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Assessment of Microplastic Contamination in Farmed Oysters (*Crassostrea gigas*) from the Lima River Estuary and the Effectiveness of Depuration

Cláudia Moura

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SEDE ADMINISTRATIVA INSTITUTO DE **CIÊNCIAS BIOMÉDICAS ABEL SALAZAR**

FACULDADE DE CIÊNCIAS

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Abstract

Bivalve production is a growing sector in Portugal, accounting for 67% of the country's total aquaculture output (INE 2020). This activity mainly takes place in estuaries and lagoons, which are vulnerable to anthropogenic pressures, including microplastics (MPs) pollution. MPs have already been detected in shellfish from aquaculture farms in Portuguese estuaries, raising concerns about seafood safety. The risks posed by contaminated shellfish are influenced by physiological and environmental factors, which vary spatially and seasonally. This study, for the first time, investigates the temporal variations in MPs concentrations in Pacific oysters (*Crassostrea gigas)* farmed in the Lima River estuary, comparing oysters collected in early autumn (October) and in winter (February). MPs contamination in the surrounding environment (water and sediment) was also evaluated. MPs were visually characterized by color and size under a stereomicroscope and chemically identified via Fourier Transform Infrared Spectroscopy (FTIR). MPs concentrations in Pacific oysters ranged from 0 to 38 MPs ind^{-1} and 0 to 1.60 MPs g^{-1} wet weight, with significant temporal differences (Kruskal-Wallis test, $p < 0.001$). In contrast, MPs contamination in water and sediment showed no significant temporal variation (Kruskal-Wallis test, $p > 0.05$), despite an overall decreasing trend, with higher MPs concentrations in autumn than in winter. The characterization of MPs by color, size, and polymer type revealed that oysters do not accurately reflect the contamination present in their surrounding environment, suggesting they might not be reliable bioindicators of MPs pollution. The predominant polymers identified were cellulose and polyethylene terephthalate (polyester, PET), likely originating from anthropogenic sources such as wastewater treatment plants (WWTPs) effluents. The presence of MPs in oysters intended for human consumption raises concerns about food safety. To address this, the efficacy of depuration in removing MPs was assessed through experiments conducted in both laboratory and commercial depuration settings. Oysters were sampled at different times (0h, 24h, 48h in both lab and commercial trials, and 96h in the commercial trial; n=15 per time point) to investigate possible MPs reduction over time. In the laboratory, MPs concentrations in oysters decreased significantly (Kruskal-Wallis test, $p < 0.05$) by 78% after 48 hours (from 12.9 ± 14.9 MPs g^{-1} to 3.1 ± 3.2 MPs g^{-1}). A similar decreasing trend was observed in commercial depuration facilities; however, this reduction was not statistically significant (Kruskal-Wallis test, $p > 0.05$), achieving only a 59% decrease in MPs concentrations (from 2.9 ± 3.1 MPs ind^{-1} to 1.1 ± 1.7 MPs ind^{-1}). These findings indicate that controlled conditions, specifically in terms of managing external contamination, can be more effective at reducing MPs contamination. However, residual contamination remained in both cases,

underscoring the need for further research to improve MPs removal from bivalves before their consumption.

Resumo

A produção de bivalves é um setor em ascensão em Portugal, representando 67% da produção total de aquicultura (INE 2020). Esta atividade ocorre predominantemente em estuários e lagoas, suscetíveis a pressões antrópicas, incluindo a poluição por microplásticos (MPs). Os MPs já foram detetados em bivalves cultivados noutros estuários portugueses, suscitando preocupações em relação à segurança alimentar. Os riscos associados à contaminação dos bivalves são influenciados por fatores fisiológicos e ambientais que variam espacial e sazonalmente. Este estudo investiga, pela primeira vez, as variações temporais nas concentrações de MPs em ostras do Pacífico (*Crassostrea gigas)* cultivadas no estuário do Rio Lima, comparando os períodos de outono (outubro) e inverno (fevereiro). A contaminação por MPs no ambiente circundante (água e sedimento) também foi avaliada. Os MPs detectados foram caracterizados visualmente, por cor e tamanho, através de um estereomicroscópio e identificados quimicamente por Espectoscopia no Infravermelho com Transformado de Fourier (FTIR). As concentrações de MPs encontradas nas ostras do Pacífico variaram de 0 a 38 MPs ind^{-1} e de 0 a 1.60 MPs g^{-1} de peso húmido, apresentando diferenças temporais significativas (Kruskal-Wallist test, $p < 0.001$). Em contrapartida, a contaminação por MPs na água e no sedimento não evidenciou variações temporais significativas (Kruskal-Wallis test, $p > 0.05$), embora tenha demonstrado uma tendência geral de concentrações superiores no outono relativamente às do inverno. A caracterização dos MPs por cor, tamanho e tipo de polímero revelou que as ostras não refletem a contaminação existente no ambiente circundante, sugerindo que podem não ser bons bioindicadores da poluição por MPs. Os polímeros dominantes identificados foram celulose e polietileno tereftalato (poliéster, PET), provavelmente provenientes de fontes antrópicas, como efluentes de estações de tratamento de águas residuais (ETARs). A presença de MPs em ostras destinadas ao consumo humano gera preocupações sobre a segurança alimentar. Para investigar esta problemática, foi avaliada a eficácia da depuração na remoção de MPs por meio de ensaios realizados em ambientes distintos, laboratorial e comercial. As ostras foram amostradas em diferentes intervalos de tempo (0h, 24h, 48h em ambos os ensaios, e 96h apenas no ensaio comercial; n=15 por amostragem) para medir uma possível redução de MPs ao longo do tempo. No laboratório, as concentrações de MPs diminuíram significativamente (Kruskal-Wallist test, $p < 0.05$), cerca de 78% após 48 horas de depuração (de 12.9 ± 14.9 MPs g^{-1} para 3.1 ± 3.2 MPs g^{-1}). Uma tendência análoga foi observada na depuração em instalações comerciais. Embora a redução não tenha sido estatisticamente significativa (Kruskal-Wallis test, $p > 0.05$), houve uma diminuição de

59% nas concentrações de MPs após 96 horas de depuração (de 2.9 ± 3.1 MPs ind^{-1} para 1.1 ± 1.7 MPs ind^{-1}). Estes resultados indicam que condições controladas, especialmente no que diz respeito à gestão da contaminação externa, parecem induzir melhores resultados na redução da contaminação por MPs. No entanto, a contaminação residual permaneceu em ambos os contextos, evidenciando a necessidade de mais investigação para se obter melhores resultados em relação à remoção de MPs de bivalves cultivados antes do seu consumo.

Scientific outputs

1. Moura, C., Almeida, C. M. R., Ramos, S., Freitas, V. (2024). Seasonal Assessment of Microplastics (MPs) contamination in Farmed Oysters (*Crassostrea gigas*) from the Lima River estuary. Poster presentation in IJUP – Investigação Jovem da Universidade do Porto, Faculdade de Economia da Universidade do Porto – 08th May 2024.

2. Moura, C., Almeida, C. M. R., Ramos, S., Freitas, V. (2024). Evaluation of the Potential Depuration of Microplastics (MPs) By farmed Pacific Oysters (*Crassostrea gigas*). Poster presentation in BTC – Bue Think Conference, CIIMAR - Interdisciplinary Center of Marine and Environmental Research, Terminal de Cruzeiros de Leixões, Av. General Norton de Matos, Matosinhos – 10th and 11th September 2024.

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List of Abbreviations

ABS – Acrylonitrile butadiene styrene
ANOVA – One-way analysis of variance
BPA – Bisphenol A
FAO – Food and Agriculture Organization of the United Nations
FTIR – Fourier Transform Infrared Spectroscopy
H_2O_2 – Hydrogen peroxide
HDPE – High-density polyethylene
MPs – Microplastics
NW – Northwest
PA – Polyamide
PAK – Polyacrylate
PAHs – Polycyclic Aromatic Hydrocarbons
PBDEs – Polybrominated diphenyl ethers
PE – Polyethylene
PET – Polyethylene terephthalate
POPs – Persistent Organic Pollutants
PS – Polystyrene
PVC – Polyvinyl chloride
PVS – Polyvinyl stearate
PTT – Poly trimethylene terephthalate
PP – Polypropylene
SD – Standard deviation
UV – Ultraviolet radiation
WHO – World Health Organization
WWTPs – Wastewater treatment plants

Chapter I - Introduction

1.1 Environmental impact of plastic production

Over the years, human lifestyle has negatively impacted ecosystems, leading to the widespread environmental contamination from both chemical and solid waste. One of the most pressing environmental concerns is plastic pollution, which has become an urgent and growing problem (UNEP, 2021) (Figure 1). This concern results from the persistence of plastic in the environment, its ability to adsorb and release other pollutants (Hanvey et al., 2017), and its harmful effects on wildlife and potentially on human health (Franzellitti et al., 2019; Mallik et al., 2021; Wang et al., 2020a; Wang et al., 2020b). Since its introduction in the 1950s, plastic has become ubiquitous in the environment, a consequence of its mass production driven by its durability, versatility, low cost, and mechanical strength (Andrady, 2011; Jambeck et al., 2015). Despite these advantages, only a small percentage of the plastic produced is recycled, with the vast majority entering the environment as plastic waste. Research has identified wastewater treatment plants (WWTPs), landfills, direct human disposal, wind, and urban runoff as the main pathways of entry into aquatic ecosystems (Galvão et al., 2020; Kumar et al., 2021; Sridharan et al., 2021). Of the 7 billion tons of plastic waste generated globally, less than 10 % has been recycled (Geyer, 2020). Global plastic production increased from 1.5 million tonnes in 1950 (European Comission, 2013) to 359 million tonnes in 2018 (Plastic Europe, 2019) and it is expected to reach 34 billion metric tonnes by 2050 (Fig. 1) (Geyer, 2020; UNEP, 2021).

Plastics are synthetic polymers derived from fossil materials, and during their manufacturing, additives such as flame retardants (PBDEs), heat stabilizers, and UV stabilizers may be incorporated to enhance their performance (da Costa et al., 2016; Smith et al., 2018). Due to their lightweight, plastics can be transported by winds and currents, leading to widespread accumulation across various environments (Lavers & Bond, 2017; Lebreton et al., 2017; Nunes et al., 2023). Plastics are highly resistant to aging and have minimal biological degradation, making them non-biodegradable. Depending on the type of polymer, plastics can persist in aquatic environments for extended periods without decomposing (Wang et al., 2020b). However, over time, exposure to UV radiation, atmospheric oxidation, and the hydrolytic effects of water can weaken these polymers, causing them to fragment into smaller pieces (Andrady, 2011; Moore, 2008).

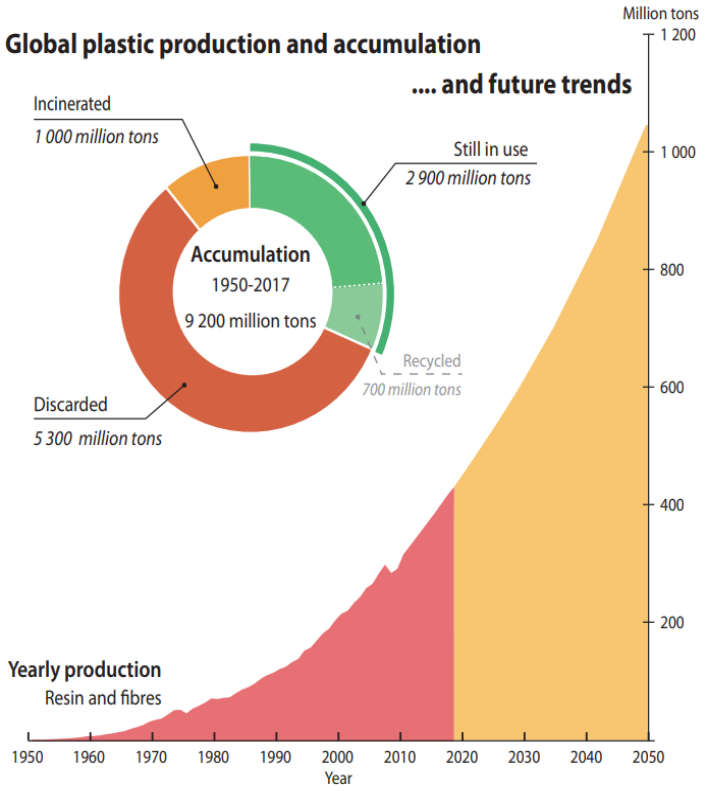

Figure 1. Plastic production, accumulation and trends worldwide (authorized by United Nations Environment Programme (2021). From Pollution to Solution: A global assessment of marine litter and plastic pollution. Nairobi. Figure i: Global plastic production, accumulation and future trends; Illustrated by GRID-Arendal).

1.2 Microplastics (MPs)

Plastics degrade in the environment through physical, chemical, and biological processes, with photodegradation (driven by sunlight) being the most efficient method, especially for plastics exposed to air or lying on beaches where conditions are most favorable (Andrady, 2011; da Costa et al., 2016). Plastic litter can be found on beaches, in surface waters, and in deep-sea environments, but the rate of degradation varies significantly among these locations. On beaches, plastics are exposed to higher temperatures, which accelerate light-initiated oxidative degradation and enhance photodegradation. In contrast, floating plastics in surface waters degrade at reduced rates due to lower temperatures and biological interactions that lead to the formation of a biofilm that protects the plastic surface from sunlight (Andrady, 2011). Additionally, the lack of UV radiation in deeper ocean waters contributes to reduced degradation rates (da Costa et al., 2016). As plastics break down, they produce smaller fragments less than 5 mm in size, known as microplastics (MPs) (Kazmiruk et al., 2018). MPs are classified as primary or secondary based on their origin (Olivatto et al., 2018). Primary

MPs are intentionally produced to be smaller than 5 mm and are commonly found in cosmetics (e.g., scrubs, soaps, toothpaste) or result from the abrasion of larger plastics during production or use (e.g., abrasion of synthetic fabrics during washing) (Kannan & Vimalkumar, 2021). Secondary MPs result from the degradation of larger plastics discarded in the environment (Kazmiruk et al., 2018). Due to their small size, MPs are easily ingested by marine organisms with different feeding mechanisms, promoting their transfer along the food chain (Amato-Lourenço et al., 2021; Andrady, 2011; Wang et al., 2020b; Worm et al., 2017). MPs can enhance the bioavailability of pollutants by adsorbing other contaminants and accumulating them within organisms and ecosystems. They act as vectors of exposure and transfer of Persistent Organic Pollutants (POPs), Polycyclic Aromatic Hydrocarbons (PAHs), heavy metals and other environmental contaminants (Amelia et al., 2021; Li et al., 2020; Wang et al., 2020b) increasing their toxicity and bioaccumulation (Wang et al., 2020b). In addition, MPs can serve as substrates for microorganisms, being contaminated by marine bacteria, which leads to the formation of microbial biofilms. When these biofilm-coated MPs are ingested, they can increase the consumption of hydrophobic organic contaminants (Afianti et al., 2022).

