000.

5.2.3. Sampling plastic pellets using drone images

In this section, we only consider the largest beach sampled, Itaguaré beach to guarantee a varied dynamic of plastic pellets input along the entire beach. The aerial

photography of Itaguaré Beach was performed using a DJI MAVIC PRO 2 quadcopter with a factory model RGB camera attached (1 inch CMOS sensor - 20 megapixel). The UAV-drone flight was operated automatically using the Pix4Dcapture mobile app, installed in an Iphone X. In the app, the flight plan was set at a fixed height of 60 m above the ground level, covering 12 polygons (dimensions on Table 5.1) distributed along the beach at high speed (flight speed cannot be specifically set), and with flight path in transects parallel to the sea line. For the images acquisition, the camera gimbal was angled in 90^{o} (bird's eye view), and the photographs were shot with 80% of front overlap and 70% of side overlap, in a ground resolution of 1.41 cm pixel^{-1} of Ground Sampling Distance (GSD). All flights occurred on the same day (27/10/2022) and within 6 h of solar noon (10 h to 16 h) to minimize daily climate changes and shadows, conditioned by battery limitations and flight autonomy (Table 5.1).

Table 5.1. Summary of UAV-drone flight for each polygon performed in Itaguaré Beach, using a DJI Mavic Pro 2 quadcopter. The information provided for each polygon was: dimensions of the polygon (length and height), the total path flown by the UAV-drone sampling the polygon, the takeoff hour, the flight time, and the total number of images photographed during the flight.

Polygon	Dimensions (m x m)	Path (m)	Takeoff time (hour)	Flight time	Images (n)
#1	218 x 173	851	9:57	5'28"	82
#2	233 x 202	1339	10:03	7'41"	109
#3	222 x 179	1006	10:24	6'41"	92
#4	230 x 179	1020	10:32	6'25"	99
#5	225 x 179	1155	10:40	6'25"	112
#6	225 x 179	1155	10:47	6'53"	112
#7	225 x 179	1155	11:02	6'13"	112
#8	225 x 179	1155	11:09	6'40"	112
#9	225 x 179	1155	15:15	6'03"	112
#10	225 x 179	1155	15:22	6'36"	112
#11	214 x 215	1184	15:35	6'36"	102
#12	181 x 249	1183	15:44	6'50"	111

The 1267 aerial images obtained (each 5472x3648 pixels in size and ~ 10MB) on the 12 flights carried out were processed using open software OpenDroneMap (WebODM) to generate the high-resolution RGB orthomosaic. All orthomosaics were interpolated and

georeferenced using QGIS software (Fig. 5.2). The RGB total orthomosaic of Itaguaré beach was analyzed in the same software by manually adding target points to the black and dark gray tones pixels identified in the high tide line. The high resolution of the UAV-drone image allowed a careful manual identification of the natural debris present on the beach, avoiding possible mistake with different sand colors (wet sand is usually darker). A heat map was created for this multipoint shapefile layer created, using the Kernel density estimation algorithm, as in the previous section. A radius of 100 m was set (following the same radius used in the previous section) for each target point. The color spectrum was divided into 5 colors with equidistant divisions.

