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MICROPLASTIC CONTAMINATION IN TWO RESIDENT SPECIES FROM THE MONDEGO ESTUARY (PORTUGAL)

THE COMMON COCKLE (*CERASTODERMA EDULE*) AND THE EUROPEAN GREEN CRAB (*CARCINUS MAENAS*)

Dissertation in MSc in Ecology, supervised by Doctor Ana Filipa da Silva Bessa (Department of Life Sciences of the University of Coimbra and Mare – Marine and Environmental Sciences Centre) and presented to the Department of Life Sciences, Faculty of Sciences and Technology of the University of Coimbra

October 2021

“For most of history, man has had to fight nature to survive,

in this century he is beginning to realize that, in order to survive, he must protect it.”

Jacques Yves Costeau

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Department of Life Sciences
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Abstract

The ingestion of microplastics (<5 mm) by aquatic species is one of the current important environmental threats, specially to human consumption. This study analyzes the presence of microplastics and their characteristics in two benthic species with high commercial interest, the European green crab (*Carcinus maenas*) and the common cockle (*Cerastoderma edule*), collected from Mondego estuary, used as a case study. The gills and digestive system of *Carcinus maenas,* were also analyzed to assess the main gateway of interaction between individuals and microplastics, by respiration or prey ingestion.

A total of 142 microplastics from both species were recovered, ranged from 0.052 mm to a maximum of 6.313 mm in length. Significant differences were observed in the number of microplastics between *Carcinus maenas* and *Cerastoderma edule* ($p<0.05$) and were higher in *Carcinus maenas* (90%) than in *Cerastoderma edule* (72%). Samples of digestive system and gills of crabs were analyzed and 73 microplastics were reported and higher quantities of microplastics were found in the digestive system compared to gills (M=58 and M=15, respectively). In general, the most common shape of microplastics observed was fibers (84.5%) and the most common color was blue (61.19%).

In addition, significant differences were observed between the levels of microplastics according to the target organ analyzed in *Carcinus maenas* ($p<0.05$), showing that the principal way of microplastic interaction in this species was via ingestion. Another relevant result was a negative correlation, in males of *Carcinus maenas*, between weight and microplastics ingestion.

The results of this study that suggests *Carcinus maenas* could be an appropriate bioindicator species for the assessment of microplastic pollution in transitional zones.

In addition, the results obtained are important as they report the presence of microplastics in species with high interest commercial.

This study shows for the first time the presence of microplastics in *Carcinus maenas* (gills and digestive systems) from Portugal.

Keywords: Microplastics, Bioindicator, Mondego estuary, *Carcinus maenas*, *Cerastoderma edule.*

Resumo

A ingestão de microplásticos (<5 mm) por espécies aquáticas é uma das actuais ameaças ambientais, especialmente para o consumo humano. Este estudo analisa a presença de microplásticos e as suas características em duas espécies de bentos com elevado interesse comercial, o caranguejo verde europeu (*Carcinus maenas*) e o berbigão comum *(Cerastoderma edule*), recolhidos no estuário do Mondego, utilizado como um estudo de caso. As guelras e o sistema digestivo de *Carcinus maenas*, foram também analisados para avaliar a principal forma de interação entre estes indivíduos e microplásticos, por respiração ou ingestão de presas.

Foram extraídos um total de 142 microplásticos, variando de 0.052 mm a um máximo de 6.313 mm de comprimento. Foram observadas diferenças significativas no número de microplásticos entre *Carcinus maenas* e *Cerastoderma edule* ($p<0{,}05$) e foram mais elevados em *Carcinus maenas* (90%) do que em *Cerastoderma edule* (72%). Foram analisadas amostras do sistema digestivo e brânquias de caranguejos e foram observados 73 microplásticos e foram encontrados números mais elevados de microplásticos no sistema digestivo em comparação com as brânquias ($M=58$ e $M=15$, respetivamente). Em geral, a forma mais comum dos microplásticos observados foram as fibras (84,5%) e a cor mais comum foi o azul (61,19%).

Além disso, foram observadas diferenças significativas entre os níveis de microplásticos de acordo com o órgão alvo analisado em *Carcinus maenas* ($p<0{,}05$), mostrando que a principal forma de interacção de microplásticos nesta espécie era através da ingestão. Outro resultado relevante foi uma correlação negativa, nos machos de *Carcinus maenas*, entre o peso e a ingestão de microplásticos.

Os resultados deste estudo sugerem que *Carcinus maenas* poderia ser uma espécie bioindicadora apropriada para a avaliação da poluição de microplásticos em zonas de transição. Além disso, os resultados obtidos são importantes, uma vez que relatam a presença de microplásticos em espécies com elevado interesse comercial.

Este estudo mostra pela primeira vez a presença de microplásticos em Carcinus maenas (brânquias e sistemas digestivos) em Portugal.

Palavras-chave: Microplásticos, Bioindicador, Estuário do Mondego, *Carcinus maenas*, *Cerastoderma edule.*

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Chapter I – Introduction

1.1 Plastic: production, use and waste
1.2 Plastic pollution
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1.1 Plastic: production, use and waste

The Humankind always made transformations in the environment to our benefit and developed materials that are not found in nature. The first synthetic material produced was Parkesine (actually celluloid) in the middle of 19th century, but the first mass produced plastic - the term "plastic" came from "*plastikos*", a Greek word, that means molding - was Bakelite on 1907 (PlasticEurope,2021). After World War II the plastic's increased exponentially (Figure 1.1).

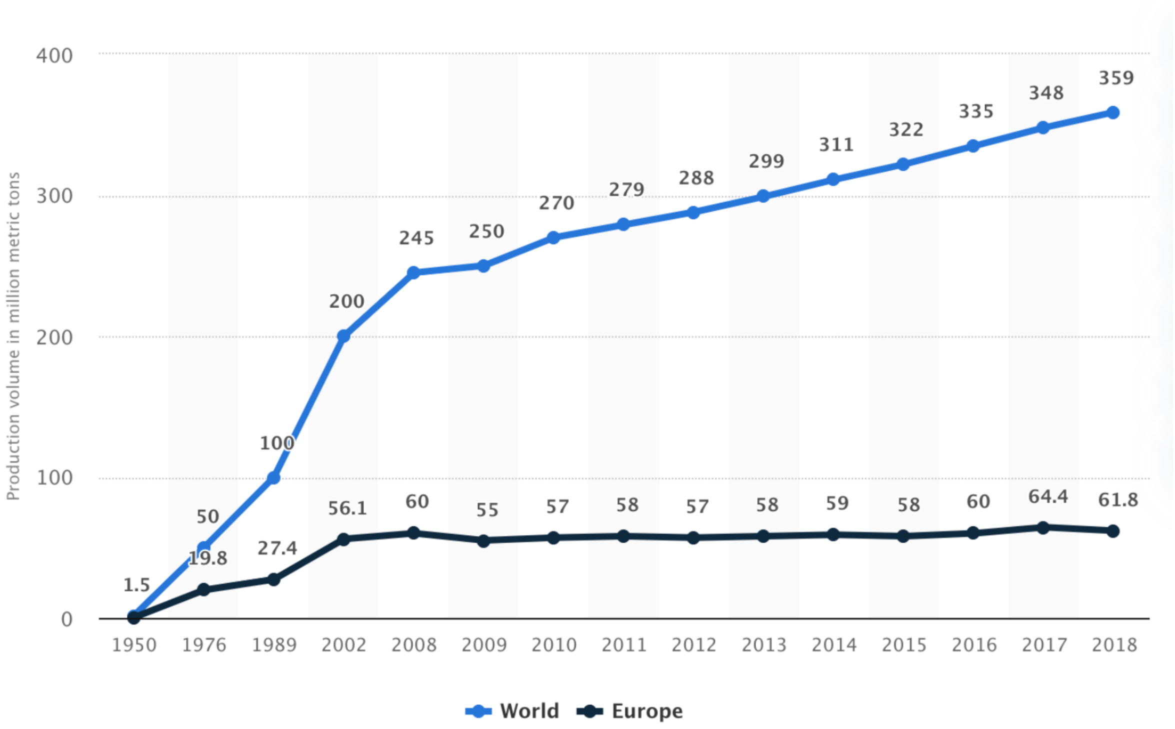

Figure 1.1-Plastic production between 1950 and 2018 in the world (blue) and Europe (black) (PlasticEurope Market Research Group, 2018).

The growth of plastic production bring many benefits to society, nowadays ranks 7th in European industrial value added, are responsible for 1.5 million jobs in Europe and enable other industries have technologies advances, like cars industry, healthcare and renewable energies industry (PlasticEurope, 2020).

In 1950, the plastic production was 1.5 million of tonnes at worldwide level and increased to 368 million of tonnes in 2019 (PlasticEurope, 2020). In Europe the production was 57.9 million of tonnes, this value suffer a decrease when compared to 2018 (PlasticEurope, 2020) but estimates are 61.8 million of tonnes.

There are two main families of plastic (PlasticEurope, 2020) that are divided in:

-Thermoplastics (high production levels, consequently occur more frequently in environment), can be melted when heated and rigid when cooled. This type of plastic can be reused by plastic industry due their capacity to be reheated and reshaped many times.

-Thermoset, suffer a chemical change wen heated. It is only possible heat this type of plastic one time.

Plastic material can be produced for different feedstock. The plastic with more demand (Figure 1.2), in Europe, are:

-Polypropylene (PP), mainly used on food packaging, automobiles industry and bank notes.