The pollution of aquatic environments by MPs pollution is escalating, with plastic constituting 80-85 % of marine litter (Barletta et al., 2019; Jambeck et al., 2015) and MPs making up 92 % of plastic debris (Santana et al., 2016). Rivers are the main source of marine litter transported to the sea (Ramos et al., 2023), with between 1.2 and 2.4 million tons of plastic waste entering the ocean annually through rivers (Lebreton et al., 2017). Around 90 % of marine litter that reaches the ocean originates from land-based sources, with estuaries being the main export route for debris, particularly plastic debris, from land to sea and this bidirectional flow promotes a significant accumulation of plastic in estuarine environments (Pinheiro et al., 2021). Estuaries are highly dynamic ecosystems characterized by significant environmental fluctuations resulting from tidal influences and the mixing of marine and freshwater (McLusky & Elliott, 2004). They are also highly productive ecosystems, providing habitat for a wide diversity of organisms and providing numerous services to humans, including those related to transportation, tourism, and industry, such as the exploitation of fish and molluscks (Conley & Schelske, 1993; Nixon et al., 1986; Piehler & Smyth, 2011). However, estuaries face various stressors due to urbanization, improper wastewater treatment, intensive agriculture, industrialization, and maritime activities (Piarulli et al., 2020), making them susceptible to contamination, particularly by MPs (Pinheiro et al., 2021). In contrast to coastal waters, there is a substantial accumulation of MPs in estuarine environments (Peng et al., 2017; Zhao et

al., 2014), with concentrations that can amount to 1000 MPs m^{-3} (Ramos et al., 2023). The presence of these particles within estuarine sediments indicates that they function not only as sources of MPs into the ocean but also as potential vectors for MPs (Qian et al., 2021). Common polymers found in aquatic environments include polyethylene (PE), polypropylene (PP), polyethylene terephthalate (polyester, PET), polyvinyl chloride (PVC), polystyrene (PS), polyamide (PA), and cellulose acetate, primarily associated with anthropogenic sources such as fishing, food packaging, and textiles (Andrady, 2011; Dhaka et al., 2022; Ding et al., 2022a; Ó Briain et al., 2020; Wang et al., 2021a). The behavior of MPs is influenced by their properties and polymer compositions. Smaller and lighter MPs have greater potential for long-distance travel and mobility, while heavier MPs tend to settle into sediment layers more rapidly (Alfonso et al., 2021). Consequently, estuaries play a crucial role in the transfer of MPs to the ocean, acting as significant sources of these pollutants.

MPs have been detected in various environmental matrices worldwide, including sediments, water, and biota (Chen et al., 2021; Ding et al., 2021; Du et al., 2022; Jang et al., 2020; Mladinich et al., 2024; Saldaña-Serrano et al., 2022; Truchet et al., 2021; Wootton et al., 2022). In Portugal, MPs have been identified in water bodies and sediments throughout the country (Almeida et al., 2023; Espincho et al., 2024; Prata et al., 2020; Rodrigues et al., 2019a), with higher contamination levels observed in the North, Center and Lisbon regions compared to the south (Alentejo and Algarve) (Prata et al., 2020). It is estimated that around 4.1 trillion MPs are discharged annually from mostly untreated wastewater sources (Prata et al., 2020). Additionally, MPs have been reported in several commercial fish species along the Portuguese coast (Bellas et al., 2016; Bessa et al., 2018; Neves et al., 2015; Rodríguez & Pham, 2017), affecting both pelagic and demersal fish and highlighting their widespread presence in both the water column and seabed sediments (Bellas et al., 2016; Neves et al., 2015). MPs contamination has also been reported in bivalves (Barboza et al., 2024; Carvalho Ferreira & Lôbo-Hajdu, 2023; Cozzolino et al., 2021; Marques et al., 2021; Pequeno et al., 2021; Vital et al., 2021). These findings underscore the urgent need for further research, as Portugal, a leading seafood consumer in Europe (Madsen & Chkoniya, 2021), faces significant concerns about food safety, marine health, and potential risks to human health from MPs contamination (Kibria, 2023).

1.3 Impacts on marine organisms

The presence of MPs in marine organisms, poses a threat to ecosystem health. Numerous studies assessing the impact of MPs have documented physical,

ecotoxicological, physiological and molecular effects across different levels of biological organization (Franzellitti et al., 2019; Ma et al., 2020; Mallik et al., 2021; Wright et al., 2013). The transfer of these particles through the throphic levels suggests potential health implications for marine organisms, the food chain and potentially humans (Alberghini et al., 2023; Bhuyan, 2022; Farrell & Nelson, 2013; Makhdoumi et al., 2023; Mercogliano et al., 2020).

Ingested MPs can cause physical damage, such as obstructing or injuring the gills and digestive tract (Wright et al., 2013). For instance, fibers, a common type of MPs found in aquatic organisms, are particularly problematic because they are difficult to remove from the digestive tract (Li et al., 2015; Rebelein et al., 2021). This suggests that fibers may cause more severe adverse effects due to prolonged exposure (reviwed by Rebelein et al., 2021). In bivalves, MPs exposure can disrupt filtration activity. Research has shown that the filtration activity of the mussel *Mytilus edulis* decreased after exposure to MPs, reducing food absorption and consequently the energy available for growth and other essential functions (Sussarellu et al., 2016). On the other hand, the oyster *Ostrea edulis* displayed a significant increase in filtration activity following MPs exposure (Green, 2016), suggesting that different species may use distinct strategies to respond to MPs exposure. Furthermore, MPs can serve as vectors for pollutants and pathogens, exacerbating their adverse effects on many marine organisms (Granby et al., 2018; Ma et al., 2020; Oliveira et al., 2013; Pittura et al., 2018; Wang et al., 2020a; Wang et al., 2020b; Zhang et al., 2019). The main responses are related to molecular, cellular, and systemic effects, such as neurotoxicity, immunotoxicity, oxidative damage, genotoxicity, structural damage, and antioxidant capacity, among other effects (Baroja et al., 2021; Bringer et al., 2022; Franzellitti et al., 2019; Oliveira et al., 2013; Wang et al., 2020b)

1.4 MPs contamination in aquaculture

Aquaculture seafood products have significantly increased their share of global aquatic food consumption, rising from 26 % in 2000 to 46 % in 2018. Within this sector, bivalve aquaculture has seen remarkable growth, with production rising from 8.3 million tons in 2000 to 15.9 million tons in 2018 (FAO, 2020). Oyster aquaculture, in particular, has been on an upward trend since 1950 (Botta et al., 2020). This industry predominantly occurs in the intertidal zones of estuaries, employing several methods that vary across locations and in response to environmental conditions (Mercer & Lovatelli, 2024). The quality of bivalve harvesting and production areas depends on the geographical characteristics of the coastline, but also on the coexistence of other anthropogenic

activities that generate pollution, consequently influencing water quality (Silva, 2008). In estuarine environments, both fishing and aquaculture activities are exposed to a wide range of contaminants, including MPs, which may lead to contamination of these products (Harmon, 2018). Regular monitoring of these environments is crucial due to significant concerns about food safety and the potential risks to human health associated with the consumption of contaminated seafood (Zhou et al., 2021). MPs can enter the aquaculture systems from two primary sources: internal sources, such as aquaculture gear and infrastructure weathering and shedding, and external sources, like anthropogenic pollution (reviewed by Wu et al., 2023). MPs also present risks to aquaculture systems themselves, potentially introducing harmful substances like chlorine (PVC), which can increased acidity in the water (Gewert et al., 2015), or organic additives such as BPA , which can act as endocrine disruptors when leached into the environment (Do et al., 2022). These risks can result in reduced growth rates, DNA damage (Pannetier et al., 2020), increased absorption of chemical contaminants (Zhang et al., 2019), neurotoxic effects, and lower overall quality of aquaculture products (Tang et al., 2021). Therefore, it is essential to monitor both the contamination of organisms and the surrounding environment, as well as determine whether aquaculture activities themselves contribute significantly to MPs pollution.

The accumulation of MPs in farmed bivalves, mostly mussels (*M. edulis*) and oysters (*C. gigas*), has been well-documented, with reported levels ranging from 0.22 to 178 MPs per individual and 0.02 to 20 MPs per gram, depending on the species and location (reviewed in Bom & Sá, 2021). Some studies have found that mussels accumulate higher levels of MPs than other species, such as oysters (Chinfak et al., 2021; Piarulli et al., 2020), which possess more efficient selective-feeding mechanisms (Ward et al., 2019). However, other studies have reported the opposite (Phuong et al., 2018; Van Cauwenberghe & Janssen, 2014). Some studies have also compared wild vs. farmed animals to assess the potential impact of MPs pollution due to aquaculture activity on seafood and the local environment. Again, contrasting results have been found, with some studies reporting higher MPs levels in farmed bivalves compared to wild ones (Anderson et al., 2016; Covernton et al., 2019; Mathalon & Hill, 2014; Phuong et al., 2018), while others found no significant differences between farmed and their wild counterparts (Birnstiel et al., 2019; Covernton et al., 2019; Zhang et al., 2022). Thus, overall it seems that the contamination of farmed bivalves with MPs may depend on a wide range of factors, including geographic location, local environmental conditions, season, farming practices, as well as the cultured species. Further research is needed to examine these variables and to clarify these issues.

1.5 Seafood consumption and human health

MPs have been detected in consumer products such as table salt, tap water, and even in the air, indicating multiple exposure pathways (Zhang et al., 2020). Human uptake from seafood is another source of exposure (Vital et al., 2021). Van Cauwenberghe and Janssen (2014) reported that heavy consumers in Europe ingest approximately 11 000 MPs per year through mollusk consumption, while lighter consumers ingest around 1 800 MPs per year. Bivalve mollusks, especially oysters, which are often consumed whole and raw without the removal of the digestive tract, represent a potential source of exposure to MPs and other contaminants for humans (Ding et al., 2022b). However, while reporting the occurrence of MPs in seafood is important, it should be noted that there is still insufficient data to assess the potential toxicity of MPs in humans, making it difficult to evaluate the risk of MPs to human health (EFSA 2016; Lusher et al. 2017).

In the context of reducing the threat to human health from seafood consumption, some studies have highlighted the importance of depuration in removing MPs contamination from seafood. Depuration is a common post-harvest practice used by the shellfish aquaculture industry to remove microbial contaminants and potential pathogens from the bivalves, ensuring its safety (McLeod et al., 2017). It involves placing bivalves in tanks filled with clean seawater under controlled conditions, which promote their natural filtration activity, allowing them to expel intestinal contents and thus remove contaminants (Lee et al., 2008). In the case of MPs, the viability and effectiveness of depuration in reducing or removing MPs from commercially farmed bivalves have been barely studied (but see Covernton et al., 2022; Paul et al., 2023). Studies conducted to date have shown that depuration can reduce MPs concentrations in oysters (Birnstiel et al., 2019; Van Cauwenberghe & Janssen, 2014). However, the effectiveness of MPs depuration appears to be influenced by various factors, including depuration time, initial concentrations, as well as the type and size of MPs (Covernton et al., 2022; Graham et al., 2019; Weinstein et al., 2022). Time is a critical parameter, as the depuration period varies depending on the species and the nature of the contaminants. For example, Van Cauwenberghe and Janssen (2014) reported a 33 % reduction of MPs in mussels and a 25% reduction in oysters after 72 hours of depuration. However, while extending the depuration period may further reduce MPs levels, it can also negatively affect bivalve health, causing stress and a decline in quality (Chen et al., 2022). Additionally, longer depuration times increase operational costs, potentially leading to more expensive products. Given the current levels of MPs contamination in commercially harvested

bivalves, further studies are needed to evaluate whether depuration is truly effective in removing MPs and ensuring food safety.

1.6 Aim

This study addresses the MPs contamination in commercial bivalves produced in aquaculture. Specifically, the aims were to: i) determine the levels of MPs in Pacific oysters (*C. gigas*) farmed in the Lima estuary (NW Portugal) and investigate possible seasonal variations; ii) examine the relationship between MPs contamination in oysters and their surrounding environment, including water and sediments; and iii) assess the effectiveness of depuration in reducing the MPs burden in commercial oysters, under both laboratory-controlled and commercial depuration conditions.

Chapter II - Methodology

2.1. Study site and sample collection

All samples (water, sediment, and oysters) were collected from an oyster farm located in an intertidal area in the Lima estuary (41°41'21"N 8°48'44"W; Fig. 2A) on the NW coast of Portugal. The sampling site is a designated bivalve production area classified as Class B (IPMA 2024), meaning that the bivalves harvested for human consumption need to be depurated, transposed or transformed in an industrial unit, before being further sold and consumed. The Lima estuary holds high ecological significance (Ramos et al., 2010), but it is vulnerable to a range of anthropogenic disturbances (Ramos et al., 2015), including agricultural activities that lead to the dispersion of pollution, industrial waste discharge, potential impact from regular dredging for navigation purposes and presence of a commercial port (Rocha et al., 2021). The oyster farm with an extent of about 3.6 ha, grows Pacific oysters (*C. gigas*) using the so-called Australian system, an adjustable longline system with suspended plastic (HDPE) baskets (Fig. 2B). This method takes advantage of the natural flow of the tides to tumble the oysters, resulting in improved shell shape. Triploid oyster seeds are obtained from commercial hatcheries and reared in Lima estuarine waters until they reach commercial size. During high tide, the aquaculture baskets are submerged in the water, and during low tide, oysters are exposed to air for a number of hours.

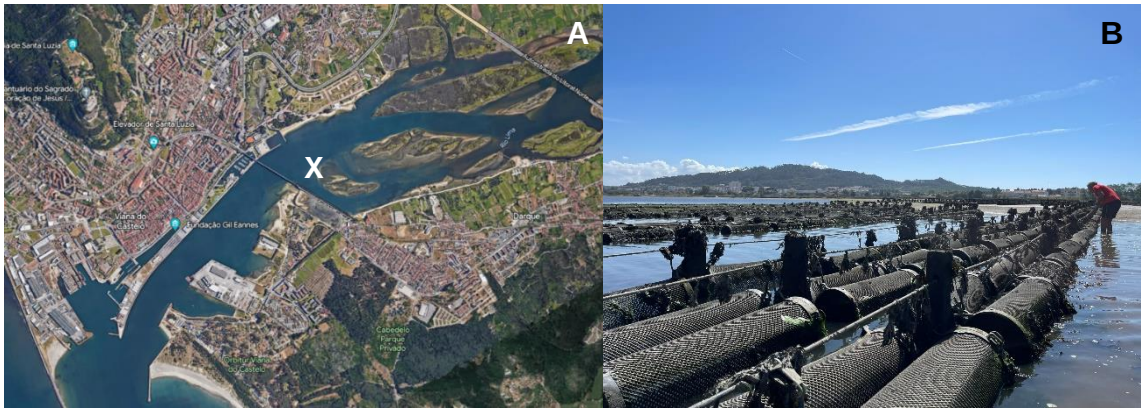

Figure 2. (A) Lima estuary (NW Portugal) with the location of the oyster farm and **(B)** general view of the oyster baskets at low tide.

For assessing MPs contamination, two sampling campaigns were conducted, one in early autumn (6 October 2023) and another in winter (27 February 2024). These sampling dates were chosen to represent two different periods of the oysters production cycle reflecting natural environmental variability (e.g. temperature, precipitation, river flow) and oysters physiological conditions. In each sampling, at low tide, the following samples were collected: i) a total of 30 adult oysters, all at the same stage of

development and of commercial size (80 ± 6 mm shell length), were randomly harvested by hand directly from the grow-out baskets, stored in tin-foil in a cool box, and transferred to the lab, being afterwards kept at -20 ºC; ii) three replicates of sediment (top 5 cm, 100 g per replicate) were collected from the same sampling site of oyster collection using a metal shovel, stored in tin foil and frozen at -20 °C in the lab for subsequent analysis; iii) three water samples were collected in 1-liter glass bottles directly from the surface and stored in the laboratory at room temperature for later analysis. Additionally, a small sample from the oyster plastic baskets and lids was collected to compare the aquaculture gear composition with MPs found in oysters and environmental samples.

