EFFECTS OF THE PRESENCE OF MICROPLASTIC PARTICLES IN PORTUGUESE COASTAL WATERS AND MARINE MUSSELS

Thesis submitted to the Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia for the degree of Doctor of Philosophy in Environmental Sciences (Environment Doctoral Programme).

Supervisor: Maria Paula Oliveira Sobral, PhD, FCT-UNL
Co-supervisor: Richard C. Thompson, PhD, Plymouth University

Panel jury:
President:
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Monte de Caparica, 2015
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
João Pedro Garcez Luís de Frias  
Master's degree (MSc)

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Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
DEDICATED TO

All the women who are part of my life,

Both family and friends.
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
INSPIRATIONAL QUOTES AND POEMS TO RAISE AWARENESS

Blue sea,
Blue sky,
Nothing other than the color of peace in there,
Just blue that embraces the Earth,
Teaching us that the world is one.

Poem by unknown Japanese author

“Dans la nature rien ne se crée, rien ne se perd, tout se transforme.”

Antoine Lavoisier

“When eu nasci, as frases que hão-de salvar a humanidade já estavam todas escritas, só faltava uma coisa - salvar a humanidade.”

José de Almada Negreiros
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
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ABSTRACT

Marine litter is one of the most pervasive environmental pollution problems that is currently affecting marine ecosystems worldwide. Synthetic plastic materials are the most common type of marine debris which according to worldwide statistics represent approximately 80% of litter stranded on beaches. Inside marine litter, microplastics represent the vast majority of findings (98%) in the Portuguese coastal area. Microplastics can cause severe impacts to wildlife through entanglement or particle ingestion and to commercial shipping, through block propellers. Plastics can also affect tourism in coastal areas by direct revenue loss.

This thesis manuscript constitute a novel approach to the marine litter problem, as it focus on microplastics in the Portuguese coastal area and surface waters. Microplastics are fragments or particles with a diameter below 5mm. The most common type are resin pellets that represent 60% of the plastic materials collected in beach monitoring surveys, over a three year period (2010-2013). Pellets are divided in four classes: white, aged, colour and black. Polymer type were identified through Fourier transform infrared spectroscopy technique (μ-FTIR), and the most common polymers were polyethylene (low and high density), polypropylene, mixtures of polyethylene and polypropylene, polyacrylates and polyurethane, being this last type only identified in black resin pellets. Marine litter is widely distributed throughout the Portuguese coast and its accumulation depends on season and weathering conditions. The beaches with higher densities are Matosinhos (362 items m⁻²), Viera de Leiria (332 items m⁻²) and Sines (84 items m⁻²), which are located near ports and industrial facilities. Regarding persistent, bioaccumulative and toxic chemicals (PBTC), aged pellets accumulated higher concentrations of pollutants, probably due to a longer exposure time in the environment. PBTC concentrations were variable, with PAH concentrations ranging between 53 and 44800 ng g⁻¹, PCB from 2 to 223 ng g⁻¹ and DDT ranging between 0.42 and 41 ng g⁻¹.

Laboratorial bioassays with marine mussels were also conducted as part of this work in order to estimate the effects of microplastic ingestion, using environmentally relevant particle concentrations (10, 100 and 1000 particles ml⁻¹), which ranged in size from 2 to 10 μm. Separate bioassays, one only with non-contaminated microparticles and another with non-contaminated and PAH contaminated particles were conducted. For the first one, no significant histopathological changes were identified which demonstrate mechanical abrasion of the tissues for the bioassay using different concentrations of non-contaminated polystyrene microparticles.

Regarding the acute contamination bioassay with PAH contaminated particles, and non-contaminated particles, results show that contaminated particles exert a higher effect in histopathology and biochemical biomarker levels, resulting in higher lipid peroxidation (LPO) in the digestive gland, with significant tubule alterations. When compared to the gills, the digestive gland of the mussels is more affected in LPO and histopathological alterations. Total glutathione levels are inversely proportional to LPO concentration, which is also visible in the results. Chronic bioassays with the same species, pollutant and polymer type are extremely important in order to evaluate long-term effects on mussels.

Two strategic management approaches to minimise the problem that marine litter represents are also included in this work. A Strength-Weakness-Opportunity-Threat (SWOT) matrix analysis and a
Drivers-Pressures-State-Impact-Response (DPSIR) model were created and developed to raise awareness and propose solutions. Awareness and outreach campaigns allied with best available practices and different economic models (circular economy) will not only engage stakeholders to take actions, but also to make safer and well informed choices. Beach clean-up activities, lectures on education for sustainable development (ESD) and campaigns to reduce plastic consumption are extremely important to minimize the problem. Solutions can only be reached with the involvement all stakeholders from management, policy, industry, fishing and maritime activities, surfers, educators, scientists and the general public.
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
RESUMO

O lixo marinho é um dos maiores problemas ambientais que atualmente afeta os ecossistemas marinhos mundiais. Materiais sintéticos de plástico encontram-se entre o tipo de lixo marinho mais comum, que de acordo com estatísticas mundiais representa cerca de 80% dos detritos recolhidos em praias. Dentro do lixo marinho, os microplásticos representam a larga maioria das incidências na área costeira Portuguesa (98%). Os microplásticos causam impactes severos à vida marinha através de aprisionamento ou ingestão de partículas; e à navegação comercial, através do bloqueio das hélices. O plástico também afeta o turismo nas áreas costeiras através da perda direta de receitas.
Este manuscrito de tese constitui uma nova abordagem ao problema do lixo marinho, uma vez que se foca em microplásticos na área costeira Portuguesa e águas superficiais. Os microplásticos são fragmentos ou partículas com diâmetro inferior a 5mm. O tipo mais comum são pastilhas de resina (vulgarmenente conhecidos por pellets), que representam 60% de todos os materiais de plástico recolhidos em campanhas de monitorização de praia num período de três anos (2010-2013). Os pellets pertencem a quatro classes: brancos, envelhecidos, coloridos e negros. Os tipos de polímero foram identificados através de uma técnica de espectroscopia de infravermelho com transformada de Fourier (μ-FTIR), e os polímeros mais comuns são polietileno (alta e baixa densidade), polipropileno, misturas de polietileno e polipropileno, poliacrilotos e poliuretano, sendo que este último, apenas foi identificado em pastilhas de resina negra.
O lixo marinho encontra-se amplamente distribuído por toda a costa Portuguesa e a sua acumulação depende da estação do ano e das condições meteorológicas. As praias com maior densidade são Matosinhos (362 items m⁻²), Vieria de Leiria (332 items m⁻²) e Sines (84 items m⁻²), que se encontram localizadas perto de portos e indústrias.

Em relação aos químicos persistentes com potencial tóxico e de bioacumulação (PBTC), os pellets envelhecidos acumulam maiores concentrações de poluentes, provavelmente devido a longos períodos de exposição no ambiente. As concentrações de PBTC são variáveis, sendo que para os PAH a concentração varia entre 53 e 44800 ng·g⁻¹, para os PCB entre 2 e 223 ng·g⁻¹ e para o DDT entre 0,42 e 41 ng·g⁻¹.
Ensaios laboratoriais com mexilhões marinhos foram ainda realizados como parte deste trabalho, de modo a estimar os efeitos da ingestão de microplásticos, usando concentrações ambientalmente relevantes de partículas (10, 100 e 1000 partículas ml⁻¹), que tinham tamanhos compreendidos entre 2 e 10 μm.
Foram realizados distintos ensaios, um usando somente partículas não contaminadas e outro com partículas contaminadas por um PAH e partículas não contaminadas.

Em relação ao primeiro ensaio, não foram encontradas mudanças significativamente estatísticas na análise histopatológica que demonstre a abrasão mecânica dos tecidos para o ensaio usando diferentes concentrações de micropartículas de poliestireno não contaminadas.
Em relação ao ensaio de contaminação aguda realizado com partículas não contaminadas e contaminadas com PAH, os resultados demonstram que as partículas contaminadas exercem um maior efeito na histopatologia e biomarcadores bioquímicos, resultando em valores altos de peroxidação lipídica (LPO) na glândula digestiva, com alterações nos túbulos significativas.
Quando comparadas com as brânquias, a glândula digestiva dos mexilhões é mais afetada pela LPO e por alterações histopatológicas. Os níveis de glutatonia total variam em proporção inversa em relação à concentração de LPO, o que é possível de verificar através dos resultados. Ensaios crónicos com a mesma espécie, poluente e tipo de polímero são extremamente importantes para avaliar os efeitos a longo prazo nos mexilhões.

Duas abordagens de gestão estratégica para minimizar o problema que o lixo marinho representa são ainda incluídas neste trabalho. Uma matriz de análise de Forças-Fraquezas-Oportunidades-Ameaças (SWOT) e um modelo de Forças motrizes-Pressões-Estado-Impacte-Resposta (DPSIR) foram desenvolvidos para consciencializar e propor soluções para mitigar o problema do lixo marinho.

Consciencialização e campanhas de comunicação aliadas com boas práticas e diferentes modelos económicos (economia circular) irão não só comprometer os atores sociais a tomarem ação, mas também a fazerem escolhas melhores e bem informadas.

Atividades de limpeza de praia, palestras sobre educação para o desenvolvimento sustentável (ESD) e campanhas para reduzir o consumo de plástico são extremamente importantes para reduzir este problema. As soluções apenas podem ser alcançadas com o envolvimento de todos os atores sociais de gestão, política, indústria, atividades piscatórias e marítimas, surfistas, educadores, cientistas e o público em geral.
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ACRONYMS AND ABBREVIATIONS

» μm – Micrometre (10\(^{-6}\) m)
» ALDFG - Abandoned, Lost or Otherwise Discarded Fishing Gear
» BaP – Benzo(a)pyrene
» BeP – Benzo(e)pyrene
» CIS – Commonwealth of Independent States
» DDD – Dichlorodiphenyldichloroethylene
» DDE – Dichlorodiphenyldichloroethane
» DDT – Dichlorodiphenyltrichloroethane
» EEA – European environmental agency
» ER – Epoxy resin
» EU – European Union
» ESD – Education for sustainable development
» FTIR - Fourier transform infrared spectroscopy
» GES – Good environmental status
» GSSG – glutathione disulphide
» GSHt – total glutathione
» HDPE – High density polyethylene
» LDPE – Low density polyethylene
» LPO – lipid peroxidation
» MARPOL - International Convention for the Prevention of Pollution from Ships
» MSW – Municipal solid waste
» m.y.o. – Million years old
» NAFTA – North American Free Trade Agreement
» PA - Polyamides
» PAH - Polycyclic aromatic hydrocarbons
» PBTC – Persistent, bioaccumulative and toxic chemicals
» PCB - Polychlorinated biphenyls
» PE - Polyethylene
» PET – Polyethylene terephthalate
» POP – Persistent organic pollutants
» PP - Polypropylene
» PS – Polystyrene
» PUR - Polyurethane
» PVC – polyvinyl chloride
» ROS – reactive oxygen species
» TBARS – thiobarbituric acid-reactive species
» WHO - World Health Organization.
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
1 General Introduction

One of the early examples of environmental awareness in society happened after Rachel Carson’s *Silent Spring* was published in 1962. The book described, among many relevant environmental man-made changes in ecosystems, the long-lasting effects and impacts of pesticides in wildlife, creating debate in society about ecosystem health. Since then, scientists and researchers have addressed and approached environmental problems such as chemical pollution, biodiversity, soil, air and water pollution, climate change and recently, plastic marine pollution. Using available methodologies and technologies to gather accurate data, scientists and researcher use that information to propose or create measures and recommendations that will enable solutions for each of these environmental problems.

Recently, mass media and the scientific community have focused on ocean pollution, particularly in floating marine litter. Although there are records of ocean pollution in the literature since the 1970’s, it was only after Captain Charles Moore discovered an accumulation zone in the North Pacific Central Gyre, in 1997, that marine litter was considered as a global problem. Though composed of different materials, plastic represents a significant share of all marine litter floating in the oceans.

In order to provide an introduction, particularly to those who are not familiar with the topic, this chapter will focus on: (1) plastic as marine litter and as vector for transport of persistent bioaccumulative and toxic chemicals; (2) ecologic and economic relevance of the Mediterranean mussel (*Mytilus galloprovincialis*) as a bioindicator species; (3) the importance of biochemical biomarkers and their relevance to this study and (4) the Marine Strategy Framework Directive (MSFD) in Europe, regarding marine litter.
1.1 Plastic as marine litter and as vectors for transport of persistent bioaccumulative toxic chemicals

1.1.1 Definition, history, production, sources and impacts

The presence of plastics in the marine environment is a common phenomenon that has been highlighted in recent decades in scientific reports, publications and even in the mass media news. Accumulation of marine litter in coastal and marine environments at a global scale, is mostly related to the excessive consumption of goods, mainly in developed countries, inappropriate waste management and low recycling rates of plastic products (Andrady and Neal, 2009; Ten Brink et al., 2009; Hopewell et al., 2009; Plastics Europe, 2011; Andrady, 2011).

According to the United Nations Environmental Programme (UNEP) and to the European Commission Task Group for the descriptor 10 of the Marine Strategy Framework Directive (hereinafter MSFD), marine litter is described as any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment. It consists of items that have been made or used by people and deliberately discarded into the sea, rivers or deposited on beaches as well as brought indirectly by winds, river run-off, drainage, sewage and storm waters or unintentionally lost at sea or coastal areas (UNEP, 2009; Galgani et al. 2010). Pursuant to this definition, marine litter consists of a wide range of materials, such as plastics, processed wood, metal, glass, clothes, rubber, and materials related to medicine. Semi-solid remains of vegetable oils, paraffin and chemicals are not included in the definition of marine litter within MSFD (Galgani et al. 2010).

It is estimated that 70 - 80% of plastic in the marine environment derive from land sources (Bowmer and Kershaw, 2010), with industrial or densely populated areas contributing mainly with plastic packaging (Derraik, 2002). Marine and maritime sources are responsible for 30-20% of low density floating plastics that result either from unintentional release by ships, degradation of macro plastics or natural disasters such as earthquakes and tsunamis (Pichel et al., 2007; Corcoran et al., 2009; Lebreton and Borrero, 2013).
Plastics materials are defined as semi-synthetic or synthetic materials that are shaped to adopt a three-dimensional stable form and that can be modified with chemical additives (Shashoua, 2008). According to moulding properties, plastics can be divided into two groups: (1) thermoplastics and (2) thermosets.

(1) Thermoplastics consist of linear or lightly branched long polymeric chains that are affected by temperature and pressure. Thermoplastic polymers are rigid at room temperature, becoming soft and more fluid when heated, and allowing moulding into useful daily products. If and/or when reheated they can be shaped and reshaped. Thermoplastics can be formed by chain growth or step growth reactions and can be easily recycled. Examples of thermoplastics are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and polyamides (PA). (Xanthos, 2005; Peacock and Calhoun, 2006).

(2) Thermosets consist of a network of interconnected cross-link chains that do not flow when heated or subjected to pressure. Thermoset polymers do not dissolve in solvents, but they can soften and swell. Upon heating, thermosetting plastics will become soft, but cannot be shaped or reshaped. These plastics are extremely strong and durable, mainly due to their cross-link chains. Thermosets can be formed by step growth or crosslinking with a thermoplastic. Often in powder or resin forms, these plastics are more difficult to recycle. Examples of thermosets are polyurethanes (PUR) and epoxy resins (ER). (Xanthos, 2005; Peacock and Calhoun, 2006).

Due to their characteristics such as high durability and low density, plastics have a wide range of uses, from storage (ideal to store solid and liquid products) to transport (competitive material when compared to heavier materials such as glass). These characteristics, besides making this material suitable for technological and medical applications, also enable plastics to have slow degradation rates, therefore increasing its persistence in the environment.

Light weight and colour contribute to its environmental dispersal by water courses (river streams, drainage systems, ocean currents) and migratory animals (birds, turtles, dolphins, seals, among others), respectively. It has been suggested that about 10% of worldwide plastic production accumulates in the oceans, where they persist for long periods of time (Thompson, 2006). Plastic materials are able to travel great distances across the globe,
being found in regions thousands of kilometres away from any known source (Bockhorn et al., 1999; Derraik, 2002; Thiel et al., 2003; Thompson et al., 2004; UNEP, 2009; Frias et al., 2010; Andrady, 2011; Heskett et al., 2012). The interaction of marine and coastal species with plastic marine litter usually results in entanglement in abandoned, lost or otherwise discarded fishing gear (ALDFG), synthetic ropes, lines, nets or packaging bands or ingestion of fragments and particles of microscopic size (Allsopp et al., 2006, Crimmins et al., 2002; Allen et al. 2012; Wright et al., 2013, Ivar do Sul and Costa, 2014).

Weathering environmental conditions also play an important role not only in transport but also in degradation of plastic particles. Solar radiation, mechanical abrasion (e.g. sand), or water and wind movements cause plastic marine debris to break and attain small sizes without substantial chemical degradation (Moore, 2008; Barnes, 2009). Microplastics (fragments and particles with a diameter below 5mm), can be divided into two classes (primary and secondary microplastics) depending on either they are manufactured to be of a microscopic scale or derived from breakdown of larger plastic debris (Cole et al., 2011; Wright et al., 2013), both at sea and/or land (Thompson et al., 2004), posing primarily mechanical threats to marine organisms due to ingestion of fragments (Browne et al., 2008; von Moos et al., 2012; Cole et al. 2013). Considerable amounts of microplastics, namely from cosmetics filled with exfoliating microparticles, have been directly introduced into the environment by human activities (von Moos et al., 2012).

But where do all this plastics come from? How many polymers are there? When were they used for the first time? Historical records provide evidence of polymer use over centuries and across the world. Throughout mankind’s history, different materials have been used to support daily activities. The first records date back approximately to 1600 B.C., when natural rubber was used to produce spheres and small statues by the Mesoamericans (Hosler et al., 1999). The 1760’s English industrial revolution that started mass production of all sorts of products and the development of organic chemistry in the 19th century, initiated and greatly contributed to the development of modern plastic production (Andrady and Neal, 2009).

The first innovation developments made in polymer science occurred in 1839, when Charles Goodyear and Thomas Hancock discovered the process that lead to the production of vulcanized rubber. A few years later in 1845, Christian Schönbein, a Swiss chemist and
professor at University of Basel, Switzerland, accidentally discovered cellulose nitrate while cleaning a table where nitric acid had been spilled, with a cotton apron. Another important discovery was made by Alexander Parks, in 1861, by further researching cellulose nitrate, Parkesine, a new material described as “solid, fluid, hard as ivory, opaque, flexible and water resistant” (Martinho and Rodrigues, 2007). In 1907, a Belgian chemist and physicist named Leo Baekeland created the first entirely synthetic plastic, Bakelite, highly contributing to the further development of material science in plastics (Martinho and Rodrigues, 2007).

Throughout the 19th and 20th centuries, many discoveries on semi-synthetic and synthetic polymers through developmental work. The most important discoveries are summarized in table 1.1.1.