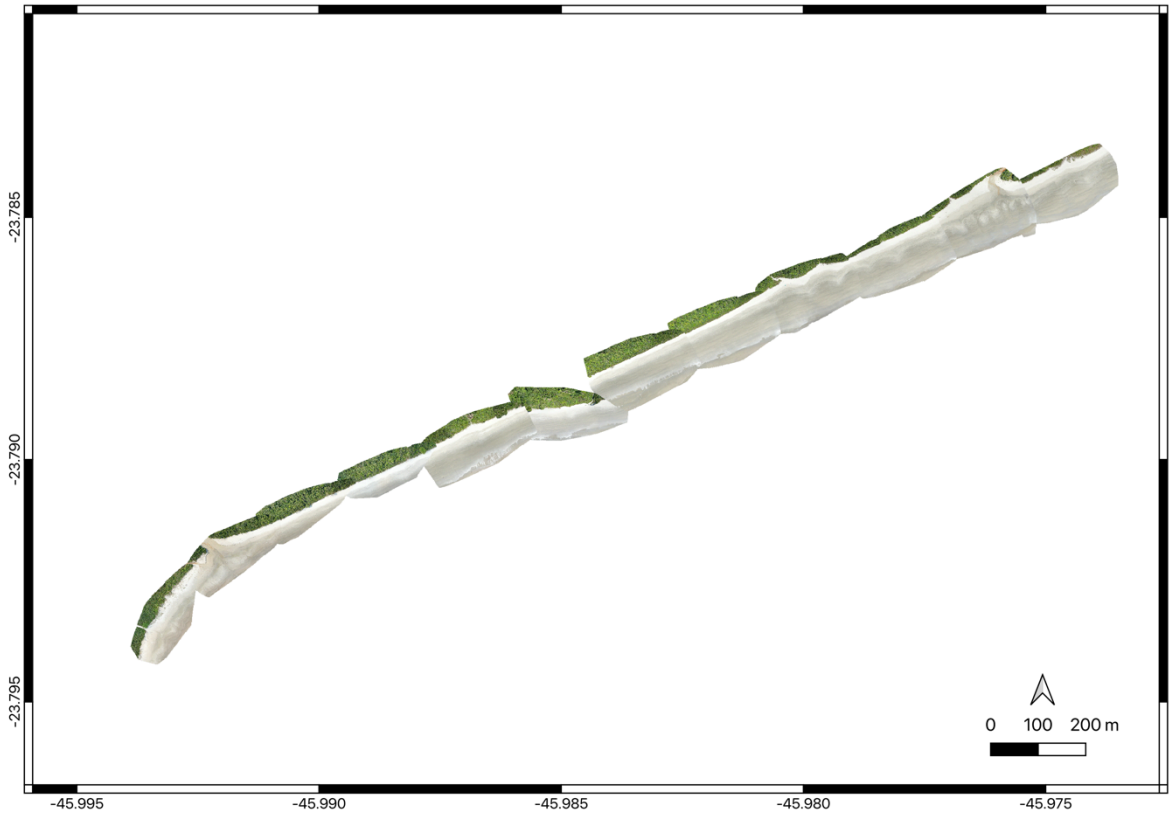

Fig. 5.2. The RGB orthomosaic of the area surveyed with the UAV-drone in Itaguaré Beach. This figure was created merging 1267 aerial pictures in 12 orthomosaics.

5.3. RESULTS

5.3.1. Natural debris vs. no natural debris

In Góes Beach, the average pellets density per square was 0.36 ± 0.63 and 13.6 ± 19.3 in treatment without and with natural debris, respectively (Fig. 5.3). In Praia Preta Beach, the

average pellets density per square without natural debris was 0.94 ± 1.01 and with natural debris, 11.0 ± 5.81 (Fig. 5.3). Lastly, it was found 2.34 ± 2.51 pellets per square in Itaguaré Beach without natural debris and 21.0 ± 9.95 with natural debris (Fig. 5.3). The three beaches sampling followed the same pattern for natural debris treatment and percentage of natural debris coverage, in which natural debris treatments had more pellets than no natural debris, increasing the pellets density as the percentage of natural wrack coverage increases (Fig. 5.3).

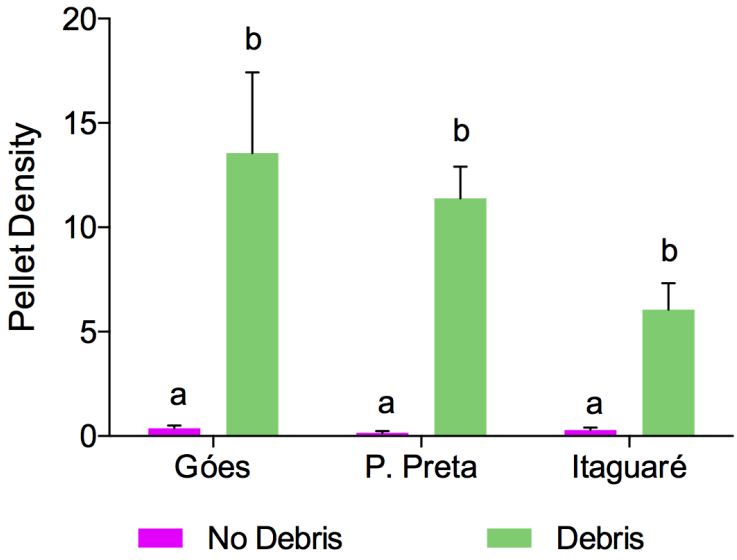

Fig. 5.3. Average plastic pellet densities per square in treatments: 1) No debris (pink bars) = without natural debris (less than 5% of coverage) and 2) Debris (green bars) = with natural debris, in the three beaches sampled: Góes, Praia Preta and Itaguaré. Error bars represent 95 % confidence intervals. Different letters indicate significant differences between treatments on the same beach.

In Góes Beach, beach-stranded plastic pellets density was 10 times higher in treatments with natural debris than in treatments with no natural debris (AIC = 313.5, R^2 = 0.76, X^2 = 78.1, $p < 0.001$, RR = 10.12, $CI_{95\%}$: 5.38 - 21.65). For the co-variable factor, at each increase of 1 unit in the percentage of natural debris coverage, plastic pellet density increased by 6% (RR = 1.06, $CI_{95\%}$: 1.05 – 1.06, $p < 0.001$). In Praia Preta Beach, pellet density was 52 times higher in the natural debris treatment (AIC = 112.3, R^2 = 0.85, X^2 = 91.4, $p < 0.001$, RR = 51.78, $CI_{95\%}$: 15.86 - 318.69) with an increase rate of 5% in pellets density, to each unit of percentage of natural debris coverage (RR = 1.05, $CI_{95\%}$: 1.02 - 1.07, $p < 0.001$). Finally, Itaguaré Beach had 12 times more pellets in natural debris treatment (AIC = 117.9, R^2 = 0.66, X^2 = 29.2, $p < 0.001$, RR = 11.94, $CI_{95\%}$: 4.41 - 41.80), and a 3% of increase

rate in pellet density, to each unit of percentage of natural debris coverage (RR = 1.03, $CI_{95\%}$: 1.01 - 1.05, p = 0.002).

