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MICROPLASTICS AND THEIR ASSOCIATED CONTAMINANTS IN THE MARINE ENVIRONMENT: FROM DETECTION TO THE MECHANISMS OF INTERACTION WITH MARINE BIOTA

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ABSTRACT

The rapid rise in global plastic production, combined with inadequate plastic waste management, has escalated into a severe environmental crisis. Plastic now accounts for the majority of anthropogenic debris in marine environments, where its chemically engineered durability enables it to persist and integrate into ecosystems. This thesis addresses the pressing issue of plastic pollution in marine environments, highlighting key gaps in our understanding of microplastics' distribution and ecological impacts.

Several key challenges are identified:

1. **Lack of Standardization**: The absence of standardized methods for detecting and quantifying microplastics complicates large-scale data comparisons and undermines effective policy-making.
2. **Sample Contamination**: Controlling sample contamination is essential, as it can lead to overestimating microplastic concentrations in environmental samples.
3. **Laboratory Limitations**: Many laboratory studies are conducted under controlled conditions that do not accurately reflect the complexities and variability of real-world marine environments.

This research aims to address these challenges by first validating and optimizing analytical methods capable of detecting microplastics as small as 25 μm, along with phthalic acid esters in marine invertebrates, expressing concentrations in meaningful units for cross-study comparability. To mitigate sample contamination, the research was conducted in a clean room compliant with ISO 6 standards, ensuring minimal interference and the generation of reliable results.

While most plastic pollution is commonly thought to come from mismanaged waste, recent research has increasingly identified tire wear as a significant source of microplastics contributing to ocean pollution.

In this thesis, we present results from experiments exposing two estuarine model species, the fish *Menidia beryllina* and the mysid shrimp *Americamysis bahia*, to an environmentally relevant multi-tire mixture provided by the U.S. Tire Manufacturers Association. This mixture incorporates a blend of three tire types - passenger car tire, light truck tire, and truck/bus tire - and was specifically designed to reflect the proportions of tire types found in the United States, determined from tire shipment data. To better simulate real-world conditions, we UV-weathered the mixture and compared the toxicity of pristine and weathered micro- (1-20 μm) and nano- (<1 μm) tire particles in both species. By addressing methodological challenges, enhancing the accuracy of microplastic assessments, and conducting laboratory experiments under realistic conditions, this work aims to improve monitoring, inform policy formulation, and develop effective strategies for mitigating plastic pollution in marine ecosystems.

Chapter 1

General introduction

1.1. The emergence and rapid expansion of plastics in the modern world

Plastic pollution has emerged as one of the most urgent and widespread environmental challenges of our time, largely driven by the rapid rise in plastic production over the past century (Geyer et al., 2017). The origins of modern plastic manufacturing can be traced back to the early 20th century when the invention of Bakelite in 1907 marked the dawn of the synthetic plastics industry (Olatunji, 2024). This invention revolutionized manufacturing by offering a durable, heat-resistant material that could be molded into various forms, paving the way for countless innovations. However, it wasn't until the 1950s that plastic production began to increase exponentially, transforming industries and everyday life across the globe. In fact, since then, plastic production has skyrocketed nearly 230-fold, reaching 368 million metric tons in 2019, and it is expected to double within the next 20 years (Lebreton and Andrady, 2019; Walker, 2021). Particularly concerning is the fact that in just the last two decades, plastic production has more than doubled, reflecting not only the growing demand for plastic across various sectors but also society's increasing reliance on this highly versatile yet environmentally persistent material. Plastics, prized for their lightweight, strong, and durable characteristics, are ideally suited for a wide array of applications. From construction materials and agricultural products to electronics, medical devices, and transportation, plastics have become essential to modern economies (Thompson, 2006). However, one of the largest sectors driving the demand for plastic is consumer packaging. Nearly one-third of global plastic resin production is dedicated to packaging materials, especially single-use items like bags, wrappers, bottles, and containers, which are often discarded within a year of being produced (Chen et al., 2021). These disposable products now dominate waste streams and frequently end up in landfills, incineration plants, or worse - littering our natural environments (Rangel-Buitrago et al., 2022). This throwaway culture has led to

a growing dependence on plastic, exacerbating the global waste crisis. The world now generates around 350 million tonnes of plastic waste annually, a figure that continues to rise alongside plastic production (OECD, 2022). The problem, however, isn't simply the volume of waste, but how it is managed or mismanaged. A significant portion of this plastic waste is not properly disposed of, with an estimated 82 million tonnes being mishandled through insecure landfills, inadequate recycling processes, or insufficient incineration (OECD, 2022). The inadequacy of waste management systems allows large amounts of plastic to escape into the environment. About 19 million tonnes of this mismanaged plastic waste leak into ecosystems annually, polluting both land and water (OECD, 2022; Ritchie, 2023). Terrestrial ecosystems, including urban and rural landscapes, are often the first to be contaminated, with approximately 13 million tonnes of plastic accumulating on land. Meanwhile, another 6 million tonnes make their way into rivers and coastal areas. From these polluted water sources, an estimated 1.7 million tonnes of plastic waste eventually flows into the oceans each year, 1.4 million tonnes through rivers, and 0.3 million tonnes from coastal activities (OECD, 2022; Ritchie, 2023). Although this accounts for only about 0.5% of the world's total plastic waste, the impact on marine ecosystems is severe and long-lasting. To combat the escalating plastic pollution crisis, nations worldwide have initiated significant measures. The European Union (EU) implemented measures to ban single-use plastic products such as plates, cutlery, straws, balloon sticks, and cotton buds from EU markets starting July 3, 2021 (European Commission. Directorate General for Environment., 2021). The United States has not yet placed a single-use plastic ban on a federal level, but states and cities have taken up this responsibility. San Francisco (California, USA) set a precedent in 2007 as the first major city to ban single-use plastic bags, followed by California's statewide ban in retail establishments in 2014 (Wang et al., 2022). By 2019, six more states (New York,

Maine, Delaware, Vermont, Oregon, and Connecticut) enacted similar laws, followed by Washington and New Jersey in 2020, and Colorado in 2021 (Wang et al., 2022). These regulations represent a significant step toward reducing plastic waste in marine environments, but global efforts must continue to expand to address the full scope of the problem. Furthermore, while legislative efforts are underway, consumer behavior plays a crucial role in this crisis. Initiatives promoting recycling and sustainable alternatives are essential for reducing demand for single-use plastics. Awareness campaigns highlighting the environmental impact of plastic use and encouraging the adoption of reusable products can help shift consumer habits and reduce plastic dependency. Marine plastic issues are addressed in the Sustainable Development Goals (SDGs) established by the UN in September 2015. Goal 14 aims to "conserve and sustainably use the oceans, seas, and marine resources for sustainable development". Specifically, target 14.1 seeks to "prevent and significantly reduce marine pollution of all kinds by 2025", with a focus on land-based sources, including marine debris and nutrient pollution. Progress will be assessed using Indicator 14.1.1, which measures the "index of coastal eutrophication and floating plastic debris density" (Walker et al., 2021). In March 2022, during the United Nations Environment Assembly in Nairobi, Kenya, 175 countries unanimously agreed to adopt a historic global treaty to tackle plastic pollution. This legally binding agreement aims to reduce plastic pollution across the entire plastic life cycle and to establish uniform standards for plastic consumption across all participating nations, setting a clear course toward a future free from plastic pollution (Aanesen et al., 2024; Brander et al., 2024).

1.2. The mystery of missing ocean plastics

One of the significant challenges researchers face when studying plastic pollution is understanding what happens to plastics after they enter the ocean. While it's estimated that at least one million tons of plastic waste enter the oceans each year, the quantity of

plastic observed floating on the ocean's surface is far lower than anticipated, creating a puzzling and concerning discrepancy (Cózar et al., 2014). This mismatch raises critical questions about the fate of the vast amounts of plastic produced and discarded globally. Based on current estimates of global plastic production and input rates, the amount of floating plastic should be much higher, particularly given the vast scale of plastic use and the billions of tons generated annually for various applications. This significant discrepancy suggests that surface waters are not the final destination for most buoyant plastic waste, and has led researchers to explore various potential sinks for the "missing" plastics, including shore deposition, nano-fragmentation, biofouling, ingestion by marine organisms, and deposition in deep-sea sediments (Andrady, 2011; Law et al., 2010). Evidence indicates that a large portion of microplastics ultimately settles on the seafloor, and their widespread detection in marine sediments worldwide suggests that ocean depths could become a long-term reservoir for these particles (García Rellán et al., 2023). In the marine environment, plastics often undergo various processes, such as photodegradation, oxidation, and hydrolysis, which can cause them to become brittle and break down mechanically over several months (Andrady, 1990; Feldman, 2002). Additionally, biofilms can grow on floating plastic debris, and marine organisms can attach to it, increasing its weight and causing it to sink to the ocean floor (Morét-Ferguson et al., 2010; Ye and Andrady, 1991). This process not only alters the physical state of the plastics but also drives their accumulation in the benthic environment, where they contribute to sediment pollution and impact benthic organisms (Bergmann et al., 2015; Semcesen and Wells, 2021). Moreover, beaches across the globe are covered with plastic debris derived either from inland sources transported to the coast by rivers, wind, man-made drainage systems, or human activity, or directly from the oceans, where low-density floating particles are transported across great distances (Corcoran et al., 2009;

Fruergaard et al., 2023; Garcés-Ordóñez et al., 2020). This accumulation of plastic on shorelines poses threats not only to marine life but also to coastal ecosystems and human health. Finally, the ingestion of plastic debris has been well-documented in various marine species, including seabirds, marine mammals, fish, and marine invertebrates, raising significant concerns about the impacts on wildlife health and food safety (Besseling et al., 2015; Naidoo et al., 2016; Raguso et al., 2022). Overall, the combination of physical, chemical, and biological factors, along with the diverse pathways that plastics can take, underscores the complexity and challenge of accurately tracking and understanding the fate of plastic debris in marine ecosystems.

1.3. Primary sources of ocean microplastics

Littering and mismanaged waste are widely acknowledged as the leading contributors to the influx of plastics into the ocean. These behaviors not only result in visible pollution along coastlines, which can have detrimental social and economic effects (Aretoulaki et al., 2021), but they also pose serious threats to marine ecosystems. Wildlife can suffer from various issues such as ingestion of plastic debris, injury, entanglement, and even suffocation (Gregory, 2009). Recent research estimates that an astonishing 8.3 billion tons of plastic have been produced since its inception (Geyer et al., 2017), with a mere 9% of this total being recycled (Geyer et al., 2017). Each year, approximately 1.7 million tonnes of plastic waste enter the oceans (OECD, 2022). This figure is derived from the annual mass of waste generated per capita, the proportion of that waste that is plastic, and the percentage of mismanaged plastic waste that poses a risk of becoming ocean pollution. Mismanaged waste, such as improperly discarded plastic bags, food containers, and various single-use plastic items, is subjected to a range of environmental weathering processes. These processes are driven by exposure to abiotic factors such as ultraviolet (UV) radiation from sunlight, mechanical forces from wind, and the physical

and chemical interactions with water. Over time, these environmental conditions initiate a series of degradation mechanisms, including photodegradation, hydrolysis, and oxidative degradation, generating smaller plastic fragments known as secondary microplastics (Andrady and Koongolla, 2022). These secondary microplastics pose a unique challenge, as their origins are often difficult to trace, complicating efforts to assess how much macroplastic has transformed into microplastic. Despite the prevailing belief that most plastic contaminating the world's oceans originates from mismanaged waste, recent studies have identified seven other major sources of microplastics that significantly contribute to ocean pollution: tires, synthetic textiles, marine coatings, road markings, personal care products, plastic pellets, and city dust (Lassen et al., 2015; Magnuson et al., 2016; Essel et al., 2015). Each of these sources significantly contributes to the proliferation of microplastics in our oceans. Microplastics are lost at various stages throughout the plastic lifecycle, with a notable proportion occurring during product use and maintenance activities, such as driving cars and washing clothes (Boucher and Friot, 2017). Therefore, it is crucial to recognize that our everyday actions directly impact ocean pollution, posing significant threats to marine biodiversity and human health. Furthermore, a closer examination of the global release of microplastics into the world's oceans reveals a striking statistic: nearly two-thirds (63.1%) of these releases stem from just two primary sources. The foremost contributor is the laundering of synthetic textiles (34.8%), closely followed by tire erosion during driving (28.3%) (Boucher and Friot, 2017). These figures underscore the importance of addressing these key sources in efforts to mitigate microplastic pollution and protect the marine environment. To effectively combat the pervasive issue of microplastic pollution, it is crucial to adopt a holistic approach that encompasses both prevention and mitigation strategies. This includes implementing stricter regulations on the production and use of plastics, as well as

enhancing waste management practices to promote recycling and reduce the generation of secondary microplastics from larger plastic waste.

1.3.1. Microfiber shedding from synthetic textiles

The shedding of microfibers from synthetic textiles during washing has become a significant contributor to marine plastic pollution (Boucher and Friot, 2017; Granek et al., 2022). These synthetic microfibers (MFs), composed of non-biodegradable polymers like nylon, polyester (PE), rayon, polyethylene terephthalate (PET), polypropylene (PP), acrylic, and spandex, measure less than 5 mm in length (Sanuj et al., 2024). During laundering, fabrics undergo mechanical agitation and chemical exposure, which causes these tiny fibers to break off (De Falco et al., 2019). Research estimated that a single garment can shed over 1,900 microfibers per wash cycle (Browne et al., 2011), while an average 6-kilogram load of acrylic fabric may release over 700,000 fibers (Napper and Thompson, 2016). Galvão et al. (2020) provided the first measurements of emissions from textile washing under real household conditions, estimating an average release of 18,000,000 synthetic microfibers per 6 kg load of synthetic fabrics. Given the global population and the frequency of laundering, this adds up to vast amounts of microfibers entering wastewater systems and, subsequently, aquatic environments. As urbanization and population growth drive increased textile consumption and water usage, microfiber pollution is projected to rise sharply in the coming decades (Devi and Devi, 2024). Thriugh wastewater treatment plants (WWTPs) microfibers can enter rivers, lakes, and oceans, where they pose significant risks to ecosystems, biodiversity, and human health (Gago et al., 2018; González-Pleiter et al., 2020; Miller et al., 2017). Even the most advanced WWTPs with microplastic treatment technologies cannot entirely remove these particles (Liu et al., 2021). Studies from regions like San Francisco Bay (USA), Sweden, Australia, and Finland confirm that significant concentrations of microfibers are present

in wastewater effluents, regardless of the treatment processes in place (Magnusson & Wahlberg, 2014; Sutton et al., 2016; Talvitie et al., 2017; Granek et al., 2022). The persistence of microfibers in the environment is a particularly challenging issue. These non-biodegradable fibers resist environmental degradation, often persisting for decades and accumulating in marine ecosystems over time (Andrady, 2011; Siddiqui et al., 2023). This widespread contamination highlights microfiber pollution as a global issue unaffected by geographic location or local infrastructure sophistication (Henry et al., 2019). Once microfibers enter aquatic ecosystems, they are readily ingested by a diverse array of marine organisms. Research has shown that crabs (Watts et al., 2015), lobsters (Murray and Cowie, 2011), fish (Siddiqui et al. 2023; Macieira et al., 2021; Lusher et al., 2013; Possatto et al., 2011), and birds (Zhao et al., 2016) all consume microfibers either intentionally during feeding or accidentally through respiration and trophic transfer within food webs (Covernton et al., 2021). The ingestion of microfibers can have serious consequences for marine organisms, including reduced nutritional intake, diminished energy availability for growth, and altered behaviors (Watts et al., 2015; Siddiqui et al., 2023; Stienbarger et al. 2021). Moreover, microfiber pollution affects not just individual species but entire marine ecosystems. As microfibers accumulate in organisms like plankton, they are transferred up the food chain, eventually reaching larger predators, including those consumed by humans (Setälä et al., 2014; Torres et al., 2023). This introduces serious public health concerns, as humans may ingest these harmful particles through contaminated seafood (Barboza et al., 2018). To combat this growing problem, interventions are needed, including more sustainable clothing design. Researchers are focusing on understanding the factors that contribute to microfiber loss during laundering. Vassilenko et al. (2021) found that fiber shedding correlates positively with fabric thickness for nylon and polyester, revealing a more than 850-fold difference in the

number of microfibers lost between low and high-shedding textiles. Additionally, the implementation of filtration systems in washing machines can effectively capture microfibers before they enter wastewater systems. Laundry lint traps have been demonstrated to retain up to 90% of polyester fibers and 46% of nylon fibers (Vassilenko et al., 2021). A community-scale pilot project in Parry Sound, Ontario, where filters were installed in approximately 10% of households connected to the municipal WWTP, revealed an average weekly lint capture of 6.4 grams, equating to between 179,200 and 2,707,200 microfibers (Erdle et al., 2021). In conclusion, addressing the pervasive issue of microfiber pollution requires a multifaceted approach that includes innovative fabric technologies, improved filtration systems in washing machines, and increased public awareness, all aimed at protecting marine ecosystems and human health from the adverse effects of plastic contamination. By implementing these solutions, we can significantly mitigate the environmental impact of microfibers and promote a more sustainable future for our oceans.

1.3.2. Tire wear: the second largest source of global microplastic pollution

Car tires are increasingly recognized as the second-largest source of microplastic pollution globally, contributing an estimated 28% of total microplastic emissions due to tire erosion during driving (Boucher and Friot, 2017; Mayer et al., 2024). On average, about 0.81 kg of tire particles per capita is released into the environment annually (Kole et al., 2017). This substantial contribution highlights the magnitude of the problem and underscores the urgent need for targeted mitigation strategies. The United States stands out as the largest emitter, releasing 1.52 million metric tonnes of tire particles annually. Other countries, including China, India, Japan, Italy, Norway, Sweden, and Denmark, report emissions ranging from 6,721 to 756,240 tonnes per year (Kole et al., 2017). Tire

particles (TPs) primarily originate from the abrasion of tires on road surfaces and consist of a complex mixture of components. These include polymeric materials, fillers, softeners, vulcanizing agents, and various additives. The predominant component, synthetic rubber, is derived from polymers such as styrene-butadiene, which constitutes 40-50% of tire composition (Johannessen et al., 2022). Fillers, like carbon black sourced from coal combustion, account for 30-35% of the tire's mass, serving both as reinforcements and colorants. Softeners, composed of oils and resins, make up approximately 15% of the total mass, while vulcanization agents, including sulfur and zinc oxide, are present in concentrations ranging from 2% to 5%. Additional additives, including the toxic compound 6PPD, which enhances tire durability but poses risks to aquatic life, represent 5-10% of the total mass (Sommer et al., 2018; Tian et al., 2021). TPs undergo aging processes as they travel through different environments (Wagner et al., 2021). Initially, after being generated, sunlight and temperature drive changes at the road surface. These particles are then carried by wind or rain runoff, experiencing photooxidation, leaching, and microbial breakdown at the roadside. As TPs travel further - potentially reaching sewer systems, terrestrial landscapes, or aquatic environments - they experience mechanical forces that can cause them to either fragment or aggregate. When dispersed in water, these particles can release inorganic and organic materials (Wagner et al., 2021). Despite their significant contribution to micro/nanoplastic pollution (MNPs), TPs are often underrepresented in scientific literature. From 2000 to 2019, only 1% of microplastic studies incorporated data on rubber-based TPs, revealing a significant gap in research and environmental monitoring efforts (Knight et al., 2020). This underreporting is concerning, given that TPs are released into various environmental compartments. For instance, Lead and Weinstein (2019) found that in the sediment and surface water of three major tributaries within the Charleston Harbor estuary in South

Carolina, TPs were the second most abundant type of microplastic, comprising 17.1% of the total microplastics identified. Additionally, Parker et al. (2020) provided the first evidence of TP consumption in field-collected organisms, revealing that suspected tire wear particles were found in 14% of individuals across five fish species in Charleston Harbor, a densely populated urban estuary along the southeastern Atlantic coast of the United States. In urban stormwater runoff from 12 watersheds surrounding San Francisco Bay (USA), some samples were composed of as many as 64% black rubbery fragments, potentially tire and road wear particles (Werbowski et al., 2021). Wik and Dave (2009) performed a comprehensive meta-analysis on the presence of tire components in the environment, demonstrating that the maximum Predicted Environmental Concentrations (PECs) of tire wear particles in road runoff entering surface waters can range from 0.03 to 56 mg/L. In sediments from areas with heavy traffic or where driving patterns lead to accelerated tire wear, the maximum PECs were found to be between 0.3 and 155 g/kg dry weight. TPs exhibit varied behaviors in aquatic environments, with factor such as salinity, pH, particle size, and aggregation significantly influencing the release of toxic substances. Gualtieri et al. (2005) demonstrated that greater particle aggregation reduces zinc leaching, while Hartwell et al. (2000) observed that tire leachate toxicity decreases with increasing salinity up to 15 ppt, suggesting a potential salt-toxicant interaction that could reduce toxicity in estuarine and marine habitats. TPs in the environment pose significant risks to wildlife due to both the particles themselves and the toxic leachates they release. Turner and Rice (2010) showed that TP leachates impair photochemical efficiency in the marine alga *Ulva lactuca*, with organic compounds driving toxicity more than zinc. Exposure to tire particle leachates negatively affects the early life stages of three key echinoderm species (*Paracentrotus lividus, Arbacia lixula,* and *Diadema africanum*), leading to abnormal development, increased mortality, and a concentration-

dependent reduction in body size (Rist et al., 2023). Although leachate is a primary contributor to toxicity, there is substantial evidence that tire particles can independently cause harm to organisms (Cunningham et al., 2022; Siddiqui et al., 2022). In zebrafish, exposure to nano-tire particles resulted in adverse effects such as mortality, hatching delays, and malformations, which were not observed in exposures to leachate or larger microparticles. TP leachates also caused specific developmental abnormalities, including malformed eyes, snout, and jaw, along with yolk sac edema in zebrafish embryos. The only effect uniquely observed in micro tire particle exposure was a developmental delay at 24 hours post-fertilization (hpf). These findings underscore the diverse effects TPs exert on aquatic ecosystems and emphasize the need for targeted environmental assessments to comprehend and mitigate their impacts. Given their intricate composition and extensive environmental distribution, TPs present a significant yet often overlooked environmental challenge, necessitating urgent attention from researchers and policymakers alike to address their risks effectively.

1.4. Microplastics as sources and sinks of environmental contaminants

The environmental impacts of microplastics are further compounded by their ability to absorb and transport contaminants from their surroundings. In aquatic systems, microplastics act as carriers for a range of chemical pollutants (Fred-Ahmadu et al., 2020). Their hydrophobic nature and high surface area-to-volume ratio allow them to easily adsorb persistent organic pollutants (POPs) and trace metals (Ashton et al., 2010). Studies reveal that organic contaminants on plastic surfaces can be found at concentrations several orders of magnitude higher than in surrounding waters, facilitated by the significant water-polymer partition coefficient (KP/W [L/kg]), which promotes pollutant retention in plastics (Atugoda et al., 2021). The sorption process is further

influenced by factors such as surface weathering and the chemical properties of the contaminants (Fred-Ahmadu et al., 2020). Aging processes, such as exposure to thermal, mechanical, biological, radiative, and oxidative pressures, further enhance the ability of plastics to adsorb harmful substances like metals and organic compounds (Albertsson et al., 1987). For instance, Liu et al. (2019) demonstrated that UV-accelerated aging significantly increases the adsorption capacity of polystyrene and polyvinyl chloride for ciprofloxacin, a hydrophilic organic contaminant. Aging also reduces the hydrophobicity of plastics, enhancing their capacity to absorb hydrophilic pollutants (Hüffer and Hofmann, 2016). Plastics also contain various chemical additives, such as reinforcing fibers, fillers, plasticizers, and stabilizers, which improve their performance but may leach into the environment (Deanin, 1975; Sridharan et al., 2022). Because these additives generally do not form covalent bonds with polymers, they can easily leach from plastics, especially as microplastics break down. This exposes marine organisms to a complex mixture of chemicals through the ingestion of plastic debris (Hermabessiere et al., 2017). Marine organisms can ingest plastic materials along with the absorbed chemicals and metals they contain. These substances may accumulate within the organisms and subsequently move up the food chain, eventually reaching humans (Seltenrich, 2015). Key plastic additives commonly found in marine species include polybrominated diphenyl ethers (PBDEs), phthalates (PAEs), and nonylphenols (NPs) (Hermabessiere et al., 2017). PBDEs and PAEs have been detected in fish (Mariussen et al., 2008; Peng et al., 2007; Kelly et al., 2008; Panio et al., 2020), marine mammals (Yogui et al., 2011; Rotander et al., 2012; Andvi et al., 2024; Routti et al., 2021), mussels (Johansson et al., 2006; Dosis et al., 2016), and corals (Raguso et al., 2022). NPs have been detected in zooplankton, algae, and fish (O'Halloran et al., 1999; Naylor, 1995; Graff et al., 2003). Several studies have demonstrated the acute toxicity of plastic

leachates on marine life. For instance, leachates from various recyclable plastics, such as high-density polyethylene (HDPE), polypropylene (PP), and polycarbonate (PC), negatively impact the survival and settlement of barnacle larvae (*Amphibalanus amphitrite*) (Li et al., 2016). Bejgarn et al. (2015) investigated the toxicity of leachates from 21 different weathered plastic products on the marine copepod *Nitocra spinipes*. They discovered that leachates from 38% of the plastic samples (8 out of 21) were toxic to the copepods. Of these, four plastic samples became significantly more toxic after UV irradiation, indicating that sunlight exposure can enhance the release of harmful substances from certain plastics. Cappucci et al. (2024) observed that *Drosophila melanogaster* exposed to leachate from virgin and oxo-degradable polypropylene and polyethylene exhibited genomic instability and an increased likelihood of loss of heterozygosity (LOH), thereby promoting tumor growth. These findings underscore the environmental hazards associated with microplastic pollution, particularly the role of plastic additives and leachates in marine ecosystems. As these pollutants bioaccumulate in marine food webs, they pose long-term risks to both marine life and human health.

