Universidade de Aveiro

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Departamento de Química

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Avaliação do potencial de *Zalerion maritimum* e *Nia vibrissa* para a biodegradação de poliestireno expandido (EPS)

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Industrial e Ambiental realizada sob a orientação científica do Doutor João Pinto da Costa, Investigador em Pós-Doutoramento do Departamento de Química da Universidade de Aveiro e da Doutora Teresa Rocha Santos, Investigadora Principal do Departamento de Química e do Laboratório Associado CESAM (Centro de Estudos do Ambiente e do Mar) da Universidade de Aveiro

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Azul poético

Ela está morta. Morta em azul poético, azul revolucionário. Morte consciente em azul profundo. Ela, o ponto azul-(claro). O fim do mundo é sempre poético. E distante. Humano – *Oryza sativa, Bacillus subtilis, Drosophila melanogaster, Zalerion maritimum, Loxodonta africana, Canis lupus, Quercus suber, Vulpes vulpes, Homo sapiens, Cuculus canorus, Prunus spinosa, Canis rufus, Danio rerio, Birgus latro*. Aprende-se no jardim-escola. A semear nuvens, porque já não chove e os oceanos afogam-se em lágrimas de sereia, (os microplásticos, ironicamente, dão-lhes esse nome) que existem em maior número do que estrelas na galáxia. É estupidamente… poético! Mágico, trágico, real – o deserto árido onde nevou, alterações climáticas. Frio (!), o apocalipse serve-se frio, no prato. Não sofrem, não sentem as dentadas, o polvo tem três corações! Para de me humanizar, civilizar, educar. Desiste que eu tenho casca de maçã e o azul é um pigmento raro na natureza. Extração, abuso descartável, a discriminação por espécie testada. A arte dos polegares requer pólen, abelhas, borboletas e pirilampos no estômago para o peito estalar em magnólias, a polinização. Colisão, micro-poesia, bosão de Higgs, o átomo, a aranha, o violino, viola d'arco, violoncelo, contrabaixo, o Kilimanjaro, a Terra, a nébula hélix, a existência. A escorrer-te em sumo de limão das veias grossas do gomo-embrião. Os sistemas vasculares, o xilema, o floema são relâmpagos. Metades, somos. Somos colónias de bactérias, microbiomas, microclimas, micro-nano-climas. Somos o que comemos, (que não sabemos de onde vem), o que vestimos, o combustível do carro, o exfoliante. Não sabemos (!), temos de voltar ao jardim-escola. Salvar o planeta, banal (ainda há pouco o fiz – SALVEI A GALÁXIA- num jogo de smartphone!).

Ela, o ponto azul, (claro) está viva.

Aos que existem, sonham e fazem em azul-elétrico.

o júri

presidente

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palavras-chave

Poliestireno expandido (EPS), *Zalerion maritimum*, *Nia vibrissa*, biodegradação, microplásticos

resumo

A baixa densidade e durabilidade do poliestireno expandido (EPS) colocam-no entre os plásticos mais versáteis e com maior quota de mercado. Estas mesmas propriedades levantam preocupações ambientais, uma vez que o tornam facilmente transportável até aos oceanos, onde é suscetível à fragmentação, originando microplásticos. Apesar de existirem soluções convencionais para a reciclagem deste plástico, apresentam limitações, sendo necessária a procura de métodos alternativos eficientes e mais sustentáveis. Soluções biotecnológicas têm sido investigadas. No presente trabalho, o potencial de biodegradação do EPS pelos fungos marítimos *Zalerion maritimum* e *Nia vibrissa* foi avaliado através da quantificação de variações de massa apresentadas pelas amostras de partículas de plástico e biomassa de fungo usadas, durante ensaios de exposição ao plástico. Na avaliação preliminar, em 28 dias, a melhor % de remoção atingida pelo fungo *Z. maritimum* foi de 66.2%, sendo a mais baixa de 25.0%. Num segundo ensaio, as percentagens de remoção apresentaram-se inferiores. Assim, num seguinte ensaio de otimização, utilizando o modelo Central composto (CCD), pretendeu-se maximizar a resposta e avaliar a influência das variáveis – concentração de EPS, tamanho das partículas e concentração de extrato de malte, na resposta - percentagem de remoção de microplásticos. Obtiveram-se, respetivamente, os seguintes valores ótimos - 0.1458 g/L, 1-1.40mm e 20 g/L. Apesar de apresentar potencial para a biodegradação do EPS, o processo com o fungo *Z. maritimum* mostrou-se variável e exigindo elevadas quantidades de malte. Consequentemente, o fungo *N. vibrissa* foi também avaliado, sendo que, num primeiro ensaio, atingiu percentagens superiores de remoção de microplásticos. Um novo ensaio foi realizado com este fungo em meio otimizado, tendo-se obtido como melhor percentagem de remoção 47±16%.