5.3.2. Plastic pellets vs. natural debris - Is there a relationship?

In Itaguaré Beach, the percentage of natural debris coverage was statistically significant (AIC = 437.9, R^2 = 0.13, X^2 = 9.87, p < 0.001) with an increase of 4% in pellet density for each unit of the percentage of natural debris coverage (RR = 1.04, $CI_{95\%}$: 1.01 - 1.07 - Fig. 5.4A and B). The model correctly predicted pellet densities in 19 (33%) of 57 sampled points and marginally in 6 (10.5%) sampled points. In Góes Beach, the percentage of natural debris coverage was also statistically significant (AIC = 219.6, R^2 = 0.67, X^2 = 105, p < 0.001), with an increase of 12% in pellet density to each unit of percentage of natural debris coverage (RR = 1.12, $CI_{95\%}$: 1.09 - 1.16 - Fig. 5.4C and D). The model correctly predicted pellet densities in 14 (28%) of the 50 sampled points and marginally in 5 (10%) sampled points. Both models had a good fit to low and medium values of debris coverage and a large error margin for extreme values, higher than 40% of natural debris coverage (Table 5.S1 - Supplementary data).

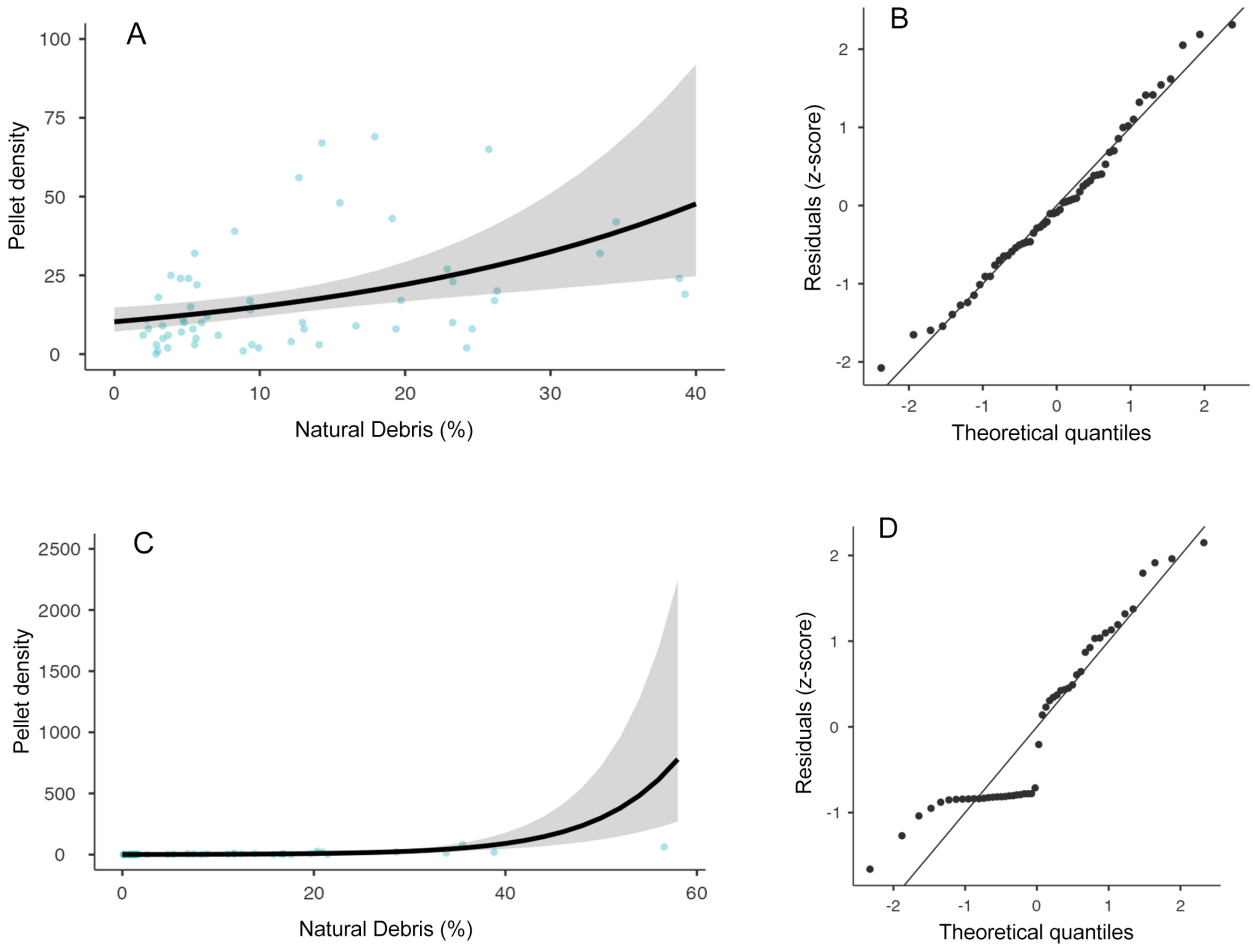

Fig. 5.4. Regression model (Negative Binomial - Log link function) of natural debris coverage (%) and plastic pellet density per square on A) Itaguaré Beach and C) Góes Beach. Dots represent each sample data, and gray areas represent 95 % confidence intervals. QQ normal plots of the residuals from the regression model, in z-score, for B) Itaguaré Beach and D) Góes Beach.

Itaguaré Beach presented a greater pellet density in the Western portion of the beach and at the Eastern end (Fig. 5.5A), while the greatest coverage of natural debris occurred in the eastern portion, close to the mouth of the Itaguaré River. Also, a considerable amount was found on the western side of the beach (Fig. 5.5B), in line with the pellets hot spot on this beach. Góes Beach also had a greater pellet density on its western side (Fig. 5.5C). However, the greatest accumulation of natural debris coincided in the same location, with significant accumulations also in the eastern part of the beach (Fig. 5.5D).