1.4.1. Phthalates acid esters (PAEs)

Phthalates (PAEs) are a group of organic compounds derived from phthalic acid esters. Since the 1930s, they have been the dominant plasticizers in the polymer industry, added to plastics in concentrations ranging from 10% to 60% by weight (Giuliani et al., 2020). Their primary function is to improve the flexibility, transparency, elasticity, and durability of plastic materials. However, because of their low water solubility and lack of chemical bonding to the plastic matrix, phthalates can leach out of products over time. Once released into marine environments, they can migrate, transform, and become bioavailable to marine organisms, where they may accumulate, raising significant concerns about their environmental impact. Phthalates have been detected at varying

concentrations across marine ecosystems worldwide, with trace amounts even found in the Arctic (Xie et al., 2007). Among the different PAEs, Di (2-ethylhexyl) phthalate (DEHP), Di-n-butyl phthalate (DnBP), and Diisobutyl phthalate (DiBP) are frequently identified as the most prevalent in seawater, marine sediments, and biota, along with their primary metabolites - Mono(2-ethylhexyl) phthalate (MEHP), Mono-n-butyl phthalate (MnBP), and Mono-isobutyl phthalate (MiBP) (Hidalgo-Serrano et al., 2022). While the detection frequencies of phthalates and their metabolites are generally similar across various species, significant variations in PAE concentration levels have been observed. Some studies indicate a decrease in PAE concentrations with rising trophic levels, while others suggest the opposite trend (Hu et al., 2016; Lo Brutto et al., 2021; Routti et al., 2021). Variations in the distribution of contaminants may be associated with the feeding behaviors and habitats of different species, along with the degree of microplastic contamination present in their environments. Higher concentrations of phthalates have been documented in mollusks and fish that consume benthic organisms (Hu et al., 2016), as well as in corals located in areas severely affected by plastic pollution (Saliu et al., 2019). In this regard, phthalates have been proposed as indicators of marine organisms' exposure to microplastics. Baini et al. (2017) established a positive correlation between the levels of phthalates and microplastics in neuston and plankton samples, suggesting that phthalate esters could function as tracers for plastic exposure and ingestion in marine wildlife. Phthalates are known to negatively affect health and reproductive functions in various organisms, consequently influencing ecosystem dynamics. Their anti-androgenic and estrogen-mimicking properties classify them as significant endocrine disruptors. These compounds can disrupt hormonal balance, adversely affecting reproductive systems and interfering with crucial developmental processes, including masculinization (De Toni et al., 2017). Research conducted by Ye et al. (2014) demonstrated that

exposure of marine medaka (*Oryzias melastigma*) to DEHP and its metabolites from hatching through adulthood resulted in endocrine disruption with sex-specific effects. Specifically, males displayed greater sensitivity than females, showing a significant decrease in testosterone (T)/E2 ratios (Ye et al., 2014). In addition to environmental contamination, phthalates have been detected in a variety of consumer products, such as soft drinks, milk, mineral water, wine, oil, and ready-to-eat meals (Beltifa et al., 2017; Chatonnet et al., 2014; Hayasaka et al., 2014; Lin et al., 2015; Luo et al., 2020). Human exposure to phthalates has been linked to numerous health issues, including obesity, type 2 diabetes, hypertension (Muscogiuri and Colao; 2016), asthma, and allergies (Jaakkola and Knight, 2008). Due to these health and environmental concerns, PAEs are now recognized as priority pollutants by major environmental protection agencies worldwide, including the United States Environmental Protection Agency (US-EPA) (Net et al., 2015). Regulatory measures have been put in place to limit PAE use. In the European Union, DEHP, BBP, DBP, and DiBP are banned in toys and childcare products if concentrations exceed 0.1% by weight. Phthalates are also heavily restricted in food contact materials and are mostly banned in cosmetics. Similarly, China has imposed a 0.1% limit on DBP, BBP, DEHP, DNOP, DiNP, and DiDP in plastic toys, with DEHP, BBP, and DBP prohibited in cosmetics (Monti et al., 2022).

1.5. Challenges in the microplastic research field

One of the significant challenges in detecting microplastics is effectively controlling sample contamination, which is frequently caused by airborne microplastics, including synthetic fibers from clothing, that settle as atmospheric fallout. These airborne particles can easily infiltrate environmental samples, leading to misleading results if proper precautions are not taken during the sampling and analysis processes (Brander et al., 2020). Without stringent protocols in place, environmental samples become highly

susceptible to contamination from plastic particles originating from external sources. This risk of cross-contamination is particularly concerning, as overlooking it can result in substantial overestimations of microplastic prevalence and abundance in the environment, ultimately distorting our understanding of their ecological impacts (Bogdanowicz et al., 2021). Another key issue is the difficulty of conducting large-scale spatial and temporal comparisons, given the diverse range of methods employed to identify and quantify microplastics in various studies. The lack of consistency in methodological approaches leads to complications when attempting to compare results across different geographical locations and time frames. This inconsistency not only hampers the scientific community's ability to establish reliable datasets but also undermines the efficacy of long-term monitoring programs. As such, establishing standardized methodological criteria for assessing microplastic abundance and distribution is essential for facilitating accurate comparisons and effective long-term monitoring efforts (Hidalgo-Ruz et al., 2012; Thornton Hampton et al., 2023). Additionally, microplastic concentrations and quantities are often reported using a variety of units, which can create further challenges when attempting to compare findings across different studies. While adopting convertible units can aid in harmonizing data (Hidalgo-Ruz et al., 2012), certain environmental measurements, such as items per square meter, cannot be converted, thus limiting the utility of those data (Cunningham and Sigwart, 2019). Therefore, it is crucial to express concentrations in meaningful units that enable straightforward comparisons with effect study data, which predominantly report findings in terms of mass or particles per volume (Burns and Boxall, 2018). Moreover, most laboratory experiments have utilized microplastic concentrations that are several orders of magnitude higher than those typically found in natural environments. This discrepancy raises significant concerns regarding the ecological validity of

laboratory findings, as the ingestion and associated effects observed in exposed organisms may not accurately reflect conditions encountered in the wild. However, given that we lack accurate measurements of micro and nanoplastic concentrations in the wild, understanding how organisms will respond at higher concentrations may be important in the future. Furthermore, the situation is complicated in the realm of nanoplastic research due to the current lack of available tools and instrumentation capable of measuring environmental concentrations effectively (Koelmans et al., 2015). Although, in the coming decades, it is unlikely that the levels of microplastics in the environment will diminish, given the increasing reliance on plastic due to its many benefits. Consequently, it is becoming increasingly crucial to investigate the biological effects of elevated concentrations of microplastics on living organisms. This need is underscored by the potential for significant ecological disruptions, including impacts on marine life and the broader food web. Additionally, most studies tend to commercially acquire particles and rely on a single type of polymer, often characterized by a specific size and uniform shape, whereas microplastics encountered in the field consist of a diverse mixture of polymers that vary widely in size, shape, and composition. This variability complicates the extrapolation of laboratory results to real-world scenarios, as the responses of organisms to these heterogeneous mixtures may differ markedly from their responses to single, uniform polymers. On the other hand, conducting exposure studies with a single type of polymer, characterized by specific size and shape, facilitates the identification of the specific effects induced by that polymer. However, this approach may not fully capture the complexities of environmental interactions. Furthermore, it is important to note that microplastics in marine environments are not pristine; they undergo extensive weathering and absorb various organic compounds from their surroundings, which alters their chemical and physical properties. These environmental conditions are challenging to

replicate accurately in laboratory settings, underscoring the need for more ecologically relevant experimental designs to enhance our understanding of microplastics' behavior and impacts in the natural world (Phuong et al., 2017; Thornton Hampton et al., 2022).

1.6. Thesis outline

The pervasive contamination of marine environments by microplastics poses a significant threat to environmental health, with far-reaching implications that extend beyond marine life to impact entire ecosystems and human populations that rely on these vital resources. As discussed in Section 1.5, numerous challenges persist in the study of microplastics, ranging from methodological inconsistencies to difficulties in accurately assessing their ecological effects.

This thesis aims to tackle several of these pressing challenges by providing new insights across three key areas: a) developing effective strategies to control sample contamination, which is crucial for obtaining reliable data; b) creating and validating robust analytical methods for detecting microplastics and phthalic acid esters in marine invertebrates, thereby enhancing our ability to monitor and assess contamination, and c) investigating the effects of one of the major source of microplastics on model estuarine species, utilizing environmentally relevant microplastics that more accurately reflect real-world conditions.

The thesis is structured into four distinct chapters to address these objectives. **Chapter 1** provides essential background information through a comprehensive review of the relevant literature, clearly outlining the research objectives that guide the investigation. Following this foundational chapter, the core of the thesis consists of a collection of four research papers, two of which have been published in reputable journals, while the other two are currently in preparation for submission. Finally, **Chapter 4** provides a synthesis

of the overall findings. Following Chapter 4, the thesis includes an appendix detailing national and international conferences attended, awards received, and peer-reviewed publications produced over the course of the Ph.D. program.

Chapter 2 features two research papers, published in *Marine Pollution Bulletin* Journal, that report analytical methods for efficiently detecting microplastics and phthalic acid esters in two marine invertebrate species: sea urchins and scleractinian corals. The first paper, titled "*First Detection of Microplastics in Reef-Building Corals from a Maldivian Atoll*" represents a collaborative effort with the MaRHE Center (Marine Research and High Education Center) and sheds light on the alarming presence of microplastics in coral ecosystems. This discovery raises urgent concerns about the health and resilience of these critical habitats, which are already under stress from climate change and other anthropogenic pressures. The second paper, titled "*Detection of Microplastics and Phthalic Acid Esters in Sea Urchins from Sardinia (Western Mediterranean Sea)"* is a collaborative study with the International Marine Centre (IMC) in Otranto, Italy. This research aims to elucidate the presence of microplastics and associated phthalic acid esters in the sea urchin *Paracentrotus lividus*, providing important insights into the potential human exposure to phthalate acid esters through seafood consumption. By investigating these marine invertebrates, the study highlights not only the ecological implications of microplastic pollution but also the potential health risks to humans, emphasizing the need for continued research and monitoring in this area.

Chapter 3 includes one collaborative paper with the Brander lab at Hatfield Marine Science Center, Oregon State University, USA. This study investigates the effects of tire wear, a major source of microplastics in marine environments, on model estuarine species. In this study, the microplastics used for exposure experiments were prepared in

the lab and subjected to UV weathering to simulate real-world environmental conditions, enhancing the ecological relevance of the findings. The paper, titled *"Impact of Pristine and Weathered Environmentally Relevant Multi-Tire Mixture on Two Model Estuarine Species",* explores the effects of a cryo-milled tire tread (CMTT) composite provided by the U.S. Tire Manufacturers Association. This composite realistically reflects environmental conditions by incorporating a blend of three tire types (passenger car tire, light truck tire, and truck/bus tire) in proportions typical of those estimated in the United States. The study examines how both pristine and UV-weathered forms of this multi-tire mixture, as well as their associated leachates, influence the growth and locomotory behavior of two key estuarine species: the fish *Menidia beryllina* and the mysid shrimp *Americamysis bahia*. By focusing on these two species, the research sheds light on the broader ecological implications of tire-derived microplastics on estuarine ecosystems, with an emphasis on behavioral and physiological effects.

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Chapter 2

Detection of microplastics and phthalic acid esters in marine invertebrates

First detection of microplastics in reef-building corals from a Maldivian atoll

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Abstract

The presence of microplastics in the world's oceans and their effects on marine habitats are highly concerning. As suspension feeders, corals are very exposed to microplastics, compromising the health of coral reef ecosystems. In this study, we surveyed for the first time the presence of microplastics in Maldivian reef-building corals. Aiming to determine the influence of exposure and depth on microplastic distribution, analyses were carried out on 38 individuals belonging to three different species. 58% of the investigated colonies were contaminated with particles within the 25–150 μm size range. The maximum concentration was encountered in a *Pocillopora verrucosa* colony sampled from a shallow inner reef (8.9 particles/g of coral). No significant differences in microplastic concentration were observed between different depths, exposures, sites, and species. Overall, this study confirmed microplastic presence in coral reefs of the Maldivian archipelago including foundation species.

1. Introduction

Synthetic polymers, due to their chemical stability and widespread use, have become the predominant human-made debris in the ocean, accounting for 60–80% of marine litter (Moore, 2008; Sharma and Chatterjee, 2017). The fact that microplastics pose a threat to marine habitats is widely accepted (John et al., 2021). Due to their small size, microplastics are easily ingested by a wide range of marine organisms (Cole et al., 2013; Iannilli et al., 2019; Van Cauwenberghe et al., 2015).

Ingestion of plastic particles <5 mm in size has been documented in a variety of marine invertebrates, including zooplankton (Cole et al., 2013), amphipods (Iannilli et al., 2019), mussels and lugworms (Van Cauwenberghe et al., 2015). Moreover, plastic additives, such as phthalic ester acids (PAEs), may leach out from the plastic compound and

become bioavailable to the organisms (Saliu et al., 2019), displaying adverse effects, *i.e.* acting as endocrine disruptors, inducing oxidative stress and immunotoxicity (Oehlmann et al., 2009). Only recently, the possible impacts of plastic pollution on the health of reef-building corals, the most iconic inhabitants of coral reef ecosystems, have been started to be investigated (Lamb et al., 2018). Two major mechanisms of Interaction were highlighted: active incorporation and passive adhesion (Corona et al., 2020; Martin et al., 2019). In the former, plastic particles carried by water currents end up trapped by the crown of tentacles and then ingested. This is a result of the suspension feeding habits of corals. For the latter, the role played by coral skeletons in removing microplastics from the surrounding waters is considered more relevant (Corona et al., 2020; Martin et al., 2019). Feeding experiments carried out in tanks have demonstrated that stony corals can actively ingest microplastics (Hall et al., 2015; Reichert et al., 2018), mistaking them as food items (Savinelli et al., 2020), and face health impacts such as bleaching, tissue necrosis, alteration of the photosynthetic performances (Reichert et al., 2019; Syakti et al., 2019), and decrease of the skeletal growth rates (Chapron et al., 2018). It also has been demonstrated that chemoreception could play a role in coral plastic consumption; microplastics, releasing phagostimulants, are treated similarly to prey (Allen et al., 2017). Since these studies were carried out in laboratories, by employing artificial conditions with high concentrations of commercial plastic particles, in several cases with no alternative food provided, it was questioned if the results may be totally representative of what happens in the "real" environment, where the stress is supposed to be lower (Vencato et al., 2021). Under this view, there is an urgent need for studies carried out around the world and *on the field* to evaluate the impact of microplastics in coral reef ecosystems. At this time, most of the investigations come from Southeast Asia (Huang et al., 2021) and confirm that coral reefs are already highly threatened by plastic mi-

microparticles. In large part, these studies deal with coral reef sediment contamination (Cheang et al., 2018; Cordova et al., 2018; Saliu et al., 2018; Zheng et al., 2019; Utami et al., 2021) and microplastic abundance in surface seawater (Saliu et al., 2018; Huang et al., 2019; Tan et al., 2020). Ding et al. (2019), analyzing seawater samples within a depth of 10 to 40 m from outer reef slopes and at depths of 5 and 15 m in the lagoons, found that microplastic abundance tends to decline with increasing depth. Moreover, a much higher average concentration of microplastics was detected in the lagoons (15.9 items L^{-1}), in comparison with those in outer reef slopes (2.4 items L^{-1}). In the current literature, only a few studies investigate the presence of microplastics in wild-collected scleractinian corals (Ding et al., 2019; Rotjan et al., 2019; Tang et al., 2021). In the coral reef systems from Xisha Islands of the South China Sea, microplastics were extracted from 24 different coral species, and particles were detected within the size range 24–4729 μm in 100% of the samples with a concentration of 0.02–1.3 items/g, revealing the soft coral *Sinularia* to be the most contaminated with fibers prevalent among all shapes (87.6%) (Ding et al., 2019). Rotjan et al. (2019) investigated the distribution of microplastics in 4 colonies of *Astrangia poculata* at Fort Wetherill Park in Jamestown, Rhode Island, finding in all of them an average of 112 particles $polyp^{-1}$ larger than 60 μm, of which 73.4% were fibers. Around Hainan Island (China), the small-polyp coral *P. damicornis* and the large-polyp coral *G. fascicularis* showed an average of 3.68 ± 3.94 particles cm^{-2} and 5.89 ± 5.15 particles cm^{-2}, respectively, mostly represented by fibrous materials (93.17%) (Tang et al., 2021).

Here, we aimed to preliminary assess the presence of microplastics in a real case scenario, by surveying the contamination of reef-building corals in the Maldives, an archipelagic state, owning the seventh largest coral reef system in the world (Spalding et al., 2001),

and, recently, suggested to be a potential sink of missing plastics due to the seasonally reversing monsoonal currents that dominate the Northern Indian Ocean (van der Mheen et al., 2020). Particularly, we looked to investigate if the presence of microplastics in the coral colonies can be affected by the different wave exposure, site location, depth, and species. To the best of our knowledge, this is the first report regarding the detection of plastic microparticles in the foundation species of the Maldivian coral reef.

2. Materials and methods

2.1. Sample collection

The sampling was carried out between February and March 2019 in the water surrounding the Magoodhoo island, Faafu Atoll, Maldives (3° 4′ 49.08″ N, 72° 57′ 57.19″ E), using the MaRHE center (Marine Research and High Education Center) as logistic station. Coral fragments approximately 2–3 cm in length were broken off with a side cutter, then wrapped individually in preheated (500 °C) aluminum foil, held on ice while in the field, and finally stored at -16 °C. A total of 38 individuals belonging to the species *Porites lutea* (n = 13), *Pavona varians* (n = 14) and *Pocillopora verrucosa* (n = 11) (Fig. 1) were collected across 8 sites (Fig. S1), two depths (shallow 5–10 m and deep 10–20 m), and two exposures: four locations were outside the atoll rim and the remaining ones onto inner reefs (Table 1). Inner reef locations, mainly lagoons facing sides of the atoll rim, are expected to be subjected to land-based pollution derived from the anthropic activities on islands, while the outer reef sites representing the ocean-facing side of the atoll rim, characterized by strong hydrodynamic conditions, are expected to be subjected to pollution derived from the open ocean (Saliu et al., 2018).

2.2. Sample preparation

Each sample was freeze-dried and weighted onto an analytical balance (Gibertini® E50S). Specifically, the total wet weight of the coral fragment (including coral tissue and skeleton) was recorded. The material was then crushed and hydrogen peroxide solution (30%) was added (2 mL per gram *d.w.)*. Sample digestion was carried out for 2 h at 75°C, applying magnetic stirring (150 rpm). At the end of the treatment, all the bioorganic matrix (proteins and lipids) resulted dissolved. The sample was then let to cool to room temperature, filtered onto a stainless steel filter (Paco Filter, 25 μm pore size), and submitted to density separation. Specifically, zinc chloride solution (density 1.7 g/cm^3) was added and separation between plastic particles and mineral fraction was induced by vigorously stirring the mixture for five minutes and then waiting for the settling of the denser material. As demonstrated in a previous work, this procedure enables an easy recovery on the top of the solution of the most common polymers characterized by density ranging between 0.9 and 1.5 g/cm^3. When the procedure was completed, the supernatant was collected and filtered onto a cellulose nitrate filter (Whatman™, 3 μm), using a vacuum glass apparatus. Collected filters were then submitted to Micro FTIR analysis.

2.3. Microplastic detection

The filters were visually inspected by using a stereomicroscope (Leica® S9E Leica Microsystems GmbH, Germany) capable of magnification of up to 40×. This preliminary screening was aimed at obtaining a general description of the isolated particles and their characterization by color and shape. Then a μ-FTIR Spotlight 200i FTIR (Perkin Elmer), equipped with a liquid nitrogen-cooled mercury cadmium telluride (MCT) single detector, was used to collect infrared spectra from each particle (Saliu et al., 2021).

ID sample	Species	Site	Lat N	Long E	Exposure	Depth
1	*Porites lutea*	1	3°04′37.8″	72°58′23.8″	Outer reef	Deep
2	*Pavona varians*	1	3°04′37.8″	72°58′23.8″	Outer reef	Shallow
3	*Pocillopora verrucosa*	1	3°04′37.8″	72°58′23.8″	Outer reef	Shallow
4	*P. lutea*	1	3°04′37.8″	72°58′23.8″	Outer reef	Shallow
5	*P. lutea*	2	3°04′29.2″	72°57′58.6″	Outer reef	Deep
6	*P. varians*	2	3°04′29.2″	72°57′58.6″	Outer reef	Deep
7	*P. verrucosa*	2	3°04′29.2″	72°57′58.6″	Outer reef	Deep
8	*P. varians*	2	3°04′29.2″	72°57′58.6″	Outer reef	Shallow
9	*P. lutea*	2	3°04′29.2″	72°57′58.6″	Outer reef	Shallow
10	*P. lutea*	3	3°03′30.5″	72°55′29.6″	Outer reef	Shallow
11	*P. verrucosa*	3	3°03′30.5″	72°55′29.6″	Outer reef	Shallow
12	*P. varians*	3	3°03′30.5″	72°55′29.6″	Outer reef	Shallow
13	*P. varians*	3	3°03′30.5″	72°55′29.6″	Outer reef	Deep
14	*P. lutea*	3	3°03′30.5″	72°55′29.6″	Outer reef	Deep
15	*P. verrucosa*	3	3°03′30.5″	72°55′29.6″	Outer reef	Deep
16	*P. varians*	4	3°04′49″	72°58′04″	Outer reef	Shallow
17	*P. verrucosa*	4	3°04′49″	72°58′04″	Outer reef	Shallow
18	*P. lutea*	4	3°04′49″	72°58′04″	Outer reef	Deep
19	*P. lutea*	5	3°07′14.2″	72°58′46.9″	Inner reef	Shallow
20	*P. varians*	5	3°07′14.2″	72°58′46.9″	Inner reef	Shallow
21	*P. varians*	5	3°07′14.2″	72°58′46.9″	Inner reef	Deep
22	*P. verrucosa*	5	3°07′14.2″	72°58′46.9″	Inner reef	Deep
23	*P. verrucosa*	6	3°05′24.3″	72°58′04.5″	Inner reef	Shallow
24	*P. lutea*	6	3°05′24.3″	72°58′04.5″	Inner reef	Shallow
25	*P. varians*	6	3°05′24.3″	72°58′04.5″	Inner reef	Shallow
26	*P. verrucosa*	6	3°05′24.3″	72°58′04.5″	Inner reef	Deep
27	*P. lutea*	6	3°05′24.3″	72°58′04.5″	Inner reef	Deep
28	*P. varians*	6	3°05′24.3″	72°58′04.5″	Inner reef	Deep
29	*P. varians*	7	3°06′46.1″	73°01′15.9″	Outer reef	Deep
30	*P. varians*	7	3°06′46.1″	73°01′15.9″	Outer reef	Shallow
31	*P. verrucosa*	7	3°06′46.1″	73°01′15.9″	Outer reef	Shallow
32	*P. lutea*	7	3°06′46.1″	73°01′15.9″	Outer reef	Shallow
33	*P. varians*	8	3°08′34.6″	73°00′43.4″	Outer reef	Deep
34	*P. lutea*	8	3°08′34.6″	73°00′43.4″	Inner reef	Deep
35	*P. verrucosa*	8	3°08′34.6″	73°00′43.4″	Inner reef	Deep
36	*P. varians*	8	3°08′34.6″	73°00′43.4″	Inner reef	Shallow
37	*P. verrucosa*	8	3°08′34.6″	73°00′43.4″	Inner reef	Shallow
38	*P. lutea*	8	3°08′34.6″	73°00′43.4″	Inner reef	Shallow

Table 1: Coral samples collected and description of the related sampling sites.

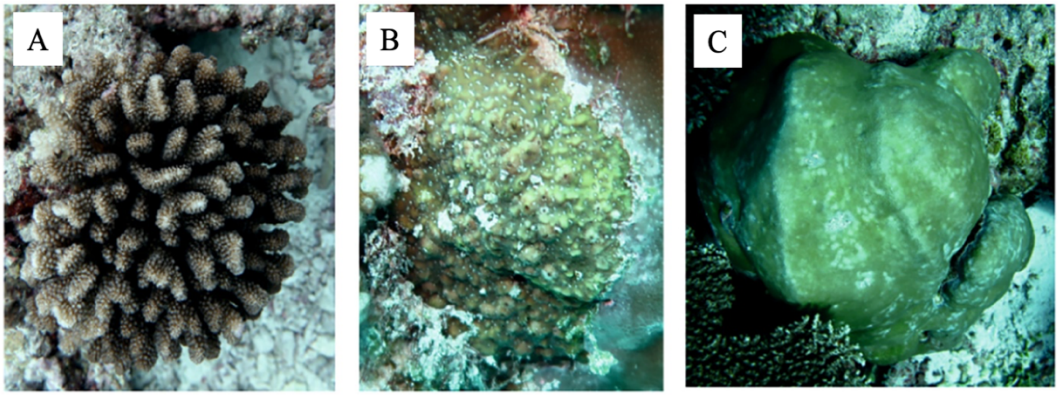

Figure 1: Photos of three specimens of the collected species: *Pocillopora verrucosa* (A), *Pavona varians* (B)*, and Porites lutea* (C).

For each particle, three measurements were carried out by operating in attenuated total reflectance (ATR) with 32 co-added scans in the wave-number range 4000–600 cm^{-1}. Background spectra were collected before each acquisition. The patented COMPARE™ spectral comparison algorithm provided by Perkin Elmer was used to confirm the assignment of the particles. The Hummel spectra library was employed as a reference and matches were accepted with confidence levels of 80% or greater (Saliu et al., 2018). Examples of detected plastic particles, with the relative spectra, are provided in Fig. 2. Since corals are colonial, to reflect the microplastic abundance in the entire individual, data were expressed as items detected per g of coral (TDW).

2.4. PAEs analysis

Analyses of di-methyl phthalate (MEP), di-ethyl phthalate (DEP), di-butyl phthalate (DBP), benzyl butyl phthalate (BBzP), bis(2-ethylhexyl) phthalate (DEHP) in scleractinian coral samples were carried out according to a SPME-LC-MS/MS method previously described (Saliu et al., 2020a, 2020b). Briefly, SPME was carried out by inserting C18 fibers into the coral tissue for 15 min and by eluting the extracted analytes with a 90:10 methanol: water mixture. LC-MS analysis was performed with a TSQ

Quantum Access Max LC/MS instrument (ThermoScientific) equipped with an ESI interface and a triple quadrupole mass analyzer, applying a selected reaction monitoring approach including one qualifier and one quantifier for each phthalate.