keywords

Expanded polystyrene (EPS), *Zalerion maritimum, Nia vibrissa,* biodegradation, microplastics

abstract

Expanded polystyrene (EPS) is among the most demanded plastic commodities due to its attractive properties of lightness and durability. Such desirable characteristics present, however, an environmental threat, as it is easily transported until it reaches the ocean, where it is likely to be fragmented into microplastics. Despite the efforts for EPS waste management, sustainable and efficient solutions are needed. Biotechnology-based solutions have been investigated for their potential. Herein, *Zalerion maritimum* and *Nia vibrissa* were screened for their potential in the biodegradation of EPS, based on the quantified mass differences in both the fungus and the microplastic pellets. In a preliminary evaluation, the highest removal percentage obtained by *Z. maritimum*, in 28 days, was 66% and the lowest was 25%. In a second assay, the removal percentages were lower. An optimization assay, using Central composite design (CCD), was conducted to obtain optimum values for EPS concentration, pellet dimension and malt extract concentration. These were, respectively, 0.1458 g/L, 1-1.40mm and 20 g/L. Although *Z. maritimum* showed ability to degrade EPS, the process appeared to be variable and required high amounts of malt extract. Therefore, *N. vibrissa* was screened for its potential also. In a preliminary assay, *N. vibrissa* achieved higher microplastic removal percentages. Consequently, a biodegradation assay in optimized medium was conducted. However, the best removal percentage obtained was of 47±16%.

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List of abbreviations

EPS – expanded polystyrene

LDPE – low density polyethylene

HBCD – hexabromocyclododecane

HDPE – high density polyethylene

NOAA – National Oceanic and Atmospheric Administration

PA – polyamide

PCCPs - personal care and cosmetic products

PCL- Polycaprolactone

PE – polyethylene

PHA - polyhydroxyalkanoate

PLA – Polylactic acid

PET – polyethylene terephthalate

POPs - persistent organic pollutants

PP – polypropylene

PS - polystyrene

PU - polyurethane

PVC – polyvinyl chloride

Chapter 1. The problem of marine debris

1.1 Garbage patches around the world's oceans

Marine debris is defined by NOAA (National Oceanic and Atmospheric Administration) as any persistent solid material intentionally or unintentionally discarded or abandoned in marine environments. These include plastics, paper, glass, metal, ceramics, textiles, cloth, rubber, and wood originating from both land and ocean based sources [1]. Having twice the size of Texas and up to 2.7 million tonnes located in the North Pacific Ocean, lies an island not immediately visible to the naked eye. It is not a real island however, it is a real problem – The Great Pacific Garbage Patch, the biggest marine debris vortex. It is estimated that about 80% of the debris is originated from land-based activities and 20% from ocean-based sources [2,3]. Comprising waters from the West Coast of North America to Japan, the Great Pacific Garbage Patch includes the western Garbage Patch and the Eastern Garbage Patch connected by the North Pacific Subtropical Convergence Zone, which allows debris to move between both patches. The formation of these patches along the ocean arises from a combination of factors, the input of large and persistent amounts of waste generated in anthropogenic activities plus the wind and wave action leading to the rotation of waters in a cyclone-like way. The Pacific Garbage Patch, represented on **Figure 1**, is actually dispersed through several regions in the North Pacific Ocean and their precise content and size is not accurately known [3-5].

