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2018/19

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Mestrado em Biologia Marinha

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Declaração de autoria de trabalho

Service or disservice? The role of marine coastal bioengineers in plastic debris trapping

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Lorenzo Cozzolino)

ACKNOWLEDGMENTS

I am thankful to Dr. Katy Nicastro, Carmen B. de los Santos and Gerardo Zardi for their constant availability, great support and wise guidance, which allowed the realisation of this work.

To the team of the laboratory of “Biogeographical Ecology and Evolution” and to the ALGAE group for the lovely working environment and to encourage knowledge sharing and mutual aid.

To Badr Elmahrad for the help with ArcGIS software and the realization of the maps.

To my family, as without them this would not have been possible.

RESUMO

Hoje em dia, a poluição plástica é cada vez mais reconhecida como uma séria ameaça ambiental e económica que compromete a biodiversidade dos ecossistemas marinhos e os serviços que estes prestam. A crescente entrada de lixo plástico nos oceanos exige esforços imediatos para coletar dados sobre a ocorrência, abundância e dispersão de detritos plásticos para uma melhor avaliação ambiental e para impulsionar práticas de gestão. Para alcançar esses resultados, é necessário um foco importante em ecossistemas-chave, como os formados por bioengenharia. Os bioengenharia são espécies que modulam a disponibilidade de recursos diminuindo o estresse ambiental ou aumentando a complexidade do habitat, influenciando positivamente as espécies em sua comunidade, resultando em um aumento geral da biodiversidade.

No entanto, há uma lacuna de conhecimento sobre o papel potencial dos bioengineiros costeiros marinhos na captura de detritos plásticos que exige atenção. Ao aumentar a complexidade do habitat, os engenheiros dos ecossistemas costeiros, tais como ervas marinhas, marismas e macroalgas rizófitas, podem agir indirectamente como sumidouros de plástico dos oceanos. Além disso, se o acúmulo de detritos plásticos em ecossistemas formados por bioengenheiros for realmente promovido, isso pode ter, por sua vez, efeitos deletérios sobre sua fauna associada. A exposição direta dos organismos que vivem dentro das estruturas dos bioengineiros a altas concentrações de poluentes plásticos pode ter efeitos negativos sobre sua saúde. Em particular, o risco de ingestão acidental de microplásticos pode ser aumentado em bivalves com hábitos de alimentação por filtração que ocorrem em habitats bioengenharia. É importante notar que, em zonas onde as espécies comerciais de bivalves são directamente colhidas de ecossistemas naturais, como os prados de ervas marinhas, o potencial destes habitats para actuarem como sumidouros de plástico pode, em última análise, afectar a saúde humana.

Aqui, eu investiguei o papel potencial dos bioengenharia costeira como sumidouros de macro e microplástico. Assim, quantifiquei a ocorrência, tipologia e abundância de detritos plásticos em ervas marinhas intertidais (*Zostera noltei*), ervas marinhas subtidais (*Cymodocea nodosa* e *Zostera marina*), macroalgas rizófitas (*Caulerpa prolifera*) e sapais intertidais (*Sporobolus maritimus*) habitando uma lagoa costeira antropizada. Hipotetizei que estes bioengenheiros acumulam mais macroplásticos entre as frondes das copas e microplásticos no sedimento superficial do que as manchas adjacentes de sedimentos nus. No geral, não encontrei diferenças significativas entre a capacidade de aprisionamento de bioengenharia vegetal e sedimentos nus laterais. Uma diferença significativa foi detectada apenas entre a abundância de macroplásticos em *S. maritimus* e seu sedimento pelado intertidais lateralmente. No entanto, apesar da falta de diferença estatística

significativa, os sedimentos coletados em *Z. noltei* intertidais (0.019 ± 0.017 n MPs g^{-1}) e *S. maritimus* (0.024 ± 0.019 n MPs g^{-1}) resultaram ligeiramente menos contaminados por microplásticos do que os das espécies sub-mareais *C. nodosa* e *C. prolifera* (0.035 ± 0.027 n MPs g^{-1} e 0.034 ± 0.025 n MPs g^{-1}). Em geral, o microplástico mais abundante detectado foi a fibra (86,5%) e a cor mais comum foi o azul (45%).

Por outro lado, a avaliação macroplástica revelou *S. maritimus* (0,220 ± 0,157 macroitems m^{-2}) e *C. prolifera* (0,048 ± 0,061 macroitems m^{-2}) com a maior concentração macroplástica por número (n macroitems m^{-2}) e a menor por massa (0,513 ± 0,428 g macroitems m^{-2} e 0,848 ± 1.056 g macroitems m^{-2}, respectivamente), enquanto *C. nodosa* (0,013 ± 0,021 macroitems m^{-2}) e *Z. noltei* (0,013 ± 0,020 macroitems m^{-2}) apresentaram a menor abundância macroplástica (n macroitems m^{-2}) e a maior massa (16,431 ± 39,752 g macroitems m^{-2} e 6,326 ± 14,827 g macroitems m^{-2}, respectivamente). No total, 61,4% dos detritos macroplásticos encontrados eram fragmentos.

Em seguida, avaliei se os bioengineiros intertidais e subtidais aprisionaram diferentes quantidades e tipologias de macro e microplásticos. Para tanto, comparei as espécies intertidais *Z. noltei* e *S. maritimus* com as espécies subtidais *C. nodosa*/*Z. marina* e *C. prolifera*. Hipotetizei que o padrão de distribuição do tipo plástico difere entre-marés e bioengenharia sub-marítima de acordo com a forma como a sua formação estrutural modifica as condições ambientais locais. Em geral, eu esperava encontrar mais plástico em espécies sub-mareais devido à sua maior exposição aos poluentes presentes na coluna de água.

No entanto, nenhuma diferença significativa foi detectada entre a quantidade e a tipologia dos macro e microplásticos acumulados em habitats intertidais e subtidais.

Para fornecer uma declaração completa da deposição de plástico nos prados dos bioengineiros, eu também quantifiquei a ocorrência, tipologia e abundância de microplásticos aderidos nas copas dos bioengineiros. Para atingir este objectivo, analisei as lâminas das folhas dos cinco bioengineiros visados: *Z. noltei*, *S. maritimus*, *Z. marina*, *C. nodosa* e *C. prolifera*. Hipotetizei que parte dos microplásticos aprisionados nos bioengineiros não atingiria a superfície do sedimento, mas poderia potencialmente aderir às folhas de ervas marinhas. A expectativa era encontrar um número maior de MPs na superfície das folhas das espécies sub-mareais devido à sua maior exposição aos poluentes presentes na coluna de água. Diferenças significativas na abundância de microplástico nas folhas dos bioengineers intertidais e subtidais confirmaram a minha hipótese, com as espécies subtidais aprisionando mais detritos do que as intertidais. Em geral, a maior concentração microplástica foi encontrada nas folhas de *C. prolifera* (0,0559 ± 0,0936 MPs cm^{-2}) e *Z. noltei* (0,0529 ± 0,1238 MPs cm^{-2}), seguido por *C. nodosa* (0,0198 ± 0,0308 MPs cm^{-2}) e *Z. marina* (0,0114 ± 0,0113 MPs cm^{-2}).

A menor contaminação foi registrada em S. maritimus com 0 MPs cm^{-2}. No geral, os microplásticos detectados nas folhas eram todas fibras e as cores mais comuns eram o azul (36%).

Finalmente, para entender se altas concentrações de plástico nos bioenginheiros e nos sedimentos laterais nus podem afetar a fauna associada, avaliei a ocorrência e abundância de microplástico ingerido por espécies bivalves rentáveis (*Ruditapes decussatus*, *Polititapes* sp. e *Cerastoderma* sp.), comumente colhidas na Ria Formosa. Encontrei que MPs ocorrem em altas concentrações em todas as espécies bivalves investigadas. Especificamente, *Ruditapes decussatus* continha em média 18,4 ± 21,9 MP itens g^{-1} (u.i.) tecido e exibiu a maior concentração de MP por peso, *Cerastoderma* sp. e *Polititapes* sp. seguidos de 11,9 ± 5,5 MP itens g^{-1} (u.i.) e 10,4 ± 10,4 MP itens g^{-1} (u.i.), respectivamente. Globalmente, 88% das MP encontradas eram fibras sintéticas, a maioria das quais eram azuis (51,6%). Os polímeros mais representados foram o polietileno (PE) e o poliestireno (PS). O número inesperadamente elevado de microplásticos registados em bivalves sugere que este sistema lagunar semi-fechado está a sofrer uma pressão antropogénica superior à dos sistemas costeiros abertos e afirma um esforço imediato para reduzir os resíduos plásticos e melhorar a gestão da eliminação de águas residuais na Ria Formosa.

Os resultados desta investigação ajudariam a preencher as lacunas de conhecimento existentes e a definir novas zonas potenciais de acumulação de plástico em habitats costeiros chave para a vida selvagem marinha, mas é necessária investigação futura para melhor inferir o padrão de deposição de plástico nestes ecossistemas vitais.

Palavras-chave: Ambientes costeiros, engenheiros de ecossistemas, lixo marinho, poluição plástica, Ria Formosa, mariscos.

ABSTRACT

The rapid rise of plastic pollution in the world's oceans demands immediate efforts to investigate the occurrence, abundance and dispersal of plastic debris in the marine environment. The identification of plastic accumulation areas or sinks in key habitat for marine wildlife, such as foraging or nursery areas, is a high priority for the implementation of management strategies. Habitats formed by coastal bioengineers play a crucial role in modifying local environmental conditions and in maintaining a high biodiversity. Here, we investigated the potential role of coastal vegetated bioengineers as sink for macro- and microplastics. We focused on intertidal (*Zostera noltei*) and subtidal seagrasses (*Cymodocea nodosa and Z. marina*), rhizophytic macroalgae (*Caulerpa prolifera*) and intertidal saltmarsh (*Sporobolus maritimus*) from the Ria Formosa lagoon. We found no significant differences in microplastic (MP) abundance or type between any of the bioengineer and its side bare sediments as well as between intertidal and subtidal habitats. A similar pattern was observed for macroplastics abundance, type and mass except for *S. maritimus* that had significantly more macroplastic than its intertidal bare sediment. The most abundant microplastic type and colour was fibre (86.5%) and blue (45%) respectively while 61.4% of macroplastics found were fragments. Subtidal bioengineers trapped significantly more MPs than intertidal species on their leaves, with 100% MPs being fibres, 36% of which blue in colour. To understand if eventual high concentrations of plastic in the bioengineer can affect its associated fauna, we also assessed the occurrence and abundance of microplastic ingested by profitable bivalve species (*Ruditapes* decussatus*., Polititapes* sp. and *Cerastoderma* sp.). MPs occurred at high concentrations in all the bivalve species investigated. Specifically, *Ruditapes decussatus* contained on average 18.4 ± 21.9 MP items g^{-1} (w.w.) tissue followed by *Cerastoderma* spp. and *Polititapes* spp. with 11.9 ± 5.5 MP items g^{-1} (w.w.) and 10.4 ± 10.4 MP items g^{-1} (w.w.), respectively. Our findings suggest that this semi-closed lagoon system is experiencing high anthropogenic pressure and claim for immediate effort to reduce plastic waste and improve the management of wastewater disposal.

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Keywords: Coastal environments, ecosystem engineers, marine litter, plastic pollution, Ria Formosa, seafood.

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PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and microplastic abundance as the dependent variable.

Table 3.1. Frequency of occurrence (%) of MPs in the samples of the three commercial bivalve species, their MP abundance as items g^{-1} (w.w.) (mean ± SD), total number of MP items found in each species, the percentage of occurrence for each MP types (fibres, foams and films) and polymer (PE - Polyethylene, PS - Polystyrene, PET - Polyethylene terephthalate, PP - Polypropylene).

LIST OF ABBREVIATIONS

MPs - Microplastics

Macro - Macroplastics

PVC - Polyvinyl chloride

PS - Polystyrene

PET - Polyethylene terephthalate

PP - Polypropylene

PCBs - Polychlorobiphenyls

ZN - *Zostera noltei*

ZM - *Zostera marina*

SM - *Sporobolus maritimus*

CN - *Cymodocea nodosa*

CP - *Caulerpa prolifera*

BSI - Bare sediment intertidal

BSS - Bare sediment subtidal

AG – Above-ground biomass

CHAPTER 1. INTRODUCTION

Plastic pollution is ubiquitous in ecosystems worldwide. Since plastics have been discovered in the 1950s, the production of this relatively inexpensive and long-lasting material has increased exponentially exceeding 320 million tons in 2015, 40% of which consumed as single use packaging (Plastics Europe, 2016). Plastics are synthetics polymers mostly derived from petrochemical sources that have desirable characteristics (lightness, softness, ductility, durability, low cost, etc.) for many applications (Andrady and Neal, 2009; Cauwenberghe et al., 2015; Costa et al., 2016). However, it is because of its resistance to degradation, along with its extensive use and challenging disposal, that plastic is accumulating in the environment (Sivan, 2011). Plastic debris have been found not only in areas close to the plastic pollution sources, but also in the most remote and supposedly pristine areas of the planet, from the Arctic (Obbard et al., 2014) to the sub Antarctic (Eriksson et al., 2013), and in remote uninhabited islands (Lavers et al., 2019). Plastic debris has been reported for a wide variety of ecosystems, such as beaches (Fok and Cheung, 2015), deep ocean (Van Cauwenberghe et al., 2013), lakes (Faure et al., 2012), rivers (Williams and Simmons, 1996; Moore et al., 2011) and estuaries (Morritt et al., 2014; Sadri and Thompson, 2014).

Plastic debris are generally divided into two size categories: macro- (> 5 mm) and microplastics (< 5 mm). Microplastics (MPs) are the most common debris in the marine environment (Hidalgo-Ruz et al., 2012) and can be further sub-categorized into: (1) primary, such as industrial pellets used in the production of larger plastic items or microbeads included in a number of industrial and household products, and (2) secondary, originating from the fragmentation and degradation of larger plastic items (Cole et al., 2011; Dubaish and Liebezeit, 2013). Secondary microplastics occur in a variety of forms, including fragments, films and fibres. Generally, the degradation time is strictly linked to the material (Guo et al., 2012), and to the physical and chemical features of the surrounding environment. Heat and solar radiation, wave and wind action, the biological activity of microbial communities and potential deep-water high pressure (Tosin et al., 2012) are all recognized as factors favouring plastic degradation.

As plastic waste is undoubtedly linked to human activities, the most industrialized and highly populated areas are also the most polluted (Derraik, 2002). Importantly, land-based sources of plastic debris account for the 80% of the litter in marine environment (Gregory and Ryan, 1997). It has been estimated that only in 2010, 4.8-12.7 million metric tons of plastic entered the world's ocean from lands (Jambeck et al., 2015). Despite some wind-blown debris and recreational litter left into the environment (Coe and Rogers, 1997), most of the debris enter the sea via rivers and storm-water run-

off (Browne et al., 2010; Cole et al., 2011). Other relevant sources include wastewater effluent and household sewage discharge (Gregory 1996; Browne et al., 2010). These last inputs of pollution mostly carry polyethylene (PE), polypropylene (PP) and polystyrene (PS) derived from health and beauty products (Andrady, 2011), as well as polyester and acrylic in the form of plastic fibres from synthetic cloths (Oerlikon, 2009). Indeed, one single clothing item can release more than 1900 fibres per wash (Browne et al., 2011; Woodall et al., 2014). Despite removal efficiency of wastewater treatment facilities can be very high (95–99%), municipal wastewater effluents remain a conspicuous pathway for microplastics to reach aquatic systems (Browne et al., 2007; Murphy et al., 2016). The remaining portion (20%) of marine litter that is not land-based enters the ocean mostly as accidental dumping from cargo ships and discarded fishing gear from fishing vessels (Good et al., 2010). This last source is of raising concern due to the increasing fishing effort of the last decades and increased durability of fishing gears. The abandoned, lost or discarded fishing equipment, known as "ghost fishing", has a further impact on the environment by affecting fish stocks and benthic environments and thus adding substantial social and economic costs (Macfadyen et al., 2009).

Worldwide, the "marine plastic" is negatively impacting marine ecosystem services with a medium to high degree of irreversibility. These services include the provision of fisheries, cultural and recreational opportunities as well as tourism (Beaumont et al., 2019). As consequence, human welfare is also challenged. According to the 2011 ecosystem services values, the ecological, social and economic costs deriving from marine plastic pollution corresponded to an annual loss of $500-$2500 billion in the value of benefits derived from the communal marine natural heritage (Beaumont et al., 2019). This estimate is expected to increase significantly in future years as the flux of plastic in the ocean is expected to grow (Jambeck et al., 2015). The increasing input of plastic litter into the oceans demands immediate efforts to collect data of the occurrence, abundance, type and fate of plastic debris for better environmental risk assessments and functional management practices. Indeed, many data gaps about dispersal, distribution and accumulation of plastic debris in aquatic environments still exist.

Overall, the distribution and movement of marine litter depend on a wide range of environmental and anthropogenic factors. These include physical forces such as winds, currents and coastline profiles (Law et al., 2010) but also human-linked influences such as the vicinity to urban and industrialized areas and shipping routes (Barnes et al., 2009), as well as the amount of sewage overflow (Free et al., 2014). Generally, the unpredictable action of natural and physical forces together with multiple diffuse inputs of plastic debris into the sea, results in great temporal and spatial

variability of accumulation patterns (Ryan et al., 2009). In the last decade, numerous studies have been carried out to define the occurrence of plastic debris and its pattern of deposition in coastal areas. Intertidal sandy shorelines have been widely described (Cole et al., 2011; Lima et al., 2014) and wind, currents and seasonality specified as dominant factors driving deposition, regardless of human activities (Ryan et al., 2009). The knowledge acquired also provides information regarding the most common types of debris beached. Among these are expanded polystyrene (Fok and Cheung, 2015), pellets (Gregory, 1978; Obbard, 2006; Tuner and Holmes, 2011) and fibres (Claessens et al., 2011), reflecting location-specific people's lifestyle. Compared to other coastal ecosystems, transitional waters such as estuaries and coastal lagoons, have been poorly described despite their central role as transport route of plastic debris to the ocean (but see Zhao et al., 2015; Naidoo et al., 2015). These transitional environments between fresh and saltwater habitats present particular dynamics, such as salt wedges, that might influence the patterns of plastic deposition by acting as barrier for the movement of high-density polymers, thus enhancing their settlement (Acha et al., 2003). Furthermore, coastal areas house key ecosystems such as those formed by bioengineers that ensure important functions and services to human society.

Ecosystem engineers (i.e. bioengineers or foundation species; Jones, 1994) are species that modify local environmental conditions creating new habitat and influencing species richness and composition. They modulate the availability of resources by decreasing environmental stress or increasing habitat complexity, positively influencing species within their community resulting in an overall increased biodiversity (Jones et al., 1997). Coastal vegetation such as seagrasses and saltmarshes are essential bioengineers in coastal environments as they control erosion by enhancing wave attenuation and promote sediment stabilization through their canopies or roots/rhizomes (Gedan et al., 2011). These structures also maintain a high biodiversity, including commercially exploited fish and invertebrate species, by providing habitat, nursery area, feeding area, and/or refuge from predation (Jackson et al., 2001). Furthermore, the spatial growth of bioengineer's structures, together with its infauna assemblages, increase the sedimentation of suspended particles and decrease their resuspension, improving water quality and reducing turbidity (Gacia and Duarte, 2001; Short et al., 2007; Van der Heide et al., 2007).