-Polyethylene (PE), which includes Low-Density Polyethylene (PE-LD), Linear Low-Density Polyethylene (PE-LDD) and High-Density Polyethylene (PE-HD), mainly used in agricultural films, toys, cosmetic package, reusable bags.

-Polyvinyl chloride (PVC), mainly used in pipes, cables, inflatable pools.

-Polyurethane (PUR), mainly used in insulating foams for fridge building insulation.

-Polyethylene terephthalate (PET), mainly used in bottles for water, juices and cleaners.

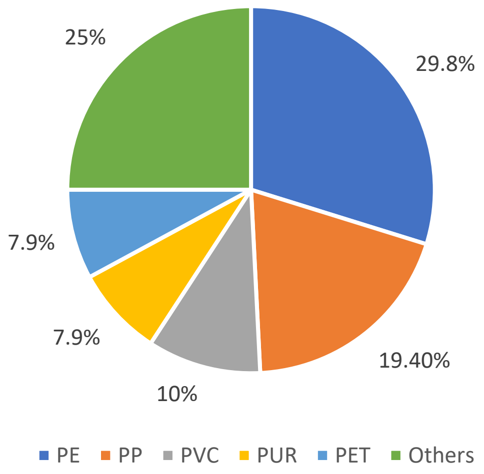

Figure 1.2-European plastics demand distribution by resin type in 2019(Source: PlasticEurope, 2020), PE-Polyethylene, PP- Polypropylene, PVC- Polyvinyl chloride, PUR- Polyurethane, PET- Polyethylene terephthalate.

These categories of plastics, together, are responsible for 75% of plastic demand in Europe (PlasticEurope, 2020).

It is undeniable that plastic is part of our lives and replaced other materials like glass or metal and have many benefits, in consideration demand by segment (Figure 1.3):

1)Food and drink packaging, 50% European goods are packaged in plastics and the plastic weight has been reduced 28%;

2)construction industry, plastic durability and cheaper and easier to install than traditional materials;

3) transport industry, pieces on plastic make car lightweight;

4)electronics gadgets due plastic have plastic flame retardants;

5)agriculture in greenhouse, mulching because helps maintain humidity and improves thermal conditions, in silage and irrigation systems;

6)Household, leisure activities and sports material;

7)others, like medical supplies, furniture, etc…

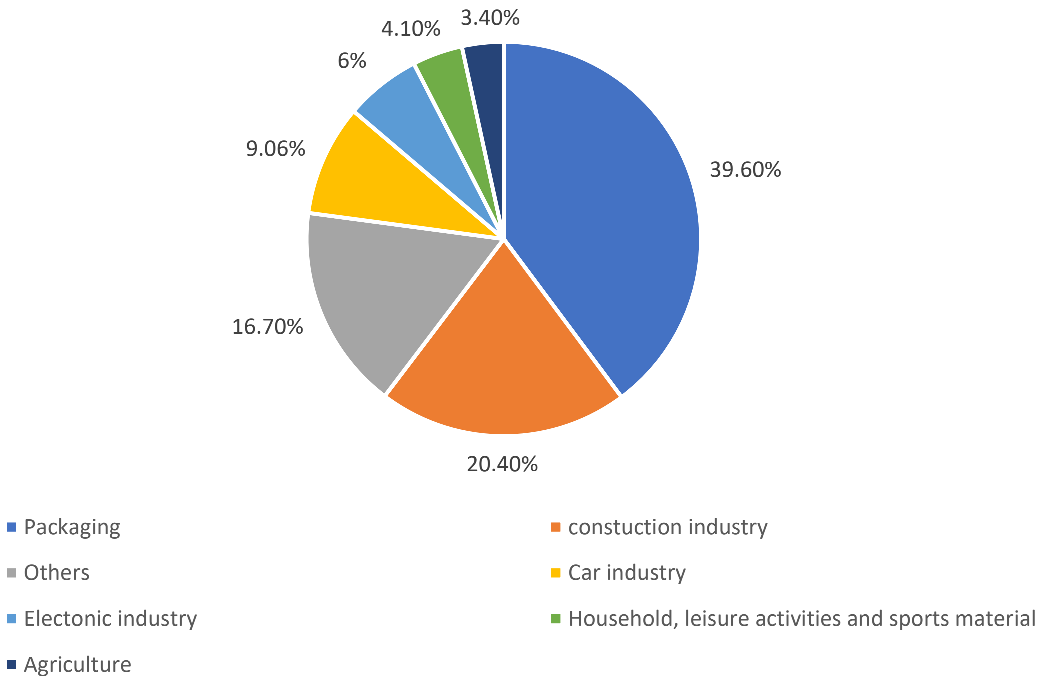

Figure 1.3-Distribution of European plastic demand by segment in 2019 (Source: PlasticEurope, 2020).

The majority of plastic products may contain chemicals added during their production such as UV stabilizers, antioxidants, colorants (Turner, 2016), flame-retardants and plasticizers.

The plastic is not a problem by itself, in 2018 a total of 29.1 million of tonnes of plastic waste were collected and only 32.5% was recycled and 25% of plastic waste was sent to landfill and a great part was single-use plastic (PlasticEurope, 2020). In the same year, 17.8 million of tonnes of plastic packaging were collected and 3.3 million of tonnes were disposal in landfill (PlasticEurope, 2020).

Approximately 50% of consumer plastics are single use (UNEP, 2020) and associated with linear economy (production-transport-consumption-discard), an inefficient waste management and plastic's properties, mainly low-cost production, extremely durable and resistant consequently have low rate degradation in environmental conditions (Cole et al., 2011), and for all of these reasons, plastic is one of the biggest environmental concern.

1.2 Plastic Pollution

Since Industrial Revolution impacts of anthropogenic pollution have been documented, for example the famous case of peppered moth, *Biston butelaria* in United Kingdom (Cook et al., 2012). The term pollution presupposes entrance or presence in ecosystems of any material or chemical compound that has adverse effects on environment and biota (National Geographic, 2020).

In 1972, Carpenter and Smith (Carpenter et al., 1972) were the first to alert to the presence of plastics in the environment, in particular in the North Atlantic. Plastic litter in marine environment is considered a threat to global marine diversity (Ozturk et al., 2020) and contamination with plastic debris was observed in diverse environments such as rivers (Van Emmerik et al., 2020), ocean (Luna-Jorquera et al., 2019), agricultural farmlands (Piehl et al., 2018) consequently, many studies report ingestion of plastic by animals, turtles (Santos et al, 2016), by aquatic birds (Basto et al., 2019) ,including penguins (Bessa et al., 2019a; Le Guen et al., 2020), by whales in European coast (Panti et al., 2019) and over 50 freshwater species are reported to ingest plastic (Jams et al., 2020).

Since 1950, 9200 million metric tons of plastic were produced and 5000 million metric tons of plastic were waste and this value show the problem of linear economy, low recycling efficiency and this associated with deficit waste management result in accumulation in ecosystems (Barnes et al., 2009). The main plastic debris observed in the environment come from human consume and use, for example, from food packaging, cigarettes, beverage containers (Sheavly and Register, 2007) and the plastic litter on the streets enter in rivers through surface runoffs, sewers and illegal disposal and consequently arrive to the sea, leisure activities on the beach and near to coastline are responsible too for plastic litter arrive to the sea (Sheavly & Register, 2007).

According to the United Nations report (UNEP, 2020), tonnes of plastic leak into the ocean per year and become exposed to abiotic conditions and biota interactions, therefore, the plastic litter suffers degradation by different ways. The main cause of plastic fragmentation is photodegradation that allows for oxidative degradation of the polymers chain (Andrady et al., 1996), the other ways of plastic degradation are by biological (animal bite and human activities) and physical factors (wind, wave, temperature) (Cole et al., 2011).

Over time, plastic suffer degradation and turned into plastic pieces with small size classified according to size as microplastics and/or nanoplastics (Table 1.1).

Table.1.1 Characterization by size range of plastic litter (Van Cauwenberghe et al., 2015)

Tipology	Size
Macroplastic	>2.5 cm
Mesoplastic	5 mm-2.5 cm
Microplastic	1µm-5 mm
Nanoplastic	<1µm

1.3 Microplastic pollution

Microplastics (MPs) are plastic particles from 1µm to 5 mm (GESAMP, 2016) and have been documented in many environmental compartments, as in the atmosphere (Cai et al., 2017), soil (Guo et al., 2020), water (Alam et al., 2019) and biota (Nan et al., 2020) and in many habitats around the globe as oceans, rivers, estuaries, and lakes (Vianello et al., 2013, Cincinelli et al., 2017; Firdaus et al., 2020; Pan et al., 2020; Kanhai et al., 2018; Patria et al., 2020) (Table 1.2).

Table 1.2 Examples of studies reporting the levels of microplastics concentrations reported for habitat from around the world.