<table>
<thead>
<tr>
<th>Plastic</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural polymers (cellulose, wool, natural rubber)</td>
<td>Pre-1800</td>
</tr>
<tr>
<td>Cellulose nitrate</td>
<td>1846</td>
</tr>
<tr>
<td>Celluloid</td>
<td>1870</td>
</tr>
<tr>
<td>Phenol-formaldehyde (Bakelite)</td>
<td>1907</td>
</tr>
<tr>
<td>Regenerated cellulose sheet (Cellophane)</td>
<td>1912</td>
</tr>
<tr>
<td>Polyvinyl chloride (PVC)</td>
<td>1927</td>
</tr>
<tr>
<td>Polystyrene (PS)</td>
<td>1937</td>
</tr>
<tr>
<td>Polyurethane (PUR)</td>
<td>1937</td>
</tr>
<tr>
<td>Nylon 6,6</td>
<td>1938</td>
</tr>
<tr>
<td>Low density Polyethylene (LDPE)</td>
<td>1941</td>
</tr>
<tr>
<td>Polyesters</td>
<td>1942</td>
</tr>
<tr>
<td>Teflon</td>
<td>1943</td>
</tr>
<tr>
<td>High density Polyethylene (HDPE)</td>
<td>1957</td>
</tr>
<tr>
<td>Polypropylene (PP)</td>
<td>1957</td>
</tr>
<tr>
<td>Poly(phenylene oxide)</td>
<td>1964</td>
</tr>
<tr>
<td>Poly(butylene terephthalate)</td>
<td>1970</td>
</tr>
<tr>
<td>Polyethylene terephthalate (PET)</td>
<td>1977</td>
</tr>
</tbody>
</table>
At this point in time, there are approximately 50 different basic types of polymer included in sixty thousand plastic formulations (Shashoua, 2008). The most common plastics produced worldwide can be divided into six main categories:

1) Polyethylene terephthalate (PET);  
2) High density polyethylene (HDPE);  
3) Low density polyethylene (LDPE);  
4) Polypropylene (PP);  
5) Polyvinyl chloride (PVC); and  
6) Polystyrene (PS).

These six categories correspond to 80.2% of the European plastics demand by resin type, as possible to see in figure 1.1.1. (PlasticsEurope, 2013). The code PUR represents polyurethane.

**Figure 1.1.1** - European plastics demand in the EU-27+N/CH by resin type in 2012  
(Source: PlasticsEurope, 2013).

Worldwide mass production of plastics date back to the 1930’s (Shashoua, 2008). PlasticsEurope provides records of growth in Europe since 1950, year when were produced 1.7 Mtonnes of plastic worldwide, to which Europe contributed with 0.35 Mtonnes to the global figure. In 2012, worldwide plastic production was approximately 288 Mtonne, to
which Europe contributed with approximately 57 Mtonne (PlasticsEurope, 2013), as it is possible to see in figure 1.1.2. The annual growth rate since the 1950 is 8.63%.

![World and European plastic production](image)

*Figure 1.1.2 - World and European plastic production*

(Source: PlasticsEurope, 2013).

Between 2006 and 2011, European plastic production decreased almost 5%, reaching 20.4% of global production, while, during the same period of time, China increased its production by 13.3%, reaching almost 24% of worldwide production, Figure 1.1.3. (PlasticsEurope, 2010; 2012, 2013). In the figure, NAFTA stands for North American Free Trade Agreement and CIS stands for Commonwealth Independent States. In 2009, the amount of manufactured plastic corresponded to approximately 8% of global oil production (Cole et al., 2011). The exponential increase in production is mainly due to the high demand for plastic products, particularly in Western countries.
The history of waste management mirrors the relationship between societies that produce waste and the environment. Alongside with the large increase of products and goods worldwide, rises the need for proper solid waste management. Mobilisation of resources for daily activities leads to huge amounts of waste being discarded, particularly solid waste (Melosi, 1981). Significant changes occurred after the Industrial Revolution, and figure 1.1.4, illustrates how municipal solid waste composition of New York City and USA changed across time, where red bars represent plastic products in the form of packaging.

Figure 1.1.3 - World Plastic Production changes between 2009 and 2012 by region.
(Adapted from: PlasticsEurope, 2010 – 2013)

Figure 1.1.4 - Change in municipal solid waste composition in Kg of waste per capita (Adapted from: Melosi, 1981; US EPA, 2005)
Similar trends occurred in Europe, where packaging products increased both in number and in percentage along time. Figure 1.1.5 shows the municipal waste per capita in the EU-27 member states, and Croatia, Iceland, Norway, Switzerland and Turkey, in 2001 and in 2010 (EEA, 2013).

![Municipal waste generated per capita, between 2001 and 2010 in Europe](image)

**Figure 1.1.5** - Municipal waste generated per capita, between 2001 and 2010 in Europe (Source: EEA, 2013)

Landfill of municipal solid waste has been the predominant option for several years in the European Union + Norway and Switzerland. The average landfill rate of municipal solid waste (MSW) has fallen from 68% in 1995, to 40% in 2007, as possible to see in figure 1.1.6 (Bakas et al., 2010), due to incineration and recycling efforts of municipal solid waste.
The cumulative worldwide plastic production (represented in figure 1.1.2) and waste management are intrinsically connected. In order to minimize the problem, it is important to understand the flow of materials throughout the world system. Approximately 275 million metric tons of plastic waste were generated in 192 coastal countries, with 4.8 to 12.7 million entered the marine environment in 2010 (Jambeck et al., 2015). According to Jambeck et al., it is unlikely that the world is going to reach a global litter peak before 2100. Also, it has been suggested that about 10% of worldwide plastic production accumulates in the oceans, where they persist for long periods of time (Thompson, 2006), therefore it is important that the cumulative problem that plastics already represent, must also be dealt with international and national legislation.

In order to increase recycling and recovery of packaging waste and to divert biodegradable municipal waste from landfills, two legal frameworks (Packaging and Packaging Waste Directive and Landfill Directive), were introduced in Europe, in 1994 and 1999, respectively. A revision of the Waste Framework Directive, included targets for increasing waste recovery through recycling or production of energy.
According to the previsions of Bakas et al., 2010, for 2020, it is assumed that 28% of generated waste will be deposited in landfills and that 23% will be incinerated in order to recover energy from waste.

PlasticsEurope, the European plastic production association, created a campaign entitled “Zero Plastics to Landfill” that aims to reduce the amount of plastics deposited in landfills, and eventually remove all plastics from landfill by 2020. Currently this campaign is available in five countries (United Kingdom, Italy, Spain, France and Poland), and PlasticsEurope hopes to reach many other European countries. Incineration of plastic materials enables the production of high amounts of energy. Side by side with the high energy production are the by-products of incineration, such as dioxins, that also have environmental impacts (McKay, 2002). Nonetheless, incineration that follows European guidelines and health and safety regulations for air treatment, contribute to a safe energy production.

In Portugal, waste management is becoming more efficient with the increase of recycling rates and minimisation of waste deposited in landfills (Bakas et al., 2013). Yet, due to the geographical location of Portugal and its islands, as well as extensive coastal area and prevailing winds, the country is vulnerable to plastic accumulation on beaches, with these materials coming from land or the ocean. One important source of plastic marine debris results from maritime activities such as fishing and recreational activities or from the high number of commercial vessels and cruise ships (approximately 800) that daily route in Portuguese waters (Martins and Sobral, 2011). Surface sampling in coastal waters (using manta trawls to collect plankton samples) enables us to identify the amounts of plastic marine debris floating in the ocean.

Land sources are still accountable for the majority of plastics found in beach surveys conducted in Portugal. Many of these plastics are polluted with chemical substances present in the environment.
1.1.2 Plastics as vectors for transport of Persistent Bioaccumulative and Toxic Chemicals

Despite international regulations, environmental compartments are continuously receiving inputs of xenobiotics, man-made organic chemicals, which even if in small quantities, are released by urban communities and industries all around the world and cause environmental impacts. In the 20th century, many thousands of organic trace pollutants, such as polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), organochlorine pesticides (OCP), polychlorinated dibenzofurans (PCDF) and dibenzop-p-dioxins (PCDD) have been produced, and consequently, part of them have been released into the environment. Awareness of the potential long-term adverse effects of these chemicals and their potential risks for aquatic and terrestrial ecosystems in particular, started in the early 1960’s (Van der Oost et al., 2003).

Plastic marine debris, including microplastics, have the capacity to adsorb persistent bioaccumulative toxic chemicals (PBTC) and persistent organic pollutants (POP) from sediment or water, and the capacity to release additives to the environment (e.g. flame retardants, antioxidants, plasticisers, light stabilisers) along with plastic degradation (Endo et al., 2005; Takada et al., 2005, Frias et al., 2010). Plastic marine debris fragments and microparticles in sediment and in the water column, are a potential vector of contamination for marine animals who mistake them for food. There is the assumption that the ingestion of contaminated microplastics might to accumulation of toxic chemicals in lipid reserves of marine organisms. This effect may eventually lead to an accumulation and transfer of PBTC along the food chain and possibly into human diets, as some authors suggest (Ryan et al., 1988; Zarfl and Matthies, 2010; Tanaka et al., 2005; Rios et al., 2007; Teuten et al., 2007, Hirai et al., 2011; Andrady, 2011, Bakir et al., 2012).

PBTC are organic compounds that resist photolytic, biological and chemical degradation. As characteristics they are semi-volatiles, have low water solubility and high lipid solubility. They are able to move long distances in the atmosphere before deposition, and also that are able to bioaccumulate in fatty tissues (Ritter et al., 2007) or adsorb onto plastics. POP are represented by two important subgroups including PAH and some halogenated hydrocarbons, which are known for environmental and human health impacts (Binková et
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al., 2004; Ritter et al., 2007). Semi-volatility and persistence coupled with environmental factors result on the presence of PAH and PCB across the world, even in regions where were have never been used before.

PAH are composed of three or more fused benzene rings in linear, angular or agglomerate form. They can be formed in nature by geologic or thermic processes, e.g. forest fires, however, the main sources are anthropogenic, resulting from fossil fuel combustion, pyrolysis processes, oil spills and waste incineration (Blumer, 1976). According to its sources, they can be divided into two groups: pyrogenic and petrogenic (Boonyatumanond et al., 2006). When originated through incomplete combustion of organic matter (e.g. coal, petroleum, wood), PAH origin is said to be pyrogenic (referred as well as pyrolytic origin). Most of pyrogenic PAH are emitted to the atmosphere as gas or soot and enter the marine environments through rain or surface run-off. When the PAH derive from crude oil and petroleum products such as kerosene, gasoline, diesel fuel, lubricant oils or asphalt, the source is said to be petrogenic (Boonyatumanond et al., 2006). Due to their low solubility in water and high partition coefficient (K ow) PAH adsorb onto the surface of particles associated with organic matter (Schorer, 1997).

PCB are manufactured commercially by the progressive addition of chlorine atoms to biphenyl in presence of a catalyst (e.g. iron chloride). PCB have high thermal and chemical stability, resistance to acids and bases and high liposolubility. Due to their characteristics, PCB had been widely used for several purposes, namely in industrial processes, such as dielectric fluids in transformers and capacitors, in lubricants, hydraulic oils, paints and adhesives. These compounds pose several risks to the environment and human health, affecting the nervous, reproductive and immune systems, and may cause cancer and act as endocrine disruptors (Hanlon, 2004). In the 1970s several countries banned PCB production (WHO, 1993; Stockholm Convention, 2001), although currently, there are still significant amounts in circulation. These synthetic substances are highly resistant to degradation and once in the environment tend to accumulate in the organisms and undergo processes of biomagnification along the food chain (Brown and Wagner, 1990).

DDT, an insecticide developed in the 1930’s, is another concerning pollutant that illustrates the capacity for environmental persistence of chemical substances. DDT is
insoluble in water and soluble in organic compounds and degrades into two primary metabolites, dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE) (US EPA, 2002). Banned in Europe and in U.S.A. in the 1970’s, DDT is still used in some African countries as malaria vector control (FAO, 1995; Channa et al., 2012). In Portugal, it was officially banned in 1988 (Decreto-Lei n. 347/88 and Portaria n. 660/88), but traces of DDT are still found in the environment (Takada et al., 2005; Frias et al., 2010; Mizukawa et al., 2013).

Previous studies conducted with plastic pellets collected in Portuguese beaches (Frias et al., 2010; Antunes et al., 2013) shown that every sample of plastic pellets had adsorbed PBTC as was also reported by Mizukawa et al., 2013.

Spatial patterns of PBTC, from urban and rural areas, were identified in plastic pellets collected over 700 km along the Portuguese coast. In that study DDT was found but its source resulted from agricultural activities from the 1970’s and not from a new pollution agent known as dicofol (Mizukawa et al., 2013).

In order to estimate the effects of xenobiotics in the environment and ecosystem health, scientists usually use key indicator species. In this thesis the organism selected was the Mediterranean mussel, and the next sub-chapter will provide some information about this species.
1.2 The mussel *Mytilus*

1.2.1 Taxonomy, distribution and characteristics

The genus *Mytilus* is believed to have had its origins at approximately 400 million years ago (m.y.o.) in the Devonian period of the Paleozoic Era. This genus belongs to Mollusca, the largest marine phylum that represents 23% of marine organisms (Gosling, 1992). Members of the genus *Mytilus* are metazoan semi-sessile epibenthic bivalve molluscs with several species and subspecies. In the Iberian Peninsula there are two predominant species, *Mytilus edulis* Linnaeus, 1758, and *Mytilus galloprovincialis* Lamarck, 1819 (figure 1.2.1), which have different characteristics concerning shell and mantle edge colour, flattening of the ventral margin and downturned umbones (Hayward and Ryland, 1995).

*M. galloprovincialis* is believed to have diverged from *M. edulis* when the Mediterranean Sea was separated from the Atlantic during the Pleistocene ice age, about 1-2 m.y.o. (Gosling, 1992). *Mytilus* sp. are widely distributed throughout cooler and temperate waters of both northern and southern hemispheres, except in polar zones. *M. edulis* is widely distributed in the northern hemisphere from Scandinavia to the United Kingdom and north-west of France. *M. galloprovincialis* is found in the Mediterranean Sea and in the Black Sea, on the coasts of Portugal, Spain, Italy, Greece, but also south-west of France and in the United Kingdom (Gosling, 1992).

![Figure 1.2.1 – Mytilus galloprovincialis](Source: Hayward and Ryland, 1995)

Marine mussels live anchored to a secure substrate, or attached to other mussels through byssus threads secreted from glands located in the foot. Mussels can achieve a limited degree of movement by secreting new threads and adjusting the lengths of others. The visceral
mass is protected by two hinged halves or valves shells, secreted by the mantle that protect the organism from predators and against desiccation. The two valves of the shell are equal in shape and size and are held tightly closed by a large posterior adductor muscle when the mussel is exposed to air. When the valves are closed, the byssus threads pass through a small notch, the pedal gape, in the middle of the ventral junction of the two valves. Mussel shells are almost always wedge shaped or asymmetrical, often longer than wide, which protect mussels from predators and support soft tissue inside (Newell, 1989).

Mussels are filter feeders; they feed on plankton and other microscopic sea organisms which are free-floating in seawater, by drawing water in, through its inhalant siphon. The water is then brought into the brachial chamber by the actions of the cilia located on the gills for ciliary-mucus feeding. The effluent exits through the exhalant siphon. The labial palps finally funnel the food into the mouth, where digestion begins (Gosling, 1992).

It has been reported in numerous studies that bivalves only efficiently retain particles larger than 3 to 7 µm, depending on the species feeding structures and size of the individual. In the mussel *Mytilus edulis*, particles larger than 4 µm are reported to be retained with a nominal efficiency of 100% (Møhlenberg and Rüsgård 1978) and retention efficiency rapidly declines as particle size decreases below this threshold (Strohmeier et al., 2012).

They present separate sexes and fertilization occurs outside the organism. Larvae float around from three weeks to six months before settling and becoming a young mussel. The reproductive cycle of any mussel population is the result of a complex balance between exogenous factors such as temperature, salinity, food availability, position in the intertidal zone, and endogenous factors such as nutrient reserves and genotype. Interaction between these factors requires the synchrony of gamete development within the population. Such synchrony is important for an oviparous species and ensures that larvae are in the water at the optimum time for their growth and survival (Gosling, 1992).
1.2.2 Importance of mussels for ecotoxicological assays

Marine mussels have been used as model organisms to various physiological, biochemical and genetic investigations due to their characteristics (Gosling, 1992). They are often used to evaluate the effect of contaminants in bio-monitoring programs due to their ability to survive under polluted conditions and to accumulate both organic and metal pollutants (Aarab et al., 2011).

Therefore, there is a set of attributes that have led to the use of bivalves as ‘sentinel’ or ‘indicator’ organisms in environmental monitoring programmes throughout the world (Gosling, 1992), such as:

1. **Geographical distribution**
   Mussels are dominant members of coastal and estuarine communities and are widespread distributed, which minimizes the data comparison problems if made with different species;

2. **Sedentary organisms**
   Due to the fact mussels are semi-sessile organisms they act as better integrators of chemical contamination in a given area, when compared to mobile species;

3. **Tolerance to environmental conditions**
   They are relatively tolerant, and not insensitive, to a wide range of environmental conditions and moderately high levels of contaminants;

4. **Filter feeder organisms**
   They are suspension-feeders that pump large several litres of water per hour and concentrate many chemicals in their tissues, by factors of 10 to $10^5$, relative to seawater concentration;

5. **Bioaccumulation of chemicals**
   Bivalve tissue provide an assessment of biological availability of chemicals that might not be apparent in other environmental compartments (water, suspended particles and sediment).
6. **Bioconcentration of chemicals**
   
   Due to its low level enzymatic activity, mussels are less capable, when compared to fish or crustacean, to metabolize organic contaminants such as hydrocarbons and polychlorinated biphenyls. Contaminant concentrations in the tissues of bivalve reflect more accurately the magnitude of environmental contamination.

7. **Population stability**
   
   Mussel populations are relatively stable and can be large enough for repeated sampling, proving data on different time scales and changes in contaminant levels.

8. **Adaptation**
   
   Mussels can be relocated and maintained in cages to sites of interest either in intertidal zone, subtidally in moorings or in laboratories.

9. **Economic importance**
   
   Mussels are a commercially important seafood species, therefore the measurement of chemical contamination is of interest for public health considerations.

   The latter attributes were the main reasons for the selection of this organisms to the conducted bioassays described in chapter 3.
1.3 Biomarkers

The mere presence of xenobiotics in segments of the ecosystem, by itself, does not indicate injurious effects whatsoever. Nonetheless, relationships can be established between environmental concentrations and internal concentrations on organism to relate adverse effects (Van der Oost et al., 2003). Pollutants in the environment have the ability to create synergetic effects by mutually interacting, difficulting environmental risk assessment. In order to measure biological effects on environmental quality assessments, researchers often resort to the use of biomarkers, which consist in the identification of early-warning signals that might reflect adverse biological responses towards xenobiotics.

There are several definitions of biomarker, but the most common is a change in a sub-individual biological response related to exposure to environmental chemicals or their toxic effects (Martín-Díaz et al., 2004). The biological changes might range from molecular through cellular and physiological responses to behavioural changes, and they can be identified inside an organisms or in its biological products (urine, faeces, hair, feathers, bone marrow or other tissues) (Van der Oost et al., 2003). Three classes of biomarkers have been identified so far (NRC, 1987; WHO, 1993):

1) Biomarkers of exposure: which cover the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between a xenobiotic agent and some target group, individual, molecule or cell that is measured in a compartment within an organism. A connection is established between external exposure and the quantification of internal exposure to the exogenous substance;

2) Biomarkers of effect: a measurable biochemical, physiological, behavioural or any other alteration within an organism that depending upon the magnitude, can be recognized as associated with an established or possible health impairment or disease. These biomarkers are used to document health adverse effects, resulting from exposure and absorption of a chemical substance. The connection between biomarkers of exposure and biomarkers of effect contributes to the dose-response relationship;

3) Biomarkers of susceptibility: an indicator of an inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance. These
biomarkers allow us to establish the exposure of individuals. They might reflect acquired or genetic factors that influence the organism’s response to a certain chemical substance. These factors are pre-existent and independent from the exposure. They are predominantly genetic, however there might be other environmental agents that can alter individual susceptibility (Van der Oost et al., 2003).