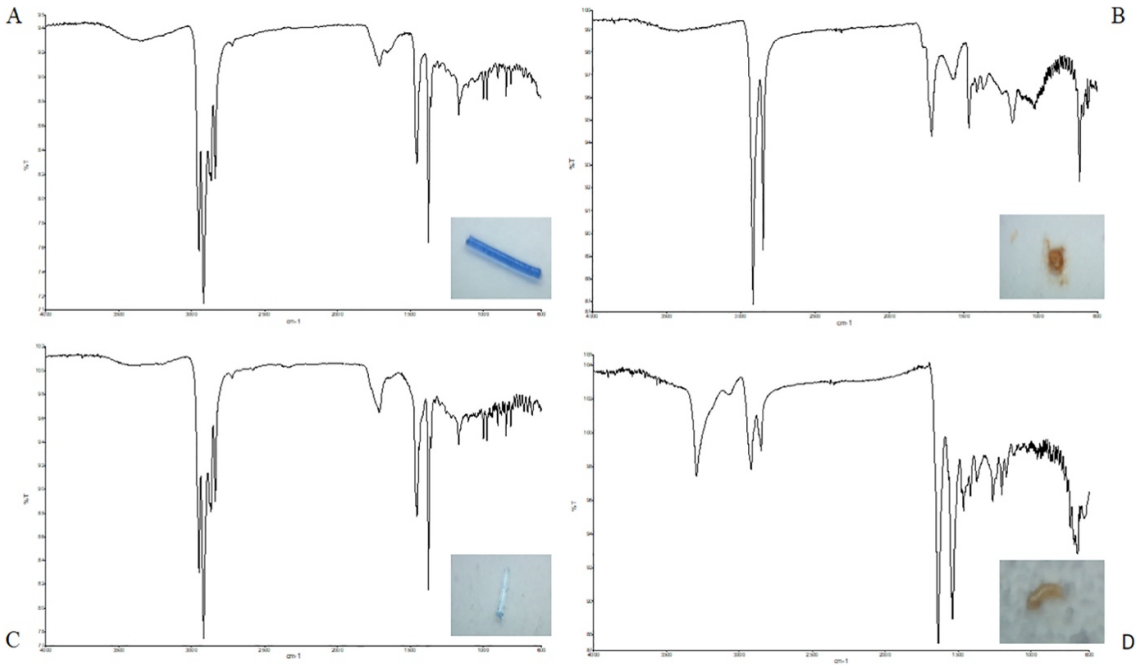

Figure 2: Representative picture of microplastics detected in corals with relative spectra: (A) polypropylene + $CaCO_3$, (B) oxidized polyethylene, (C) polypropylene, (D) polyamide.

2.5. Quality control

To reduce the contamination derived from the surrounding laboratory environment, scrupulous measures were implemented. Glassware and tools were rinsed with bi-distilled water three times and dried at 300° in the muffle furnace before being used. The hydrogen peroxide, used for the digestion of the biological matrix, was pre-filtered onto a glass microfiber filter (Whatman GF/D 2.7 µm). No plastic materials were used during the sample preparation workflow and cotton laboratory coats were worn. In addition, background contamination was measured by running twelve procedural blanks, determining the limit of quantitation as the average of the procedural blanks plus six times their standard deviation.

2.6. Statistical analysis

The data reported as microplastic particles per gr of coral (TDW), were statistically analyzed to explore the effect of site, exposure, and depth on microplastic content in corals. According to a Shapiro–Wilk test of normality, microplastic concentration was not normally distributed. The Kruskal-Wallis test was applied to explore differences in particle concentration between studied factors and levels. Spearman correlation was performed to study the relationship between plastic particles, five PAEs (DBP, BBzP, DEP, DEHP, DMP), and their total (Σ5PAEs). Statistical analyses were performed using SPSS ver. 27 (IBM, New York). All data are expressed as mean ± standard error (SE).

3. Results

3.1. Microplastics in scleractinian corals

Analysis showed the presence of 60 plastic particles within the size range of 25–150 μm distributed in 22 (58%) of the surveyed colonies. The remaining 16 (42%) colonies were free of particles. The average concentration was 1.8 ± 0.4 particles/g (mean ± SE). *P. verrucosa* showed the greatest average concentration of plastic particles (2.8 ± 0.9 particles/g) and was also the species displaying the maximum particle concentration in a colony (8.9 particles/g), while *P. varians* was the least impacted species with 1.2 ± 0.7 particles/g. The most abundant polymers (Fig. 3a) were polyethylene (PE) followed by polypropylene (PP), making up 63% of the total particles. Smaller amounts of polystyrene (PS), polyamide (PA), polyester (PL), ethylene-propylene-diene monomer (EPDM), and alkyl acrylate copolymer (ACM) were also present. Brown and pink were the predominant colors (35% and 23% respectively), followed by grey (12%), white (13%), black (7%) and blue (10%) (Fig. 3c). Considering the shape, four different types were detected (Fig. 3b): films (42%), foams (32%), fragments (18%) and fibers (8%).

The average concentration observed in corals sampled on inner reefs was 1.8 ± 0.75 particles/g, while in colonies from outer reefs was 1.8 ± 0.50 particles/g. Corals sampled at site 4 and site 5 were the most contaminated (3.9 ± 2.1 particles/g and 4.0 ± 1.8 particles/g, respectively), while the ones from site 8 were the least affected (0.28 ± 0.28 particles/g). Shallow corals showed 1.9 ± 0.56 particles/g and deep corals 1.8 ± 0.50 particles/g. Statistical analysis (Fig. 4) showed no significant differences among the eight sampling sites (Kruskal-Wallis H test = 8.1, df = 7, p = 0.322), the two different exposures (Kruskal- Wallis H test = 0.378, df = 1, p = 0.539), the three surveyed coral species (Kruskal-Wallis H test = 2.3, df = 2, p = 0.319) and the two depths (Kruskal-Wallis H test = 0.224, df = 1, p = 0.636).

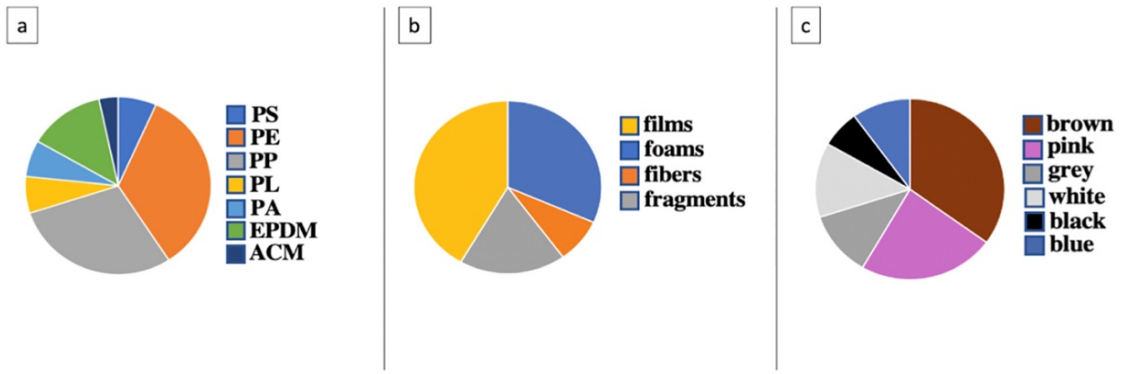

Figure 3: Relative abundance of microplastics in terms of (a) polymer type (PS = polystyrene, PE = polyethylene, PP = polypropylene, PL = polyester, PA = polyamide, EPDM = ethylene-propylene-diene monomer rubber, ACM = polyacrylic elastomer), (b) shape and (c) color.

3.2. Comparison with PAE data

The 22 coral samples displaying microplastics were also surveyed for PAE contamination. For each individual, the concentration of di-methyl phthalate (MEP), di-ethyl phthalate (DEP), di-butyl phthalate (DBP), benzyl butyl phthalate (BBzP), bis(2-ethylhexyl) phthalate (DEHP) and the sum of all PAEs (Σ5PAEs) were correlated with the concentration of plastic particles (items/g) (Table 2). The analysis showed no significant correlation between different PAE contamination (Spearman coefficient,

MEP ρ = 0.000; DEP ρ = 0.363, p = 0.097; DBP ρ = 0.335, p = 0.128; BBzPρ=0.064,p=0.777; DEHPρ= −0.075,p=0.739; Σ5PAEs ρ = 0.143, p = 0.526).

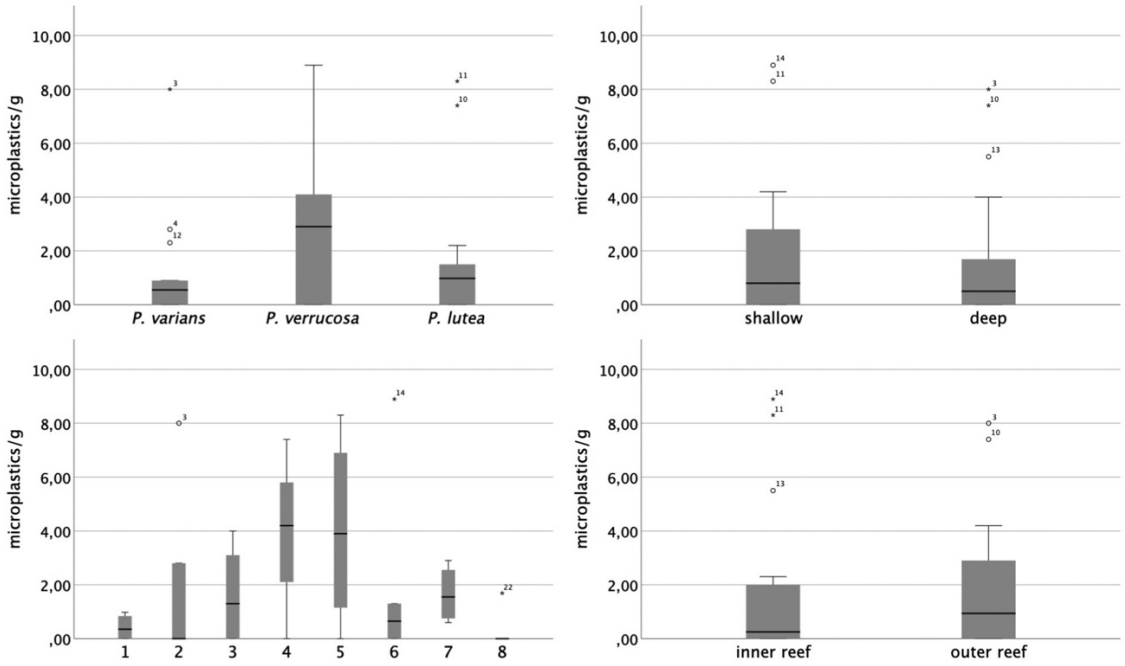

Figure 4: Box plots reporting the microplastic concentrations, according to the different sites, species, exposures and depths. Line in box = median of sampled con- concentrations; Box = 25th to 75th percentiles: bars = min and max values excluding outliers.

4. Discussion

4.1. The Maldivian coral reefs MPs contamination at geographical level

To the best of our knowledge, this study is the first to report the detection of microplastics in reef-building corals from the Maldivian archipelago, and the Indian Ocean overall. A recent work has demonstrated that Maldives could be a potential sink of missing plastics, being among the most affected countries in the Indian Ocean by beaching of plastic particles, as a result of the monsoonal regime, which dominates the Northern Indian Ocean by beaching of plastic particles, as a result of the monsoonal regime, which dominates the Northern Indian Ocean atmospheric and oceanic dynamics (van der Mheen et al., 2020). Here we showed that a great part of the analyzed reef-building coral colonies

of the area are affected by microplastic pollution. The contamination did not display a clear pattern across sites, species, and environments.

ID sample	MPs	DEHP	MEP	DEP	DBP	BBzP	Σ_5PAEs
1	0.70	n.d.	n.d.	n.d.	19.9	1.40	21.3
2	0.98	8.9	n.d.	2.0	11.3	0.70	22.8
3	8.00	n.d.	n.d.	7.3	10.9	n.d.	18.2
4	2.80	n.d.	n.d.	10.0	12.4	1.40	23.8
5	1.50	n.d.	n.d.	5.4	8.5	1.10	15.1
6	3.10	29.7	n.d.	3.7	18.6	0.80	52.7
7	1.10	172.4	n.d.	n.d.	n.d.	n.d.	172.4
9	4.20	0.0	0.0	14.1	16.6	0.70	31.5
10	7.40	11.4	n.d.	5.1	19.3	1.3	37.3
11	8.30	n.d.	n.d.	n.d.	5.9	0.9	6.7
12	2.30	n.d.	n.d.	n.d.	3.9	0.1	4.0
13	5.50	n.d.	n.d.	2.0	13.8	0.2	15.9
14	8.90	n.d.	n.d.	4.2	24.6	0,80	29.7
15	0.80	n.d.	n.d.	n.d.	43.8	2.4	46.0
16	1.30	n.d.	n.d.	3.4	5.3	0.3	9.0
17	0.61	4.0	n.d.	n.d.	3.1	0.2	7.3
18	0.92	15.9	n.d.	6.8	11.4	0.5	34.6
19	0.64	n.d.	n.d.	2.5	4.3	0.4	7.2
20	2.90	64.7	n.d.	n.d.	16.4	n.d.	81.1
21	2.2	n.d.	n.d.	7.7	4.8	0,7	13.3
22	1.7	n.d.	n.d.	n.d.	2,50	0.8	3.3

Table 2: Concentration of microplastics (items/g) and PAEs (ng/g) detected in each sample.

A possible explanation for this outcome may be the large number of sources of plastic debris present along the Maldivian archipelago, the additional contribution of long-distance transport (Ryan et al., 2009), and the mechanism of vertical mixing or biofouling that involves the plastic particles in tropical waters (Obbard, 2018). The average concentration found in this survey (1.8 ± 0.4 particles/g) is close to those reported in previous reports. Ding et al. (2019) investigated 24 different coral species from the Xisha Islands (South China Sea), an area characterized by higher population density, finding concentrations ranging between 0.02 and 1.3 items/g of coral. In Hainan Island, average concentrations of 3.68 ± 3.94 particles cm^{-2} and 5.89 ± 5.15 particles cm^{-2} were found in the small-polyp coral *P. damicornis* and the large-polyp coral *G. fascicularis*,

respectively (Tang et al., 2021). At Fort Wetherill Park in Jamestown, Rhode Island, the scleractinian coral *A. poculata* was also contaminated by microplastics (112 ± 5.01 particles/polyp) (Rotjan et al., 2019). Moreover, it should be pointed out that in these surveys most of the microplastics were in the form of fibrous materials, whereas in our survey only 8% of the total detected particles were fibers. Because of the different sample processing methods and units of measure adopted in these studies, is difficult to compare the information provided. This highlights the lack of harmonization in microplastic studies as an issue to be urgently solved.

4.2. MP characteristics and distribution within the atolls system

Considering the spatial distribution of microplastics, no significant difference between inner and outer reefs was highlighted. Moreover, we found that site 4 (near Magoodhoo island) and site 5 (near Bileddhoo island) showed the highest average particle concentration (3.9 ± 2.1 particles/g and 4.0 ± 1.8 particles/g respectively), while site 8 (Adanga island) resulted in the least impacted (0.28 ± 0.28 particles/g). Being Magoodhoo and Bileddhoo inhabited islands (~ 900 inhabitants), where the solid waste management system is based on “collective collection” and on-site or open-air burning (Saliu et al., 2018), it's reasonable to think that this waste management contributes to coral contamination in these sites. On the other hand, Adanga is a desert uninhabited island, less subject to anthropic pressure. Because the detected microplastics showed clear signs of surface oxidation (presence of hydroxyl and carbonyl groups), we can assert that their genesis was likely to be *via* photo-oxidative degradation of larger plastic items (secondary micro-plastics) transported from the open ocean and trapped in the corals of the inner atoll rim. This finding is in line with our previous study, in which even microplastic contamination of the sea surface in the inner atoll rim (0.31 particles/m^3)

resulted in relatively higher than outside (0.02 particles/m^3), suggesting that the inner atoll rim may represent an accumulation zone (Saliu et al., 2018). In the South China Sea, a similar pattern was observed, with a much higher amount of microplastics in the lagoons (15.9 items L), in comparison with those in outer reef slopes (2.4 items L^{-1}) (Ding et al., 2019). A slightly higher concentration of microplastics was detected in individuals sampled at shallow depth (5–10 m), in accordance with the low density characterizing the pre-dominant detected polymers, polyolefin (PE and PP), already recognized as the most abundant polymer types in the marine environment overall (Erni-Cassola et al., 2019) and in the water surrounding the Magoodhoo island, Faafu atoll (Saliu et al., 2018). To support our result, Ding et al. (2019) found that microplastic abundance declines with increasing depth. However, since even deep coral samples showed relatively high contamination, somehow these polymers were removed from the seawater surface. Possible mechanisms involved in the removal of plastic microparticles from seawater surface are transported by marine snow (Kvale et al., 2020; Long et al., 2015; Porter et al., 2018) and turbidity currents (Pohl et al., 2020), but both have not been examined in the Maldivian area yet. Finally, the highest microplastic concentration, found in *P. verrucosa*, may be explained by considering the branching morphology of this species, which is likely to enable a higher degree of particle entrapment than the massive morphology, typical of the other two analyzed species.

4.3. Correlation between MPs and PAEs

No significant correlations were found between PAEs and particle concentrations. These results are in line with previous studies conducted on other marine invertebrates, such as mussels (Hermabessiere et al., 2019) and solitary ascidians (Vered et al., 2019). Differently, Baini and coauthors (Baini et al., 2017) demonstrated a correlation for

neustonic/ planktonic samples, but analyses were performed after solvent extraction. Under solvent extraction conditions, the bio-organic matrices become “swelled” and the analytes are detected in their bound and unbounded form. Saliu et al. (2020a, 2020b) showed that solvent extraction brings to the release of PAEs from plastic microparticles since they are not covalently bound to the polymers (Saliu et al., 2020a, 2020b). Thus, if large amounts of PAEs are directly released by the ingested plastic material only during the extraction, and the contribution of the amount accumulated in the tissue as the effect of prolonged exposition is negligible, the correlation is undoubtedly more robust even if less informative. In our case, with SPME, only the “unbounded” PAEs were extracted. In fact, the C18 extracting phase of the fibers may only “capture” the “free” species capable of migrating from the matrix (here the coral tissue). Starting from this analytical consideration, it is very likely that in the examined corals the rate of ingestion/egestion of the plastic particles and the rate of the absorption and excretion of the phthalates may not be comparable, and this can be considered the reason for the lack of correlation in the data. Finally, it cannot be excluded that the contamination of PAEs in the tissue is affected by uncontrolled covariates, for instance, repartition with PAEs in the water and the particulate matter. This is a matter that deserves further investigation.

Conclusion

In conclusion, we reported for the first time the presence of microplastics in wild-collected corals from the archipelago of the Maldives. Plastic particles up to 25 μm in size were found in 58% of the analyzed colonies and this may be taken as an alarming sign of the current contamination of the Indian Ocean. The availability at present of only four studies concerning the problem of microplastic contamination in reef-building corals summarizes the scarcity of data and recalls the need for further effort in determining the impacts of this growing issue on the health of coral reef ecosystems worldwide.

Supplementary materials

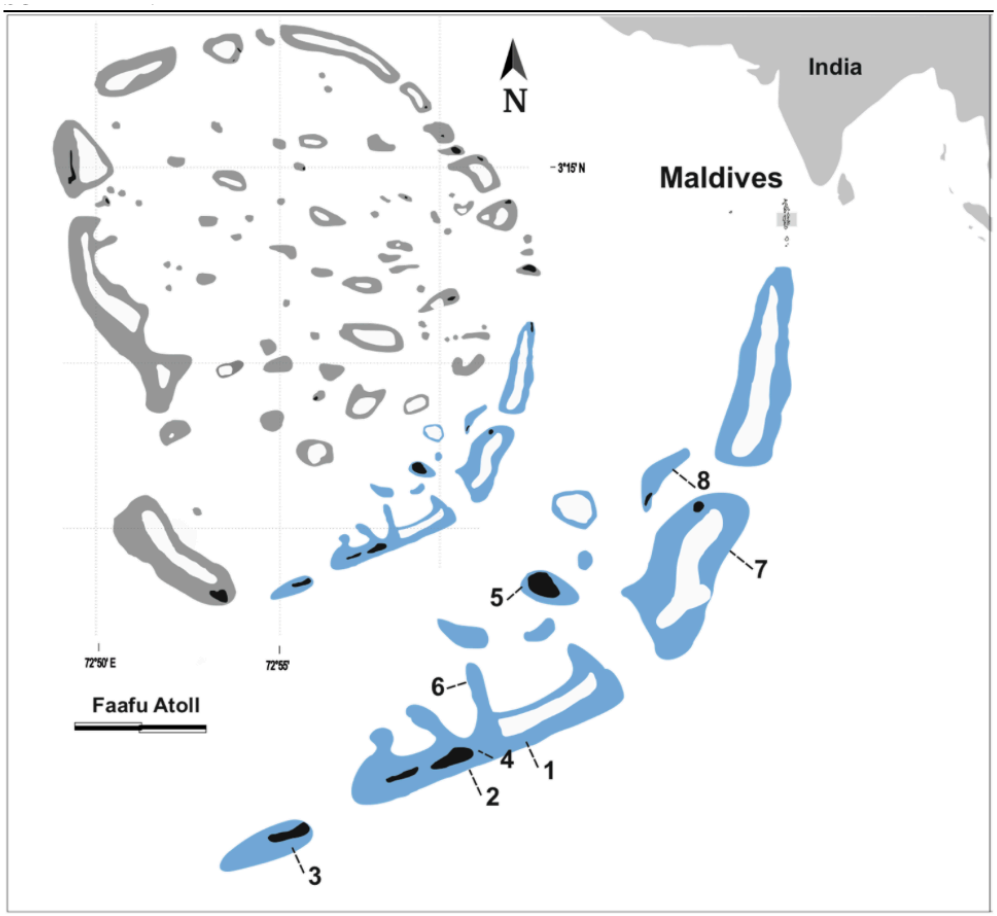

Figure S1: Map of the Faafu Atoll and the sampling area. Numbers indicate the code related to the different sampling location (see Table 1 for a detailed description).

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Detection of microplastics and phthalic acid esters in sea urchins from Sardinia (Western Mediterranean Sea)

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Abstract

The occurrence of microplastics (MPs) and phthalic acid esters (PAEs) in wild purple sea urchins (*Paracentrotus lividus*) of Sardinia (Italy, Western Mediterranean Sea) was surveyed. Specifically, MPs were analyzed in the digestive tract by μFTIR and PAEs in the gonads by SPME-LC-MS/MS. 9 out of 22 specimens resulted contaminated with MPs, and 20 displayed levels of PAEs over the quantification limit. A total of 23 MPs were detected with a maximum concentration of 4 microplastics/individual in the commercially undersized specimens. PAEs displayed an average concentration of 32 ng/g, $\sigma = 5.3$ with a maximum value of 77 ng/g. The most abundant congeners were DEHP (17 ng/g, $\sigma = 4.3$) and DBP (10 ng/g, $\sigma = 2.5$). Statistical analysis showed a correlation between DEHP and fiber concentrations and among the concentrations of MEP, DEP, DBP, and BBzP. Due to the local use of sea urchin gonads as a gourmet delicacy, the potential human exposition to MPs and PAEs by consumption is also discussed.

1. Introduction

The exponential growth of plastic production worldwide and the mismanagement of plastic waste are leading this human-made material to be ubiquitous in the marine environment (Browne et al., 2007). Analysis of a 60-year time series confirms in fact that a significant increase in open ocean plastics occurred in the last decades (Ostle et al., 2019). Concern has been mainly addressed to micro and nanoparticles (Andrady, 2011), which may derive both from the photo-oxidative degradation of the larger plastic items (secondary microplastics) floating on the ocean surface or by the direct discharge in the waterways of plastics that have been tailored at the microscale by the producer to exert specific functions (primary microplastics).

All these particles may threaten marine biota by direct physical interaction, *i.e.* by the entanglement or blocking of the digestive tract after ingestion (Wright et al., 2013), by acting as vectors for alien rafting species, microbial communities, and diseases (Zettler et al., 2013; Lamb et al., 2018) or by leaching out toxic substances associated to the plastic material (Teuten et al., 2007; Koelmans et al., 2013; Atugoda et al., 2021). Since MPs occupy the same size fraction of sediments and of some planktonic organisms, they are potentially bioavailable to a wide range of organisms, actively ingested by low trophic suspension, filter and deposit feeders, detritivores, and planktivores. To date, MPs have been found in a large variety of wild marine animals, such as foraging sea- birds (Wilcox et al., 2015), marine mammals (Fossi et al., 2016; Lusher et al., 2015), fish (Boerger et al., 2010; Foekema et al., 2013), crustaceans (Devriese et al., 2015; Murray and Cowie, 2011), worms and mollusks (Van Cauwenberghe et al., 2015), reef-building corals (Raguso et al., 2022) and even deep-sea inhabitants (Taylor et al., 2016). Recently, it has been also highlighted that microplastic ingestion frequency is slightly higher in grazers and omnivores (33 %) than in filter feeders (17 %), carnivorous and detritivores (21–23 %) (Avio et al., 2020). Besides their physical impact, the finer fractions of MPs and nanoplastics may display an intrinsic toxicity correlated to their capacity to overcome biological barriers (Lai et al., 2022). Specifically, Mattsson and coauthors showed that nanoplastics are transferred through a three-level food chain and can cause brain damage in top consumers, penetrating their blood-to-brain barrier (Mattsson et al., 2017). Toxicity could also arise from the leaching of the other constituents of the plastic material (that are present in the formulation with the plastic polymer) such as residual monomers and plastic additives, or by environmental pollutants concentrated onto the MP surface, since plastic displays a greater affinity to hydrophobic molecules compared to seawater, and the partition on the plastic particle surface may be greatly enhanced by the larger

surface area to volume ratio. Under this view, MPs represent a possible and alternative route of exposure of organic micro-pollutants to marine organisms (Wright et al., 2013).

Among the plastic ingredients that may be leached from MPs, phthalic acid esters (PAEs) have recently gained attention. PAEs are used as plasticizers (*e.g.* to increase the flexibility, transparency, and longevity of the final material) and may represent up to 60 % of the total plastic product weight (Teuten et al., 2007). Since they are not covalently bound to the plastic polymers, they can be released from the plastic material to the surrounding environment, becoming bioavailable to marine organisms (Fossi et al., 2018; Saliu et al., 2019). As for other plastic ingredients, their release may be greatly enhanced during the generation of secondary MPs by the weathering-induced breakdown of the larger plastic debris (Yuan et al., 2022). A recent toxicological assessment showed that PAEs may display endocrine disruptor activity, induce oxidative stress and immunotoxicity in various aquatic organisms (Oehlmann et al., 2009; Zhang et al., 2021); surveys highlighted their occurrence in the marine biota from plankton (Browne et al., 2007) to organisms at the higher level of the trophic chain (Net et al., 2015), including marine mammals and large filter feeders (Fossi et al., 2014; Fossi et al., 2018; Saliu et al., 2022). It was also shown that the concentration of PAEs may display a correlation with the concentration of MPs and of larger plastic items, suggesting that PAEs may serve as plastic tracers (Fossi et al., 2018; Paluselli et al., 2018; Saliu et al., 2019; Panio et al., 2020). However, at present, little is known about the influence of the various environmental factors (*e.g.* temperature, pressure, microbial colonization) on the kinetic of the release of plastic ingredients, especially in the benthic environment (Fauvelle et al., 2021; Isa et al., 2022). Thus, caution in postulating the correlation between MPs and PAEs is required.