Region	Country	Environmental compartments	Nº Microplastics	Reference
Antarctica		Water	0.17 ± 0.34 MP m^{-3}	Cincinelli et al., 2017
Arctic		Deep-sea sediments	4356±675 MP kg^{-1}	Bergmann et al., 2017
Asia	Japan	Sediments	60–2020 MPs m^{-2}	Fisher et al., 2015
Europe	Ireland	Biota (*Nephrops norvegicus)*	1.75±2.01 MPs per individual	Hara et al., 2020
South America	Ecuador	Sediments (River)	1.3 MPs m^{-2}	Lucas-Solis et al., 2021
North America	United States of America	Water (Pacific Ocean)	0.448 MPs m^{-2}	Goldstein et al., 2013
Africa	Uganda	Water (Lake Victoria)	0.73 MP m^{-2}	Egessa et al., 2020
Oceania	Australia	Sediments	2 – 147 MPs Kg^{-1}	Townsend et al., 2019

It is possible to divide microplastic in two groups according to their origin: primary and secondary microplastics (Cole et al., 2011). Microplastics produced to be smaller than 5 mm, are appointed as primary origin, those included in cosmetic products and toothpaste, resin pellets, which are used as units for plastic industries (Auta et al., 2017) and the main entry point in the environment is mainly during transportation and domestic wastewater treatment plants, microplastics from secondary origin results from the fragmentation of larger items (plastic litter, plastic bags, fishing material and packaging). According to their shape, microplastic can be divided in groups, for example fibers, fragments, films, pellets, microbeads, filaments and ropes, sponge and foam (Arthur and Baker, 2008; Thompson et al., 2004; Bessa et al., 2019b). Microplastics entry the aquatic environments from different ways: from domestic wastewater treatment plants, from washing synthetic clothes, for instance, about 0 to 2g of fibers are released per wash (Hartline et al., 2016) and plastic litter decomposition (Auta et al., 2017). The wastewater treatment plants are considered one of the main point source for microfibers (Gouveia, 2018) since for example, one piece of clothes can release more than 1900 fibers per wash (Browne et al., 2011).

Estuaries are considered the main route of microplastics to the oceans, with estimates of about, 1.15 to 2.41 million tonnes of plastic litter (macroplastic and microplastic) are released in the oceans from all rivers, every year (Lebreton et al., 2017).

One of the biggest concern of these particles is the fact that microplastics have the capacity to adsorb and transport heavy metals (Turner, 2015) and hydrophobic organic contaminants (HOC), as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), for example, (Bakir et al., 2014). Despite the most part of these contaminants were banned for a long time, it is possible to find them in the environment due to their persistence and stability character (Kelce et al., 1995). These contaminants and plastic additives, added during plastic production, are liable to bioaccumulation and microplastics are a pathway for their transference to biota (Bakir et al., 2016; Wang et al., 2016; Brennecke et al., 2016).

Due to the small size of microplastics can be easily ingested by biota. Many studies have shown that microplastics have ubiquitous distribution (Waller et al., 2017; Di et al., 2018; Hall et al., 2015; Pegado et al., 2018; Khan et al., 2020) and animals from different trophic levels are affected too, as zooplankton, turtles, bivalves, seals (Cole et al., 2014; Caron et al., 2018; Li et al., 2015; Hernandez-Milian et al., 2019). However, filter feeders and planktonic suspension species are the most prone to microplastic ingestion due to the unselective feeding way (Lusher, 2015).

There are two possible ways to ingest microplastics, from trophic transfer (Nelms e tal., 2018) and directly from the environment (Van Cauwenberghe et al., 2014). Accidentally microplastic ingestion occurs due the similar size between microplastics particles and plankton.

1.4 Effects of microplastics on species and ecosystems

Microplastics have low-rate degradation and for that reason not disappears from the environment and it is frequently ingested by biota. The size of microplastics is very similar to the plankton size and consequently are accidentally ingested (Wright et al., 2013). Another factor that increase the probability of ingest microplastics, is the ability of dimethyl sulfide (DMS) algae colonize them and the presence of DMS indicates the presence of palatable prey (Procter et al., 2019).

After ingestion, microplastics can be quickly excreted without causing damage to biota (Browne et al., 2008), but interactions between biota and microplastics can be possible and physical damage can occur.

Microplastic ingestion can result in less energy for growth (Galloway et al., 2017), behavior alterations, on (Tosetto et al., 2016) verify changes in anti-predator behavior, affect reproduction rate (Sussarellu et al., 2016), in cellular level, reduced enzymatic activity and increase oxidative stress (Sun et al., 2021), all of these consequences were observed in laboratory. In addition, microplastics can have the ability to adsorb toxic chemicals and can pose serious threats to aquatic life (Vo and Pham, 2021). HOCs present many harmful effects to biota, as modify gene expression (Rochman et al., 2014), affect male rats endocrine system (Kelce et al., 1995).

1.5 The pathways of microplastics from the river to the sea

Estuaries are among the most productive and economically important aquatic ecosystems (Paerl, 2006) and provide key goods and services, including food for migratory and resident species, fisheries resources, habitat, an important nursery zones for fish species (Martinho et al., 2007). Estuaries are transitional zones between rivers/land and sea, consequently freshwater and marine ecosystems. Estuaries are divide in three sections, 1) upper estuary (linked with river, mainly freshwater), 2) middle estuary (mix between freshwater and saltwater) and 3) lower estuary (mainly brackish water) (Dris et al., 2020), the upper estuary is an important point source of plastic pollution in marine environment, from which approximately 80% of plastic litter are from land-base (Li et al., 2016) and rivers are responsible for the transport to marine environment. The other 20% of plastic litter are from ocean-base, due tidal cycle saltwater enters in estuary, consequently can bring plastic debris from ocean (Dris et al., 2020). From land-base the main sources (Figure 1.4) are floods, water discharge from wastewater treatment plant, industrial activities, mismanagement of solid urban waste.

In coastal cities near rivers, when heavy rain periods occurs, the litter present on streets are transported to the rivers (Best, 2019).

Industrial activities and water discharge from wastewater treatment plant, WWTP can remove 78% (Murphy et al., 2016), are the main source of primary microplastic. One example of industrial activity pollution is plastic resin pellet, between 2 and 5 mm, that are used as feedstock to plastic industry (Derraik, 2002).

All offshore activities are potential sources of marine litter and can be unintentional or illegal discard. From ocean-base the main sources include commercial fishing, recreational activities, debris from diversity ships, debris from oil and gas platforms.

Litter resulting from commercial fishing are nets, polystyrene foam boxes, lines and ropes and small pieces from degradation of materials.

Estuarine zones are under influence of river flow, responsible for water exchanges between estuaries and marine environments (Dris et al., 2020). Tidal cycles that alternate between low and high tide. New or full moon are responsible for the higher range between low and high tide and consequently the progress of salt water to estuaries.

Another important characteristic is the residence time of water in estuarine systems, which is the average time that a water portion stay in a water body. The short residence time decreases the probability of pollutants standing in water body due to water exchanges and the long residence time the increases the probability of pollutants standing and depositing on sediments (Kenov et al., 2012). The sediments in transitional ecosystems such as estuaries are considered an important sink of pollutants, including microplastics (Cozar et al., 2014). Microplastics that exceed densities of 1.2 g cm^{-3} will sink and accumulate in the sediments (Van Cauwenberghe et al., 2015) and are able to benthos ingestion and for this reason it is important analyze benthos to provide real data of environmental health conditions (Santana et al., 2016).

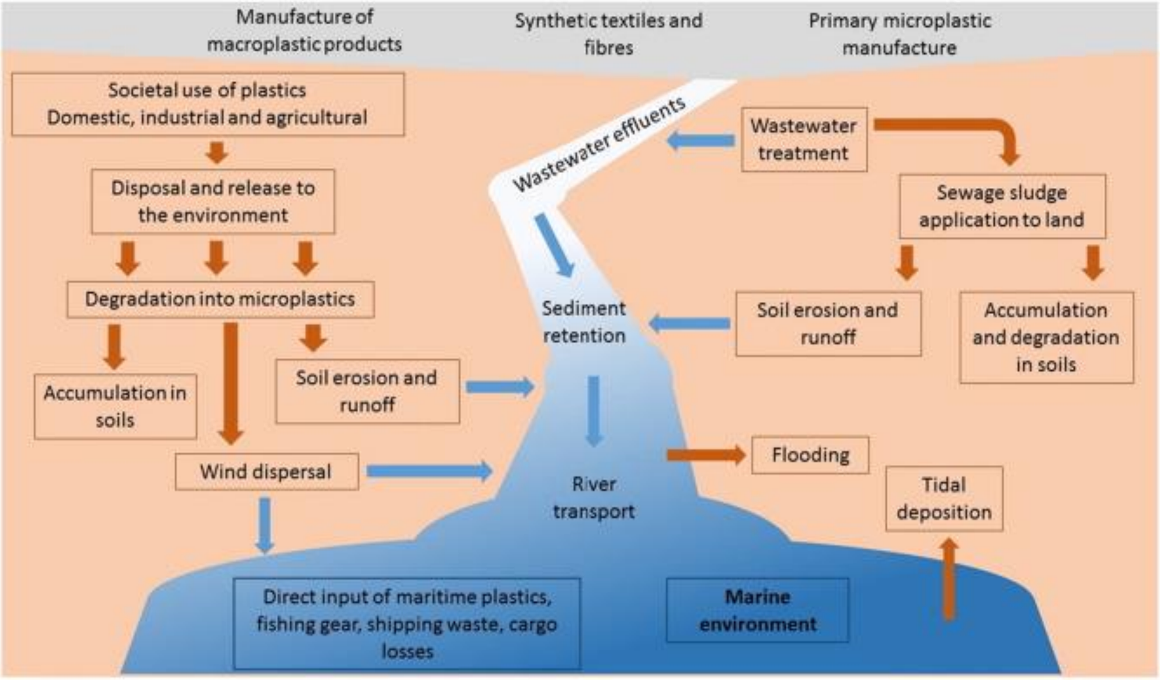

Figure 1.4- Potential pathways of microplastic pollution in transitional zones such as estuaries (Horton et al.2017).