The fact that seagrass beds, as well as rhizophytic algae and saltmarsh beds, show a positive effect on particles deposition (Hendriks et al., 2010) suggests also their potential in plastic particles trapping. Yet, there is a knowledge gap on the potential role of marine vegetated coastal bioengineers in plastic debris trapping. The presence of MPs in seagrasses have been recently demonstrated in the

leaf blades of *Thalassia testudinum*, reporting 75% of samples contaminated by fibres (Goss et al., 2018). The potential role of vegetated ecosystem engineers as ocean plastic sink would highlight critical trade-offs in ecosystem function: what is considered as an ecosystem service (water purification), can turn into an ecosystem disservice (plastic accumulation).

If the accumulation of plastic debris is promoted in ecosystem engineers, this may have, in turn, deleterious effects on the associated fauna. Indeed, although the water purification service is largely modulated by the interaction between water flow and leaf canopy (Koch et al., 2001), the associated fauna living within seagrasses also play a major role. The active filtering performed by the suspension-feeders and the excretion of sticky exopolymers by the epiphytes leaving on leaves (Gacia et al., 2003) result in active and passive particles trapping. This highlight a direct risk of exposure of the organisms living within bioengineers' structures to microplastics, particularly for bivalves with filter-feeding habits. By filtering the water, these bivalves have an important role of reducing phytoplankton and bacteria biomass and particulate organic matter, attenuating turbidity and favouring light penetration (Vaughn and Hakenkamp 2001). It has been shown that ingestion of MPs by bivalves can provoke physical damage to the gut tissues with subsequent harsh inflammatory responses (von Moos et al., 2012), reduced nutrient uptake and effects on feeding behaviour (Wegner et al., 2012; Sussarellu et al., 2016). In addition to the physical effect of ingesting the particles, plastic debris contain harmful chemicals (i.e. Bisphenol A and Phthalates) part of which are used in the production process (Meeker et al., 2009) while others, such as PCBs and heavy metals, are absorbed in the natural environment (Mato et al., 2001; Ashton et al., 2010; Rochman et al., 2014). These substances can be absorbed by the epithelial cells of the intestinal tract (von Moos et al., 2012) and sequentially translocate to the circulatory system (Browne et al., 2008). As consequences, bivalve can experience DNA damage, neurotoxicity, effect on the immune and reproductive system (Avio et al., 2015; Sussarellu et al., 2016; Ribeiro et al., 2017). Furthermore, bivalves constitute an important food source for many marine organisms such as snails, crabs, fishes and marine birds, as well as for humans (Peitso et al., 1994; Dudas et al., 2005). Overall, since benthic biota contribute up to 90% of fish prey biomass, its contamination could affect higher trophic levels in nature (Schindler and Scheuerell, 2002) including humans. Hence, quantifying the microplastics abundance in biota, particularly at the lowest trophic level, is central to assess the impact of plastic pollution and to determine potential food-webs pathways and sinks.

Apart from the filter-feeders, grazers living within seagrass meadows could also ingest MPs. The adherence of MPs to the leaves of seagrasses (Goss et al., 2018) and algae (Yokota et al., 2017) could

favour MPs deposition in the vegetated bioengineer and in turn represent a relevant threat to herbivores. The particle deposition process is usually promoted by the excretion of sticky exopolymers by the epiphytes leaving on leaves (Gacia et al., 2003) therefore, it is expected that seagrass leaves with higher concentration of epiphytes are more prone to accumulate MPs. Evidences show that incrusted MPs on algae impact algal productivity directly reducing photosynthesis, growth and morphology (Yokota et al., 2017). Moreover, the accumulation of MPs on leaves could also potentially constitute a new pathway of microplastics introduction in the coastal food chain by lower trophic levels (Gregory, 1996).

1.1 OBJECTIVES AND HYPOTHESES

The general aim of this thesis is to assess the potential role of coastal habitats formed by ecosystem engineers as a sink for macro- and microplastic debris and understand the potential risks for the associated fauna. To achieve this objective, I focused on intertidal and subtidal habitats from an anthropized area. These included three seagrasses species (*Zostera noltei*, *Zostera marina*, *Cymodocea nodosa*), a rhizophytic algae (*Caulerpa prolifera*), and a saltmarsh species (*Sporobolus maritimus*).

The study area of this research is Ria Formosa lagoon, experiencing high concentrations of pollutants including macro and microplastic debris due to the high urbanization and population density. Furthermore, in this coastal lagoon all the targeted species are concurrently present. These ecosystems are vital to coastal environments and contribute to human well-being through the provision of critical ecosystem services. Thus, as semi-enclosed system housing an invaluable biodiversity but suffering at the same time from a high anthropogenic pressure, the region represents a suitable case study to test whether marine coastal bioengineers act as sink for marine plastic debris. The thesis is organized in two papers addressing different objectives in the framework of the general aim. *Paper 1* (chapter 2) assesses the role of coastal vegetated ecosystems on macro- and microplastic trapping, and *Paper 2* (chapter 3) assesses the ingestion of microplastics by infauna species. The specific objectives for each paper are:

Paper 1 (chapter 2)

(a) Quantify the occurrence, typology and abundance of plastic debris in habitats formed by coastal vegetated bioengineers. To do so, we had a multispecies approach focusing on intertidal seagrasses

(*Zostera noltei*), subtidal seagrasses (*Cymodocea nodosa* and *Zostera marina*), rhizophytic macroalgae (*Caulerpa prolifera*) and intertidal saltmarsh (*Sporobolus maritimus*). We hypothesized that marine bioengineers accumulate more macroplastics in their canopy and microplastics in the superficial sediment than adjacent bare sediment areas. The expected result was to find more plastic particles in the ecosystem engineers because of their trapping capacity than in the sided bare sediment.

(b) Assess whether intertidal and subtidal bioengineers trap different amounts and typology of macro and microplastics. To do so, I compared intertidal species *Z. noltei* and *S. maritimus* with subtidal species *C. nodosa*/*Z. marina* and *C. prolifera*. I hypothesised that the distributional pattern of plastic type differs intertidal and subtidal bioengineers according to the way their structure formation modifies the local environmental conditions. However, changes in plastics trapping among inter- and subtidal habitats also depend on debris type (floatability) and local environmental conditions (i.e. hydrodynamic). Overall, I expected to find more plastic in subtidal species due to their longer exposition to the particles present in the water column.

(c) Quantify the occurrence, typology and abundance of microplastics adhered on the bioengineers' canopies. To achieve this objective, I analysed the leaf blades of five species: *Z. noltei, Z. marina, C. nodosa, C. prolifera and S. maritimus.* I hypothesised that part of the microplastics trapped into the bioengineers would not reach the sediment surface but could potentially stick on seagrass leaves. The expectation was to find a higher number of MPs on the leaves surface of subtidal species due to their longer exposition to the particles present in the water column.

Paper 2 (chapter 3):

(a) Assess the occurrence and abundance of microplastic ingested by three profitable bivalve species commonly harvested in the Ria Formosa lagoon. Specifically, I will focus on the clams *Ruditapes decussatus* and *Polititapes* spp. inhabiting seagrass meadows (*Zostera noltei*) of the Ria Formosa and on cockle *Cerastoderma sp.* because they are edible species and economically important for the region. My hypothesis was that bivalves living in the Ria Formosa lagoon are exposed to high concentration of microplastics in the water column, hence they are prone to ingest plastic particles.

1.2 TARGET SPECIES

1.2.1 Ecosystem engineer species

This thesis focuses on five species of coastal autogenic bioengineers that can potentially act as plastic sinks: intertidal saltmarsh (*Sporobolus maritimus*, SM), intertidal seagrass (*Zostera noltei*, ZN), subtidal seagrasses (*Cymodocea nodosa*, CN; *Zostera marina*, ZM) and rhizophytic macroalgae (*Caulerpa prolifera*, CP). All these species occur within the Ria Formosa lagoon, occupying different areas and displaying different canopy properties. The stiff leaves of *S. maritimus* dissipate the hydrodynamic forces of the marine environment more successfully than the flexible leaves of *Z. noltei* (Bouma et al. 2005), yet both species exhibit poor epiphyte communities (Lebreton et al., 2009; Schanz et al., 2002). *Zostera marina* and *C. nodosa* are subtidal species which form commonly mixed meadows in the Ria Formosa lagoon (Billingham et al., 2003) and present long and flexible leaves commonly covered with epiphytes (Borowitzka et al., 2006). *Caulerpa prolifera* is a rhizophytic macroalgae presenting a siphonous thallus structure and forming short but dense meadows over mud and fine sand (Verlaque and Fritayre, 1994). This species is a strong competitor of seagrasses due to its faster growth, lower light compensation point and ability to establish from small fragments (Smith and Walters, 1999), and it is presently spreading fast in the Ria Formosa lagoon (ALGAE research group, personal communication).

My general expectation was that structural properties of the bioengineers, such as stiffness, canopy height, meadows density and leaves morphology would represent key elements in defining their capability in plastic debris trapping.

1.2.2 Ecosystem engineer's infauna species

This thesis focuses on three important commercially exploited bivalve species inhabiting the Ria Formosa lagoon: the clams *Ruditapes decussatus* and *Polititapes* spp. and the cockle *Cerastoderma* spp.

Polititapes spp. and *Ruditapes decussatus* belong to the family *Veneridae*. Their geographical distribution ranges from Southern and Western England to the Iberian Peninsula and Mediterranean Sea (Bourne 1982; Hamza-Chaffai et al., 2003) including South to western Morocco, Senegal and West Africa (Poppe & Goto, 1991). Both genera, *Ruditapes* decussatus and *Polititapes* sp., commonly inhabit intertidal and subtidal sheltered shallow areas where they live buried in sand, muddy gravel or clay bottoms; *R. decussatus* have been also found in leaf litter beds (Como et al., 2008).

The cockles *Cerastoderma* spp. belong to the family *Cardiidi*. These species are widely distributed in the Eastern Atlantic, Mediterranean and Black sea from Portugal to Egypt, in Norway, Russia and Senegal. They are commonly found in shallow intertidal and subtidal coastal areas and estuaries (Desroy et al., 2002; Obolewski and Piesik 2005) in association with *Z. noltei* and *C. nodosa* meadows as well as in sandy and muddy flats (Brun et al., 2009) with some input of fresh water.

All of the above targeted species are the most profitable molluscs in coastal areas; hence, if they accumulate plastic particle, the microplastics might end up in the human food chain potentially posing serious risks for human health.

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CHAPTER 2. Service or disservice? The role of marine vegetated coastal bioengineers in plastic debris trapping

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2.1 ABSTRACT

Plastic pollution is jeopardizing biodiversity worldwide. Of major concern are coastal vegetation habitats such as seagrass meadows, macroalgae forests, and saltmarshes as these provide several ecosystem services that contribute to human well-being. Here, we assess the potential role of coastal vegetation in plastic debris trapping and potentially, in turning a well-valued water purification ecosystem service into an ecosystem disservice of plastic accumulation. We targeted three seagrass species (*Zostera noltei*, *Zostera marina*, *Cymodocea nodosa*), a rhizophytic algae (*Caulerpa prolifera*), and a saltmarsh species (*Sporobolus maritimus*) inhabiting intertidal and subtidal habitats from Ria Formosa, an anthropized coastal lagoon. We found no significant differences in microplastic (MP) abundance or type between any of the bioengineer and its side bare sediments as well as between intertidal and subtidal habitats. Similar patterns were observed for macroplastics abundance, type and mass except for *S. maritimus* that had significantly more macroplastic than its intertidal bare sediment. Moreover, *S. maritimus* and *C. prolifera* showed the highest macroplastic concentration by number but the lowest by mass whereas *C. nodosa* and *Z. noltei* had the lowest macroplastic number yet the highest mass. However, differences in macroplastic mass were also not significant. Overall, the most abundant microplastic type and colour was fibre (86.5%) and blue (45%) while 61.4% of macroplastics found were fragments. Subtidal bioengineers trapped significantly more MPs than intertidal species on their leaves. The highest microplastic concentration was found on *C. prolifera* and *Z. noltei* followed by *C. nodosa* and *Z. marina* while no MPs were detected on *S. maritimus* leaves. MPs detected were all fibres and the most common colour was blue (36%). Overall, these results reveal bioengineers acting as plastic sinks, however the high variability observed emphasize

the need for further research to infer the key biotic and abiotic factors triggering the patterns of plastic deposition within these ecosystems.

Keywords: Coastal environments, ecosystem engineers, marine litter, plastic pollution, Ria Formosa.

2.2 INTRODUCTION

Coastal vegetation such as seagrass meadows, macroalgae forests, and saltmarshes constitute complex habitats that positively influence the biological, chemical and physical properties of coastal environments acting as ecosystem engineers (Jones et al., 1997). By doing so, these habitats provide a number of services to marine systems and human populations, contributing to human well-being (Barbier et al., 2011; Cullen-Unsworth et al., 2014). Seagrasses are among the most productive ecosystems on the planet (Duarte and Cebrián, 1996) as well as hotspots for carbon sequestration (Santos et al., 2019). In addition, seagrasses, together with saltmarshes, protect coastlines from erosion by enhancing wave attenuation and promoting sediment stabilization through their structural components (Gedan et al., 2011; Duarte et al., 2013). These ecosystems provide also sheltered habitats, nursery and feeding grounds for a wide variety of economically important finfish and shellfish species (Jackson et al., 2001). Furthermore, the engineering properties of coastal vegetation have a central role in water purification through nutrients' uptake (Short and Short, 1984; Moore, 2004). The physical structure of the bioengineers (above- and below-ground architecture), together with the associated infauna assemblages, increase the sedimentation of suspended particles and decrease their resuspension, improving water quality and reducing turbidity (Gacia and Duarte, 2001; Short et al., 2007; Van der Heide et al., 2007; Hendriks et al., 2010). The crucial role of coastal vegetation as provider of key ecosystem services for humans requires management actions against factors threatening these ecosystems, including marine plastic pollution. Indeed, seagrass beds, as well as rhizophytic algae, enhance particles deposition suggesting a potential sink for plastic particles too. The plastic trapped may then be physically detrimental both to the bioengineer itself and to the associated fauna by limiting gas exchange, and also chemically harmful by leaching toxic chemicals absorbed by or industrially added to plastic items (Cole et al., 2011). Here, we explore the role of coastal vegetation in trapping macro- and microplastics and, potentially, in turning what is considered as ecosystem service (water purification) into an ecosystem disservice (plastic accumulation).

Plastic has become ubiquitous in ecosystems worldwide. Due to its resistance to degradation, along with its extensive use and challenging disposal, plastic is accumulating in the environment (Sivan, 2011) from the Arctic (Obbard et al., 2014) to the sub Antarctic (Eriksson et al., 2013) including remote and supposedly pristine areas. Plastic debris has been reported on beaches (Fok and Cheung, 2015), deep ocean (Van Cauwenberghe et al., 2013), lakes (Faure et al., 2013), rivers (Williams and Simmons, 1996; Moore et al., 2011), and estuaries (Morritt et al., 2014; Sadri and Thompson, 2014). Worldwide, plastic pollution has a negative impact on the marine ecosystem services with a medium to high degree of irreversibility (Beaumont et al., 2019). According to the 2011 ecosystem services values, the ecological, social and economic costs deriving from marine plastic pollution corresponded to an annual loss of $500-$2500 billion in the value of benefits derived from the communal marine natural heritage (Beaumont et al., 2019).

Despite the exponential number of studies about plastic pollution in marine ecosystems, only a few very recent works tested the capacity of coastal ecosystem engineers in plastic debris trapping. Indeed, mangrove forests are acting as sink for marine litter with forest density driving debris capture abundance (Garcés-Ordóñez et al., 2019; Martin et al., 2019; Riascos et al., 2019). However, only marginal attention has yet been paid to the fate of macro- and microplastics stranded in intertidal and subtidal habitats colonized by marine vegetation such as seagrasses, saltmarshes, and rhizophytic macroalgae. The presence of microplastics (MPs) have been recently reported on algae (Yokota et al., 2017) and on the leaf blades of the seagrass *Thalassia testudinum,* with 75% of samples contaminated by fibres (Goss et al., 2018). This process could be promoted by the excretion of sticky exopolymers by the epiphytes leaving on seagrass leaves (Gacia et al., 2003) thus, it is expected that vegetated bioengineers with higher epiphyte cover are more prone to act as adhesion surface to plastic particles. The adherence of microplastics to the marine vegetation may represent a new process favouring MPs deposition in the ecosystems they form and, in turn, a potential threat to herbivores (Gutow et al., 2015).

In this study, we investigated the role of marine vegetated coastal bioengineers living on tidal and subtidal flats of a coastal lagoon as sink for marine litter. Five species were targeted: intertidal saltmarsh (*Sporobolus maritimus*), intertidal seagrass (*Zostera noltei*), subtidal seagrasses (*Cymodocea nodosa* and *Zostera marina*), and subtidal rhizophytic macroalgae (*Caulerpa prolifera*). These intertidal bioengineers display different canopy properties with *S. maritimus* having stiffer leaves that dissipate hydrodynamic forces in the marine environment more successfully than *Z. noltei* which has flexible leaves (Bouma et al. 2005). Subtidal bioengineers used in this study also have

different structural properties: seagrasses *Z. marina* and *C. nodosa* have long flexible leaves, whereas the rhizophytic subtidal macroalgae *C. prolifera* is characterised by siphonous thalli and form dense but short meadows. The specific objectives were to assess whether (i) marine vegetated coastal bioengineers accumulated more and different types of macro and microplastic debris than nearby bare sediment areas; (ii) intertidal and subtidal bioengineers trapped different amounts and typology of macro and microplastics, and (iii) subtidal and intertidal species displayed different typology and abundance of microplastics adhering to their canopies.

2.3 MATERIAL AND METHODS

2.3.1 Study site and characterization

Meadows of intertidal seagrasses (*Zostera noltei*, ZN), intertidal saltmarshes (*Sporobolus maritimus*, SM), subtidal algae bed (*C. prolifera*, CP), and subtidal seagrasses (*Cymodocea nodosa* and *Zostera marina*, CN), and bare sediment adjacent to the intertidal (BSI) and subtidal (BSS) habitats, were sampled in November 2018 and March 2019 in Ria Formosa, a sheltered, large mesotidal lagoon located in Algarve, southern Portugal (Figure 2.1). Ria Formosa system is characterized by a complex network of channels and tidal sand flats dominated by coastal vegetation and it comprises two peninsulas and five sand barrier islands that protect the lagoon from the Atlantic Ocean, to which the lagoon is connected through six inlets. The lagoon extends for 55 km in length and 6 km at its widest, with a mean depth of 3 m and a tidal range of 1.5-3.5 m. Water circulation is mostly driven by tides, but wind and bottom morphology can also affect the transport of nutrients, sediments and contaminants (Canu et al., 2003; Carafa et al., 2006; Roselli et al., 2013).