The impact of toxic xenobiotics on marine organisms can be assessed by various types of exposure and effect biomarkers. Many environmental contaminants and their metabolites have proved to exert toxic effects related to oxidative stress, mainly due to injuries caused cytotoxic reactive oxygen species (ROS), which are oxygen free radicals. The reduction of molecular oxygen react with critical cellular macromolecules, possibly leading to enzyme inactivation, lipid peroxidation (LPO), DNA damage and, ultimately cell death (Van der Oost et al., 2003). As in aquatic ecosystems, dissolved oxygen and temperature are environmental variables that influence oxidative processes, therefore it is important to determine the effects of ROS on organisms.

Generally, the most sensitive effect biomarkers are alterations in levels and activities of biotransformation enzymes. These enzymes are divided into two major types: a) Phase I enzymes and b) Phase II enzymes and cofactors. The first phase of metabolism involves oxidation, reduction or hydrolysis, and for the majority of xenobiotic compounds the phase I reactions are catalysed by microsomal monooxygenase enzymes, also known as mixed function oxidase system (for example, cytochrome P450). The second phase of metabolism involves a conjugation of xenobiotic parent compounds or its metabolites with an endogenous ligand. The majority of phase II enzymes catalyse these synthetic conjugation reactions, thus facilitating the excretion of chemicals by the addition of more polar groups (for example, reduced glutathione-GSH). Phase II enzymes play an important role in homeostasis, detoxification and clearance of many xenobiotic compounds. The major pathway for electrophilic compounds and metabolites is the conjugation with GSH (Van der Oost et al., 2003).

In opposition to most of the biochemical parameters, physiological and morphological parameters represent higher-level responses following chemical and cellular interactions, which are generally indicators of irreversible damage. And, in this case, the feasibility of
histopathological parameters have been confirmed as a biomarkers for aquatic pollution (Van der Oost et al., 2003).

In this work two biomarkers, lipid peroxidation and histopathology, were used. LPO can be basically described as the oxidative degradation of lipids (oxidation of polyunsaturated fatty acids as a direct consequence of oxidative stress). The process of lipid peroxidation proceeds by a chain reaction, and as in the case of redox cycling, demonstrates the ability of a single radical oxygen species to propagate a number of deleterious biochemical reactions with nefarious consequences (Van der Oost et al., 2003). LPO levels can be determined by measuring of degradation products, like for example malondialdehyde.

Histopathology is a biomarker of effect and may be used to identify pathological lesions in tissues induced by environmental contamination and disease. These changes reflect disturbances at the molecular level and can aid in the understanding of the overall health of the animals. Histopathology is often the simplest method for assessing both short- and long-term toxic effects in the field by providing an overall assessment of the general health status of marine organisms, such as mussels (Aarab et al., 2011).

A successful implementation of biomarkers requires a good understanding of the mechanisms underlying biological responses, but like any other method or technique there are limitations to the responses. Biomarker responses are powerful because they integrate a wide array of environmental, toxicological and ecological factors that control and modulate exposure to, as well as effects of, environmental contaminants. However, these same factors may also difﬁcult interpretation of the biomarker responses significance in ways that may not always be anticipated (Van der Oost et al., 2003).

Organisms’ health, condition, sex, age, nutritional status, population density are some of the other factors that may cause alterations to biological response.
1.4 **Marine Strategy Framework Directive**

The enormous intrinsic value of seas and oceans is currently under threat due to unsustainable anthropogenic uses such as overfishing or pollution. Traditional and modern sea activities, like fishing, tourism, mineral extraction, production of waves and wind energy, are affecting and being affected by drastic environmental changes in the marine environment. Some of these changes occur naturally, other are strongly influenced by human activity.

In order to protect marine-related economy and its social activities more effectively across Europe, the European Union (EU) created the Marine Strategy Framework Directive (MSFD) on the 17th June 2008. This framework aims to achieve good environmental status (GES) on the EU’s marine waters by 2020 (Directive 2008/56/EC) and it is the first EU legal instrument related to the protection of marine biodiversity, and it contains the explicit regulatory objective to ensure biodiversity is maintained by 2020, as a cornerstone for achieving GES (Galgani et al., 2010; Santos et al., 2012). The legal framework integrates concepts of environmental protection and sustainable use, by using an ecosystem approach to the management of human activities that have impact on the marine environment.

The Annex V of the International Convention for the Prevention of Pollution from Ships (MARPOL) had already created a basis for the prevention of pollution by garbage from ship (when entered into force on 31st December 1988), yet the MSFD is the first legislative document that regulates and addresses specifically plastic marine litter, which this thesis refers to. The Directive establishes European marine regions and sub-regions on the basis of geographical and environmental criteria, in order to achieve GES, divided by 4 marine regions – Baltic Sea, North-east Atlantic Ocean, Mediterranean Sea and Black Sea – located within the geographical boundaries of the Regional Seas Convention. Each member state is required to develop a national strategy for its marine waters and coastal areas in order to address the set of indicators of the MSFD.

The directive also classified *harm* in three general categories: 1) ecological (mortality or sub lethal impacts to plants and animals through entanglement, physical damage and ingestion including uptake of microplastics, accumulation of chemicals from plastics, facilitating the invasion of alien species, or altering the benthic community structure); 2)
economic (e.g. cost to tourism, damage to vessels, fishing gear and facilities, losses to fishery operations, cleaning costs); and 3) social (reduction in aesthetic value and public safety).

In order to estimate acceptable levels of harm it is necessary to consider impacts of marine litter in different compartments of the marine environment (seabed, sea surface, water column, coastline, and deep sea), ecological effects of the litter (ingestion of plastic; entanglement rates), problems associated with degradation of litter (microplastics) as well as social and economic aspects. Tourism, as an example, is one of the sectors that is negatively affected by the presence of marine litter (Galgani et al., 2010, Cauwenbergh et al., 2013; Schlining et al., 2013; Jang et al., 2014).

The given examples are related to one of the descriptors that the MSFD contemplates, marine litter (descriptor 10), which is also the most relevant for this work, that provides detailed information for managers and decision makers to create strategies to minimize the marine litter problem in Portugal. This thesis aims to contribute to the goals of the MSFD through providing information about the situation in Portuguese coastal waters and coastal areas.

1.5 Research outline and thesis structure

The scope and research questions of this thesis address and are linked with goals of the project “Microplastics and persistent pollutants: a double threat to marine organisms (POIZON) reference PTDC/MAR/102677/2008”, which aimed to assess microplastic abundance on the Portuguese coast and the potential transfer of contaminants to the ocean food-chains from ingestion of microplastics. Microplastics are the main focus of this work, where its evidence in Portuguese coastal areas and ocean surface waters from several locations is identified.

The assessment was made through field and laboratorial work, where microplastics were collected, measured, weighted, and identified according to their according to their polymer type. In what concern resin pellets, PBTC adsorbed to them was also quantified.

This work also focus on microplastic ingestion bioassays with marine mussels. The bioassays used non-contaminated and benzo(e)pyrene (BeP) contaminated polystyrene particles. Alterations were assessed through measuring oxidative stress and histopathologic
biomarkers on the gills and digestive gland of the Mediterranean mussel - *Mytilus galloprovincialis* – which were collected from a location far from any pollution source, in the West of Portugal (Praia da Légua, Alcobaca Municipality).

Concentrations of microplastics were also determined in zooplankton samples collected along the Portuguese coast, from surveys to assess fish stocks. For this experiment, microplastics were retrieved from the water and not from the zooplankton organisms.

Data is integrated according to two different strategic approaches to marine litter (SWOT analysis and DPSIR model).

The main achievements are described in the final chapter that addresses the concluding remarks and future work concerning this research topic. The scope and research questions posed act as hypotheses for the work are divided by the two main chapters of the thesis:

Chapter 2)

Determination of quantities of microplastics in Portuguese beaches and PBTC adsorbed to resin pellets.

- Are there accumulation areas in the Portuguese coast for plastics and microplastics?
  What are the beaches with higher concentrations of microplastics along the Portuguese coastline?

- What is the concentration range of PAH, PCB and DDT in Portuguese beaches? Do plastic pellets accumulate PBTC, such as PAH, PCB and DDT?

- What are the sources of PAH in resin pellets?

Chapter 3)

Effects of different size ranges and concentrations in the gills, gut cavity and gonads of mussels exposed to non-contaminated and BeP-contaminated polystyrene microparticles.

- Will there be damage in the gut cavity of the mussels, as an effect of ingestion? Will there be damage in the gills as a result of exposure?

- Will different microplastic concentrations and/or different size ranges influence or have different effects/damage on the organisms?
- How will specific concentrations of Benzo(e)pyrene (BeP) adsorbed to microplastic particles affect organisms exposed to it? Will there be significant changes in tissues?

Chapter 4)
Detect and quantify microplastic debris in zooplankton samples and identify plastic polymers using spectroscopy techniques such as micro-FTIR.
- Are there microplastic particles along Portuguese coastal waters?
- If particles are found, what types of polymers are commonly present?

Chapter 5)
Strategic approaches to minimise marine litter. In this chapter is presented a SWOT matrix and a DPSIR model to assess and reduce marine litter at a global scale.

The following chapters will explore in higher detail these topics and aim to provide answers to these questions.
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
2 Evidence of microplastics in Portuguese beaches

Marine litter is considered a global environmental problem that directly impacts coastal communities. This section will focus on microplastics from Portuguese beaches, based on a case study that report on sizes, abundances and polymer types of the debris commonly collected at the Portuguese marine coastal areas. Surveys were conducted between 2011 and 2013 and concentrations of persistent bioaccumulative and toxic chemicals (PBTC) adsorbed to resin pellets were also measured, in order to assess contaminant levels and to understand the pollutant’s source or origin. This case study has set the foundation for the following chapters.
2.1 Resin pellets and microplastics from the Portuguese coast: quantification and determination of persistent bioaccumulative and toxic chemicals adsorbed to pellets

Abstract

Plastic marine litter accumulation was investigated along the Portuguese coastline, between 2011 and 2013, in order to determine amounts and size ranges of resin pellets and microplastics, with a large number of marine litter fragments registered (2757 items m\(^{-2}\) on average; 98% were plastic). Microplastics account for 70-83% of total items collected, to which resin pellets, the most abundant category in all beaches, represented 58%. Concentrations of adsorbed persistent bioaccumulative and toxic chemicals (PBTC) were also determined, namely polycyclic aromatic hydrocarbons (PAH); polychlorinated biphenyls (PCB) and dichlorodiphenyltrichloroethane (DDT). Matosinhos (Mt) and Vieira de Leiria (VL) presented the highest number of items-m\(^{-2}\) (362 and 332, respectively), and resin pellets with 4 mm in diameter were the most abundant size class, accounting for 50% of pellets. PBTC concentrations were variable, with PAH concentrations ranging between 53 and 44800 ng-g\(^{-1}\), PCB from 2 to 223 ng-g\(^{-1}\) and DDT ranging between 0.42 and 41 ng-g\(^{-1}\). Generally, aged and black pellets registered higher concentrations for all contaminants. Matosinhos (Mt), Vieira de Leiria (VL) and Sines (Si), near industrial areas and port facilities, were the most contaminated beaches. Research efforts to assess entry points of microplastics in the marine environment (e.g. rivers) and actions to prevent new inputs, particularly during the transfer and transport of resin pellets from plastic manufacturers to converters, are urgently needed. Riverine and estuary inputs should be assessed to proper estimate the amount of microplastics arriving from land sources. The creation and implementation of prevention guidelines, or enforcement of the MSFD should be further addressed to prevent microplastics from reaching the oceans, minimizing the impacts on wildlife and local economies.

Keywords
Plastic debris, marine litter, PBTC, Portugal

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\(^{1}\) Data relative to 2012 adapted from: Antunes, J. C., Frias, J. P. G. L., Micaelo, A. C., Sobral, P., (2013). Resin pellets from beaches of the Portuguese coast and adsorbed persistent organic pollutants. Estuarine, Coastal and Shelf Science 130, 62-69. DOI:10.1016/j.ecss.2013.06.016.
2.1.1 Background and objectives

Approximately eighty percent of plastic marine debris derives from land sources, being transported by water courses and migratory animals throughout the globe (Thompson et al., 2004; UNEP, 2009; Heskett et al., 2012). Do to their malleable characteristics, durability and low weight, plastic materials are suitable for multiple applications, making them easy and cheaper for transport and product storage. Some of the before mentioned characteristics, might contribute to the widespread distribution of plastics in the environment and to their slow degradation over time (Bockhorn et al., 1999; Andrady, 2011).

Derived from crude oil, resin pellets are the raw material used to produce all plastic materials, through specific melting and moulding processes at high temperatures. Resin pellets are small cylindrical granules, generally ranging between 2 and 6 mm (Andrady, 2011), that might be introduced in the ocean through accidental spills during transport (Ogata et al., 2009). These small granules and other plastic fragments are easily mistaken for food and ingested by marine organisms and birds.

Also, due to their capacity of adsorb persistent, bioaccumulative, and toxic chemicals (PBTC), such as polycyclic aromatic hydrocarbons (PAH) and pesticides, there is an increased concern of bioavailability and persistence of these pollutants in the marine environment.

This work provides data of the first national coverage of marine litter in Portugal as part of the POIZON project (Reference number: PTDC/MAR/102677/2008). The aim of this work was to determine accumulation areas for resin pellets and microplastics stranded along the Portuguese coast and to estimate the concentrations of PBTC (PAH, PCB and DDT) adsorbed to resin pellets. Data comprises the time period between 2011 and 2013. PBTC concentrations correspond to 2012-2013.

2.1.2 Methodology

Stranded marine debris were collected from ten beaches of the Portuguese coast (Figure 2.1.1), in the winter months, immediately after spring tides, on the last tidal mark on shore, between 2011 and 2013. Sample sites were selected according to three main criteria: (1) influence/exposure to dominant northerly winds, (2) proximity to industry sites and port facilities and (3) proximity to estuaries/river mouths.
Table 2.1.1 – Sampling sites on the Portuguese coast: Matosinhos (MT), Espinho (ES), Mira (MI), Vieira de Leiria (VL), Paredes de Vitoria (PV), Peniche (Pen), Cresmina (CR), Fonte da Telha (FT), Sines (SI) and Bordeira (BOR). Code, sampled sites names, GPS coordinates and sites of activities/influences (industry, river and ports) are represented on the table. (Source: Antunes et al., 2013).

2.1.2 Sample collection and processing

Sediment samples were collected at the tidemark on shore, from the top 2 cm of two different size square areas (50 x 50 cm and 2 x 2 m) along the shore line. At each site, three to five sediment replicates were collected and stored in paper bags. At all times contact with plastic materials was avoided to prevent contamination.

In the laboratory, samples were processed according to the methodology described in Frias et al. (2010) and Martins and Sobral (2011), and resin pellets were separated from the remaining marine debris, counted, weighted and divided in four classes (white, aged, coloured and black) according to a classification adapted from Endo et al. (2005). In Endo et al. classification, white pellets are translucent white virgin pellets and aged pellets correspond to yellow-brown pellets which assumedly are present in the marine environment for a longer period of time. Coloured pellets included all resin pellets with various pigments, apart from the black pellets, who represent a different category due to their heterogeneous composition – black pellets were μ-FTIR identified as PP, PE and as polyurethane (PU). In translucent white pellets it is easy to distinguish the virgin
from the aged pellets, however in the case of coloured pellets, it is not possible to determine whether pellets are aged or not, consequently both coloured and black classes include virgin and aged pellets.

Any wet samples were first dried in a glass desiccator with silica gel before sorting according to Hirai et al. (2011). All items were weighed and counted, according to the size classes adopted by Ogi and Fukumoto (2000) and kept in covered glass Petri dishes until pollutant analysis.

2.1.2.2 Contaminants analysis

Approximately 1 g of resin pellets of each qualitative category (white, aged, coloured and black) from each site, was analysed for contaminant content and concentration. Regarding PAH analysis, resin pellets were spiked with 1 ml surrogate standards (SUPELCO) containing acenaphthene-d10 (0.408 µg ml⁻¹), phenanthrene-d10 (0.397 µg ml⁻¹), chrysene-d12 (0.397 µg ml⁻¹), perylene-d12 (0.433 µg ml⁻¹). The extraction was made in an accelerated solvent extractor Dionex® ASE 200 with a mixture of hexane:acetone (1:1, v:v) at 100 °C and 1500 psi for 5 min, followed by static extraction for 5 min. The extract was fractioned with a silicaalumina (1:1), glass column. The first fraction, corresponding to aliphatic hydrocarbons, was eluted with 20 ml of n-hexane and not analysed. The second fraction, containing the PAH compounds, were collected by eluting 30 ml of n-hexane:dichloromethanomethane (9:1; v:v) and 40 ml n-hexane:dichloromethane (4:1, v:v). The solvent was evaporated by a rotator evaporator and concentrated to 0.5 ml under a gentle stream of N₂ for prior analysis. PAH concentration determination was performed on a Thermo® DSQ Trace GC Ultra gas chromatography-mass spectrometry (GC-MS) system with a 30 m × 0.25 mm × 0.25 mm film thickness with capillary column J&W, DB5mn (Argilent, USA) in selected ion monitoring mode (SIM), according to the methodology of Martins et al., 2012. Injection was performed by auto sampler in the splitless mode, at 280 °C and, interface line and ion source temperature maintained at 220 °C. Helium was used as carrier gas at a flow of 1.0 ml min⁻¹. Initial oven temperature was 70 °C, then ramped to 140 °C at 30 °C min⁻¹, followed by another ramp step to 270 °C at a rate of 3 °C min⁻¹, and held for 15 min. Relevant standards were run to check column performance, peak height and resolution, before analysis. Concentrations of individual PAH was conducted using the internal standard peaks area method, ion ratio (m/z) of a standard PAH solution NIST (SRM 2260a) and using two calibration curves with nine points each, for each compound ranging 0.1e 0.7 ng g⁻¹ (dry weight basis) (Martins et al., 2008). With each set of samples to be analysed, a solvent blank, a standard mixture and a
procedural blank were run in sequence to check for contamination, peak identification and quantification. Seventeen individual PAH were analysed (Table 2.1.1). Identification of PAH compounds was based on the comparison of their GC retention times and mass spectrum with appropriate individual standards. Concentrations of these individual PAHs was done by the internal standard peaks area method and using two calibration curves with nine points each, for each compound ranging 0.05-3.0 ng g\(^{-1}\) and 1.3-54 ng g\(^{-1}\). Total PAH (tPAH) is the sum of all the analysed compounds and all results are expressed on a dry weight basis.