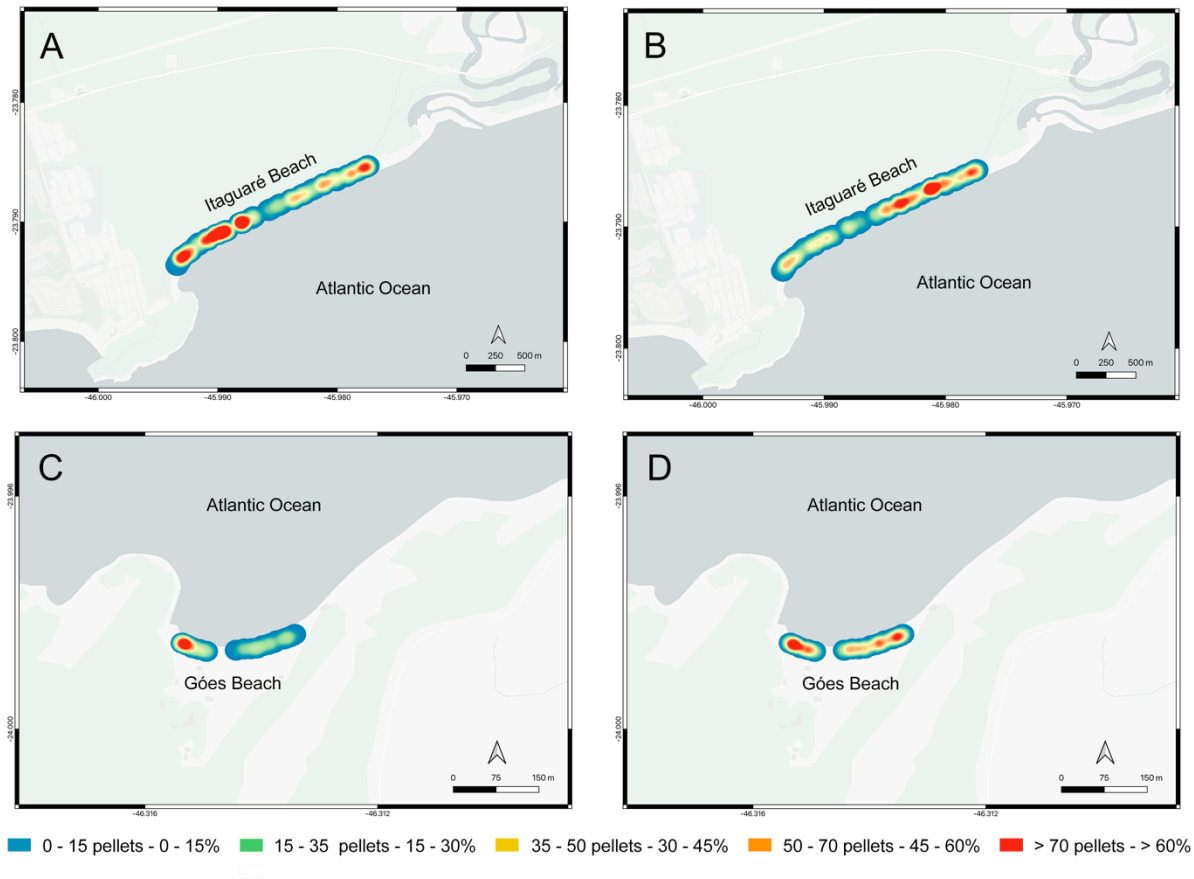

Fig. 5.5. Heat maps representing: A and C - pellets densities per square sampled (50 x 50 x 5 cm - 0.0125 m^3) and B and D - the natural debris coverage (%) on Itaguaré (A and B) and Góes (C and D) beaches. Warm colors represent high concentrations, as presented by the legend.

5.3.3. Sampling plastic pellets using drone images

At Itaguaré Beach, 78 min flight covered approximately 500.000 m^2 of beach area. A total of 2041 target points were added to the Itaguaré Beach orthophoto (Fig. 5.6A). The distribution of natural debris obtained by UAV drone aerial image analysis followed the pattern found in field sampling but with a greater intensity of accumulation on the west side of the beach and another accumulation point near the Eastern limit (Fig. 5.6B). Hotspots of natural debris accumulation were identified close to the mouths of rivers and small streams.