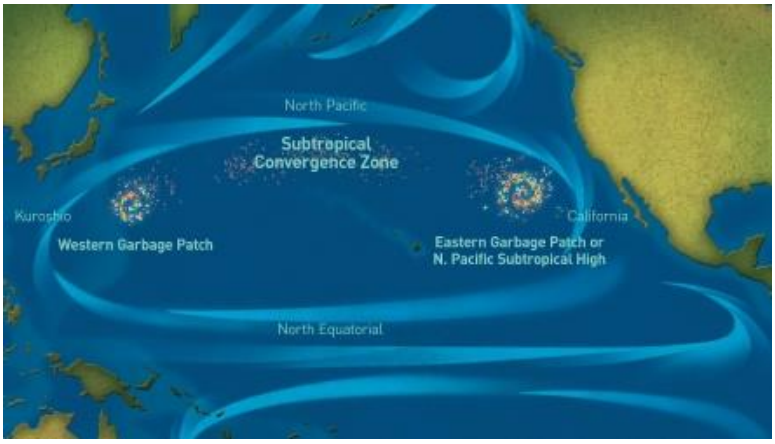


Figure 1 - The Great Pacific Garbage Patch – available from [1]

When sailing through those areas, contrary to the common assumption, not much litter can be seen, which is explained by the fact that these patches are mostly composed of smaller fragments of one of the most common types of marine debris - microplastics.

Marine debris is an ubiquitous type of pollution. Not only is it accumulating in the Pacific Ocean but also other marine garbage patches exist in the Atlantic and Indian Oceans. Furthermore, these are also forming in smaller water bodies. Marine debris is as easily perceived a global, since the ocean dynamics favours its dispersal, and relevant problem [3].

1.2 Characterization of the most common type of marine debris

Up to 60-90% of the collected marine debris from shorelines and both from the surface and bottom of the sea is composed of plastic [6], justifying the urge in finding solutions for reducing its presence in the oceans.

1.2.1 Physical and chemical characterization of plastics

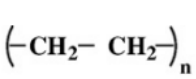
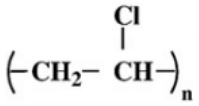
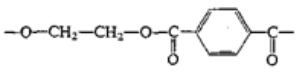
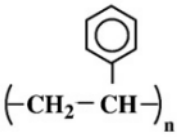
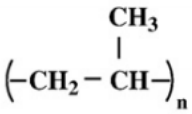
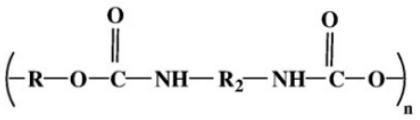
According to the International Union of Pure and Applied Chemistry (IUPAC) plastics are defined as a "polymeric material that may contain other substances to improve performance and/or reduce costs" [7]. Plastics may refer to synthetic or natural occurring polymers which are prone to be moulded when soft and retain its form when rigid. Plastics can be further divided in two categories: 1) thermoplastics, having a simpler molecular structure arranged in a chain-like structure, which are susceptible to be recurrently moulded when heated, and 2) thermoset plastics, having a three dimensional network arrange of monomers, which cannot. Examples of thermoplastics are polypropylene (PP), polyethylene (PE), polyvinylchloride (PVC), polystyrene (PS), polyethylene terephthalate (PET) which differ in their density and crystalline organization. There are also thermoset plastics which can only be heated and shaped once, such as epoxy or polyester resins. Their chemical nature gives them properties of high durability, strength, low density and impermeability [8]. Although not representing a significant percentage of the plastics market, there are also bioplastics and biodegradable plastics, which will be further discussed in Chapter 3.

1.2.2 Consumer trends and plastic applications

The aforementioned physical and chemical properties of plastics make these a very convenient and versatile manufacturing material in a broad range of applications. Plastic demand has been increasing since 1959, when its global production was of 1.5 million tonnes [6]. In 2017, according to a recent detailed report on the annual global production of plastics, it exceeded 335 million tonnes [9], reflecting the industry's response to the

contemporary lifestyle, marked by increasing consuming rates. If these consumer trends remain, by 2050, plastic production will reach up to 1200 mt, annually. [10] Among the several existing types of plastics, PE, PET, PP, PVC, PS, including expanded polystyrene (EPS) and polyurethane (PUR) (listed on **Table 1**) are those mainly produced, taking into account market fluctuations plus the proliferation of new polymers and co-polymers [11, 12] According to their specific properties, their applications vary. For example, PET is often found in the composition of soda bottles, PS in clothing, PE in plastic bags, high-density polyethylene (HDPE) in detergent bottles; PVC in plumbing pipes, PP in drinking straws, and PS in food packaging [8].