**Table 2.1.1 – PAH analysed in the GCMS method, their code and CAS number.**

<table>
<thead>
<tr>
<th>Number of aromatic rings</th>
<th>Name</th>
<th>Code</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 aromatic rings</td>
<td>Acenaphthylene</td>
<td>Any</td>
<td>208-96-8</td>
</tr>
<tr>
<td></td>
<td>Acenaphthene</td>
<td>Ana</td>
<td>83-32-9</td>
</tr>
<tr>
<td></td>
<td>Fluorene</td>
<td>F</td>
<td>86-73-7</td>
</tr>
<tr>
<td></td>
<td>Phenanthrene</td>
<td>P</td>
<td>85-01-8</td>
</tr>
<tr>
<td></td>
<td>Anthracene</td>
<td>A</td>
<td>120-12-7</td>
</tr>
<tr>
<td>4 aromatic rings</td>
<td>Fluoranthene</td>
<td>Fl</td>
<td>206-44-0</td>
</tr>
<tr>
<td></td>
<td>Pyrene</td>
<td>Py</td>
<td>129-00-0</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)anthracene</td>
<td>Ba</td>
<td>56-55-3</td>
</tr>
<tr>
<td></td>
<td>Chrysene</td>
<td>C</td>
<td>218-01-9</td>
</tr>
<tr>
<td>5 aromatic rings</td>
<td>Benzo(b)fluoranthene</td>
<td>BbF</td>
<td>205-99-2</td>
</tr>
<tr>
<td></td>
<td>Benzo(b)fluoranthene</td>
<td>BkF</td>
<td>207-08-9</td>
</tr>
<tr>
<td></td>
<td>Benzo(a)pyrene</td>
<td>BaP</td>
<td>50-32-8</td>
</tr>
<tr>
<td></td>
<td>Benzo(e)pyrene</td>
<td>BeP</td>
<td>192-97-2</td>
</tr>
<tr>
<td></td>
<td>Dibenzo(ah)anthracene</td>
<td>DbA</td>
<td>53-70-3</td>
</tr>
<tr>
<td></td>
<td>Perylene</td>
<td>Per</td>
<td>198-55-0</td>
</tr>
<tr>
<td>6 aromatic rings</td>
<td>Indeno(1,2,3-cd) pyrene</td>
<td>In</td>
<td>193-39-5</td>
</tr>
<tr>
<td></td>
<td>Benzo(g,h,i)perylene</td>
<td>BpE</td>
<td>191-24-2</td>
</tr>
</tbody>
</table>

For PCB determination, pellet organochlorines were Soxhlet extracted with hexane for 17 h. The extraction was fractioned with a Florisil glass column, and then eluted with n-hexane, followed by a clean-up with sulfuric acid (H\(_2\)SO\(_4\)). The extracts were then injected in a Hewlett Packard\(^\text{®}\) chromatographer (ECD), model 6890 with capillary column J&W, DB5 (60 m) and
automatic sampler. Eighteen PCB congeners were analysed (Table 2.1.2). Following the procedure for PCB analysis, a second extraction was made to determine pp'-DDE, pp'-DDD and pp'-DDT. Eighteen PCB congeners with several degrees of chlorination, together with pp'-DDE, pp'-DDD and pp'-DDT were analysed using a standard solution and the internal standard peaks area method with two calibration curves with seven points each. The detection limit for these compounds is 0.01 ng g⁻¹ (dry weight basis) (Ferreira and Vale, 2001).

<table>
<thead>
<tr>
<th>Number of chlorine atoms</th>
<th>Name</th>
<th>Code</th>
<th>CAS No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 chlorine atoms</td>
<td>2,2',5-trichlorobiphenyl</td>
<td>CB18</td>
<td>37680-65-2</td>
</tr>
<tr>
<td></td>
<td>2,3',5-trichlorobiphenyl</td>
<td>CB26</td>
<td>38444-81-4</td>
</tr>
<tr>
<td></td>
<td>2,4',5-trichlorobiphenyl</td>
<td>CB31</td>
<td>16606-02-3</td>
</tr>
<tr>
<td>4 chlorine atoms</td>
<td>2,2',3,5'-tetrachlorobiphenyl</td>
<td>CB44</td>
<td>41464-39-5</td>
</tr>
<tr>
<td></td>
<td>2,2',4,5'-tetrachlorobiphenyl</td>
<td>CB49</td>
<td>41464-40-8</td>
</tr>
<tr>
<td></td>
<td>2,2',5,5'-tetrachlorobiphenyl</td>
<td>CB52</td>
<td>35693-99-3</td>
</tr>
<tr>
<td>5 chlorine atoms</td>
<td>2,2',4,5,5'-pentachlorobiphenyl</td>
<td>CB101</td>
<td>37680-73-2</td>
</tr>
<tr>
<td></td>
<td>2,3,3',4,4'-pentachlorobiphenyl</td>
<td>CB105</td>
<td>32598-14-4</td>
</tr>
<tr>
<td></td>
<td>2,3',4,4',5-pentachlorobiphenyl</td>
<td>CB118</td>
<td>31508-00-6</td>
</tr>
<tr>
<td>6 chlorine atoms</td>
<td>2,2',3,3',4,4'-hexachlorobiphenyl</td>
<td>CB128</td>
<td>38380-07-3</td>
</tr>
<tr>
<td></td>
<td>2,2',3,4,4',5'-hexachlorobiphenyl</td>
<td>CB138</td>
<td>35065-28-2</td>
</tr>
<tr>
<td></td>
<td>2,2',3,4,5,6-hexachlorobiphenyl</td>
<td>CB149</td>
<td>38380-04-0</td>
</tr>
<tr>
<td></td>
<td>2,2',3,5,5',6-hexachlorobiphenyl</td>
<td>CB151</td>
<td>52663-63-5</td>
</tr>
<tr>
<td></td>
<td>2,2',4,4',5,5'-hexachlorobiphenyl</td>
<td>CB153</td>
<td>35065-27-1</td>
</tr>
<tr>
<td>7 chlorine atoms</td>
<td>2,2',3,3',4,4',5-heptachlorobiphenyl</td>
<td>CB170</td>
<td>35065-30-6</td>
</tr>
<tr>
<td></td>
<td>2,2',3,4,4',5,5'-heptachlorobiphenyl</td>
<td>CB180</td>
<td>35065-29-3</td>
</tr>
<tr>
<td></td>
<td>2,2',3,4,5,5',6-heptachlorobiphenyl</td>
<td>CB187</td>
<td>52663-68-0</td>
</tr>
<tr>
<td>8 chlorine atoms</td>
<td>2,2',3,3',4,4',5,5'-octachlorobiphenyl</td>
<td>CB194</td>
<td>35694-08-7</td>
</tr>
</tbody>
</table>
2.1.2.3 Determination of the sources of PAH contamination

Ratio phenanthrene/anthracene (Phen/Ant) was used to determine the sources of PAH. Phenanthrene and anthrancene are thermodynamically dependent, and if Phen/Ant >10 the source is petrogenic while if Phen/Ant <10 the source is pyrogenic. Likewise considerations can be applied to the ratio fluoranthene/pyrene (Fluo/Pyr) ratio where values greater than 1 are classically related to pyrogenic origins, namely coal combustion.

2.1.2.4 Statistical analysis

Due to invalidation of the homogeneity of variances, as determined by Levene’s test, data was analysed by nonparametric statistics. The Mann-Whitney U test was used for pairwise comparisons between: A) sampling sites (beaches), pellets size and items m² in each color class and B) contaminants in each color class. The Spearman’s R statistic was computed for correlation analysis. The significance level for all analysis was set at 95% (α = 0.05). All calculations were performed with the software Statistica 7.0 (Statsoft Inc., Tulsa, OK, USA).

2.1.3 Results

2.1.3.1 Marine debris

Between 2011 and 2013, the average marine debris was 2757 items m² (corresponding to 371.24 g m⁻²) and 98% were plastic (2702 items m², 364 g m⁻²). The most representative class of plastic marine debris was resin pellets representing 58% (Figure 2.1.2) of total marine debris items collected (59061 items m²; 1374.94 g m⁻²) followed by plastic fragments with 25% of the global figure.

![Figure 2.1.2 - Marine debris items collected in Portuguese beaches between 2011 and 2013.](image_url)
2.1.3.2 Resin pellets distribution

Resin pellets accumulation was variable along the Portuguese coast and along the years, as it depends on various environmental and climatologic parameters. The results on this section refer to 2012-2013. As it was expected, significantly higher amounts of resin pellets were collected in beaches near industrial sites or port facilities (Figure 2.1.3) - Matosinhos (Mt) (362±41 items m$^{-2}$), Vieira de Leiria (VL) (332±174 items m$^{-2}$) and Sines (Si) (84±35 items m$^{-2}$) than in the remaining sampling sites (Mann-Whitney U test, p <0.05).

Densities of resin pellets for each class (Figure 2.2.4) were dependent of the proximity to river mouth and to industries or ports. Espinho (Es) (3.4±1.7 items m$^{-2}$) and Mira (Mi) (1.5±1.1 items m$^{-2}$) presented significant lower number of items than the remaining sites (Mann-Whitney U test, p <0.05).

The most common pellet classes were white (37%) and aged (24%). Concerning size, resin pellets generally ranged from 3 to 6 mm, and the higher abundant classes were 3, 4 and 5 mm, with 25%, 50% and 20% of total pellets collected, respectively. Among sample sites no significant differences between pellet size were identified (Mann-Whitney U, p<0.05). White and aged pellets were dominant in all sites, but were significantly higher in Matosinhos (Mt) (136±18 items m$^{-2}$ and 154±3 items m$^{-2}$, respectively), Vieira de Leiria (VL) (199±107 items m$^{-2}$ and 55±23 items m$^{-2}$, respectively) and Sines (Si) (48±22 items m$^{-2}$ and 26±10 items m$^{-2}$, respectively) (Mann-Whitney U, p < 0.05). Vieira de Leiria (VL) and Sines (Si) beaches showed lower variability when compared to the remaining sites (low standard deviation).

Coloured and black pellets had significantly higher densities in Vieira de Leiria (VL) (24±6 items m$^{-2}$ and 55±32 items m$^{-2}$, respectively) and Matosinhos (Mt) (20±7 items m$^{-2}$ and 51±4 items m$^{-2}$, respectively) than in remaining sites (Mann-Whitney U, p < 0.05).
Figure 2.1.3 – Average resin pellets (items m$^{-2}$ ± SD) for different classes, collected in Portuguese beaches Matosinhos (Mt) (n = 3), Espinho (Es) (n = 4), Mira (Mi) (n = 4), Vieira de Leiria (VL) (n = 3), Peniche (Pen) (n = 4), Cresmina (Cr) (n=5), Fonte da Telha (FT) (n = 3), Sines (Si) (n = 4) and Bordeira (Bor) (n = 5); white pellets (A); aged pellets (B); coloured pellets (C) and black pellets (D). Sites with different letters are significantly different (Mann-Whitney U test, p < 0.05). (Source: Antunes et al., 2013)

2.1.3.3 Contaminants analysis

PAH concentrations (Figure 2.1.4) ranged between 53 ng g$^{-1}$ in Cresmina beach (Cr) to 44800 ng g$^{-1}$ in Sines (Si), which was the most PAH contaminated beach (686 ng g$^{-1}$ in white pellets, 2552 ng g$^{-1}$ in colored pellets, 11860 ng g$^{-1}$ in aged pellets and 44800 ng g$^{-1}$ in black pellets) contrasting with the remaining beaches. Peniche (Pen) (541 ng g$^{-1}$ colored pellets), Bordeira (Bor) (554 ng g$^{-1}$ black pellets) and Matosinhos (Mt) (377 ng g$^{-1}$ black pellets) also had high PAH concentrations. The majority of PAH contaminated pellets comes from petrogenic sources (Figure 2.1.5).
**Figure 2.1.4** - PAH concentrations (ng g⁻¹) recorded in resin pellets collected in Portuguese beaches (2012-2013).  
(Source: Antunes et al., 2013)

**Figure 2.1.5** – Sources of PAH in resin pellets (W- white; A- aged; C- coloured; B – black)  
(Source: Antunes et al., 2013)
PCB contamination was higher in aged pellets than in any of the other classes (Figure 2.1.6). Matosinhos (Mt) (223 ng g\(^{-1}\)), Peniche (Pen) (77 ng g\(^{-1}\)), Cresmina (Cr) (69 ng g\(^{-1}\)) and Fonte da Telha (FT) (52 ng g\(^{-1}\)) beaches recorded the highest concentrations. However, high concentrations of PCB were also found in black pellets from Sines (Si) (89 ng g\(^{-1}\)) and colored pellets from Matosinhos (Mt) (108 ng g\(^{-1}\)). White pellets showed significantly lower PCB concentrations than black and aged pellets (Mann-Whitney U test, p < 0.05). PCB congeners 138, 153 and 180 presented the highest concentrations in Matosinhos (Mt) (71 ng g\(^{-1}\), 69 ng g\(^{-1}\) and 71 ng g\(^{-1}\)).

![Figure 2.1.6 - PCB concentrations (ng g\(^{-1}\)) recorded in resin pellets collected in Portuguese beaches (2012-2013). (Source: Antunes et al., 2013)](image)

PCB congeners concentrations for each site are presented in figure 2.1.7. Concentrations of the less chlorinated congeners were higher near rural and less populated areas - Mira (Mi) and Sines (Si) - and the more chlorinated congeners recorded higher concentrations in the proximity of urban areas – Matosinhos (Mt), Espinho (Es), Paredes de Vitória (Pv) and Cresmina (Cr).
The highest total DDT (tDDT) concentrations (Figure 2.1.8) were observed in aged pellets. Matosinhos (Mt) (41 ng g$^{-1}$) recorded highest levels of DDT contamination. Fonte da Telha (FT) (25 ng g$^{-1}$) and Peniche (Pen) (21 ng g$^{-1}$) also recorded significantly higher values. White pellets recorded significantly lower concentrations than the other classes (Mann-Whitney U, p < 0.05).
Figure 2.1.8 - DDT concentrations (ng g⁻¹) recorded in resin pellets collected in Portuguese beaches. (Source: Antunes et al., 2013)

DDT composition is shown in figure 2.1.9. Percentages of pp'-DDE, pp'-DDD and pp'-DDT represented higher concentration in those three sites.

Figure 2.1.9 - Compositional patterns of DDT in total resin pellets, for each sampled site. (Source: Antunes et al., 2013)
2.1.4 Discussion

This study shows that there is a highly variable distribution of resin pellets and microplastics along the Portuguese coast and that the vast majority of microplastic marine debris in Portugal, correspond to plastic (98%), and that of these 60% are resin pellets.

Plastic marine debris densities were higher near ports and industrial areas, such as Matosinhos (Mt) (362 items m\(^{-2}\)), Vieira de Leiria (VL) (332 items m\(^{-2}\)) and Sines (Si) (84 items m\(^{-2}\)), which might be related to leakages that occur during transfer and transport activities, as plastics are transported either unpacked in bulk, or in big bags. This study did not identified a correlation between the high number of resin pellets and the proximity to industrial facilities. Further research is needed to determine whether there is a correlation or not between these variables. The high number of resin pellets in Vieira de Leiria (VL) may be influenced by the proximity of several plastic packaging production units, but further investigation should be carried out before guaranteeing this assumption.

The sites with the lowest number of pellets are Mira (Mi), in a sparsely populated area, and Espinho (Es) close to the river Douro mouth. In Espinho, coastal south-north currents may transport microplastics to Matosinhos, located in the north of the river mouth (Coelho et al., 2002).

Seventy five per cent of the resin pellets analysed belonged to the size range 3-4 mm, which may contribute to a higher toxic level of the resin pellets collected in Portugal. Also, the close proximity to industrial sites and ports (Sines and Matosinhos) seems to be related with the increased concentrations of PBTC in pellets.

Polymer identification was not performed on all resin pellets, however selected randomly samples were analysed using a Fourier transform infrared spectroscopy (FTIR) technique, confirming that the most common polymers found were polyethylene (PE) and polypropylene (PP) were the most common polymers found. This is not a surprising result as PE and PP are used daily for worldwide manufacture of plastics. Polyurethane was occasionally identified in black pellets.

Aged pellets accumulated higher concentrations of PBTC, which is probably linked to the longer exposure time in the environment (Takada et al., 2005; Endo et al., 2005; Ogata et al., 2009; Frias et al., 2010). Although it is still uncertain the time period that plastic are likely to remain in the environment, it is believed that they may last for a minimum of 10 years, being this time period extended up to 50 years, if the plastic contains chemical additives (Gregory, 1978; Derraik, 2002).
Resin pellets of smaller dimension have higher surface area for PBTC adsorption and as a consequence have potentially higher toxicity (Vlietstra and Parga, 2002). PAH concentration in aged pellets in Fonte da Telha (710 ng g⁻¹), might be an example of such influence from near industrial and highly populated areas. In this region that is close to the Portuguese capital city, Lisboa, several maritime activities take place, such as cruise and freight ship routes, motorized leisure activities (jet skis and boating), and traditional seine-haul fishing boats and tractors. High PCB concentrations in aged pellets from urban areas (figure 2.1.6) is consistent with data from atmospheric monitoring (Totten et al., 2006).

Lower chlorinated PCB congeners (18 and 31) were more abundant in rural sites such as Mira (Mi), probably due to atmospheric dispersion (Mizukawa et al., 2013). On the other hand, higher chlorinated biphenyls (138, 153 and 180) showed the highest concentration in urban areas (Figure 2.2.7) due to their tendency to remain closer to their source (Meijer et al., 2003). However, when in the ocean, plastic pellets can act as a vector for dispersion of chlorinated congeners, which could reach remote areas.

In Sines, the highest PCB concentrations were recorded in black pellets, probably due to the polyurethane fraction, as polyurethane characteristics provide a higher surface area for adsorption of these contaminants (Zia et al., 2007).

DDT and PCB use was banned in 1970s (WHO, 1993; Stockholm Convention, 2001), however pellets show traces of DDT, with the highest concentrations being registered in Matosinhos (Mt) (41 ng g⁻¹), Fonte da Telha (FT) (25 ng g⁻¹) and Peniche (Pen) (21 ng g⁻¹), reflecting contaminants environmental persistence. Percentages of pp’-DDE, pp’-DDD and pp’-DDT can be explained by the proximity to urbanized areas (Peniche (Pen) and Fonte da Telha (Fr), near Lisboa and Matosinhos (Mt) near Porto) and river mouths (Tejo river, near Lisboa and Douro river near Porto).

The results presented for PCB and PAH in aged pellets were higher than those reported by Rochman et al. (2013), in a 12 month study with virgin resin pellets. Longer exposure periods and weathering conditions to which plastics may be experiencing while in the environment, may lead to increased concentrations of PBTC. Although the concentrations in this study were higher, there are no evidence of the risk of harm to marine species.

According to the same study, depending on polymer type, PAH with few aromatic rings and lighter congeners of PCB with lower hydrophobicity reached adsorption equilibrium faster than the heavier ones. Moreover, antagonistic/synergistic effects are known to occur and affect
sorption capacities as shown by Bakir et al. (2012) for the phenantherene (PAH) and DDT. In environmental conditions, mixtures of substances and their effects are still unknown for most of the PBTC available.

Plastic marine debris contamination and accumulation on beaches has been approached through several awareness activities at the community level, such as beach cleaning campaigns, education for sustainable development (ESD) lectures and awareness campaign to reduce plastic consumption. Despite the efforts of individual stakeholders or groups of stakeholders, solutions must be reached, by involving all the stakeholders in the process and producing guidelines and preventive measures to tackle this global issue.

2.1.5 Conclusion

The assessment of plastic pollution on the Portuguese coast reveals a great quantity of stranded resin pellets. Proximity to urban areas or industrial and ports facilities (Sines and Matosinhos) seems to be related to increased concentrations of adsorbed contaminants found in pellets. High concentration of PAH probably result from the engine oils spills from ships and industrial activities such as petrochemical refining and chemical manufacturing (Li et al., 2007).

When compared to other studies, high PCB and DDT concentrations were recorded in aged pellets, reflecting their ubiquity and environmental persistence. These concentrations are not described as harmful.

Further research efforts must be undertaken regarding adsorption/sorption studies of individual PBTC and their synergetic effects once adsorbed onto plastic, as well as, their effects on the biota. The results from this studies could lead to guideline values important for environmental risk assessment of plastic marine debris.
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
3 Ingestion of microplastics by marine mussels

When exposed to weathering conditions, plastic marine debris can undergo degradation and fragmentation into smaller pieces of various shapes and colours, which are likely to be mistaken for food particles by marine organisms.