2.2. Microplastics analysis

2.2.1 Contamination control

Throughout all the laboratory stages, procedures were carried out to prevent and reduce airborne and cross-contamination namely: i) all the materials used (glassware and metal tools, i.e. non plastic) were pre-washed with filtered deionized water and 70 % ethanol; ii) disposable nitrile gloves and 100 % cotton gray laboratory coats were worn during all procedures; iii) capping all samples and solutions (e.g., with aluminum foil); and iv) washing materials between samples. Additionally, the exterior of the oyster shells was washed with filtered deionized water to remove any particles and organic material that might been attached to the surface, before oyster measurement and dissection. Moreover, procedural blanks (open petri dishes with deionized water) were placed during all phases to monitor airborne MPs contamination and were subsequently examined under a stereomicroscope at the end of each procedure.

2.2.2 Oyster sample preparation

Oysters were removed from the freezer and carefully rinsed off with deionized water. While still frozen, each oyster was individually measured for shell length (umbo to longest edge) to the nearest milimeter (mm), using a digital caliper (Fig. 3A). The oysters were then thawed at room temperature (for approximately one hour), and weighed on a digital scale (Radwag, d = 0.01 g) both with the shell (total wet weight) and without shell (soft tissue wet weight) (Fig. 3B). The specimens sampled in both seasons were of similar size, with a mean length of 80 ± 7 mm and an mean tissue wet weight of 21 ± 5 g in autumn, and a mean length of 77 ± 4 mm and an mean tissue wet weight of 25 ± 3 g in winter (Table 1).

Table 1. Number of specimens (N), shell length (mm), total weight (g), and soft tissue wet weight (g) of oysters collected in Lima estuary. Mean values ± SD.

	N	Shell length (mm)	Total weight (g)	Soft tissue weight (g)
6 October (autumn)	30	80 ± 7	60 ± 12	21.4 ± 5.2
23 February (winter)	30	77 ± 4	60 ± 7	24.7 ± 3.0

Both the oyster soft tissues and intervalvular fluids were transferred into a pre-weighed, clean aluminium container. The oyster soft tissues were not rinsed, as the aim of the research was to investigate contamination from the perspective of humans, who consume the bivalve tissues whole. Each aluminium container (oyster tissue + intervalvular fluid) was properly sealed with aluminium foil to prevent contamination, and frozen at -20 ºC until the MPs extraction protocol.

Figure 3. Biometric parameters: **(A)** digital calliper used to measure the oyster shell length (mm) and **(B)** digital scale for weighing oyster samples.

2.2.3 MPs extraction

MPs extraction from oyster samples was carried out following the protocol developed by Silva et al. (2022). In short, the oysters' tissues were placed in an oven at 40ºC overnight to dry out. The dried tissue was covered with a 30 % H_2O_2 solution and kept at 65 ºC for 48 hours to digest the organic matter (Fig. 4A). After digestion (Fig. 4B), the samples were filtered through a cellulose nitrate membrane filter (pore size 0.45 µm, 47 mm diameter) using a vacuum filtration glass system in a laminar flow cabinet (Fig. 4D). Filtered deionized water was used to rinse out any remaining tissues in the sample containers. The filters were placed in properly labeled and covered glass petri dishes and allowed to dry at room temperature until subsequent MPs characterization.

For sediment samples, MPs extraction was conducted adapting an optimized protocol (Rivoira et al., 2020). Initially, the sediment was defrosted at room temperature, properly protected with aluminum foil to prevent airborne contamination. Subsequently, a known mass of sediment (100 g) was divided into replicates of about 20 g each, depending on the amount of organic matter. A density separation step followed using a saturated NaCl solution (Fig. 4C). After discarding the bottom fraction, the supernatant was treated with 30 % H_2O_2 solution to digest organic matter and was kept overnight in an oven at 65 °C. The samples were then filtered through a cellulose nitrate membrane filter, as described before.

The extraction of MPs from water samples was carried out based on the protocol established by Rodrigues et al. (2019b). The water samples were treated with 30 % H_2O_2 solution to digest organic matter, and then the samples were filtered through a cellulose nitrate membrane filter, as described before.

2.2.4 MPs characterization and identification

All filters, once properly dried at room temperature, were examined for visual enumeration and characterisation of MPs using a stereomicroscope (Leica EZ4W, C-W 15x/16mm) (Fig. 4F) equipped with an integrated camera connected to a computer running LAS X Office 1.4.4 software. To avoid overcounting of potential MPs, the filters were examined from left to right, top to bottom. The potential MPs recovered from each sample (oysters, water, and sediment) were counted and classified based on their shape (e.g., fragment, film, or fiber) and color, following Karami et al. (2017). Whenever a potential MPs exhibited two colors, for example, mostly transparent but with a small blue portion, the particle was considered blue, as the blue part could indicate that it was originally blue and that lost its color due to environmental factors (Cho et al., 2021; Jang et al., 2020). Potential MPs were measured approximately along their longest axis and classified into 3 size categories: < 0.5 mm, 0.5 - 2 mm, and > 2 - 5 mm.

Subsequently, a subsample (at least 10% of the total) of the most representative particles was selected for Fourier Transform Infrared Spectroscopy (FTIR) to determine the polymer type (Fig. 4E), following the guidelines of the European Union's Marine Strategy Framework Directive, which recommends analyzing 10 % of all samples to verify the accuracy of visual identification in identifying plastic microparticles (Galgani et al., 2023). Due to technical limitations with the in-house FTIR, MPs smaller than 0.5 mm could not be analyzed. Besides size, color was also used as a selection criterion for FTIR analysis, with priority given to the most commonly observed colors. Polymer spectra

were captured using a PerkinElmer FT-IR Spectrum 2 instrument (Waltham, MA, USA) equipped with attenuated total reflectance (ATR), capable of detecting particles as small as 10 µm. The spectra were recorded with an average of 4 scans over the range of 4000–800 cm^{-1}. The resulting spectra were compared with the instrument's reference library. For sediment and water samples, matches with a confidence level of 75 % or higher were accepted (Almeida et al., 2023; Rodrigues et al., 2020) while for oyster samples, matches with confidence levels of 65 % or higher were considered acceptable. A lower match threshold was selected to account for the potential effects of MPs passing through the oyster gut, which may cause further wear on the particles.

Figure 4. Extraction procedures: **(A)** Before and **(B)** after digestion of organic material with H_2O_2 solution; **(C)** separation by density of the sediment in a saturated NaCl solution; **(D)** vacuum filtration glass system; **(E)** Fourier-transform infrared spectroscopy (FTIR) and **(F)** stereomicroscope;

2.3 Depuration trials

Two depuration trials were conducted to assess the removal of MPs by farmed oysters under controlled laboratory conditions and realistic commercial depuration conditions. These trials were conducted in November 2023 and April 2024, respectively. All oysters were sampled from the same oyster farm in Lima estuary, following the previously described sample collection and transport methods.

2.3.1 Laboratory trial

A pilot-scale depuration system was set up at CIIMAR aquatic animal facilities (Matosinhos, Portugal) (Fig. 6A). The experiment consisted of a static system with eight 30-L glass aquarium tanks in a water bath system made of three plastic boxes filled with water and connected to a cooling unit (two chillers) to stabilise the experimental temperature (15.6 ± 0.2 ºC) across tanks (Fig. 6D, 6E). Each aquarium was filled with 16-L of natural seawater (29-30 ‰), which had been pre-filtered through a 50 µm filter, followed by an activated carbon filter to adsorb chemicals, and then treated with UV light. Before filling the tanks, the pre-treated seawater was filtered through a cellulose nitrate membrane filter (pore size 0.45 µm, diameter 47 mm) using a glass vacuum filtration system. The tanks were then covered with glass lids and aluminum foil to prevent airborne contamination for 1-2 days before the experiment began and throughout its entire duration. To ensure adequate aeration in each tank, glass Pasteur pipettes connected to aeration tubes were used (Fig. 6B, 6C).

The oysters were hand-harvested from an oyster farm in the Lima estuary (see section 2.1) to obtain 60 individuals of similar size (shell length: 74 ± 6 mm and sof tissue wet weight: 19 ± 3 g) and were immediately transported to the laboratory in cool boxes within 1 hour. Upon arrival at the laboratory, the oysters were carefully scrubbed with a metal brush for removing attached barnacles and other fouling organisms, washed with tap water and then finally washed with deionized water. Oysters were individually measured and sorted to get 4 groups of 15 similar sized individuals. One group of 15 oysters was immediately wraped in tin foil and frozen at -20 ºC to determine initial MPs concentration (control, not subjected to depuration), while the other 45 individuals were used in the depuration experiment. No acclimation was performed as the experimental temperature was similar to the field temperature. To mimic the industry depuration practices, oysters were not fed throughout the experiment.

Fifteen oysters were placed in each of the three experimental tanks assigned to the 24h depuration treatment. This set up allowed for providing at least 1-L of water per

oyster as recommended for laboratory experiments (Shumway et al. 2023). To account for background contamination, a control tank with the same volume of filtered water but without oysters, was placed next to the experimental tanks (Fig 5).

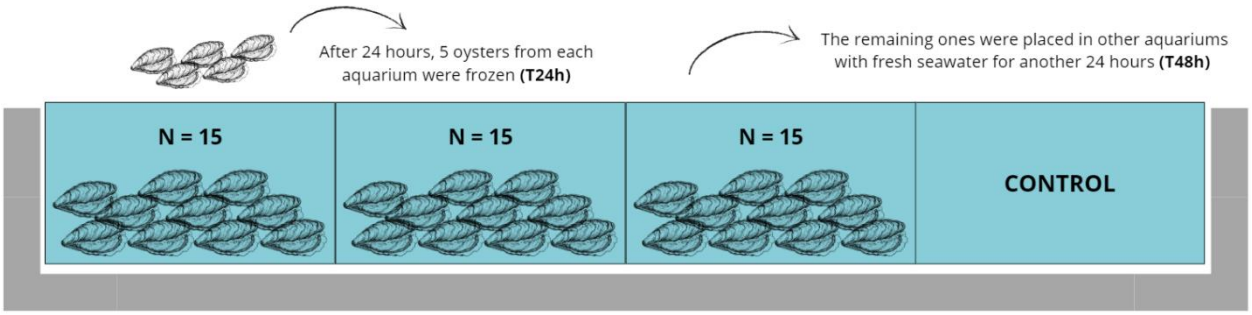

Figure 5. Static depuration setup. Three experimental tanks with oysters and one control tank without oysters (to account for background contamination).

After 24h, 5 oysters from each of the three tanks (n=15) were removed, wraped in tin foil and stored at -20 ºC for later processing. The remaining 10 oysters per tank were then transferred to three other experimental tanks with clean and filtered seawater assigned to the 48h depuration trial, and left for another period of 24h until final sampling. Due to logistical constraints, it was not possible to conduct the 72-hour period, as initially planned. Consequently, the remaining oysters (5 per tank) were frozen but were not used in this study. The set up used was meant for ensuring that any previously egested particles at the end of the first 24h would not become re-suspended in the water column and re-filtered by the oysters. Similar to the 24h treatment, a control tank was used to account for background contamination in the 48h treatment.

At the end of the 48h period, all the water from each aquaria (16-L) was filtered using a sequential filtration process. First, the water was passed through a cellulose nitrate membrane filter with a pore size of 0.8 µm, followed by filtration through a cellulose nitrate membrane with a pore size of 0.45 µm. This approach allowed for the recovery of particles present in water and in oysters' biodeposits (feces and pseudofeces), for subsequent extraction and characterization of MPs.

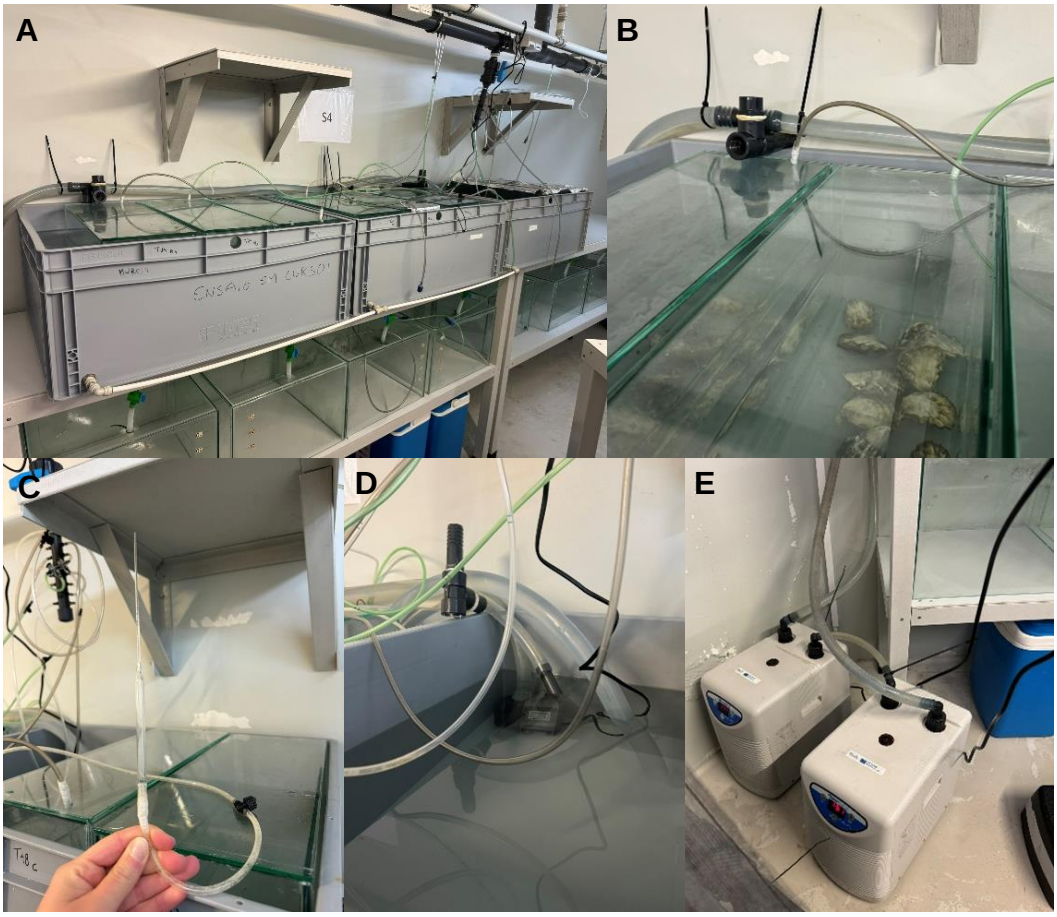

Figure 6. Experimental set-up including **(A)** plastic boxes used as a water bath system for maintaining the water temperature stable; **(B)** and **(C)** aeration set up; **(D)** water circulation pump; and **(E)** water chillers.

MPs analysis was conducted as described in section 2.2, with some additional steps implemented to prevent contamination. These steps included: i) filtering the entire water volume from each aquaria prior to the trial through a cellulose nitrate membrane filter (pore size 0.45 µm, 47 mm diameter) using a vacuum filtration glass system; ii) aerating the aquaria with glass pipettes to avoid external plastic contamination; and iii) covering the aquaria with glass lids lined with aluminum foil to prevent airborne contamination.

2.3.2 Commercial depuration

A depuration experiment was conducted in a commercial depuration facility to assess the natural reduction of MPs contamination in farmed oysters under realistic conditions. Sixty commercial-size oysters (shell length: 69 ± 5 mm and soft tissue wet weight: 13 ± 3 g) were randomly collected from the oyster farm in Lima estuary, immediately refrigerated, and transported to the depuration facility (Vila do Conde) in refrigerated boxes. Fifteen oysters were immediately frozen to serve as the control group (non-

depurated), while the remaining 45 oysters were placed on a perforated plastic tray, which was then submerged in a tank with recirculated seawater. The recirculated seawater was disinfected with chlorine at room temperature, following the normal practices of the commercial depuration operator. Oysters were sampled directly from the commercial depuration facility (n=15 per sampling interval) after 24, 48 and 96 hours of depuration. At each sampling interval, the oysters were frozen at -20 ºC until further processing.