1.6 Biota as bioindicator of microplastic pollution

In ecosystems, there are different trophic levels (producers, primary consumers, secondary and tertiary consumers) and to understand pollution levels in habitats is necessary know interactions among these levels (Thompson et al., 2008).

Bioindicators are living organisms, as plants (Matsuoka et al., 1998), unicellular organisms (Roe and Patterson, 2014) and animals (Camedda et al., 2014; Capillo et al., 2018) which provide information about the state of ecosystems.

Bioindicator species are able to indicate direct chemicals exposure and accumulation, in addition, are able to sign potential ecologically adverse effects (Bryan et al., 1985). Bioindicators might be easy to identify, abundant and easily available for sampling all year and have a wide distribution (Rainbow, 1995).

It is important to find bioindicator species for microplastic pollution. Use bioindicator species as sentinels become a common method for the assessment of microplastic pollution in certain environmental compartments and become a common established way for comparisons between different specimens in different countries, rivers and oceans (Beyer et al., 2017; Zhu et al., 2019).

In many studies, bivalves are used as a common bioindicator species due to their habitat features being sessile species (Li et al., 2019), that can present the levels of certains local rather that mobile species. Decapoda was also used as bioindicators of heavy metals contamination in several studies (Beltrame et al., 2011; Ghedira et al., 2016). It´s important regard the way of feeding and dependence of river sediments.

Some criteria are commonly established to select appropriate bioindicator species for plastic litter ingestion (Fossi et al., 2018), as background information to understand biology and ecology of the selected species, habitat information and natural distribution of species, trophic and feeding behavior information, as feeding mechanisms and feeding behavior (feeding on schooling, benthivorous feeding, etc.), spatial distribution to allow adequate spatial coverage, social-economic interest and conservation status and presence, in literature, of data and statistics from plastic litter ingestion.

It is necessary, identify bioindicators of microplastic pollution to assess the real extension in aquatic ecosystems.

1.7 Goals of the study

The main goal of this study is to assess the levels of microplastic pollution in two different species from an estuarine environment: a filter-feeder and top-predator *Cerastoderma edule* and *Carcinus maenas*, respectively, using the Mondego Estuary (Portugal) as a case study.

In additional, secondary goals were to:

1. Assess the presence and characteristics of microplastic in *Cerastoderma edule* and *Carcinus maenas*.
2. Verify if the presence of microplastic varies between gills and the digestive system in the green crab.
3. Assess if these species can act as bioindicator of microplastics pollution in estuarine ecosystems.

Chapter II- Materials and Methods

2.1 Study site
2.2 Study species
2.3 Sample collection
2.4 Microplastic extraction
2.5 Microplastic verification
2.6 Statistical analysis
2.7 Contamination control

2.1 Study site

The Mondego estuary (Fig.2.1), located on the Atlantic coast of Portugal is about 7 km long, approximately 2-3 km length and 1072 ha of wetland (Lopes et al., 2000). The Mondego estuary is a relatively small estuary (860ha) and it consists of two arms, north and south separated by Murraceira island formed by deposition of detritus transported by the river. The north arm is deeper (5m-10m, high tide) (Marques et al., 2003), is the main navigation channel and the location of Figueira da Foz Harbor. The south arm (2m-4m, high tide) (Marques et al., 2003) is shallower and the water circulation depends on tidal activity and freshwater input from small tributary, the Pranto river which is controlled by a sluice and is regulated depending on the water needs in rice production (Cardoso et al., 2004). The residence time (RT) in the northern arm is 2 days and the RT in the southern arm is 9 days (Flindt et al., 1997).

The Mondego Estuary is the biggest hydrographic basin that is exclusively Portuguese and for this reason all pollutants observed have origin in Portugal. There are many stressors located upstream, as agricultura areas (Lopes et al., 2000), industrial activity, mainly cellulose and paper industry, aquaculture farms and many wastewaters treatment plant along the river.

Mondego estuary was affected by anthropogenic pressures and pollution, as (Nunes et al., 2011) documented the presence of PCDD/Fs in sediments and biota, high levels of nitrogen compounds (nitrites, nitrates, ammonia) that have origin in agricultural fertilization (Marques et al., 2003) and the presence of microplastics in fish, north arm (Bessa et al., 2018).

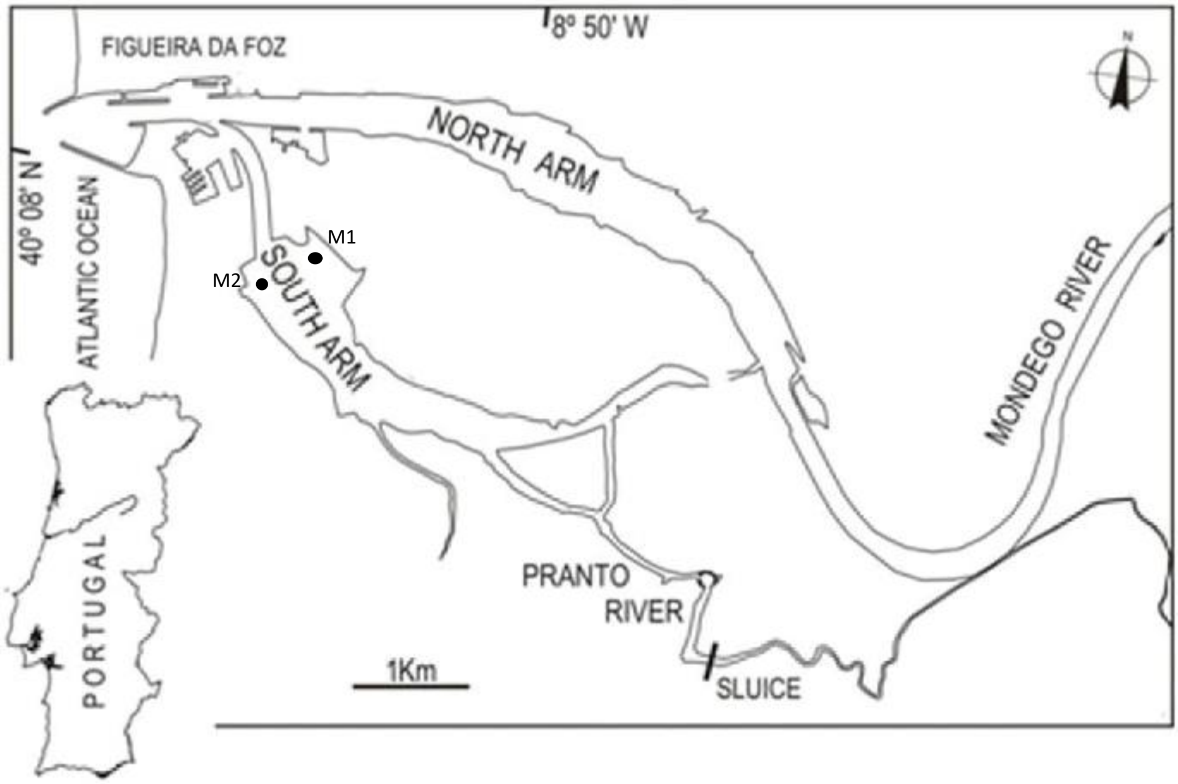

Figure 2.1- Study area, Mondego estuary, and respective sampling sites M1 and M2 in the intertidal area of the Mondego estuary (Portugal) (adapted from Costa et al., 2013).

2.2 Study species

The species were selected using some criteria based in the protocol developed by (Bessa et al., 2019b). The first criteria was the high abundance and wide distribution, the second criteria was feeding behavior, in this case, two different ways of feeding, filter-feeder and predator, and two different position in food web, the third criteria was the commercial importance, the fourth criteria was related with the abundance of studies that documented microplastic ingestion (Watts et al., 2015) and the fifth criteria was the presence of studies in literature that use the species as bio-indicator/bio-monitor (Jebali et al., 2011; Cheung et al., 2006). For these reasons the selected species were *Carcinus maenas* and *Cerastoderma edule*.

The European green crab, *Carcinus maenas* (Fig 2.2) is an omnivorous predator with diversity prey food, like molluscs, crustacean and polychaetes (Crothers, 1968; Calvez, 1987). In the food web of Mondego estuary, the green crab, is considered a main top-predator (Baeta et al., 2006) and can affect the abundance of commercially bivalves (Raffaelli et al., 1989). In this study, the crabs were selected due their importance in the food web (top-predator) and all the criteria mentioned.

Figure 2.2- Examples of individuals of *Carcinus maenas* collected from Mondego estuary presenting sexual dimorphism (A-Female; B-Male)

Bivalvia sp. have an important role in food webs in estuarine ecosystems because they are the connection between different trophic levels, they are the link between primary producers and consumers (Verdelhos et al., 2015) and the principal prey of crustaceans (Sanchez-Salazar et al., 1987) and other species such birds. Bivalvia sp. are commonly filter-feeders that can ingest phytoplankton, zooplankton and bacteria and accidentally can ingest microplastic (Hermabessiere et al., 2019), toxins and heavy metals (Pipe et al., 1999), because they are not selective.