Table 1 – Commonly produced polymers and their chemical structures, adapted from [13,14]

Polymer	Chemical structure		
PE		PVC	
PET		PS	
PP		PUR	

1.2.3 Sources of plastic debris, fate and behaviour in the environment

Most sources of plastic waste are, understandably, in land. Relevant land-based sources of marine debris are agriculture, as well as the construction sector, though, as construction-related plastics are designed to be as durable as possible, they account for a smaller share. Trough discharge in domestic and industrial wastewaters, transport by wind and surface run-off, approximately 80% of the land originated plastic waste ends up in the oceans [7]. Plastic leakage into the environment may occur due to debris released

by anthropogenic activities which is washed by surface run-off or transported by the wind into watercourses ultimately ending up in the ocean, discharged from sewage plants into rivers, not sufficiently treated wastewaters. Furthermore, from the plastic waste ending up on oceans, plastic from packaging has the highest share being considered one of the most relevant sources of waste, followed by intermediate lifespan items. In Europe, in 2012, about 62% of the plastic waste collected from waste streams was from single use packaging plastic items. This might be due to its low-value market, the discrepancy between the cost of the item and the cost of its proper waste treatment, which leads to its uncontrollable disposal [6,12].

Regarding ocean-based sources of plastic waste, coastal tourism is an example, both by deliberate or accidental littering. Other sources are the fisheries sector, shipping, aquaculture and recreational fishing.

The fate of a plastic item in the environment depends also on its intrinsic properties, such as its density. Less dense plastics float being more susceptible to the action of wind and currents which spans them widely across the ocean. They are also more exposed to solar radiation and air. Denser plastics have the tendency to sink to the bottom and accumulate there or to be redistributed with sedimentary particles, by means of bottom sedimentary processes. Also, plastics on the surface will start to sink when subjected to thermohaline circulation. Plastic debris once in the ocean is susceptible to water circulation patterns and subtropical gyres which enhance its vertical dispersion [6].

When in the environment, plastics may undergo different fates as they will be exposed to weathering agents. Plastics may undergo mechanical disintegration caused by pressure changes, water turbulence, attrition and the action of organisms or degradation. However, these two phenomena are different, as in mechanical breakdown there are only morphological changes and molecular bonds are not affected, contrary to what occurs in degradation [7]. Polymer degradation may take place in the environment as photo, thermal, mechanical, chemical or biological degradation [15], which will be further discussed in Chapter 3. Photodegradation, viewed as an efficient degradation mechanism, occurs when the polymer is able to absorb tropospheric solar radiation, being the more common case the absorption of higher energy radiation, such as UV-B and UV-A, which induces oxidation and cleavage. Chain scission and cross-linking reactions mediate the degradation process. Atmospheric pollutants, agrochemicals and particularly oxygen are major agents of chemical degradation. Thermal or photoinduced oxidation may also occur, consisting in the introduction of oxygen into the polymer matrix. Ozone is an

example of a strong oxidant, that although in small concentrations in the atmosphere leads to crosslinking reactions and chain scissions resulting in the production of free radicals [13, 16-18].

1.3 Focus on microplastics as marine debris

Marine debris exists in several sizes, from meters-long to nanometres. However, more recently, microplastic pollution has been gathering increasing attention and concern due to its ubiquitous presence in marine environments [19-21] Furthermore, they are widely dispersed around the globe, in other environments, such as rivers and in remote locations, as the Antarctic islands [22]. Owing to their small size, it is difficult to access their precise concentration in the environment. Moreover, the extent to which they are integrated in trophic chains and their toxicological impacts are not yet fully understood [6,7].

1.3.1 Definition, classification and sources of microplastics

In more recent papers, microplastics have been defined, according to NOAA (and other authors have been using this definition as well), as plastic debris with a diameter between 1 and 5mm in size [2]. According to their origin, as illustrated on **Figure 2**, they are classified into two categories: primary microplastics, which are manufactured as microplastics, or secondary microplastics, which originate from the fragmentation of larger plastic debris, commonly occurring in the ocean due to several mechanisms, such as weathering or (bio)degradation. Primary microplastics are manufactured in the form of pellets, microfibers used in textiles, capsules or microbeads which are often found in the composition of cosmetics and personal care products. Hence, sources of primary microplastics include facial cleansers, toothpaste and exfoliating creams, as well as drug vectors used in medical applications. Microplastics used in personal care and cosmetic products (PCCPs) can be referred to as microbeads and their function in those products is improving the cleaning function. Additional sources of primary microplastics are industrial abrasives, raw material for the production of plastics. These microplastics might reach the environment through wastewater collection and treatment systems. Sources of secondary microplastics are fibres arising from synthetic textiles when washed, particles and by-products originated during industrial production, abrasion in landfill and recycling facilities and the other sources of plastic that suffer fragmentation and any other particles arising from cutting, polishing or moulding a plastic-based product. A relevant source of secondary microplastics is the already abundant existing

debris in the ocean, which will fall under the action of the previously described environmental agents, such as UV radiation and heat, wind and waves leading to the progressive fragmentation of plastic items into microplastics. Other source of marine microplastics may arise from the remobilization of plastic polluted sediments or soils [6,7,12].