There are documented records of encounters between marine organisms and plastic debris since the 1970s. Recent scientific reports have highlighted to the fact that at least 660 marine species are negatively affected by the presence of marine litter in the environment. These species that have different habitats and behaviours, are threatened by microplastics ingestion.

In this section focus will be given to bioassays conducted with Mediterranean mussels (*Mytilus galloprovincialis*) that were fed with different concentrations of commercial polystyrene microparticles. Two case studies using non-contaminated and PAH-contaminated microplastic particles were used in separate bioassays. The first assay reflects on particle size and in environmentally relevant concentrations of plastic particles in water, according to three scenarios. For the second assay, non-contaminated and BeP-contaminated particles were used with a high concentration of particles. For both assays, health impacts were assessed by biochemical biomarkers.

Particle sizes, concentrations and the pollutant used were chosen according to the present state-of-the-art for microplastic research throughout the world and relevant data for Portugal.

Results aim to address organism health which may have potential negative consequences at population and ecosystem levels, and for that reason histological and biochemical biomarkers were measured.
3.1 **Biomarker and histopathological effects resulting from the ingestion of different concentrations of microplastic particles by *Mytilus galloprovincialis***

**Abstract**

Microplastic debris represents a global environmental threat to coastal communities, economies and wildlife. The ingestion of microplastics has been previously documented, but only a few studies used environmentally realistic particle concentrations. This work provides a novel insight to the topic as it addresses environmentally relevant microplastic concentrations in seawater, in three distinct scenarios, and as it uses a biomarker approach to estimate oxidative stress associated with the ingestion. In the bioassay three different particle sizes (2, 6 and 10 μm), and three concentrations in seawater, which represented a low, an average and a high concentration scenario (10, 100 and 1000 part. ml⁻¹) were used. Mussels ingested microplastics in the same size range of the plankton they usually ingest (2-10 μm) and microplastics were commonly found inside the digestive gland and sporadically around the gills, particularly for 6 and 10 μm in the higher particle concentrations. No significant histopathological changes that demonstrate mechanical abrasion were found. Lipid peroxidation levels were higher for higher concentrations of particles and for smaller dimensions. Accumulation of particles in the gut cavity exerted oxidative stress in the organisms. The fact that the particles used in this experiment were calibrated and of spherical shape, might have had some influence in the lower LPO levels. Further research of the effects of non-contaminated particles and of contaminated particles must be performed, in order to fully understand the impacts of microplastics towards the biota and consequences at population and ecosystem level.

*Keywords*

Microplastics, ingestion, mussels, biomarkers, Portugal

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3.1.1 Background and objectives

Although comprised of different materials (paper, glass, fabric, plastic, ceramic), marine litter is predominantly plastic (60 to 90% of total items collected). In Portugal, microplastics correspond to 98% of total debris collected in beach accumulation areas (Antunes et al., 2013).

Microscopic debris represents a global environmental threat with well-documented socio-economic and environmental impacts (Ballance et al., 2000; Matsuoka et al., 2005; Mouat et al., 2010; Frias et al., 2014; Jang et al., 2014).

According to its source, microplastics can be essentially divided into primary or secondary, depending if they were intentionally produced to have microscopic dimensions or if they result from fragmentation and degradation of larger pieces (Cole et al., 2011). The most common polymers are polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS). Combined, this polymers correspond to 90% of worldwide production and it is widely accepted that the vast majority of plastic items accumulating in coastal and marine environments are comprised of the aforesaid polymer types (Martins and Sobral, 2011; Engler, 2012), polystyrene (PS) being the sixth (7.4%) in the world production rank (Hidalgo-Ruiz et al., 2012; PlasticsEurope, 2013; Ivar do Sul and Costa, 2014).

Regardless of the polymer type, all microplastics pose threats of ingestion and entanglement that will harm organisms and eventually cause their death (Cole et al., 2013; Ivar do Sul and Costa, 2013) Filter feeders such as mussels are particularly affected (Browne et al., 2008; von Moos et al., 2012). The Mediterranean mussel (Mytilus galloprovincialis) was selected as it is a well-studied model organism and also for its relevant economic value in Portuguese markets. Mussel aquaculture represented 13% (1.8 million tons) of worldwide aquaculture production in 2010 (Oliveira et al., 2014), and 5% of national aquaculture production, in 2008.

This work will focus on the effects of the ingestion of primary microparticles made of polystyrene and aims to provide data on biochemical and histopathological effects towards marine mussels and to determine if the intake of different size and concentrations of PS microplastic particles will have significant health effects on marine mussels, based on lipid peroxidation levels.
3.1.2 Methods and materials

3.1.2.1 Experimental design

Approximately 300 healthy mussels (*Mytilus galloprovincialis*) were collected from Légua beach (39° 39’ 21” N, 9° 4’ 6” O) in Alcobaça municipality (West of Portugal), in October 2013, from an area unimpacted by pollutants.

Mussels were acclimatized to laboratory conditions for 48h before the experiment, in flow-through tanks containing filtered and constantly aerated, recirculated seawater.

The experimental assay consisted of a static arrangement of 2L-capacity beakers filled with filtered seawater, for each experimental condition - Blank, Control, and three different concentrations of polystyrene smooth shaped microplastic particles (10; 100; 1000 particles mL⁻¹), described in figure 3.1.1. The different concentrations represent three different plastic concentration scenarios 1) low, 2) average and 3) high, based on Norén, (2008).

![Table and diagram showing experimental design](image)

**Figure 3.1.1** – Experimental design of the conducted bioassay.

Three size ranges of particles were used in the bioassays (2, 6 and 10μm) purchased from Polysciences and Sigma Aldrich, made of polystyrene (PS). Aeration provided that mussels were always in contact with particles. The bioassay had the duration of 28 days, and was performed in duplicate, with sampling at the fourteenth day (T14) and at the twenty-eighth day (T28) for all experimental conditions.
The physicochemical parameters of the seawater were also monitored at T0, T7, T14, T21 and T28 and were found similar (temperature = 16 ± 1°C; salinity = 30.83 ± 0.89 ‰; pH = 7.50 ± 0.10; total ammonia = 0 – 4 mg L⁻¹).

Six randomly selected mussels (5.36 ± 0.88 cm shell length; 2.74 ± 0.44 cm shell width and 5.40 ± 3.71 g whole-body weight) were placed in each tank. Mussels were fed daily with microalgae specifically cultured for the experiment (*Isochrysis galbana*).

The gills and the digestive gland of each mussel were excised and divided for histopathological analysis or stored at -80°C for subsequent biochemical analysis (lipid peroxidation). These organs were chosen as target for their roles in microplastic particles ingestion or uptake.

### 3.1.2.2 Histopathological analysis

Animals were euthanized and organ samples (gut and gills) were immediately excised and divided for biochemical biomarkers and for histopathological analysis.

Gills and gut samples were fixed in Bouin-Hollandé’s solution (10% v/v formalin; 7% v/v acetic acid; picric acid added to saturation) for 48 h, at room temperature. Samples were then washed in ultrapure distilled water (24 h) to remove excess fixative and afterwards dehydrated through a progressive series of ethanol (70%, 96% and 100% v/v) and embedded in paraffin (xylene was employed for intermediate impregnation). Histological preparations were obtained according to Martoja and Martoja, 1967. After deparaffination and rehydration, paraffin-embedded sections (5–7 μm thick) were obtained for both organs. These sections were stained with haematoxylin and eosin (H&E) for structural analysis. All slides were cleared with xylene and mounted with DPX resin (VWR, Germany). Observations were carried out on about eight sections per slide, and on at least two slides of each organ per specimen. Histological examinations were performed using a DMLB model microscope equipped with a DFC480 digital camera (Leica microsystems).

### 3.1.3 Biochemical biomarker analysis

#### 3.1.3.1 Tissue handling and preparation

Gut samples were homogenized in cold phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 9000 x g for 5 min. The clear homogenates were divided in aliquots. In order to
determine total protein content, one aliquot was diluted (1:1) and used to determine total protein content according to the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard, in order to normalize all biochemical biomarker data. Lipid peroxidation (LPO) was determined from sample aliquots deproteinized and diluted (1:1) with 10% (m/v) metaphosphoric acid and centrifuged to remove debris and precipitated protein.

### 3.1.3.2 Lipid peroxidation

Lipid peroxidation determination was conducted using thiobarbituric acid-reactive species (TBARS) protocol adapted to microplate reader by Costa et al., 2011. Succinctly: 1% (m/v) thiobarbituric acid solution was added to the supernatant and incubated in a boiling water bath (100°C) for ten minutes, to conjugate thiobarbituric acid with lipid peroxides (in heat and low pH), forming a pink pigment. The absorbance was measured at 532 nm using a Biochrom UVM 340 microplate reader (figure 3.1.2). Malondialdehyde bis(dimethylacetal), from Merck, was used as standard to build an eight-point calibration curve. The results are expressed in nmol TBARS per mg of protein.

![Figure 3.1.2 – Lipid peroxidation determination methodology](image-url)
3.1.3.3 Statistical analysis

Data was analysed by non-parametric statistics after invalidation of homogeneity of variances, determined by Levene’s test. The Mann-Whitney U test was used for pairwise comparisons between experimental conditions (time and concentrations) and particle size. The significance level for all analysis was set at 95% (\( \alpha = 0.05 \)). All calculations were performed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA).

3.1.4 Results

3.1.4.1 Histopathological analysis

Results confirmed the presence of PS microparticles in the digestive gland of the marine mussels. Particles were commonly found in the digestive gland and sporadically found around the gills. Although particles were found in samples from all treatments, they appear to be more frequent in the average (100 part. ml\(^{-1}\)) and high (1000 part. ml\(^{-1}\)) concentration scenarios, where higher inflammatory responses were also registered. Even so, results show that there are no significant histopathological changes that demonstrate mechanical abrasion in the organs processed, which means that there is no evidence that smooth spherical shaped particles cause damage to organisms once ingested.

In what concerns particle size, 6 and 10 \( \mu \text{m} \) particles were commonly seen throughout the digestive gland (Figure 3.1.3), while particles of smaller dimensions (2 \( \mu \text{m} \)) were more difficult to identify. Nonetheless, figure 3.1.4., shows an example of 2 microns particles in the digestive tubules.

In this experiment the mortality rate was low (1%), affecting 3 individuals that were not in contact with microplastic particles (control groups).
Figure 3.1.3 – Tubules of the digestive gland with 6 and 10 µm microplastic particles, H&E stain. (a. and b. fluorescent 6 µm microplastic particle in the treatment with 10 part. ml⁻¹; c. and d. accumulation of 10 µm in the treatment with 1000 part. ml⁻¹).

Figure 3.1.4 – Tubules of the digestive gland with 2 µm polystyrene microparticles, H&E stain.
3.1.4.2 Lipid peroxidation

Statistically significant differences were found for lipid peroxidation (LPO), between T14 and T28, namely in the control group (*) for 2μm particles, in the 100 part. ml⁻¹ (***) for 6μm particles, in the control group (*) and in the 10 part. ml⁻¹ (*) for 10μm (Figure 3.1.5) (Values of p: * p < 0.05; ** p < 0.01; *** p < 0.001).

The highest LPO value occurred after 28 days exposure to the smaller particle size (2μm) at the highest particle concentration (1000 part. ml⁻¹). Other high lipid peroxidation values, when compared to the blank (T0), were found at T28 both for 6μm at 100 part. ml⁻¹ concentration and for 10μm at 1000 part. ml⁻¹ concentration.

![Graph showing lipid peroxidation levels](image)

**Figure 3.1.5** - Lipid peroxidation measured in gut cavity. Mean values ± SD (concentrations in duplicate); Values of * p < 0.05; ** p < 0.01; *** p < 0.001 (MannWhitney U-test).
3.1.5 Discussion

This experiment shows that *M. galloprovincialis* ingest microplastic particles that are in the same range as the food they usually filter (between 2 and 10 μm), as already demonstrated in previous studies (Browne *et al.*, 2008; von Moos *et al.*, 2012). The main difference is that this study takes into account three different scenarios with environmentally relevant particle concentrations, which were based on the report from Nóren, (2008).

The microplastic concentration in seawater that would best describe the current situation in Portuguese coastal water, would be a scenario between the lower (10) and the average (100 particle ml⁻¹) concentrations.

Microplastics were commonly found inside the digestive gland and sporadically around the gills, particularly for particles of 6 and 10 μm in the higher particle concentrations. Regarding smaller particles, their identification was harder, but was still possible as it is possible to see in figure 3.1.4.

The particles used in this bioassay, are commercial microparticles, calibrated and of perfect smooth spherical shape. One of the disadvantages of working with these particles is that they do not reflect the wide range of shapes or sizes that presumably exist in the environment. It is believed that most of the microplastics in the environment are secondary microplastics, resulting from degradation and fragmentation of larger pieces, and that might have sharp edges, therefore contributing to internal cuts in the digestive tract and associated extensive inflammatory response (Browne *et al.*, 2008; von Moos *et al.*, 2012).

In this study, no significant histopathological changes that demonstrate mechanical abrasion were found, and the probable main cause for this is, as well, the smooth shape of the particles used.

In the whole experiment, the mortality rate was very low, 1% (3 individuals), accounts for the good health and condition status of the mussels. Also, none of the mussels died as a consequence of ingesting plastic, as mortality affected animals in beakers without microparticles.

The results of this study show that there are statistically significant differences between T14 and T28 for 6 and 10 μm, as seen in figure 3.1.5., and that for 6 μm a longer period of exposure to 100 particles ml⁻¹ exerted negative effects in terms of lipid peroxidation. For 2 and 10 μm, the higher concentration scenario showed high concentrations of LPO at T28, which means that under long periods of time, microplastics will have negative effects on lipid peroxidation of marine mussels. Statistically significant differences were found between the 14th and 28th day (T14 and T28). Results show that in 28 days animals exposed to 2 and 10 particles sizes and high particle concentrations will have higher oxidative stress effects than control organisms. Organisms in the
control groups suffer higher oxidative stress at T14, which may be a result of their adaptation to laboratory conditions. LPO decreases its concentration after 28 days.

Considering the smaller particle size (2 μm), the highest LPO value was registered at T28, for the highest particle concentration. This means that after 28 days, if mussels are exposed to a high concentration of particles in the water, this will lead to an increase in inflammatory responses, leading to an eventual cell death that will result in alterations of their health.

Also, due to the surface/area ratios, for the same concentration of particles, particles of 2 μm will represent a higher threat to mussel tissues, when compared to 10 μm particles. This might be the reason why the value for 2 μm particles was high.

### 3.1.6 Concluding Remarks

This work shows that *M. galloprovincialis* ingests microplastic particles of 2, 6 and 10 μm, which is in the same size range as particles they feed upon. No significant histopathological changes were identified, which demonstrate mechanical abrasion of the tissues. However, lipid peroxidation levels were higher for smaller particles and for higher concentrations of microplastic particles in the water. Accumulation of particles in the gut cavity exerted oxidative stress in the organisms.

The fact that calibrated smooth microplastics were used in experiment, may have influenced in the lower LPO levels. Further research of the effects of non-contaminated particles and of contaminated particles must be done, in order to fully understand the impacts of microplastics in the environment towards the biota and consequences at populations and ecosystem level.

### 3.1.7 Acknowledgments

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Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
3.2 Biomarker and histopathological effects of the ingestion of non-contaminated and PAH contaminated microplastic particles by *Mytilus galloprovincialis*³

Abstract

Plastic marine litter tends to fragment into microplastic particles when exposed to weathering conditions, and distributed throughout the world’s oceans, posing an ingestion risk to marine organisms. The ingestion of microplastics has been reported before in marine species, as part of laboratorial experiments with high concentrations of microplastics in water. This work provides a novel insight to the topic as it addresses non-contaminated and PAH contaminated microplastics particles, and the biochemical effects associated with the ingestion. Bioassays were conducted using a high concentration of microplastic particles in seawater (1000 part. ml⁻¹), of three different sizes (2, 6 and 10 μm) of particle. In order to simulate an acute exposure, microplastic particles were contaminated with a high concentration (100 μg L⁻¹) of a benzo(e)pyrene, a polycyclic aromatic hydrocarbon. Digestive glands presented higher oxidative stress and inflammatory responses when compared to gills. The most prevalent histopathological lesions and alterations were related structural changes in the tubules of the digestive gland. Generally, PAH contaminated particles were responsible for higher lipid peroxidation levels in the digestive gland. In the case of gills, total glutathione levels were higher for contaminated particles. Statistically significant differences in biomarkers were found between the non-contaminated and BeP contaminated exposure, meaning that animals exposed to BeP have higher oxidative stress and are likely suffer health consequences. Although these results constitute a novelty in the field microplastic ingestion experiments by marine filter feeders, they must be carefully interpreted as they result from acute exposure experiments. Further research and chronic exposure assays with more species and pollutants are needed to fully assess the influence and impact of ingestion of contaminated microplastic debris towards the biota.

Keywords

Microplastics, oxidative stress, PAH, Portugal

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3.2.1 Background and objectives

Plastic is, undoubtedly, one of the most useful materials in modern society, particularly due to the wide range of uses, from food preservation to medical and/or technological applications. Production and consumption of plastic materials has been intensely rising since the 1950s, driven by the low price and malleable characteristics of this material (Thompson et al., 2009; PlasticsEurope, 2013).

One remarkable characteristic of this material is its capacity to adsorb persistent, bioaccumulative and toxic chemicals (PBTC) such as polychlorinated biphenyls (PCB), polycyclic aromatic hydrocarbons (PAH), dioxins and aldrins, from sediments or water (Takada et al., 2005; Rios et al. 2007; Teuten et al. 2007, Hirai et al. 2011; Andrady, 2011, Bakir et al. 2012). Having into consideration this particularity of plastic materials, this work aims to determine the effects of the ingestion of PAH contaminated microplastic particles.

PAH are semi-volatiles compounds with low water solubility and high lipid solubility. These characteristics, coupled with environmental factors result in the presence of these pollutants all over the world, even in regions where they have never been used before (Engler, 2012), as they are able to travel long distances in the atmosphere, before deposition. Because they are PBTC, they have also the capacity to bioaccumulate in fatty tissues of living organisms (Ritter et al., 2007; Tanaka, et al., 2013) or adsorb onto plastic (Takada et al., 2005; Frias et al., 2010; Mizukawa et al., 2013).

Marine organisms have been reported to ingest microplastics (Browne et al., 2008; von Moos et al., 2012; Cole et al., 2013), and obstruction of the digestive tract is always a concern, particularly if the microplastics are contaminated with PTBC, as there might be a potential chemical transfer along the food web (Ryan et al., 1988; Zarfl and Matthies, 2010; Bakir et al., 2012; Engler, 2012, Tanaka et al., 2013). PBTC adsorbed onto plastic are likely to cause chronic diseases to organisms who ingest them (Tanaka et al., 2013). Benzo( pyrene (BeP) was selected for being the PAH with highest adsorbed concentration onto resin pellets, in studies conducted in Portugal between 2010 and 2014 (Frias et al., 2010; Antunes et al., 2013, Mizukawa et al., 2013).

In order to measure biological effects, researchers often resort to the use of biomarkers, which are early-warning signals that might reflect adverse biological responses towards xenobiotics. Defined as change in a sub-individual biological response related to exposure to environmental chemicals or their toxic effects (Martin-Diaz et al., 2004), biomarkers are a useful tool to measure biological changes and damage. The biological changes might range from molecular through cellular and physiological responses to behavioural changes, and can be identified inside an organism or in its biological products (Van der Oost et al., 2003).
Organisms are able to metabolize some organic xenobiotics, such PAH, into a more water-soluble form which is easy to excrete than the original compound. Some xenobiotic derivatives like aromatic diols and quinones, nitroaromatics, aromatic hydroxylamines, and bipyridyls may be accompanied by a burst in the production of reactive oxygen species (ROS) as a consequence of biotransformation. When ROS exceeds the cellular defence systems, alterations like lipid peroxidation can occur, since the redox state in cells is defined by a balance between antioxidants (reducing) and hazardous (oxidizing) agents (Van der Oost et al., 2003; Martín-Díaz et al., 2004; Martins et al., 2012). The ingestion impact of non-contaminated and PAH-contaminated microplastic particles by marine mussels was investigated in order to reduce information gaps regarding biochemical effects.