Cerastoderma edule, the common cockle, is abundant in the Mondego estuary and it is commercially important, they are found from the North of Africa to north Europe and along European Atlantic coast (FAO, 2021), it is a suspension-feeder living in intertidal zone in the first centimeters of sediments (Nilin et al., 2012). Due to be a suspension-feeder, consequently is an unselective feeder, associated with the ability to accumulate pollutants and is widely used as bioindicator of contaminants (Domingos et al., 2007; Ricciardi et al., 2006).

If transfer of microplastic from cockle, *Cerastoderma edule*, to crabs, *Carcinus maenas,* occurs could be implications for the rest of the food web.

2.3 Sample collection

Specimens were collected by hand in the south arm of the Mondego estuary because this is a deposition zone and the water residence is higher than in north arm.

Individuals from both species, eighty individuals, samples were collected from two different sites, from each site were collected twenty-five cockles and fifteen crabs specimens, alive and not damaged and were frozen (-18ºC) whenever possible in the laboratory for further processing.

2.4 Microplastic extraction

For the common cockles, *Cerastoderma edule*, for all the individuals collected, the shell were measured and the biological material were removed from the shell, after defrosted, and weighed (g). The crabs, *Carcinus maenas,* were weighed, the carapace measured at the widest point (cm), sexed and the occurrence of females carrying eggs registered.

After defrost, the carapace was removed and digestive system and gills (Fig.2.3) were removed and placed separately, that way was possible distinguish between environmental contamination (gills) or prey contamination (digestive system).

Figure 2.3-*Carcinus maenas* during removal process of digestive system (blue) and gills (red).

The entire individuals (for common cockle) and the organs of the crabs were placed on 250ml glass beakers, one individual per glass and a potassium hydroxide (KOH 10%) was added, at least three times the volume of the sample (Fig.2.4A). The digestion process occurred during 24 h (fig.2.4B) at 40ºC and was covered with Petri dish. After digestion, the solution was passed through a 63 µm steel sieve, cleaned using distilled water, and then the digested solution were filtered using a vacuum filtration system through 1.2 µm Whatman GF/C microfiber filter and each filter were placed into a clean Petri dish, closed until stereoscope inspection was complete, and dried at 40ºC for 24h (according to the protocol of Bessa et al., 2018; Bessa et al., 2019a).

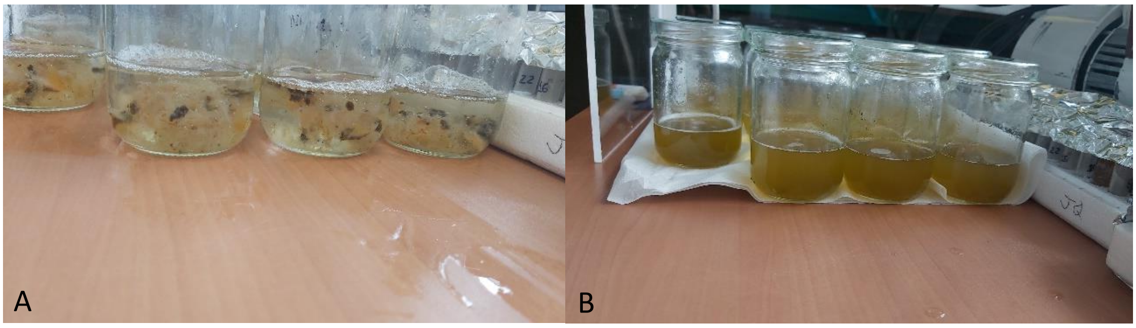

Figure 2.4- **(A)** *Cerastoderma edule* after added KOH (10%) and **(B)** *Cerastoderma edule* after 24h in KOH (10%)

The filters were visually inspected under a stereomicroscope Leica M80 in closed Petri dish, to reduce airborne contamination, to identify possible microplastics and all particles recovered were photographed using IC80 HD Camera with Leica Application suite (LAS). Particles collected were classified by color, shape (fiber, tangle of fibers, fragments and films) and were measured using ImageJ (Image Processing and Analysis in Java) an open source software.

2.5 Microplastic Verification

Visual identification is an essential step to identify potential microplastics although is prone to false identification (Lusher et al., 2017) thus microplastics must be verified.

(Kapp et al., 2018), recommend the verification techniques in at least 5% to 10% potential microplastics, but in this study, all potential microplastics were submitted to verification techniques. In the first moment, forceps were used and if particles easily broke, were exclude for the next process and were removed. The second step was the hot needle test, that consist in expose a heated needle in contact with potential microplastics, if melted are consider a synthetic particle (Campbell et al., 2017). This technique cannot identify the polymer type, which is commonly confirmed using a FTIR technique or similar, as Raman spectroscopy due to logistical constraints.

2.6 Statistical analysis

After analyzing all filters, data was reported as number of microplastics per individual and then characterized according to shape, color and size and divided in six classes: lower than 1mm, 1 to 2mm, 2 to 3mm, 3 to 4mm,4 to 5mm and higher than 5mm. A frequency table was performed to verify which class was dominant in the samples of european green crab and the common cockle. ANOVAs were used to determine if there were significant differences between the length of microplastic from European green crab and common cockle.

All data was analyzed for normality using Kolmogorov-Smirnov test and Levene's test for homoscedasticity. Statistical analysis were made using a significance level $\alpha=0.05$. The data were not normally distributed (Kolmogorov-Smirnov: $p<0.05$) and not homoscedastic (Levene's test: $p<0.05$), therefore non-parametric tests were performed. The number of microplastic were compared between species using permutational multivariate analysis of variance (PERMANOVA test) (Anderson, 2001). Kruskal-Wallis test were performed to assess significant differences between gills and digestive system from *Carcinus maenas*. All tests were performed using Past software. Spearman correlation analyses were performed to test possible relations between width (carapace and shell) and the number of microplastics in each species and between weight and the number of microplastics. Subsequently, Spearman correlation tests were performed, analyzing males and females separately.

Statistical analyses were made using significance level $\alpha=0.05$. The data were analyzed using Past software.

2.7 Contamination controls

To avoid any airborne contamination by fibers the samples were analyzed in a clear and restricted laboratory room and nitrile gloves and cotton coats were used during specimen and sample handling to reduce possible airborne contamination. In addition, all laboratory surfaces, materials (glass materials were submitted to 1% nitric acid bath) and equipments were clean using distilled water and ethanol. Even with these procedures, airborne contamination may occur and to quantify this contamination blank filters in Petri dish were placed in the laboratory during samples processing and used as control. During the digestion process was used one control glass beaker, only with the solution KOH 10% and then filtered as described before.

In the end of each day of processing all blank filters were analyzed and if exist contamination only the individuals processed on that day will be affected by this contamination and the number of particles/fibers were subtracted to the total number of particles founded in filters of the samples

The specimens were only manipulated with glass or metal materials.

Chapter III- Results

3.1 Occurrence of microplastic in *Cerastoderma edule* and *Carcinus maenas*

3.2 Characterization of microplastics

3.3 Comparison between *Carcinus maenas* and *Cerastoderma edule*

3.4 Comparison between Females and Males of *Carcinus maenas*

3.5 Comparison between organs of *Carcinus maenas*

3.1 Occurrence of microplastics in *Cerastoderma edule* and *Carcinus maenas*

A total of 142 microplastics (Table 3.1) were found in, *Carcinus maenas* and *Ceristoderma edule*), two species from the Mondego estuary (Table 3.1) (Figure 3.1). Of the two species collected from south arm of Mondego estuary, 63 of 80 individuals (78.75%) had ingested microplastics, 27 of 30 green crab (90%) and 36 of 50 common cockle (72%), with a maximum of 9 microplastics in one individual (*Carcinus maena*).

Table3.1 Number of sample size (N), number (M) and frequency of occurrence of microplastic found from *Carcinus maenas* and *Cerastoderma edule*

Species	Sample size N	Average individual weight (g)	Average individual length (mm)	Number of microplastic M	Frequency of MP occurrence (%)	Mean microplastics *per* individual
Carcinus maenas	30	49.42±11.38	54.633±4.84	73	90	2.43±1.91
Cerastoderma edule	50	3.82±0.67	24.840±1.57	69	72	1.38±1.23