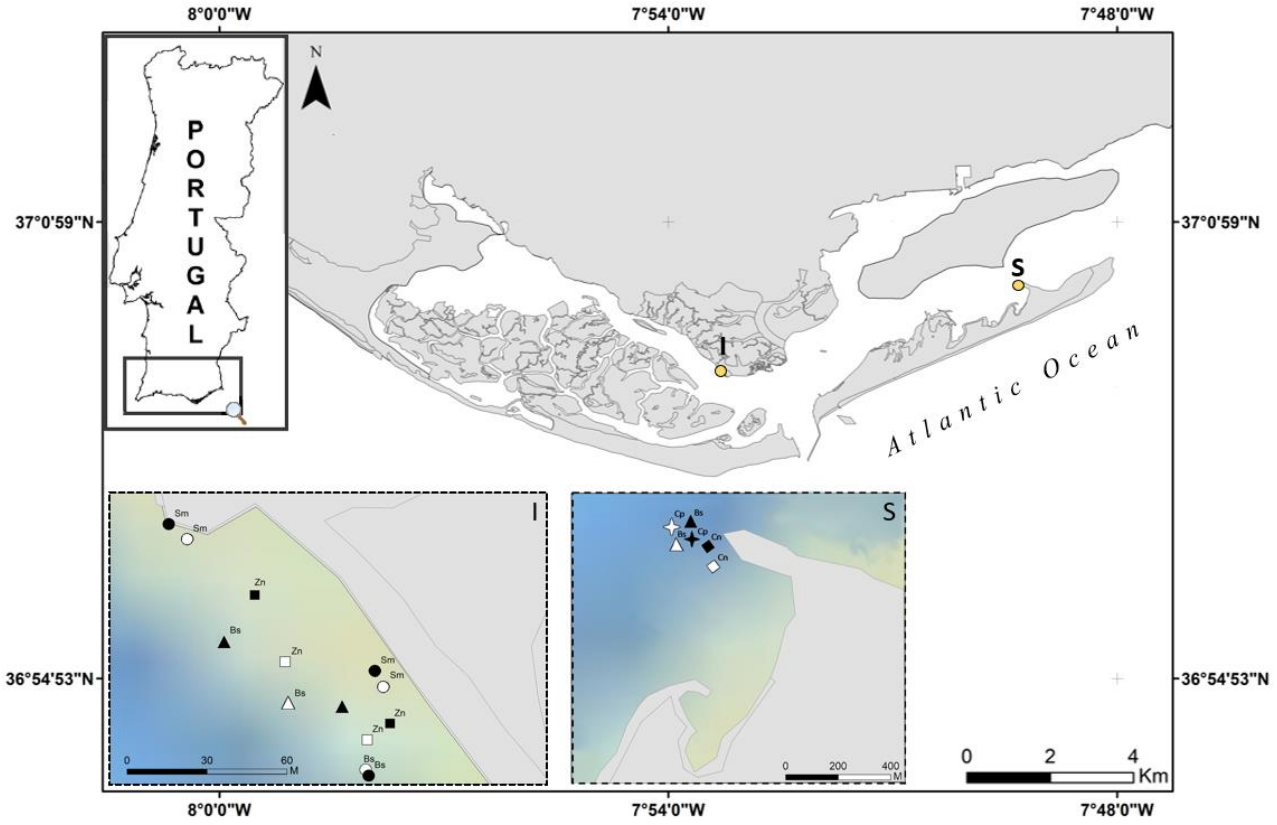

Figure 2.1. Location of the Ria Formosa lagoon, and intertidal (I) and subtidal (S) sampling areas. Habitats code: *Zostera noltei* (ZN); *Sporobolus maritimus* (SM); *Cymodocea nodosa/Z. marina* (CN); *Caulerpa prolifera* (CP); *Bare sediments* (BS). Symbols represent habitats and colours represent macroplastics (black) microplastics (white). In the subtidal area plots were close to each other thus were displayed only once to avoid overlapping.

ZN and SM were sampled at an intertidal mud flat in the Ria Formosa lagoon (Figure 2.1). The area is subjected to the water flow of the Faro-channel and exposed to the Faro-Olhão inlet, overall responsible for 60% of the total tidal prism in the lagoon. Due to its position, the area result in a potential accumulation zone for various debris including macro and microplastics, hence it is particularly suitable for testing the trapping abilities of the intertidal bioengineer species. CN and CP were sampled at Culatra Island (Figure 2.1), an area subjected to the water flow of the Olhão-channel and influenced by both the Armona-inlet and Faro-Olhão inlet. Furthermore, this location represents an important fishing ground for the local community, hence it is potentially exposed to various debris including discarded fishing gears such as nets, ropes and traps.

Biomass samples (n = 3 in each plot, two plots per habitat) were collected for characterization of the vegetation in terms of above-ground biomass (g d.w. m^{-2}), shoots density (shoots m^{-2}), and

canopy height (cm). Biomass samples of *Z. noltei* and *C. prolifera* were collected using a 12-cm diameter core (0.0113 m^2), rinsed of sediments and transported to the laboratory in cool dark conditions. Total number of shoots in each sample was counted and above-ground biomass (AG, leaves) was separated from below-ground biomass (rhizomes and roots) and weighted after oven-dried (60 ºC, 48 h). Shoots of *S. maritimus* and subtidal seagrasses (*C. nodosa* and *Z. marina*) were counted *in situ* using quadrats (30x30 cm and 25x25 cm, respectively). Five shoots were cut and transported to the laboratory and they were oven-dried (60 ºC, 48 h) to calculate the specific shoot biomass (g d.w. $shoot^{-1}$) which, along with shoot density, was used to estimate the AG biomass for these species. Canopy height (cm) was estimated based on the length of five shoots/leaves for each habitat.

2.3.2 Microplastic assessment in the sediment

Superficial sediments samples were collected in each habitat and in their adjacent bare sediment during low tide (intertidal samples) or by scuba diving (subtidal samples). For each bioengineer, the sampling area was divided into two plots in which replicated quadrats (n = 5; 0.5x0.5 m) were randomly selected. In each quadrat, two sediment samples were collected using a polypropylene (PP) plastic box corer (14 cm diameter) gently buried in the first 2-3 cm of sediment surface then gathered with the help of a clean metal shovel. Sediment samples were transported to the laboratory where the wet weight (g w.w.) was recorded using a microbalance (± 0.001 g). Samples were oven-dried at 60 °C and reweighted (g d.w.), then stored for further analysis.

Microplastics extraction from sediment was based on the density separation principle (Hidalgo-Ruz et al. 2012), using a hypersaline solution prepared with ultrapure water (350 g NaCl L^{-1}). Before extraction, the solution was filtered through a GF/C Whatman glass fibre filter (47 mm of diameter and 1.2 µm pore size). A subsample of 200 ± 50 g d.w. of sediment was placed in a glass jar (3.3 L) containing 2 L of the hypersaline solution and mixed for 3 min using a metal spoon. The whole jar was shaken vigorously for 2 min, then the sediment was allowed to settle down for 18/24 h. After sedimentation, *ca*. 250 ml of the overlying water was collected using a glass pipette and filtered over a GF/C Whatman filter (47 mm of diameter and 1.2 µm of pore size) using a vacuum system. To avoid clogging, several filters were used when necessary. Mixing and filtering was repeated twice to allow the flotation of denser polymers and/or of plastic particles potentially trapped by organic matter (Hidalgo-Ruz and Thiel, 2012; Claessens et al., 2013; Mathalon, 2014).

Filters were placed in pre-labelled glass petri dishes and dried in oven at 40 °C for 24 h, followed by visual examination under a stereomicroscope (Leica S8 APO). Each identified microplastic item was counted and photographed. The particles were categorized according to their shape as fragment, fibre, film, foam, granules and microbeads (Gündoğdu and Çevik, 2017) and based on their colour (blue, white transparent, white opaque, black, brown, violet, green, yellow and red).

Several measures were adopted while handling and processing the microplastic samples to minimise contamination with airborne fibres. During the entire analysis, a 100% cotton lab coat and latex gloves were worn. In addition, glassware and other materials were always rinsed three times with saturated ultrapure water (purified by an Elix® equipment) before use and covered with clean aluminium foil. The hypersaline solution of sodium chloride was entirely filtered through a GF/C Whatman glass fibre filter to prevent potential microplastics contamination by salt. The salt used during this investigation was lab gradient salt (PanReac AppliChem) reporting a maximum limit of impurities of 0.005%. Procedural blanks with the pre-filtered NaCl solution were performed in parallel to each extraction batch, resulting in one control for each habitat plot.

2.3.3 Microplastics assessment in vegetation canopy

Replicated samples (n = 12) of canopy structures (fronds for seaweed *C. prolifera*, shoots for seagrass and saltmarsh species) were taken in the same area where sediment samples were collected. The structures were carefully cut at their base and transferred to a plastic zip-lock bag. Seagrass and saltmarsh shoots were carefully separated in old and young leaves according to their position within the shoots (outer leaves being the oldest one). The foliar structures were observed under the stereomicroscope (Leica S8 APO) and any identified potential microplastic particle was counted and transferred into a filter for further analysis. Particles were classified according to their colour and shape as previously explained. Abundance of microplastics was expressed as number of items per unit of foliar structure area (items cm^{-2}), after measuring the leaf or frond area using image analysis (ImageJ; Schneider et al., 2012).

2.3.4 Macroplastics assessment

Assessment of macroplastics was conducted in the same areas and plots where sediment samples were collected. Three quadrats (5x5 m, 25 m^2, intertidal habitats during low tide) or underwater transects (6x4 m, 24 m^2, subtidal habitats by scuba diving) were haphazardly selected and carefully

checked to ensure that all the visible macroplastics were taken. Collected macroplastics were transported to the laboratory where they were washed, counted, measured in their maximum length (± 0.1 cm), and weighted using an analytical balance (± 0.001 g) or a scale balance for large items (± 0.01 g). The macroplastics were then classified according to their shape (fibre, fragment, foam, film), colour (blue, white transparent, white opaque, black, brown, grey, green, yellow and red), and functional origin (e.g. beverage bottles, cups, shopping bags; Lippiatt et al., 2013).

2.3.5 Data analysis

A series of univariate and multivariate permutational analysis of variance (PERMANOVA) were used to test our hypothesis. For all datasets, a Bray–Curtis dissimilarity matrix was used on square root transformed univariate and multivariate measures. All PERMANOVAs in this study were run for unrestricted permutation of raw data and, because power and precision of the tests increase with increases in the number of permutations (Hope, 1968), all tests were run with 9999 permutations. Monte Carlo P-value was preferred over permutation P-value when only very few unique permutations were possible (Anderson, 2005). For the multivariate testing, a separate test for homogeneity of dispersions (PERMDISP), was run to identify differences in dispersion when the PERMANOVA p-value resulted significant (Anderson, 2004). This test helped to uncover the nature of the differences among habitats or plots detected by PERMANOVA. Finally, to spatially visualize the datasets and the distances between samples, a non-metric multidimensional scaling analysis (nMDS) based on the Bray–Curtis similarity resemblance matrix was performed. PERMANOVA, PERMDISP and nMDS analysis were all performed using PRIMER 6.1.15 & PERMANOVA+ 1.0.5 software (PRIMER-E Ltd 2012).

To assess whether marine vegetated coastal bioengineers accumulated more and different types of micro and macroplastic debris than nearby bare sediment patches, each bioengineer was compared with its corresponding adjacent bare sediment. A univariate analysis was used to test for differences in the overall microplastic abundance (n MPs g^{-1} d.w. sediment) while a multivariate analysis was used to test for differences in the abundance of shape classes (n fibre, fragment, foam or film g^{-1} d.w. sediment). A series of separate two-way PERMANOVA was designed with presence/absence of each bioengineer as fixed factor (levels: ZN, SM, CN or CP and BSS or BSI), plot (levels: 1, 2) as the random factor nested in habitat, and either total abundance (univariate) or type of microplastics (multivariate) as the dependent variable(s).

For macroplastics, a similar approach was used. In addition to abundance (n Macro m^{-2}; items fibre, fragment, foam or film m^{-2}), datasets with macroplastics expressed as mass (g Macro m^{-2}; g fibre, fragment, foam or film m^{-2}) were also tested, totalling to two univariate and two multivariate analyses for each bioengineer.

To assess whether intertidal and subtidal bioengineers trapped different amounts and typology of micro and macroplastics**,** the adjacent bare sediment areas were excluded from the analyses. A univariate analysis was used to test for differences in the overall abundance (n MPs g^{-1} of sediment) of microplastics between intertidal and subtidal habitats, while a multivariate analysis was used to test for differences in the type of microplastics found (n fibre, fragment, foam, film g^{-1}). A two-way PERMANOVA designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN and SM for intertidal; CN and CP for subtidal) as the random factor nested in habitat and either total abundance (univariate) or type of microplastics (multivariate) as the dependent variable(s). For the macroplastics, a similar approach was used, but, in addition to abundance, datasets with macroplastics expressed as mass (g) were also tested. Differences in macroplastics abundance (n) and mass (g) among the different bioengineers and plots were tested running two univariate (n Macro m^{-2}; g Macro m^{-2}) and two multivariate analysis (n fragment m^{-2}, n film m^{-2}; g fragment m^{-2}, g film m^{-2}).

To assess whether subtidal and intertidal species displayed different typology and abundance of microplastics adhering to their canopies, differences between habitats were tested using univariate analysis on MPs abundance (n MPs cm^{-2} leaf). Two-way PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor and species (ZN, SM, CN, ZM and CP) as the random factor nested in habitat and MPs abundance on leaves as the dependent factor.

2.4 RESULTS

2.4.1 Habitat characterisation

ZN meadows showed an above ground (AG) biomass equal to 185.8 ± 43.6 g d.w. m^{-2} (mean ± SD) and a density of 13189 ± 2475 shoots m^{-2}, whereas the mean canopy height was 24.5 ± 7.9 cm. The meadows of SM had a higher AG biomass (221.6 ± 87 g d.w. m^{-2}) and a lower density (1344 ± 408 shoots m^{-2}) than ZN, whereas the mean canopy height was equal to 24.3 ± 2.6 cm. The subtidal mixed meadows of CN and ZM showed an AG biomass of 114.1 ± 57.3 g d.w. m^{-2} with percentages of

occurrence of 55.5% CN and 43.4% ZM, based on mean biomasses. The average meadow density was 312 ± 132 shoots m^{-2} with 69.2% being CN and 30.8% ZM, based on percentages of shoots density; the mean canopy height of the mixed meadow was 40.6 ± 9.4 cm. The meadows of CP showed the lowest values of AG biomass (85.7 ± 22.8 g d.w. m^{-2}) and a density of 2608 ± 486 fronds m^{-2}, whereas the mean canopy height ranged was equal to 9.17 ± 1.3 cm (Table 2.1).

Table 2.1. Meadow properties of the bioengineers included in the study. Values are given as mean ± standard deviation (n = 6).

Bioengineer	**Code**	**Above-ground biomass (g d.w. m^{-2})**	**Shoot or frond density (shoots or frond m^{-2})**	**Canopy height (cm)**
Zostera noltei	ZN	185.8 ± 43.6	13189 ± 2475	24.5 ± 7.9
Sporobolus maritimus	SM	221.6 ± 87.1	1344 ± 408	24.3 ± 2.6
*Cymodocea nodosa**	CN	114.1 ± 57.3	312 ± 132	40.6 ± 9.4
Caulerpa prolifera	CP	85.7 ± 22.84	2608 ± 486	9.17 ± 1.3

* *mixed meadow of* C. nodosa *and* Z. marina.

2.4.2 Micro and macroplastic debris in vegetated and bare sediment areas

2.4.2a Microplastics assessment

Microplastics were present in the superficial sediment at all habitats. Of the total 385 microplastic items recorded, 225 items (58%) were found in subtidal habitats and 160 items (42%) in the intertidal ones. Each vegetated bioengineer was compared with its side bare sediment (Figure S2.1). The intertidal species ZN (0.019 ± 0.017 n MPs g^{-1}; Table 2.2) and SM (0.024 ± 0.019 n MPs g^{-1}) resulted the least contaminated and showed no significant differences in MPs abundance compared to their side intertidal bare sediment (0.030 ± 0.015 n MPs g^{-1}; Table S2.1 for ZN and Table S2.2 for SM). Subtidal species CN and CP (0.035 ± 0.027 n MPs g^{-1} and 0.034 ± 0.025 n MPs g^{-1}) were the most contaminated bioengineers and showed no significant differences in MPs abundance (n MPs g^{-1}) when compared with their side subtidal bare sediments (0.022 ± 0.014 n MPs g^{-1}; Table S2.3 for CN and Table S2.4 for CP). However, plots were significantly different within subtidal bioengineers CN (0.022 ± 0.019 n MPs g^{-1} in P1; 0.049 ± 0.028 n MPs g^{-1} in P2; P(perm) = 0.0089) and *CP* (0.027 ± 0.015 n MPs g^{-1} in P1; 0.041 ± 0.032 n MPs g^{-1} in P2; P(perm) = 0.0064). The series of multivariate analyses using microplastic type (fibre, fragment, foam and film; Table S2.5 for ZN, Table S2.6 for SM, Table S2.7 for CN, Table S2.8 for CP) did not detect any significant differences between each

bioengineer and its side bare sediments but revealed slightly significant variability among plots in ZN, CN and CP (P(perm) = 0.039; P(perm) = 0.04; P(perm) = 0.046). The distribution of the samples was displayed by non-metric multidimensional scaling analysis and was coherent with the results of the PERMANOVA (Figure S2.2). Overall, the most abundant microplastic shape was fibre (86.5%), followed by fragment (8.8%) and film (3.9%). Only 1 microbead (0.3%) and 2 foams (0.5%) were found. Regardless of the microplastic shape (Figure 2.2), the most common colours were blue (173 items; 45%) and transparent (95 items; 25%), while the least represented colours were yellow (4 items; 1%) and red (3 items; 1%; Figure 2.3).

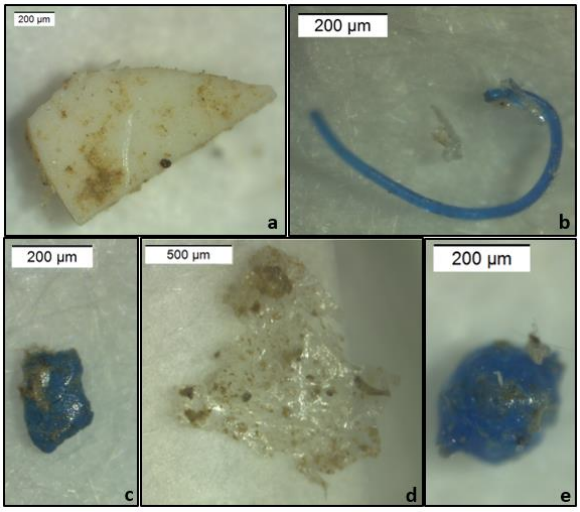

Figure 2.2. Examples of the microplastic types identified: Fragment (a) fibre (b) foam (c) film (d) microbead (e).

Table 2.2. Total number of microplastic items (n MPs g^{-1}; ± SD) found in intertidal and subtidal bioengineers and side bare sediment, and the average abundance (n g^{-1}; ± SD) for each microplastic types (fibres, fragments, foams, films and microbeads).

Bioengineer	**Code**	**Total MPs (n MPs g^{-1})**	**Fibres (n g^{-1})**	**Fragments (n g^{-1})**	**Foams (n g^{-1})**	**Films (n g^{-1})**	**Microbeads (n g^{-1})**
Intertidal bare sediment	BSI	0.0298 ± 0.0149	0.0254 ± 0.0101	0.0009 ± 0.0020	0.0005 ± 0.0016	0.0030 ± 0.0065	0
Zostera noltei	ZN	0.0192 ± 0.0172	0.0157 ± 0.0156	0.0010 ±0.0021	0	0.0020 ± 0.0025	0.0005 ± 0.0015
Sporobolus maritimus	SM	0.0235 ± 0.0194	0.0226 ± 0.0194	0.0009 ± 0.0019	0	0	0
Subtidal bare sediment	BSS	0.0220 ± 0.0140	0.0168 ± 0.0111	0.0044 ± 0.0051	0	0.0008 ± 0.0017	0
*Cymodocea nodosa**	CN	0.0352 ± 0.0267	0.0307 ± 0.0212	0.0033 ± 0.0078	0.0004 ± 0.0013	0.0008 ± 0.0017	0
Caulerpa prolifera	CP	0.0340 ± 0.0245	0.0304 ± 0.0225	0.0036 ± 0.0030	0	0	0

* mixed meadow of *C. nodosa* and *Z. marina*.