The purple sea urchin *Paracentrotus lividus* (Lamarck, 1816) (Echinodermata: Echinoidea) is a keystone species playing a central role in the Mediterranean coastal benthic habitats and trophic cascade (Giakoumi et al., 2012), regulating the organization and structure of shallow macroalgal assemblages (Boudouresque and Verlaque, 2020). Sea urchins are voracious herbivores and are capable of ingesting large amounts of macroalgal biomass, thus acting as an energetic link from shallow water macroalgae to benthic communities (Dethier et al., 2019). It also displays an important commercial value and it is widely exploited as seafood in the Mediterranean and Atlantic Europe, owing its worldwide fame to its gonads, a delicacy generally indicated as "roe" in the fishery and sushi market (Carboni et al., 2012; Furesi et al., 2016). Being a sensitive species, *P. lividus* is exposed to and damaged by several anthropogenic factors that affect the marine environment, such as temperature rise, ocean acidification, toxic algal bloom, and pollution (Migliaccio et al., 2016, 2019; Yeruham et al., 2015; Sukhn, 2010). As reported by recent investigations, the abundance of *P. lividus* wild stocks is declining due to a combination of several factors such as overexploitation, cascading effects related to the overfishing of predators (Ceccherelli et al., 2022; Farina et al., 2022; Grech et al., 2022) and introduction of new contaminant entities in the environment, lately including MPs (Yeruhametal., 2015; Fernández-Boo et al., 2018). Regarding the possible impacts of MPs on sea urchins, it was recently shown by exposition experiments in seawater that they accumulate more MPs in the 45 μm size class in the digestive system (61.39 ± 27.90 particles/g) than in the gonads (18.17 ± 8.27 particles/g), while smaller plastic particles (10 μm) are mostly stored in the water vascular system, specifically in the ring canal (28 ± 7.34 particles/g) and in the stone canal (33 ± 8.98 particles/g) (Murano et al., 2020). Effects of MPs (and nanoplastics) on *P. lividus* embryonic development (Gambardella et al., 2018; Martínez-Gómez et al., 2017; Messinetti et al., 2018; Piccardo et al., 2020;

Thomas et al., 2020) and immune system (Murano et al., 2020, 2021) have been documented, but very limited information is currently available on their occurrence on native wild specimens populating Mediterranean coastal areas. The few available reports point to 1.0 MPs/individual in the Adriatic Sea, almost exclusively represented by synthetic polymers such as PE, PP, PS and PA (Avio et al., 2020), 26 ± 19 MPs/individual in the Aegean Sea (Greece), mainly represented by fibrous particles (97 %) (Hennicke et al., 2021) and 2.6 fibers/individual in the Gulf of Naples, where 67 % was cotton-based 33 % synthetic polymers (polyester) (Murano et al., 2022).

Starting from this basis, in this work, we aimed to assess the occurrence of MPs and PAEs in wild sea urchin specimens sampled on the west coast of Sardinia (Italy), where the sea urchin is an appreciated gourmet delicacy, and its gonads are largely exploited in the local seafood market. Our goal was to determine possible correlation in the occurrence of both these contaminants, and more specifically: a) to investigate the levels of exposure to MPs of *P. lividus* through ingestion in his natural habitat b) to determine the possible contribution of MPs ingestion to the contamination of *P. lividus* gonads by leaching of PAEs c) to establish the PAEs exposure levels in humans caused by consumption of fresh sea urchin gonads.

In our experimental work, we decided to focus on the occurrence of MPs in the digestive tract because it is generally accepted that ingestion is one of the main pathways of MPs uptake: many marine organisms exert limited selectivity between particles and capture anything of appropriate size, while organisms in the higher levels of the trophic chain could passively ingest micro-plastics during normal feeding activity or mistake the plastic particles for their natural prey (Moore, 2008). In the current literature, it was already hypothesized that *P. lividus* may come in contact and accumulate MPs at least

through two different pathways: food ingestion and the water vascular system. Moreover, it was also verified by a lab exposition experiment in seawater that the digestive tract is the organ displaying the higher MP accumulation for larger sizes (Murano et al., 2020). However, at present, the contribution of MP ingestion to the accumulation of PAEs in sea urchin tissues due to leaching (the possible role of MPs as a "*Trojan Horse"* for PAEs) is still scarcely considered in the literature. Under this view, we decided to also research the occurrence of PAEs in the gonads. These organs display a higher concentration of lipids among sea urchin tissues (Montero-Torreiro and Garcia-Martinez, 2003) related to their dual function as storage tissue and reproductive organ (Hughes et al., 2006), and consequently they are expected to be the main target for the accumulation of PAEs as lipophilic contaminants (Sugni et al., 2007). Moreover, being gonads consumed by humans, they may represent a possible way PAEs enter into humans, and the possible associated risk must be carefully assessed, by starting from the evaluation of the dietary intake as proposed in the here presented work.

2. Material and methods

2.1. Samples

Sampling activities were carried out by scuba diving in July 2020. Specifically, 22 specimens of wild purple *P. lividus* were sampled across five sites along the west coast of Sardinia (Italy), representative of its common rocky habitat. A map of the sampling area is reported in Fig. 1, while additional information regarding the sample stations is reported in Table 1. The specimens were divided into two classes according to their test diameter (TD) and the threshold established by Italian national regulation that identifies sea urchins that may be consumed by those that may not: individuals of commercial size

Site and sampling site I.D.	Number of samples	LAT N	LONG E	Mean wind speed±SE (m/s)
Putzu Idu (1)	5	40° 01′44″	8°23′19″	6.1±1.1
Torre dei Corsari (2)	5	39°40′54″	8°26′45″	6.3±1.0
Santa Caterina (3)	6	40°07′24″	8°28′42″	6.5±1.1
Su Pallosu (4)	3	40° 02′57″	8°25′49″	4.4±1.1
Alghero (5)	3	40°34′58.0″	8°15′43.8″	6.1±1.1

Table 1: Sampling site description (with number of samples, coordinates and wind exposure).

(diameter without spines, TD ≥ 50 mm, briefly considered "large" in our work) and the undersized individual (30 ≤ TD < 50 mm, briefly considered "small" in our work). More specifically, the size of the sampled sea urchins ranged between 32 mm and 59 mm indicating specimens that have lived in their respective sites approximately from 3 to 5 years (Loi et al., 2017). In all the specimens the developmental status was sufficient to collect > 0.1 g of gonad sample and perform the SPME extraction, indicating that the individuals were in the most advanced gonad maturation stage. After sampling, the samples were stored at − 20 °C until analysis.

2.2. Sample preparation

To control any possible source of external contamination during sample manipulation, the sample preparation workflow was performed in a dedicated controlled air ISO 6 cleanroom laboratory (temperature: 22 °C; SAS pressure: +15 Pa; SAS brewing rate: 30 vol/h; lab pressure: +30 Pa; brewing rate: 50 vol/h) equipped only with glass and metal apparatus and furniture. The use of plastic material was carefully avoided, and all personnel wore only cotton clothes. From each sea urchin individual, the digestive system and gonads were removed and processed separating one from the other. During the gonad

dissection particular care was taken to avoid contamination from surrounding organs. The digestive tract was submitted to MP's isolation as described further in this paragraph. Gonads were submitted to PAE extraction as described in Section 2.4. The choice of processing only these two organs and with two dedicated procedures was dictated by the need to control the external contamination in the matrices. According to our experience, extensive handling of the samples and application of different procedures on the same matrix display a high risk of contaminating it (Saliu et al., 2020; Raguso et al., 2022). Specifically, the isolation of MPs from the digestive tracts was carried out by following the protocol described by Avio and coauthors (Avio et al., 2015) with slight variations. Briefly, the digestive tracts were dissected by using a stainless-steel scalpel, weighted onto an analytical balance, and then the mixture was heated at 50 °C for 2 h. The completion of the digestion process was assured by the clear appearance of the solution with no traces of residual tissues. Finally, the digestate was filtered onto a cellulose nitrate filter (Whatman^{TM}, 3 μm) and submitted to μFTIR analysis.

2.3. Identification of MPs by μFTIR

MPs were identified and characterized by using a Spotlight 200i FT- IR (Perkin Elmer), following the point mode acquisition procedure previously described (Saliu et al., 2021). IR spectra were recorded by using the diamond attenuated total reflectance (ATR) unit and a liquid nitrogen-cooled mercury cadmium telluride (MCT) single detector, applying a wave-number range 4000–400 cm^{-1}, resolution of 4 cm^{-1} and 32 co-added scans. The patented COMPARE^{TM} spectral comparison algorithm (by Perkin Elmer) was used to confirm the synthetic origin of the particles as previously reported (Saliu et al., 2018). Examples of the detected items with the respective spectrum are reported in Fig. 2.

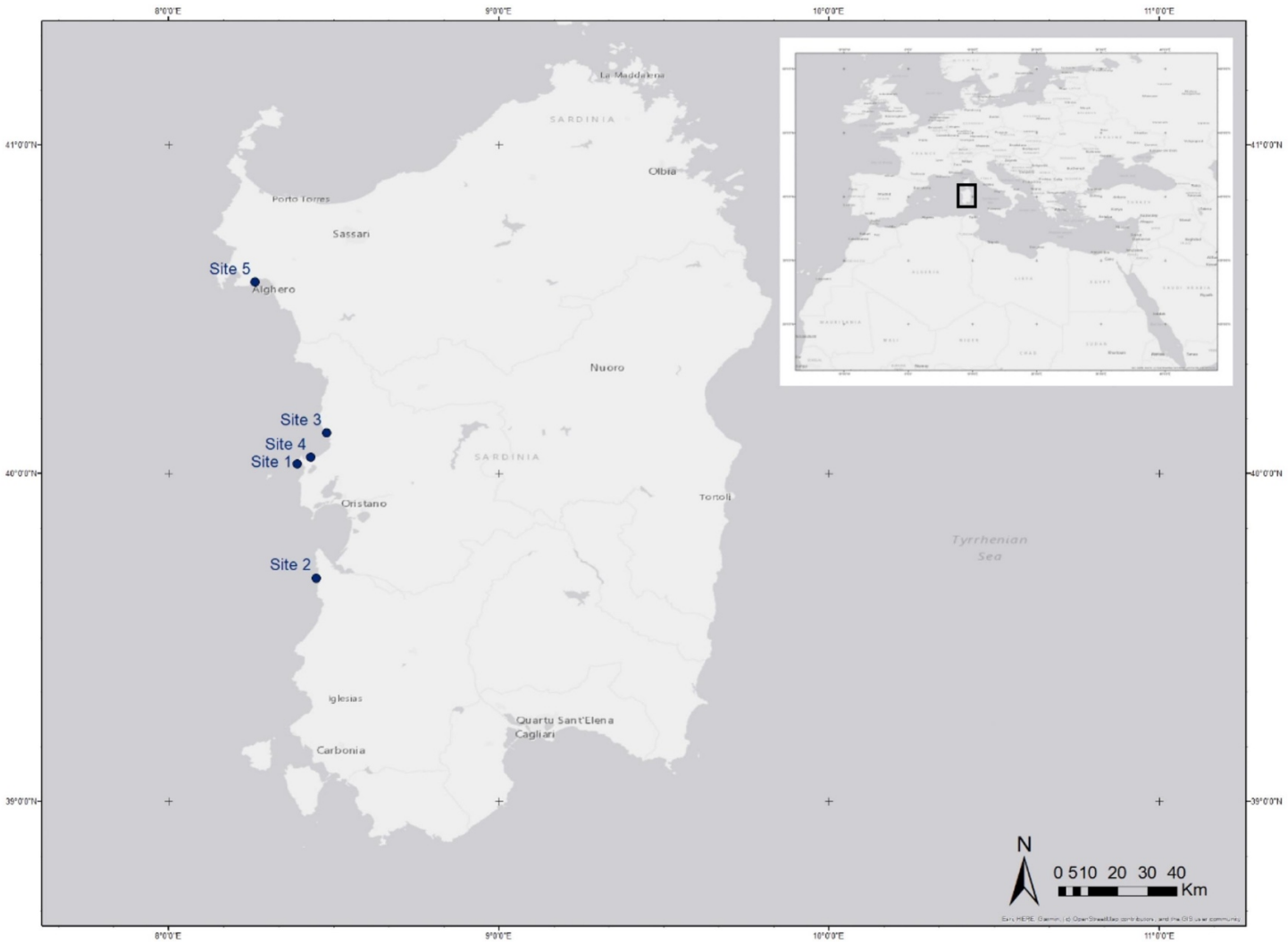

Figure 1: Area of study. The map shows the sampling stations in Sardinia (*Putzu Idu* = 1; *Torre dei Corsari* = 2; *Santa Caterina* = 3; *Su Pallosu* = 4; *Alghero* = 5).

The complete list of the identified MPs is reported in the supplementary material associated with this article.

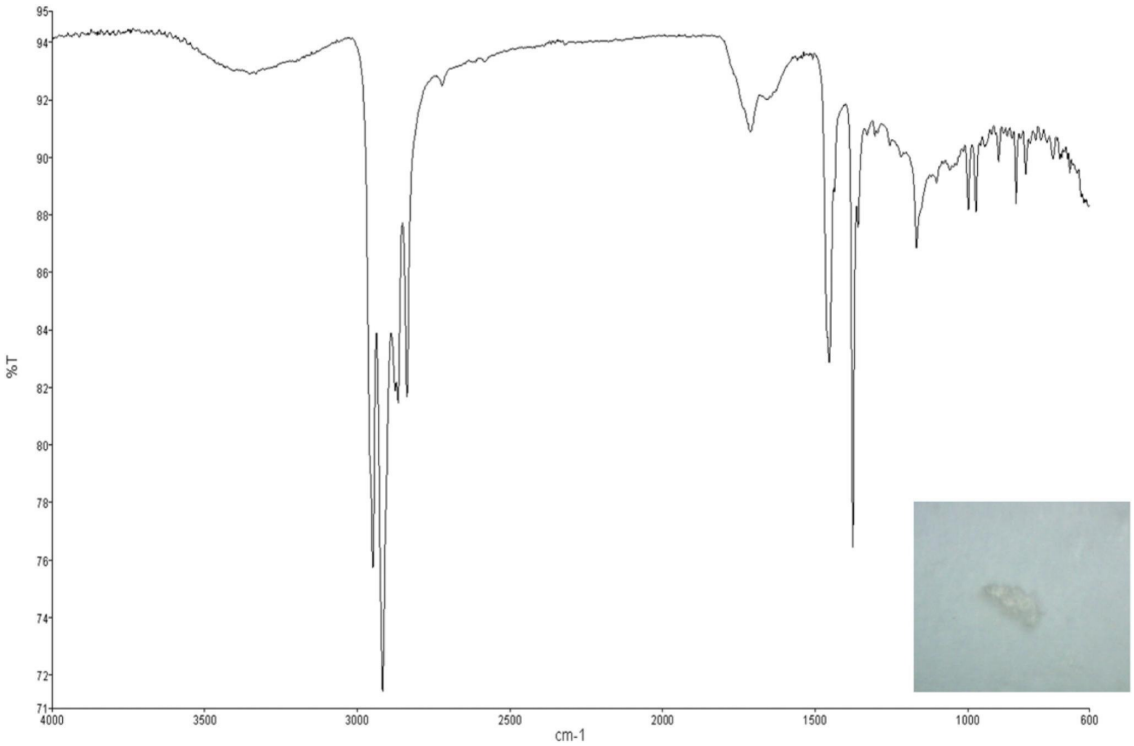

Figure 2: Representative picture of a plastic microparticle with the respective μATR-FTIR spectrum.

2.4. Determination of PAEs by SPME-LC-MS/MS

Dimethyl phthalate (MEP), di-ethyl phthalate (DEP), di-butyl phthalate (DBP), benzyl butyl phthalate (BBzP), bis(2-ethylhexyl) phthalate (DEHP) were researched in the sea urchin gonads by applying a SPME-LC-MS/MS method previously described in Saliu et al. (2020) with slight variations. C18 SPME fibers (purchased from Supelco) were inserted into the gonads and left for 30 min to enable the extraction of the analytes, running the procedure at the pre-equilibrium condition of the time extraction curve. After that, the fibers were placed into a glass vial insert containing 100 μL of methanol: water 90:10 and stirred for 40 min at 40 °C to perform the elution of analytes. Then, the fiber was taken out from the vial and the vial was directly transferred into the instrument

autosampler for the subsequent analysis. LC-MS/MS analysis was performed by using a ThermoScientific TSQ quantum access max instrument. A ThermoFisher Accucore C18 column was used for performing the chromatographic separation of the phthalates, by eluting at 0.6 mL/min with a gradient program from 85 to 96 % of methanol (solvent B) and acidified water (solvent A, 0.1 % of formic acid). The injection volume was set up at 20 µL. PAEs were detected by applying a selected reaction monitoring (SRM) of the target ions, with one qualifier and quantifier for each phthalate. Calibration of the system was obtained by using a standard calibration mixture and labeled internal standards as described in Saliu et al., 2021.

2.5. Statistical analysis

Statistical analyses were performed using SPSS ver. 28 (IBM, New York). All data are expressed as mean ± standard error (SE). Shapiro–Wilk test of normality highlighted the non-normal distribution of microplastic concentrations. The Kruskal-Wallis test was applied to explore differences in particle concentration across classes (sites and sizes). Spearman correlation was performed to investigate the relationship between plastic particles, five PAEs (DBP, BBzP, DEP, DEHP, DMP), and their total (Σ5PAEs).

3. Results

3.1. MPs identification by µFTIR

Table 2 reports the results of the MP analysis carried out on the 22 specimens of *Paracentrotus lividus* surveyed in this study. MPs were found in 9 specimens with a concentration of up to 4 particles/individual, while 13 resulted in not being contaminated. Specifically, a total number of 23 particles in the size range 28–803 µm were observed, determining an average of 1.0 ± 0.30 particles for individuals in the whole data set and of 11 ± 4.2 particles for g of the digestive tract. The size distribution of the detected

particles is reported in Fig. 3. The 25–50 μm class was the most abundant in the whole dataset and it's easily visible that the more the size class increases, the more the number of detected particles decreases. Moreover, particles larger than 250 μm were present only in "*big"* specimens, while particles in the class range 25–50 μm were most abundant in "*small"* individuals (Fig. 4). By shape, 22 % of the particles were fibers and 78 % were non-fibers. Specifically, 56 % were fragments, 13 % were foams and 9.0 % were films. Four different colors were observed: grey (65 %), brown (22 %), black (9.0 %), and blue (4.0 %). Considering the constituting polymers, 52 % of the plastic particles resulted be made of polyolefin (PE/PP), 13 % of synthetic rubber (EPDM), and 8.0 %, of polyurethane (PU). A same percentage equal to 9.0 % resulted in polystyrene (PS), polyamide (PA), and rayon/cellulose. In addition to the plastic particles, we identified some mineral particles (mostly calcite and smithsonite), probably derived from the grazing activity of sea urchins (as they were found in the digestive tract). Fig. 5 reports the distribution of MPs obtained by considering the total individuals for each different site and the distribution of MPs considering the two size classes adopted in this survey. Specifically, site 2 named *Torre dei Corsari* displayed the highest level of contamination (2.4 ± 0.68 items/individual), while *Su Pallosu* resulted in the least impacted site with no MPs found in any of the sampled sea urchins. Considering the concentration as particles per gram of the digestive tract, *Su Pallosu* was confirmed as the least polluted, whereas the highest concentration was found in *Putzu Idu* (23 ± 16 items/g). However, no statically significant differences were highlighted among the sites both in terms of items/ individual and items/g (Kruskal-Wallis H test = 8.1, df = 4.0, ρ = 0.09; Kruskal-Wallis H test = 7.5, df = 4.0, ρ = 0.11). Considering the different sizes of the specimens, the "*small*" sea urchins (under the commercial size) showed an average concentration of 1.4 ± 0.45 items/individual and of 16 ± 7.1 items/g, while the "*big*" specimens (in the

commercial size) showed an average concentration of 0.60 ± 0.40 items/individual and of 3.7 ± 2.5 items/g. However, the statistical analysis did not highlight any significant difference for these two size classes both in terms of plastic particles per gram of digestive tract and per individual (Mann Whitney *U* test, ρ = 0.09 and ρ = 0.12).

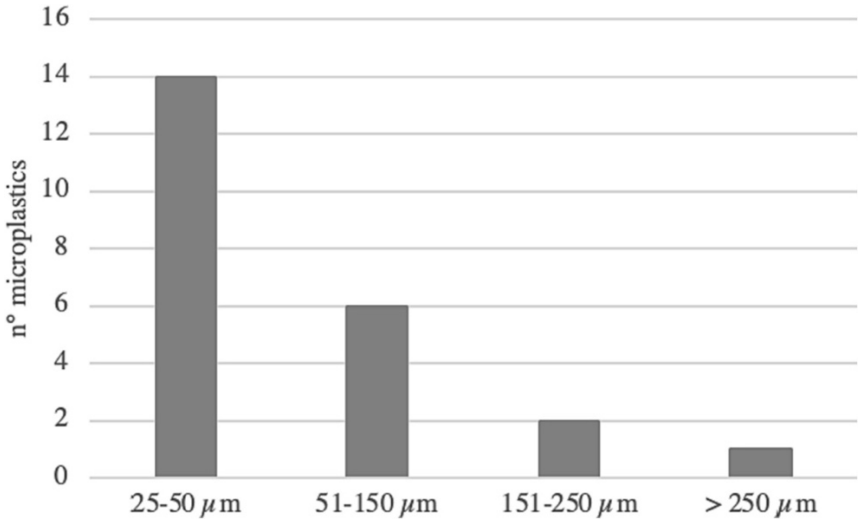

Figure 3: The size distribution of all the microplastics detected in the digestive systems of the collected sea urchin specimens. The size of each item was calculated as the geometric mean of the longest and widest dimension perpendicular to the length, measured using the digital images collected by the Spectrum 10 ESTM software (Perkin Elmer).

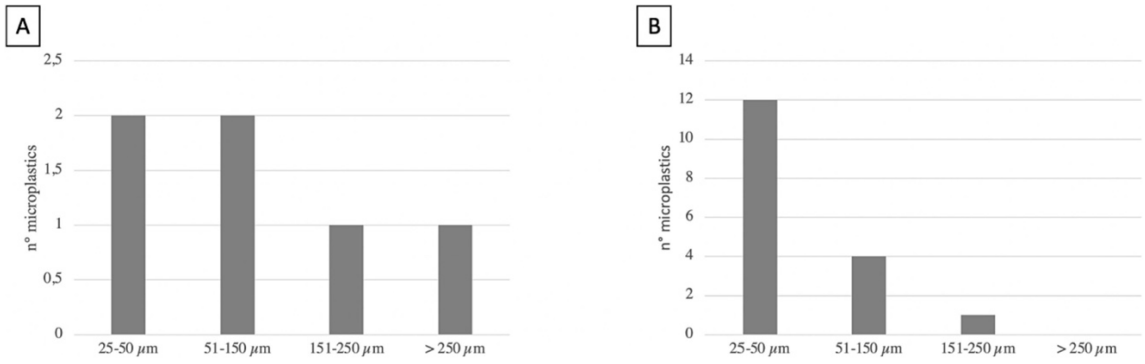

Figure 4: The size distribution of the microplastics detected in the digestive systems of the sea urchin *small* (A) and *large* (B) specimens analyzed.

Sample I. D.	Size	Sampling site	MPs/ g	MPs/ individual	Polymer, shape, color
p.idu1	Small	*Putzu Idu*	22.2	2	1 PU fiber, grey 1 PE film, grey
p.idu2	Small	*Putzu Idu*	9.1	1	1 EPDM fiber, brown
p.idu3	Small	*Putzu Idu*	80.0	4	1 PE film, brown 1 PE film, grey 1 PE film, grey 1 EPDM film, grey
p.idu4	Small	*Putzu Idu*	0.0	0	/
p.idu5	Big	*Putzu Idu*	0.0	0	/
t.cors1	Small	*Torre dei Corsari*	16.6	2	1 EPDM film, grey 1 PP foam, grey
t.cors2	Small	*Torre dei Corsari*	40.0	4	1 PU fragment, brown 1 PE film, grey 1 PE film, grey 1 PE film, grey
t.cors3	Small	*Torre dei Corsari*	20.0	3	1 PE film, grey 1 PS film, brown 1 PE fragment, black
t.cors4	Big	*Torre dei Corsari*	15.0	3	2 Rayon fiber, grey 1 PA fiber, grey
t.cors5	Small	*Torre dei Corsari*	0.0	0	/
s.cat1	Big	*Santa Caterina*	0.0	0	/
s.cat2	Big	*Santa Caterina*	0.0	0	/
s.cat3	Big	*Santa Caterina*	0.0	0	/
s.cat4	Big	*Santa Caterina*	0.0	0	/
s.cat5	Big	*Santa Caterina*	0.0	0	/
s.cat6	Small	*Santa Caterina*	7.1	1	1 PP foam, blue
p.usu1	Small	*Su Pallosu*	0.0	0	/
p.usu2	Small	*Su Pallosu*	0.0	0	/
p.usu3	Small	*Su Pallosu*	0.0	0	/
al.ro1	Big	*Alghero*	21.4	3	1 PA foam, brown 1 PE film, grey 1 PS film, black
al.ro2	Big	*Alghero*	0.0	0	/
al.ro3	Big	*Alghero*	0.0	0	/

Table 2: Occurrence of MPs in the digestive tracts of the surveyed sea urchin specimens. (size: big = >50 mm, small = <50 mm; polymers: PU = polyurethane, PE = polyethylene, PS = polystyrene, PA = polyamide, PP = polypropylene).

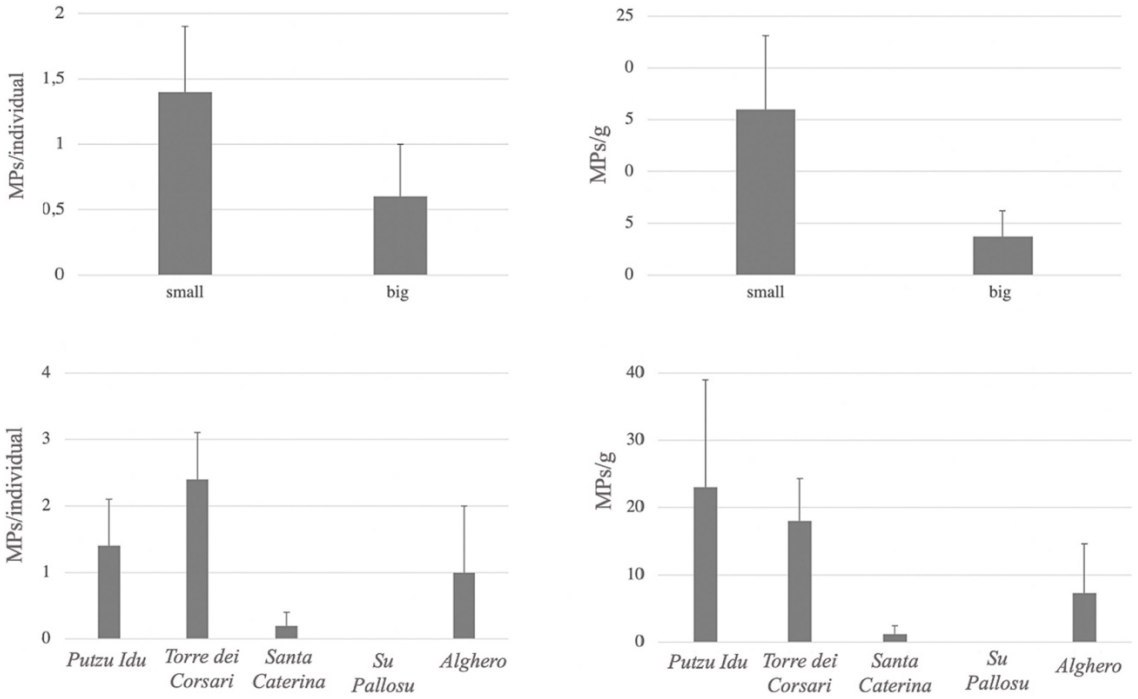

Figure 5: Comparison of the microplastic distribution detected in *small vs big* individuals expressed as items/individual (up left) and items/g (up right) and of the distribution detected in the five different sites as items/individual (down left) and items/g (down right).