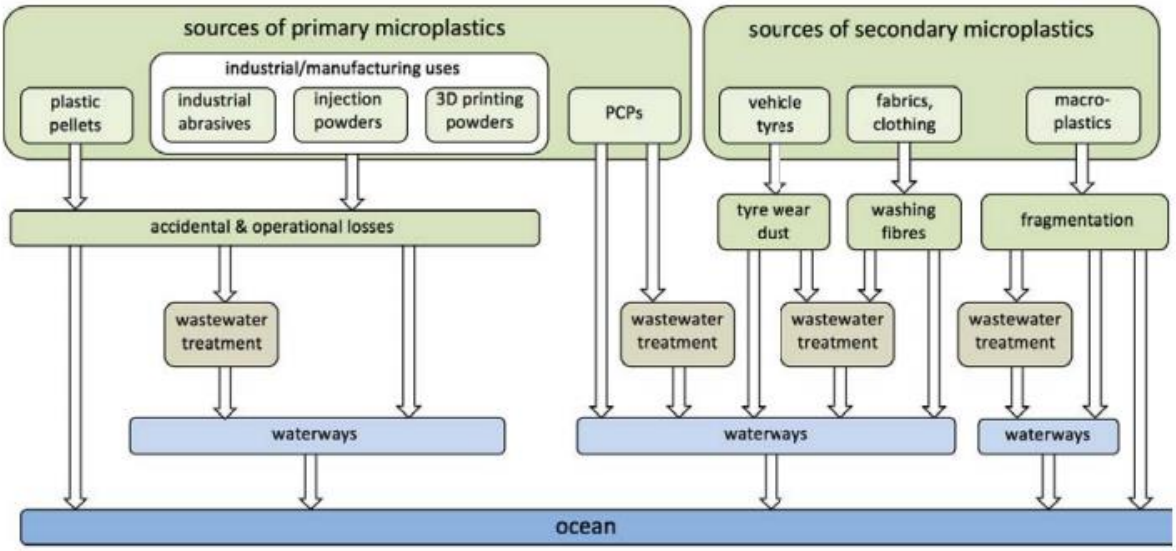


Figure 2 – Examples of primary and secondary sources of microplastics and their leakage into the ocean, available from [12]

1.3.2 Environmental, economic and social impacts of microplastics

Plastics are economically very important, but pose several environmental threats. The most commonly applied types of plastic can be found throughout the water column. Moreover, the accumulation of plastic debris and microplastics in deeper parts of the ocean has also been reported [6].

The toxicological impact of microplastics in organisms is not yet fully unveiled. However, toxicological studies have shown that microplastics can be ingested by several organisms and the pointed mechanisms for their toxicological impact were related to the stress caused by its ingestion, the release of additives and the exposure to contaminants adsorbed by them [7].

The size of the marine debris determines which animals are more affected. According to the debris size, different animal groups will be more exposed, for example, mega and macroplastics with metre sizes will have a bigger impact on seabirds, whales, dolphins and turtles. A report from Kuhn *et al*., (2015) document the presence of marine

debris in 100% of marine turtles, 59% of whales, 36% of seals and 40% of seabirds examined [6].

Obvious environmental impacts of macroplastic waste are entanglement, suffocation and ingestion by marine organisms. The ingestion of macroplastics will lead to physical effects such as digest tract blockage, resulting in less food intake that may result in starvation and loss of energy. Mesoplastics are likely to be ingested by invertebrates, fishes and birds, and microplastics by fishes and invertebrates as well, but also by filter feeding organisms. Microplastics can also lead to physical effects, such as digestive tract blockage, behaviour alterations in mobility, morphological changes and difficulty in breathing. Not only does the size has influence in microplastics uptake but also their shape, density and colour. There can be effects on reproduction and in the level of hormones and enzymes produced. Other negative effects on organisms, that can be sublethal or lethal, are neurotoxicity and heartbeat alterations [6,7, 23-25].