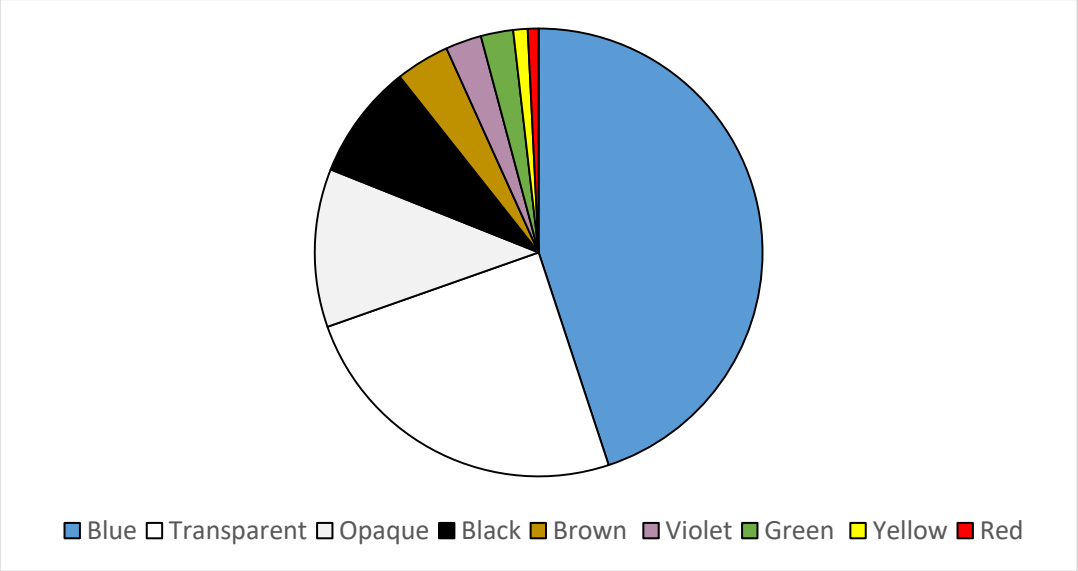

Figure 2.3. Overall percentage of occurrence of microplastics by colour. Values in percentages: blue (45%), transparent (25%), opaque (11%), black (8%), brown (4%), violet (3%) green (2%) yellow (1%) and red (1%).

2.4.2b Macroplastics assessment

Macroplastics were found in all the vegetated bioengineers but were absent on the adjacent bare sediments (Figure S2.3). The series of univariate analyses using data of macroplastics abundance (n Macro m^{-2}) showed significant differences only between SM (0.220 ± 0.157 macro-items m^{-2}) and its side subtidal bare sediment (P(MC) = 0.031; Table S2.10 for SM). A significant variability was identified among plots in the subtidal bioengineer CP showing an abundance of 0.097 ± 0.048 macro-items m^{-2} in P1 and 0 macro-items m^{-2} P2 (P(perm) = 0.031; Table S2.12 for CP).

The series of multivariate analyses using macroplastic types as the dependent factor (fragment and film) showed the same results (Table S2.13 for ZN, S2.14 for SM, S2.15 for CN, S2.16 for CP; Figure S2.4). However, the PERMDISP test using habitat (*S. maritimus*) as group factor was not significant (P(perm) = 0.051), highlighting no differences in dispersion of samples between bioengineer and its side (intertidal) bare sediment. In general, SM (0.220 ± 0.157 macro-items m^{-2}; Table 2.3) was the bioengineer that trapped more macroplastics followed by CP (0.048 ± 0.061 macro-items m^{-2}) and CN (0.013 ± 0.021 macro-items m^{-2}). ZN instead was the least contaminated bioengineer (0.013 ± 0.020 macro-items m^{-2}). Overall, 61.4% of macroplastic debris found were fragments and the remaining portion were films (38.6%).

A second datasets with macroplastics expressed as mass (g macroplastic m^{-2}) was tested to identify differences in macroplastics mass (g) among the different bioengineers and side bare sediment (Figure S2.5). CN had the highest macroplastic mass (16.431 ± 39.752 g macro-items m^{-2}) followed by ZN (6.326 ± 14.827 g macro-items m^{-2}). Although having the highest macroplastic abundance (n macro-items m^{-2}), CP and SM showed the lowest macroplastic mass (0.848 ± 1.056 g macro-items m^{-2} and 0.513 ± 0.428 g macro-items m^{-2}, respectively). Overall, no significant differences based on macroplastic mass were detected among each bioengineer and its side bare sediment (Table S2.17 for ZN, S2.18 for SM, S2.19 for CN, S2.20 for CP). However, significant variability among macroplastic mass in the plots of CP (1.695 ± 0.798 g macro-items m^{-2} in P1; 0 g macro-items m^{-2} in P2; P(perm) = 0.034; Table S2.20) was detected. The series of multivariate analyses using macroplastic type (g fragment m^{-2}; g film m^{-2}) did not reveal any significant difference between each bioengineer and its side bare sediment (Table S2.21 for ZN, S2.22 for SM, S2.23 for CN, S2.24 for CP; Figure S2.6).

Table 2.3. Total macroplastic abundance (n Macro m^{-2}; ± SD in intertidal and subtidal bioengineers and side bare sediment areas, and abundance for each macroplastic types (fragments n m^{-2} and films n m^{-2}; ± SD).

Bioengineer	**Code**	**Total macroplastics (n m^{-2})**	**Fragments (n m^{-2})**	**Films (n m^{-2})**
Intertidal bare sediment	BSI	0	0	0
Zostera noltei	ZN	0.0133 ± 0.0207	0.0133 ± 0.0207	0
Sporobolus maritimus	SM	0.2200 ± 0.1575	0.1133 ± 0.1086	0.1067 ± 0.0935
Subtidal bare sediment	BSS	0	0	0
*Cymodocea nodosa**	CN	0.0139 ± 0.0215	0.0139 ± 0.0215	0
Caulerpa prolifera	CP	0.0486 ± 0.0613	0.0417 ± 0.0527	0.0069 ± 0.0170

* *mixed meadow of* C. nodosa *and* Z. marina.

2.4.3 Micro and macroplastic debris in intertidal and subtidal bioengineers

2.4.3a Microplastics assessment

The univariate analysis testing for differences in overall MP abundance did not detect any significant difference between intertidal and subtidal vegetated habitats (Figure S2.7; Table S2.25; P(MC) =

0.127). No variability between intertidal (ZN = 0.019 ± 0.017 n MPs g^{-1}; SM = 0.024 ± 0.019 n MPs g^{-1}) and subtidal species (CN = 0.035 ± 0.027 n MPs g^{-1}; CP = 0.034 ± 0.025 n MPs g^{-1}; P(perm) = 0.874) was further detected. The multivariate analyses using microplastic type (fibre, fragment, foam and film) did not highlighted any significant differences between intertidal and subtidal habitats (Table S2.26; P(MC) = 0.077) neither between intertidal and subtidal species (P(perm) = 0.701). These outcomes were confirmed by the distribution of samples revealed by the nMDS (Figure S2.8).

2.4.3b Macroplastics assessment

An average of 0.116 ± 0.152 n macro-items m^{-2} were found on intertidal vegetated bioengineers and 0.0313 ± 0.0474 n macro-items m^{-2} in subtidal vegetated bioengineers (Figure S2.9). Univariate analysis testing for differences between habitats in the overall macroplastic abundance (n Macro m^{-2}) detected no significant differences (P(MC) = 0.6908; Table S2.27). Furthermore, no variability between intertidal and subtidal species in macroplastic abundance was observed (P(perm) = 0.1159). A similar pattern was observed with the multivariate analyses using macroplastic types as depended factors (fragment n m^{-2} and film n m^{-2}; P(MC) = 0.6652; Table S2.28; Figure S2.10).

Another univariate analysis was run with macroplastics expressed as mass (g macroplastic m^{-2}) to test potential differences in total macroplastics mass (g) among intertidal and subtidal vegetated bioengineers (Figure S2.11). Overall, intertidal vegetated bioengineers trapped 3.420 ± 10.451 g macro-items m^{-2} whereas subtidal vegetated bioengineers trapped 8.639 ± 28.018 g macro-items m^{-2}, yet no significant differences were observed (P(MC) = 0.7709; Table S2.29). Multivariate analyses using macroplastic type mass (fragment g m^{-2} and film g m^{-2}; Table S2.30; Figure S2.12) also did not detect any significant differences.

2.4.4 Microplastic adhered to the canopies of intertidal and subtidal bioengineers

Differences between trapping capacity of intertidal and subtidal bioengineers' leaves were tested using univariate analysis on MPs abundance (n MPs cm^{-2} leaf). An average of 0.0264 ± 0.0898 MPs cm^{-2} and 0.0290 ± 0.0589 MPs cm^{-2} were detected on the leaves of intertidal (ZN and SM) and subtidal bioengineers (CN, ZM and CP) respectively (Figure S2.13), being significantly different (P(MC) = 0.015). Although no significant variability was observed among species (P(perm) = 0.111; Table S2.31), the highest MPs abundance was found on the leaves of the subtidal CP (0.0559 ± 0.0936 MPs cm^{-2}; Table 2.4; Figure S2.14) and intertidal ZN (0.0529 ± 0.1238 MPs cm^{-2}) followed by the subtidal CN (0.0198 ± 0.0308 MPs cm^{-2}) and ZM (0.0114 ± 0.0113 MPs cm^{-2}). The lowest MPs

contamination was recorded in SM with 0 MPs cm^{-2}. Overall, the microplastics detected on the leaves were all fibres (Figure 2.4) and the most common colours were blue (36%) and black (36%) whereas the least represented colour was red (2%; Figure 2.5).

Table 2.4. Total microplastic abundance (n MPs cm^{-2}; ± SD) on the canopies of intertidal and subtidal bioengineers.

Bioengineer	**Code**	**Total microplastics (n cm^{-2})**
Zostera noltei	ZN	0.0529 ± 0.1238
Sporobolus maritimus	SM	0
Cymodocea nodosa	CN	0.0198 ± 0.0308
Zostera marina	ZM	0.0114 ± 0.0113
Caulerpa prolifera	CP	0.0559 ± 0.0936

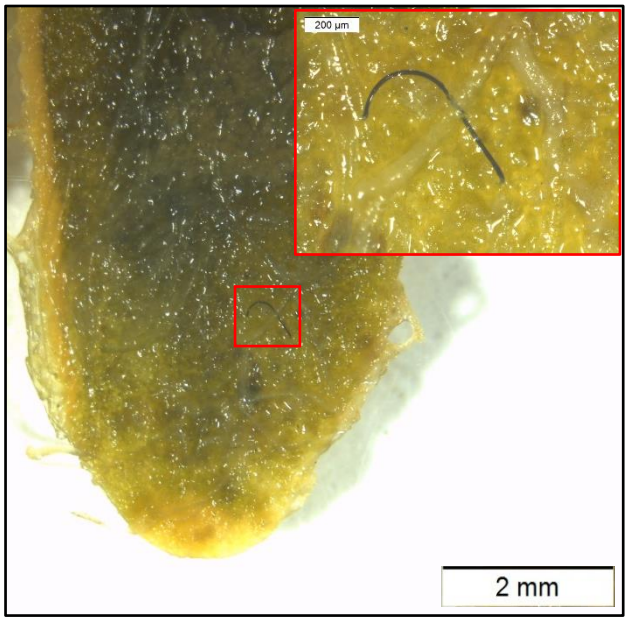

Figure 2.4. Microplastic fibre on a leaf of *Caulerpa prolifera*.

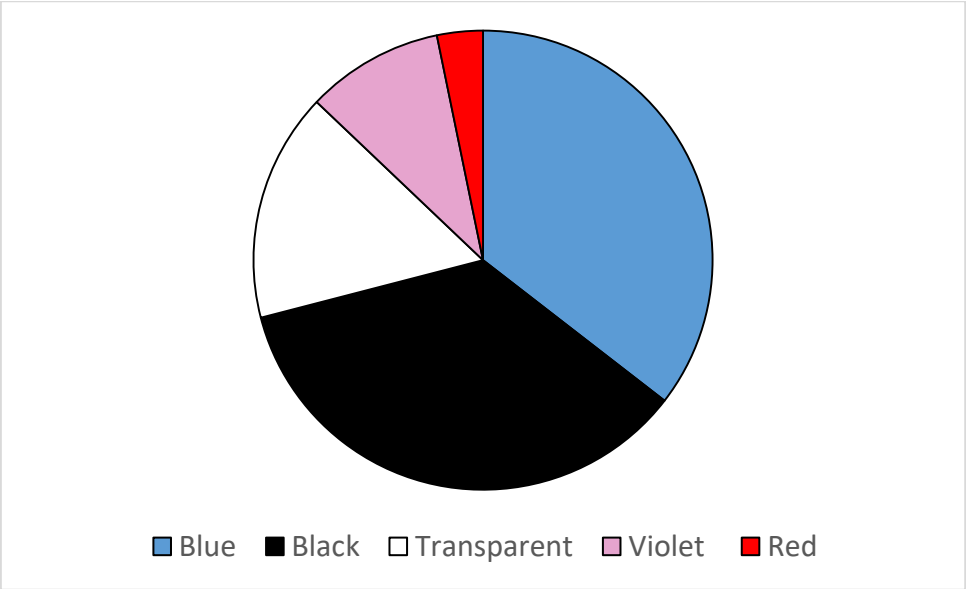

Figure 2.5. Percentage of occurrence of microplastics by colour found on intertidal and subtidal bioengineers' leaves (*Z. noltei*, *S. maritimus*, *C. nodosa*, *Z. marina*, *C. prolifera*). Values in percentages: blue (36%), black (36%), transparent (16%), violet (10%) and red (2%).

2.5 DISCUSSION

Here we investigated for the first time whether plastic, a global threat for marine ecosystems, accumulated in coastal vegetated marine habitats formed by seagrasses, salt marshes and macroalgae.

It was hypothesized that marine coastal bioengineers accumulate more macroplastics among canopy and microplastics in the superficial sediment than adjacent bare sediment. Our results, however, did not support this hypothesis, since microplastic abundance in the sediment of bioengineers did not differ from the abundance found in their adjacent bare sediments. However, despite the lack of differences, significant variability was detected in the MPs concentration between plots within subtidal bioengineers CN and CP. This variability highlights the relevance of meadow's location in influencing MPs accumulation within the same bioengineer species and in turn, the importance that physical factors such as depth, currents and bottom morphology might have in shaping MPs dispersal and deposition.

It was also hypothesized that intertidal and subtidal habitats differ for the amount and typology of macro- and microplastic debris they accumulate. Our results do not support this hypothesis as no significant differences were detected among the trapping capacity of bioengineers in intertidal and subtidal habitats. In general, the non-significant relationships detected in this thesis are possibly a consequence of the high variability in plastic depositional patterns within ecosystems engineers.

The MPs research in marine coastal vegetated ecosystems is still in its infancy, thus there is a limited number of studies to compare our findings with. In general, we found average MPs concentrations of 0.019 ± 0.017 n MPs g^{-1} and 0.023 ± 0.019 n MPs g^{-1} in intertidal species *Zostera noltei* and *Sporobolus maritimus*, respectively. Slightly higher concentrations were found in *Cymodocea nodosa* (0.035 ± 0.026 n MPs g^{-1}) and *Caulerpa prolifera* (0.0340 ± 0.0245 n MPs g^{-1}). The MPs concentration in *C. nodosa* was compared with this found in the Northern Adriatic Sea (0.170 items g^{-1} d.w.), yet this study included both macro and microplastics debris (Renzi et al., 2018). Similar studies in coastal mangrove forests reported MPs concentrations of 0.036 ± 0.023 items g^{-1} d.w. in Singapore (Nor et Obbard., 2014) that are consistence with the concentrations herein found in *C. prolifera*. However, the concentration reported in mangroves from other location such as the Persian Gulf of Iran (0.125 ± 0.025 items g-1 d.w.; Naji et al., 2017) or from the Colombian Caribbean (ranging between 0.031 and 2.86 items g^{-1}; Garcés-Ordóñez et al., 2019) appeared higher. The MPs concentrations we reported are also lower than those found in beach sediments (0.092 ± 0.037 items g-1 d.w.; Claessens et al., 2011). Overall, these comparisons together with the statistical results we observed, may support the fact that marine coastal vegetated bioengineers would not act as major microplastic sink.

The adherence of MPs to the leaves of seagrasses and algae has been recently suggested as a new potential process favouring microplastic deposition in the vegetated bioengineers (Yokota et al., 2017; Goss et al., 2018). Thus, we also investigated the MPs abundance on the canopies of the targeted species, reporting microplastic particles being present on the leaves of all bioengineers tested except for the saltmarsh species *S. maritimus*. This species inhabits the upper level of the intertidal zone and thus is rarely completely submerged. Furthermore, *S. maritimus* lack epiphytes and has thinner leaves that offer a lower area for microplastic adherence compared to seagrass species. Given that the adherence of microplastics might be promoted by the exopolymers that epiphytes excrete on leaves (Gacia et al., 2003), the absence of epiphytes on *S. maritimus* may be a major factor explaining the lack of MPs.

Indeed, the other intertidal species, *Z. noltei*, nearly exposed to the same environmental conditions yet exhibiting epiphytes on its shoots, showed the highest level of leaf contamination (Table 2.4) among the seagrasses tested. This is unexpected results given that the subtidal species *C. nodosa* and *Z. marina* apart from exhibiting rich epiphyte communities, are continuously exposed to the microplastics in the water column and have larger and longer shoots than *Z. noltei*. However, the surface area does not appear as a driving factor considering the high concentration of microplastic on the short fronds of *C. prolifera*.

Overall, subtidal species exhibited a higher concentration of MPs adhered to leaves than intertidal species. This might be explained by their longer exposition to the particles present in the water column and by specie-specific traits of the bioengineer. Specifically, these findings might be linked to meadow density. In general, the microplastics transported by the water flow, trapped in the bioengineer's meadow, in a higher shoots' density scenario, might be easily caught on the bioengineer' leaf surface. The particles eventually trapped on the leaves would not be able to reach the sediments resulting in smaller amount of MPs reaching the sediment. This hypothesis could also explain the lower concentration of MPs on the surface sediments of *Z. noltei* while higher MPs' concentration was found on its leaves. However, further research is needed to validate this conclusion.

In general, incrusted microplastics on algae and seagrass species can impact algal productivity directly reducing photosynthesis, growth and morphology (Yokota et al., 2017). Furthermore, grazers commonly prefer to feed on vegetation with higher densities of epibionts (Goss et al., 2018), which could also be the ones with higher plastic accumulated. Hence, even if these are generally assumed to be less vulnerable to microplastic impacts because not subjected to biomagnification dynamics, also herbivores would result expose to the MPs contamination (Gutow et al., 2015).

The MPs assessment in bioengineer species reported fibres as the most represented MPs shape. Specifically, 86.5% of MPs detected in the bioengineer's sediments were fibres whereas 100% of MPs found on leaves were fibres. These outcomes are in agreements with similar studies on sediments from coastal habitats such as beaches, estuaries and mangrove forests (Thompson et al., 2004; Claessens et al., 2011; Nor and Obbard., 2014; Naji et al., 2017) and with studies on seagrass leaves (Goss et al., 2018).