3.2. Identification of PAEs by LC-MS/MS

The results of the analysis carried out on the sea urchins by SPME- LC/MSMS are reported in Table 3. PAEs were detected in all the samples with an average concentration of 32 ng/g, $\sigma = 5.3$. The maximum concentration was 77 ng/g and was found in a sample from site 5 (sample 20). The most abundant congeners were DEHP with an average of 17 ng/g, $\sigma = 4.3$, and DBP with an average of 10 ng/g, $\sigma = 2.5$. The maximum concentration of DEHP was found in sample 20, site 5 with 73 ng/g whereas the maximum concentration of DBP was found in sample 15, site 3 with 33 ng/g. Statistical analysis did not highlight any significant differences among the different sites for the concentration retrieved of the surveyed congeners (DEP: Kruskal-Wallis H test = 6.3, df = 4.0, $\rho = 0.18$; DBP: Kruskal-Wallis H test = 1.3, df = 4.0, $\rho = 0.86$; BBzP: Kruskal-Wallis H test = 2.7, df = 4.0, $\rho = 0.62$; MEP: Kruskal- Wallis H test = 3.3, df = 4.0, $\rho = 0.50$; DEHP: Kruskal-Wallis H test = 3.3, df = 4.0, $\rho = 0.51$; ΣPAEs: Kruskal-Wallis H

test = 3.5, df = 4.0, ρ = 0.48). Significant differences were instead highlighted for the relative concentration of each congener (*i.e.* MEP, DEP, BBZP, DBP, and DEHP) considering the sampling sites. No significant differences were found among the PAE concentration detected in small and big individuals (DEP: Kruskal-Wallis H test = 0.50, df = 1.0, ρ = 0.48; DBP: Kruskal- Wallis H test = 0.16, df = 1.0, ρ = 0.69; BBzP: Kruskal-Wallis H test = 1.2, df = 1.0, ρ = 0.27; MEP: Kruskal-Wallis H test = 1.3, df = 1.0, ρ = 0.26; DEHP: Kruskal-Wallis H test = 1.0, df = 1.0, ρ = 0.31; ΣPAEs: Kruskal-Wallis H test = 0.74, df = 1.0, ρ = 0.39).

Sample I.D.	DEP	DBP	BBzP	DEHP	MEP	$\sum_5$PAEs
p.idu1	0.0	1.6	0.0	44.0	0.0	45.0
p.idu2	0.0	0.0	0.0	48.0	0.0	48.0
p.idu3	0.0	5.6	0.0	0.0	0.0	5.6
p.idu4	11.0	39.0	0.0	16.0	14.0	79.0
p.idu5	8.8	34.0	0.0	20.0	7.8	70.0
t.cors1	0.0	5.6	0.0	18.0	0.0	24.0
t.cors2	4.1	10	0.0	0.0	0.0	14.0
t.cors3	3.6	3.4	0.0	0.0	0.0	7.0
t.cors4	11.0	28.0	0.0	20.0	1.8	61.0
t.cors5	14.0	12.0	0.0	0.0	0.0	26.0
s.cat1	0.0	1.7	0.0	33.0	0.0	35.0
s.cat2	3.4	0.0	0.0	0.0	0.0	3.4
s.cat 3	0.0	4.1	0.0	30.0	0.0	34.0
s.cat4	7.9	12	0.0	0.5	0.0	20.0
s.cat5	5.4	33	3.5	2.7	7.0	52.0
s.cat6	3.3	15	0.0	4.0	0.0	22.0
p.usu1	0.0	0.0	0.0	37.0	0.0	37.0
p.usu2	0.0	6.2	0.0	0.0	0.0	6.2
p.usu3	0.0	4.6	0.0	0.0	0.0	4.6
al.ro1	0.0	4.6	0.0	73.0	0.0	77.0
al.ro2	3.2	5.7	0.0	20.0	0.0	29.0
al.ro3	0.0	4.4	0.0	0.0	0.0	4.4

Table 3: PAE concentration (ng/g) detected in the gonads of the different sea urchin samples.

3.3. Multivariate statistical analysis on MPs and PAEs dataset

To highlight a possible leaching of PAEs from ingested MPs into the sea urchin tissues, we tested by rho Spearman the correlation between PAEs and MPs in all the surveyed individuals. No correlation was found between the sum of PAEs and the total MP concentration. A significant correlation was highlighted between DEHP and fiber concentration (rho = 0.44, alpha = 0.05) instead. Moreover, a correlation between DMP,

DEP, and DBP was highlighted (rho = 0.70, 0.67, 0.61 respectively) and be- between BBzP and MEP (rho = 0.43). In addition, the datasets including the PAEs and MPs concentration retrieved from the 22 specimens were submitted to principal component analysis (PCA). The variables related to the polymer and size distributions were also considered in additional runs (all the data were expressed as counts/g for uniformity). As reported in Fig. 7, the two principal components of the PCA explained 43 % of the total variability. Specifically, the PC1 and PC2 scatter plots (Fig. 7) displayed a similar pattern among big and small specimens. A large part of the small individuals (samples 10,17, 16, 18, 19) showed high values of BBzP and was mostly characterized by items blue in coloration, as well as most of the big individuals (samples 15, 12, 22, 11, 13, 21, 14). Concerning the different sites, PCA highlighted that site 1 was mostly influenced by PC1 where the major contribution was given by MEP and PU concentrations (variable contribution 33 % and 49 % respectively) and was characterized by the presence of non-fibers within the size range 51–150 μm. Even site 2 showed higher influence from PC1 and a major contribution was given by BBzP (31 %), EPDM (35 %), PP (12 %), and PE (89 %) concentrations and displayed microplastics mainly fragments and foam in shape. Site 3, 4, and 5 resulted to be very similar, being characterized by the high value of BBzP and presenting mostly blue items. Samples 3 and 9 were well separated from the others, characterized by low levels of BBzP; particularly sample 3 presented a high value of EPDM and particles in the 25–50 μm class range, while sample 9 was characterized by the high presence of particles larger than 250 μm and rayon.

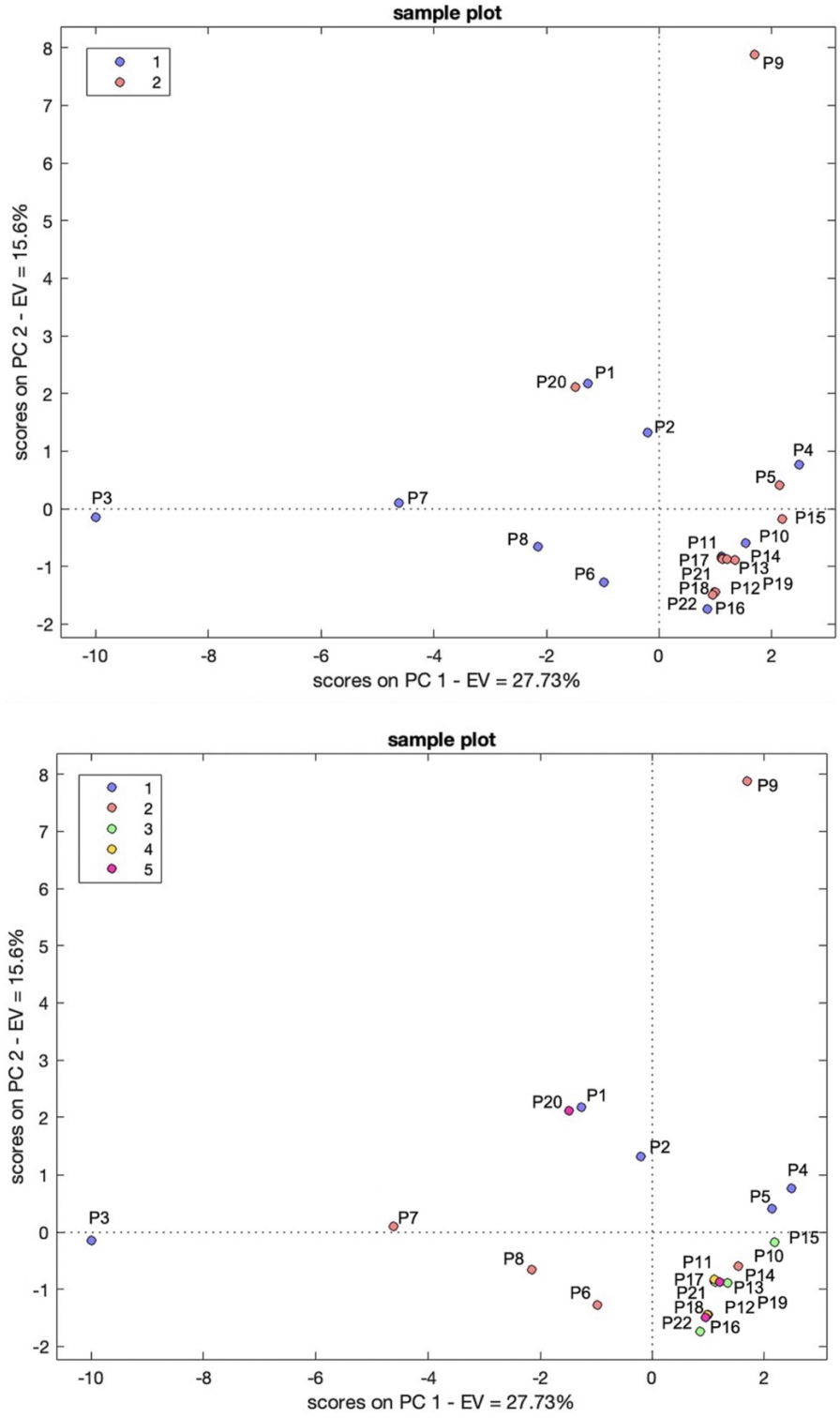

Figure 7: Loadings and scores plots from the PCA on PAE and MP concentration dataset of the 22 sea urchin specimens. A slight differentiation along the F1 is observed.

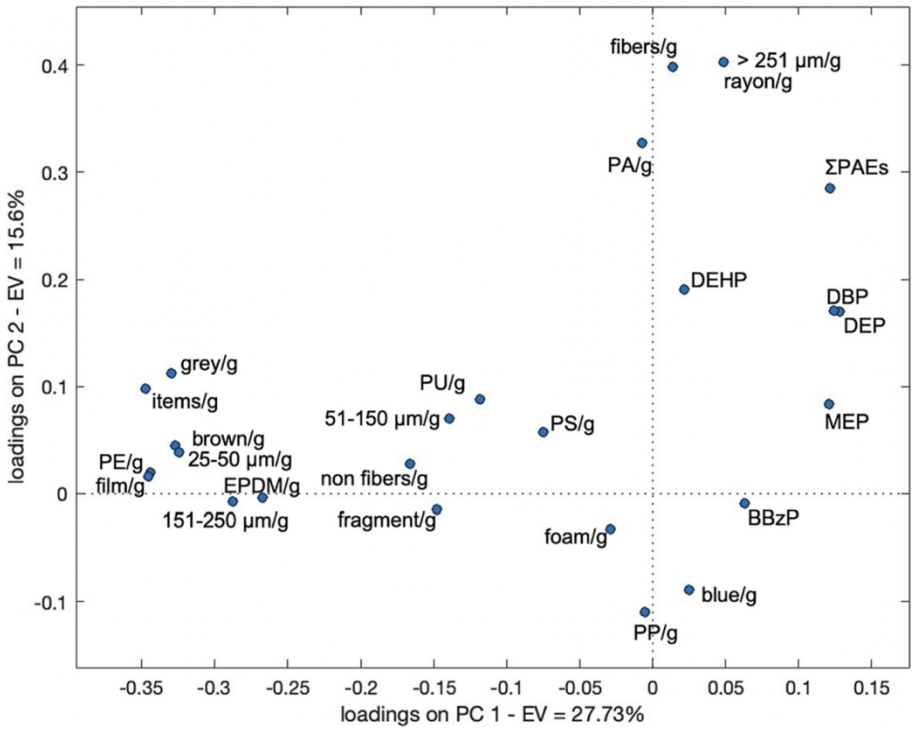

Figure 7: (*continued*).

4. Discussion

4.1. MPs as contaminant in *P. lividus*

In the current scientific literature, it is widely reported that marine organisms may ingest plastics as a result of visual or tactile misidentification (Moore, 2008). In several cases, it was shown that they may be attracted by flavoring organic compounds on the plastic surface (Procter et al., 2019). Being sea urchin benthic animals, colonizing rocky substrates, and characterized by a grazing feeding behavior, they are naturally exposed to MPs lying on the seafloor. Moreover, sea urchins are one of the most common macro-grazers of seagrasses, which recently have been suggested as a potential sink of plastic particles and as an efficient way for microplastics to enter coastal food webs (Gutow et al., 2016). Thus, it is very likely that micro and nanoplastics are ingested by sea urchins because wrongly recognized as food. The results of the here presented study confirm the ingestion of MPs by a wild sea urchin, as we found a total of 23 plastic particles within the size range 28–803 µm in 9 (41 %) of the surveyed specimens, determining an average

of 11 ± 4.2 items/g and 1.0 ± 0.31 items/individual with a maximum concentration of 4 items per individual detected in 3 individuals. These concentrations are in line with the ones previously found in two of the three available surveys in the current literature, which were carried out in other areas of the Mediterranean Sea and specifically in the Gulf of Naples by Murano and coauthors (Murano et al., 2022), reporting a mean of 2.6 fibers/individual, obtained by analyzing the gonads, coelomic fluid and digestive system of *P. lividus* and a survey in the Adriatic Sea carried out by Avio and coauthors (Avio et al., 2020), reporting an average concentration of 1.0 MPs/individual retrieved from the soft tissues. The third available report by Hennicke et al. (2021) obtained by surveying specimens sampled in the Eastern Aegean Sea, reported a concentration of 26 ± 19 MPs/individual body content, which is one order of magnitude higher than our results and the ones of the other two cited works. This difference, besides a possible level of contamination in the area, may be also related to the different filter analysis approach performed by Hennicke et al. (2021), since the items/individual were determined by extrapolating the number of microplastics found on 10 filters. In our survey, no significant difference in spatial distribution was highlighted, except for site 4 where no contaminated individuals were detected. This finding seems in line with the more sheltered position of site 4 with respect to the similar exposure to the winds dominating the west coast of the island of the other sites (Table 3). Looking at the two different sizes characterizing the analyzed specimens, higher levels of contamination were found in *small* individuals both in terms of items/g and items/individual (16 ± 7.1 items/g and 1.4 ± 0.45 items/individual respectively). This finding is in line with previous studies in which it has been demonstrated that young individuals may ingest more plastic particles than older ones, because of two reasons: they can feed three times as fast as older ones, and the discharge from their bodies can be prevented by the smaller anus (Moore and

McPherson, 1965; Feng et al., 2020). Considering that the commercial size of *Paracentrotus lividus* is limited by law to specimens larger than 50 mm diameter Sardinia (TD > 50 mm), from November to April the most contaminated sea urchins should not be consumed by humans. However, despite regional decrees concerning fishing periods issued to safeguard the stocks and avoid the population collapse (that in the last year became even tighter with a complete ban on sea urchin fishing till 2025), the illegal collection and sale of undersized specimens are unfortunately frequent and largely documented together with the occasional removal by recreational fishermen (Pais et al., 2007).

Regarding the size of the particles, we noticed that in the digestive tract, particles ranged between 801 and 25 μm with most of them detected in the 51–150 μm range (Fig. 3). The distribution does not follow the classical 1D-fragmentation model expected in the marine environment (the slope in the logarithmic scale does not follow a linear trend) and it is observed a substantial shortage of particles in the smaller sizes compared to the distribution commonly observed in the surface seawater (Cózar et al., 2014), especially for the larger specimens (Fig. 4). This is in agreement with the results obtained by Murano and coauthors (Murano et al., 2020), indicating a major accumulation of the smaller particles (10 μm) in the water vascular system, and a lack of these particles in the digestive system. According to the authors, these differences in the uptake could be related to a potential sorting action of the madreporite, facilitating the penetration, as well as excretion mechanism, of the smaller particles but also to the dynamics of the retention and egestion processes and the capability of organs to retain the particles of different size. It must be pointed out that at present data regarding the transfer of MPs from the digestive tract to the other tissues (rates and amounts) are not available. In our study, since we used μFITR for MP identification, due to the physical limitation of the infrared radiation, we

were not able to identify particles smaller than 20 μm. However, data still clearly confirm the lack of smaller size, which may indicate a possible active excretion and/or translocation process. Finally, from the plastic polymers identification additional consideration may be drawn. We found that polyolefin (PE/PP) was the most abundant polymer, corresponding to 52 % of the total encountered. They are characterized by low density (0.90 g/cm^3) and expected to float in the seawater, therefore, since sea urchins are benthic organisms, it is reasonable that mechanisms such as biofouling, marine snow or turbidity currents brought them on the seabed (Kvale et al., 2020; Pohl et al., 2020). In addition, an important role as a pathway for MPs may be played by seaweeds and seagrass: for instance, the collection of suspended MPs and transfer to the marine benthic herbivore *Littorina littorea* was described (Gutow et al., 2016). In this case, it was asserted that the snail does not distinguish between algae with adherent microplastics and clean algae without microplastics. To date, the role of seaweed and seagrass in determining sea urchin MP contamination in environmental conditions has still not been totally elucidated. By comparing *Paracentrotus lividus* with *Psammechinus miliaris*, a sea urchin with strong omnivore dietary habits, Suckling recently highlighted the species-specific response to MPs in sea urchins (Suckling, 2021), and how *P. lividus,* being a strong herbivore and consuming softer items (*e.g.* biofilms, algae), results more likely sensitive to MPs. With the current available data, the possibility that some selection is operated during the grazing activity cannot be excluded as well as the fact that also the development stage may play an important role. Under this respect, recently Thomas et al. (2020) showed in a feeding study carried out *in vitro* with 1–230 μm particles that the larvae of *Paracentrotus lividus* more readily ingested polymethylmethacrylate than polystyrene particles. It must be pointed out that, to the best of our knowledge, at present no data obtained by feeding experiments with adult *P. lividus* individuals are available,

and most of the information is related to experiments carried out with specimens exposed to MPs in seawater but not fed.

4.2. Correlation between MPs and PAEs

As reported in Section 3.3, our data did not show a clear correlation between the total concentration of MPs in the digestive tract and the levels of PAEs in the gonads, and thus did not confirm the contribution of MPs by leaching to the contamination of sea urchin tissues. On the other hand, a correlation between the concentration of fibers and DEHP was highlighted. In addition, MEP, DEP, BBzP, and DBP resulted correlated, but not correlated with MPs. This may suggest that the concentration of some PAEs in the sea urchin tissues might be more influenced by the environmental background levels (concentration in the seawater), whereas DEHP may be carried out by the ingested fibers and act as a "*Trojan Horse"* for PAEs. Even if we did not collect seawater samples during our sea urchin monitoring activities, several reports indicated that DEHP is the most abundant congener found in the seawater in this area: *e.g.* de Lucia et al. (2014) reported concentrations of DEHP ranging from 9.00 ng/to 13.74 ng/g in surveys carried out in July 2012 and July 2013, Fossi et al. (2012) levels of DEHP equal to 23.42 ± 32.46 ng/g; data collected by Paluselli et al., 2018, in the northwestern Mediterranean Sea along the whole water column till 30 m depth displayed values for ΣPAEs ranging from 130 to 1330 ng L^{-1} (av. 520 ng L^{-1} ± 313.3 ng L^{-1}) with maximum near the bottom and the lowest values at the sea surface. In this case, DEHP (9.3–91.6 %) was the most abundant PAE, followed by DiBP (3.8–78 %) and DnBP (2.1–44.8 %), while DMP, DEP, and BzBP were the least abundant (0.2–2 %). Considering the partition coefficient of DEHP and the reported concentration, the expected levels of DEHP in the sea urchin gonads (assuming an average lipid content in the gonad of 20 %) should be several orders of magnitude higher than the average concentration found in our study. This might indicate

that passive diffusion is not the mean of PAE contamination in sea urchins, that possible mechanisms of PAE excretion and metabolism are occurring, and that the contribution of fibers in sequestering PAEs from the seawater and/or the occurrence of leaching when fibers are ingested might be relevant. All these hypotheses must be carefully examined in future lab feeding experiments. Under this view, also the residence time of MPs in the digestive tract and kinetic rates in the metabolization of PAEs are important variables that may cause the differences observed in the MPs and PAEs profiles. In this context also the choice of the analytical procedure for the extraction of PAEs is highly relevant. In fact, by SPME only the "free and unbounded" fraction of PAEs is extracted, thus the detected PAEs are those that accumulated in the tissue (in our specific case in the gonads) due to the uptake from seawater or by leaching after ingestion (this second hypothesis needs confirmation by feeding experiments). Differently, when solvent extraction is used, a strict correlation is more easily highlighted, since PAEs might be chemically extracted directly from the MPs occur- ring in the sample matrix. However, in this case, the analysis does not demonstrate the occurrence of the leaching process and ultimately the role of MPs as Trojan horses for PAEs in the marine organism. As underlined in the introduction, at present, for *Paracentrotus lividus* there are no data available that support the hypothesis of the leaching of PAEs from ingested MPs to the other organs. Also, the toxicity and the synergistic effect of MPs and PAEs have still not been elucidated. To the best of our knowledge, only a very recent investigation carried out by Beiras and coauthors (Beiras et al., 2021), highlighted how the leaching of contaminants might be the key factor in determining toxicity in sea urchins. In this study, the authors compared the toxicity measured by the sea-urchin embryo test (SET) of plastic manufactured with conventional oil-based and alternative formulations. Among plasticizers, they only tested

Diisodecyl-phthalate (DIDP) at 2.5 g/L concentration level but they found no toxicity associated with the leaching (EC 50 < 100 mg/L).

In this study, we didn't perform analysis to distinguish among male and female specimens and neither to determine the gonad maturation stage because we wanted to avoid an excessive sample manipulation that may easily end up in external contamination and a possible overestimation of MPs and PAEs (Saliu et al., 2020). For the same reason, we did not perform an analysis of the phospholipid content in the gonads and of the related fatty acid profiles. This analytical determination requires the use of organic solvent for the extraction and the use of considerable amounts of the available biological material. Instead, SPME is carried out solventless and with the direct contact of the SPME fiber with the gonad matrix in a non-destructive manner (Saliu et al., 2019). Other analytical techniques may be adopted to obtain a simultaneous determination of all these different molecule classes (polymers, lipids, and PAEs) in the same chromatographic run *e.g.* pyrolysis-GC/MS (Saliu et al., 2022), however a method for the sea urchin gonads is not available in the current literature and must be implemented and validated. As suggested by an anonymous reviewer, the information regarding the sex of the sea urchin specimens and the maturation stage of the gonads may be relevant to explain the patterns observed in the PAE profiles. To comment on the data obtained in the here presented study it is possible to rely on the information provided by a recent study carried out in the same area, which highlighted a sex ratio of almost 1:1 for males and females (Loi et al., 2017). Thus, we may consider a realistic equal distribution in our samples. Moreover, we carried out the sampling activities in May–June before the summer spawning season was identified to be recurrent in this area (Loi et al., 2017). Moreover, all the individuals submitted to analysis showed the presence of large amounts of gonad matrices available for the SPME extraction and this information might indicate an advanced maturation

stage of the gonads. On the other hand, it must be pointed out that the seasonal and environmental factors influencing the number of spawning events occurring along the year and the lipid evolution in sea urchin gonads are debated issues in the literature (Siliani et al., 2016). Also, the sex-induced differences in fatty acid profiles in the gonads are reported in some studies (Rocha et al., 2019) while other studies did not highlight any statistically significant gender-related relationship (Carboni et al., 2013). Thus, it will be highly relevant to establish in further studies the temporal variability of the PAE profile in the gonads together with sex and gonad maturation.

4.3. Possible human exposition to MPs and PAEs by sea urchin gonad consumption

Sea urchin gonads are very appreciated in the south of Italy and especially in Sardinia for their taste that recalls the aromas and flavors of the sea. Known since ancient times, they were widely consumed by the Romans, as indicated in various written records *i.e.* they were included as an ingredient created in recipes described by the famous Roman gastronome Marco Gavio Apicius (*De re coquinaria*). Nowadays, in Italy the product is mostly consumed raw: in the season in which the gonads are most abundant from September to April, with a peak in January and February, the freshly fished sea urchins are opened with a special tool designed to deprive their apical part, and the gonads are eaten at the seaside accompanied with bread, “focaccia” or “taralli”. In the restaurant or at home, the most famous recipe is “Spaghetti con i ricci di mare”, where the pulp becomes a precious dressing for pasta. For commercial products available in the market, the pulp is extracted, sterilized, preserved in water and salt, and sealed in plastic or glass recipients. Outside Italy, sea urchin eggs are popular in Japan to make sushi known as “Uni Nigiri” and in some other European countries *i.e.* Portugal, Spain, Greece, and France. Here the gonads are mostly eaten raw. In France, three recipes are famous that

have sea urchin as an ingredient: “l'Oursinado” a fish soup similar to “Bouillabaisse”, in which the sea urchin is used to prepare the accompanying sauce, the “Daurade à la crème d’oursin” in which the cooked sea bream is served with a Dutch sauce enriched with sea urchin eggs and the “Omelette d'oursins” made with sea urchin eggs. As indicated by a recent study, the perception of sea urchins as a novel and trendy product is growing worldwide (Baião et al., 2021), and aquaculture production has been fast developing since stocks faced overfishing in the last years in several geographical areas (including Sardinia). According to FAO data, the world consumption of sea urchins (live weight) in 2018 amounted to 58.58 tons (Baião et al., 2021). Together with gastronomic, economic, and ecological considerations, unfortunately also marine pollution is becoming a new issue to be included in the overall evaluation of seafood.