The goals of this exposure study were to 1) evaluate whether the ingestion of microplastics would exert negative biochemical effects (cell death) on mussels; 2) evaluate whether different size of microplastic particle have diverse effects on mussels; 3) determine if there are significant changes in the tissues as a result from exposure.

### 3.2.2 Methods and materials

#### 3.2.2.1 Particle spiking

Commercial polystyrene (PS) smooth spherical fluorescent (excitation 441 nm/emission 486 nm) labelled microparticles (Polysciences, Inc., Germany) of different sizes (2, 6 and 10 μm) were spiked with a concentration of 100 μg L⁻¹ of Benzo(e)pyrene (BeP; Sigma Aldrich, Germany) previously dissolved in dimethyl sulfoxide (DMSO), to simulate an acute bioassay. The concentration used was based in literature review on the No Observable Effect Level (NOEL) concentration levels for benzo(a)pyrene (BaP), an isomer of BeP. BeP was the pollutant selected for this experiment because it was the PAH with highest concentration adsorbed to pellets collected from Portuguese beaches.

Figure 3.2.1 shows the experimental design of the bioassay. Treatments with 2 replicates for the three different sizes (2, 6 and 10 μm), at a concentration of 1000 part. mL⁻¹, as well as the non-contaminated (NCP) and BeP contaminated (CP) microplastic particles are represented. In order to make comparisons with references, three control treatments without microplastic particles (blank, DMSO and BePCtrl) were used. Because BeP had to be dissolved in DMSO, it is important to have a control group for DMSO alone, to estimate what are the effects of this substance.
PS concentrations were selected based on the experiment described in sub-chapter 3.1. Each exposure beaker was equipped with one aeration stone in order to ensure constant aeration and the even distribution of PS spheres in suspension. After exposure, histological techniques were used to determine the presence of microplastics in the gut.

3.2.2.2 Experimental design

Approximately 150 healthy mussels (*Mytilus galloprovincialis*) were collected from Légua beach (39° 39’ 21” N, 9° 4’ 6” O) in Alcobaça municipality (West of Portugal), in October 2013, from an area unimpacted by pollutants. Mussels were acclimatized to laboratory conditions for 48h before the experiment, being introduced in flow-through tanks containing filtered and constantly aerated recirculated seawater. Three size ranges of polystyrene (PS) particles were used in the bioassays (2μm, 6μm and 10μm) purchased from Polysciences Inc., Germany.

The experimental assay consisted of a static arrangement of 2L-capacity beakers, each with 2L filtered seawater (figure 3.2.1.). Treatments were blanks (BL), the controls (DMSO, BePCtl, 2NCP, 6NCP and 10NCP) and experimental particle spikes (2CP, 6CP and 10CP). As BeP had to be previously dissolved in DMSO, there are two pollutant controls (DMSO and BePCtl) that were dissolved into water, in the same concentrations used for the experimental controls. The particle controls represent the effect of particles without the contaminant. The bioassay had the duration of 7 days and was performed in duplicate. The physicochemical parameters of water were also monitored at T0 and T7 and were found similar (temperature = 16 ± 1°C; salinity = 30.58 ± 0.73 ‰; pH = 7.53 ± 0.04; total ammonia = 0 - 4 mg L⁻¹).

Six randomly selected mussels (6.59 ± 0.53 cm shell length; 3.29 ± 0.28 cm shell width and 6.24 ± 2.23 g whole-body weight) were placed in each tank. Mussels were fed daily with microalgae specifically cultured for the experiment (*Isochrysis galbana*) and sampling was performed at the seventh day (T7) for all experimental conditions.

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The gills and the digestive gland of each mussel were excised and divided for histopathological analysis or stored at -80°C for subsequent biochemical analysis (lipid peroxidation, glutathione activity). These organs were chosen as target for their roles in apical entry of toxicants or microplastic particles ingestion.

3.2.2.3 Histopathological analysis

Histological preparations were obtained according to Martoja and Martoja, 1967. Briefly: tissue samples were fixed in Bouin-Hollande’s solution (10% v/v formalin; 7% v/v acetic acid; picric acid added to saturation) over 48 h, at room temperature. Samples were afterwards washed in ultrapure distilled water (24 h) to remove excess fixative and dehydrated through a progressive series of ethanol (70%, 96% and 100%; v/v). Xylene was employed for intermediate impregnation. After deparaffinization and rehydration, paraffin-embedded sections (5–7 µm thick) were stained with haematoxylin and eosin (H&E). All slides were mounted with DPX resin (VWR, Germany). Observations were carried out on about eight sections per slide, with at least three slides of each organ being prepared per specimen. Histological examinations were performed using a DMLB model microscope equipped with a DFC480 digital camera (Leica microsystems).

3.2.3 Biochemical biomarker analysis

3.2.3.1 Tissue handling and preparation

Gut and gill samples were homogenized in cold phosphate-buffered saline (PBS), pH 7.4, and centrifuged at 9000 x g for 5 min. The clear homogenates were divided in aliquots. In order to determine total protein content, one aliquot was diluted (1:1) according to the Bradford method (Bradford, 1976), using bovine serum albumin (BSA) as standard, in order to normalize all biochemical biomarker data. The absorbance was measured at 595 nm using a Biochrom UVM 340 microplate reader.

GSH-related biomarkers and lipid peroxidation (LPO) were determined from sample aliquots deproteineized and diluted (1:1) with 10% (m/v) metaphosphoric acid and centrifuged to remove debris and precipitated protein (Figure 3.2.2.).
3.2.3.2 Lipid peroxidation

Lipid peroxidation determination was conducted using thiobarbituric acid-reactive species (TBARS) protocol adapted to microplate reader by Costa et al., 2011. Briefly: 1% (m/v) thiobarbituric acid solution was added to the supernatant and incubated in a boiling water bath (100°C) for ten minutes to conjugate thiobarbituric acid with lipid peroxides (in heat and low pH), forming a pink pigment. Activity was determined spectrophotometrically using the aforesaid microplate reader (absorbance was measured at 532 nm). Malondialdehyde bis(dimethylacetal) (MDA), from Merck, was used as standard to build an eight-point calibration curve. The results are expressed in nmol TBARS per mg of protein.

3.2.3.3 Glutathione determination

The glutathione (total, reduced and oxidized) was measured in the mussel digestive gland and in the gills, using the Glutathione Assay Kit (Cayman Chemical Company, MI, USA), following manufacturer instructions. The assay allows quantification of total glutathione (GSHt) and glutathione disulphide (GSSG) by derivatising GSH in samples with 2-vinylpyridine (from Sigma-Aldrich). Total GSH and GSSG were calculated from a calibration curve obtained with GSH. Activity was determined spectrophotometrically using the aforesaid microplate reader (absorbance was measured at 410 nm). The results were expressed as nmol per mg of protein. The ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) was calculated using the equation GSH/(GSSG/2).

3.2.3.4 Statistical analysis

After data failed to comply with parametric assumptions, namely homogeneity of variances,
as determined by the Levene’s test, data was applied non-parametric statistical tests. The Mann-Whitney U test was used for pairwise comparisons between experimental conditions and particle size. The significance level for all analysis was set at 95% ($\alpha = 0.05$). All calculations were performed using the software Statistica® 7.0 (Statsoft Inc., Tulsa, OK, USA).

3.2.4 Results

3.2.4.1 Histological analysis

PS particles were commonly found in the digestive gland and sporadically seen around the gills. Digestive gland samples consistently yielded more histopathological changes and inflammatory responses than gill samples, particularly in the treatments with BeP contaminated particles. Figure 3.2.3 shows examples of the inflammatory responses in the digestive gland with 2$\mu$m non-contaminated and contaminated particles and figure 3.2.4 shows examples of 6$\mu$m particles in the digestive gland and gonads.

In figure 3.2.3., it is possible to see the digestive gland without tubule alterations (a. and c.) and with inflammatory responses or necrotic digestive diverticula (b. and d.) after exposure to 2 $\mu$m particles. Alterations are indicated by arrows in the figure.

![Image](image-url)

Figure 3.2.3 – Tubules of digestive gland exposed to 2 $\mu$m NCP (a. and c.) and CP (b. and d.). H&S stain.
Figure 3.2.4., shows the presence of microparticles in the digestive gland and in the gonads, stained with haematoxylin and eosin and also under fluorescence. Particles are located where the arrows point.

![Figure 3.2.4 – NCP 6 μm particles in tubules of the digestive gland (a. and b.) and in the gonads (c. and d.). H&E stain and fluorescence light.](image)

Granulocytes (white cells) and lipofuscin deposits were sporadically identified in all BeP samples, either control or experimental treatments. Figure 3.2.5., show more examples of inflammatory responses in the digestive gland and in gonads, an in the case of d. granulocytes are visible in the digestive diverticula.
Figure 3.2.5 – Digestive gland and gonads after exposure to 6µm CP particles. a. microplastic particle in the gut cavity; b. inflammatory response surrounding the digestive diverticula; c. inflammatory response surrounding the male gonads, d. granulocytes in the digestive diverticula. H&E stain.

Figure 3.2.6 shows examples of the digestive gland of the mussels exposed to 10 µm BeP contaminated particles, where it is possible to see inflammatory responses and particles entrapped in the diverticula.
Figure 3.2.6 – Tubules in the digestive gland after exposure to 10μm CP. a. and b. inflammatory response with necrotic tubules; c. microplastic particle in the digestive diverticula; and d. detail of inflammatory response in the digestive diverticulum. H&E stain.

Figure 3.2.7 compares the two particles sizes regarding their controls and experimental treatments. It is possible to see that when BeP is present the inflammatory response in this tissue is higher.

Figure 3.2.7 – Gills exposed to NCP and CP. a. 6BeP; b. 10Ctl, c. 6Ctl and d. 10BeP. H&E stain.
3.2.4.2 Biochemical biomarker analysis

Concentrations of TBARS (lipid peroxidation), total glutathione (GSHt), glutathione disulphide (oxidised glutathione; GSSG) and the ratio of reduced glutathione to oxidised glutathione (GSH/GSSG) glutathione are shown in figure 3.2.8 for the digestive gland and in figure 3.2.9 for the gills.

Regarding the digestive gland, lipid peroxidation concentrations were higher for the BeP contaminated microplastic particles, which was an expected result, as particles would be in direct contact with this tissue after ingestion. Statistically significant differences between non-contaminated and BeP contaminated particles for particle size 2 μm (* p<0.05) and 6 μm (** p<0.001) were found. This means that mussels exposed to BeP contaminated particles, within these experimental conditions, suffered higher oxidative stress than mussels exposed to non-contaminated microplastic particles.

Total glutathione (GSHt) concentrations were higher for non-contaminated particle treatments, as expected, since GSHt is inversely proportional to TBARS. No statistically significant differences were found between treatments, for GSHt, GSSG or the ratio GSH/GSSG.
Figure 3.2.8 – Lipid peroxidation and glutathione levels measured in the gut cavity. Mean values ± SD (concentrations performed in duplicate). Values of * p < 0.05; ** p < 0.01; *** p < 0.001 (Mann-Whitney U-test).

Treatments with different letters are significantly different (Mann-Whitney U-test, p< 0.05).

In the case of gills, biochemical biomarker results varied in inverse proportion with the ones described before. TBARS concentrations were higher for treatments with non-contaminated particles.

Regarding microplastics input, or even direct contact, the digestive gland (where microplastics can be retained) and the gills are very distinct structures. BeP might contribute to a reduction in metabolic activity, leading to lower concentrations of TBARS. Regardless of the reason that contributed to a change in the values, further research is needed to determine the mechanisms that lead to this result, particularly through chronic bioassays. Statistically significant differences were found to 6 μm and to 10 μm particles (figure 3.2.9, d and e, respectively).

In what concerns the GSHt, concentrations were higher for the contaminated particles, meaning that there are higher concentrations of oxidised glutathione and therefore lower ratio GSH/GSSG, when compared to the results in 3.2.8.
Comparing both tissues, concentrations of TBARS and ratio GSH/GSSG show that the digestive gland suffered higher oxidative stress than the gills.

3.2.5 Discussion

In this study, the effects of the ingestion of non-contaminated and BeP contaminated microplastic particles were assessed through an acute bioassay. Histopathologic analysis and biochemical biomarkers were used to determine inflammatory responses and oxidative stress levels (TBARS and GSHt, GSSG, ratio GSH/GSSG).

Thiobarbituric acid reactive substances (TBARS) are formed as a by-product of lipid peroxidation and can help measure the damage produced by oxidative stress. The TBARS assay used measures malondialdehyde (MDA) that is present in samples or that results from reaction of the lipid hydroperoxides.

The tripeptide glutathione (GSH) has an important role, as it serves as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics, being an essential
electron donor to glutathione peroxidases in the reduction of hydro peroxides. Glutathione is present in the cells in both the reduced (GSH) and oxidized (GSSG) forms. Because of the action of the NADPH-dependent enzyme GSSG reductase (GR), the cellular content of glutathione is predominantly in favour of GSH under normal physiologic conditions. However, pathophysiologic conditions causing oxidative stress result in changes in the GSH/GSSG ratios (Figure 3.2.10).

![Figure 3.2.10 — GSH recycling (Source: Caymen Glutathione assay Kit)](image)

Levels of TBARS and GSHt result from metabolic processes that are inversely proportional, which is in accordance with the results presented in figures 3.2.8 and 3.2.9. While TBARS concentration levels are increasing, GSHt levels are decreasing. GSSG, glutathione disulphide, a form of oxidised glutathione will follow a similar trend to TBARS. The ratio of reduced glutathione to oxidised glutathione (GSH/GSSG) within cells is often used as a measure of cellular toxicity and was also used to assess oxidative stress in this study.

Higher concentrations of TBARS were registered for treatments with BeP contaminated particles. In the digestive gland statistically significant differences were found between non-contaminated and BeP contaminated particles for particle size 2μm (* p<0.05) and 6 μm (** p<0.001), meaning that the particles that were contaminated with a PAH had a strong influence in the oxidative stress and lipid peroxidation in this tissue. Statistically significant differences were also found in the gills, namely for 6 and 10 μm particles. Although there were no difference either if the particles were contaminated or not, both 6 and 10 μm particles exerted higher oxidative stress, than 2 μm particles. Animals exposed to contaminated particles had higher oxidative stress and visible inflammatory responses and/or granulocytes in the digestive gland than animals exposed to non-contaminated particles. The fact that the gills are not in direct contact for long periods of time with the contaminated micropastics, might suggest a reason for the lower TBARS concentration levels, which could be explained if the BeP contributed to a reduction of the metabolic activity, yet, further investigation is needed to determine correctly this assumption. By comparing both tissues, concentrations of TBARS and ratio GSH/GSSG show that the digestive gland suffered higher oxidative stress than the gills.
3.2.6 Concluding Remarks

This study contributes to understand the impacts of the ingestion of contaminated microplastic particles by the Mediterranean mussel. According to this acute bioassay, the digestive gland of the mussels is more affected in terms of oxidative stress and histopathological lesions, than the gills. In this 7 days bioassay a high concentration of pollutant (100 µg L⁻¹ of Benzo(e)pyrene) was used, which does not reflect the actual concentration of this pollutant in the environment. This pollutant was selected having into consideration the studies of PBTC adsorbed to plastic resin pellets collected in Portuguese beaches since 2008.

Regarding the organs used, in the digestive gland, lipid peroxidation values were higher for BeP contaminated particles, which is expected as contaminated microparticles are in direct contact with internal tissues after ingestion. Another result is the fact that BeP contaminated particles exert higher lipid peroxidation levels, meaning that contaminated particles have higher risk of oxidative stress after an exposure of 7 days. As total glutathione levels are inversely proportional to TBARS, treatments using non-contaminated microparticles had higher GSHt concentrations. Concerning the gills, biomarker results were opposite to the ones described for the digestive gland.

One of the limitations of this work is the fact that is an acute bioassay with a high concentration of BeP, and therefore these results must be carefully interpreted before making general assumptions and conclusions. Nonetheless this assays are also important to estimate the effects of sudden high concentrations of pollutants (e.g. oil spill). The way to overcome this limitation, is through further research and conducting chronic bioassays with the same pollutant and species. Also, it is important to conduct similar studies with species from functional groups and with a wider range of pollutants to fully verify and assess the impacts of contaminated microplastic particles that are ingested by marine organisms.

3.2.7 Acknowledgments

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Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
4 Evidence of microplastics in Portuguese coastal waters

Due to its light weight, plastics can travel great distances once in the open ocean. Besides accumulation areas in beaches and in gyres, there is a growing concern among researchers to identify the amount of plastics floating in the ocean.

This chapter provides some data about Portuguese coastal waters, particularly evidences of microplastics collected from annual water sampling conducted by the Portuguese Institute of the Sea and Atmosphere (IPMA) to assess fish stocks.

This work focus on polymer types of floating debris and on the microplastic:zooplankton ratios for several locations in Portuguese waters.
4.1 **Evidences of microplastics in Portuguese coastal waters**

**Abstract**

Records of high concentrations floating plastic marine debris in the ocean have led to investigate the presence of microplastics in samples of zooplankton from Portuguese coastal waters. Zooplankton samples collected at four offshore sites (Aveiro, Lisboa, Costa Vicentina and Algarve), in surveys conducted between 2002 and 2008, with three different sampling methods, were used in this preliminary study. A total of 152 samples were processed with microplastics identified in 93 of them, corresponding to 61% of the total. Costa Vicentina, followed by Lisboa, were the regions with higher microplastic concentrations (0.036 and 0.033 no. m\(^3\)) and abundances (0.07 and 0.06 cm\(^3\) m\(^3\)), respectively. Microplastic: zooplankton ratios were also higher in these two regions, which is probably related to the proximity of densely populated areas and inputs from the Tejo and Sado river estuaries. Microplastics polymers were identified using Micro Fourier Transformed Infrared Spectroscopy (\(\mu\)-FTIR), as polyethylene (PE), polypropylene (PP) and polyacrylates (PA). The present work is the first report on the composition of microplastic particles collected with plankton nets in Portuguese coastal waters. Plankton surveys from regular monitoring campaigns conducted worldwide may be used to monitor plastic particles in the oceans and constitute an important and low cost tool to address marine litter within the scope of the Marine Strategy Framework Directive (2008/56/EC).

**Keywords**

Microplastics, plastic, zooplankton, MSFD, FTIR, Portugal

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4.1.1 Background and objectives

The presence of plastic marine debris, as well as its sizes and potential threat to marine fauna through ingestion and/or entanglement, have been documented throughout the world’s oceans. Plastic marine debris are widely spread in the ocean as a result of their light weight that causes these materials to float, being transported by ocean surface currents into remote areas.