The higher abundance of synthetic fibres might be linked to two major sources in the Ria Formosa, the wastewater and domestic discharges and the fibrous pollution from fishing gears. In the western side of the lagoon are located four wastewater treatments sites (WWTPs) that although being very efficient in effluent depuration, may remain a major microplastics input in the aquatic environment (Murphy et al., 2016). Furthermore, the models of water and particles circulation in the Ria Formosa suggest long particles retention, up to 18 days, before the contaminants get washed out through the Armona and the Faro-Olhão inlets (Fabião et al., 2016). Such high retention times along with the route that the microparticles follow to get out of the lagoon, result in an extended contamination exposure for the ecosystem engineers living in the area. In addition to this major source, the fishing gears such as ropes, nets and traps commonly used by fishermen, is often lost or discarded in the lagoon eventually resulting in fibrous pollution due to their degradation (Browne et

al., 2011). Only lower concentrations of film (3.9%), foam (0.5%) and microbead (0.3%) were found during the microplastic analysis.

Although the microplastic colour did not represent a major driver of deposition within the bioengineers, it can still result a useful tool to identify the source and/or the nature of the polymers. Overall, the most common colour among the fibres detected in sediments within bioengineers and on bioengineer's canopy was blue, accounting for 45% and 36% respectively.

The results of the macroplastics assessment within the bioengineers suggest that these species might have a higher influence in macroplastic trapping than in microplastics, and that intertidal and subtidal vegetated bioengineers have similar macroplastic trapping capacities. Plastics of size > 5 mm were found in all the vegetated bioengineers but were absent on the adjacent bare sediments. Despite this evidence, significant differences were detected only between *S. maritimus* and its side intertidal bare sediment. The intertidal salt marsh species was the bioengineer that trapped the highest number of macro-items (0.220 ± 0.157 macro-items m^{-2}). Main factors driving to this result were identified in its specific location in the upper intertidal and in the morphology of its stiff shoots successfully dissipating the hydrodynamic forces of the marine environment and acting as a small barrier to the debris transported by the ocean or by the wind. However, the plastics trapped within the bioengineer canopy are likely to re-enter the aquatic environment, eventually re-suspended by the tide or blown by the wind. In alternative, the macro litter may be transported to the uppest layer of the intertidal zone where saltmarsh species including *Sarcocornia* spp., *Atriplex* spp. and *Suaeda* spp. have been observed to act as efficient barriers against the redistribution of litter in the marine environment by wind and wave action, resulting in a major accumulation zone. Similar dynamics have been observed in mangrove habitats eventually acting as marine litter traps (Martin et al., 2019; do Sul et al., 2014).

The other intertidal species, *Z. noltei,* did not show a great capacity in macroplastic debris trapping resulting the least contaminated bioengineer (Table 2.3). This finding was linked to its flexible and thin leaves which under strong current conditions tend to bend with the flow and appear not stiff enough to act as barrier for the macro debris. For what concern the subtidal species, the green algae *C. prolifera,* characterized by siphonous thalli forming meadows with short canopies, accumulated more macroplastics than its "neighbours" *C. nodosa* and *Z. marina*. The significant variability detected among *C. prolifera* plots (0.097 ± 0.048 macro-items m^{-2} in P1 and 0 macro-items m^{-2} P2) were likely related to physical factors of the marine environment such as currents, tides, depth and bottom morphology, influencing the pattern of plastic deposition and dispersion within the same bioengineer meadows. Overall, our findings are in agreements with studies on mangrove soils

reporting average macro-litter concentration ranging between 0.054 ± 0.013 and 0.003 ± 0.002 items m^{-2} close and far populated areas respectively (Garcés-Ordóñez et al., 2019).

The mass of the macroplastics detected was used to test differences among bioengineers and side bare sediment. Despite having the lowest macroplastic number, *Cymodocea nodosa* and *Z. noltei* showed 16.4 ± 39.7 g macro-items m^{-2} and 6.3 ± 14.8 g macro-items m^{-2} respectively. These masses were likely related to the presence of heavy fishing gears within their canopies, confirming in turn the plastic pollution linked to fishing activities in the lagoon. On the other hand, *C. prolifera* and *S. maritimus* showed lower macroplastic mass, despite the highest macroplastic abundance. However, no significant differences were detected among each bioengineer and its side bare sediment.

Overall, 61.4% of macroplastic debris found were fragments and the remaining portion were films, most of which originated as food and beverage packaging (i.e. cans, bottles, cups, bags) hence, highly related to the close urban centres of Faro and Olhão. A recent study by Balestri et al., (2017) reported biodegradable bags trapped within subtidal seagrasses promoting the spatial segregation of their clones and influencing species coexistence shifting intra and interspecific interactions from neutral to competitive and changing growth form. Thus, it is possible that the plastics trapped in seagrass and macroalgae meadows represent a physical impediment potentially affecting growth and photosynthesis, in addition to jeopardize the health of the associated fauna through ingestion or adsorption of plastic leaching chemicals (Cole et al., 2011).

2.6 CONCLUSION

Plastics deposition in the marine environment is dictated by highly variable (in time and space) interactions among environmental factors, community traits and plastic debris characteristics. The overall lack significant differences in plastic trapping among distinct habitats and between bare sediment and vegetated sites found in this study indicates that the effect of complex biotic and abiotic interactions overcomes that of bioengineers' species-specific traits. Our capacity to detect distributional patterns largely dependent on the scale at which we conduct our observations and on the characteristics of the observed dependent variable. Further research focusing on specific temporal scales (e.g., daily and seasonal) and taking into consideration distinct polymers (e.g., distinct densities) and sizes (within the broader micro and macro categories) is required to highlight potential distributional patterns of macro and microplastic debris within engineering organisms and the keys factors triggering such patterns.

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2.8 SUPPLEMENTARY MATERIAL

2.8.1 TABLES

Table S2.1. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by microplastic abundance (n MPs g^{-1}). PERMANOVA was designed with ZN and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	1141	1141	1.484	0.3336	3	0.3288
Plot	2	1537.8	768.88	1.1339	0.2578	9895	0.3488
Res	16	10850	678.11				
Total	19	13528					

Table S2.2. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by microplastic abundance (n MPs g^{-1}). PERMANOVA was designed with SM and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	1526.3	1526.3	1.9921	0.3438	3	0.232
Plot	2	1532.4	766.22	0.69413	0.7675	9879	0.611
Res	16	17662	1103.8				
Total	19	20720					

Table S2.3. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by microplastic abundance (n MPs g^{-1}). PERMANOVA was designed with CN and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	372.94	372.94	0.20343	0.6716	3	0.751
Plot	2	3666.5	1833.2	6.3875	0.0089	9951	0.004
Res	16	4592.1	287				
Total	19	8631.5					

Table S2.4. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by microplastic abundance (n MPs g^{-1}). PERMANOVA was designed with CP and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	610.94	610.94	0.46472	1	3	0.6026
Plot	2	2629.3	1314.6	5.9648	0.0064	9932	0.0079
Res	16	3526.4	220.4				
Total	19	6766.6					

Table S2.5. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by microplastic type (fibres, fragments, foams, films). PERMANOVA was designed with ZN and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of microplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	1973.4	1973.4	0.86765	1	3	0.5162
Plot	2	4548.9	2274.5	1.8375	0.022	9919	0.103
Res	16	19805	1237.8				
Total	19	26327					

Table S2.6. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by microplastic type (fibres, fragments, foams, films). PERMANOVA was designed with SM and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of microplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	1610.2	1610.2	1.0704	0.6672	3	0.43
Plot	2	3008.7	1504.3	1.1914	0.2812	9919	0.3111
Res	16	20202	1262.7				
Total	19	24821					

Table S2.7. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by microplastic type (fibres, fragments, foams, films). PERMANOVA was designed with CN and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of microplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	962.24	962.24	0.45431	0.6649	3	0.6569
Plot	2	4236	2128	3.0102	0.029	9944	0.0356
Res	16	11258	703.61				
Total	19	16456					

Table S2.8. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by microplastic type (fibres, fragments, foams, films). PERMANOVA was designed with CP and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of microplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	719.27	719.27	0.4561	1	3	0.6504
Plot	2	3154	1577	3.2264	0.031	9956	0.0362
Res	16	7820.5	488.78				
Total	19	11694					

Table S2.9. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic abundance (n Macro m^{-2}). PERMANOVA was designed with ZN and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5833.3	5833.3	1	1	1	0.4703
Plot	2	11667	5833.3	1.2727	0.1825	3	0.2238
Res	8	36667	4583.3				
Total	11	54167					

Table S2.10. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic abundance (n Macro m^{-2}). PERMANOVA was designed with SM and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	12927	12927	3.5566	0.3347	2	0.0314
Plot	2	7269.2	3634.6	1.0818	0.4359	125	0.3843
Res	8	26877	3359.7				
Total	11	47073					

Table S2.11. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic abundance (n Macro m^{-2}). PERMANOVA was designed with CN and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5833.3	5833.3	1	1	1	0.476
Plot	2	11667	5833.3	1.2727	0.1808	3	0.2239
Res	8	36667	4583.3				
Total	11	54167					

Table S2.12. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic abundance (n Macro m^{-2}). PERMANOVA was designed with CP and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	7382	7382	1	1	1	0.4755
Plot	2	14764	7382	1.938	0.0177	7	0.0314
Res	8	30472	3809				
Total	11	52618					

Table S2.13. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic type (fragments n m^{-2} and films n m^{-2}). PERMANOVA was designed with ZN and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5833.3	5833.3	1	1	1	0.4792
Plot	2	11667	5833.3	1.2727	0.1826	3	0.2294
Res	8	36667	4583.3				
Total	11	54167					

Table S2.14. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic type (fragments n m^{-2} and films n m^{-2}). PERMANOVA was designed with SM and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	12291	12291	3.3421	0.3314	2	0.0331
Plot	2	7355.2	3677.6	1.0484	0.4477	125	0.4196
Res	8	28063	3507.8				
Total	11	47709					

Table S2.15. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic type (fragments n m^{-2} and films n m^{-2}). PERMANOVA was designed with CN and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5833.3	5833.3	1	1	1	0.4728
Plot	2	11667	5833.3	1.2727	0.1758	3	0.2188
Res	8	36667	4583.3				
Total	11	54167					

Table S2.16. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic type (fragments n m^{-2} and films n m^{-2}). PERMANOVA was designed with CP and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	7214	7214	1	1	1	0.4719
Plot	2	14428	7214	1.8531	0.0188	10	0.0391
Res	8	31144	3893				
Total	11	52786					

Table S2.17. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic mass (g Macro m^{-2}). PERMANOVA was designed with ZN and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5454.7	5454.7	1	1	1	0.4782
Plot	2	10909	5454.7	1.1429	0.1862	3	0.3132
Res	8	38181	4772.7				
Total	11	54545					

Table S2.18. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic mass (g Macro m^{-2}). PERMANOVA was designed with SM and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	10417	10417	2.187	0.3315	2	0.1006
Plot	2	9526.3	4763.2	1.2856	0.1493	125	0.2445
Res	8	29640	3705				
Total	11	49583					

Table S2.19. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic mass (g Macro m^{-2}). PERMANOVA was designed with CN and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5280.1	5280.1	1	1	1	0.4745
Plot	2	10560	5280.1	1.0864	0.1839	3	0.376
Res	8	38880	4860				
Total	11	54720					

Table S2.20. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic mass (g Macro m^{-2}). PERMANOVA was designed with CP and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and total abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	7442.2	7442.2	1	1	1	0.4668
Plot	2	14884	7442.2	1.9694	0.0176	10	0.0348
Res	8	30231	3778.9				
Total	11	52558					

Table S2.21. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic type (fragments g m^{-2} and films g m^{-2}). PERMANOVA was designed with ZN and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5454.7	5454.7	1	1	1	0.4706
Plot	2	10909	5454.7	1.1429	0.1828	3	0.3187
Res	8	38181	4772.7				
Total	11	54545					

Table S2.22. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal vegetated bioengineers and side intertidal sandbanks. Data were grouped by macroplastic type (fragments g m^{-2} and films g m^{-2}). PERMANOVA was designed with SM and BSI as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	10277	10277	2.1495	0.3313	2	0.1103
Plot	2	9562.5	4781.2	1.28	0.1378	125	0.2404
Res	8	29883	3735.4				
Total	11	49723					

Table S2.23. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic type (fragments g m^{-2} and films g m^{-2}). PERMANOVA was designed with CN and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	5280.1	5280.1	1	1	1	0.4769
Plot	2	10560	5280.1	1.0864	0.1749	3	0.3712
Res	8	38880	4860				
Total	11	54720					

Table S2.24. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of subtidal vegetated bioengineers and side subtidal sandbanks. Data were grouped by macroplastic type (fragments g m^{-2} and films g m^{-2}). PERMANOVA was designed with CP and BSS as the fixed factor, plot (1 and 2) as the random factor nested in habitat and type of macroplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	7266.5	7266.5	1	1	1	0.4754
Plot	2	14533	7266.5	1.8792	0.0193	10	0.0385
Res	8	30934	3866.8				
Total	11	52734					

Table S2.25. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal vegetated bioengineers. Data were grouped by microplastic abundance (n MPs g^{-1}). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and total MPs abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	1533.2	1533.2	2.659	0.3306	3	0.1271
Species	2	1153.2	576.6	0.56671	0.8742	9924	0.7778
Res	36	36629	1017.5				
Total	39	39315					

Table S2.26. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal vegetated bioengineers. Data were grouped by microplastic type (fibre, fragment, foam and film). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and type of microplastics as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	3062.9	3062.9	2.848	0.3266	3	0.0777
Species	2	2150.9	1075.5	0.7646	0.7016	9905	0.645
Res	36	50637	1406.6				
Total	39	55850					

Table S2.27. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal vegetated bioengineers. Data were grouped by macroplastic abundance (n Macro m^{-2}). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and total macroplastic abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	3584.6	3584.6	0.69091	1	3	0.6908
Species	2	10376	5188.2	1.3644	0.1159	9353	0.1633
Res	20	76049	3802.5				
Total	23	90010					

Table S2.28. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal vegetated bioengineers. Data were grouped by macroplastic type (fragment n m^{-2}, film n m^{-2}). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and macroplastic type as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	4466.2	4466.2	0.73564	1	3	0.6652
Species	2	12142	6071.2	1.5636	0.058	9627	0.0834
Res	20	77657	3882.8				
Total	23	94265					

Table S2.29. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal vegetated bioengineers. Data were grouped by macroplastic mass (g Macro m^{-2}). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and total macroplastic mass as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	3202.8	3202.8	0.62073	1	3	0.7709
Species	2	10319	5159.7	1.2461	0.178	9814	0.2201
Res	20	82812	4140.6				
Total	23	96334					

Table S2.30. Permutational multivariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal vegetated bioengineers. Data were grouped by macroplastic type (fragment g m^{-2}, film g m^{-2}). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and macroplastic type as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	3321.3	3321.3	0.63704	1	3	0.7682
Species	2	10427	5213.5	1.2496	0.1746	9836	0.212
Res	20	83443	4172.1				
Total	23	97191					

Table S2.31. Permutational univariate analysis of variance (PERMANOVA) results for comparisons of intertidal and subtidal bioengineers' leaves. Data were grouped by microplastic abundance (n MPs cm^{-2}). PERMANOVA was designed with habitat (Intertidal and Subtidal) as the fixed factor, species (ZN, SM; CN, CP) as the random factor nested in habitat and microplastic abundance as the dependent variable.

Source	Df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Habitat	1	11861	11861	2.1917	0.1013	10	0.0156
Species	3	16235	5411.8	1.2041	0.1113	9790	0.1409
Res	55	2.4721E5	4494.6				
Total	59	2.753E5					

2.8.2 FIGURES

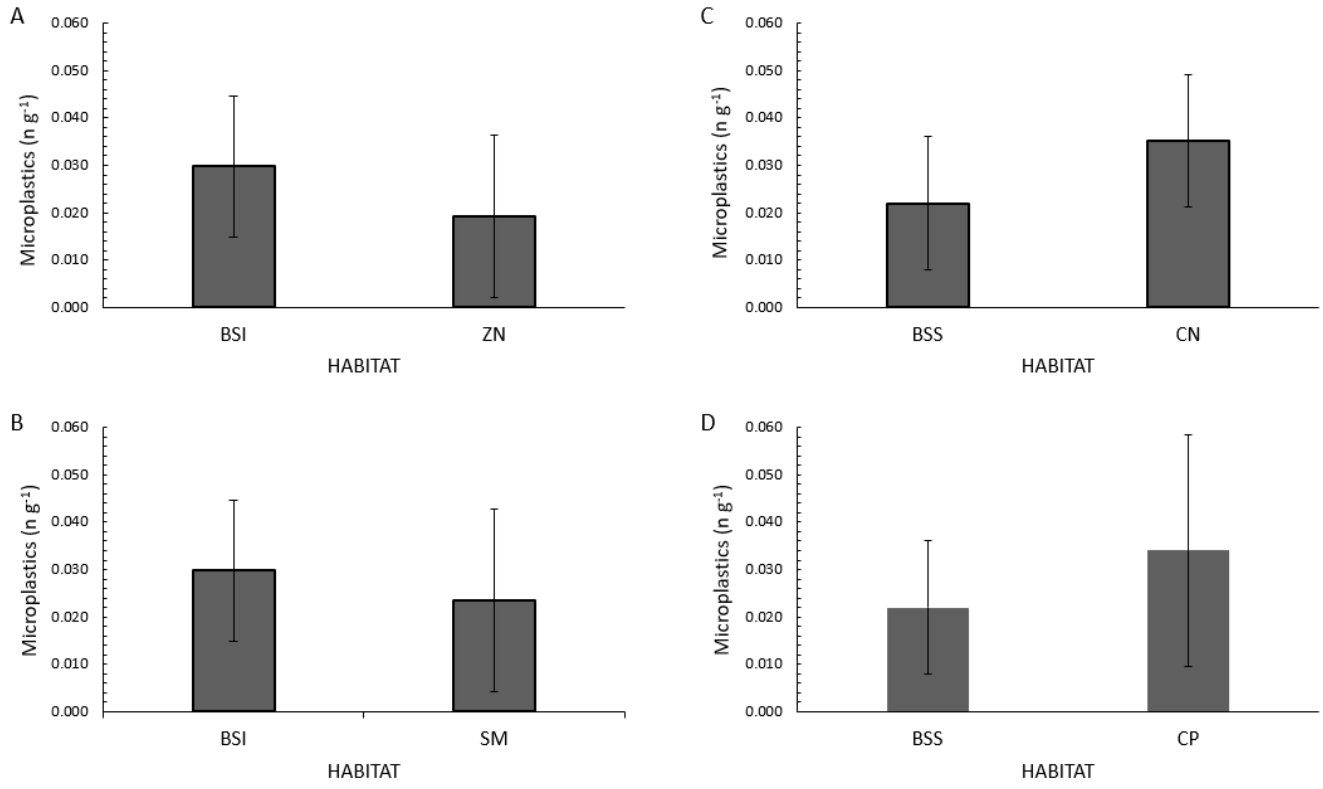

Figure S2.1. Average microplastics abundance (n MPs g^{-1}; ± SD) in the bioengineer species *Zostera noltei* (ZN; A), *Sporobolus maritimus* (SM; B), *Cymodocea nodosa* (CN; C), *Caulerpa prolifera* (CP; D) and side bare sediment intertidal (BSI) and bare sediment subtidal (BSS).