In contrast to the laboratory trial, no measures were taken to prevent MPs contamination in the commercial depuration facilities. Oyster sample preparation, MPs recovery, characterization and identification were performed as described in section 2.2.

2.4 Data analysis

A descriptive analysis of the data was conducted considering the potential MPs, defined as microparticles that are similar in size and visual characteristics to MPs but have an unknown composition, as it was not possible to confirm the chemical composition of all microparticles. MPs abundances in oysters were expressed both as the number of MPs per g wet soft tissue weight (MPs g ww^{-1}) and per individual oyster (MPs ind^{-1}). The number of MPs in water and sediment was standardized to volume (MPs L^{-1}) and sample dry weight (MPs g dw^{-1}), respectively. Statistical analyses were employed to detect differences in MPs abundances across different matrices (oysters, water, and sediment) and seasons (autumn and winter) (field assessment), as well as among different depuration times (depuration trials). Whenever data fulfilled the requirements of normality and homogeneity of variances, one-way analysis of variance (ANOVA) was employed. Alternatively, the non-parametric Kruskal-Wallis test was used instead. For multiple comparisons, Tukey's Honest Significant Difference (HSD) test and Dunn's KW post-hoc test were applied based on whether or not the ANOVA assumptions were met, respectively. Fisher's Exact Test was used to analyze the distribution of MPs colors and sizes in oysters, water and sediment, and along depuration times. When significant differences were identified, pairwise tests of independence were conducted to determine which specific groups differed from each other. All statistical analyses, were performed using R software with a significance level set at $p < 0.05$. Graphical representations were generated using Prism software and R software version 4.4.1 (R Core Team, 2024). Boxplots were made in R using library ggplot 2 (Wickham, 2016). For Dunn’s KW test, dunnTest() from library FSA (Ogle et al., 2019) was used. The pairwise tests of independence were conducted using function pairwiseNominalIndependence() from library rcompanion (Mangiafico, 2024).

Chapter III - Results

3.1 Field assessment

3.1.1 MPs contamination

Contamination in the procedural blanks (controls) during laboratory work was considered low (Table 2). A range of 0 to 7 fibers was detected in the blanks, with the highest count observed during the visual analysis of the filters. This phase is the most time-consuming, and thus, it was exposed to airborne contamination for the longest period. Despite strict measures taken to prevent contamination, the fibers detected are likely from airborne sources. Therefore, only the fibers found during the MPs characterization phase were considered. If the type, color, and size of the microparticles in the procedural blanks matched those found in the samples (e.g., a black fiber), those particles were excluded from the total microparticle counts for the sample being analyzed.

Table 2. Airbone contaminations detected in procedural blanks across different laboratory stages. Numbers indicate the range of fibers found per blank.

	Transparent	Black
Sample preparation	0 - 3	0 - 1
MPs extraction	0 - 2	0
MPs characterization	0 - 5	0 - 2

3.1.2 Presence and characterization of MPs

A total of 293 potential MPs were found in oyster samples collected in autumn (n=30), with a range of 0 to 38 MPs per individual and 0 to 1.60 MPs per gram. This corresponds to mean concentrations of 9.8 ± 7.5 MPs ind^{-1} and 0.48 ± 0.34 MPs g^{-1} ww. In contrast, 65 potential MPs were identified in oysters sampled during winter, with a range of 0 to 7 MPs per individual and 0 to 0.29 MPs per gram. This resulted in mean concentrations of 2.2 ± 1.7 MPs ind^{-1} and 0.09 ± 0.07 MPs g^{-1} ww (Fig. 7). The Kruskal-Wallis test revealed significant differences ($p < 0.001$) in the concentration of MPs between the autumn and winter samples.

In the environmental samples collected in the autumn, a total of 40 and 408 potential MPs were found in water and sediment, corresponding to mean concentrations of 13.3 ± 4.6 MPs L^{-1} and 1.4 ± 0.7 MPs g^{-1} dw, respectively. In the winter, a total of 23 and 306 potential MPs were found in the water and sediment, respectively, corresponding to mean concentrations of 11.5 ± 3.5 MPs L^{-1} and 0.64 ± 0.10 MPs g^{-1} dw (Fig. 8). No statistically significant differences (Kruskal-Wallis test, $p > 0.05$) were observed in the concentration of MPs in water and sediment between the autumn and winter samples. However, a downward trend was observed, with MPs concentrations being higher in autumn than in winter.

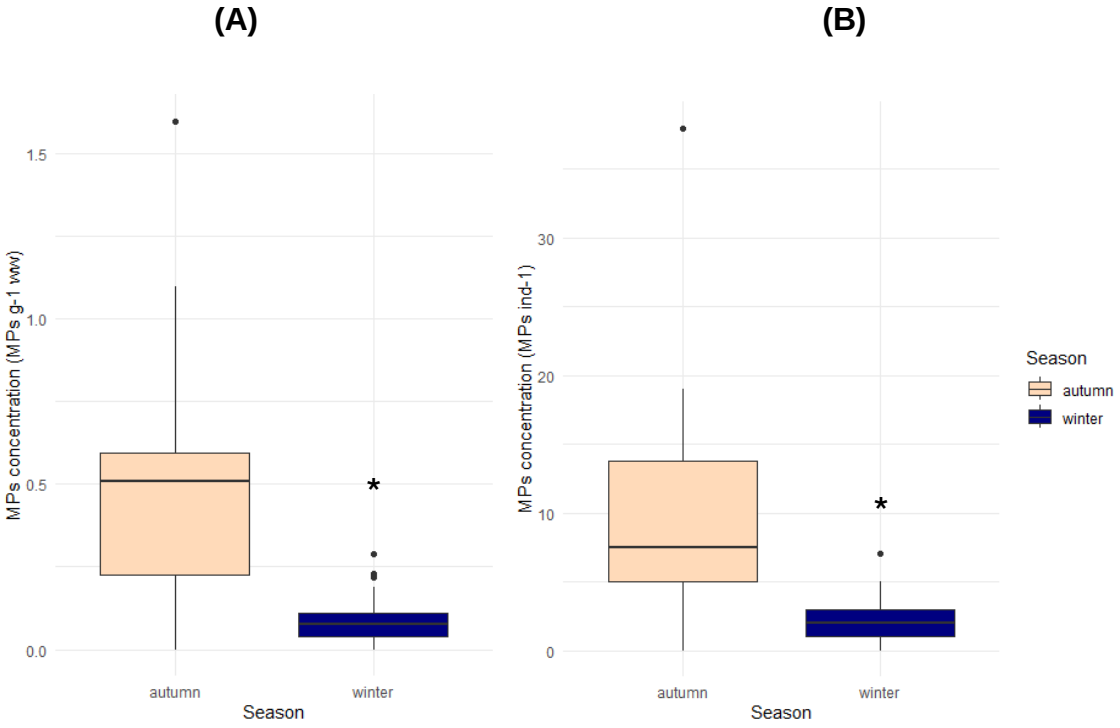

Figure 7. Boxplot showing the seasonal variation of potential MP fibers concentration **(A)** per gram of wet weight and **(B)** per individual of all potential MPs detected in oyster tissues, between autumn and winter. The boxplots represent the median (central line), interquartile range (IQR; box), and outliers (individual points beyond the extended lines). * indicates significant differences (Kruskal-Wallis test, $p < 0.001$)

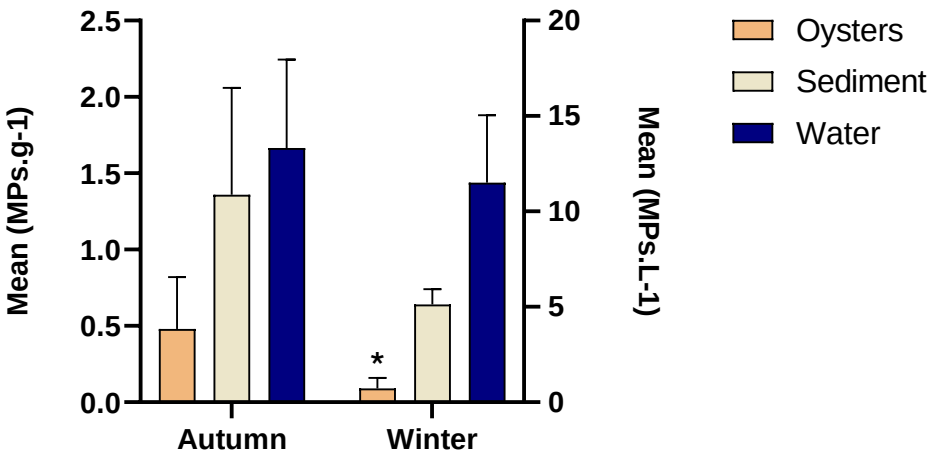

Figure 8. Mean concentration of potential MPs fibers in oysters, water and sediment samples in autumn and winter. In oysters and sediment, concentrations are expressed as MPs g-1 ww and MPs g^{-1} dw, respectively, while in water, concentrations are express as MPs L^{-1}. * indicates significant differences between autumn and winter (Kruskal-Wallis test, $p < 0.05$).

Fibers were the only type of MPs detected in oysters, water and sediment samples, across both sampling seasons. In oysters collected during autumn, transparent fibers were the most common, making up 61 % of the total, followed by blue (14 %), black (11 %), and gray (10 %). Other colors, including red, green, white, and yellow, were also found, but each represented 1 % or less of the total observed MPs. In water and sediment samples, transparent MPs were also dominant, accounting for 62 % in water and 71 % in sediment. These were followed by blue (19 % in water and 14 % in sediment), black (10 % and 9 %), and gray (3 % in both). In sediment, additional colors like red, green, and pink were detected, while in water, red and purple were also found, each representing 3 % or less of the total MPs (Fig. 10A). Statistically significant differences in the color distribution of MPs between oysters and sediment samples were observed (Fisher's exact test, $p < 0.05$), but not between the other groups ($p > 0.05$).

In oysters collected in winter, transparent MPs were also the dominant type (60 %), followed by black (19 %), and gray (14 %). Blue fibers were not present, and other colors, such as red, green, and yellow, were present in small amounts (≤ 3 %). In water and sediment samples, transparent MPs continued to dominate, making up 44 % in water and 70 % in sediment, followed by blue (22 % in water and 18 % in sediment), gray (13 % and 3 %), and black (13 % and 7 %). Additional colors found in sediment included red, green, and pink, while in water, red, orange, green, pink, and yellow were observed, each accounting for ≤ 4 % of the total MPs (Fig. 10A). Significant differences in MPs color distribution were found across matrices (Fisher's exact test, $p < 0.001$), with notable

differences between oysters and sediment ($p < 0.05$) and between sediment and water ($p < 0.05$), but not between oysters and water ($p > 0.05$).

Regarding the size distribution of MPs found, in both sampling seasons, MPs between 0.5 and 2 mm were the most abundant across all matrices. In autumn samples, MPs in this size category represented 72 % in oysters, 81 % in sediment, and 60 % in water. MPs smaller than 0.5 mm were the second most common size in oysters, making up 23 %, but were much less frequent in sediment (5 %). In sediment, MPs larger than 2 mm were the second most abundant, accounting for 14 % of the total MPs. In water, MPs smaller than 0.5 mm and those larger than 2 mm each made up 20 % of the total fibers (Fig. 10B). Significant differences in MPs size distribution were found across the matrices (Fisher's exact test, $p < 0.001$), with notable differences between oysters and sediment ($p < 0.05$) and oysters and water ($p < 0.05$).

In the winter samples, MPs between 0.5 and 2 mm accounted for 66 % of the total in oysters, 77 % in sediment, and 70 % in water. MPs smaller than 0.5 mm were also relatively common, representing 17 % in oysters, 13 % in sediment, and 17 % in water. MPs larger than 2 mm constituted 17 % of the total in oysters but were less frequent in sediment (10 %) and water (13 %) (Fig. 10B). No significant differences in MPs size distribution were observed among the three matrices during this season (Fisher's exact test, $p > 0.05$).

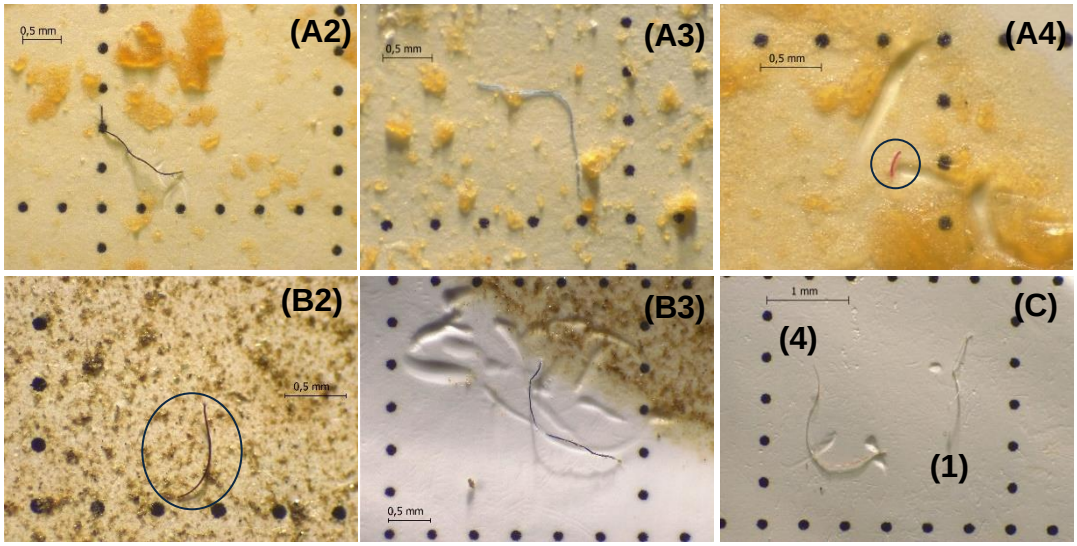

Figure 9. Variety of colors and sizes of MPs fibers found in the samples collected: oysters **(A)**, sediment **(B)** and water **(C)**. Example of fiber colors detected: transparent **(1)**, black **(2)**, blue **(3)** and red **(4)**.