According to our measurements carried out on specimens not submitted to PAE analysis, about 0.70 g of gonads were obtained from a sea urchin in the Italian commercial size which displayed an average weight of 80 g. Since the gonads contained an average value of PAEs of 32 ± 5.3 ng/g, considering a consumption of 10–20 gonads for serving corresponding to around 7.0–14 g, this accounts for a total of 0.2–0.4 μg of PAEs. This content is lower compared to that found in other seafood, *i.e.* salmon fillet, tuna, raw shrimp, chopped clams, and sardines, where mean values from 0.30 to 32 μg/g have been reported (Schecter et al., 2013). Cheng (2013) also reported PAE values from 2 to 425 μg/kg in fish from the Hong Kong market while in Norwegian frozen and fresh seafood products levels from beyond the quantification limit to 55 μg/kg have been found (Sakhi et al., 2014), thus the possible level of exposition for a 100 g serving is comprised between 0.2 and 43.5 μg. In a recent study on cultured seabass and rainbow trout, we evaluated that with an average fillet portion of 150 g, humans could be exposed to an average of 0.3–9.4 μg of PAEs (Panio et al., 2020). Of course, the consumption of sea

urchin gonads must be considered occasional, but as already pointed out it depends on the traditional habits; in Sardinia, it has been reported that annual pro-capita consumption is about 1.1 kg (Carboni et al., 2012). According to a study by Serrano et al. (2014), in which they examine the *per capita* total DEHP dietary intake for eight food groups (dairy, meat, egg, fish, grain, vegetable, fruit, fat) in average diets of US infants, adolescents and females of reproductive age, dairy intake was reported at the highest rate (30.8 μg/kg-day in infants, 3.9 μg/kg-day in adolescents, 5.7 μg/kg-day for female of reproductive age 13–49), while fish and egg consumption was minimal (fish: 0.05 μg/kg-day in infants, 0.02 μg/kg-day in adolescents, 0.03 μg/kg-day in females of reproductive age; egg: 0.03 μg/kg-day in infants, 0.01 μg/kg-day in adolescents and 0.01 μg/kg-day in females 13–49 years old) (Serrano et al., 2014). Therefore, based on actual dietary patterns, for women of reproductive age the greatest contribution to DEHP exposure (47.2 %) derived from dairy products, and being the total dairy intake for DEHP equal to 5.7 μg/ kg-day, for a 70 kg woman, this translates to a total exposure of 399 μg/ day (Serrano et al., 2014), which is significantly higher than the intake derived by occasionally consuming sea urchin gonads. Similarly, Sakhi et al. (2014) investigated the exposure to 10 PAEs (DMP, DEP, DiBP, DnBP, BBzP, DEHP, DCHP, DnOP, DiNP, DiDP) in the Norwegian adult population, analyzing foods and beverages commonly consumed in a typical Norwegian diet. They found that the food items with the highest concentrations for $\sum_{10}$PAEs were buns (835.59 μg/kg fresh weight), chocolate spreads (472.5 μg/kg fresh weight) and margarine (441.1 μg/ kg fresh weight). Considering the estimated daily dietary exposure, they found levels under the TDI values established by EFSA, with a larger relative contribution from grain and meat (seafood excluded). DEHP with 384 ng/kg bw/day and DiNP with 402 ng/kg bw/day DiDP resulted in the highest contributor among the ten congeners surveyed. In this study, it was also evaluated the

difference in the dietary intake of other countries worldwide, and the importance of including country-specific data for PAE dietary exposure calculations (*e.g.* the author found similar levels of exposition worldwide except in Germany where the intakes were estimated to be 10 times higher). In summary, all this data confirms that the relative intake of PAEs due to the occasional consumption of a sea urchin serving must be considered negligible if compared to the average dietary intake.

For MP's exposition, it must be considered that the preparation of gonads for human consumption necessarily requires breaking the sea urchin, dissecting the alimentary tract, and removing the gonads from the inner side of the arterial body. It is therefore highly likely that during the preparation, plastic particles are released from the digestive tract onto the gonads. In our study, we found up to 4 particles for individuals and thus determined possible gonad contamination of up to 6 particles for gram. The only available data, obtained by direct determination in gonads were provided by Murano and coauthors (Murano et al., 2020) and indicated a concentration around 5.5–19.0 particles/g thus comparable with our estimation. Since at present, there are no indications of processes capable of removing the residual plastic particles from the digestive tract (*i.e.* longer starvation), as pointed out by other studies (Suckling, 2021), it is advisable to implement in the industrial processing a washing stage with filtered water aimed to remove excess of MPs.

Conclusion

In conclusion, the impact of MPs and PAEs on the benthonic sea urchin community living in Sardinia (West Mediterranean Sea), and the level of exposition to humans of these associated contaminants due to the consumption of sea urchin gonads as gourmet delicacy, were assessed for the first time. MPs were found in 40 % of the analyzed

specimens with concentrations up to 4 particles/individual, and with the maximum concentrations found in individuals under the commercial size. Since the detected particles resulted mostly in the size 50–150 μm, displaying a distribution deviating from the classical 1d-fragmentation model, it was hypothesized the presence of a selection mechanism, probably operating during the sea urchin grazing activity. PAEs were detected in all the samples, with DEHP resulting in the most represented congener and resulting significantly correlated to the presence of the microfibers in the digestive tract (but not with fragments). According to our evaluation, the intake of PAEs associated with a sea urchin serving is one to two orders of magnitude lower than the intake associated with the average fish fillet serving. This is mostly due to the smaller quantity consumed.

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Chapter 3

Impact of tire wear as a major source of microplastics

Impact of pristine and weathered environmentally relevant multi-tire mixture on two model estuarine species

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In preparation

Abstract

Tire erosion from driving is the second largest source of secondary microplastics in the oceans. Tire particles consist of a diverse array of components that vary depending on the tire type, potentially influencing their toxicity. However, most toxicological studies have predominantly focused on single-tire types, overlooking the environmental relevance of multi-tire mixtures. This study investigates, for the first time, the behavioral toxicity and growth-related effects of pristine and weathered environmentally relevant multi-tire mixtures on two model estuarine species: the fish Inland Silverside (*Menidia beryllina*) and the mysid shrimp (*Americamysis bahia*). We exposed 5-day post-fertilization fish embryos and 7-day-old mysid shrimp to micro (1–20 μm) and nano (<1 μm) tire particles at four concentrations (10, 100, 1000, and 10,000 particles/mL), as well as to their associated leachates. Both species showed a positive correlation between tire particle concentration and ingestion rate, with significantly higher ingestion rates observed for weathered tire particles. Growth responses were species-specific, with the most pronounced impacts occurring in response to weathered tire particles. Both species exhibited growth reductions when exposed to weathered tire particles, but only *A. bahia* was affected by weathered leachate and pristine tire particles. Behavioral toxicity also varied by species, with weathering amplifying the toxicity of tire particles in *A. bahia*, but not in *M. beryllina*. This study highlights the broader ecological impacts of multi-tire mixtures, emphasizing the need to move beyond single-tire particle assessments and incorporate multiple test species to account for species-specific responses.

1. Introduction

Plastic pollution has escalated into a critical global environmental issue, primarily driven by the substantial rise in plastic production over the past century (Borrelle et al., 2020; Geyer et al., 2017). The growing production and mismanagement of plastic waste have turned the oceans into a waste repository (Ostle et al., 2019). Mismanaged waste, along with the smaller fragments it generates through environmental weathering processes, known as secondary micro- and nanoplastics (MNPs), is widely recognized as a major source of plastic pollution in marine environments (Andrady and Koongolla, 2022). In addition, recent studies have highlighted the significant contributions of microfibers shed from synthetic textiles during washing and tire particle erosion during driving, which together account for 63.1% of global microplastic releases into the oceans (Boucher and Friot, 2017; Galvão et al., 2020; Granek et al., 2022; Mayer et al. 2024). Tire particle (TP) pollution is a significant environmental concern, with an estimated 3,369,698 metric tonnes of TPs released annually worldwide (Kole et al., 2017), underscoring the urgency for targeted interventions. The United States leads in annual emissions, releasing 1,524,740 metric tonnes of TPs, while other countries, including China, India, Japan, Italy, Norway, Sweden, and Denmark, have emissions ranging from 6,721 to 756,240 tonnes per year (Kole et al., 2017). TPs have only recently begun to be recognized as MNPs in environmental studies and are likely underreported. Despite the large quantities of TPs released into the environment, only 1% of microplastic studies from 2000-2019 addressed TP pollution (Knight et al., 2020). Lead and Weinstein (2019) identified TPs as the second most abundant microplastic in the Charleston Harbor estuary (USA), comprising 17.1% of the total. Parker et al. (2020) documented TP ingestion in 14% of individuals across five fish species in Charleston Harbor (USA). In urban runoff from 12 San Francisco Bay watersheds (USA), up to 64% of microplastics were black rubbery

fragments, likely from tire and road wear (Werbowski et al., 2021). Wik and Dave (2009) conducted a meta-analysis on tire components, finding that tire particle maximum Predicted Environmental Concentrations (PECs) in road runoff entering surface waters range from 0.03 to 56 mg/L, and up to 155 g/kg dry weight in sediments of high-traffic areas. TPs are primarily generated through the abrasion of tires on road surfaces and consist of a complex mixture of components. These include polymers, which make up 40-50% of a tire's composition, fillers (30-35%), softeners (15%), vulcanizing agents (2-5%), and various additives (5-10%) (Johannessen et al., 2022; Sommer et al., 2018). However, the specific composition of TPs varies depending on tire type and use, which also affects their potential toxicity. For example, passenger car tires have more synthetic rubber, while truck tires contain more natural rubber, and heavy-duty vehicle tires lack synthetic rubber entirely (Grammelis et al., 2021). Despite this variability, the limited toxicity studies in the literature examine single tire types. Cunningham et al. (2022) examined the effects of tire particles from a new, undriven standard passenger car tire on embryonic zebrafish (*Danio rerio*) and the crustacean *Daphnia magna*, exposing them to micro (1–20 μm) and nano (<1 μm) TPs along with their associated leachate. The study found that nanoparticles significantly increased toxicity, leading to higher mortality rates in both species, as well as more severe effects, including hatching delays and axis malformations in *D. rerio*. Siddiqui et al. (2022) explored the effects of tire micro- and nanoparticles, as well as their leachates, on *M. beryllina* and *A. bahia*. The study revealed significant alterations in swimming behavior for both species, with micro TPs leading to notable growth reductions in both organisms, and nano TPs specifically affecting *M. beryllina* growth. *M. beryllina* exposed to both weathered and unweathered micro- and nano-TPs showed also disruptions in muscle contraction, cell signaling, and osmoregulation, potentially linked to dysregulated gene expression (Hutton et al.,

2024a). Schell et al. (2022) observed reduced reproductive output and survival in *D. magna* after 21 days of exposure to TPs (25–75 μm) derived from end-of-life passenger tires at concentrations of 0.15 and 0.015 g/L. Khan et al. (2019) reported that TPs from a road-worn tire significantly impacted mortality, neonate production, and net growth in the amphipod *Hyalella azteca* at concentrations ranging from 500 to 2000 particles/mL after 21 days of exposure. Collectively, these six studies utilized tire particles derived from specific tire types: a new, undriven standard passenger car tire (Cunningham et al., 2022; Siddiqui et al., 2022; Hutton et al., 2024a), a road-worn tire (Khan et al., 2019), and end-of-life passenger tires (Schell et al., 2022). In this study, we assess, for the first time to our knowledge, the toxicity associated with an environmentally relevant multi-tire mixture, comparing the difference in toxicity between pristine and weathered TPs. We hypothesize that the multi-tire mixture will have more pronounced effects compared to studies using single tire types, potentially due to its more diverse chemical composition, which may increase its toxicity. Our research focuses on the early life stages of two model estuarine species recommended by the U.S. Environmental Protection Agency: *M. beryllina* and *A. bahia*. Both species play a crucial role in estuarine food webs on a global scale and are extensively used in ecological research due to their rapid spawning and swift attainment of reproductive maturity. The TP concentrations used in this study do not reflect the levels currently observed in estuarine environments. However, it's widely recognized that the concentrations reported in the literature, especially those linked to smaller particles, are likely underestimated (Knight et al., 2020; Weber and Kerpen et al., 2023). This is primarily due to the current lack of advanced tools and technologies capable of accurately measuring nanoparticle concentrations in the environment (Koelmans et al., 2015). Furthermore, given the growing reliance on plastics due to their numerous advantages, it is unlikely that the levels of MNPs in the

environment will decrease in the coming decades. Therefore, understanding how organisms will respond at higher concentrations may be important in the future.

2. Materials and methods

2.1. Tire particle and leachate preparation

Cryo-milled tire tread (CMTT) composite provided by the U.S. Tire Manufacturers Association (USTMA) was used for this experiment. The composite represented a typical mixture of tire materials that may be encountered in the environment, specifically designed to reflect the proportions of tire types found in the United States. USTMA developed this estimate based on tire shipment data from the USTMA Fact Book for 2016, 2017, and 2018. The blend consisted of three primary types of tire materials: 41% passenger car tire, 14% light truck tire, and 45% truck/bus tire. The preparation of the tire particles followed the detailed methodology outlined by Cunningham et al. (2022) and McColley et al, (2023). Briefly, 3.25 grams of the cryo-milled tire particles were combined with 300 milliliters of water containing 50 milligrams per liter of natural riverine organic matter (Suwanee River NOM, obtained from the International Humic Substances Society), to minimize particle aggregation during the preparation process. To further refine particle size, glass beads were added to the tire particle suspension, which was then autoclaved, cooled, and placed on a shaker table for 72 hours to promote size reduction. After shaking, the glass beads were carefully removed, and the suspension was sequentially filtered through a 20 µm sieve, followed by a 1 µm Adantec mixed cellulose filter. This filtering process was critical in isolating tire particles smaller than 1 µm. For the subsequent recovery of tire particles ranging from 1 to 20 µm in size, the 1 µm filter was backflushed with fresh, particle-free water. Following this, the suspension containing tire particles smaller than 1 µm underwent further filtration. A 0.02 µm

Whatman Anotop filter was used in a centrifuge set to 7500 rpm for 5 minutes. This filtration step effectively removed the nano-tire particles from the suspension, leaving behind only the leachate components. The resulting solution, now containing the leachate free of particles, was collected as an undiluted leachate stock solution. This leachate stock was subsequently used in its concentrated form for 100% leachate exposure in further experimental analyses. Tire particle fractions were quantified in triplicate using advanced analytical techniques. A flow cytometer (Accuri C6 Flow Cytometer, BD Biosciences, San Jose, California), calibrated with appropriate size standards, was employed to perform particle counts of the micro-sized fraction. Meanwhile, the nano-sized fraction of tire particles was characterized using Nanoparticle Tracking Analysis (NTA version 3.4), performed on a NanoSight NS500 instrument (Malvern Instruments, Westborough, Massachusetts) equipped with a 405 nm laser.

2.2. UV weathering of tire particles

Half of the micro, nano, and leachate solutions were subjected to UV weathering using a SciSun Solar Simulator (AM1.5G, 150W, Class AAA, 50 × 50 mm, London, Ontario, Canada) to simulate real-world environmental conditions. Covered with clock glasses to prevent evaporation and particle contamination, the solutions were placed 380 mm beneath the simulator and exposed to full-sun equivalent irradiation (1000 W/m²) for 72 hours at 40°C. To promote uniform exposure and ensure consistent weathering of all particles, the solutions were continuously stirred using a magnetic stir plate. Temperature and light intensity were monitored throughout the exposure to ensure consistent conditions.

2.3. Animal husbandry and maintenance

At Oregon State University's Hatfield Marine Science Center, adult Inland Silversides, aged 1 to 1.5 years, were kept in accordance with the ethical guidelines outlined in Animal Care and Use Program (ACUP) protocol #4999. The fish were housed in tanks with water temperatures maintained at 21°C and salinity levels set at 15 PSU. Water quality parameters, including salinity, pH, temperature, dissolved oxygen and ammonia, were checked daily to ensure optimal living conditions. To induce spawning, a natural wool-based substrate was introduced into the tanks for 24 hours, allowing sufficient time for egg deposition before its removal. Afterward, the eggs were gently removed from the substrate using forceps and transferred to a holding tank, where salinity was also maintained at 15 PSU. The embryos remained in this tank until they reached 5 days post-fertilization (dpf), at which point they were ready for subsequent experimental procedures. Five-day-old mysid shrimp were sourced from Marinco Bioassay Laboratory (MBL) Aquaculture in Sarasota, Florida. Upon arrival, they were housed in tanks with salinity maintained at 20 PSU for a two-day acclimation period before the start of the exposure. To ensure a steady supply of food, we maintained both brine shrimp and rotifer cultures throughout the study. Brine shrimp cultures were replenished every other day using 80% brine shrimp eggs (Technical Grade, 8 oz), hatched in aerated saltwater under controlled conditions. Live marine rotifers, specifically *Brachionus plicatilis* (L-type), were purchased from Reed Mariculture in Campbell, California, and the culture was sustained with daily feedings of RGCOMPLETE™ (Reed Mariculture, Campbell, California), a premium microalgal feed specifically formulated to provide optimal nutrition for rotifers. This feeding regimen ensured a continuous and high-quality live food source for both the fish and shrimp throughout the experimental period.

2.4. Experimental design

Inland Silverside embryos, at 5 dpf, and 7-day-old mysid shrimp were placed in 250 mL beakers for exposure to both pristine and UV-weathered micro (1–20 μm) and nano (<1 μm) tire particles (TPs), adapted from guidelines set by the Environmental Protection Agency (EPA 2002). The organisms were exposed to four sublethal concentrations of TPs: 10, 100, 1000, and 10,000 particles/mL, as well as to 100% pristine and weathered leachates. The exposure scenarios also included river-derived natural organic matter (NOM), which was utilized during the preparation of the tire particles, as well as control treatments. Thus, each model species was exposed to a total of 19 treatment groups (Fig. S1). The exposure was conducted at 15 PSU for the embryos, lasting 96 hours, while the mysid shrimp were exposed at 20 PSU for 7 days. We utilized 10 organisms per replicate, with a total of four replicates for each treatment group, ensuring robust statistical power, and covered exposure containers to avoid any air-borne contamination. The Inland Silverside embryos hatched during the exposure period, between days 1 and 2, with no significant differences in hatching rates between control and treatment groups ($p > 0.05$, ANOVA, Dunnett's post hoc test). We daily performed 80% water changes, replacing the old water with freshly prepared exposure solutions to maintain optimal conditions. During these changes, we carefully removed any debris and assessed organism survival alongside various water quality parameters, including pH, dissolved oxygen, salinity, temperature, and ammonia (Table S2). Following the experimental maintenance, the embryos were fed rotifers, while the mysid shrimp were provided with brine shrimp. Organisms were maintained under a 14:10 light cycle. After the exposure period, we evaluated growth, particle internalization, and behavioral responses for both species.

2.5. Growth measurements and TP internalization

After the experiment, two specimens from each replicate were selected for growth assessments and imaged using a Leica EZ4 stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with Moticam visual software (Motic Inc., Richmond, Canada). Measurements of their width and length were obtained using ImageJ software (National Institutes of Health, Bethesda, Maryland; ImageJ 1.53t). Data were analyzed according to the methodology outlined by Siddiqui et al. (2022), utilizing the index $\frac{W}{L} \times d$, where W represents the organism's width, L its length, and d the number of exposure days. For *M. beryllina*, we measured the standard length (SL) - the distance from the tip of the lower jaw to the posterior end of the hypural bone - as described by Anderson and Gutreuter (1983). In contrast, the body length of *A. bahia* was measured from the base of the telson to the tip of the rostrum (Metillo and Ritz, 1994). The same organisms were subsequently fixed in 4% paraformaldehyde solution. Fish and shrimp were cleared using the method described by Siddiqui et al. (2022; 2023), enabling the quantification of ingested microparticles (1-20 μm) at each concentration. Each replicate was washed in 5 ml phosphate-buffered saline (PBS) for 30 minutes at room temperature, incubated in 5 ml of CUBIC-L at 37°C for seven days to facilitate lipid removal and decoloring, washed again in 5 ml PBS for 2 hours, and incubated in CUBIC-R at 37°C for another seven days. The number of TPs in the digestive tract of each organism was then quantified using an SZX10 Stereomicroscope (Olympus, Tokyo Japan) equipped with an SC100 camera (Olympus, Tokyo Japan).

2.6. Behavioral assay

Behavioral assays were conducted using a DanioVision Observation Chamber (Noldus, Wageningen, Netherlands), following the dark-light cycle established by Siddiqui et al.

(2022). In this experimental setup, 10 mL glass beakers were organized within a 12-well plate tray, with each well randomly assigned either one fish or one shrimp, along with 3 mL of solution drawn from each of the four replicates across all 38 treatment groups. A total of four pseudo-replicates (individual fish and shrimp) were analyzed for each replicate (Fig. S2). Before testing, organisms were acclimated in the 10 mL beakers for a minimum of 15 minutes. They were then transferred into the DanioVision Observation Chamber, where they underwent an additional 5 minutes of acclimation. Following this acclimation period, the organisms were subjected to an alternating dark-light cycle (Fig. S2), which consisted of three 2-minute dark periods interspersed with three 2-minute light periods (Siddiqui et al., 2022). Behavior was monitored and tracked with a Basler Gen 1 camera, utilizing Ethovision® XT15 software at a resolution of 1280 × 960, under lighting conditions of 10,000 lx, and at a frame rate of 25 frames per second. Behavioral tracking was conducted throughout the day designated for the takedown, specifically from 07:00 to 19:00 h. Time spent bursting (speed > 20 cm/s), cruising (speed > 0.5 cm/s and < 20 cm/s), freezing (speed < 0.5 cm/s), and velocity (cm/s) were measured, together with thigmotaxis (wall hugging) and total distance moved (cm). Thigmotaxis is a key behavioral indicator used to assess anxiety and stress in organisms exposed to environmental stressors (Hutton et al., 2023). This instinctive behavior, characterized by a preference for staying close to walls, serves as a survival mechanism, offering a sense of security from perceived threats. An elevated thigmotactic response indicates heightened anxiety, while reduced thigmotaxis suggests lower anxiety levels and a greater sense of safety. However, reduced vigilance can also increase predation risk, as less cautious organisms are more likely to roam freely in search of food or mates (Hutton et al., 2023). In addition to thigmotaxis, total distance moved (TDM) is another well-established behavioral metric that provides valuable insights into an organism's overall

level of activity, distinguishing between hypoactivity (reduced movement) and hyperactivity (increased movement). These metrics are critical for interpreting the stress responses and behavioral shifts observed in marine organisms exposed to environmental pollutants, which may have profound and long-lasting effects on reproductive success, fitness, and ecosystem dynamics (Saaristo et al., 2018).

2.7. Quality assurance/quality control (QA/QC)

Background contamination was minimized maintaining high cleanliness standards.. The glassware was meticulously hand-washed using a 1% Alconox soap solution and tap water. After removing all visible soap, the glassware was rinsed with Microplastics Analysis Grade (MAG) water, prepared by filtering reverse osmosis (RO) water through a 1 µm polycarbonate (PCTE) membrane filter (Sterlitech, Auburn, Washington, USA). The cleaned glassware was then dried in a laminar flow hood and covered with a double layer of aluminum foil, to provide extra protection. Finally, the glassware was baked in a muffle furnace at 450°C for four hours, to ensure complete decontamination. Throughout all exposure periods, the beakers were sealed with parafilm to prevent contamination, thereby enhancing the reliability of the results (Brander et al., 2020). To assess the level of background contamination in our experimental setup, control blank filters were established in petri dishes and strategically positioned near the experimental setup and the designated area for water changes to closely monitor any potential contamination sources. This control setup was run in three replicates, which allowed us to obtain a robust assessment of background levels. All experiments and analyses were conducted wearing orange cotton lab coats to ensure minimal contamination from clothing. Analysis of these filters revealed no detectable particles resembling tire particles but identified an average of 7 ± 1 microfibers (mean ± standard error).

2.8. Statistical analysis

Statistical analyses were conducted using RStudio Version 2024.04.0 (Posit, Boston, Massachusetts, USA). The normality of the survival data was assessed using the Shapiro-Wilk test. Since the data did not meet the assumptions of normality, the non-parametric Kruskal-Wallis test was employed, followed by Dunn's post-hoc test, to compare survival rates across different treatment groups. A generalized linear model (GLM) with a Poisson distribution was used to examine the relationship between particle ingestion and concentration, with 95% confidence intervals calculated for the predictions. Log transformation of the concentration variable was applied to improve model fit. Additionally, a two-way ANOVA was performed to assess the effects of concentration and weathering on particle ingestion, followed by Tukey's HSD post-hoc test for group comparisons. Growth data were normally distributed, and differences between treatment groups were analyzed using ANOVA with Tukey's HSD post-hoc test. Behavioral data, which were not normally distributed, were analyzed using the Kruskal-Wallis test, followed by Dunn's post-hoc test with Bonferroni correction to account for multiple comparisons. To visualize the behavioral results in heatmaps, z-scores were computed from the normalized behavioral data. The normalization process scaled the data to a range between 0 and 1 using the formula $\frac{x-min\,(x)}{max(x)\,-min\,(x)}$ where x represents individual data points. Heatmaps were generated using the *ggplot2* package. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Survival, TP internalization and growth reduction in *A. bahia*

No significant impact on survival was observed across any of the treatments (Kruskal-Wallis non-parametric test, Dunn's post-hoc test, $p > 0.05$). The control group showed an

average survival of 98 ± 0.1% and treatment groups - including pristine and weathered nano tire particles, micro tire particles, and leachates - exhibited a 91 ± 0.2% survival rate. Our findings revealed a clear positive correlation between TP concentration and ingestion rate (Fig. 1). *A. bahia* exposed to the highest concentration of micro weathered and pristine TPs (10,000 particles/mL) demonstrated a significant increase in particle ingestion compared to the control group (Dunnett's test ANOVA, $p < 0.001$). When comparing the internalization of pristine and UV-weathered TPs, the results revealed a significantly higher ingestion rate for weathered tire particles ($p < 0.05$, GLM analysis with a Poisson family distribution). Growth reduction was observed solely following exposure to pristine TP treatments, while exposure to pristine leachate alone did not yield a similar effect (Fig.2). Particularly exposure to 1,000 micro- and nano-TPs/mL and to 10,000 micro- and nano-TPs/mL resulted in significant growth reductions (Tukey HSD post-hoc ANOVA, $p < 0.5$ and $p < 0.001$, respectively). Conversely, when *A. bahia* was exposed to weathered TPs and weathered leachate, both treatments resulted in significant growth reductions compared to the control group (Tukey HSD post-hoc ANOVA, $p < 0.001$) (Fig. 2). The onset of growth reduction occurred at lower concentrations for the weathered TP treatments compared to the pristine counterparts. Specifically, nano TPs began to exhibit adverse effects at 100 particles/mL. These results indicate that weathered TPs, along with their associated leachates, may exacerbate the toxic effects on *A. bahia* growth, with negative impacts manifesting at lower exposure levels than those observed in pristine treatments.