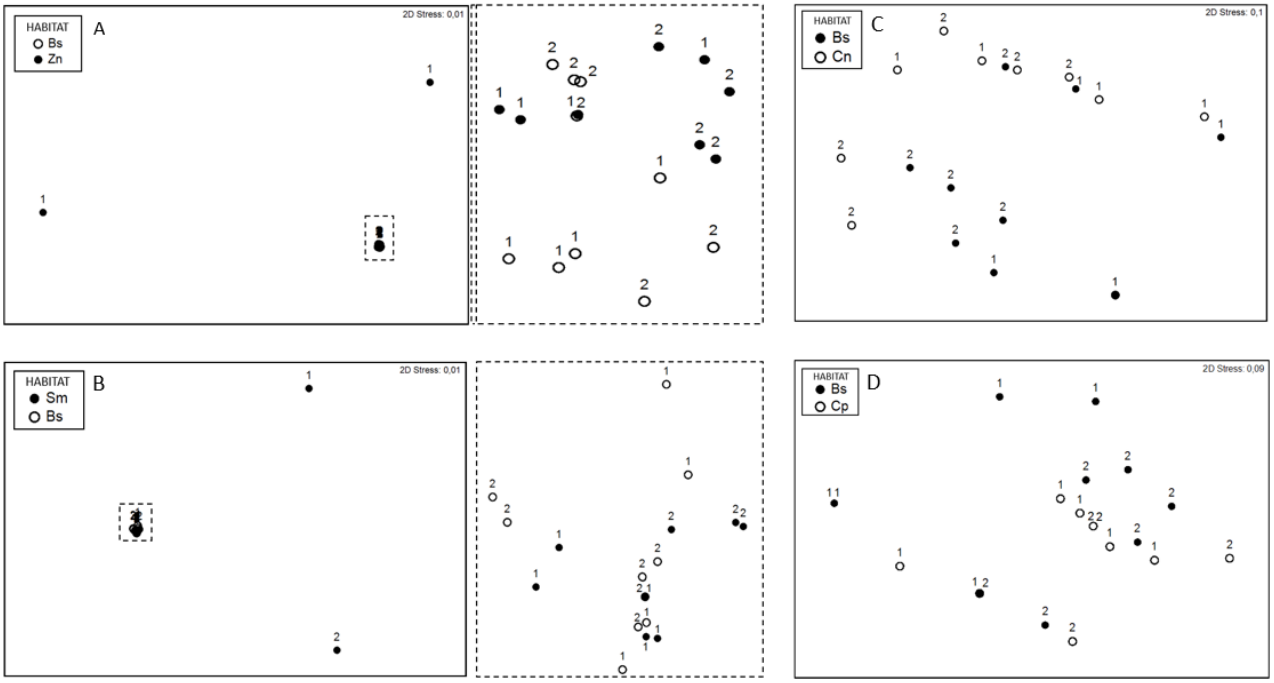

Figure S2.2. Distribution of samples by non-metric multidimensional scaling analysis for data grouped by microplastic type (fibre, fragment, foam, film) for each bioengineer and its side sandbank. *Zostera noltei* (ZN*;*

A), *Sporobolus maritimus* (SM; B), *Cymodocea nodosa* (CN*;* C), *Caulerpa prolifera* (CP; D) and side intertidal and subtidal bare sediment. Plots depicted as 1 or 2 and dashed areas represent zoomed portion of the graph.

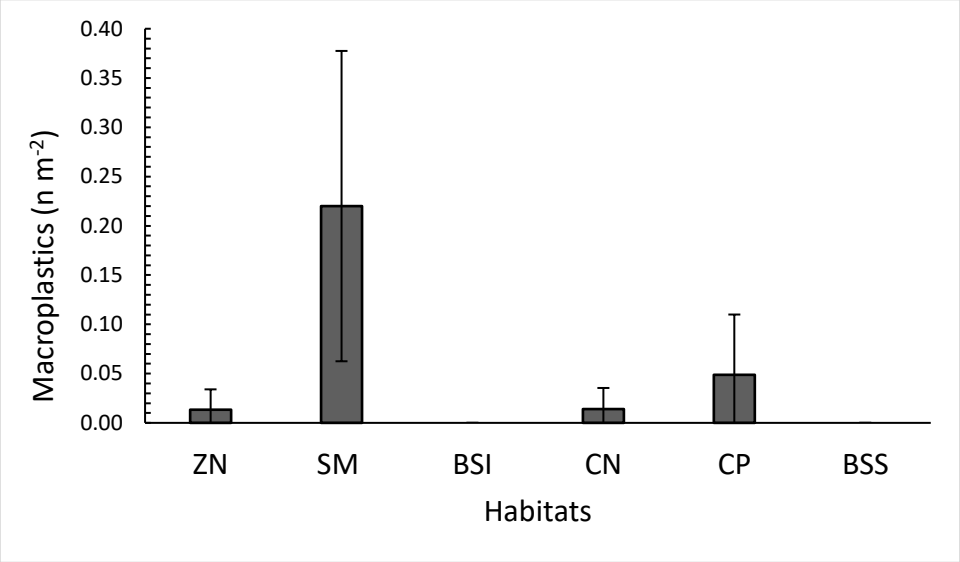

Figure S2.3. Average macroplastics abundance (n macroplastics m^{-2}; ± SD) in the bioengineer species *Zostera noltei* (ZN), *Sporobolus maritimus* (SM), *Cymodocea nodosa* (CN), *Caulerpa prolifera* (CP) and side bare sediment intertidal (BSI) and bare sediment subtidal (BSS).

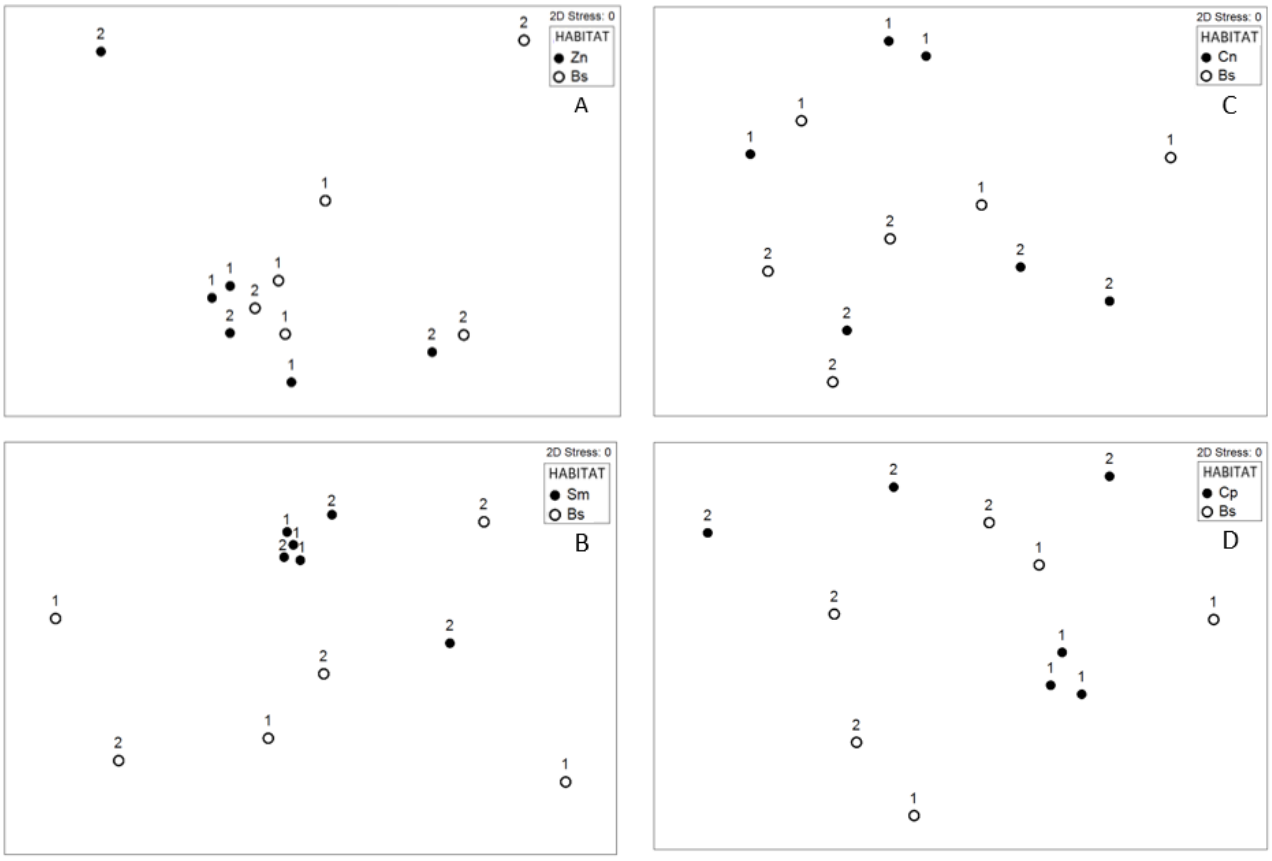

Figure S2.4. Distribution of samples by non-metric multidimensional scaling analysis for macroplastic type (fragment and film); labels (1,2) represent plots and symbols represent the habitat type (*Z. noltei* (ZN); *A*, *S. maritimus* (SM); *B*, *C. nodosa* (CN); *C*, *C. prolifera* (CP); *D*).

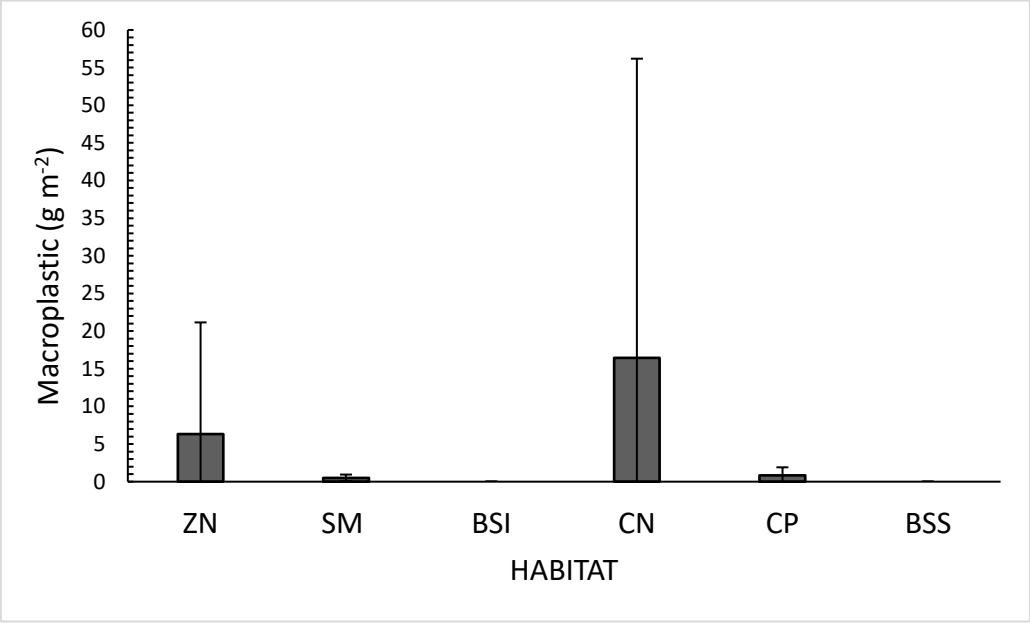

Figure S2.5. Average macroplastics mass (g Macro m^{-2}; ± SD in the bioengineer species *Zostera noltei* (ZN), *Sporobolus maritimus* (SM), *Cymodocea nodosa* (CN), *Caulerpa prolifera* (CP) and side bare sediment intertidal (BSI) and bare sediment subtidal (BSS).

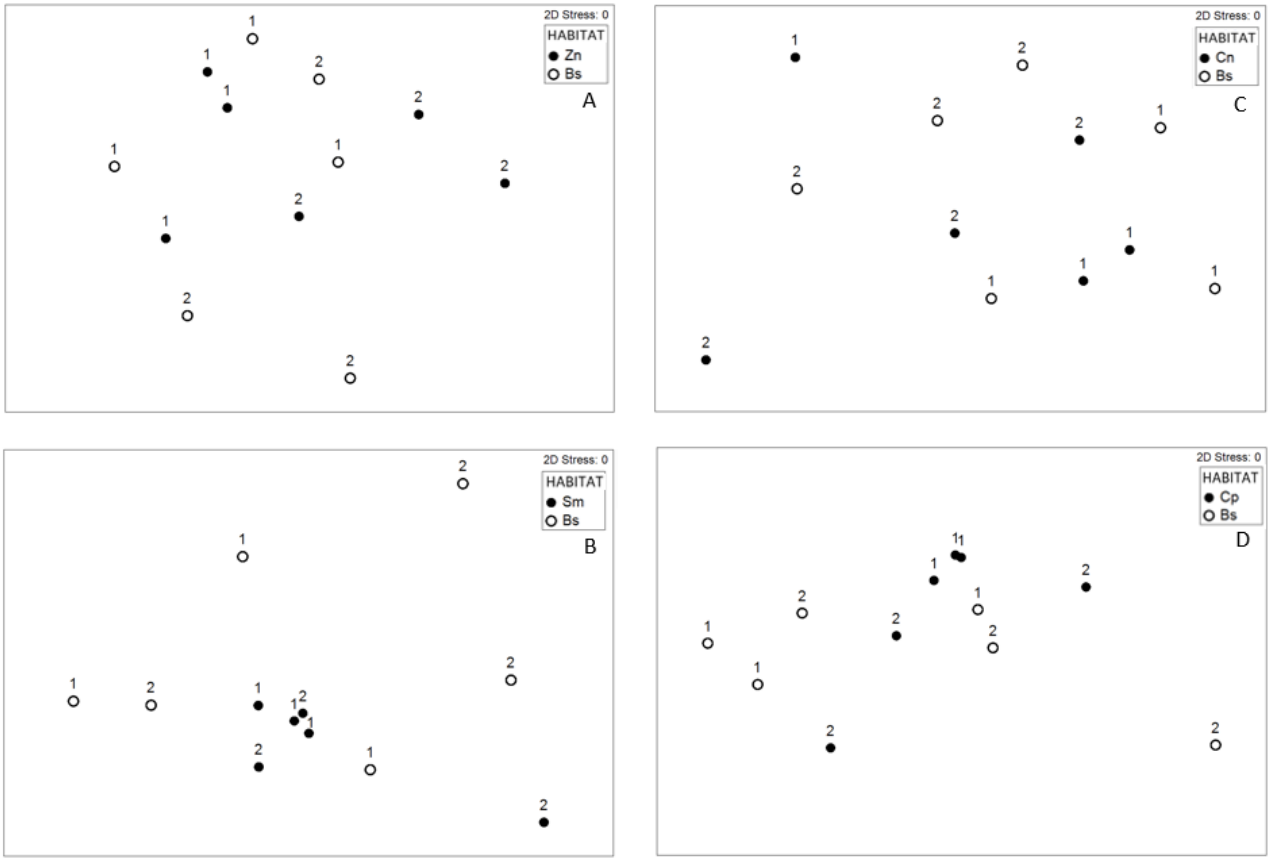

Figure S2.6. Distribution of samples by non-metric multidimensional scaling analysis for macroplastic type mass (g fragment m^{-2}, g film m^{-2}); labels (1,2) represent plots and symbols represent the habitat type (*Z. noltei* (ZN), A; *S. maritimus* (SM), B*; C. nodosa* (CN), C*; C. prolifera* (CP), D).

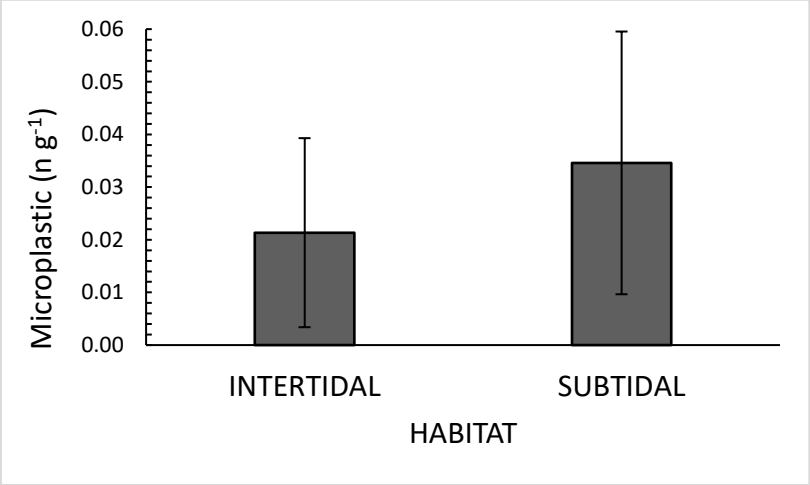

Figure S2.7. Average microplastic abundance (n MPs g^{-1}; ± SD) for intertidal were obtained from *Z. noltei* (ZN) and *S. maritimus* (SM) whereas those for subtidal from *C. nodosa/Z. marina* (CN) and *C. prolifera* (CP).

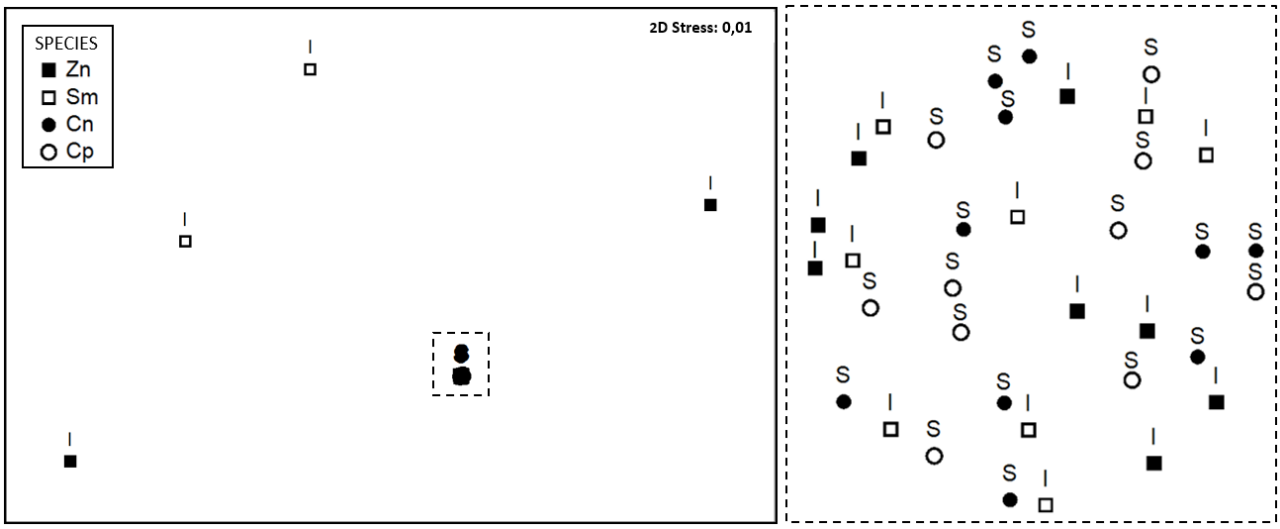

Figure S2.8. Distribution of samples by non-metric multidimensional scaling analysis for data grouped by microplastic type (fibre, fragment, foam, film); labels represent habitat type (I=intertidal; S=Subtidal) and symbols represent species (*Z. noltei* (ZN), *S. maritimus* (SM), *C. nodosa* (CN), *C. prolifera* (CP)). The dashed area represents zoomed portion of the graph.

Figure S2.9. Macroplastic abundance (n MPs m^{-2}; ±SD). Values for the intertidal habitat were obtained from *Z. noltei* (ZN) and *S. maritimus* (SM) whereas those for subtidal from *C. nodosa/Z. marina* (CN) and *C. prolifera* (CP).