Microplastics' impact can be experienced in various levels of the food chain, including at its basis, due to the ingestion of these materials by filter feeding and sediment ingesting organisms. Reports showed that zooplankton retains microplastics for several days and the presence of microplastics has been reported in amphipods, sea cucumbers, mussels and marine worms. From zooplankton, it is transferred to turtles or whales leading to bioaccumulation [6,7,12]. There are several ways in which plastic debris may leak into the food chain, as detailed in **Figure 3**. Less dense plastic at the sea surface is ingested by sea birds, pelagic fishes and zooplankton; microplastics in beach sediments

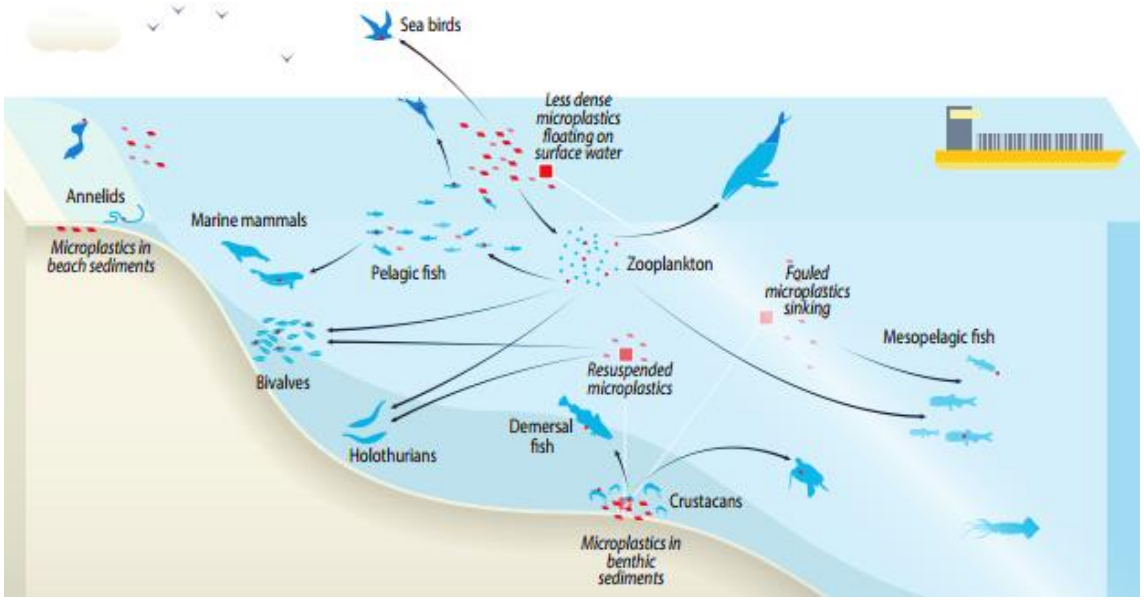


Figure 3 – Pathways of microplastic into the marine food chain, available from [6] image credits to Maphoto/Riccardo Pravettoni

are ingested by annelids; microplastics in benthic sediments by crustaceans; resuspended microplastics are ingested by bivalves and sinking debris by mesopelagic fish.

Adding to the physical risks associated with microplastic uptake, there is also the ingestion of hazardous chemicals absorbed on their surface, such as persistent organic pollutants (POPs). Usually, microplastics are not pointed as the first source of POPs in the marine environment, since other sources are highlighted, such as effluents from wastewater and sewage treatment plants, untreated industrial wastewater, urban runoff, agricultural runoff, and ship-related activities. However, reports imply that plastic use at sea and their debris should be considered an additional pollution source of hazardous chemicals. Examples of common organic contaminants which can be absorbed by plastic include Polycyclic Aromatic Hydrocarbons (PAH's), dichlorodiphenyltrichloroethane and polychlorinated biphenyls (PCB's), originating from insecticides, combustion products and insulating fuels [6, 11, 12, 26].

There are several additives added to plastic and microplastic items during its manufacture that are of concern. Examples of common chemicals added to plastics are flame retardants, such as Hexabromocyclododecane and Polybrominated Diphenyl Ethers, Bisphenol A, colour and fragrance fixers, such as Diethyl Phthalate, stabilizers and plasticizers [12].

Floating marine debris and microplastics are an artificial substrate for microorganisms, implying that it can contribute to the proliferation and spreading of invasive species, since they spread from their original habitat along with the plastic [7].