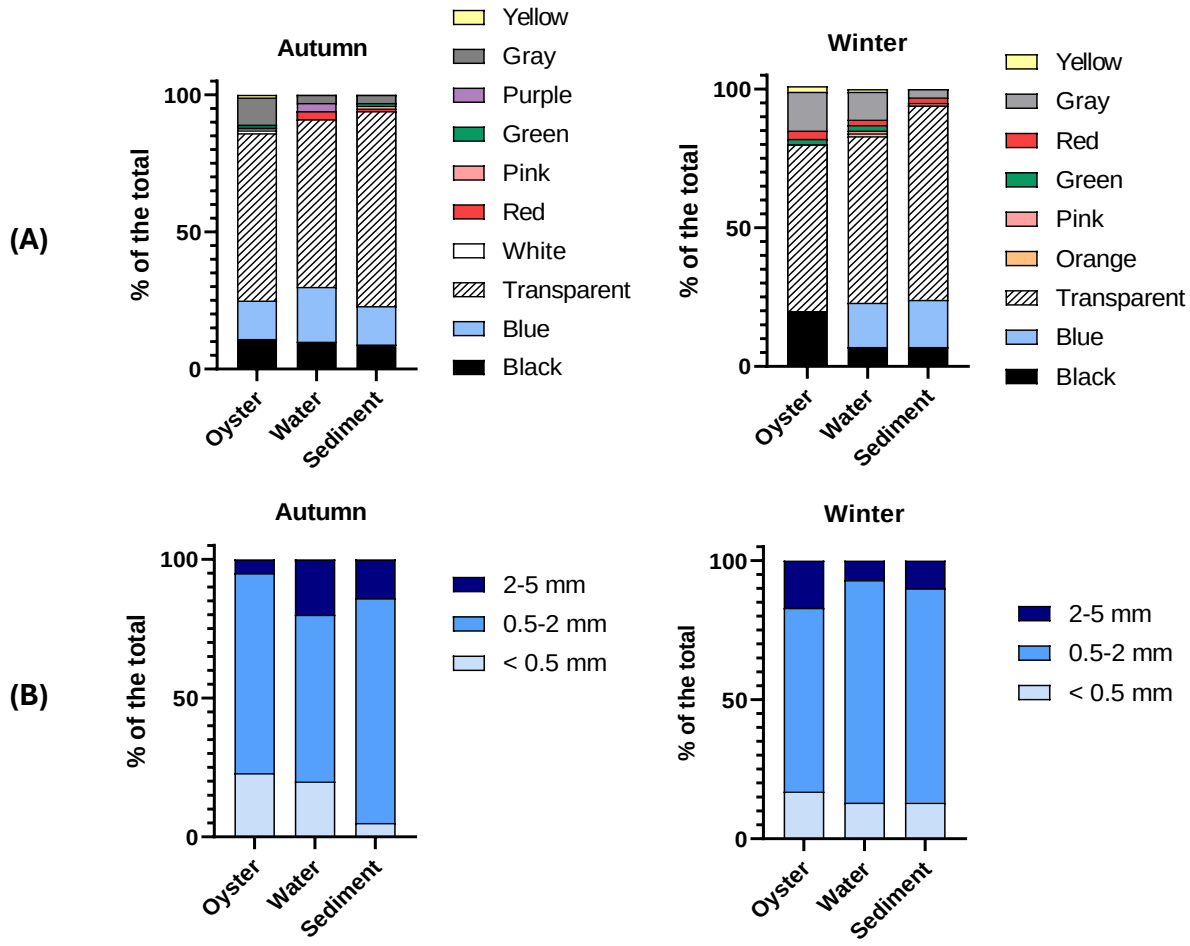

Figure 10. Illustration of the distribution of MPs by color **(A)** and by size **(B)** in the two seasons, autumn and winter.

3.1.3 Polymer identification

A total of 143 potential MPs were selected and examined under FTIR (13 % of the total). Of these, 71 were considered MPs (with confidence levels ≥ 65 % for oysters and ≥ 75 % for water and sediment samples), 29 in oysters (37 % of the total fibers analyzed by FTIR), 11 in water (48 % of the total) and 31 in sediment (46 % of the total). This analysis identified five distinct chemical categories of particles in oysters, and two in the sediment and water samples. Cellulose and PET were found across all samples and were the predominant polymers in oysters, followed by rayon, polyvinyl stearate (PVS), and PP (Fig. 11). Cellulose and cellulose-based components, such as rayon, were included as MPs, as these, especially cellulosic microfibers, are considered anthropogenic particles (Ó Briain et al., 2020). Additionally, FTIR analysis detected adipate, a widely used plasticizer (Vikhareva et al., 2021), in the different environmental matrices, accounting for 41 %, 3 %, and 9 % of the fibers examined in oysters, sediment and water samples, respectively.

The aquaculture gear samples taken from the lid and the basket were also analyzed using FTIR, which identified HDPE, a polymer that was not detected in the oysters, water, or sediment samples.

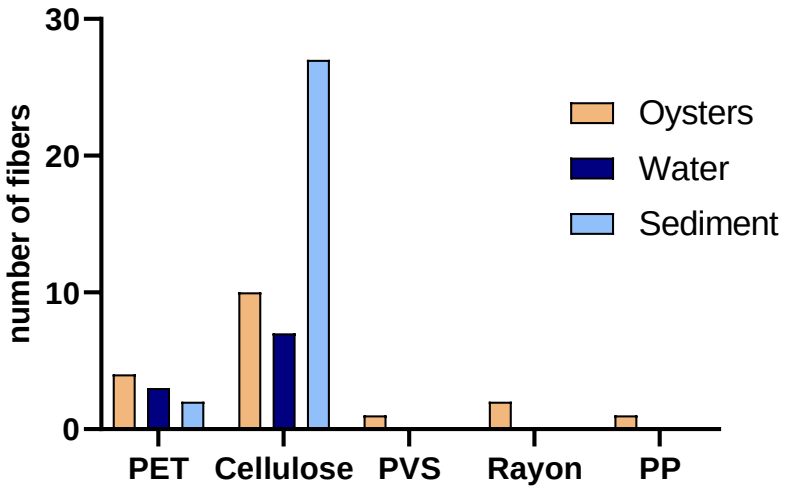

Figure 11. Chemical composition and number of each type of particle identified in oysters, water and sediment samples.

3.2 Depuration trials

Contamination in the procedural blanks (controls) during laboratory work was considered low (Table 3). A range of 0 to 6 fibers was detected in each blank, with the highest count occurring during the preparation of the seawater for the experiment and during the visual analysis of the filters. As mentioned before, these phases are the most time-consuming, resulting in extended exposure of the petri dish to airborne contamination. As a result, only fibers identified during the MPs characterization phase were taken into account for correction. If the type, color, and size of the microparticles in the procedural blanks matched those found in the samples, those particles were excluded from the total microparticle counts for the analyzed sample.

Table 3. Airbone contaminations detected in procedural blanks across different methodological stages. Numbers indicate the range of fibers found per blank.

	Transparent	Black	Blue
Seawater preparation	0 - 4	0 -2	0 - 1
Sample preparation	0 - 2	0	0
MPs recovery	0 - 2	0 - 1	0
MPs characterization	0 - 5	0 - 2	0

3.2.1 Laboratory trial

3.2.1.1 MPs characterization

Throughout all samples and treatments, only fibers were identified as potential MPs. In the control group, oysters non depurated, a total of 194 fibers were detected. The concentration of MPs per individual ranged from 0 to 55 MPs ind^{-1}, with an mean of 12.9 ± 14.9 MPs ind^{-1}. The concentration of MPs per gram of soft wet tissue ranged from 0 to 3 MPs g^{-1}, with an mean value of 0.8 ± 0.9 MPs g^{-1} ww (Fig. 13). The most observed fiber colors were transparent (61 %), blue (13 %), gray (12 %) and black (10 %). Regarding fiber sizes, those between 0.5 and 2 mm were the most prevalent, accounting for 76 % of the total, followed by MPs with sizes lower than 0.5 mm (16 %). **After 24 hours of depuration**, the concentration of MPs in the oysters decreased by 66%, resulting in an mean concentration of 4.9 ± 2.6 MPs ind^{-1} and 0.2 ± 0.1 MPs g^{-1} ww (Fig. 13). The most common colors were transparent (59 %), blue (16 %), gray (10 %), and black (10 %), reflecting a similar trend observed in the oysters that were not subjected to depuration. Likewise, the most frequent size range was between 0.5 and 2 mm (63 %), followed by

MPs smaller than 0.5 mm (27 %). **After 48 hours of depuration,** MPs were still found; however, there was a reduction of 78 % compared to the initial concentrations, with an mean concentration of 3.1 ± 3.2 MPs ind^{-1} and 0.2 ± 0.2 MPs g^{-1} ww (Fig. 13). The most abundant color of MPs observed was transparent, accounting for 63 % of the total, consistent with other treatments. However, unlike previous treatments, black and gray MPs were equally the next most abundant colors, each accounting for 11 %. Regarding fiber sizes, MPs between 0.5 and 2 mm were the most prevalent, representing 67% of the total, similar to other treatments. Notably, MPs larger than 2 mm constituted 20 % of the total, which contrasts with the findings from previous treatments. Based on the distribution of MPs colors and sizes (Fig. 12A, 12B) observed at different depuration times, no significant differences were found (Fisher's exact test, $p > 0.05$). The Kruskal-Wallis test showed significant differences ($p < 0.05$), between the control group and the 48-hour treatment group ($p < 0.05$).

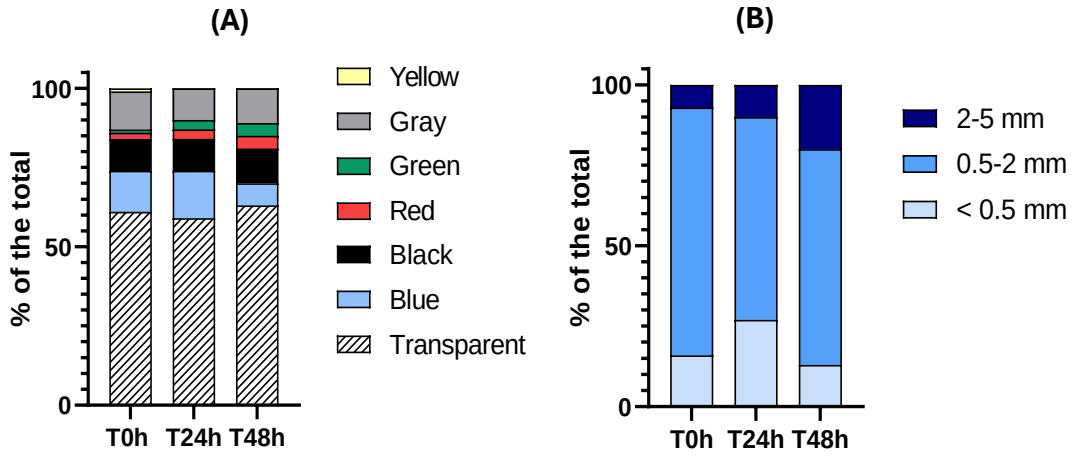

Figure 12. MP characterization by color **(A)** and size **(B)** of the fibers found in oysters across the different treatments (depuration time: 0h, 24h and 48h).

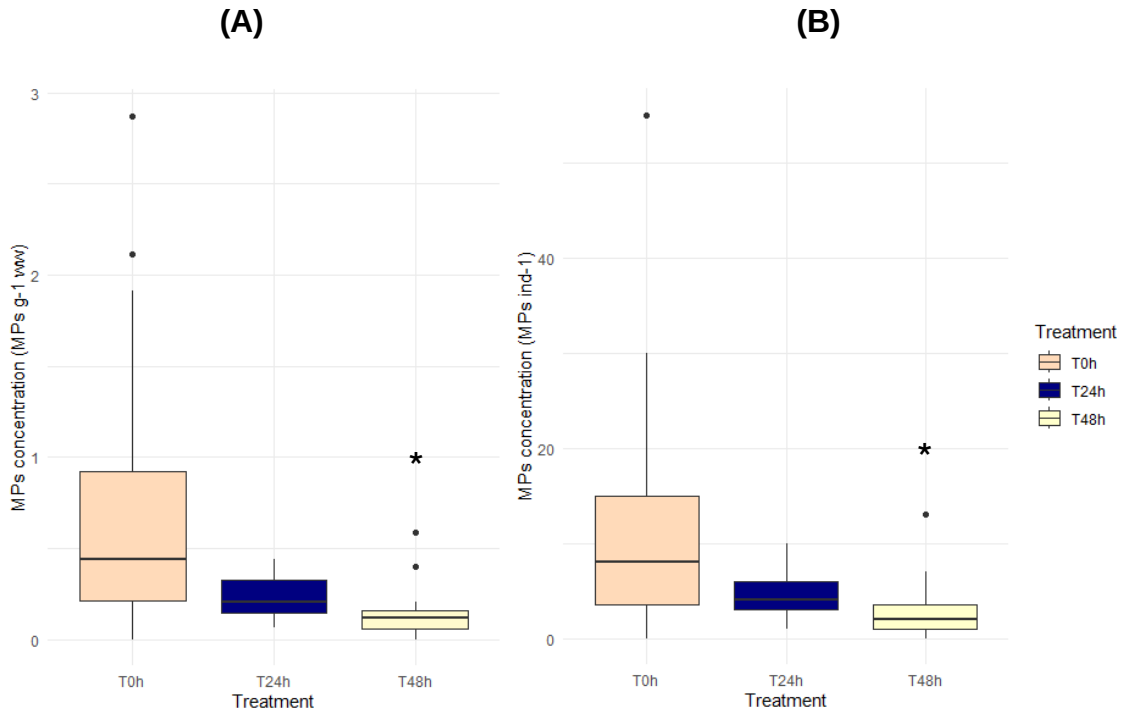

Figure 13. Boxplot showing MP concentrations **(A)** per gram of wet weight and **(B)** per individual of all potential MPs detected in oyster tissues, through the different treatments of depuration. The boxplots represent the median (central line), interquartile range (IQR; box), and outliers (individual points beyond the extended lines); * indicates significant differences (Kruskal-Wallist test, $p < 0.05$).

The analysis of the water and biodeposits at the end of the 48h depuration treatment, showed that fibers were the predominant form of MPs, though fragments were also detected (9 in total). After 24 hours, the mean MPs concentration was 27 ± 14 MPs L^{-1}. The most common colors observed were transparent (47 %), blue (20 %), gray (18 %), and black (8 %). The majority of MPs were between 0.5 and 2 mm in size (56 %), followed by those smaller than 0.5 mm (34 %). In the 48-hour depuration treatment, the mean MPs concentration in the water and biodeposits increased to 59 ± 14 MPs L^{-1}. The trends in dominant colors and sizes remained consistent with those at the 24-hour mark. These concentrations were corrected for the contamination detected in the control tanks (Mean: 19 ± 15 MPs L^{-1}), to account for potential airborne and waterborne contaminants (Fig. 14).

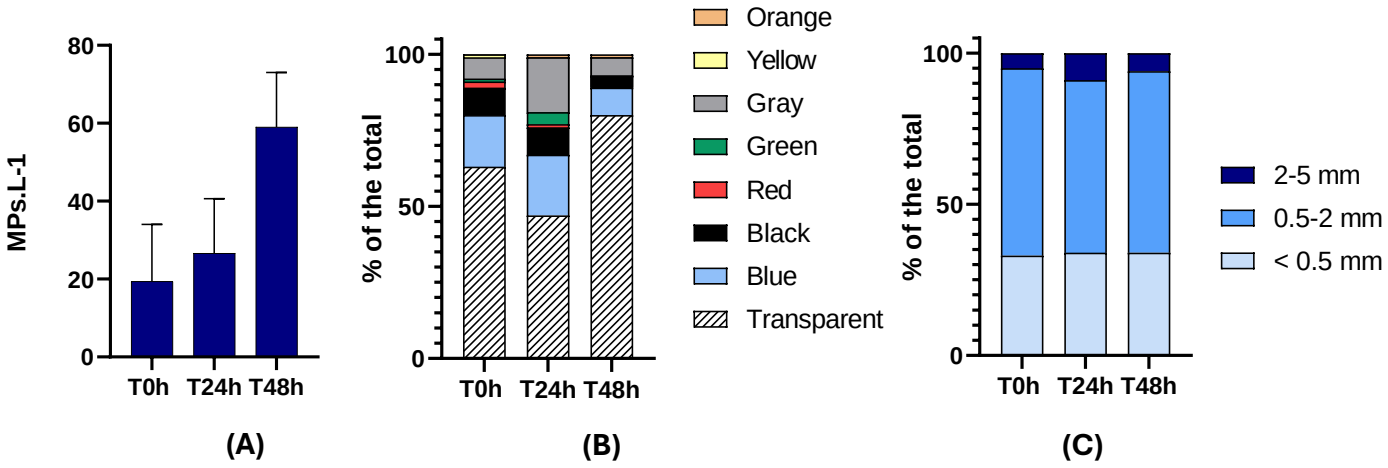

Figure 14. Mean concentration of potential MPs **(A)**, proportion of MPs colors **(B)**, and sizes **(C)** found in water from the aquaria at the start of the experiment (T0h), and after 24 hours (T24h) and 48 hours (T48h) of depuration. Results are presented as mean ± SD.