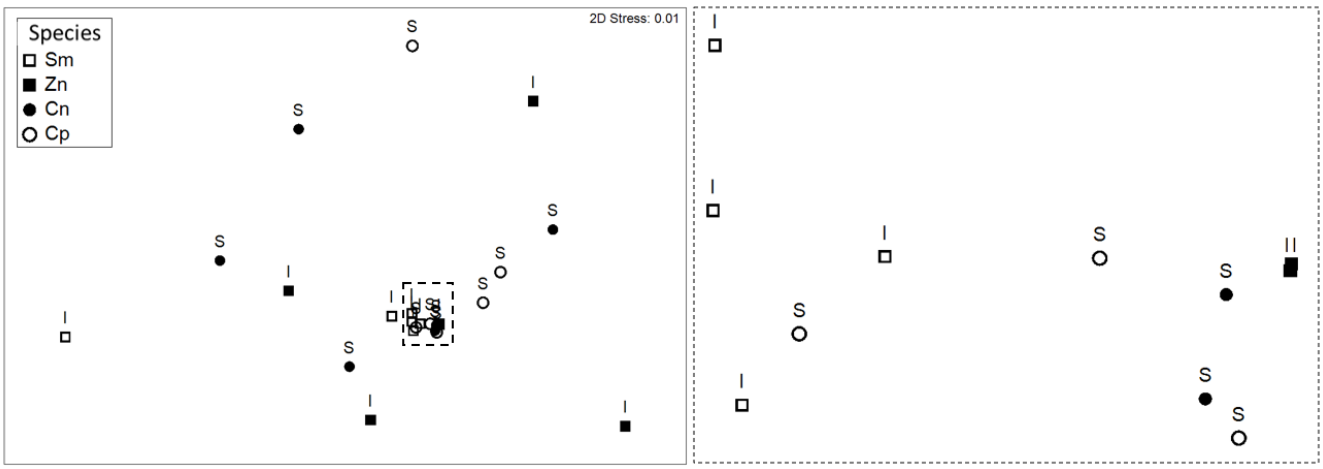

Figure S2.10. Distribution of samples by non-metric multidimensional scaling analysis for data grouped by macroplastic type (fragment n m^{-2} and film n m^{-2}); labels represent habitat type (I=intertidal; S=Subtidal) and symbols represent species (*Z. noltei* (ZN), *S. maritimus* (SM), *C. nodosa* (CN), *C. prolifera* (CP)). The dashed area represents zoomed portion of the graph.

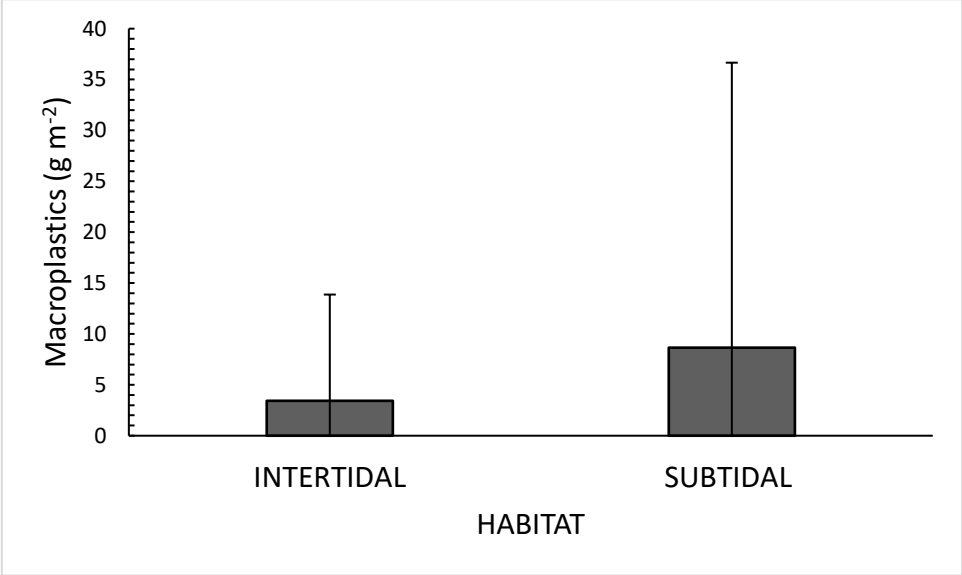

Figure S2.11. Average macroplastic mass (g MPs m^{-2}; ± SD). Values for the intertidal habitat were obtained from *Z. noltei* (ZN) and *S. maritimus* (SM) whereas those for subtidal from *C. nodosa/Z. marina* (CN) and *C. prolifera* (CP).

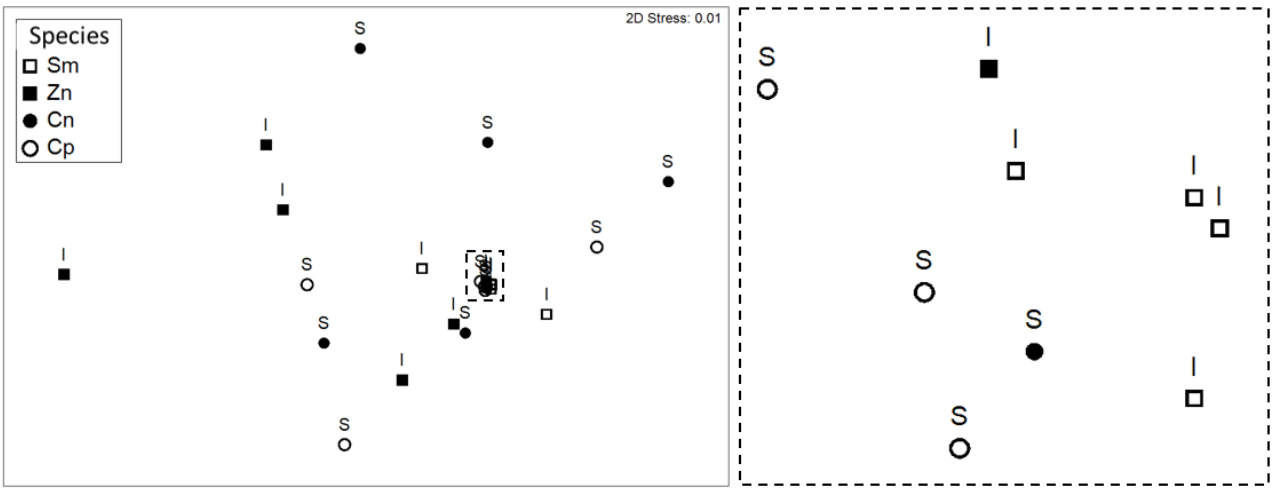

Figure S2.12. Distribution of samples by non-metric multidimensional scaling analysis for data grouped by macroplastic type mass (fragment g m^{-2} and film g m^{-2}); labels represent habitat type (I=intertidal; S=Subtidal) and symbols represent species (*Z. noltei* (ZN), *S. maritimus* (SM), *C. nodosa* (CN), *C. prolifera (*CP)). The dashed area represents zoomed portion of the graph.

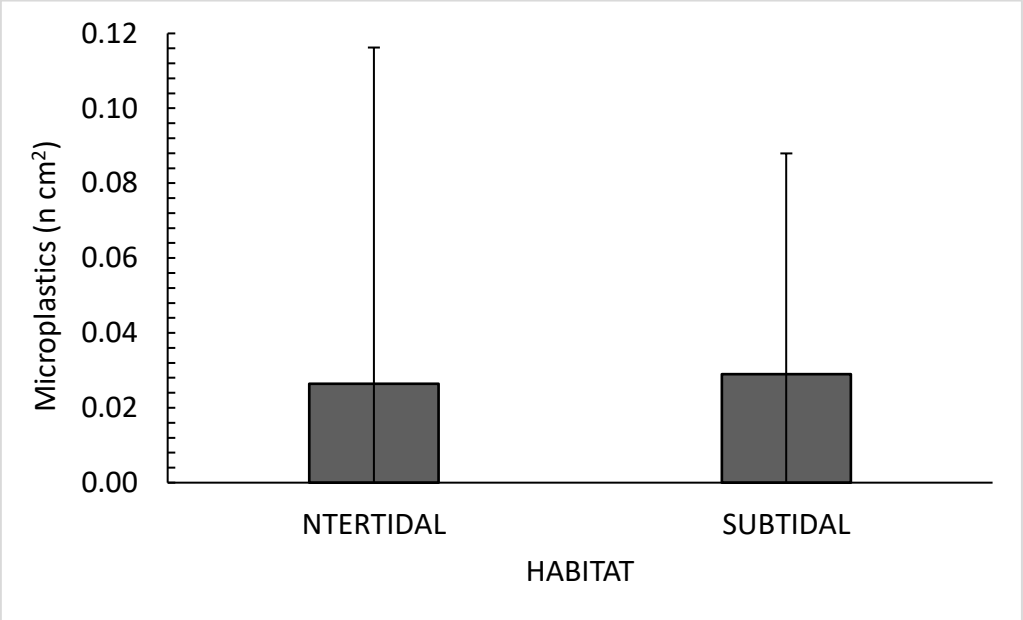

Figure S2.13. Microplastic abundance (n MPs cm^{-2}; ± SD) on the leaves of intertidal and subtidal species. Average values for intertidal species were obtained from MPs concentration on *Z. noltei* (ZN) and *S. maritimus* (SM) whereas those for subtidal on *C. nodosa* (CN), *Z. marina* (ZM) and *C. prolifera* (CP).

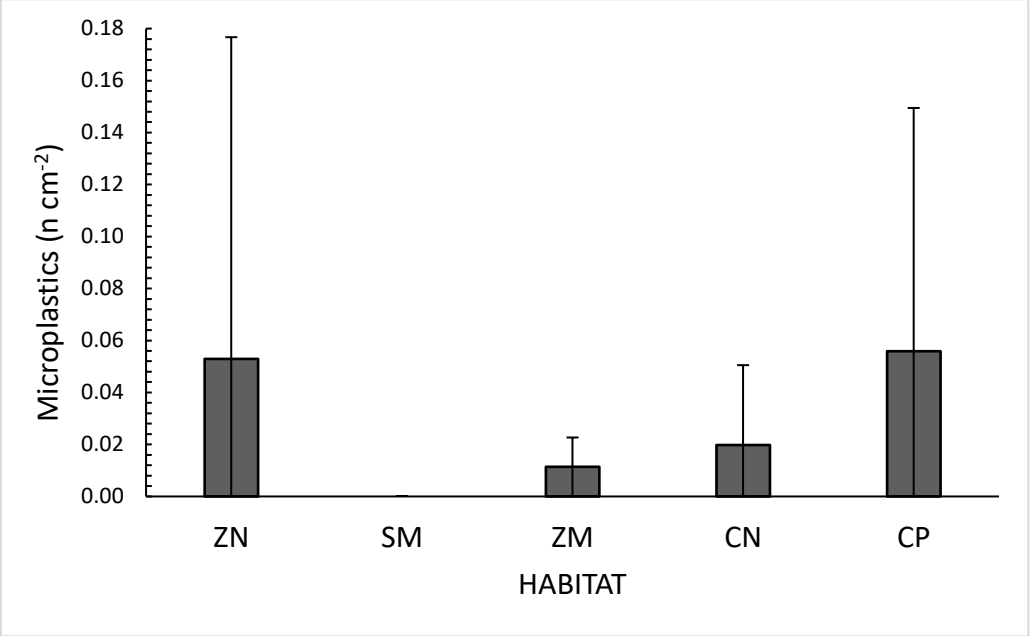

Figure S2.14. Microplastic abundance (n MPs cm^{-2}; ± SD) on the leaves of intertidal and subtidal species (*Z. noltei* (ZN), *S. maritimus* (SM), *Z. marina* (ZM), *C. nodosa* (CN) *and C. prolifera* (CP)).

-----Submitted to Marine Pollution Bulletin as a Baseline-----

CHAPTER 3. Microplastics in commercial bivalves harvested from intertidal seagrasses and sandbanks in the Ria Formosa lagoon, Portugal

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3.1 ABSTRACT

Microplastic (MP) pollution is jeopardizing human health through seafood. Of major concern are commercial bivalves because their filter-feeding activity directly exposes them to MP in the water column. We provide quantitative and qualitative baseline data on MP content in the soft tissues of three commercially important bivalves collected in the Ria Formosa lagoon, southern Portugal. *Ruditapes decussatus* contained on average 18.4 ± 21.9 MP items g^{-1} (w.w.) tissue and exhibited the highest MP concentration by weight. *Cerastoderma* spp. and *Polititapes* spp. followed with 11.9 ± 5.5 MP items g^{-1} (w.w.) and 10.4 ± 10.4 MP items g^{-1} (w.w.), respectively. Overall, 88% of MPs found were synthetic fibres, the majority of which were blue (51.6%). The most represented polymers were polyethylene (PE) and polystyrene (PS). The unexpectedly high number of MPs recorded suggests that this semi-closed lagoon system is experiencing higher anthropogenic pressure compared to open coastal systems.

Keywords: microfibers, FTIR, marine debris, seafood, environmental monitoring.

Microplastic (MP) pollution is posing severe threats to marine biodiversity and it may eventually affect human health through seafood consumption (Smith et al., 2018). Bivalves are of particular concern because, as filter feeders, they are directly exposed to natural and anthropogenically derived microparticles in the water column (Ward et al., 2019). However, bivalves are able to select among particles both at pre- and post- ingestion levels according to their size, shape and surface characteristics (Ward et al., 2019). Thus, not all the captured particles are necessarily ingested, the rejected ones are transported to the mantle and expelled as pseudofaeces (Garrido et al., 2012). Ingested particles can provoke physical damage to the digestive organs (von Moos et al., 2012) as well as neurotoxicity and negative effects on the immune and reproductive systems (Avio et al., 2015; Sussarellu et al., 2016; Ribeiro et al., 2017) due to the leaching of plastic chemicals (i.e. Bisphenol A, Phthalates, PCBs; Browne et al., 2008). Recent results have highlighted MPs in the foot and the mantle of the blue mussel *Mytilus edulis*, suggesting a novel pathway of MP uptake through direct contact or adhesion to the tissues which can account for up to 50% of total MP absorption (Kolandhasamy et al., 2018). Regardless of the uptake mechanism, presence of MP has been reported in wild, farmed or sold bivalves worldwide (De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014; Rochman et al., 2015; Vandermeersch et al., 2015; Li et al., 2015, 2018; Murphy, 2018; Karlsson et al., 2017; Digka et al., 2018). In addition to humans, bivalves are a food source for many marine organisms such as snails, crabs, fishes, and marine birds (Dame, 2016) and their contamination could affect higher trophic levels in nature (Farrell & Nelson, 2013).

In this study, we provide a baseline assessment of MP abundance and type (visual and spectroscopic) in three commercial bivalves harvested in the intertidal areas of the Ria Formosa lagoon: the clam *Polititapes* spp. (golden carpet shell) inhabiting seagrass meadows (*Zostera noltei*), the clam *Ruditapes decussatus* (grooved carpet shell) and the cockle *Cerastoderma* spp. (edible cockle) inhabiting sandbanks.

Ria Formosa is a sheltered large mesotidal lagoon located in Algarve, southern Portugal (Figure 3.1). This habitat has been recognized as an important natural wetland with high social, cultural and economic value, classified as a Natural Park since 1987 and as a Ramsar and Natura 2000 site. It comprises a complex network of channels and tidal flats dominated by coastal vegetation. Specifically, the back-barrier mudflats are largely colonised by the intertidal seagrass *Zostera noltei* and subtidal seagrasses *Cymodocea nodosa* and *Zostera marina* (Cunha et al., 2009). The intertidal seagrasses cover about 2,900 ha of the total intertidal area (Guimaraes et al., 2012) providing

important ecological functions and ecosystem services, in particular feeding, breeding, and nursery habitats, eventually supporting local fisheries (Ribeiro et al., 2006; Guimarães et al., 2012).

One of the most important economic activities in the Ria Formosa is the bivalve exploitation sector (Guimarães et al., 2012; Bernardino, 2000; Oliveira et al., 2013). About 395 ha of the intertidal area is occupied by clam or oyster farms (Guimarães et al., 2012; Oliveira et al., 2013), and about 10,000 ha are dedicated to manual harvesting of clams and cockles (Bernardino, 2000). These activities have a relevant impact on the national economy, with up to 90% of the clams consumed in the country being produced in the Ria Formosa (Cachola, 1996). In 2011, edible cockles (*Cerastoderma* spp.) harvested in the Ria Formosa represented over 51% of the national bivalve capture, accounting for €1,271,000; whereas clams represented up to 40%, corresponding to a profit of €3,534,000 (DGPA/INE, 2011). Bivalves that grow in the intertidal areas of the Ria Formosa are directly affected by pollutants from untreated sewage, industrial discharges, and agriculture and storm-water runoff (Bebianno 1995). Several wastewater treatment plant sites (WWTP) have been constructed in the lagoon area to control the discharge of wastewater and avoid the deterioration of the water quality (Almeida and Soares, 2012). In particular, the western sector of the Ria Formosa has four WWTPs: Faro-Noroeste (WWTP1), Faro-Nascente (WWTP2), Olhão-Poente (WWTP3), and Olhão-Nascente (WWTP4; Figure 3.1). These are well known sources of contamination including MPs (Murphy et al., 2016).

Paired habitats (n=3) of intertidal *Zostera noltei* meadows and sandbanks were sampled during low tide between April 2017 and January 2018 in Culatra island (Figure 3.1), an area where bivalves are manually harvested by locals. In each habitat, replicated samples (n=5) were haphazardly taken using a PVC corer (diameter 15.6 cm) at a depth of 20 cm. The core content was sieved in situ and cleaned of sediment using 1-mm black mesh and transported to the laboratory under dark cool conditions (< 3 h). In the laboratory, samples were inspected and alive individuals of three species of bivalves were selected and frozen for further analysis. While *Cerastoderma* spp. and *R. decussatus* were associated to sandbanks, *Polititapes* spp. was found within *Z. noltei* meadows. Bivalves were not subjected to purification time before analysis as we wanted to measure the absolute MP abundance including those particles recently ingested or possibly translocated to the tissues (Mathalon and Hill, 2014; Li et al., 2015).