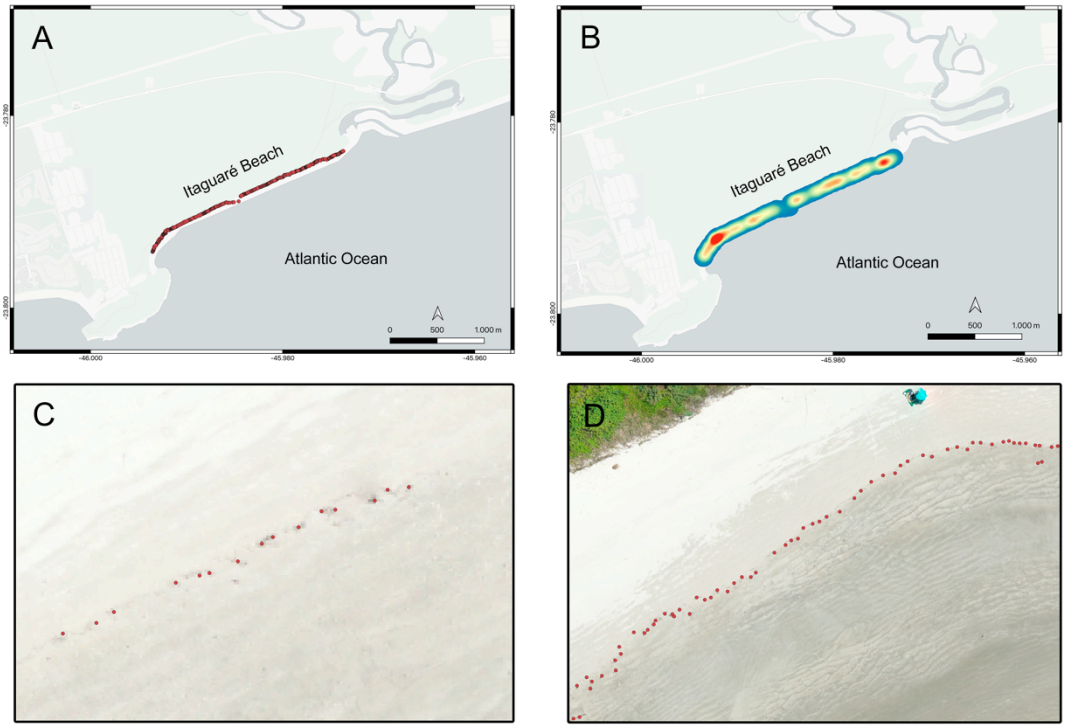

Fig. 5.6. Results of the manual identification of natural debris in the high tide line of Itaguaré Beach: A - target points of each black pixel identified as natural debris, B - heat map of these target points, C and D - examples of natural debris mapped on the orthophoto in different beach areas and zoom (1:40 and 1:100, respectively).

The heat pattern found by this aerial sampling methodology was very similar to the pattern found for plastic pellets in the field sampling methodology in the previous section (Fig. 5.5A). The analyses were focused on the high tide line, manually analyzing all dark pixels as possible stranded natural debris (Fig. 5.6C and 5.6D). Due to the resolution of the UAV drone aerial image obtained, it was not possible to accurately identify the type of marine debris, only accumulation zones. Based on the regression obtained in the previous section (highlighting the maximum accuracy limit of 40% of natural debris coverage), predictive values can be assigned to green and yellow color areas, in which expected about 72 pellets per square for green areas, and about 112 pellets per square for yellow areas. Orange and red areas can expect pellet densities above 160 pellets per square.

5.4. DISCUSSION

Plastic pellets tend to beach and accumulate along with natural debris on sandy beaches. Here, we found a very clear relationship between them in sandy beach areas with and without the presence of natural debris on the high tide line. Despite the low relationship found

in the regression models, their predictions were 40% correct. Other factors can influence the accuracy of the models in the input of pellets. One of them is the presence of river mouths since there are sources of pellets along their course (Corcoran et al., 2020). River inputs can also influence the quantity of natural debris carried to the beach, which appears to be the case at Itaguaré Beach. The proximity of populated areas, despite being a factor to be considered in the distribution of pellets (Van Cauwenberghe et al., 2015; Corcoran et al., 2020), does not seem to be a factor that affects the distribution of the pellets on the beaches sampled. Even though Góes and Itaguaré beaches are distant from each other (around 5 km) and Góes Beach is located inside Santos Bay (a region with around 1 million inhabitants), the high pellets presence in Itaguaré Beach demonstrates the influence of the Port of Santos as a source of plastic pellets to the region and goes beyond the limits of the Santos Bay (Izar et al., 2019). Wave energy and sediment composition (especially the presence of fines) seem to be the main factors to be considered in modeling pellet accumulation on sandy beaches (Browne et al., 2010; Corcoran et al., 2020).

Here, we provide the first assessment of plastic pellets using aerial images of UAV drones. The UAV drone methodology tested was effective in identifying plastic pellet input hotspots, but it was not accurate in estimating density. Identifying possible hot spots for the accumulation of plastic pellets in sandy beaches is ecologically important since high amounts of this microdebris in the sediment can modify the permeability and temperature of the sediment (Carson et al., 2011). Also, the presence of plastic debris has negative ecological effects on macrobenthic populations (Laurino et al., 2023; Izar et al., 2024). Therefore, mapping the input hot spots of the entire beach in a few hours (or minutes - depending on the length of the beach) can be an important tool for identifying areas of greatest stress and potential ecological risk for benthic macrofauna. Even though the standing stocks of plastic pellets tend to accumulate on the backshore (Van Cauwenberghe et al., 2015; Moreira et al., 2017), identifying pellets indirectly through natural debris in the high tide line proved to be a reliable, easy, and fast method that makes it possible to pinpoint the input hot spots on the beach. This can guide the location of standing stocks on the backshore. Using this methodology, a single person can sample around 500,000 m^2 in a few hours, while in conventional field methods, a team and an entire day of sampling are required to sample around 15-20 m^2, as demonstrated in this study. It is 35 thousand times more beach areas sampled than the conventional field methodology in just over 1 h flight. This methodology can be used as a first screening for an initial mapping of possible beach sampling areas. It can

also be used in monitoring programs, combined with field sampling and hydrodynamic modeling of surface currents, to understand better the dynamics of plastic pellets' entry into sandy beaches. This methodology can also be extended to microplastics since they mostly have the same pellet behavior.