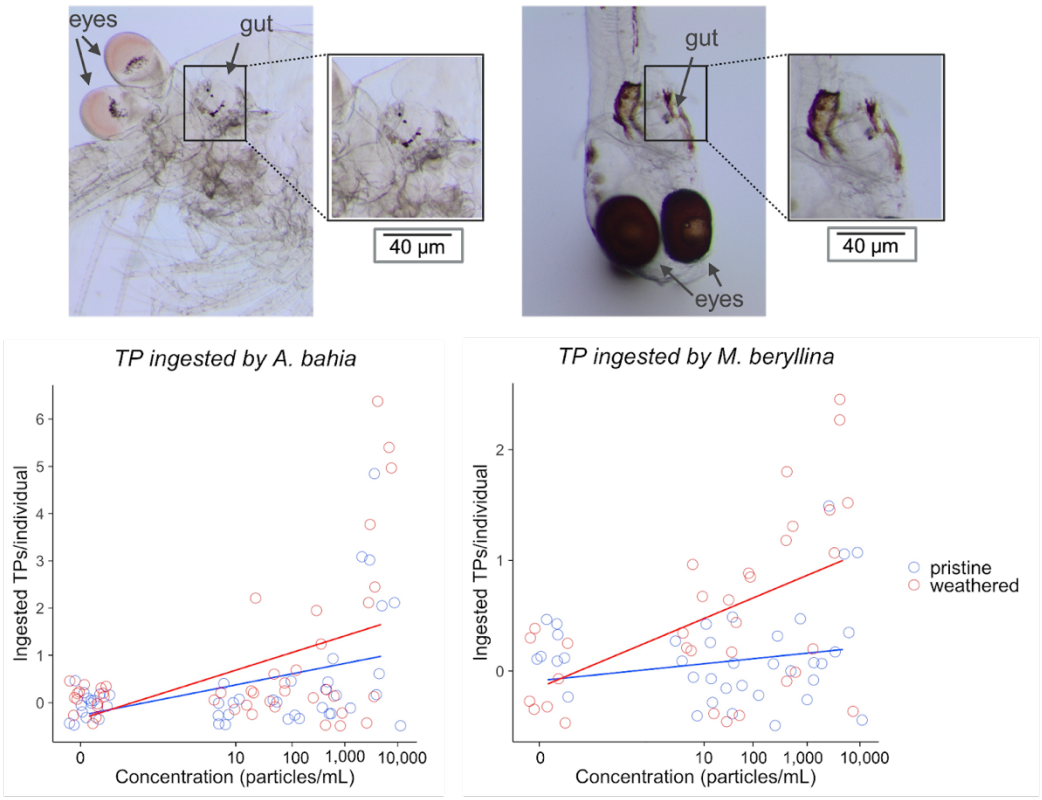

Figure 1: Top images show the gut contents of *A. bahia* (left) and *M. beryllina* (right), highlighting ingested TPs, captured using an Olympus SX10 microscope. The bottom panel presents the results of a Generalized Linear Model analysis, illustrating the relationship between TP concentration (pristine and weathered) and ingestion rates across treatment groups for both species.

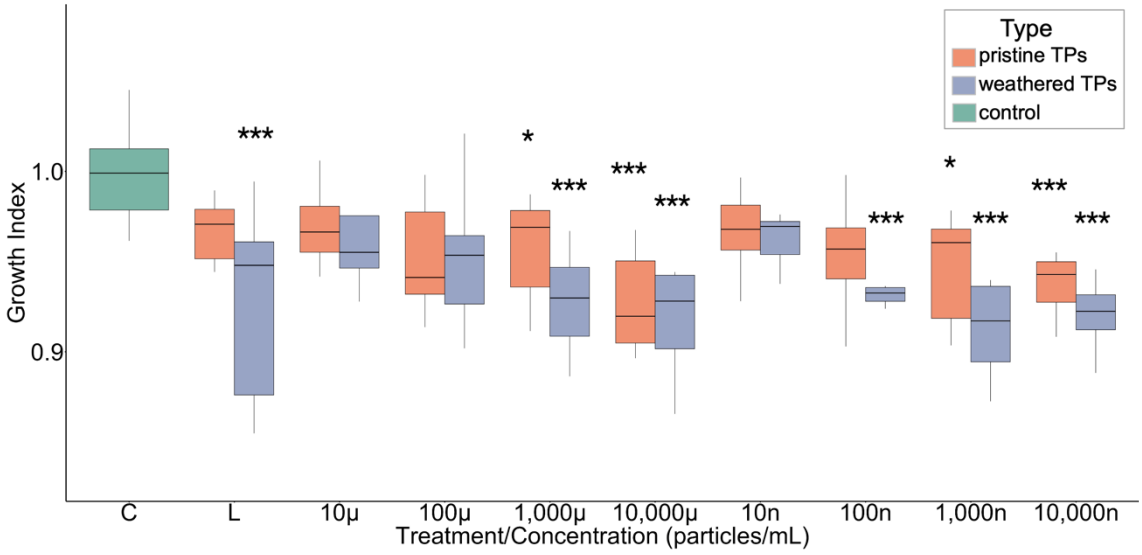

Figure 2: Growth index of *A. bahia* across varying concentrations of pristine and weathered micro- and nano-TPs and leachates. Concentrations ranged from 10 to 10,000 particles/mL. Asterisks indicate significant differences compared to the control group (*$p < 0.05$, ***$p < 0.001$). Abbreviations: L = leachate, C = control, µ = micro, n = nano.

3.2. Survival, TP internalization and growth reduction in *M. beryllina*

The average survival rates for the control and exposure treatments were 96.65 ± 4.24% and 91.64 ± 9.45%, respectively, with no significant differences observed between the treatment groups (Kruskal-Wallis test, Dunn's post-hoc test, $p > 0.05$). When *M. beryllina* was exposed to pristine TPs, a slight increase in ingestion rate was observed with rising concentrations, but no significant differences were found among the treatment groups (Dunnett's test ANOVA, $p > 0.05$). In contrast, exposure to weathered TPs revealed a clear concentration-dependent increase in particle ingestion (Fig. 1). Specifically, larvae exposed to the highest concentration of weathered TPs exhibited significantly higher ingestion rates compared to the control group (Dunnett's test ANOVA, $p < 0.001$). Furthermore, ingestion of weathered TPs was significantly greater than that of pristine TPs (GLM analysis with a Poisson family distribution, $p < 0.05$). Exposure to either weathered or pristine leachate alone did not lead to significant growth reductions in *M. beryllina*, highlighting the pivotal role of TPs themselves. While *M. beryllina* did not show significant growth reductions when exposed to pristine TPs, it exhibited sensitivity to weathered TPs (Fig. 3). Particularly, growth was significantly reduced compared to the control group by weathered micro-TPs at concentrations of 100, 1,000, and 10,000 particles/mL (Tukey HSD post-hoc ANOVA, $p < 0.01$), and by nano-TPs at the highest tested concentration (Tukey HSD post-hoc ANOVA, $p < 0.05$).

3.3. Behavioral changes during dark: light cycle in *A. bahia*

After a 7-day exposure, all six behavioral endpoints analyzed were affected. Pristine micro-TPs impacted 33.3% of the measured endpoints, while pristine nano-TPs and both weathered micro- and nano-TPs affected 100% of the endpoints. Pristine and weathered

leachate influenced 83.3% of the endpoints (Table S1). These results indicate that the weathering process to which the TPs were subjected increased their behavioral toxicity.

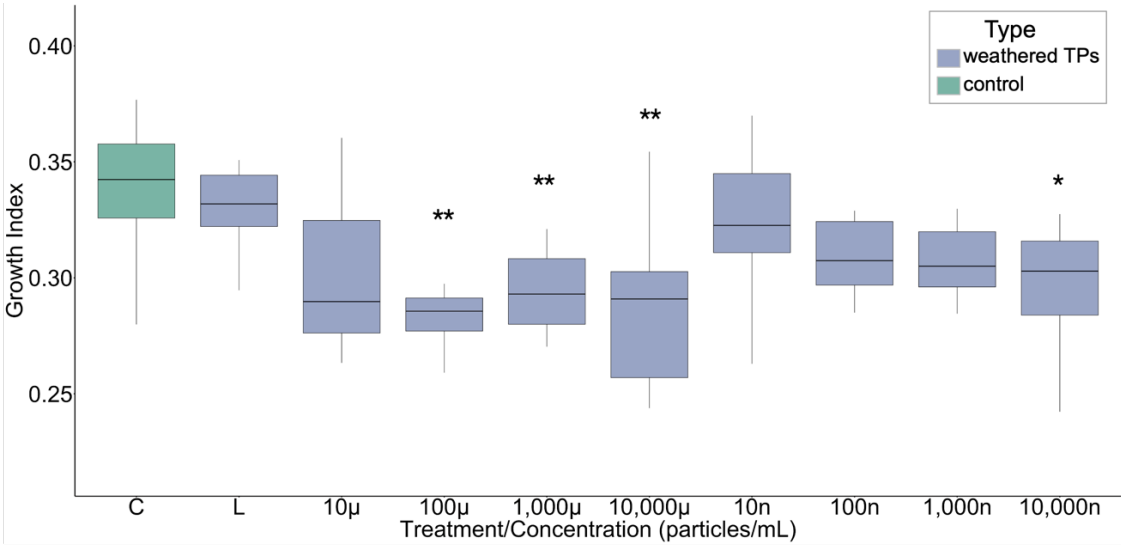

Figure 3: Significant growth reduction in *M. beryllina* when exposed to weathered TPs at varying concentrations. Asterisks indicate significant differences compared to the control group (* $p < 0.05$, **p < 0.01). Abbreviations: L = leachate, C = control, µ = micro, n = nano.

Thigmotaxis and TDM were notably altered, revealing distinct patterns under both light and dark conditions (Fig. 4). Under light conditions, exposure to pristine micro-TPs significantly reduced thigmotaxis, particularly at concentrations of 10 particles/mL and 1,000 particles/mL. Similarly, in dark conditions, a decrease in thigmotaxis was observed at 1,000 particles/mL, suggesting reduced anxiety. However, exposure to pristine micro-TPs did not result in significant differences in TDM under either light or dark conditions. In contrast, pristine nano-TPs caused fewer significant changes in thigmotaxis compared to micro-TPs, but their effects on TDM were more pronounced. Specifically, thigmotaxis increased under light conditions at a concentration of 1,000 particles/mL, while TDM showed significant increases at 10 and 1,000 particles/mL under light conditions, and at 10,000 particles/mL in the dark, indicating heightened hyperactivity. In summary, both pristine micro- and nano-TPs influenced the behavioral responses of *A. bahia*, with micro-TPs exhibiting more pronounced effects on anxiety-like behavior (thigmotaxis)

and nano-TPs driving increased overall activity (TDM). Exposure to weathered micro-TPs induced hypoactivity under light conditions, particularly at a concentration of 1,000 particles/mL, while hyperactivity was observed at 100 particles/mL. Anti-thigmotaxis occurred in both light and dark conditions at concentrations of 100, 1,000, and 10,000 particles/mL. In contrast, weathered nano-TPs primarily increased thigmotaxis in both light and dark conditions at 100, 1,000, and 10,000 particles/mL. Additionally, exposure to weathered nano-TPs resulted in hypoactivity at 10 particles/mL in the dark and at 100 and 10,000 particles/mL in the light. The observed effects were not strictly concentration-dependent and varied between light and dark environments, suggesting complex interactions among particle size, exposure concentration, and environmental conditions.

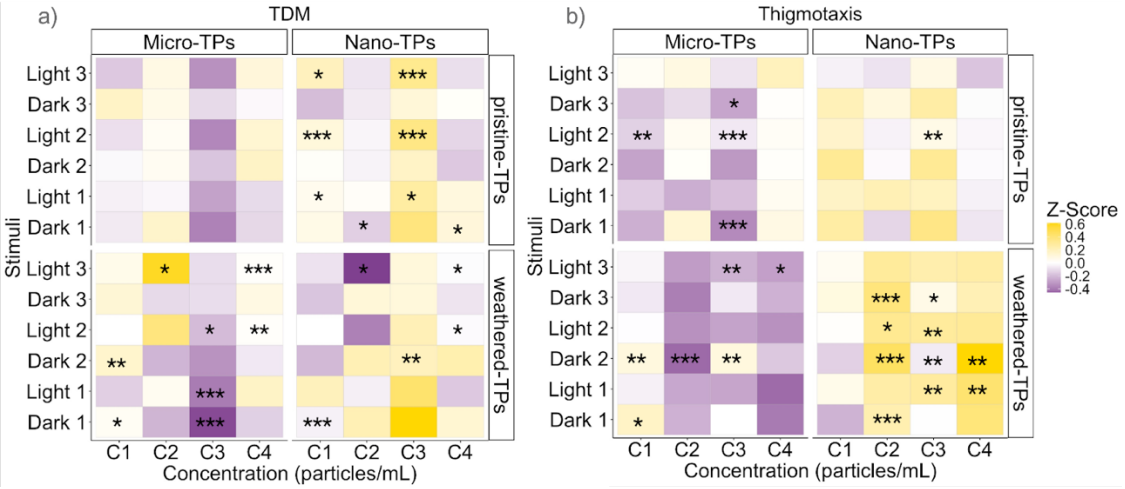

Figure 4: Heat maps illustrating *A. bahia* total distance moved (TDM) (a) and thigmotaxis (wall-hugging behavior)/anti-thigmotaxis (b), across various stimuli and concentrations of both pristine and weathered micro- and nano-TPs (C1 = 10 particles/mL, C2 = 100 particles/mL, C3 = 1,000 particles/mL, C4 = 10,000 particles/mL). In panel (a), a negative Z-score (purple) indicates reduced wall-hugging behavior (anti-thigmotaxis), while a positive Z-score (yellow) signifies increased wall-hugging (thigmotaxis), relative to the control group. In panel (b), a negative Z-score indicates decreased TDM, reflecting hypoactivity, while a positive Z-score corresponds to increased TDM, indicating hyperactivity. Asterisks show significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Additionally, the associated leachates also influenced behavioral responses. Notably, pristine leachate led to significant hyperactivity in light conditions. In contrast, weathered

leachate resulted in significant hypoactivity in the dark and hyperactivity in the light, along with thigmotaxis and anti-thigmotaxis in dark conditions.

3.4. Behavioral changes during dark: light cycle in *M. beryllina*

Following the 96-hour exposure, all six behavioral endpoints examined were significantly affected. However, the results suggest that the weathering process did not enhance the toxicity of TPs. Specifically, pristine micro- and nano-TPs were found to influence 100% of the measured endpoints, demonstrating their strong behavioral effects. In contrast, weathered nano-TPs affected 83.3% of the endpoints, indicating a substantial but reduced impact compared to their pristine counterparts. Weathered micro-TPs showed the least influence, impacting only 33.3% of the behavioral endpoints. Additionally, pristine leachate was shown to influence all measured endpoints (100%), while weathered leachate affected 66.7% of the endpoints, reinforcing the observation that weathering did not result in increased toxicity, and in fact may have decreased toxicity in some aspects (Table S1). In *M. beryllina*, both thigmotaxis and TDM exhibited significant variations in both light and dark conditions (Fig. 5). Notably, pristine micro-TPs increased thigmotaxis in the dark at concentration of 1,000 particles/mL, while also inducing hyperactivity in light conditions and hypoactivity in the dark. Conversely, pristine nano-TPs prompted anti-thigmotaxis in both light and dark conditions at concentrations 100 and 1,000 particles/mL, alongside hyperactivity in the dark at 10 and 100 particles/mL. In contrast, weathered micro-TPs resulted in decreased thigmotaxis in the light but did not significantly affect TDM. Weathered nano-TPs led to anti-thigmotaxis in the light, particularly at a concentration of 100 particles/mL, while also causing hyperactivity in both light and dark conditions and hypoactivity in the light. Similar to *A. bahia*, the observed effects were independent of concentration and varied between light and dark environments. Additionally, pristine leachate induced anti-

thigmotaxis in light conditions, as well as both hyperactivity and hypoactivity in the dark. Weathered leachate also resulted in anti-thigmotaxis in both light and dark conditions, along with hyperactivity in the dark.

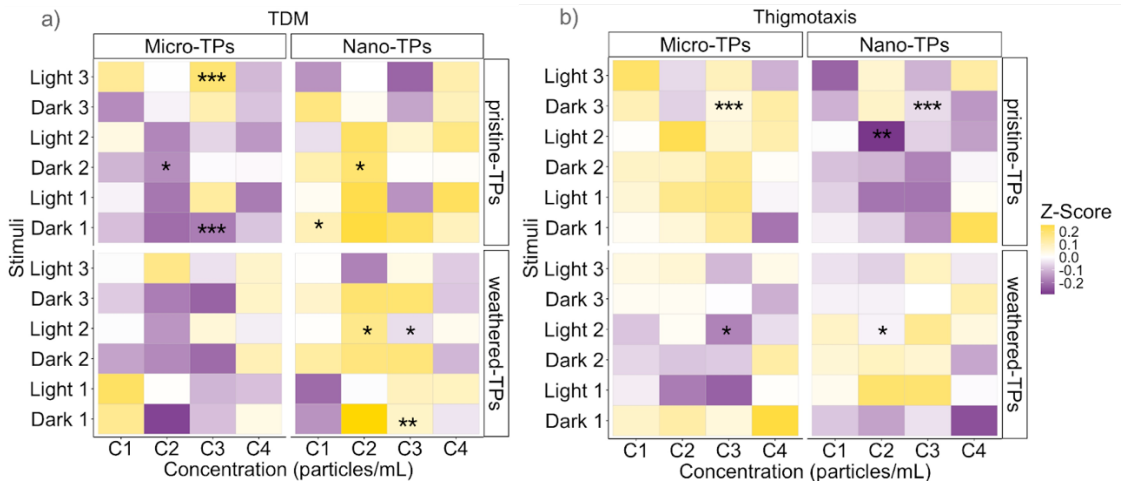

Figure 5: Heat maps illustrating *M. beryllina* total distance moved (TDM) (a) and thigmotaxis (wall-hugging behavior)/anti-thigmotaxis (b), across various stimuli and concentrations of both pristine and weathered micro- and nano-TPs (C1 = 10 particles/mL, C2 = 100 particles/mL, C3 = 1,000 particles/mL, C4 = 10,000 particles/mL). In panel (a), a negative Z-score (purple) indicates reduced wall-hugging behavior (anti-thigmotaxis), while a positive Z-score (yellow) signifies increased wall-hugging (thigmotaxis), relative to the control group. In panel (b), a negative Z-score indicates decreased TDM, reflecting hypoactivity, while a positive Z-score corresponds to increased TDM, indicating hyperactivity. Asterisks show significant differences (*p < 0.05, **p < 0.01, ***p < 0.001).

4. Discussion

4.1. Growth reduction and TP internalization

Overall, *M. beryllina* consumed fewer micro-TPs than *A. bahia*, which could be explained by differences in feeding behavior (Bour et al., 2018). As a benthic organism, *A. bahia* is more likely to ingest micro-TPs, that accumulate on the bottom of the water column. In contrast, *M. beryllina*, a pelagic species, primarily feeds on prey in the water column, where micro-TPs may be less concentrated. Internalization of both pristine and weathered TPs was observed in both study species. Particularly, individuals exposed to UV-weathered CMTT particles, the form most likely to be encountered in natural

environments, exhibited significantly higher ingestion rates compared to those exposed to pristine TPs. This increased ingestion is likely attributable to the photodegradation of TPs induced by UV radiation. This process disrupts the polymer's chemical structure by breaking down chemical bonds and introducing new surface functional groups (Fotopoulou and Karapanagioti, 2015). These functional groups can shift the particle surface from hydrophobic to hydrophilic, promoting the colonization of hydrophilic microorganisms (Shan et al., 2022). Fabra et al. (2021) demonstrated that biofilms can act as a "Trojan horse" by making the surface of microplastics more attractive to benthic filter feeders such as *Ostrea edulis* (European native oyster). Their study showed that biofilm-coated microplastics increase the likelihood of ingestion by enhancing particle palatability, leading to higher consumption rates. Both *A. bahia* and *M. beryllina,* represent potential entry points for MNPs into the food web. *A. bahia*, as a zooplankton species, occupies a critical position at the base of the food chain and serves as an essential food source for a wide array of predators, including invertebrates, fish, and marine mammals. As a lower trophic organism, it is particularly vulnerable to MP ingestion (Walkinshaw et al., 2020). This ingestion has far-reaching implications, as contamination can propagate through the food web, posing risks to higher trophic levels. Trophic transfer has been identified as the primary pathway through which large filter feeders are exposed to MP pollution, surpassing direct environmental exposure. In coastal Auckland waters (New Zealand), the mean total daily MP exposure for a large filter-feeding whale is estimated at 3,408,002 MPs (Zantis et al., 2022). Torres et al. (2023) documented MP contamination in wild zooplankton in coastal Oregon (USA) and estimated that pregnant or lactating Pacific Coast Feeding Group gray whales (*Eschrichtius robustus*) ingest between 6.45 and 21.2 million MPs daily, excluding additional ingestion from ambient water or benthic sediments. Similarly, research from coastal British Columbia (Canada)

estimates that juvenile salmon (*Oncorhynchus* spp) consuming microplastic-contaminated zooplankton ingest approximately 2-7 MP particles per day, while projections suggest that humpback whales (*Megaptera novaeangliae*) could ingest up to 300,000 microplastic particles daily (Desforges et al., 2015).

Both *A. bahia* and *M. beryllina* exhibited significant growth reductions when exposed to weathered micro- and nano-sized TPs. Additionally, *A. bahia* was also impacted by weathered leachate and pristine micro- and nano-TPs at a concentration as low as 1,000 particles/mL. In previous research, *A. bahia* showed a significant decrease in growth after 7 days of exposure to a much higher concentration of 60,000 micro-TPs/mL from a single new tire type, while no effects were observed with nano-sized TPs or leachate (Siddiqui et al., 2022). Hutton et al. (2024a) found no significant growth effects in *M. beryllina* when exposed for 21 days to pristine or weathered micro- and nano-TPs at a concentration of 50 particles/mL. These findings, combined with the results from Siddiqui et al. (2022) and Hutton et al. (2024a), suggest that both *A. bahia* and *M. beryllina* may be more adversely affected by the environmentally relevant multi-tire mixture used in this study than by exposure to a single tire type. Furthermore, the differences in sensitivity between *A. bahia* and *M. beryllina* highlight potential species-specific vulnerabilities to TP pollution, with *A. bahia* appearing more sensitive overall. Food dilution, resulting in reduced nutrient absorption and impaired growth, may provide a plausible explanation for our findings (Athey et al., 2020; Hutton et al., 2024; Uy and Johnson, 2022). The ingestion of TPs could obstruct the digestive system, impeding the normal passage of food and disrupting the digestive process. Such blockages may prevent organisms from effectively breaking down and absorbing nutrients, ultimately depriving them of the resources necessary for normal growth and development (Bucci et al., 2024). Reduced growth can negatively impact individual health and population dynamics (Marn

et al., 2020; Wang et al., 2020). Individuals with stunted growth, due to their limited development, may experience impaired escape responses, which heightens their vulnerability to predators and contributes to increased cumulative mortality (Skajaa et al. 2003). Furthermore, reduced growth often results in smaller body sizes at reproductive maturity, which can negatively impact fecundity and significantly reduce overall reproductive success (Siddiqui et al., 2022). Both study species exhibited heightened sensitivity to weathered TPs, with *A. bahia* showing additional vulnerability to weathered leachate. This may be attributed to the presence of some compounds, including some phototoxic polycyclic aromatic hydrocarbons (PAHs) species in TPs, which absorb UV light and form electronically excited molecules that are more toxic than their original state (Wik and Dave, 2005). Such compounds in the leachate could contribute to the observed adverse effects, emphasizing the role of chemical transformations in enhancing the toxicity of tire-derived pollutants.

4.2. Behavioral responses

The behavioral responses of *A. bahia* and *M. beryllina* to TP exposure reveal significant disruptions to their natural activity patterns, with potential implications for their ecological roles and survival strategies. In *A. bahia*, exposure to pristine micro- and nano-TPs under light conditions induced hyperactivity and reduced thigmotaxis, suggesting heightened stress or altered exploratory behavior that could increase vulnerability to predators. Conversely, hypoactivity observed under dark conditions with weathered nano-TPs and pristine micro-TPs represents a marked deviation from their typical nocturnal planktonic behavior, critical for feeding and reproduction. Similarly, *M. beryllina* exhibited hyperactivity and reduced anxiety under light conditions when exposed to both pristine and weathered micro- and nano-TPs. These altered behaviors may heighten predation risk, with increased activity and diminished fear responses

making prey more visible to predators. Such disruptions to prey-predator dynamics pose significant ecological concerns, potentially destabilizing the delicate balance and interconnected structure of marine food webs (Yin et al., 2018). Siddiqui et al. (2022) reported that *A. bahia* exposed to nano-TPs from a new passenger car tire exhibited increased movement at 15 PSU and 25 PSU during dark cycles, but hypoactivity under light conditions when exposed to tire leachate at 25 PSU. Hutton et al. (2024a) found that only weathered micro- and nano-TPs impacted *M. beryllina* TDM, while unweathered TPs had no effect. For thigmotaxis, they observed significant effects only with nano-TPs, regardless of weathering (Hutton et al., 2024a). The broader effects observed in our study may be attributed to the use of a multi-tire mixture rather than a single-tire type. The combined tire particles likely introduced a more diverse chemical composition, potentially increasing the toxicity. Particle size also played a critical role in driving behavioral changes in both study species. In *M. beryllina*, exposure to pristine micro-TPs in the dark increased thigmotaxis and induced hypoactivity, while pristine nano-TPs in the dark elicited anti-thigmotaxis and hyperactivity. In *A. bahia*, under light conditions, pristine micro-TPs reduced thigmotaxis, whereas pristine nano-TPs increased it. Similarly, both under light and dark conditions, weathered micro-TPs induced hyperactivity and reduced thigmotaxis, while weathered nano-TPs caused hypoactivity and increased thigmotaxis. These contrasting responses underline the significant and opposing effects of particle size on TDM and thigmotaxis, potentially reflecting the distinct mechanisms through which micro- and nano-sized TPs influence behavior. The physical presence of micro-TPs, due to their larger size, likely affects behavior by engaging sensory pathways or inducing stress from their detection in the environment. In contrast, the smaller size of nano-TPs allows them to evade direct sensory detection but enables them to enter the circulatory system (Vagner et al., 2022) and potentially cross

the blood-brain barrier. This allows them to act on the neural system, potentially disrupting normal behavioral patterns (Chen et al., 2020). The differential effects of TPs and their leachate observed between *M. beryllina* and *A. bahia* highlight species-specific sensitivities. In *M. beryllina*, pristine micro- and nano-TPs significantly affected all behavioral endpoints analyzed, whereas weathered micro- and nano-TPs had a reduced influence, impacting 33.3% and 83.3% of endpoints, respectively. Conversely, *A. bahia* exhibited a different response, with pristine micro-TPs impacting only 33.3% of the endpoints, while pristine nano-TPs and both weathered micro- and nano-TPs affected 100% of the endpoints. Both pristine and weathered leachates exhibited similar behavioral effects as the corresponding TPs in both species. These disparities may reflect intrinsic differences in how each species interacts with microplastics (Suckling et al., 2021), as well as potential variations in the relevance of specific behavioral endpoints. Additionally, the differing exposure durations, 96 hours for *M. beryllina* compared to 7 days for *A. bahia*, could further account for the variations in observed effects.