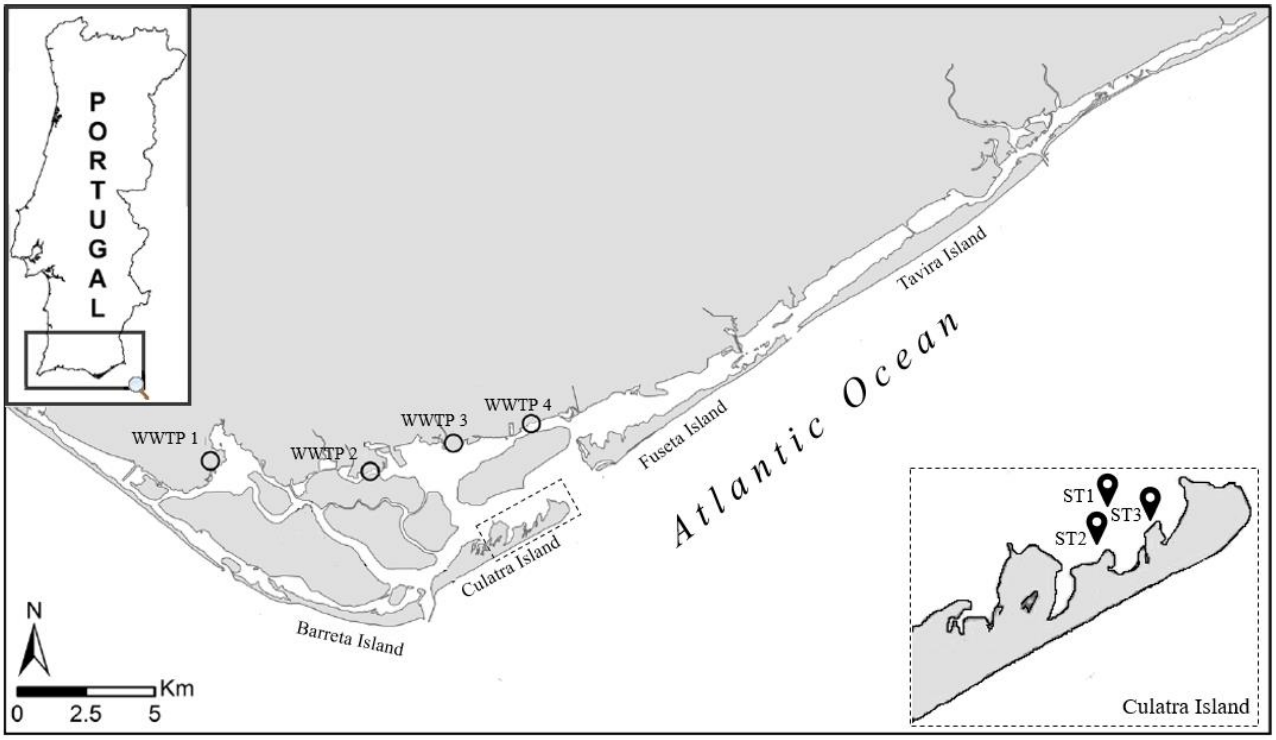

Figure 3.1. Location of the three sampling stations (ST) in Culatra island (Ria Formosa, Portugal) and the four wastewater treatment plants (WWTP1, WWTP2, WWTP3 and WWTP4) in the western sector.

Individuals of the three species were selected using a threshold of wet body weight with shell > 0.5 g. Their shell length and height were measured using a digital calliper (± 0.01 mm), and their wet body weight with shell (g w.w.) was measured using a microbalance (± 0.001 g). For each station and species, replicated samples (n=3) of 1 to 4 individuals were used. Individuals were pooled maintaining a similar final biomass (g w.w.) among replicates in each station and an appropriate ratio volume/solution to ensure a complete digestion of the organic matter and to avoid filter clogging during filtration.

Individuals in each replicate were rinsed with saturated ultrapure water (purified by an Elix® equipment) to remove external contaminants. Shells were opened, and soft tissue was extracted and weighted (g w.w.) using a microbalance (± 0.001 g). Soft tissue biomasses ranged from 0.57 g to 0.92 g in *Cerastoderma* spp., 0.37 g to 0.89 g in *Ruditapes decussatus* and from 0.51 g to 1.75 g in *Polititapes* spp. Soft tissues digestion and MPs collection were conducted using an adapted protocol from Dehaut et al. (2016). Each composite sample was placed in a 250 mL flask and KOH solution 1.8 M was added to digest the organic matter. The solution was stirred for two minutes and placed in the oven at 60 °C for 24 hours. After incubation, the solution was entirely filtered through a Whatman GF/C glass fibre filter (diameter 47 mm, 1.2 μm pore size) while still warm, using a vacuum system.

The resulting filters were dried in oven at 40 °C for 24 hours and then examined for the presence of MPs under a stereomicroscope (ZEISS SteREO Discovery.V8).

To obtain information on polymer composition and to validate MP identification, Micro Raman Spectroscopy was performed (JASCO NRS-4100, Laser Raman Spectrometer) on a subsample (n = 15) for each species. The laser beam (532 or 785 nm) was focused on the sample surface by a microscope objective. The availability of objectives with different magnification and numerical aperture (5×/0.10 N.A., 20×/0.40 N.A., and 100×/0.90 N.A.) provided the possibility to perform both spatially averaged and high-resolution analysis. The laser power was adjusted according to the characteristics of the sample in order to obtain a suitably high Raman signal yet preventing any damage. Spectra at different points of the sample surface were acquired to verify its homogeneity. To identify the polymer composition, the spectra were then compared with those of the most common polymers included in a home-made spectral database. When identification through Raman analysis was ambiguous or not possible, usually due to intense photoluminescence background, Attenuated Total Reflection Fourier-Transform Infrared Spectroscopy (ATR-FTIR) was used (JASCO FT/IR-4700).

MP items were counted and digitally measured (mm) using the software Image J (Schneider et al., 2012). Fibres were measured along their length whereas foams and films were measured for their longest dimension. Every plastic particle was assigned to one of three distinct size classes: >0.01-0.1 mm, >0.1-1 mm, >1-5 mm, and those >5 mm were excluded from the analysis. MPs were classified according to their colours (blue, violet, yellow, red, green, and colourless white) and shape (fibres, granules, foam, films, and fragments; Gündoğdu and Çevik 2017).

To eliminate post-sampling contamination 100% cotton lab coats were worn during the laboratory treatment process. In addition, all equipment used was non-plastic (i.e. glass and metal) and were rinsed twice with saturated ultrapure water between each sample extraction. Each digestion batch was made of one replicate for each species. To account for possible contamination, one procedural control was performed in parallel to each digestion batch, yielding an average procedural contamination of 1.8 ± 1.3 (mean ± SD, n=9).

MPs were present in all samples (Table 3.1). *Ruditapes decussatus* exhibited the highest level of MPs resulting on average 18.4 ± 21.9 items g^{-1} w.w. followed by *Cerastoderma* spp. and *Polititapes* spp. with averages of 11.9 ± 5.5 MPs items g^{-1} w.w. and 10.4 ± 10.4 MPs items g^{-1} w.w.,

respectively. The relatively higher levels of MP contamination found in *R. decussatus* support previous studies where this species was the most contaminated out of six molluscs harvested from the lagoon of Bizerte, Tunisia (Abidli et al., 2019).

In addition to setting a baseline for incidence of plastic in these commercially important bivalves from the Ria Formosa, our study highlights high levels of MP contamination compared to other studies. For example, MP concentrations that we observed in *R. decussatus* (18.4 ± 21.9 items g^{-1} w.w.) are an order of magnitude higher of those reported by Abidli et al. 2019 (1.4 items g^{-1} w.w.). Relatively low MP concentrations were also reported for the Manila clams *Ruditapes philippinarum* from different regions: Davidson and Dudas (2016) detected an average of 0.9 ± 0.9 items g^{-1} w.w. in Baynes Sound (British Columbia), Li et al. (2015) reported ~3 MPs items g^{-1} w.w. in China, and Cho et al. (2019) observed an average of 0.34 ± 0.31 items g^{-1} w.w. in South Korea. Similarly, the MP levels that we observed for *Cerastoderma* spp. (11.9 ± 5.5 MPs items g^{-1} w.w.) are significantly higher than the average of 0.74 ± 0.35 items g^{-1} w.w. reported by Hermabessiere et al. (2019) in the common cockle (*Cerastoderma edule*), collected in France. MPs occurrence have been documented in a variety of other bivalves including mussels, where average MP concentrations ranged between 0.36 ± 0.07 items g^{-1} w.w. in French Brittany (Van Cauwenberghe and Janssen, 2014) and ~2 ± 1 MPs items g^{-1} in China (Li et al., 2015; Ding et al., 2018).

Multiple types of MPs, including fibres, foams and films occurred in the tissues of the targeted bivalves (Table 1). Neither fragments nor plastic pellets (nurdles) were detected. Fibres were the most prevalent type category observed representing more than 80% of the total MPs. *Cerastoderma* spp. exhibited the highest concentration of fibres (94%) and the lowest of foams (1%), whereas *Polititapes* spp. had the highest amount of foams (16%) and the lowest of fibres (82%). The lowest concentration of MP films was observed in *R. decussatus* (1%; Table 3.1). In *R. decussatus* and in *Polititapes* spp., polyethylene (PE) and polystyrene (PS) were present in equal proportion followed by polypropylene (PP) and polyethylene terephthalate (PET). MPs found in *Cerastoderma* spp. were predominantly also PE while PS and PP were represented in similar proportions.

Table 3.1. Frequency of occurrence (%) of MPs in the samples of the three commercial bivalve species, their MP abundance as items g^{-1} (w.w.) (mean ± SD), total number of MP items found in each species, the percentage of occurrence for each MP types (fibres, foams and films) and polymer (PE - Polyethylene, PS - Polystyrene, PET - Polyethylene terephthalate, PP - Polypropylene).

Species	Frequency of occurrence (%)	MP abundance (items g^{-1} w.w.)	Total MP items	Fibres (%)	Foams (%)	Films (%)	PE (%)	PS (%)	PET (%)	PP (%)
Ruditapes decussatus	100	18.4 ± 21.9	95	90	9	1	33.3	33.3	13.3	20
Cerastoderma spp.	100	11.9 ± 5.5	80	94	1	5	46.7	20	6.7	26.7
Polititapes spp.	100	10.4 ± 10.4	100	82	16	2	40	40	6.7	13.3

Overall, fibres accounted for 88% of total debris found in the three species (Figure 3.2). Foams and films formed the remaining 9% and 3%, respectively. These findings are in agreement with Abidli et al. (2019) and Davidson and Dudas (2016), who reported fibres as the most abundant MPs in *R. decussatus* (91%) and *R. philippinarum* (90%), and MP films as the least abundant (3% and 5%). Fibres were also the dominant MPs in *Chlamys ferreris* and *Mytilus galloprovincialis* (84%; Ding et al., 2018) and in *Saccostrea cucullata* (69%; Li et al., 2018) from China. Although no fragments were identified in our study, their occurrence has been reported in the literature with proportions ranging from 5-6% (Davidson and Dudas, 2016; Abidli et al., 2019) to 15-20% (Ding et al., 2018; Li et al., 2018).

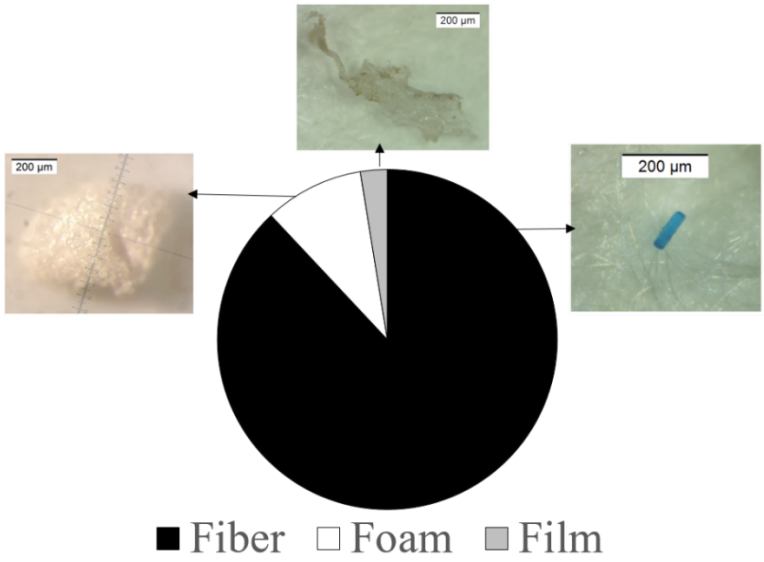

Figure 3.2. Percentage of occurrence of MPs by shape category found in the three commercial bivalve species: fibres (88%), foams (9%) and films (3%). Photos represent most common example of the indicated type of MPs.

Several recent studies have highlighted the importance of Raman and FTIR spectroscopy to discriminate between natural items for synthetic polymers and thus to avoid overestimation of MP concentration (e.g., Wesch et al., 2016). In our study, following visual identification, spectroscopy characterization revealed that 2% of putatively synthetic items were indeed of natural origin (e.g. keratin and cellulose) and these were discarded from the data (Figure 3.3).

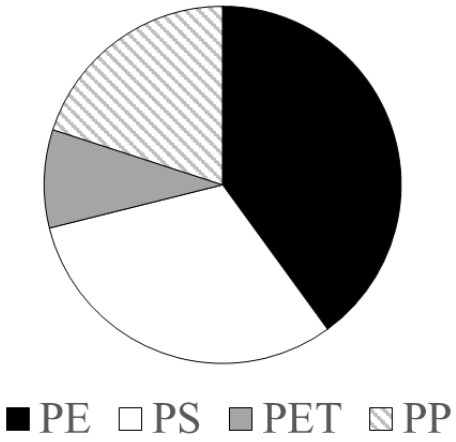

Figure 3.3. Percentage of occurrence of MPs by polymer found in the three commercial bivalve species (*Ruditapes decussatus*, *Polititapes* spp., *Cerastoderma* spp.): PE - Polyethylene (40%), PS - Polystyrene (31.1%), PET - Polyethylene terephthalate (8.8%), and PP - Polypropylene (20%).

Among all the fibres identified within the three species, blue (51.6%), white (21.9%) and violet (14.8%) were the dominant colours, whereas the least represented were green (0.4%) with only one item detected, and red (5.3%; Figure 3.4).

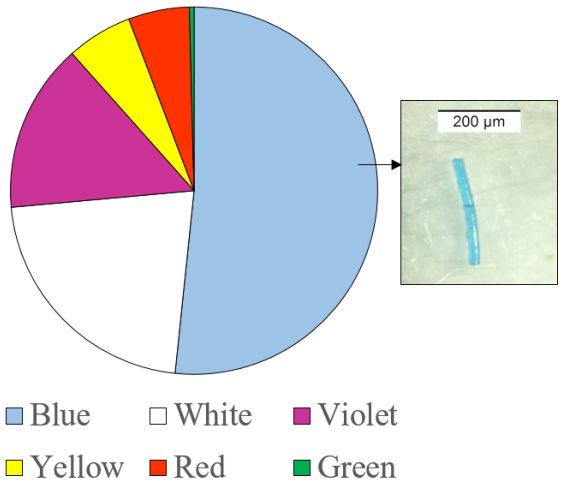

Figure 3.4. Percentage of occurrence of MPs by colour category found in the three commercial bivalve species: blue (51.6%), white (21.9%), violet (14.8%), yellow (5.7%), red (5.3%) and green (0.4%).

Size of MP items ranged from 0.01 mm to 5 mm. Size categories >0.1–1 mm and >1–5 mm were the most common, comprising 60.4% and 33.8% of the total respectively (Figure 3.5). This is consistent with previous studies assessing MP contents in commercial bivalves from other regions (e.g. Ding et al., 2018; Abidli et al. 2019).

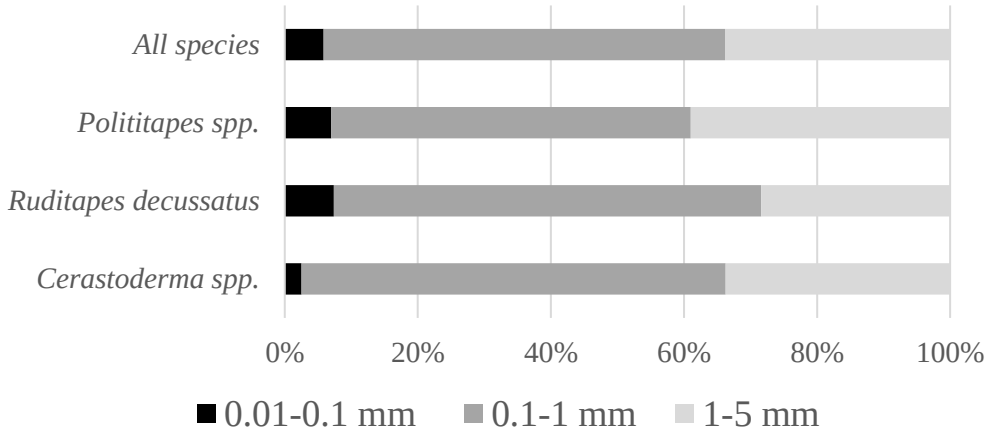

Figure 3.5. Frequency of size classes distribution (%).

The extremely high concentrations of fibres detected in this study is likely linked to the wastewater and domestic discharges in the lagoon. Despite removal efficiency of wastewater treatment facilities can be very high (95–99%), municipal wastewater effluents remain a conspicuous pathway for microplastics to reach aquatic systems (Murphy et al., 2016 and references therein). Four WWTPs are located in the western side of the Ria Formosa lagoon (Figure 3.1). According to the models of water and particles circulation, the most relevant inputs of discharge in our study area are the WWTPs of Olhão-Nascente and Faro-Nascente (Fabião et al., 2016). Importantly, the particles released from Olhão-Nascente show a mean residence time within the lagoon system of ~7 days (concentrated between Culatra and Fuseta islands) before being washed out through the Armona inlet (Fabião et al., 2016). The particles discharged from Faro-Nascente remain longer in the system (~18 days) due to a complex interconnectivity between channels in the western area of the lagoon. Such high retention times can result in an extended contamination exposure for the ecosystems in the Ria Formosa. In addition, Culatra Island is an important fishing ground for the local community. Fishing gears that include nets, ropes and traps are commonly lost or discarded during fishing activities and after long-term exposition to the physical-chemical pressures of the aquatic environment can result in fibrous pollution (Browne et al., 2011).

The small proportion of foams and films observed in our samples is likely linked to polymer density and bivalves' habitat. Plastic density is central in defining the position of the debris in the water column (Morét-Ferguson et al., 2010). Foam is usually made of expanded PS and tends to float to the surface (density 0.96–1.04 g cm^{-3}). High concentrations of PS in oysters and mussels cultured in the upper layer of the water column have been reported (Cho et al., 2019). In contrast, higher density polymers such as PE (density 1.3 g cm^{-3}) are dominant in sediments (Shim et al., 2018) where they become available to benthic organisms (Li et al., 2015, 2016; Phuong et al., 2018). This suggests that, in general, the availability of foam-shaped MPs is low for species living in intertidal sediments, while fibres constitute the main portion of the "plastic diet". In agreement with previous works on commercial bivalves (e.g. Li eta l 2015; Cho et al. 2019) PE was the most abundant polymer observed in our study. This is in agreement with other studies on commercial bivalves (Cho et al. 2019). The most probable source of this polymer is expected to be textile (Li eta l 2015). This polymer is also widely used in inexpensive items including supermarket bags and plastic bottles. PS found is mostly in solid forms as only 9% of all MPs was classified as foam. The polymer in its solid form is used in protective packaging (such snack food bars), lids, bottles, trays, disposable cutlery, and tanks. PET items were present in 8.8% of the samples analysed and most of them were blue fibres found (visual

identification). PET is used predominantly in drinks bottles, jars, plastic film, tubes, pipes, and insulation molding, and it is a potential human carcinogen (Ecology Center 1996; Luciani-Torres et al., 2104).

In conclusion, we found that MPs occur at high concentrations in clams (*Ruditapes decussatus* and *Polititapes* spp.) and cockles (*Cerastoderma* spp.) from the Ria Formosa lagoon (Portugal). The outcomes of this study urge for immediate effort to reduce plastic waste and to improve the management of wastewater disposal in the lagoon. Future research is needed to assess the potential bioaccumulation and biomagnification of chemicals in these species and to define potential risks for human health.

3.2 ACKNOWLEDGMENTS

Funding: This research was funded by Foundation for Science and Technology (FCT - MEC, Portugal (grant numbers: PTDC/MAR-EST/3223/2014, IF/01413/2014/CP1217/CT0004, UID/Multi/04326/2013, UID/Multi/04326/2019, SFRH/BPD/119344/2016) and further supported by the National Research Foundation of South Africa (grant number: 64801). Authors are thankful to A. Silva, C. Freitas, R. Texeira, A. Marçal, and N. Velez for the assistance in the field and laboratory.

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