Unlike macrodebris, in which it is possible to create precise machine-learning methodologies to identify photographed marine debris items (Acuña-Ruz et al., 2018; Deidun et al., 2018; Martin et al., 2018; Gonçalves et al., 2020; Martin et al., 2021; Taddia et al., 2021), plastic pellets have dimensions of a few milimeters. This makes automatic identification more difficult and imprecise, even with GSD close to 1.50 cm pixel^{-1}. An alternative is flights at lower altitudes to improve the orthomosaic resolution (Merlino et al., 2020; Martin et al., 2021). However, this reduces the sampling range and increases the sampling time to cover the total area, which implies drone battery limitations, double or triple the number of aerial photos shot, and a high computer processing requirement for large files. In the high tide line, manual identification, despite being laborious, is more assertive because the dark pixels in this area can also represent wet sand from the rising tide or shadows in the proximity of footprints. This can cause possible confusion and errors in automated algorithms (Fallati et al., 2019). Furthermore, UAV drone image analysis produces a 2D representation of the sampled area, which can also be a confusing factor when comparing the two methods, as we are comparing a 3D method (field sample) with a 2D representation. Infrared identification has not been tested, as we do not have the appropriate lenses and sensors in our UAV drone. But it could be a viable alternative to increase the speed and effectiveness of the methodology, elaborating indexes similar to the vegetation index NDVI widely used in agronomy and ecology (Fawcett et al., 2021; Meivel and Maheswari, 2021; Valdez-Delgado et al., 2023; Cardoso et al., 2024).

5.5. CONCLUSION

It is possible to use UAV drone images as a sampling methodology to estimate plastic pellet densities and identify their accumulation hotspots on sandy beaches since pellets tend to beach and accumulate along with marine wrack in coastal areas, especially natural debris. There is a relationship between the plastic pellets' density and the amount of marine wrack on sandy beaches. However, other factors, such as the local hydrodynamics of surface ocean currents and the volume of river input must be considered in the model for better accuracy. The UAV-drone methodology made it possible to sample plastic pellets in more beach areas

and using fewer human resources and in less time than the conventional field methodology. We strongly recommend using this tool to monitor marine debris in coastal areas.

5.6. SUPPLEMENTARY DATA

Table 5.S1. Summary of the data predicted by the negative binomial models built for each sample point of Itaguaré and Góes beaches. ND = Natural Debris in percentage, PP = the total of plastic pellets sampled, and Pred = the total of plastic pellets predicted by the model.

	Itaguaré				Góes		
	ND(%)	**PP**	**Pred**		**ND(%)**	**PP**	**Pred**
#1	2.87	0	11.4	**#1**	0.93	0	0.8
#2	5.52	3	12.6	**#2**	4.73	1	1.3
#3	8.87	1	14.4	**#3**	0.71	0	0.8
#4	25.76	65	27.5	**#4**	0.8	2	0.8
#5	2.25	11	11.1	**#5**	1.41	0	0.9
#6	4.61	7	12.2	**#6**	1.21	0	0.8
#7	3.32	9	11.6	**#7**	1.24	0	0.8
#8	15.51	48	18.6	**#8**	1.17	0	0.8
#9	12.16	4	16.3	**#9**	2.56	1	1.0
#10	3.35	5	13.3	**#10**	0.36	0	0.7
#11	5.41	8	14.0	**#11**	1.07	0	0.8
#12	9.33	14	15.6	**#12**	0.85	0	0.8
#13	19.12	43	20.2	**#13**	1.61	1	0.9
#14	6.38	12	14.4	**#14**	0.54	0	0.8
#15	3.03	18	13.2	**#15**	0.32	0	0.7
#16	17.91	69	19.6	**#16**	1.66	2	0.9
#17	5.11	24	13.9	**#17**	1.29	0	0.8
#18	2.34	8	12.9	**#18**	1.42	1	0.9
#19	12.7	56	17.0	**#19**	0.1	0	0.7
#20	5.53	32	14.1	**#20**	0.13	0	0.7
#21	4.55	24	13.7	**#21**	17.62	1	6.2
#22	8.27	39	15.1	**#22**	16.52	3	5.4
#23	3.89	25	13.5	**#23**	16.81	7	5.6
#24	14.28	67	17.8	**#24**	11.03	3	2.8
#25	1.98	6	12.8	**#25**	6.81	7	1.7
#26	4.71	11	13.8	**#26**	19.68	8	8.0
#27	5.24	15	14.0	**#27**	11.72	4	3.0

Table 5.S1 cont. Summary of the data predicted by the negative binomial models built for each sample point of Itaguaré and Góes beaches. ND = Natural Debris in percentage, PP = the total of plastic pellets sampled, and Pred = the total of plastic pellets predicted by the model.