Less is known on the toxicological impact of microplastics in fish, although, recent findings have raised the concern of the potential effect on humans consuming this contaminated fish. However, the consumption of filter feeding invertebrates, such as mussels and oysters, has been proposed as the most likely route for human consumption of microplastics.

Impacts of plastic pollution go even beyond environmental and health harm. Marine debris has also social impacts in tourism and recreation activities and other areas such as shipping, fishing, aquaculture, power generation and agriculture [12].

Besides being a transversal problem that affects a wide range of areas from economy to health, not only marine environments are affected by microplastics, they may also reach and contaminate terrestrial environments. Discarded items in landfills after suffering abrasion and atmospheric deposition accumulate in the soils, relevant sinks for microplastics.

More studies are needed to access the toxicological impacts of microplastics since it is a global and ubiquitous problem transversal to several areas [27]. For example their impact and behaviour in soils is insufficiently studied. Also, the methods for sampling microplastics are not standardized due to its size limitations and no consistent estimations of their quantity in the environment are available.

1.4 Focus on PS and EPS

1.4.1 Chemical characterization of PS and EPS

Among the most demanded and produced plastics commodity is PS, a petroleum-based polymer obtained through the polymerization of styrene monomers. The repeated units of this thermoplastic, holding the chemical structure $CH_2=CHC_6H_5$, are obtained by the reaction of ethylene with benzene in the presence of aluminium chloride to yield ethylbenzene, which is further dehydrogenated to yield styrene. The phenyl rings present in the composition of PS are relevant for its properties, such as rigidity. Chemical characterization of PS through FTIR yields typical intense peaks that may be attributable to aromatic ring vibrations [8,28].

EPS is also obtained from the polymerisation of the styrene monomer, during which a low boiling point hydrocarbon works as expansion agent, resulting in translucent spherical beads of PS [8].

1.4.2 Types, properties and applications of PS and EPS

According to the desired properties and various application purposes, there are several types of PS. These include general purpose polystyrene (GPPS), high-impact polystyrene (HIPS) and EPS. GPPS, commonly used in food packaging and disposable cutlery, is vulgarly commercialized in a transparent and rigid form of pellets with, typically, 2-5 mm size; HIPS, found in the composition of several single-use containers, refers to a more resistant copolymer obtained when PS radicals react with the double bonds of polybutadiene; EPS, employed as construction material, food trays and other applications refers to the polymer in the form of expanded beads forming a light density foam [28]. There are different types of PS foam, such as coloured foam, foam treated with flame retardants, foam as packing "peanuts", foam as medical coolers and foam packaging, which are available in a wide range of densities, leading to different physical properties suitable for distinct applications. PS foam appears informally referred to as

Styrofoam. However, this designation refers to a registered trademark, commonly used to ship fragile objects or food. Furthermore, there is also variety in the types of EPS beads: there are flame-retardant beads, mainly used in construction materials, and non-flame-retardant beads, often used for packing materials and buoys [29-31].

Why EPS is such a demanded commodity polymer can be explained by its attracting properties of lightness, insulation, durability, shock absorption and versatility. Its lightness, due to the high percentage of air in its composition, makes it an attractive and more economical choice as it reduces transportation costs and energy consumption [29]. The dimensional stability arises from its cellular structure and it is what makes it so versatile. EPS is also widely used in aquaculture buoys and fishing floats due to its resistance to moisture. While its thermal efficiency makes it ideal for shipping fresh products or pharmaceutical content, the shock absorption property is exploited to packaging electronic appliances. EPS foam is also used for construction purposes in the form of sheets or large blocks. EPS is sold as an easy to use and cost-effective solution [2].

To understand the relevance of finding solutions to remove PS from the oceans it is necessary to take into account its market share and consumer trends. According to GBI Research's report, "Polystyrene (PS) and Expandable Polystyrene (EPS) Global Market to 2020, global demand for this material is expected to grow to 23.5 million tons by 2020. Asian countries drive the global demand for EPS, which increased from 13 million tons in 2000 to around 14.9 million tons in 2010. In countries such as China, India, Iran, Saudi Arabia and Brazil, its demand is still rising. It is notable that EPS demand is expected to be higher than the PS demand, being packing and construction industries the drivers of this demand [40].

Regarding the high demand on EPS and its environmental impact, this work has the aim of finding a sustainable and efficient solution for its end-of-life, by a biotechnological approach, screening the biodegradation potential of two marine fungi.