According to the observations in Fig. 15, there was a trend of decreasing MPs concentration in the oysters tissues with increasing depuration time, while MPs concentration in the water tended to increase, in line with the rejection and/or egestion of MPs by oysters.

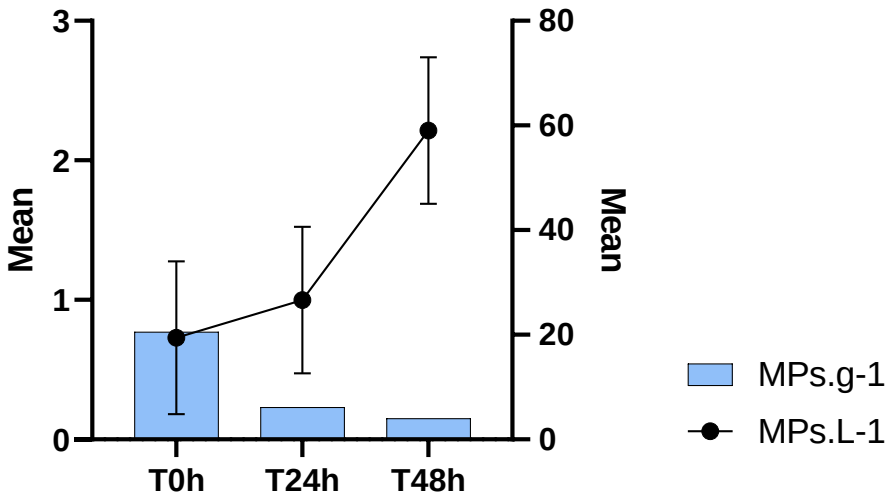

Figure 15. The mean concentration of MPs found in oysters tissues and in water (including biodeposits) at the different depuration times.

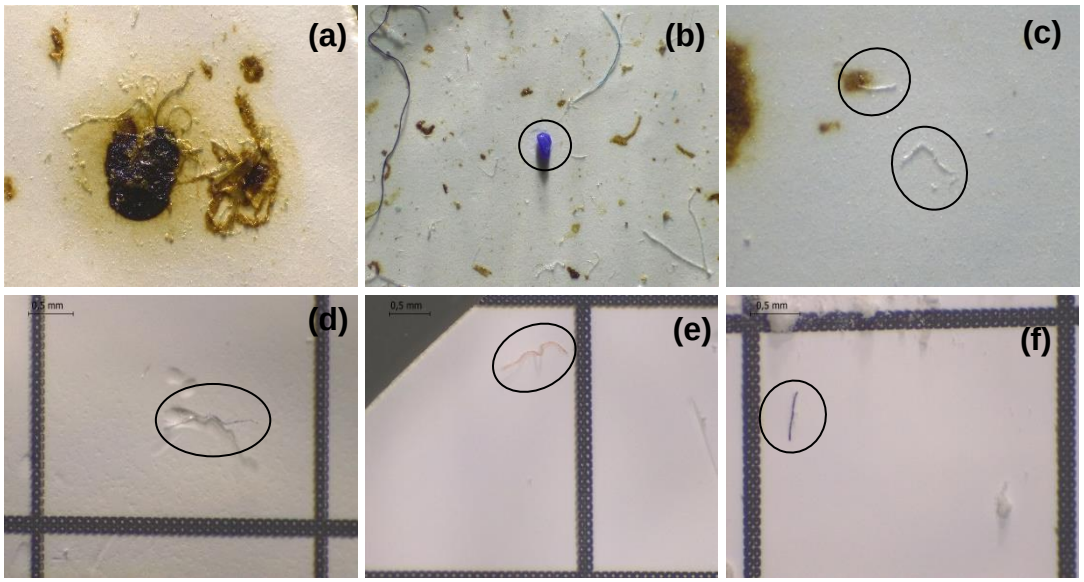

Figure 16. Examples of MPs found in water and biodeposits **(a-c),** and in oyster tissues **(d-f)** from the depuration trial. **a)** oysters' biodeposits with a variety of fibers; **b)** blue fragment; **c)** transparent fiber; **d)** gray fiber; **e)** orange fiber; and **f)** blue fiber.

3.2.1.2 Polymer identification

A total of 50 potential MPs extracted from the oysters were selected and examined under FTIR (16 % of the total). Of these, 41 were considered MPs (with confidence levels ≥ 65 %). This analysis identified five distinct types of polymers, such as cellulose and PET, which were the most common, followed by rayon, cellophane, polyacrylate (PAK) (Fig. 17). Additionally, the FTIR analysis also identified adipate, a commonly used plasticizer (Vikhareva et al., 2021), in 20 % of the fibers analyzed.

Regarding the water samples, 69 potential MPs (2 % of the total) were selected and examined under FTIR. Due to time and logistical constraints, it was not possible to conduct the recommended analysis of at least 10 %. Among the fibers examined, 27 were identified as MPs (with confidence levels ≥ 75 %), representing six different polymers. Besides cellulose fibers, also poly trimethylene terephthalate (PTT), rayon, PET, acrylonitrile butadiene styrene (ABS) and azlon, were identified.

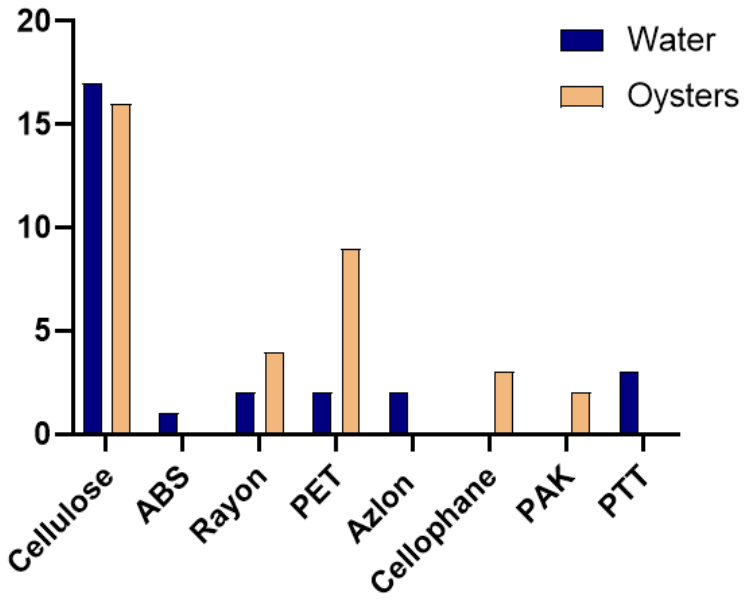

Figure 17. Chemical composition and number of each type of particle identified in oysters and water from the laboratory experiment.

3.2.2 Commercial depuration trial

3.2.2.1 MPs characterization

A total of 44 potential MPs were identified in the control group oysters (T0h), which were not depurated. The numbers of MPs per individual varied from 0 to 9 MPs, with an mean of 2.9 ± 3.1 MPs ind^{-1}. The concentration of MPs per gram of tissue wet weight ranged from 0 to 0.6 MPs g^{-1}, with an mean of 0.21 ± 0.21 MPs g^{-1}. MPs were only found in the form of fibers, and the most common colors observed were blue (48 %) and transparent (39 %). In terms of fiber size, those between 0.5 and 2 mm were the most prevalent, representing 45 % of the total, followed by fibers smaller than 0.5 mm (27 %) and larger than 2 mm (27 %). **After 24 hours of depuration**, the concentration of MPs in the oysters decreased to an mean of 2.1 ± 1.7 MPs ind^{-1} and 0.17 ± 0.16 MPs g^{-1}. The most common colors were transparent (52 %), blue (13 %), gray (10 %), black (10 %), and red (10 %). The most frequent size range was between 0.5 and 2 mm (77 %), followed by MPs larger than 2 mm (19 %). Following **48 hours of depuration**, the mean MPs concentration was further reduced to 1.9 ± 1.6 MPs ind^{-1} and 0.15 ± 0.12 MPs g^{-1}. The most abundant colors were transparent (50 %), blue (30 %), and gray (17 %), which is consistent with the previous treatments. The predominant MPs sizes were between 0.5 and 2 mm (70 %), with MPs smaller than 0.5 mm accounting for 17 %. Finally, **after 96 hours of depuration**, the oysters exhibited an MPs concentration of 1.1 ± 1.7 MPs ind^{-1} and 0.10 ± 0.15 MPs g^{-1}. The colors and sizes observed were consistent with those found in the other treatments. The most abundant color was transparent (44 %), followed by blue (25 %), black (19 %), and gray (13 %). The most common size was between 0.5 and 2 mm (75 %), with MPs smaller than 0.5 mm and larger than 2 mm each representing 13 %. No significant differences were found in MPs concentrations across the different treatments (Kruskal-Wallis test, $p > 0.05$) (Fig. 19). Similarly, the distribution of MPs sizes did not vary significantly across the treatments (Fisher's exact test, $p > 0.05$). However, the distribution of MPs colors showed significant differences (Fisher's exact test, $p < 0.05$) between treatments, with a notable reduction in blue fibers after 24 hours of depuration (Fig.18).

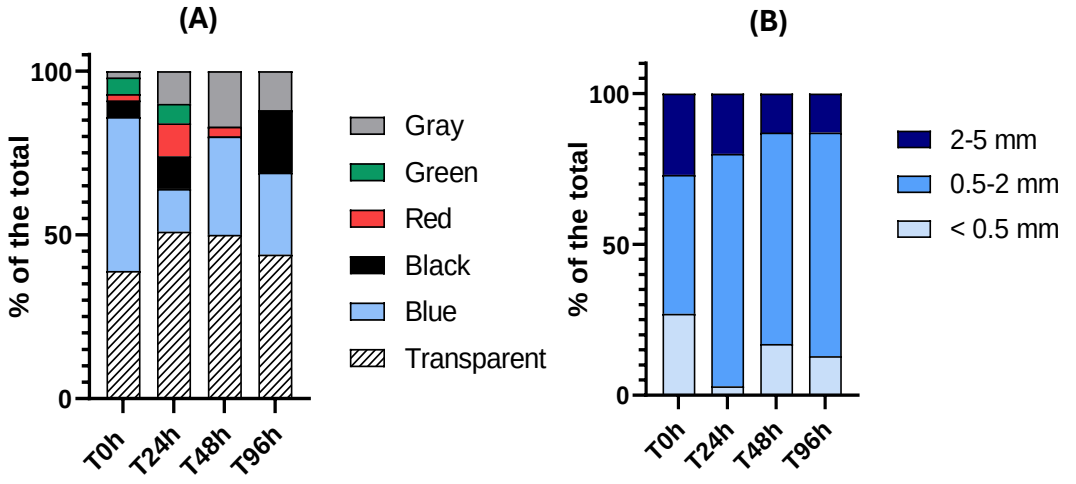

Figure 18. Mean concentration of MPs **(A)**, and proportion of MPs **(B)** colors, and **(C)** sizes found in oyster tissues across the different treatments (depuration time: 0h, 24h, 48h and 96h).

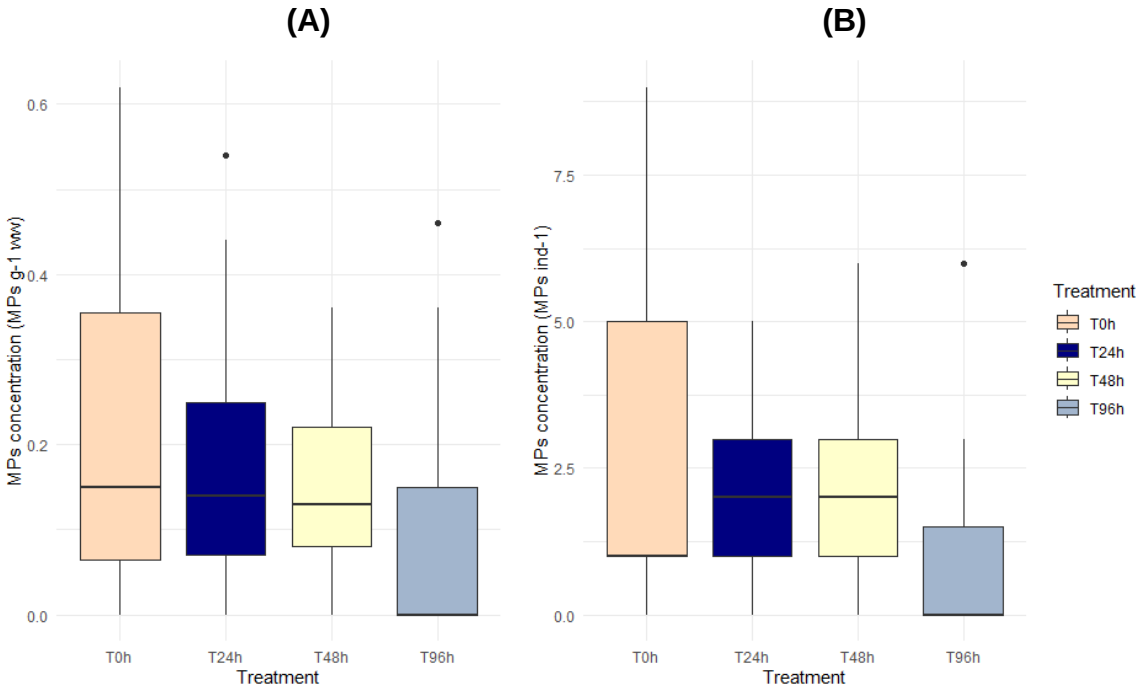

Figure 19. Boxplot showing MPs concentrations **(A)** per gram of wet weight and **(B)** per individual of all potential MPs detected in oyster tissues, through the different treatments of depuration. The boxplots represent the median (central line), interquartile range (IQR; box), and outliers (individual points beyond the extended lines).

3.2.2.2 Polymer identification

A total of 31 potential MPs were selected and examined under FTIR (26 % of the total). Of these, 18 were considered MPs (with confidence levels ≥ 65 %) in oysters belonging to 4 distinct types of polymers: cellulose, PET, PP and rayon (Fig. 20).

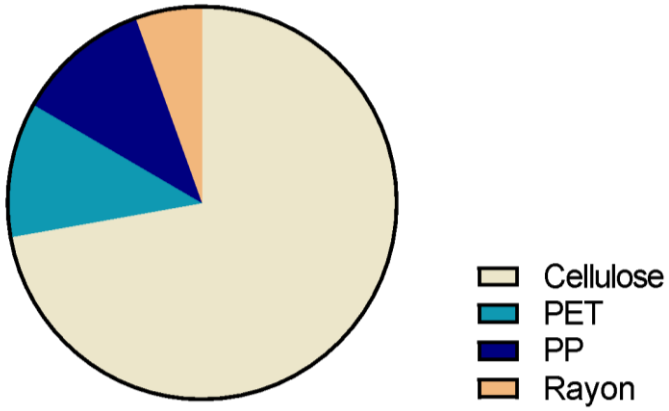

Figure 20. Chemical composition of each type of particle identified in oysters from the commercial depuration experiment.

Chapter IV – Discussion

4.1 MPs contamination in oysters and abiotic matrices

This study documented the presence of MPs in Pacific oysters from the Lima estuary in northern Portugal, which are produced for human consumption. Overall, 90 % of the oysters analyzed during the two sampling periods (n=60) contained at least one MPs particle in their tissues. In oysters sampled for depuration trials (non-depurated oysters, n=30), this percentage remained similar. This is the first study to report the presence of MPs in Pacific oysters farmed in the Lima estuary, adding to previous research that has documented MPs contamination in other components of this estuary, including water, sediments, and biota (such as zooplankton) (Almeida et al., 2023; Espincho et al., 2024).