	Itaguaré				Góes		
	ND(%)	**PP**	**Pred**		**ND(%)**	**PP**	**Pred**
#28	14.08	3	17.7	**#28**	16.86	5	5.7
#29	3.7	6	13.4	**#29**	15.76	1	5.0
#30	2.89	3	13.1	**#30**	20.29	13	8.6
#31	12.94	10	17.2	**#31**	7.45	6	1.8
#32	13.05	8	17.2	**#32**	28.62	22	23.2
#33	24.24	2	23.7	**#33**	13.84	7	3.9
#34	19.71	17	20.6	**#34**	33.82	13	43.3
#35	24.61	8	23.5	**#35**	21.43	3	9.8
#36	26.32	20	24.6	**#36**	20.9	20	9.2
#37	23.28	23	22.6	**#37**	38.82	20	78.8
#38	2.98	1	13.1	**#38**	20.36	27	8.6
#39	26.15	17	24.4	**#39**	56.59	63	658.6
#40	4.81	10	13.8	**#40**	35.53	82	53.1
#41	3.67	2	13.4	**#41**	0.75	0	0.8
#42	19.37	8	20.4	**#42**	0.78	0	0.8
#43	38.86	24	34.3	**#43**	1.16	0	0.8
#44	23.27	10	22.6	**#44**	0.13	0	0.7
#45	39.25	19	34.7	**#45**	1.01	1	0.8
#46	33.41	32	29.7	**#46**	5.34	3	1.4
#47	9.46	3	15.6	**#47**	8.28	0	2.0
#48	9.92	2	15.8	**#48**	8.83	5	2.1
#49	16.62	9	18.9	**#49**	12.43	6	3.3
#50	6.00	10	14.2	**#50**	11.64	10	3.0
#51	5.69	22	14.1				
#52	22.9	27	22.4				
#53	7.14	6	14.7				
#54	5.61	5	14.1				
#55	34.52	42	30.6				
#56	9.51	15	15.7				
#57	9.32	17	15.6				

FINAL CONSIDERATIONS: WHAT DID WE LEARN HERE?

In this present thesis, we performed experiments to better understand the potential toxicity of benthic macrofauna in sandy beaches. Using real-world environmental conditions in ecotoxicological experiments with plastic pellets has provided important information about the toxic effects of these pollutants on benthic macrofauna. Mortality was observed at the lowest plastic pellet densities tested, and an increase in intraspecific interactions of cannibalism and aggressiveness of the *E. armata*. However, organisms with a fast life cycle tend to acquire resistance to these stressors. Additionally, the behavior of plastic pellets as carriers of hydrophobic contaminants and the dynamics of the dispersion and accumulation of these particles were also observed.

Toxicity was observed for the first time in *E. armata*, an isopod of the abundant and highly resistant benthic macrofauna, *in situ* and under laboratory conditions. The simple exposure of pellets to organisms leads to behavioral changes, increasing the cannibalistic behavior of the species. This finding raises the discussion about intraspecific interactions in ecotoxicological assays. It is extremely necessary to consider these interactions since each individual acts as a factor in the ecotoxicological assay, which can confuse or mask the results if not considered. Here, ecotoxicology's relentless search for acute, chronic, physiological, or molecular results is important. Specific and deep results are sought in the body's system to explain or find toxic effects, while changes in behavior can mask all these effects mentioned and must also be considered a toxicological effect. In our case, *E. armata* individuals were more resistant to plastic stress cannibalizing the less resistant and lethargic individuals, which can be considered mortality and classified as acute mortality if it is not well analyzed at a behavioral level. This is only a finding because of the disappearance of the bodies, which surprised us. Differences between populations must also be considered since organisms have the ability to adapt to stress, especially species with a fast life cycle.

Biomarker results also deserve special attention, as they showed no difference between treatments and, consequently, no apparent sublethal toxic effects. This probably occurred because biomarker analyses were performed on organisms that survived at the end of the tests; therefore, those most resistant to the stress tested. This may represent bias in the analysis. Thus, the organism is cannibalized before it can be analyzed as a biomarker. Furthermore, as they are biomarkers of end-of-chain oxidative stress (mainly LPO and DNA damage), we can hypothesize that behavioral changes and ecological effects occur before the

physiological effects in these organisms. Alternatively, one is a consequence of the other, which we do not have the chance or time to analyze. Here, we emphasize the importance of analyzing toxicity at all organism levels, including the intra and interspecific ecological levels.

Plastic pellets are commonly lost to the environment as raw material from the plastic industry. As long the culture of high plastic consumption continues, especially single-use plastics, the input of this type of microplastic will be constant and will tend to accumulate more and more, mainly in the coastal environment. This has some environmental implications. In addition, plastic pellets can carry hydrophobic contaminants, especially PAHs. The high adsorption capacity of these compounds by pellets makes transporting these contaminants to remote and free-contaminated regions possible. Here, we identified naphthalene, phenanthrene, and chrysene as the main priority PAHs carried by pellets, which deserve greater attention due to their potential impacts and toxicities in the marine environment.

The simple presence of plastic pellets can cause toxic effects on marine and coastal organisms, especially smaller species (invertebrates) that live in habitats in direct proximity to these particles. This has already been shown in my master's degree (Izar *et al.*, 2019) and in many other studies in the literature that corroborate this premise. Therefore, accurately and quickly identifying plastic pellet hotspots on sandy beaches becomes increasingly necessary from an ecological point of view. We helped demonstrate that it is possible and scalable to use UAV drones' aerial images to monitor plastic pellets on beaches, which is a more effective method on a thousand scales than traditional field methods. This finding is ecologically important and fits as a conclusion and is the main product of the PhD thesis, as it is a passive method that can identify and monitor zones of accumulation of plastic pellets carrying hydrophobic contaminants (i.e., PAHs) and with potential ecotoxicological risk for benthic macrofauna on sandy beaches. However, this method still has limitations and is only an essential first step in guiding future consistent monitoring and conservation programs along the Brazilian coast. Therefore, as a final message, we suggest that extensive microplastic monitoring programs be created in the country and that researchers be alert to variations in the densities of these particles within the same beach, since populations from different beach areas may be unequally affected by the microplastic stress. Such task can help to better understand the impact of microplastics on the microscale and their ecotoxicological dynamics and can be useful as a basis for guiding conservation programs.