High concentrations of microplastics tend to accumulate in vortex convergence zones known as ocean gyres (Pichel et al., 2007), where reports of the high incidence of plastic marine debris in the North Pacific Central Gyre (Moore et al., 2001, 2002; Moore, 2008; Goldstein et al., 2012), and in other gyres have raised concern and an unprecedented interest for research on the topic in the areas of marine sciences as well as in social sciences (Bravo et al. 2009, Hinojosa and Thiel, 2009, Luís and Spinola, 2010). Recent studies have shown the ability for filter feeders and zooplankton to ingest plastic particles ranging from 1.7 – 30.6 μm (Crimmins et al., 2002; Browne et al., 2008; von Moos et al., 2012; Cole et al. 2013), which may eventually increase the toxic effect risk due to accumulation of persistent bioaccumulative and toxic chemicals (PBTC) in lipid reserves. Accumulated toxic chemicals may transfer along the food chain and eventually reach human diets (Ryan et al., 1988; Tanaka et al., 2013). Several size ranges of zooplankton may incorporate the tiny pieces of plastic in their diet, potentially causing a large scale accumulation problem, with unpredictable consequences (Cole et al., 2013).

While dense varieties of plastics such as commonly used nylons, polyvinyl chloride (PVC) and polyethylene terephthalate (PET) tend to sink in the water column and reach the coastal sediment (Andrady, 2011), most microplastics from commonly used polymers, such as polyethylene (PE), polypropylene (PP) and polystyrene (PS), will float and may be collected using plankton nets and manta trawls (Vianello et al., 2012).

Although plankton nets and manta trawls provide valuable information about the amounts of plastic marine litter in the marine environment, data is still scarce and underestimated, as the real quantities are difficult to assess. In order to measure the amount of plastic marine litter in the ocean, it is necessary to have into account all the inputs since the 1950’s, plus marine litter entrapped in coastal sediments or in beaches and coastal areas and floating debris at the surface and in the water column of all oceans. This is a demanding and high costly task. Nonetheless, regular plankton surveys which are performed for monitoring fish stocks may provide data on microplastics.
in the oceans without further cost of days at sea, and contribute to the marine litter evaluation as included in the Marine Strategy Framework Directive (2008/56/EC) (MSFD) for the European seas. They can also provide data on microplastics size and polymers.

Therefore, the main goals of this exploratory work were to (1) detect and quantify microplastic debris present in zooplankton samples and assess variations among sites; (2) identify the plastic polymers present, using a spectroscopy technique – the Fourier Transform Infrared Spectroscopy (μ-FTIR).

4.1.2 Material and methods

4.1.2.1 Sample collection and processing

Zooplankton samples were collected between 2002 and 2008, in four areas of Portuguese coastal waters, – Aveiro (AV), Lisboa (LX), Costa Vicentina (CV) and Algarve (AL) (Figure 4.1.1) – as part of annual surveys performed by the Instituto Português do Mar e da Atmosfera (IPMA) to assess fish stocks. Samples were collected using three different sampling methods, W (WP2 nets), N (Neuston nets) and L (Longhurst Hardy Plankton Recorder, Pro-LHPR).

Sampling methods differ in mesh size (W – 180 μm; N – 280 μm; L - 335 μm) and in opening area (W – 0.58 m diameter; N– rectangular 0.2 x 1.0 m; L - 0.42 m diameter). W and N samples were towed horizontally for 3 min at ship speed of approximately 1.5 knots, in the upper 20 cm of the water column. L samples were collected for approximately 30 min at a ship speed of approximately 4 knots, at 25 m deep.

After collection, samples were stored in plastic jars and preserved in ~4% borax-buffered formaldehyde prepared using seawater. Volumes were determined and standardized using flow meter information. Zooplankton biomass and plastic volume were estimated by displacement volume. Samples were then filtered through Whatman® glass microfiber filters with a diameter of 47 mm using manual methods. The zooplankton was then examined under a stereoscopic microscope to sort and measure the microscopic plastic particles. Particles were photographed and recovered onto concave slides covered and stored until further analysis. During sample sorting, no fibres were found in any of the samples processed, confirming that there had been no contamination from clothing.
A microplastic zooplankton ratio was calculated, based on standardized volumes (cm$^3$ m$^{-3}$). As a method, it is more accurate to use standardized volumes than dry weight for the ratio calculation. This relationship will be more realistic and can describe better the relative abundance of microplastics in the ocean. The ratio can also be useful to compare among regions, using standardized concentrations (no. m$^{-3}$), abundances (cm$^3$ m$^{-3}$) or densities (mg m$^{-3}$).

4.1.2.2 μ-FTIR analysis

To identify the composition of polymers a spectroscopy technique was used – μ-FTIR - which is a fingerprinting technique that provides characterization at the molecular level, allowing the identification and distinction of the different materials, through the interaction between infrared radiation and matter. This interaction is different for each material resulting in a
fingerprint spectrum with specific and characteristic bands for each one (Hummel, 2002). Additionally, this method of vibrational spectroscopy is extremely sensitive to molecular structural changes. When using a microscope coupled to the μ-FTIR spectrophotometer it is possible to go to the micro-scale and work with pieces with a size range of micrometres (Afremow et al. 1969; Hummel, 2002).

To guarantee representativeness, micro samples were carefully cut under the Leica KL 1500 LCD microscope, equipped with a 12 x objective and a Leica® Degilux 1 digital camera, with external illumination by optical fibres in order. For each plastic sample, depending on its heterogeneity (including degradation status) 2-3 micro samples were analysed. These were compressed in a diamond anvil compression cell, and infrared spectra were acquired in a Nicolet® Nexus spectrophotometer coupled to a Continuum microscope (32×objective) with a MCT detector.

Spectra were collected in transmission mode in 128 scans, with a resolution of 4 cm⁻¹. The spectra are shown as acquired, without corrections or any further manipulations, except for the occasional removal of the CO₂ absorption at ca. 2300-2400 cm⁻¹ (Moura et al., 2007). The identification of polymers was first made by searching the extensive polymer spectral database, and comparison analysis of the polymer characteristic band with spectral assignments.

4.1.2.3 Statistical analysis

Data was analysed by non-parametric statistics after invalidation assumption of variance’s homogeneity by Levene’s test. The Mann-Whitney U test was used for pairwise comparisons between sampling sites. The significance level for all analysis was set at 95% (α = 0.05). All calculations were performed with the software Statistica®7.0 (Statsoft Inc., Tulsa, OK, USA).

4.1.3 Results

4.1.3.1 Microplastics analysis

The non-standardized, number of microplastics collected in all processed samples was 684 and the total volume of microplastics totalled 113.01 cm³, which corresponds to 61% of all samples. Table 4.1.1 summarizes standardized microplastic and zooplankton volumes (cm³ m⁻³, mean±sd); microplastic: zooplankton ratio and microplastic concentration (n⁰. m⁻³, mean±sd) for each sampling site.
Table 4.1.1. – Data summary for each sampling site. Mean and standard deviation (sd) are given for microplastic and zooplankton abundances and concentrations.

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<td>Samples processed (n)</td>
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<td>41</td>
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</tr>
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<td>Zooplankton abundance, mean±sd (cm$^3$ m$^{-3}$)</td>
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<td>0.51±0.43</td>
<td>0.02±0.01</td>
</tr>
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<td>Microplastic: zooplankton ratio</td>
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<td>0.12</td>
<td>0.14</td>
<td>0.05</td>
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<td>0.033±0.021</td>
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</tbody>
</table>

Microplastic: zooplankton ratios were determined for each site (Table 4.1.1) and for two sampling method – 0.02 for L (n=22) and 0.07 for N (n=69). Due to the limited number of samples (n=2), W sampling method was not included in the statistical analysis nor in the microplastic:zooplankton ratio calculations. Figure 4.1.2 shows mean microplastic and zooplankton abundances (cm$^3$ m$^{-3}$) and box-plot distributions of these two variables. Regarding zooplankton abundances, 75% of data is approximately below 0.6 cm$^3$ m$^{-3}$, while 75% of microplastic abundances data is below 0.03 cm$^3$ m$^{-3}$. There is no relationship between zooplankton and microplastic abundances.
Figure 4.1.2 – Zooplankton and microplastics abundances (cm³ m⁻³)

Sampling methods (N and L) differences are represented in figure 4.1.3, where it is possible to see that sampling method N collects higher abundance of microplastics.

Figure 4.1.3 – Microplastics abundance (cm³ m⁻³) for sampling method L (LHP) and N (Neuston)

Figure 4.1.4 shows differences in mean microplastic abundances among sites. This figure shows that CV is the region with higher microplastic abundance followed by LX.
There were statistically significant differences in microplastic abundance between sites, regarding sampling method N (Mann-Whitney non-parametric test, p<0.05), particularly between AV and CV (p = 0.0072); AV and LX (p = 0.0001) and LX and AL (p = 0.0174). In what concerns sampling method L, statistical comparisons between sites were not possible due to the small number of L samples in CV (n=2) and in LX, (n=1). The region with highest microplastic concentration (0.036 no. m⁻³) and abundance (0.07 cm⁻³ m⁻³) was CV followed by LX. The same trend is reflected in the microplasticzooplankton ratio (0.14 to CV and 0.12 to LX).

4.1.3.2 μ-FTIR analysis

Using Fourier Transformed Infrared Spectroscopy analysis, different polymers were identified by comparison with reference spectra. Table 4.1.2 shows the infrared characteristic bands (cm⁻¹) with their respective spectral assignments, which were made in accordance with the literature (Hummel, 2002) and can be used to correctly identify each sample.
Table 4.1.2 – Infrared characteristic bands (cm⁻¹) for microplastic samples. Adapted from: Hummel, 2002.

(ν - stretching; δₘ – asymmetric bending; δₛ – symmetric bending)

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Compound</th>
<th>Characteristic band (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHF95A1</td>
<td>Polypropylene</td>
<td>2960-2835</td>
<td>ν(CH₃)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1460</td>
<td>δₘ(CH₃)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1377</td>
<td>δₛ(CH₃)</td>
</tr>
<tr>
<td>NE15</td>
<td>Polyacrylate</td>
<td>2960-2870</td>
<td>ν(-CH)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1731</td>
<td>ν(C=O)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1270 / 1242 / 1150</td>
<td>ν(C-O)</td>
</tr>
<tr>
<td>NEF33</td>
<td>Polyethylene</td>
<td>2917 / 2851</td>
<td>ν(CH₂)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1471</td>
<td>δₘ(CH₂)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2928 / 2855</td>
<td>ν(-CH)</td>
</tr>
<tr>
<td>NEF47</td>
<td>Alkyd resin</td>
<td>1731</td>
<td>ν(C=O)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1265 / 1124 / 1070</td>
<td>ν(C-O) + ν(C-C)</td>
</tr>
</tbody>
</table>

Figures 4.1.5 shows some of the microplastic samples collected, with different sizes and shapes.

Figure 4.1.5 - Examples of microplastics with different sizes and shapes (a - LHF95A1, Polypropylene; b - NE15, polyacrylate; c - NEF33, polyethylene; d - NEF47, alkyd resin).
The spectra for each of these samples can be found as supplementary data in the annex (Figures A1 to A5). Three plastic pellets were collected in the samples (figure 4.1.6), and μ-FTIR shows a match of 98.1% with low density polyethylene (LDPE), when compared to the reference spectrum. This is a very common type of plastic found in Portuguese beaches.

![Infrared spectrum for the pellet micro sample and comparison with reference spectra Low Density Polyethylene (LDPE).](image)

**Figure 4.1.6** – Infrared spectrum for the pellet micro sample and comparison with reference spectra Low Density Polyethylene (LDPE).

Regarding samples and their matches with reference spectrum from the Department of Conservation and Restoration database, LHF95A1 has a match of 98.1% with polypropylene; NE15 a match of 99.1% with NeoCryl-B 745, a polyacrylate; NEF 33 a match of 98.2% with polyethylene and NEF47 a match of 96.1% with an alkyd resin. The polyacrylate of sample NE15 is a copolymer of methyl methacrylate (MMA) with n-butyl methacrylate (BMA) (Flick, 1991), which is widely used as a glass substitute, and NEF47, the alkyd resin is a polyester resin modified with a drying oil (Lanson et al., 1986; Stevens, 1990; Learner, 2004).
4.1.4 Discussion

Results confirm the presence of microplastics the aforesaid zooplankton samples from Portuguese coastal waters, with some differences in concentrations and abundances among sites (Table 4.1.1, figures 4.1.3 and 4.1.4). As expected, sampling method L (LHPR) collected low concentrations of microplastics, as it is deployed at a depth range of 25 m in the water column. As consequence, sampling method L had also the lower microplastic:zooplankton ratios. Floating plastics and microplastics are more likely to be collected by N (Neuston net), which in fact, showed higher ratios.

Microplastics were present in 61% of zooplankton samples, and those collected in the proximity of densely populated areas such as Aveiro (AV) and Lisboa (LX) contained 66 and 91% of microplastics respectively, which suggests their land based origin. Similar results (62% of samples with plastic marine debris) were observed in surveys conducted in the Atlantic Ocean and Caribbean Sea for 22 years (Law et al., 2010).

For Costa Vicentina (CV) and Algarve (AL), less than 50% of samples contained microplastics. CV is close to a natural park and therefore a less populated area. However, this region showed the highest microplastic concentration, abundance and microplastic: zooplankton ratio, probably in relation to the proximity of the industrial and port facilities of Sines.

In contrast, Algarve, a region of intensive tourism where strong efforts in beach clean-ups were developed, presented lower microplastic:zooplankton ratios as well as microplastic concentrations. Results are in agreement with national beach litter assessments, where the lowest values of microplastics were found in Algarve (Antunes et al., 2013). The lowest plastic concentrations and microplastic: zooplankton concentrations were registered in AV (0.04) and AL (0.05).

Another reason for the lower microplastic concentrations in the southern region of Portugal, may be the transportation by offshore surface currents from the Atlantic Ocean to the Mediterranean Sea (Lobo et al., 2000), through the Strait of Gibraltar, into the Alboran Gyres where they are likely to be retained, but further research would be necessary to verify this assumption. CV and LX show similar microplastic abundances and concentrations, which may be related to inputs from nearby populated areas and from the Tejo and Sado river estuaries.

It is not certain how long microplastics last in the ocean. According to Derraik (2002),
microplastics last at least 10 years, being this time period extended to 50 years if the plastic has additives. Plastics, and particularly microplastics are ingested by marine invertebrates, thus all microplastics described in this work may pose risk of ingestion.

Polymers were identified using μ-FTIR analysis by comparing their chemical spectra with reference spectra and through comparison of its characteristic bands with references in literature. The chemical spectra of sample NEF47, was described as an alkyd resin, usually used as paint for ships and vessels, suggesting that degradation of ship paints may contribute to introduce different types of polymers in the ocean.

Regarding plastic pellets, they may have reached the ocean by leakage from land-based sources as they are the raw material for plastic industries. The composition of the polymers collected was expected, due to trends in plastic production and consumption.

μ-FTIR results were consistent with reports and scientific papers from all over the world (Ng and Obbard, 2006; Cooper and Corcoran, 2010; Fotopoulou and Karapanagioti, 2012).

The present study is the first μ-FTIR identification of plastic marine debris in Portugal, and provides valuable data about the types of debris floating in Portuguese coastal waters.

4.1.5 Conclusions

This work identified several types of commonly used plastic polymers in Portuguese coastal waters. Sixty one per cent of all zooplankton samples had microplastics. The total number of microplastics identified was 684 in 93 samples, corresponding to a volume of 113.01 cm³. Costa Vicentina (CV) and Lisboa (LX), were the regions with the highest microplastic concentrations, abundances and microplastic: zooplankton ratios. In the case of Costa Vicentina these values are probably related to the proximity to industries and port facilities at Sines, and in Lisboa close to the metropolitan area of the capital city, high population densities and discharges from the Tejo and Sado river estuaries play an important role in microplastic accumulation. Three plastic pellets were collected in the plankton samples which were identified as low density polyethylene (LDPE). Floating pellets have been found in several places worldwide, and this is the first record of plastic pellets in Portuguese surface waters. No plastic fibres were collected in this work. The other plastics identified using μ-FTIR analysis proved to be polyethylene (PE), polypropylene (PP), mixtures of both PE and PP, and polyacrylates and these results are consistent with FTIR results throughout
the world, due to their present and past decade’s industrial production and intensive use. This is the first work to quantify and identify microplastic particles in surface waters of Portugal and also the first contribution to assess the state of marine litter within the scope of the Marine Strategy Framework Directive (2008/56/EC) for the European Seas. This work likewise contributes to enforce the role of routine plankton surveys performed by the national authorities as a useful tool in the assessment of marine litter, and particularly microplastics, without further costs of days at sea. In order to minimize the impacts of marine litter and especially plastic debris, best practices in waste management and in daily human activities should be enforced. Information and outreach activities involving stakeholders of coastal and maritime activities must be carried out in order to modify perceptions and behaviour towards the reduction of marine litter.
5 Strategic approaches to minimise marine litter

Once an environmental problem has been correctly identified it is necessary to develop strategic approaches to solve that particular problem. This chapter focus on management proposals to address and reduce marine litter, taking into consideration different strategic approaches such as a SWOT (Strengths-Weaknesses-Opportunities-Threats) matrix analysis and a DPSIR (Drivers-Pressures-State-Impact-Responses) model analysis. These are holistic methodological tools widely used to manage environmental and social problems, and were developed in order to minimise the effects of marine litter.
5.1 Reduction of marine litter: SWOT matrix approach

There are plenty challenges associated with marine litter management, particularly with responsibility towards this global environmental problem. Stakeholder engagement is necessary to minimise the environmental and economic direct impacts caused by marine litter. In many cases it is necessary to translate the science to decision-makers and usually this is solved by using strategic tools. The Strengths-Weaknesses-Opportunities-Threats (SWOT) matrix analysis is a common strategic planning tool that is used to identify positive and negative attributes to assess environmental and socio-economic problems. The SWOT creates the mechanisms to deal with the internal (strengths and weakness) and external (threats and opportunities) attributes associated, in this case, to the reduction of marine litter, and to make decisions based on that information. This is dynamic tool also provides a framework to identify and formulate strategies that match the attributes, in order to achieve the initial goal (Hay and Castilla, 2006; Çelik et al., 2012, Scolozzi, et al., 2014). The SWOT matrix provided in table 5.1.1., was designed to be used in decision-making stakeholder meetings, in order to shape the future of marine litter policies and regulations. Table 5.1.1., focus on the reduction of litter in the global marine environment. The internal attributes that can be controlled by the manager are either helpful (strengths) or harmful (weaknesses) to achieve the goal, and the external conditions are also either helpful (opportunities) or harmful (threats) to achieve the objectives (Hay and Castilla, 2006).

First and foremost it is important to clearly identify what are the positive and negative outcomes at short and long-term ranges, so that legal frameworks act upon reducing the marine litter problem without compromising sustainability, economic growth or other political goals. As previously described, marine litter is a global problem with several stakeholders responsible for acting towards its reduction. This is not a problem of governments alone, but also of waste managers, science researchers, non-governmental organisations and the general public. In this issue, as in most of the environmental pollution problems, active education and engagement is one of the key aspects and elements to deal with the lack of information that the general public usually have.