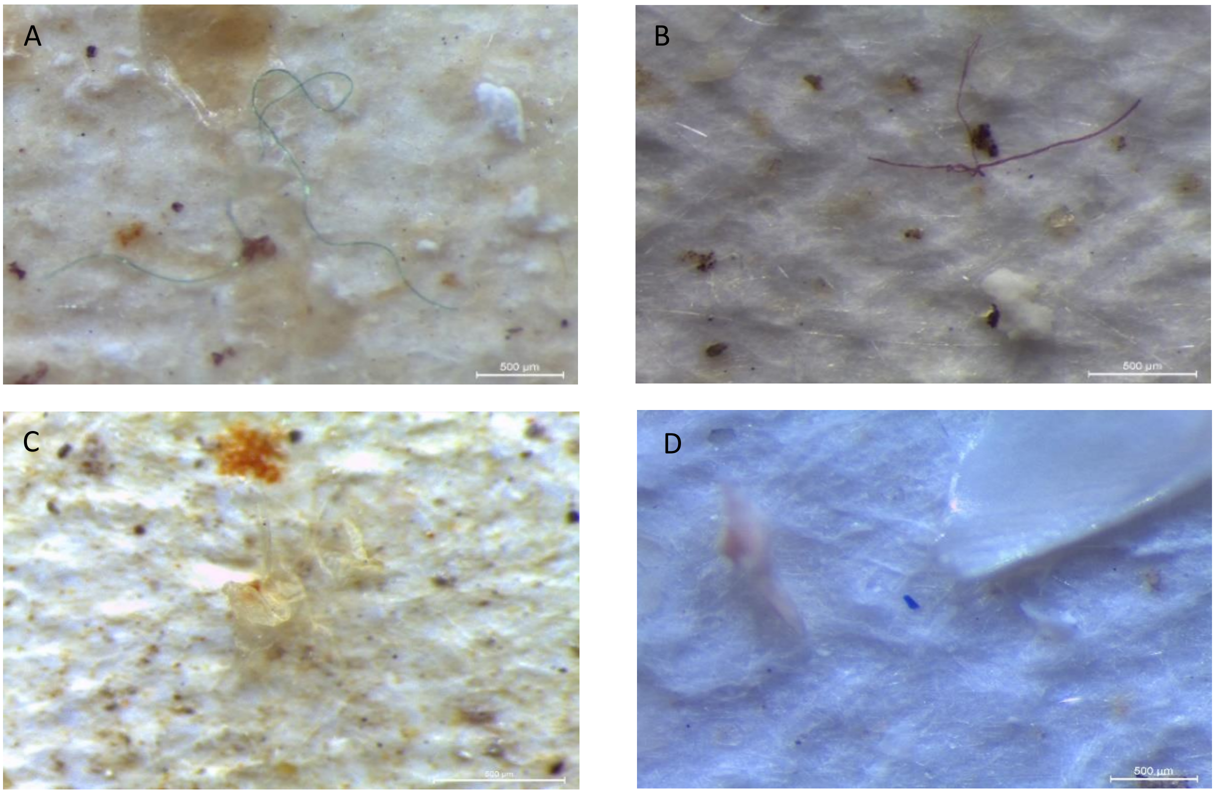

Figure 3.1- Examples of microplastics collected from *Carcinus maenas* and *Cerastoderma edule* representing the four shapes found: (A) green fiber, (B) red tangle of fibers, (C) white film and (D) blue fragment.

3.2 Characterization of microplastics

A total of 142 microplastics were recovered from both species and characterized according to their shape (Fiber, fragment, film, tangle of fibers), color and length.

In total, microplastics were categorized as fibers (84.5%), followed by fragments (11.1%), films (2.08%) and tangle of fibers (2.08%) (Figure 3.2). The color distribution of those particles was similar on two species, being blue the most common color (63.19%), followed by red (20.83%), green (6.94%), black (5.56%) and white (2.08%). In common cockle the predominant microplastic type was fibers (87%), followed by fragments (9%) (Figure 3.3B). As mentioned above, blue was the most common color (70%), followed by red (22%) and the other colors as white, green and black were above 5% each (Figure 3.4A). The microplastics recovered in green crab, were mainly fibers (82%), but we found also, fragments (14%), films (3%) and tangle of fibers (1%) (Figure 3.2A). A similar pattern was also found for the green crab blue was the most common color (59%) followed by red (21%), green (11%), black (7%) and white (3%) (Figure 3.4B).

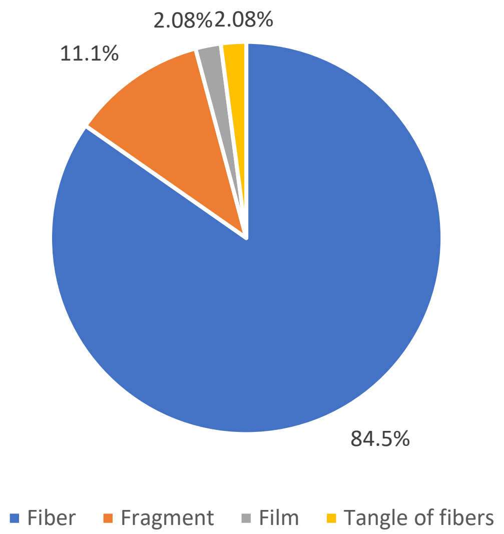

Figure 3.2-Microplastic shape distribution collected from *Carcinus maenas* and *Ceratoderma edule*.

Items recovered from common cockle ranged from 0.052mm to 4.271mm (average:1.94 ± 1.02 mm). 87% of items were fibers ranging from 0.067 to 4.21mm. Items recovered from green crab ranged from 0.079mm to 6.313mm (average:1.77 ± 1.51 mm) and 82.2% of items were fibers (Figure 3.3).

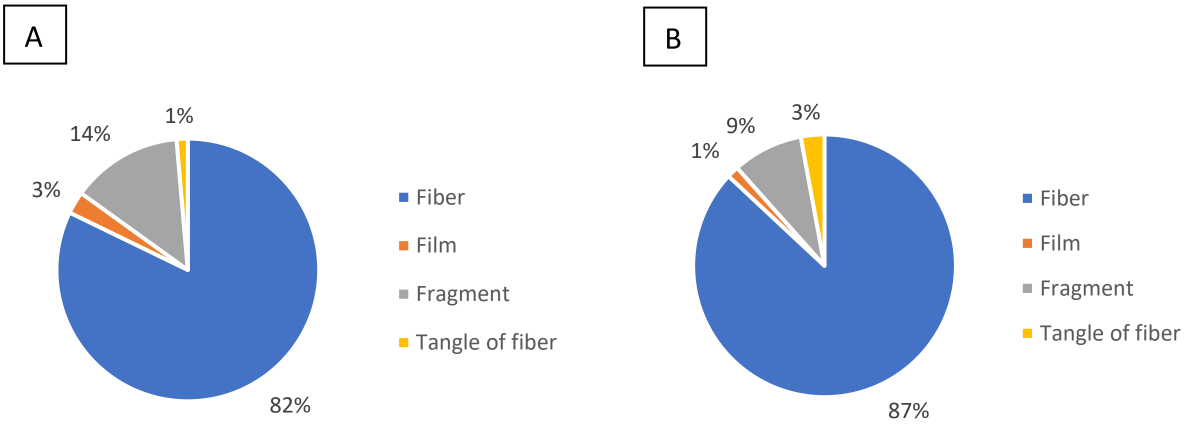

Figure 3.3- Microplastic shape distribution extracted from (A) *Carcinus maenas* and (B) *Cerastoderma edule*.

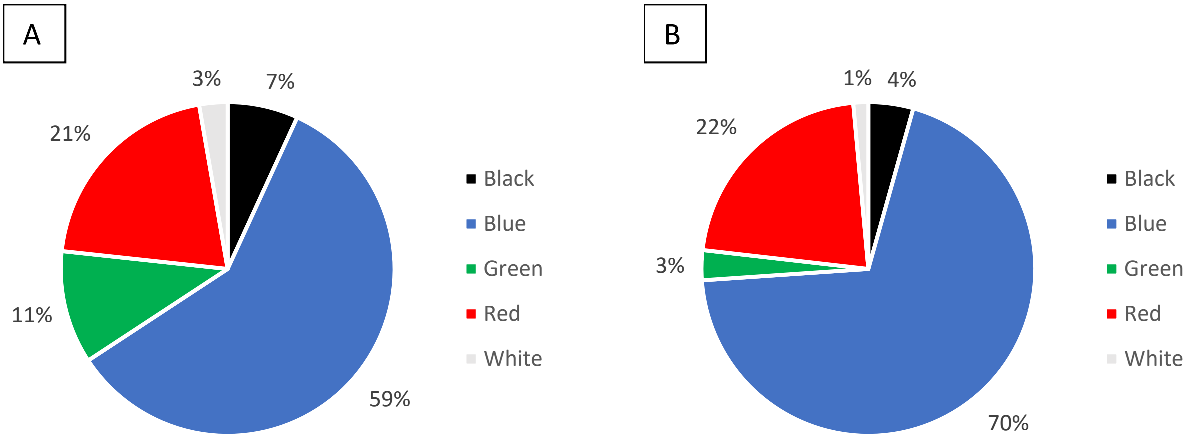

Figure 3.4- Microplastic color distribution from (A) *Carcinus maenas* and (B) *Cerastoderma edule.*

3.3 Comparison between *Carcinus maenas* and *Cerastoderma edule*

When analyzing the number of microplastic collected from green crab and common cockle (Figure 3.5), significant differences were found in the number of microplastics between the two species (PERMANOVA: pseudo-F=8.764 and p=0.0038), the number of microplastics in green crab (M=73) were higher when compared with the number of microplastics in common cockle (M=69). However, the number of microplastic per gram (MP/g) were higher in common cockle (0.361 MP/g) than in green crab (0.049 MP/g) (Table 3.2).