MPs contamination has been reported for several bivalve species from estuarine and coastal waters in Portugal, including clams (*Scrobicularia plana*, *Ruditapes decussatus*), cockles (*Cerastoderma* spp.) and mussels *Mytilus* spp. (Barboza et al., 2024; Cozzolino et al., 2021; Marques et al., 2021; Vital et al., 2021), however, there is limited information for Pacific oysters. To the best of our knowledge, there is only another study reporting MPs in *C. gigas* from Portuguese estuaries by Manthopoulos (2022). In this study, oysters from an intertidal farm in the Sado estuary presented around 20 MPs per individual, corresponding to an mean concentration of 0.1 MPs per gram. Similarly, oysters farmed in the Ria de Aveiro had a total of 10 MPs per individual, with an mean concentration of less than 0.1 MPs per gram (Manthopoulos, 2022). These concentrations per gram for oysters sampled in March are in line with the estimates for our winter sampling (February: 0.09 ± 0.07 MPs g^{-1} ww), but the concentrations per individual are higher than those found in this study (February: 2.2 ± 1.7 MPs ind^{-1})

In our study, the concentration of visually identified MPs ranged from 0 to 1.6 MPs g^{-1}. This range is comparable to those found in *C. gigas* in other studies worldwide (Ding et al., 2021; Jang et al., 2020; Wootton et al., 2022). The mean MPs concentration (0.3 ± 0.3 MPs g^{-1}) observed in this study aligns with previous findings for other bivalve species in different regions of Portugal (Pequeno et al., 2021) and globally (Ding et al., 2021; Du et al., 2022; Phuong et al., 2018; Saldaña-Serrano et al., 2022). However, it is lower than concentrations reported in other studies conducted in Portugal (Botelho et al., 2023; Marques et al., 2021; Vital et al., 2021), South Korea (Jang et al., 2020), and Australia (Wootton et al., 2022). When expressed as MPs per individual, the concentrations in this study (5.9 ± 6.6 MPs ind^{-1}) were significantly higher than those reported in most other studies worldwide (see Table 4), though Renzi et al. (2018) reported even higher concentrations.

The oysters exhibited significant individual and seasonal variation in MPs concentrations. Specifically, the mean concentration of MPs in oysters was significantly higher in autumn, aligning with findings from other similar studies (Du et al., 2022; Ruangpanupan et al., 2023). MPs concentrations in oysters also seem to vary inter-annually. In a very preliminary study of MPs contamination in oysters from Lima estuary in February 2023 (own unpublished data), concentrations were much higher, suggesting a possible decrease in MPs levels from one winter to the next (see Table 4). In temperate areas, summer and autumn are periods of high metabolic activity in oysters due to elevated temperatures and increased food availability (Dridi et al., 2007; Mao et al., 2006). These conditions may lead to higher filtration rates, potentially resulting in greater ingestion of MPs particles. In contrast, during winter, the low phytoplankton availability, combined with low water temperatures, cause a decrease in metabolic activity, which may explain the lower MPs levels in oysters. Additionally, periodic precipitation events and high river flow, characteristic of this time of the year, may further affect oyster filtration activity (Barr et al., 2024).

MPs concentrations in water were lower than those reported by Almeida et al. (2023) for other unvegetated areas of the estuary, while MPs levels in sediment were also lower compared to other areas within the estuary. There was a general trend of higher MPs levels in water and sediments during autumn compared to winter, though the seasonal differences were not statistically significant. Previous studies in the Lima estuary showed significantly higher MPs contamination in water and sediments during autumn (November) compared to winter (February), attributing this pattern to increased rainfall, which might have facilitated the transport of MPs from terrestrial and riverine sources into the estuary (Almeida et al., 2023; Espincho et al., 2024). Similarly, in the Douro estuary, Rodrigues et al., (2019a) observed higher MPs concentrations in water during autumn (November), attributing this to increased MPs during rainy periods. Other studies conducted in China observed a similar pattern, where MPs concentrations were higher in autumn compared to winter (Chen et al., 2021; Du et al., 2022). However, they attributed the lower concentrations in winter (rainy season) to increased runoff caused by rainfall and flooding, which can resuspend and transport MPs towards the sea, leading to lower MPs concentrations. Variations in hydrological and climatic conditions between seasons may also help explain our results. Our autumn sampling (late summer to early autumn) occurred during a period of low precipitation (0.2 mm and 95 mm of accumulated rainfall in the week and month before sampling, respectively), in contrast to winter, which recorded higher rainfall levels (68 and 205 mm, respectively) (SNIRH, 2024). This seasonal variation in MPs concentrations can thus be also related to

increased river flow in the estuary during the more rainy period (winter), which may enhance MPs resuspension, preventing their deposition in sediments and promoting their transport out to sea.

With respect to MPs characterization, only fibers were detected across all three matrices - oysters, water, and sediment - in this study. The predominance of fibers as the primary form of MPs is consistent with global reports on different environmental matrices (Almeida et al., 2023; Chen et al., 2021; Du et al., 2022; Espincho et al., 2024; Rodrigues et al., 2018; Truchet et al., 2021; Zhang et al., 2019; Zhu et al., 2019). Specifically, in commercial species of bivalves, fibers account for over 50 % of the detected MPs, possibly because they are more challenging to remove from the digestive tract compared to other types of MPs (Li et al., 2015; Zhang et al., 2019).

Fibers have been found in twelve different colors (transparent, blue, black, white, red, orange, pink, green, purple, gray and yellow). Color should not be used as the main category for MPs characterization due to its susceptability to chemicals used in sample digestion, temperature, and environmental conditions (Shumway et al. 2023). However, it remains useful for sample comparison among matrices, and aids in identifying sources of contamination from procedural blanks. Among the fibers observed in the three matrices, transparent fibers were the most frequently recorded, followed by black, gray, and blue fibers. The prevalence of transparent fibers in the three matrices may result from the fading or degradation of their original colors due to environmental weathering (Jang et al., 2020).The predominant fiber colors observed in this study align with global findings (Cho et al., 2021; Hossain et al., 2022; Truchet et al., 2021) and previous research in the Lima estuary (Almeida et al., 2023; Espincho et al., 2024). Almeida et al. (2023) identified blue and black fibers as dominant in sediment and water samples, while transparent MPs were much less abundant compared to this study. Additionally, Espincho et al. (2024) also reported blue and transparent fibers as main MPs colors in water samples. Gray fibers, which were frequently observed in oysters, were much less common in water and sediment samples. Although other studies have reported gray as an infrequent color of MPs, it was still present in some studies (Almeida et al., 2023; Cho et al., 2021; Espincho et al., 2024). Color distribution of MPs differeed significantly between oysters and their environment, particularly sediment, with gray fibers prevalent in oysters, but rare in the environmental samples. Seasonal variations were also noted, as blue fibers were absent in oysters in winter sampling but present in water and sediment. This mismatch between biota and abiotic samples was also noted in other studies (Jahan et al., 2019; Wang et al., 2021b). For example, in the Jackson estuary, in

Australia, the predominant MPs color in oysters was black, while MPs in the sediment were mostly white (Jahan et al., 2019). Although we assumed that sediment ressuspension may contribute to MPs ingestion, this may not be the main source of MPs in oysters. Significant differences in the distribution of MPs colors between water and sediment were observed during winter. This may be attributed to the higher flow rates in estuaries during this season, which may increase the resuspension of MPs, leading to a greater variety of MPs colors in the water compared to the sediment (Almeida et al., 2023). These variations suggest that oysters may not fully represent the contamination levels in their surrounding environment, indicating they might not be reliable bioindicators.

In the present study, most of the encountered fibers were within the size interval 0.5 – 2 mm, both in oysters and in environmental samples, which is consistent with findings from other global studies (Almeida et al., 2023; Du et al., 2022; Espincho et al., 2024; Hossain et al., 2022). Specifically, for *C. gigas*, studies have shown that the main size of MPs present in oysters tissues including gut is less than 2 mm (Botelho et al., 2023; Ding et al., 2021; Du et al., 2022; Renzi et al., 2018; Wootton et al., 2022). As with MPs color distribution, differences were observed in the proportion of MPs size classes between oysters and sediment, but only in autumn. This difference was primarily due to small MPs (< 0.5 mm), which constituted a minor fraction in sediment samples in autumn compared to those in oysters, but became more prevalent in winter. This seasonal shift in MPs size in sediments in the Lima estuary was also reported by Almeida et al. (2023), with particles ranging from 3-5 mm in autumn (November), to 1 - 3 mm in winter (February).

Fibers can be made of natural materials (e.g., cellulose) or synthetic plastics, but they are often visually similar, which makes them difficult to differentiate. Spectroscopic analysis is therefore necessary to identify them chemically. In the present study, FTIR analysis revealed the presence of five polymers in oysters, while only two were found in the water and sediment samples. The higher diversity of polymers in oysters compared to the surrounding environment contrasts with findings from other studies on oysters and mussels, where the types of polymers found in organisms generally matched those in the surrounding environment (Cho et al., 2021; Du et al., 2022; Zhu et al., 2019). Similar to this study, Mladinich et al. (2024) also identified a wider variety of polymers in oyster guts compared to seawater. This greater variety of polymers in oysters may result from their longer exposure to contaminants during feeding, as they filter large volumes of water daily (Newell, 2004). In contrast, water and sediment samples collected and analysed in the present study only represent a snapshot of environmental conditions at

a specific moment. This extended exposure period may explain why oysters reflect a broader range of MPs polymers. Further research should include a larger temporal dataset in order to better examine the relationships between MPs in oysters and the environment.

The spectroscopic identification of the chemical composition of the fibers found allows us to gain insight into the possible sources of contamination, helping to distinguish between natural and synthetic materials and their respective origins. Of the fibers analyzed by FTIR, only 71 were identified as MPs, with cellulose accounting for 64% of all polymers considered, making it the dominant polymer across all environmental matrices. Cellulose and cellulose-based components (e.g., rayon and cellophane) were included in the results as potential MPs. Cellulose fibers were present in various colors, including transparent, blue, black, and gray, suggesting that they may originate from anthropogenic sources. The inclusion of cellulose-based fibers in MPs counts is controversial with some studies excluding themfrom their results (Cho et al., 2021; Mladinich et al., 2024), while others include them (da Costa et al., 2016; Ding et al., 20224). Our decision to include cellulose-based components was based on the following considerations: i) cellulose fibers have been reported globally in numerous studies (da Costa et al., 2016; Ding et al., 2022a; Ribeiro et al., 2023; Truchet et al., 2021); ii) these fibers have been linked to anthropogenic sources and are frequently found in combination with synthetic polymers such as PP and PE (Ó Briain et al., 2020); and iii) synthetically altered cellulose, such as rayon, should be classified as MPs due to their artificial composition (Hartmann et al., 2019).

As previously mentioned, cellulose was the most frequently detected polymer, consistent with other studies (da Costa et al., 2016; Ding et al., 2022a; Ribeiro et al., 2023; Truchet et al., 2021). It has already been reported and associated with household cleaning products such as wet wipes and sanitary towels, which are commonly mixed with synthetic polymers like PET and PP (Ó Briain et al., 2020). PET was the second most frequently detected polymer across the three matrices, aligning with findings from other studies (Cho et al., 2021; Ding et al., 2022a; Du et al., 2022). Its likely sources include land-based activities, given its widespread use in textiles, polyester fibers, and food packaging (Dhaka et al., 2022; Ding et al., 2022a). These fibers can enter the environment through WWTPs effluents (Ó Briain et al., 2020), and some may also result from the degradation of other plastic materials (Ramos et al., 2023). Other polymers were also detected in oysters, including PVS, PP and rayon. PVS is not commonly reported (as reviewed by Wang et al., 2021), however, it has already been detected in

fish and sediment samples (Hussien et al., 2021; Peng et al., 2018) and is used in the plastics industry (Gooch, 2011). PP fibers may be linked to hygiene products associated with cellulose fibers (Ó Briain et al., 2020). PP is also commonly related to fishing activities (Wang et al., 2019). Rayon, a semi-synthetic fiber, is frequently detected (Ding et al., 2021; Ding et al., 2022a; Zhu et al., 2019), with its contamination likely originating from the breakdown of textiles or hygiene products, which are released into the environment through WWTPs effluents (Ding et al., 2018). Its presence alongside with PET and PP polymers further supports the idea that textiles are a significant source of pollution. Small fibers can escape during washing, pass through WWTPs effluents, and accumulate in water bodies (Browne et al., 2011; Hernandez et al., 2017). These polymers are among the most commonly reported in other studies (Almeida et al., 2023; Chen et al., 2021; Ding et al., 2021; Ding et al., 2022a; Espincho et al., 2024; Mladinich et al., 2024; Truchet et al., 2021). Although cellulose was not previously reported at this site by Almeida et al. (2023) and Espincho et al. (2024), it has been documented in other global studies (da Costa et al., 2016; Ding et al., 2022b; Ribeiro et al., 2023; Truchet et al., 2021). Overall, the detection of these polymers suggests that human activies are likely the primary source of MPs contamination in the studied environment.

Concern about the presence of MPs in cultured bivalves has drawn attention to aquaculture gear as a potential contributor to plastic pollution. This study suggests that aquaculture gear (HDPE) did not contribute to MPs pollution at the oyster farm, as this polymer was not found in oysters nor in environmental samples. This aligns with the findings of Mladinich et al. (2024), who showed that aquaculture gear does not contribute to MPs contamination in *C. virginica*. Covernton et al. (2019) and Zhu et al. (2019) identified textiles as the main source of MPs pollution in aquaculture sites for *C. gigas*, consistent with the primary polymers found in this study.

4.2 Depuration efficacy in removing MPs

Oysters are often consumed whole and raw, raising significant food safety concerns. Bivalves intended for human consumption are usually subjected to a depuration process that has been shown to remove microbial contaminants through their natural filtration activity. Therefore it is important to assess depuration as a potentially effective method for removing MPs. Results from short-term depuration experiments showed an overall reduction in MPs concentrations, but a statistically significant reduction was observed only under controlled laboratory conditions, where levels decreased by 78 % after 48 hours. Depuration in the commercial facility was not as effective; despite a 59 %

decrease in MPs levels after 96 hours, the MPs concentrations at different time points were no significantly different.

The results of the laboratory trial correspond to what could be achieved under "clean" conditions – in which considerable efforts to minimize airflow and particle exposure are taken, including filtering water, covering tanks, preventing re-ingestion of egested particles through changes of water. Although we demonstrated that a 2-day depuration would be enough to significantly reduce MPs levels in oysters under relatively strict measures, it would be difficult to completely remove all MPs from oysters under realistic commercial depuration conditions.

Comparing with other laboratory-controlled studies of depuration in bivalves, our results stand out with a higher percentage of removal in the shortest period of time (Table 5). Van Cauwenberghe and Janssen (2014) reported reductions of 33 % in mussels (*M. edulis*) and 25 % in oysters (*C. gigas*) after 72 hours; Birnstiel et al. (2019) found a 29 % reduction in mussels (*Perna perna*) after 93 hours; Covernton et al. (2022) report a 73 % reduction in oyster (*C. gigas*) anthropogenic particles concentration after 5 days; and Paul et al. (2023) observed a 77% reduction in oysters (*C. gigas*), but only after 96 hours.

These differences may be related to the specific conditions used during laboratory setups in the different studies, namely temperature, which has been shown to affect bivalve gut depuration (Bayne et al. 1987). Differences in initial MPs contamination in oysters could also affect the results; however, the study by Paul et al. (2023), which had similar MPs concentrations to ours, showed a lower reduction percentage and required longer depuration times. Further research is needed to understand the factors affecting variation in microplastic reduction rates and to refine the depuration time needed.