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APPENDIX 1

PUBLISHED POINTS OF REFERENCE - Izar, G.M. et al., 2022. Difficulties in comparison among different microplastic studies: the inconsistency of results and lack of guide values. *Environmental Toxicology and Chemistry*, *41*(4), 820-821.

Difficulties in Comparison Among Different Microplastic Studies: The Inconsistency of Results and Lack of Guide Values

In literature and international media, there is a significant approach about microplastics (MP) in recent years. MP are the results of the constant disposal of plastic waste in aquatic ecosystems, which are commonly fragmented by weathering into smaller particles whose sizes vary between 1 μm and 5 mm. MP can also be originated by the plastic industry such as fibers from industrial textile washing and plastic pellets which are raw material of manufactured plastic objects, and microspheres used in the cosmetic sector.

The MP pollution assessment due to its abundance in the environmental compartments and biota is essential in the diagnosis to better understand the ecological and human health impacts and risks. In addition this could be a good way to make pressure on stakeholders, requiring actions to combat the irregular disposal of solid waste into the environment and fundamental to monitoring programs. However, how can we assess MP pollution if there are no guide values to conduct studies on the abundance of MP?

Intuitively, we accept that the presence of MP in the environment should be undeniable. So, it is necessary to set limits on the levels of MP pollution. As it is a relatively new area, researchers have established several analytical methodologies in laboratory. Theses techiniques range from the simplest and rudimentary to the most sophisticated methodologies, which are not always avaliable for reproducibility. This infinity of analyzes allows for different forms of data representation regarding inconsistency of the results, making it

difficult to compare them between different studies. Moreover, different sampling sites, geographical regions, ecosystems, and biomes enable the variety of field sampling methodologies and the lack of consensus in defining MP (about size) make it even more difficult to compare studies.

When reporting the density of MP in environmental samples, some authors consider high MP pollution. However, high in relation to which parameter? Compared to what? Different studies have different units and methodologies (i.e. - it would not be correct to compare the total amount of MP with the number of plastic particles per gram of sediment). There are at least eight different units used to report MP pollution in water and sediment samples (Lu *et al.,* 2021), which leads to the presentation of MP pollution results in totally different ways that can be misinterpreted.

The most suitable for these comparisons would be a standardization with an environmental quality index according to the quantity of MP found in the place. Alkalay *et al.* (2007) proposes the use of plastic debris as beach cleanliness indicators, the Clean-Coast Index – CCI, since plastic represents about 90% of the total marine debris. However, this index only considers debris larger than 2 cm, which does not include MP. Fernandino *et al.* (2015), adapted the CCI for plastic pellets, Pellets Pollution Index – PPI, but without taking into account the new proportions for plastics debris when considering debris smaller than 2 cm. To address MP, a new index should be standardized considering the sampling unit (area, volume, or mass) that corresponds with the sampling matrix (water, sediment or biota) and polymeric density ranges of the main plastics found in the samples. In addition, an index is required for each type of MP: fragments and fibers/lines, as both are morphologically different and consequently have different behaviors in the environment. Moreover, fibers are not just made by polymers (many fibers can be of natural origin, such as cotton and vegetable fibers), and only few laboratories are capable to identify them.

The limits of MP pollution should consider studies in long-term ecotoxicological tests with toxicity endpoints (Van Cauwenberghe *et al.,* 2015). These studies have to be conducted using model species of aquatic and terrestrial environments, and to address parameters of physical damage and toxicity of additives and contaminants sorbed in plastic particles. In this way, by determining the biological and ecological impacts of different MP' densities in each environmental compartment, it is possible to establish the "tolerant" density values of MP in the environment. Tolerant MP' densities combined with a new index specific for MP can clarify gaps regarding MP pollution levels.

In order to facilitate sampling and comparison, we suggest to establish standardized results in particles per cubic meter (particles m^{-3}) for beach sediments, since MP can be found in different layers of deep in sandy beaches (Turra *et al*, 2014), while MP in muddy sediments can be estimated in volume (particles L^{-1}) which facilitates the conversion to particles m^{-3} and allows comparison with the densities of MP present in the water column. In water surface, as some MPs have low density and floats on water, it is reasonable that this standardization is done in particles per square meter (particles km^{-2}). And finally, particles per grams wet weight for biomass, following the most recent studies. We suggest particles as an unit instead of MP mass or surface, to enable anyone to produce data on this topic, aiming citizen science programs that usually have few financial and technological conditions.

Given the heterogeneity of MP analysis methods, we encourage future research to consider the development of protocols to establish guide values so that it can be established a standard in the representation of the results and a MP pollution parameter. In this way, with standardized MP pollution results, it will be possible to use these results as a tool in environmental assessment and monitoring programs. Thus, with the attempt to standardize methods, interlaboratory analyzes will be necessary to validate the reproducibility of the proposed methods with sentinel organisms and environmental samples from different ecosystems (using similar units) in order to obtain an overview of the global situation of MP pollution.

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