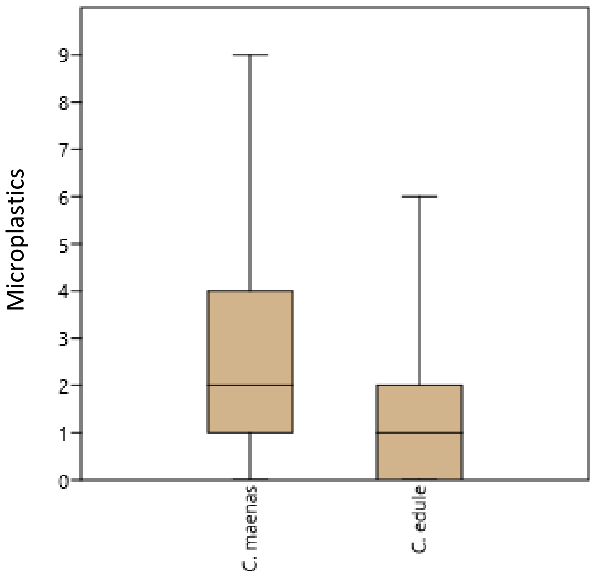

Figure 3.5- Boxplot comparing microplastic distribution between *Carcinus maenas* and *Cerastoderma edule*.

Table3.2 Total number of microplastics (M) and respective number of Microplastics per gram (MP/g).

Species	Total number of microplastics (M)	Total weight (g)	Mean Microplastic per gram (MP/g)
Carcinus maenas	73	1482.53	0.049
Cerastoderma edule	68	191.1	0.361

The microplastic collected from both species, after type and color characterization, were divided in classes according to the size (Figure 3.7). The most common class of microplastics found in green crab is <1mm (36.99%), followed by 1 to 2 mm (34.5%) and the less frequent class was >5mm (2.74%). In common cockle, the most common class was 1 to 2 mm (42%), followed by 2 to 3 mm (24.64%), the less frequent class was >5mm (0%). The classes followed a normal distribution (Kolmogorov-Smirnov; p>0.05), consequently, an ANOVA test were performed and significant differences were found (ANOVA test: F=7.004; p=0.01726).

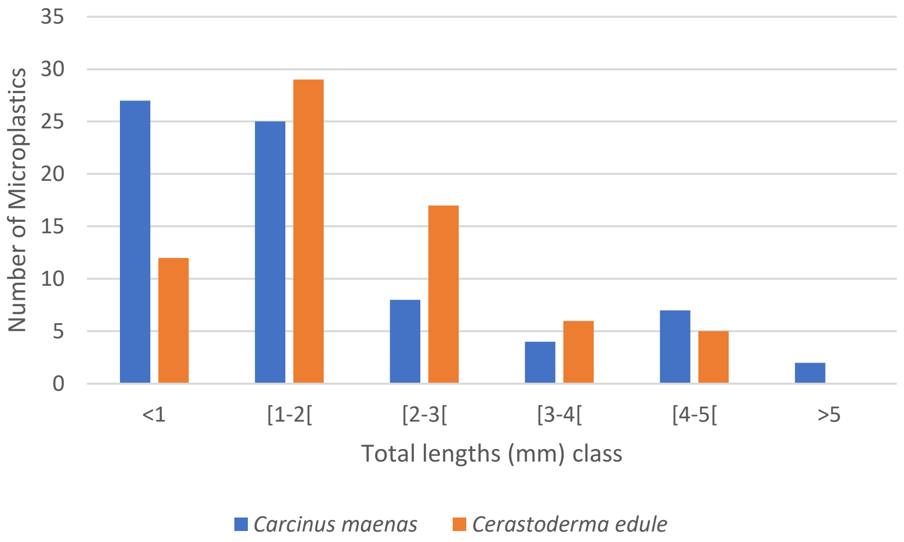

Figure 3.6- Comparison between classes of microplastics length (mm) by number of microplastics from *Carcinus maenas* and *Cerastoderma edule*

3.4 Comparison between Females and Males of *Carcinus maenas*

30 green crabs (*Carcinus maenas*) were collected from Mondego estuary and 73 microplastics were extracted from their contents. From the total, 13 were females and 17 were males (Figure 3.7). For the total number of males, there were observed 35 microplastics in the digestive system and 8 microplastics in their gills. In females, there were observed 23 microplastics in digestive systems and 7 microplastics in gills.

Nevertheless, were no significant differences in the number of microplastics between males and females (Kruskal-Wallis: H=0.7382; p=0.3902) was observed.

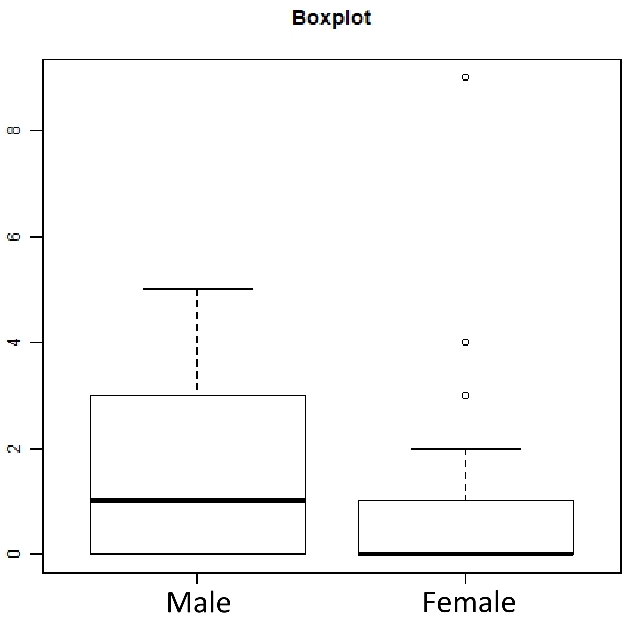

Figure 3.7- Boxplot comparing microplastic distribution between *Carcinus maenas* genre

Additionally, a Spearman correlation test was performed, to verify a possible link between the size of individuals (using the carapaces carapace width as a proxy) and microplastic ingestion and the test revealed a positive correlation between carapace width and microplastic ingestion (Spearman's test: rho=0.67; $p<4.92*10^{-5}$).

In the order hand, the Spearman correlation test between the weight and microplastic (Spearman's test: rho=-0.216; p>0.05) showed no significant correlation (Table 3.3).

Table 3.3 Spearman tests to assess possible relations between MP*Width and MP*Weight for males and females

Correlation	r	p
MP*WidthM	0.61	<0.01
MP*WeightM	-0.62	<0.01
MP*WidthF	0.082	<0.001
MP*WeightF	-0.25	>0.4

The Table 3.3 showed a correlation in MP*WidthF and MP*WidthM as expected, but showed a negative correlation in MP*WeightM when microplastic ingestion increase, the weight of male crab decrease.

3.5 Comparison between organs of *Carcinus maenas*

Microplastics were found in both organs analyzed for the green crab, the stomach and gills (Figure 3.8) with 15 microplastics collected from gills and 58 from digestive system resulting in 90% of green crab contained at least one microplastic in their digestive system and 46.7% containing microplastic in their gills. The mean number of microplastics in the digestive system was 2±1.59 microplastic per individual and in the gills was 0.5±0.56 microplastic per individual. In the total of 58 microplastics recovered from the digestive system, the predominant type was fibers (81%), followed by fragments (14%), films (3%), tangle of fibers (2%) and in gills the predominant types were fibers (87%) and fragments (13%).

Analyzing the data from gills and digestive system significant differences were found (Kruskal-Wallis: H=18.34; p<0.01).

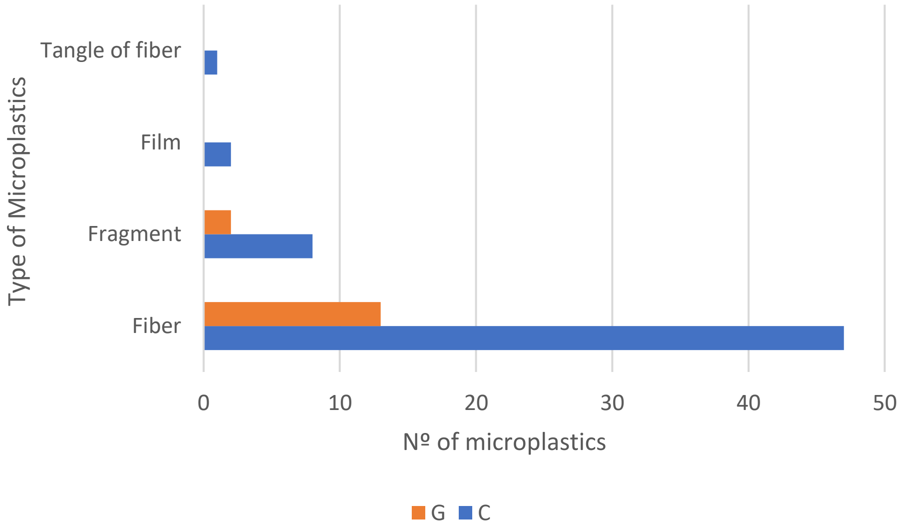

Figure 3.8- Comparison between microplastic shape and *Carcinus maenas* organs (G-gills, C-digestive system).

Chapter IV- Discussion

4.1 Feeding mode and microplastics distribution

4.2 Types and characteristics of microplastics observed in *Ceristoderma edule* and *Carcinus maenas*

4.3 Microplastic pollution in resident species from the Mondego estuary

4.4 Bioindicator species

4.5 Final remarks and future recommendations

The main goal of my thesis was to assess, for the first time, the occurrence of microplastics in two different species occupying different niches in the food web of an estuarine environment. The species selected were a filter-feeder, *Ceristoderma edule*, and a top-predator, *Carcinus maenas,* both collected from the Mondego estuary during summer 2021. In addition, the study aims at assessing if there was an interrelationship of microplastic pollution among them. In the literature it is well known, the importance of estuaries to the transport of microplastics from land to sea and due to the characteristics of these ecosystems, microplastics tends to remain in these habitats for long periods increasing the probability of ingestion by several species (Vermeiren et al., 2016).