All identified MPs were in the form of fibers, in both depuration trials, which aligns with previous reports where fibers were the dominant type of MPs in oysters (Birnstiel et al., 2019; Paul et al., 2023). Throughout the depuration process, no significant changes in the predominant color or size classes of the MPs were observed, and the most common colors and sizes remained constant, suggesting that there was no selective removal of particles under this time frame. However, in commercial depuration it is noteworthy that green fibers were not found after 48 hours, while red fibers were no longer present after 96 hours. This might suggest some selectivity in the removal of MPs or may simply reflect variability in MPs color categories across the sampled oysters.

The water used in the lab-controlled trial was analyzed, and as expected, alongside the reduction of MPs in the oyster tissues, an increase in MPs was observed in the water

and bio-deposits, comprising particles that were rejected (pseudofeces) and egested (feces) However, it is important to note that the water itself (i.e.control) contained MPs contamination, and therefore, we cannot exclude the oysters have ingested and egested these environmental particles present in the water.

A total of seven different polymers were identified in both oysters (from both trials) and water. The primary polymers found in oysters were PET and cellulose, which aligns with the findings from the field assessment (autumn and winter oyster samples). Additionally, PP, PAK, cellophane, and rayon were detected, polymers which have been frequently reported in bivalve molluscs worldwide as reviewed by Ding et al. (2022). Unlike rayon, PAK and cellophane were not detected in the other oysters sampled from the Lima estuary (field assessment study). The absence of these polymers in the seawater used in the laboratory depuration trial suggests that initial oyster contamination originated from the field at the time of sample collection in November. Cellophane and rayon are widely found in bivalves around the world (Baechler et al., 2020; Ding et al., 2021; Du et al., 2022; Wakkaf et al., 2020). These two polymers are considered modified natural polymers derived from cellulose and are often used in packaging and textiles, respectively (Ding et al., 2018; Wu et al., 2020). PAK, on the other hand, is a synthetic polymer commonly used in textile fibers (Della Torre et al., 2023). In the water, cellulose was the most abundant polymer, followed by PTT, rayon, PET, azlon, and ABS. Azlon is a fiber widely used in the textile industry (Mohamed et al., 2023). PTT is a type of polyester (Shruti et al., 2020) derived from textiles and packaging materials (Liu et al., 2013; Yang & Wang, 2016). ABS is not commonly reported but has been found in bivalves (Radhakrishnan et al., 2024). It is used in textiles, fashion, toys, and domestic products (Olivera et al., 2016). Once again, a large portion of the identified polymers were linked to textile fibers, reinforcing the idea that laundry washing and WWTPs effluents could be significant sources of MPs contamination, suggesting that the primary source of contamination is anthropogenic.

4.3 Limitations

Throughout the study, several limitations were encountered. One major challenge is the lack of standardized methods for extracting and categorizing MPs, making comparisons with existing research difficult. Various digestion methods - acidic, enzymatic, and alkaline - are commonly used, each with its own advantages and disadvantages. Acidic, oxidative, and alkaline techniques can degrade certain polymers (Kühn et al., 2017; Shumway et al. 2023). While oxidative and alkaline methods are widely used, they can cause discoloration of MPs. However, in oxidative digestions, color

changes were only observed after seven days, much longer than the 24-48 hours duration used in this study. Temperature (> 60 ºC) also affects polymer integrity, and differences in filter pore sizes can impact the detection of smaller particles, further complicating cross-study comparisons. Degradation and discoloration of MPs may lead to underestimating their abundance, favoring the detection of more resistant polymers. Further research is needed to refine extraction protocols, as reagent choice and temperature greatly affect recovery rates.

Additionally, the digestion of whole oyster tissues is particularly challenging due to their biochemical composition, which can hinder the proper breakdown of organic matter and lead to an underestimation of MPs. In this study, we used 30 % H_2O_2 solution for oxidative digestion, a method demonstrated by Silva et al. (2022) to effectively recover high amounts of MPs from shellfish with varying fat content, without comprimising the integrity of MPs.

Regarding the FTIR analysis results, due to time constraints, it was only possible to analyze the recommended minimum (10 % of the total), and the percentages of identified MPs were generally low compared to the total number of potential MPs analyzed. This indicates that some of the analyzed fibers did not yield matches in the reference library that complied with the acceptance criteria. This could be explained by the small size of the analyzed MPs, requiring the use of a micro-Fourier transform infrared spectroscopy (μFTIR), or by the fact that the reference library spectra are derived from virgin polymers, whereas the particles analyzed may have undergone environmental degradation (e.g., UV light, bacterial degradation) and potentially some degradation from the oyster's digestive system. Using customized libraries with aged polymer spectra could improve particle identification. Additionally, particles smaller than 0.5 mm were not analyzed, which further limits our study. Although FTIR can detect particles as small as 10 μm, Espincho et al. (2024) reported that when using the same FTIR system to analyze MPs smaller than 0.5 mm, they rarely achieved confidence levels equal or higher than 75 %.

In the sediment samples, some MPs polymers are denser, and using NaCl may not effectively separate materials by density (Almeida et al., 2023). Organic matter can also aggregate and retain MPs in sediment. Although recoveries rates exceeded 80 % for various MPs polymers, the optimized methodology (Rivoira et al., 2020) may still underestimate some MPs.

Challenges arose during the laboratory depuration experiment. Despite the thorough filtration of all the water used, particles were still present at the begining of the

experiment, as observed in the control tanks. Additionally, the filtration of aquarium water at the end of the trial caused significant clogging of the filters, which affected the duration of the process and limited the possibility of taking a larger number of particles to FTIR for proper chemical identification.

4.4 Conclusions

This study provides the first evidence of MPs contamination in oysters farmed in the intertidal areas of the Lima estuary in northern Portugal, establishing a baseline for MPs abundance and types using visual and spectroscopic analysis. Seasonal variations in oyster MPs contamination suggest that greater MPs accumulation occurs at the end of summer – early autumn, coinciding with periods of more intense metabolic activity and growth in oysters. However, the higher contamination in oysters does not appear to reflect MPs concentrations in the surrounding water and sediment. No selectivity in the oysters' filtration process was observed concerning the size or type of MPs, but a wider diversity of polymers were detected in the oysters than in their environment. Overall, the polymers identified were primarily related to textile fibers and anthropogenic products (e.g., food packaging, wipes), suggesting that the main source of contamination in the oysters potentially comes from anthropogenic sources namely WWTPs effluents. Additionally, this study demonstrates that aquaculture gear does not significantly contribute to MPs pollution in the oyster farm and the surrounding estuarine area.

To the best of our knowledge, this is the first study investigating the depuration efficiency of MPs in farmed oysters in Portugal. Depuration proved effective in reducing MPs contamination levels under laboratory conditions, but in a commercial setting, 96 hours were insufficient to achieve a significant decrease in MPs levels. Further research is needed to understand the factors influencing MPs contamination reduction and to provide guidelines to the industry, as laboratory studies achieved reductions of up to 80 %.

Table 4. Studies reporting MP's contamination in different bivalve species worldwide. Location, type of digestion, identification method of MPs, MPs predominant shape, size, color and type found, concentrations, and corresponding references, are indicated. Orange represent data from the present study and green represents the results for *C. gigas*. *(-) no information found

	Country	Species	Digestion protocol	Identification	Main colors	Main size (mm)	Main shape	Polymer types	Concentration (MPs g-1)	Concentration (MPs ind-1)	References
America	Argentina	*Crassostrea gigas*	HNO3 10% H2O2	Microscope FTIR	Blue	0.17 - 5.0	Fibers	PP and PE	-	2.0 - 7.0	(Fernández et al., 2019)
		Brachidontes rodriguezii	30% H2O2	Microscope, FTIR and SEM/EDX	Black and blue	< 0.5	Fibers	Cellulose, PA and polyacrylates	0.33 ±0.1	-	(Truchet et al., 2021)
		Amarilladesma mactroides			Black and transparent	01/mai			0.17 ±0.07	-	
	Brazil	*Crassostrea gigas*	10% KOH NaCl	Microscope SEM-EDS	Blue and black	< 5.0	Fibers	-	0.27 - 0.64	0.33 - 0.75	(Saldaña-Serrano et al., 2022)
Europe	France	*Crassostrea gigas*	10%KOH	Microscope μ-FTIR	Grey, green and black	< 1.3	Fragments	5 types	0.18 ± 0.16	2.10 ± 1.71	(Phuong et al., 2018)
		Mytilus edulis			Grey and black	< 1.0	Fragments	7 types	0.23 ± 0.20	0.60 ± 0.56	
	Germany	*Mytilus edulis*	69% nitric acid	Microscope micro-Raman spectroscopy	Blue and Red	0.005 - 0.02	-	-	-	0.36 ±0.07	(Van Cauwenberghe and Janssen 2014)
	Portugal	*Mytilus galloprovincialis*	10%KOH	Microscope FTIR	Blue and black	0.07 - 4.68	Fibers	PET, PP and PS	0.18 ± 0.31	0.45 ± 0.67	(Pequeno et al., 2021)
		Scrobicularia plana						PET and PVC	0.07 ± 0.15	0.30 ± 0.63	
		Mytilus galloprovincialis	10%KOH	Microscope FT-MIR	Colourless and yellow	0.2 - 1.4	Fibers and fragments	9 types	0.77 - 4.3	-	(Botelho et al., 2023)
		Cerastoderma edule							0.83 - 5.1		
		Ruditapes decussatus	10% KOH	-	Black and blue	0.1 - 0.5	Fibers and fragments	-	0 - 0.6	0 - 30	(Manthopoulos 2023)
		Mytilus galloprovincialis							0 - 0.10	0 - 5	
		Crassostrea gigas							0 - 0.15	0 - 20	
		Mytilus galloprovincialis	10% KOH	Microscope μFTIR	Blue	> 0.03	5 shapes	PE, PP and PA	0.83 ±0.31	0.6–2.2	(Vital et al., 2021)
		Mytilus spp.	10% KOH	Microscope FTIR	Colourless	0.1 - 5	Fibers and fragments	9 types	0.54 to 3.0	-	(Marques et al., 2021)
		Crassostrea gigas	30% H2O2	Microscope FTIR	Transparent, blue black and gray	0.5 - 2	Fibers	5 types	0.48 ± 0.34	9.8 ± 7.5	This study (autumn 2023)
					Transparent, black and gray				0.09 ± 0.07	2.2 ± 1.7	This study (winter 2024)
		Crassostrea gigas	30% H2O2	Microscope FTIR	Transparent, blue black and gray	0.1 - 4.8	Fibers	5 types	2.2 ± 2.0	28 ± 17	Unpublished study (winter 2023)
				Microscope	Transparent, white, black and blue	0.2 - 4.6	Fibers	-	0.3 ± 0.3	6 ± 5	Unpublished study (winter 2022)
		Mytilus galloprovincialis	10% KOH	Microscope Raman spectroscopy	White, blue, red and black	0.15 - 1.49	Fibers, Pellet and Foam	5 types	0.46 ± 0.04	0.83 ± 0.08	(Barboza et al., 2024)
		Ruditapes decussatus	1.8 M KOH	Microscope Raman spectroscopy	Blue and white	0.1 - 5	Film	PE, PET, PP and PS	-	18.4 ± 21.9	(Cozzolino et al., 2021)
		Cerastoderma spp.							-	11.9 ± 5.5	
		Polititapes spp.							-	10.4 ± 10.4	
	Italy	*Mytilus galloprovincialis*	30% H2O2	Microscope	Blue and black	1.7 - 1.9	Fibers	-	3.6 ± 12.4	8.33 ± 3.58	(Renzi et al., 2018)

Asia	China	*Crassostrea* spp.	10% KOH 30% H2O2	Microscope µ-FTIR	-	< 1.5	Fibers	CP, PE and PET	0.62	2.93	(Teng et al., 2019)
		Crassostrea gigas	10% KOH	Microscope µ-FTIR	Transparent and blue	0.070 - 5.0	Fibers	18 types	0.3 - 3.0	1.2 - 3.3	(Ding et al., 2021)
		Mytilus galloprovincialis							1.6 - 2.6	0.8 -2.1	
		Ruditapes philippinarum							4.5 - 20.1	1.2 - 3.2	
		Chlamys farreri							1.6 - 2.6	0.8 - 2.1	
		Crassostrea gigas	10% KOH 30% H2O2	Microscope µ-FTIR	-	0.03 - 2	Fibers and fragments	> 9 types	0.36 ± 0.02	2.92 ± 0.10	(Du et al., 2022)
		9 bivalve species	30% H2O2	Microscope µ-FTIR	Black, red, blue, white and transparent	< 0.25	Fibers and pellets	PE, PET and PA	2.1 to 10.5	4.3 to 57.2	(Li et al., 2015)
	Korea	*Crassostrea gigas*	35% H2O2	Microscope µ-FTIR	Transparent	< 0.3	Fragments	12 types	1.13 ± 0.84	-	(Jang et al., 2020)
		Mytilus edulis							1.43 ± 1.45	-	
		Crassostrea gigas	10% KOH	Microscope µ-FTIR	Colourless	< 0.3	Fragments	PP, PE and PES	0.33 ± 0.23	1.21 ± 0.68	(Cho et al., 2021)
		Mytilus edulis									
		Ruditapes philippinarum							0.43 ± 0.32	2.19 ± 1.20	
	India	*Perna viridis*	30% H2O2	Microscope FTIR	Transparent, red, blue and green	0.1 - 0.3	Fragments	5 types	-	2.31 ± 0.93	(Radhakrishnan et al., 2024)
		Villorita cyprinoides							-	2.41 ± 0.17	
Oceania	Australia	*Crassostrea gigas*	10%KOH	Microscope FTIR	Blue and pink	0,04 - 1,0	Fibers	7 types	1.03 ± 0.33	-	(Wootton et al., 2022)

Table 5. Studies reporting depuration of MPs in different bivalve species. Species, depuration time, shell length, initial and final MPs concentration (expressed as MPs ind^{-1} and MPs g^{-1}, respectively) and the percentage of MPs reduction, are indicated. Blue represent data from the present study. Fibers are the dominant type of MPs in all the studies. (-) no information found; * results expressed as mean ± SD

Species	Depuration time	Shell lenght (cm)	*Initial MP concentration		*Final MP concentration		Percentage of MP reduction	References
Crassostrea gigas	**48h**	**7.4 ± 0.6**	12.9 ± 14.9	0.77 ± 0.86	3.07 ± 3.2	0.15 ± 0.15	78% post 48h depuration	This study (lab-controlled trials)
Mytilus edulis	**72h**	5.2 ± 0.4	-	0.36 ± 0.07	-	0.24 ± 0.07	33% post 72h depuration	(Van Cauwenberghe & Janssen, 2014)
Crassostrea gigas	**72h**	9.0 ± 0.5	-	0.47 ± 0.16	-	0.35 ± 0.05	25% post 72h depuration	
Perna perna	**93h**	-	12.9 ± 14.12	4.12 to	3.07 ± 3.2	2.83	30% post 93h depuration	(Birnsteil et al., 2019)
Crassostrea gigas	**96h**	**6.9 ± 0.5**	12.9 ± 14.13	0.21 ± 0.21	3.07 ± 3.2	0.10 ± 0.15	59% post 96h depuration	This study (commercial trials)
Magallana gigas	**96h**	7.8 ± 0.6	12.9 ± 14.14	0.6 ± 0.3	3.07 ± 3.2	0.2 ± 0.2	70% post 96h depuration	(Paul et al., 2023)
Ostrea edulis	**96h**	7.2 ± 0.8	12.9 ± 14.15	0.4 ± 0.4	3.07 ± 3.2	0.1 ± 0.1	80% post 96h depuration	

Chapter V - References

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