Conclusion

To our knowledge, this is the first study utilizing an environmentally relevant multi-tire mixture, providing a more accurate reflection of real-world tire pollution. Our results showed a positive correlation between TP concentration and ingestion rates. Both species had higher ingestion rates when exposed to weathered TPs, with *A. bahia* consuming more overall, likely due to its benthic lifestyle. We observed species-specific responses, with weathered TPs having the most pronounced effect on growth reduction in both species. Both *M. beryllina* and *A. bahia* experienced growth reductions when exposed to weathered micro- and nano-sized TPs, but *A. bahia* was also affected by pristine TPs and weathered leachate. Notably, we started to observe growth reductions in *A. bahia* at a lower concentration when exposed to weathered TPs. Although the weathering process did not

necessarily increase behavioral toxicity. While weathered TPs increased behavioral toxicity in *A. bahia*, no such effect was observed in *M. beryllina*. This discrepancy highlights the intricate ecological impacts of tire pollution, demonstrating that certain species may be disproportionately vulnerable, and emphasizes the critical need to consider species-specific responses when evaluating the ecological risks associated with tire pollution. On a larger scale, these findings shed light on the role of TPs in exacerbating the growing crisis of microplastic contamination, with potential cascading effects on biodiversity, food webs, and ecosystem functioning. In conclusion, by comparing our findings to existing literature (Hutton et al., 2024a; Siddiqui et al., 2022), we highlighted that multi-tire mixtures exhibit broader behavioral toxicity and growth-related effects, likely due to the wide variety of associated chemicals. Future studies employing multigenerational testing are essential for gaining a deeper understanding of long-term effects, thereby strengthening risk assessment and management strategies.

Supplementary materials

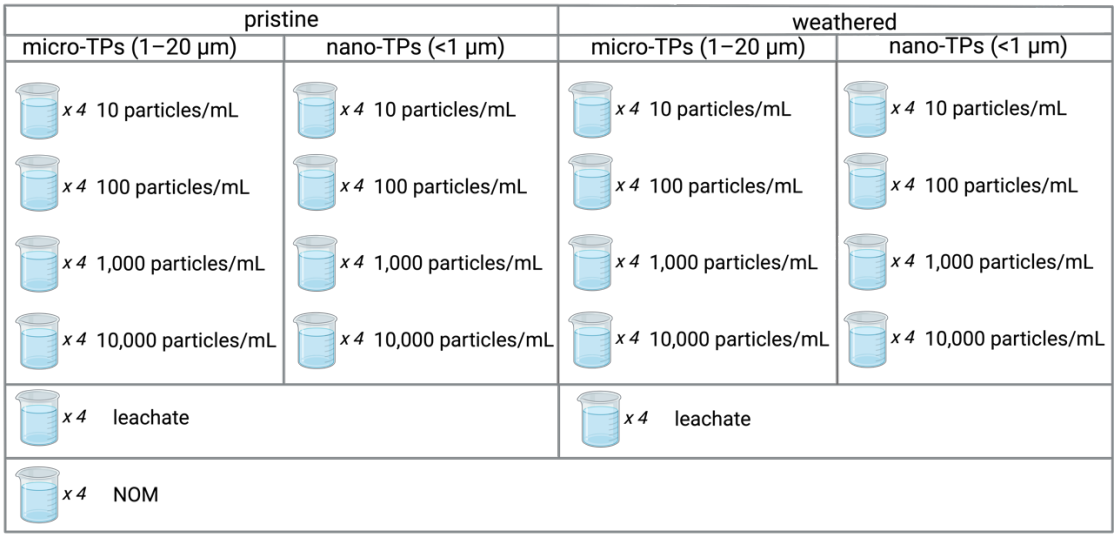

Figure S1: Experimental setup consisting of 38 treatment groups and 2 control groups, with 19 treatment groups and 1 control group assigned to each of the two study species. For each species, the treatment groups included: pristine micro- and nano-TP treatments at four concentrations (10, 100, 1,000, 10,000 particles/mL), weathered micro- and nano-TP reatment at four concentrations (10, 100, 1,000, 10,000 particles/mL), pristine leachate, weathered leachate and natural riverine organic matter (NOM). Each treatment group was replicated four times (x4).

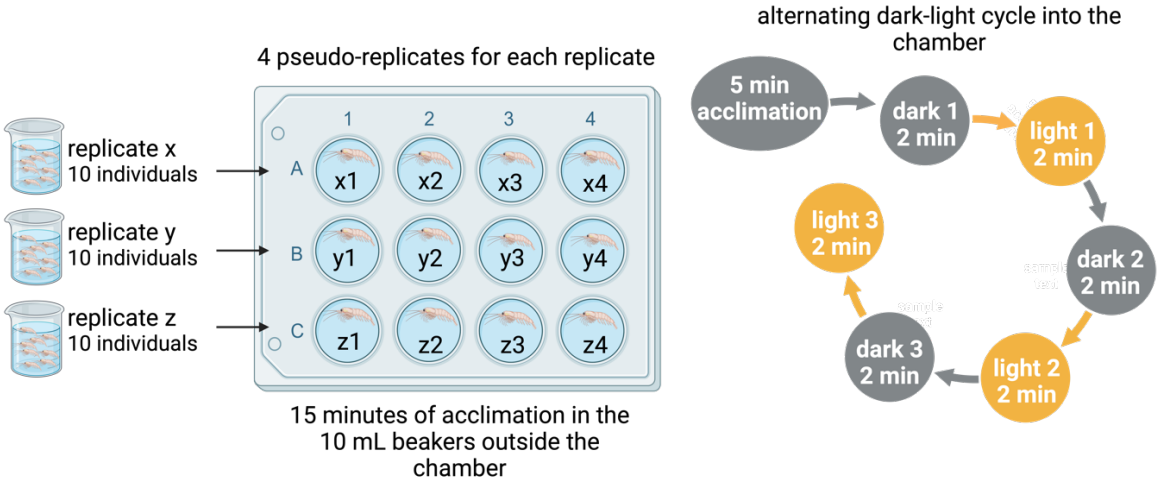

Figure S2: (left) Simplified overview of the behavioral assays. From each of the 4 replicates per treatment and control group (generalized as x, y, z), 4 individuals were randomly selected from the 10 individuals in each beaker. Behavioral assays were conducted on these 4 individuals, resulting in 4 pseudo-replicates per replicate (e.g. x1, x2, x3, x4 for replicate x). (right) Schematic representation illustrating the alternating dark-light cycle to which the organisms were subjected. Abbreviations: min = minutes.

A. bahia						
Endpoint	Pristine Micro-TPs	Pristine Nano-TPs	Weathered Micro-TPs	Weathered Nano-TPs	Pristine TP Leachate	Weathered TP leachate
Cruising (speed > 0.5 cm/s and < 20 cm/s)	None	C0/C2 in dark 1 C0/C1 in light 1, light 2 C0/C3 in light 1, light 2, light 3	C0/C3 in dark 1 C0/C4 in dark 1 C0/C1 in dark 2	C0/C1 in dark 1, dark 2 C0/C3 in dark 2	C0/leachate in light 3	C0/leachate in dark 1, dark 2, dark 3, light 1
Freezing (speed < 0.5 cm/s)	None	C0/C2 in dark 1 C0/C1 in light 1, light 3 C0/C3 in light 1, light 2, light 3 C0/C1 in light 2	C0/C3 in dark 1, light 1 C0/C4 in dark 1, light 2, light 3 C0/C1 in dark 2 C0/C2 in light 3	C0/C1 in dark 1, dark 2 C0/C3 in dark 2 C0/C4 in light 2, light 3 C0/C2 in light 3	C0/leachate in light 3	C0/leachate in dark 1, dark 2, light 1
Bursting (speed > 20 cm/s)	C0/C4 in dark 1 C0/C1 in dark 3	C0/C4 in dark 1, light 1 C0/C3 in light 1, light 2, light 3 C0/C1 in light 2	C0/C3 in dark 1, light 1, light 2 C0/C1 in dark 2, light 1 C0/C4 in light 2, light 3 C0/C2 in light 3	C0/C2 in light 3	C0/leachate in dark 2, light 1, light 2, light 3	None

Velocity (cm/s)	None	C0/C2 in dark 1 C0/C4 in dark 1 C0/C1 in light 1, light 2, light 3 C0/C3 in light 1, light 2, light 3	C0/C1 in dark 1, dark 2 C0/C3 in dark 1, light 1, light 2 C0/C4 in light 2, light 3 C0/C2 in light 3	C0/C1 in dark 1 C0/C3 in dark 2 C0/C4 in light 2, light 3 C0/C2 in light 3	C0/leachate in light 3	C0/leachate in dark 1, dark 2, light 1
Thigmotaxis (wall hugging)	C0/C3 in dark 1, dark 3, light 2 C0/C1 in light 2	C0/C3 in light 2	C0/C1 in dark 2 C0/C3 in dark 2 C0/C3 in light 3 C0/C4 in light 3	C0/C2 in dark 1, dark 2, dark 3, light 1, light 2 C0/C3 in dark 2, dark 3 C0/C4 in dark 2, light 1	None	C0/leachate in dark 1, dark 2, dark 3
Total distance moved (cm)	None	C0/C2 in dark 1, light 3 C0/C4 in dark 1 C0/C1 in light 1, light 2 C0/C3 in light 1, light 2, light 3	C0/C1 in dark 1, dark 2 C0/C3 in dark 1, light 1, light 2 C0/C4 in light 2, light 3 C0/C2 in light 3	C0/C1 in dark 1 C0/C3 in dark 2 C0/C4 in light 2, light 3 C0/C2 in light 3	C0/leachate in light 3	C0/leachate in dark 1, dark 2, light 1

M. beryllina						
Cruising (speed > 0.5 cm/s and < 20 cm/s)	C0/C3 in dark 1 C0/C2 in dark 2 C0/C3 in light 3	C0/C1 in dark 1	None	C0/C3 in dark 1, light 2 C0/C1 in light 3	C0/leachate in dark 1	None
Freezing (speed < 0.5 cm/s)	C0/C3 in dark 1, light 3 C0/C2 in dark 2	C0/C1 in dark 1 C0/C2 in dark 2	None	C0/C3 in dark 1, light 2	C0/leachate in dark 1, dark 3	None
Bursting (speed > 20 cm/s)	C0/C3 in dark 1, light 3 C0/C4 in dark 2	C0/C2 in dark 2	C0/C1 in light 2, light 3 C0/C3 in light 2 C0/C2 in light 3	None	C0/leachate in dark 1	C0/leachate in, dark 2, dark 3, light 2, light 3
Velocity (cm/s)	C0/C1 in dark 1 C0/C3 in dark 1, light 3	C0/C1 in dark 1 C0/C2 in dark 2	None	C0/C3 in dark 1, light 2 C0/C2 in light 2	C0/leachate in dark 1, dark 3	C0/leachate in dark 2
Thigmotaxis (wall hugging)	C0/C3 in dark 3	C0/C3 in dark 3 C0/C2 in light 2	C0/C3 in light 2	C0/C2 in light 2	C0/leachate in dark 3, light 3	C0/leachate in dark 3, light 3, light 2, light 3

Total distance moved (cm)	C0/C3 in dark 1, light 3 C0/C2 in dark 2	C0/C1 in dark 1 C0/C2 in dark 2	None	C0/C3 in dark 1, light 2 C0/C2 in light 2	C0/leachate in dark 1, dark 3	C0/leachate in dark 2

Table S1: Summary of behavioral endpoints examined in *A. baia* and *M. beryllina*, showing significant differences for each treatment group (pristine and weathered micro- and nano-tire particles, and pristine and weathered tire particle leachate) compared to the control group. Differences are shown across dark-light cycle stimuli for each endpoint, including cruising, freezing, bursting, velocity, thigmotaxis, and total distance moved. Abbreviations: C0 = control; C1 = 10 particles/mL; C2 = 100 particles/mL; C3 = 1,000 particles/mL; C4 = 10,000 particles/mL. None = no significant differences.

	Salinity (ppt)	pH	Temperature (°C)	Ammonia (ppm)	Dissolved Oxygen (mg/L)
A. bahia	20.08 ± 0.04	7.74 ± 0.04	23.1 ± 0.08	0.01 ± 0.08	7.05 ± 0.05
M. beryllina	15.07 ± 0.04	7.8 ± 0.02	23.2 ± 0.04	0.03 ± 0.05	7.12 ± 0.04

Table S2: Water-quality parameters for *A. bahia* and *M. beryllina* exposures. Data are expressed as mean ± standard deviation.

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Chapter 4

Conclusions

As concerns about plastic pollution continue to grow, a comprehensive initiative has emerged, advocating for regulatory measures and strategies to reduce plastic waste, including bans on single-use plastics, improved waste management practices, and increased recycling efforts. Despite the growing urgency and attention devoted to addressing this critical issue, our understanding of the distribution and behavior of plastics and microplastics in marine environments remains alarmingly insufficient. The dynamic and complex nature of the ocean further complicates this issue, making it exceptionally difficult to capture an accurate and comprehensive picture of the abundance and distribution of plastic pollution. This knowledge gap is particularly concerning, as it limits our ability to fully assess the ecological impacts and risks these pollutants pose to marine life, ecosystems, and human health.

One major challenge in microplastic detection is mitigating sample contamination, which can lead to overestimation of microplastic prevalence and abundance. To address this, a clean room environment compliant with ISO 6 standards was established, characterized by very low background contamination. This ensured that the results provided a true and reliable representation of microplastic concentrations in analyzed samples. Equally critical in tackling plastic pollution is the lack of standardized methods for identifying and quantifying microplastics and the absence of long-term monitoring frameworks to inform effective policy decisions. This thesis makes a valuable contribution to this critical effort by validating and optimizing analytical methods for detecting microplastics down to 25 μm in size, as well as phthalic acid esters in two marine invertebrate species. We presented essential data on microplastic concentrations and included information on their shape, size, color, and polymer composition. Furthermore, we emphasized the lack of a significant correlation between phthalic acid ester concentrations and microplastic

concentrations. As a result, these compounds cannot be considered reliable tracers for plastic pollution because the rates of plastic ingestion and egestion may not align with the rates of phthalate absorption and excretion. Additionally, it cannot be ruled out that the presence of phthalates in tissues may also derive from sources other than microplastics, such as the surrounding environment, further complicating their use as indicators. This knowledge represents an important framework for future studies examining the impacts of microplastics and their associated contaminants.

Once microplastics and nanoplastics enter the environment, they pose significant threats to marine life and ecosystems. Laboratory experiments are essential for understanding these impacts. However, while extensive research has focused on single types of particles, there is limited knowledge about the effects of heterogeneous mixtures that mirror the variability in shape, size, and composition of microplastics found in the real environment. To address this gap and enhance the ecological relevance of laboratory findings, this thesis, in collaboration with the Brander lab at Hatfield Marine Science Center, Oregon State University, conducted exposure studies utilizing an environmentally relevant multi-tire mixture provided by the U.S. Tire Manufacturers Association. This approach allows for a more accurate simulation of the diverse chemicals associated with tire particles typically found in natural settings. Additionally, by subjecting these tire particles to UV-weathering, we enhanced their ecological relevance, ensuring that our findings reflect the complexities of environmental interactions. Our critical review of existing literature revealed that previous tire particle studies have predominantly examined single tire types. By comparing our findings, we highlighted that multi-tire mixtures exhibit broader behavioral toxicity and growth-related effects, likely due to the diverse array of associated chemicals. We observed that weathered TPs had the most pronounced effect on growth in both species, although the

weathering process did not necessarily increase behavioral toxicity. Additionally, we underscore the importance of incorporating multiple study species in toxicity research to account for species-specific responses.

In conclusion, this thesis addresses the pressing need for improved methodologies in the study of microplastic pollution, emphasizing the critical gaps in our understanding of their distribution, behavior, and ecological impacts. By implementing rigorous analytical techniques and effectively controlling for sample contamination, this research lays a solid foundation for accurately assessing microplastic concentrations in marine environments. The use of an environmentally relevant multi-tire mixture, subjected to UV-weathering, not only bolsters the ecological validity of the laboratory study but also allows for more reliable extrapolation of findings to real-world scenarios. Ultimately, this work contributes significantly to the expanding body of knowledge essential for informed policy-making and the development of effective management strategies aimed at mitigating the widespread threat of plastic pollution in our oceans. By fostering a deeper understanding of microplastic presence and its ecological implications, this thesis aspires to contribute to a more sustainable future for our oceans.

Appendix A

A.1. National and International conferences

1. Raguso C., Kashiwabara L., Arriola S., Harper S., Harper B., Lasagni M., Brander S.M. (2024) Effects of Tire Wear Micro- and Nanoparticles in the Model Estuarine Species Fish *Menidia beryllina*. Poster: PNW SETAC 33rd annual conference, May 13 – 15 th, Troutdale, Oregon, USA.
2. Raguso C., Kashiwabara L., Arriola S., Harper S., Harper B., Lasagni M., Brander S.M. (2024) Impact of Pristine and Weathered Environmentally Relevant Multi-Tire Mixture on the Model Estuarine species Fish *Menidia beryllina* and Mysid shrimp *Americamysis bahia.* Oral communication: SETAC Europe 34 th Annual Meeting, May 5 – 9 th, Seville, Spain.
3. Raguso C., Kashiwabara L., Harper S., Harper B., Brander S.M. (2023) Effects of Tire Wear Micro- and Nanoparticles in the Model Estuarine Species Fish *Menidia beryllina*. Oral communication: SETAC North America 44th Annual Meeting, November 12 – 16 th. Online.
4. Raguso C., Kashiwabara L., Arriola S., Harper S., Harper B., Brander S.M. (2023) Effects of Tire Wear Micro- and Nanoparticles in the Model Estuarine Species Fish *Menidia beryllina*. Poster: State of the Coast, Oregon's coastal conference, November 3 – 4th, Newport, Oregon, USA.
5. Raguso C., Kashiwabara L., Harper S., Harper B., Brander S.M. (2023) Effects of Tire Wear Micro- and Nanoparticles in the Model Estuarine Species *Menidia*

beryllina. Oral communication: Microplastics and Marine Debris Workshop, October 5 – 6 th, Seattle, Washington, USA.

6. Raguso C., Saliu F., Galli P., Clemenza M., Montano S., Lasagni M. (2022) First detection of microplastics in reef-building corals from a Maldivian Atoll. Oral communication*:* IX Congresso Nazionale della Divisione di Chimica dell'Ambiente e dei Beni Culturali, June 20 – 23rd, Torino, Italy.
7. Raguso C., Saliu F., Galli P., Clemenza M., Montano S., Lasagni M. (2022) First detection of microplastics in reef-building corals from a Maldivian Atoll. Poster: Fourth Marine Science Symposium, August 13 –14th. Online.

A.2. Awards

1. Winner of the Maryann Bozza Scholarship Hatfield Marine Science Center, Oregon State University, USA. (2024)
2. Winner of the student travel award to attend PNW-SETAC 33rd annual conference – Troutdale, Oregon, USA. (2024)
3. Winner of the student travel award to attend SETAC North America 44th Annual Meeting – Online. (2023)
4. Winner of the student travel award to attend XIX Congresso Nazionale della Divisione di Chimica dell'Ambiente e dei Beni Culturali – Torino, Italy. (2022)

A.3. Abstracts of Peer-reviewed Publications Produced During the PhD Program (Unrelated to Thesis Topic)

I. Extraction of microplastic from marine sediments: A comparison between pressurized solvent extraction and density separation

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Due to the ecotoxicological effects, microplastics are considered a threat to the marine environment. Recent reports indicate their presence not only in subsurface water and in coastal beach sediments but also in the deep- sea. Notwithstanding, there is still not a scientific consensus about the analytical procedure to be applied for their determination. In this work, we compared the performance of two extraction methods: pressurized solvent extraction (PSE) and density separation. Sea sand and seafloor sediments were spiked with known amounts of polystyrene (PS), polyethylene (PE), and polypropylene (PP) microplastics and submitted to both extraction procedures. Results showed that the PSE ensured higher recoveries for the smaller size particle fractions (89,2 ± 1.1% in the 50–150 μm range) whereas the density separation enabled precise recoveries for the larger size particles (SD = 1,5%). No significant differences in terms of blanks control were highlighted.

II. The release process of microfibers: from surgical face masks into the marine environment

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Due to the COVID-19 pandemic, the use of disposable face masks has been adopted worldwide as a precautionary measure to slow down the transmission of the virus. This has determined an unprecedented rise in the production of these protective equipment, and unfortunately to a new form of environmental contamination due to improper disposal. To provide a preliminary estimation of the release of microfibers by a surgical mask dumped in the marine environment, we carried out artificial weathering experiments. Results indicated that a single surgical mask submitted to 180 hours of UV-light irradiation and vigorous stirring in artificial seawater may release up to 173,000 fibers/ day. Moreover, SEM and micro-FTIR analysis carried out on surgical masks collected from Italian beaches highlighted the same morphological and chemical degradation signature observed in the masks subjected to the artificially weathering experiments, confirming the risks of a similar microfiber release into the marine environment.

III. Detection of plastic particles in marine sponges by a combined infrared micro-spectroscopy and pyrolysis-gas chromatography-mass spectrometry approach

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Plastic pollution threatens the marine environment, especially due to the adverse effects caused by micro and nano particles interacting with the marine biota. In order to provide reliable data regarding micro and nanoplastic contamination and the related impacts, efficient analytical solutions are needed. We developed a new analysis workflow that uses marine sponges to monitor plastic pollution by characterizing the plastic particles accumulated in their tissue. Specimens of cf. Haliclona (Haplosclerida) were sampled in the Maldivian archipelago. The aim was to optimize the method and to carry out a pilot study of the contamination of the related reef habitat. Particles were isolated, size fractioned, counted, and submitted to morphological and chemical characterization. The constituting polymer was identified by infrared microspectroscopy for particles >25 μm, and by pyrolysis coupled with gas chromatography-mass spectrometry for those <25 μm. Method recoveries were between 87 and 83% and limits of quantitation (LOQs) were between 6.6 and 30.2 ng/g. Analyses showed that 70% of the sponges presented plastic contamination, with an average of 1.2 particles/g tissue for the 25–150 μm size range, and a total plastic concentration of up to 4.8 μg/g in the 0.2–25 μm size range, with

polyolefin being the most represented polymer in both size ranges. Overall, the study demonstrated the reliability of the proposed analytical workflow and of the use of sponges as biosamplers for plastic particles.

IV. Phthalates bioconcentration in the soft corals: Inter- and intra-species differences and ecological aspects

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The bioconcentration of dimethyl phthalate (DMP) diethyl phthalate (DEP) dibutyl phthalate (DBP) butyl benzyl phthalate (BBzP), di-(2-ethy hexyl) phthalates (DEHP), mono-butyl phthalate (MBP), mono-benzyl phthalate (MBzP), mono-(2-ethy hexyl) phthalate (MEHP) in the soft corals *Coelogorgia palmosa*, *Sinularia* sp., *Sarcophyton glaucum*, and *Lobophytum* sp. was investigated. Specimens were cultured in a microcosm environment built up at the Genova Aquarium and analyses were carried out by *in vivo* SPME-LC-MS/MS. The distributions of the phthalates among the four surveyed species were significantly different. Calculated bioconcentration factors (BCFs) showed values spanning over two orders of magnitude, from a minimum of log10 BCFDEP = 1.0 in *Sarcophyton glaucum* to a maximum of log10 BCFDBP = 3,9 calculated for *Coelogorgia palmosa*. Moreover, the calculated BCFs of the long-chain phthalates resulted up to three orders of magnitude lower than theoretically predicted (from log*Kow*), whereas BCF of short-chain phthalates resulted higher. This, together with the detection of phthalic acid monoesters, suggests the presence of species-specific different metabolic transformations among the surveyed soft coral species that involve DEHP.

V. Microplastics in the first-year sea ice of the Novik Bay, Sea of Japan

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Sea ice is heavily contaminated with microplastic particles (MPs, <5 mm). First-year sea ice cores (38–41 cm thick) were taken in the beginning of spring in a narrow populated bay of the Sea of Japan. Two ice cores were examined (layer-by-layer, excluding surface) for MP content: one using μ-FTIR for 25–300 μm (SMPs), and another one – with visual+Raman identification for 300–5000 μm particles (LMPs). The integral (25–5000 μm) bulk mean abundance of MPs was found to be 428 items/L of meltwater, with fibers making 19 % in the SMPs size range and 59 % in LMPs. The integral mean mass of MPs was estimated at 34.6 mg/L, with 99.6 % contribution from fragments of LMPs. Comparison with simple fragmentation models confirms a deficit of SMPs (especially of fibers in the size range 150–300 μm), suggested to result from their leakage with brine. Multivariate statistical analysis indicates a strong positive correlation between large fiber (>300 μm) counts and ice salinity.

VI. Hotspots of microplastic accumulation at the land-sea transition and their spatial heterogeneity: The Po River prodelta (Adriatic Sea)

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Deltas are the locus of river-borne sediment accumulation, however, their role in sequestering plastic pollutants is still overlooked. By combining geomorphological, sedimentological, and geochemical analyses, which include time-lapse multibeam bathymetry, sediment provenance, and μFT-IR analyses, we investigate the fate of plastic particles after a river flood event providing unprecedented documentation of the spatial distribution of sediment as well as of microplastics (MPs), including particles fibers, and phthalates (PAEs) abundances in the subaqueous delta. Overall sediments are characterized by an average of 139.7 ± 80 MPs/kg d.w., but display spatial heterogeneity of sediment and MPs accumulation: MPs are absent within the active sandy delta lobe, reflecting dilution by clastic sediment (ca. 1.3 Mm3) and sediment bypass. The highest MP concentration (625 MPs/kg d.w.) occurs in the distal reaches of the active lobe where flow energy dissipates. In addition to MPs, cellulosic fibers are relevant (of up to 3800 fibers/kg d.w.) in all the analyzed sediment samples, and dominate (94 %) with respect to synthetic polymers. Statistically significant differences in the relative concentration of

fiber fragments ≤0.5 mm in size were highlighted between the active delta lobe and the migrating bedforms in the prodelta. Fibers were found to slightly follow a power law size distribution coherent with a one-dimensional fragmentation model thus indicating the absence of a size-dependent selection mechanism during burial. Multivariate statistical analysis suggests traveling distance and bottom-transport regime as the most relevant factors controlling particle distribution. Our findings suggest that subaqueous prodelta should be considered hot spots for the accumulation of MPs and associated pollutants, albeit the strong lateral heterogeneity in their abundances reflects changes in the relative influence of fluvial and marine processes.