The results of this study showed the presence of microplastics in green crab (90%) and common cockle (72%) and confirms their ubiquity in the Mondego estuary (Portugal).

4.1 Feeding mode and microplastics distribution

The present study revealed that common cockle and green crab, collected from the Mondego estuary, ingested microplastics, followed previous results from studies that reported microplastic pollution in Portuguese estuaries (Bessa et al., 2018; Rodrigues et al., 2019; Lourenço et al., 2017) and in green crab and common cockle in other habitats (Vital et al., 2021; McGoran et al., 2020; Cozzolino et al., 2021). Comparing these results with the literature, it is possible to verify that the number of microplastics obtained in the present study for the green crab (90%) was higher than those reported from Ria Formosa lagoon (Vital et al., 2021) that, only one microplastic were found. The present results are more related with the percentage obtained from C. *maenas* collected in the Thames estuary by McGoran et al. (2020) with 71.3% of individuals with microplastics.

As far as I know, my study is the first to detect microplastic pollution in crabs from Portuguese estuaries and from the Mondego Estuary, with noticeable levels of microplastics debris in their content.

Regarding the levels of microplastics pollution recorded in this study for the common cockle (72%) is lower than the results obtained in (Cozzolino et al., 2021) in Ria

Formosa lagoon, Portugal (100%) and lower than in Tejo estuary, Portugal (90%) (Lourenço et al., 2017).

In the literature, the effect of feeding mode on microplastic frequency in species is somehow contradictory. The number of microplastic obtained for the Baltic Sea (Setala et al., 2016) was higher in filter-feeders than in predators in contrast to (Bour et al., 2018) that found more microplastics were found in *Enchelyopus cimbrius,* fish from bottom dwellers, and *Crangon allmanni,* is a shrimp that lives sandy or muddy sea bottom, from shoreline close to a small marina at Jeloya, Norway comparing with filter-feeders, from the same site. These results are in line with the results obtained in this thesis. Microplastics size observed in common cockle (1.94±1.02 mm) were higher than microplastics in green crab (1.77±1.51 mm)*,* the differences in prey sizes can explain these results, crabs ingest larger preys then cockle, mostly phytoplankton. Feeding mode are a variable that affects microplastics ingestion. When comparing omnivorous, predatory and filter-feeder crabs, omnivorous were the most contaminated by microplastic, followed by predatory crabs and finally filter-feeder crabs (Not et al., 2020). Like green crab are omnivorous are more vulnerable to plastic ingestion.

In this study, significant differences were found between filter-feeders (C. *edule*) and predator (C. *maenas*) in the number of microplastics, concluding that feeding mode may have influence the uptake of microplastics, these results are in agreement with the results of Bour et al. (2018).

4.2 Types and characteristics of microplastic observed in *Ceristoderma edule* and *Carcinus maenas*

The only comparable studies that observed microplastic pollution in crabs from Portugal are studies of *Eriocheir sinensis* (15.6%) (Wójcik-Fudalewska et al., 2016) and the only study using green crab, the microplastic occurrence was 0% (Vital et al., 2021).

Before this study, microplastic ingestion in Mondego estuary had been recorded only for fish (Bessa et al., 2018).

From the total 142 microplastics observed in common cockle and green crab in this study, fibers were the predominant type of microplastic extracted, presenting a 84.5%

frequency of occurrence, followed by fragments with 11.10% frequency of occurrence. These results are in line with those reported for the Mondego estuary, in commercial fish, with 96% of the microplastics detected were also fibers (Bessa et al., 2018). But this fact is also demonstrated for several estuarine environments and marine systems, for example, in green crab from the Thames estuary, 78% of microplastics observed were fibers, in fish from Charleston Harbor, estuary on southeastern Atlantic coast of the United States of America,77.4% of microplastic observed were fibers (McGoran et al., 2020, Parker et al., 2020).

This result can be explained by the hypothesis described in (Jabeen et al., 2017), that consider that transitional systems are more prone to fiber contamination due to the proximity to point discharges of WWTPs and along the Mondego River, the presence of WWTPs (considered a significant source of fiber pollution) are constant because two cities are in the vicinity of the estuary.

The predominant color observed from microplastics were identical to other studies, with blue (63.19%) being the most common color followed by red (20.83%) and green (6.94%) (Duncan et al., 2019, Giani et al., 2019).

The high percentage of blue color can be explained by the breakdown of trawl nets or lost fishing equipments in estuary (Bessa et al., 2018).

4.3 Microplastic pollution in resident species from the Mondego estuary

Despite that plastic ingestion and their effects had been widely reported in laboratory conditions (Watts et al., 2014), the knowledge is reduced about ingestion in the wild, with no evidences from the Mondego estuary regarding those species. The microplastics observed from green crabs could have originated from their habitat and prey. Microplastic pollution were found in gills and digestive system of the green crab analyzed, demonstrating that microplastics may enter the individuals directly from the environment from respiration and/or via prey consumption. In this study, 14 of 30 gills samples were contaminated with microplastics, corresponding to 47%. In digestive system, 25 of 30 samples had microplastics, corresponding to 83%, suggesting that digestive system are more prone to microplastics pollution than gills.

Microplastics recorded in gills could have been retained during irrigation of the gills with contaminated water and the most common type was fibers (87%; M=13), suggesting that fibers were abundant in Mondego estuary. Green crab have the ability to uptake and retain microspheres (10µm) (Watts et al., 2014). The mean microplastic pollution on the gills was low, with in general, one microplastic per individual and in digestive system was two microplastics per individual. Consequently, it is possible to consider the ingestion is the main way of exposure. Murray and Cowie, (2011), suggested that most of the plastic ingested by crabs in Clyde Sea is derived from fishing gears. This hypothesis is also possible for the Mondego estuary, due to the presence of fishing activities, aquacultures and industrial areas.

No significant differences were found between males and females, although males (58.9%) ingest more microplastics than females (41.1%). The green crab present sexual dimorphism, females are smaller than males (carapace width, claw, mouth parts) that can result in decrease of food ingestion rate and consequently reduce the microplastic ingestion probability (Wójcik-Fudalewska et al., 2016). There was a correlation between carapace width and microplastic ingestion (Spearman's test: $rho=0.67$; $p<4.92*10^{-5}$), that proof size can be a deciding factor to microplastics ingestion. A negative correlation was established between weight and microplastics ingestion in males (Spearman's test: $rho=-0.62$; $p<0.01$), this are in line with the results obtained by (Watts et al., 2015), that reported decline in their growth potential and reduced feeding in green crabs. Although significant differences were detected, is necessary evaluated with higher sampled sizes and individuals from different sizes.

4.4 Bioindicator species

The two analyzed species could be potential bioindicators for microplastics pollution due biological and ecological characteristics, widely geographic distribution and they have a great commercial and economic interest. The common cockle is an unselective feeder then are susceptible to pollutants, including microplastics.

The green crab is a potential bioindicator for microplastics pollution since it is vulnerable to microplastic pollution, 90% of individual had at least one microplastic, have a great dietary flexibility (Crothers, 1967; Chaves et al., 2010), as molluscs, crustaceans and polychaetes.

Common cockle and green crab are abundant in their habitats and prone to microplastics and their ecology are well documented (Baeta et al., 2006; Rufino et al., 2010).

The frequency occurrence of microplastic was higher in green crab than common cockle and in the particular for the green crab, it is possible to establish a profile of microplastic pollution in the food chain (analyzing digestive system) and in the water (analyzing their gills) and might be sensitive to microplastic pollution (negative correlation between weight and microplastic). In addition, since it is considered a model organism used as bioindicator to heavy metals and other contaminants (Rodrigues and Pardal, 2014; Leignel et al., 2014) could be a relevant bioindicator for microplastic pollution in transitional environments.

4.5 Final remarks and future recommendations

In my thesis, microplastics were documented in two resident species from Mondego estuary, typically in the form of microfibers possibly coming from wastewater treatment plants or fishing gears. For the first time microplastic were observed in crabs from Mondego.

More microplastics and higher frequencies of occurrence were observed in green crab than in common cockle*,* however common cockle has higher value of MP/g, consequently more microplastics in predator than filter-feeder. It is possible that these microplastic could be transferred via predation to other animals in the Mondego estuary.

Microplastic pollution were noted in digestive system and in gills. Microplastics levels in green crab were higher in digestive system than gills, suggesting ingestion is the main way of interaction between this species and microplastics.

This study suggest green crab could be good bioindicator to microplastic pollution because it is widely distributed and have commercial interest. Although more studies are necessary using sediments and water analysis and compare with green crab to understand if the microplastics levels in green crab are similar with the sediments which are considered a microplastics sink.

In the future, it is recommended a higher number of individuals for assessing temporal and spatial trends and including chemical analysis (µ-FTIR) to identify polymer types.

Despite these limitations, this master thesis try to fill the gap of research in transitional zones from Portugal and with this study it is encouraged to improve knowledge of microplastics effects in biota from transitional zones and how microplastics can affect the balance of food chain.

In addition, to prove that there are microplastic transfer in food chain of Mondego estuary is important assess all trophic level, from plankton to birds.

Further research is also needed to understand the effects of microplastics ingestion in biota and potential effects to humans health, in order to prepare programs to mitigate the plastic pollution in these ecosystems.

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