Managers that are currently dealing with marine litter problems in the oceans or in coastal areas must take into consideration the external attributes of the SWOT analysis (threats), and engage with other stakeholders to reduce or mitigate each topic described. A detailed analysis of the SWOT matrix is described after table 5.1.1.
### Table 5.1.1 – SWOT analysis for reducing marine litter in the environment

<table>
<thead>
<tr>
<th>Strengths</th>
<th>Weaknesses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Creation and implementation of legal frameworks and regulations at international and EU level;</td>
<td>1. Lack of financial, management and funding opportunities</td>
</tr>
<tr>
<td>2. Technological developments and increase of knowledge (peer-reviewed academic papers and reports);</td>
<td>2. Lack of effective measures to reduce land-based debris and enforcement of measures reduce sea-based marine litter.</td>
</tr>
<tr>
<td>3. Increase of methodological approaches on land or in maritime activities</td>
<td>3. Lack of awareness of other sectors (waste management, retail, general public)</td>
</tr>
<tr>
<td>4. Increased perception of the general public via movies, exhibitions, beach clean-ups or outdoor art.</td>
<td>4. Lack of leadership and cooperation among entities (NGOs, local authorities)</td>
</tr>
<tr>
<td>5. Increase of best practices at all stakeholder levels (ex: KIMO - Fishing for litter and Marine Litter Solutions -Operation Clean Sweep)</td>
<td>5. Lack of implementation, surveillance and control of international and EU legislation.</td>
</tr>
<tr>
<td>6. Increased efforts of waste management.</td>
<td>6. Insufficient cooperative activities at regional, national, local levels and between stakeholders</td>
</tr>
<tr>
<td>7. Increased perception of marine debris as resources instead of litter.</td>
<td>7. Technological and management gaps between countries, at academic, research and industrial levels.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Opportunities</th>
<th>Threats</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Experience and expertise using market based instruments such as taxes, fines and deposit refund schemes.</td>
<td>1. Transboundary accumulation and distribution of marine debris at a global level</td>
</tr>
<tr>
<td>2. Activities implemented by local stakeholders (ex. Beach clean-ups)</td>
<td>2. Deep-sea accumulation</td>
</tr>
<tr>
<td>3. Concerted efforts to build partnerships among researchers, NGOs, governments and local authorities (ex. Marine Litter Network)</td>
<td>3. Ghost-fishing</td>
</tr>
<tr>
<td>4. Awareness raising through active education and engagement for sustainable development.</td>
<td>4. Socio-economic and environmental impacts</td>
</tr>
<tr>
<td>5. Circular economy models</td>
<td>5. Possibility of bioaccumulation and biomagnification of PBTC throughout the food web with unpredictable consequences for wildlife and man</td>
</tr>
<tr>
<td></td>
<td>6. Lack of commitment among all stakeholder levels to take action.</td>
</tr>
</tbody>
</table>
As strengths, which can positively contribute to a change, the analysis proposes the creation and implementation of international legal frameworks with feasible goals that can contribute to the reduction of impacts in wildlife and the ocean economies, based on the increase of knowledge. This puts two important stakeholders, governments or local authorities and scientists working closely on the solutions to reduce the problem. One important stakeholder is the plastic production and recycling industry that can contribute with implementing the best available techniques on their daily activities, and by seeing resource opportunity in plastics instead of a threat. NGOs and the general public can also be actively engaged through awareness and outreach activities such as movies, exhibitions, beach clean-ups or outdoor art.

As negative internal attributes, or weaknesses, the lack of funding to run environmental policies and projects or the lack of appropriate knowledge regarding the effects and impacts of marine litter can be one of the reasons for the lack of action at individual, national, regional or international levels. Regarding environmental policy goals, the lack of effective measures to reduce land and sea based origins of marine litter and the lack of effective waste management may contribute to an increase of the amount of plastics available in the marine environment.

An insufficient cooperation of stakeholders (divergent policy goals, distinct interests) may contribute to an increase in difficulty to solve the problem. Also, not all countries have the same awareness towards marine litter which may result in technological, research or policy goals to solve this particular recent environmental problem.

As opportunities regarding this topic, the knowledge, experience and expertise provided so far by waste management and plastic production industries, as well as, NGOs and academia can provide important advances in the mitigation of marine litter as a global problem. At a governmental level it is possible to apply market based instruments such as taxes, fines and deposit refund schemes to foster technological developments to increase reusing and recycling rates. Market based instruments such as these will contribute a more efficient economic models (eg. Circular economy). The creation of national and international partnerships, where best practices and good examples are shared among members also contributes to positive outcomes.

Activities such as beach clean-ups can be a good way of interaction between different members of the coastal communities, united towards the same goal. These activities raise awareness through active education and engagement towards sustainable development goals.

Lastly, as threats there are associated risks to transboundary accumulation and distribution of debris at a global level, or in other words, a country that has the best available waste management
methodologies and technologies and with great recycling rates, might be affected by transboundary pollution with origin in other countries.

It is likely that there are plastic materials in the environment since the 1950’s as the long chemical chains enable plastics to be highly persistent. This will lead to accumulation at the surface and at deep-sea levels. The amounts of debris, such as nets contribute to a phenomenon described as ghost-fishing, which means that nets that were abandoned at sea continue to fish for long periods of time, entangling marine animals in the middle of the ocean. Some of these nets and ropes are responsible for blocking propellers causing severe economic problems to the fishing industry.

Also, due to the capacity of PBTC bioaccumulation and biomagnification throughout the food web, there might be unpredictable consequences for wildlife and mankind that future generations will have to deal with, in a not so distant future.

Finally the lack of commitment among all stakeholder levels to take action, might compromise the entire policy goals to tackle the problem in the first place.

Although this SWOT matrix is just a tool to approach this topic, there are plenty possibilities that arise from this exercise. By carefully identifying the internal and external positive and negative attributes associated to marine litter, it is possible to think ahead into taking action to solve the problem.

5.2 Reduction of marine litter: DPSIR model approach

The drivers-pressure-state-impact-response (DPSIR) model is a framework developed by the European Environmental Agency (EEA) that describes interactions between society and the environment. Initially based on the P-S-R (Pressures-State-Response) model proposed by the Organisation for Economic Co-operation and Development (OECD), this model approach has been applied to the organisation of statistics and indicators, regarding policy aims. After gathering information on all the different elements that are part of the DPSIR chain, it is possible to establish connections between the elements and create solutions (responses) to a specific environmental problem. Figure 5.1.1., shows an example of a DPSIR model, where the big arrows represent casual flows and the small arrows represent lines of influences.
The model is composed by five elements 1) Driving forces or drivers, 2) Pressures, 3) State, 4) Impacts and 5) Responses, further explained here:

1) Drivers are the changes in the socio-economic and institutional systems that have direct or indirect influence on the environmental state. The EEA’s definition for drivers is ‘the social, demographic and economic developments in societies and the corresponding changes in lifestyles, overall levels of consumption and production patterns’ (EEA, 2007). Drivers create Pressures.

2) Pressures are the anthropogenic factors that induce environmental changes (Impacts). They correspond to the release and emission of chemical substances, physical and biological agents and the use of land or resources by anthropogenic activities. Pressures influence the State.

3) State may correspond to the natural environmental system or to a natural and socioeconomic system. Indicators of state depend on the focus of the problem addressed. State can refer to a wide range of features, from the qualitative and the quantitative characteristics of ecosystems, the quantity and quality of resources, to even larger socio-economic issues. The combination of the current State and the existing Pressures explains Impacts.
4) Impacts are changes in environmental functions that affect the social, economic and environmental dimensions. They are caused by changes in the State of the system. Impacts can include changes in environmental functions such as water and air quality, health and social cohesion or access to resources. These Impacts trigger Responses.

5) Responses are the policy actions which are directly or indirectly created by the perception of Impacts. Responses attempt to prevent, eliminate, mitigate, compensate or reduce consequences and can come from different levels of the society, such as groups of individuals, governments or non-governmental sectors. These Responses can in turn influence trends in the Driving Forces, Pressures, State and Impacts (Spangenberg and O’Conner, 2009).

This model approach can be used to help understand the nature of the problem and to evaluate potential management strategies and figure 5.1.2 shows one possible DPSIR model addressing this topic. In the figure, ML stands for marine litter and ALDFG stands for abandoned, lost, or otherwise discarded fishing gear.

And just like in the previous figure, the big arrows represent casual flows and the small arrows represent lines of influences.
Figure 5.2.2 – DPSIR model for the worldwide reduction of marine litter.
The DPSIR model represented in figure 5.1.2., represents one possible model for the reduction of marine litter. Drivers have a casual flow to the Pressures, but influence also State and Impact. The driving forces related to the marine litter problem are the population and its growth, the inefficiency of waste management and the use and exploitation of natural resources. This will lead to the Pressures, who have a direct flow into the State and influence State, Impact and Responses. The excess of consumption will lead to a high generation of waste. One of the topics that appears in the Pressures is low recycling rates, which one can understand the importance of being one of the pressures after reading the general introduction in chapter 1 of this manuscript. Although there are recycling goals in several countries, most of the countries have failed to reach the proposed overall recycling in its countries (Bakas et al., 2010). The last pressure on the global system is the development of coastal zones, and industries and tourism in these areas. Approximately 1 billion of the world’s population lives within 100 km of the coast (Small and Nicholls, 2003), a number that is likely to rise between 40 to 50% by 2030 (Adger et al., 2005), causing a high demand pressure on coastal areas. The State expresses the global system at his moment. It has a direct flow into Impact and influence Drivers and Responses. It is basically the state-of-the-art concerning all the knowledge we have so far about microplastics in the environment, the evidences of plastic accumulation on gyres, on beaches, on deep-sea environments, and the direct economic revenue loss of the fishing industry and the tourism sector.

Impacts have a direct flow into Responses and influence Drivers and Pressures. They represent all the challenges of all the activities and all the potential threats, such as emission of greenhouse gases in extraction and production of plastics from fossil fuels, accumulation of plastic materials and other debris in ocean gyres, as well as every unknown ecosystem of human health effect. Responses have a direct flow to Drivers, are influenced by Impacts and influence Drivers, Pressures and State. Usually the responses will create new driving forces and move the pressures and State to achieve new Impacts that will lead to new Responses. To a certain degree, solutions or responses are initially uncertain. It is only through experiencing the entire system, with all the stakeholders, activities and threats that actions to solve the initial problem can be addressed. Awareness and outreach will lead to a new way of thinking in society, and it will eventually change the everlasting dynamic DPSIR model.
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
6  Concluding remarks and perspectives for future work

Research is the systematic investigation and study of materials, sources and theories, in order to establish facts and reach new conclusions, previously not known. Research projects gather new information and data and provide answers to specific problems. This is particularly important in the field of environmental sciences. Through further studying the ever-changing surrounding environment, it is possible to understand patterns and make estimations.

Concerning marine litter, the last decade has been very rich in research efforts and contributions to understand the ecological, social and economic consequences caused by this global pollution problem.

Current knowledge and international legislation, particularly the Marine Strategy Framework Directive in Europe, are already making proposals to reduce the impacts of what has been recognised and described as a new global problem and one of the key challenges in this century.
6.1 Main results and concluding remarks

The hereby thesis provides a state-of-the-art information that constitutes a novel approach to the marine litter problem, particularly microplastics for Portuguese relevant coastal and marine systems and species. This manuscript focus on plastics marine debris, to which microplastics are the vast majority with approximately (98%) of all findings, to which resin pellets constitute a significant part (-60%). Microplastics were randomly collected on the last tidal mark on shore, providing opportunistic samples of microplastics from several beaches from north to south of the country. In order to characterise the current situation, polymer were identified using Fourier transform infrared spectroscopy (μ-FITR), and results include polyethylene (low and high density), polypropylene, mixtures of polyethylene and polypropylene, polycrylates and polyurethane, as common polymers.

Marine litter is widely distributed in the Portuguese coast and coastal waters, and its accumulation is variable and dependent on season and weathering conditions. Regarding beach areas, the regions with higher plastic marine debris densities were Matosinhos (Mt) (362 items m⁻²), Vieira de Leiria (VL) (332 items m⁻²) and Sines (Si) (84 items m⁻²), which are areas near ports and industrial facilities where leakages in transfer and transport may occur. The regions with lower plastic marine debris densities are Mira (Mí), in a sparsely populated area, Espinho (Es) close to the river Douro mouth; Cresmina (Cr) in Guincho, North of Lisbon and Fonte da Telha (Ft) south of Lisbon. Regarding coastal waters, the regions with higher microplastic concentrations, abundances and microplastic:zooplankton ratios are close to the metropolitan area of Lisboa, the capital city, where are registered high population densities. Also, discharges from the Tejo and Sado river estuaries play an important role in microplastic transport and accumulation.

Regarding persistent, bioaccumulative and toxic chemicals (PBTC), the class with higher concentrations registered was aged pellets, probably as a result of the long exposure time in the environment. In terms of PAH concentrations, concentrations adsorbed to pellets ranged from 53 ng g⁻¹ in Cresmina beach (Cr) to 44800 ng g⁻¹ in Sines (Si), being Sines, according to provided data, the beach with the higher concentration of PAH adsorbed to pellets in Portugal, highly contrasting with pellets collected from the remaining beaches. The majority of PAH adsorbed to pellets result from petrogenic sources and from all chemical species, BeP was the PAH with higher concentration in pellets in Portugal, registered between 2010 and 2013. This was also the reason for selecting this
pollutant for the ingestion bioassays. In what concerns PCB, lower chlorinated congeners (18 and 31) were more abundant in rural sites, and higher chlorinated congeners (138, 153 and 180) were more common in urban areas due to their tendency to remain closer to their sources. The highest PCB concentration was also registered in Sines. Regarding DDT, higher concentrations were found in Matosinhos, Fonte da Telha and Peniche, which might have connections to agricultural use from the 1970’s.

With this background information in mind, it was possible to design the ingestion bioassays with live organisms collected from the sea. Two series of bioassays described in chapter 3 (sections 3.1 and 3.2) were conducted with different size ranges and concentrations of non-contaminated particles (3.1) and with different size ranges of non-contaminated and BeP contaminated microplastic particles. In the first study (sub-chapter 3.1.), no significant histopathological changes that demonstrate mechanical abrasion were found, probably due to using commercial particles with smooth spherical surface. Relevant statistically significant differences were found in what concerns lipid peroxidation, particularly for higher for higher concentrations of microparticles (1000 part. ml⁻¹) of the smaller particle size used (2 μm). The accumulation of particles in the gut cavity was registered, and it is believed that the low LPO levels might have been influenced by the smooth shape of the non-contaminated particles. For the second bioassay (sub-chapter 3.2) higher concentrations of TBARS were registered for treatments with BeP contaminated particles. Statistically significant differences were found between non-contaminated and BeP contaminated microplastics for particle size 2μm (* p<0.05) and 6 μm (** p<0.001), which means that these PAH contaminated particles have a strong influence in the oxidative stress and lipid peroxidation in this tissue. Statistically significant differences were also found in the gills, namely for 6 and 10 μm particles. Although there were no difference either if the particles were contaminated or not, both 6 and 10 μm particles exerted higher oxidative stress, than 2 μm particles, most likely due to the area/surface ratios and adsorption rates of BeP onto plastic. The digestive gland had higher concentrations of TBARS when compared to the gills, which could be explained if the BeP contributed to a reduction of the metabolic activity, nonetheless further investigation is needed to determine correctly this assumption.
6.2 Perspectives of future work

Plastic marine debris is a problem that has been addressed by many researchers worldwide, in the last decades. There are remarkable examples of cooperation and collaboration between laboratory and research facilities throughout the world, trying to create more knowledge or a better understanding of the true dimension of this problem. Plastic litter accumulation on beaches and on gyres is well-known and there is a wide number of scientific papers and reports that provide information on the quantities of marine litter currently present on the ocean. In order to continue providing state-of-the-art methodologies to deal with marine litter, it is necessary to understand what the next challenges are after the classification and categorisation of marine litter (accumulation areas, quantities, types of polymers, PBTC adsorbed). The next step in the field of ecotoxicology is very likely the continuation of bioassays with a wide range of the most common pollutants adsorbed to pellets and species of diverse functional groups. After conducting several studies on ecotoxicology with robust data, will be possible to share that information with political decision-makers in order to implement and incorporate this date into international and EU legislation.

At a society level, awareness and outreach campaigns are the best way to reach the general public, such as beach cleaning campaigns, education for sustainable development (ESD) lectures and awareness campaigns to reduce plastic consumption. The cooperation between actively engaged stakeholders from all sectors of society will create a momentum that will lead to the mitigation and reduction of the plastic marine pollution problem. Plastic is an incredible resource that should be recovered to recycle and to produce energy. When stakeholders are actively engaged, it is possible to create solutions to reduce this problem, positively influence legislation and regulation enforcement and education for sustainable development as a core principle in society. It is vital to understand that human health is directly connected to environmental and organism health. Raising awareness and conducting outreach education campaigns is, on a science communication research level, very important to change behaviours and implement good practices in society. It is also important to acknowledge and take into consideration that not all countries have the same industrial or societal development, so policy and economic goals should be created having this in mind.
In my opinion it is also necessary to educate all generations to the global threat that marine litter represents and acknowledge the positive projects from NGOs, the fishing industry or plastic production industries in order to minimise marine litter in the ocean.

It is not beyond our capacity or intellect as humans to solve this problem, that started with our species. Each individual can positively contribute to the society with small changes in his/hers daily live behaviour. Citizens know the importance of recycling, and they need to be reminded of the benefits of reducing consumption, refusing certain habits and rethinking life styles.

Responses such as the ones described on the DPSIR model approach, will contribute to new driving forces, and eventually to new states, where hopefully society will take into consideration the principle of sustainability described in the Brundtland Report, that in simple terms says: “think globally, act locally.”
Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels
7 References

Chapter 1:

Section 1.1 – Plastic as marine litter and potential vector of contamination


† Blumer, M., 1976. Polycyclic aromatic compounds in nature. Scientific American 234 (3), 35-45. DOI: 10.1038/scientificamerican0376-34


Effects of the presence of microplastic particles in Portuguese coastal waters and marine mussels

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† Rios, L.M., Moore, C., Jones, P.R., (2007). Persistent organic pollutants carried by synthetic polymers in the ocean environment. Marine Pollution Bulletin 54, 1230-1237. DOI: 10.1016/j.marpolbul.2007.03.022

Section 1.2 – The Mytilus mussel

Section 1.3 – Biomarkers


Section 1.4 – Marine Strategy Framework Directive

Chapter 2:

Section 2.1 – Resin pellets and microplastics from the Portuguese coast: quantification and determination of persistent bioaccumulative and toxic chemicals adsorbed to pellets


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Chapter 3:

Section 3.1 – Biomarker and histopathological effects resulting from the ingestion of different concentrations of microplastic particles by *Mytilus galloprovincialis*

† Frias, J.P.G.L, Otero, V., Sobral, P. (2014). Evidence of microplastics in samples of zooplankton from Portuguese coastal water. Marine Environmental Research 95, pp. 89-95. DOI: 10.1016/j.marenres.2014.01.001

Section 3.2 – Biomarker and histopathological effects of the ingestion of non-contaminated and PAH contaminated microplastic particles by Mytilus galloprovincialis

† Frias, J. P. G. L., Otero, V., Sobral, P., (2014). Evidences of microplastics in samples of zooplankton from Portuguese coastal waters. Marine Environmental Research 95, 89-95. DOI: 10.1016/j.marenvres.2014.01.001

Chapter 4:

Section 4.1 – Evidences of microplastics in Portuguese coastal waters


Chapter 5:

Section 5.1 – Reduction of marine litter: SWOT matrix approach

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Section 5.2 – Reduction of marine litter: DPSIR model approach

8 Annexes

8.1 Peer-review publication list


8.2 Other annexes

Figure A8.2.1 – Infrared spectrum for the micro sample LHF95 A1 and comparison with reference spectra for polypropylene.

Figure A8.2.2 - Infrared spectrum for the micro sample NE15 and comparison with reference spectra the acrylate Neocryl B-745, copolymer for methyl methacrylate (MMA) with n-butyl methacrylate (BMA).
Figure A8.2.3 – Infrared spectrum for the micro sample NEF33 and comparison with reference spectra for polyethylene.

Figure A8.2.4 – Infrared spectrum for the micro sample NEF47 and comparison with reference spectra for alkyd resin.
Figure A8.2.5 - Infrared spectrum for the micro sample LHF59A1 and comparison with reference spectra for polypropylene+poly(ethylene-propylene)