1.4.3 PS and EPS sources, behaviour and fate in the environment

As previously described, there are several properties to take into account when it comes to the plastic's behaviour and fate in the environment, such as its density relative to seawater. According to the type of PS, in the ocean, it may sink or float. Due to its low density, EPS is expected to float in seawater [6]. However, other parameters affect the

polymer's behaviour in the ocean. Salinity, temperature, water currents and turbulence also decide its fate. Still regarding EPS properties, the so desirable lightness of this material, from an environmental perspective might be a threat, as it means that wind or other atmospheric phenomena might be able to easily transport it until it reaches its final destination, the ocean [32,33].

Aside from the already described fate and behaviour that extend to all kinds of plastic debris, there are distinct intrinsic characteristics from PS and EPS that make them a priority to be removed from oceans. For example, once in the environment, EPS is more susceptible to fragmentation due to its higher buoyancy than polyethylene and polypropylene. Consequently, once in the ocean, EPS is likely to be more easily broken down, thus originating microplastics. Moreover, it may be more easily transported to other areas in the oceans, due to its lower density [34]. Being pervasive in marine environments, it may reach them carried by storm drains due to its lightweight.

1.4.4 Environmental, economic and social impacts of PS and EPS waste pollution

Besides the already mentioned environmental, economic and social impacts of marine debris that extend, in general, to all kind of plastic and microplastic, PS and EPS present specific impacts due to its distinctive properties.

One of them is related to its building block, the styrene monomer. In 2014, the International Agency for Research on Cancer (IARC) considered that styrene was a possible human carcinogen, and, presently, it is largely accepted that its carcinogenicity is likely caused by styrene oxide. This poses a threat only in cases where the polymerization did not occur completely, generating residual monomers that may leach into the environment or to food in EPS packages. If it reaches aqueous ecosystems, it is likely that it will be assimilated via the lipid-based cell membranes of aquatic organisms. Otherwise, if during the manufacture of EPS, the polymerization occurs in normal and complete conditions, the resulting polymer will possess strong covalent bonds between the monomer units and there is not the danger of leaching styrene residual monomers to the environment [31].

As other kinds of marine litter and more precisely plastic debris, some of the characteristics that make them so attractive for application purposes, from the environmental perspective, may constitute a drawback. For example, PS's high durability means that its degradation in the environment is an unsustainable slow process. Furthermore, its hydrophobic nature, porous surface and ability to adsorb persistent

organic pollutants, which can be released after ingestion by marine organisms, also pose an environmental threat. Reports pointed to the higher tendency of PS to adsorb polycyclic aromatic compounds then other abundant and ubiquitous plastics that prevail in marine debris [34-36].

A brominated flame retardant hexabromocyclododecane (HBCD), which is commonly added to EPS, is a main concern due to its persistence and (eco)toxicity. For example, Jang *et al.,* (2016) reported that mussels living in areas with EPS debris as substrate accumulated higher levels of HBCD when compared to those living in high-density polyethylene (HDPE) substrates [32]. Not only EPS debris containing HBCD was reported, but also evidences of accumulation of mercury on EPS debris found on beaches were shown. [37] As addressing the problem of plastic debris in the ocean is not only a question of the amount of plastic itself but also their vulnerability to adsorb priority pollutants, this turns the removal of EPS and PS debris from marine environment a relevant issue, even though it might not be the most abundant type of plastic present in plastic debris in all cases.

Other reports exist documenting the ecotoxicological impact of EPS and PS debris in marine environments. Kyun-Woo Lee *et al*., (2013) evaluated the effects of three different sizes of PS micro-beads in the survival, development and fecundity of the marine copepod *Tigriopus japonicas* and found that PS beads lead to a decrease in fecundity (at a concentration greater than 12.5 μg/mL, PS beads caused the mortality of nauplii and copepodites in the F0 generation) [38] Matthew Cole *et al*., (2015) reported that the ingestion of microplastics can significantly modify the feeding capacity of the pelagic Copepod *Calanus helgolandicus* [39]. Rossana Sussarellu *et al.,* 2016 evaluated the effect of PS microplastics exposure in oyster reproduction using transcriptomic and proteomic responses, fecundity and offspring development. The authors found evidences of feeding and reproductive alterations with significant impacts on offspring [40].

Chapter 2. Conventional solutions for plastic waste management

Plastic waste has been considered a priority concern by the European Commission and other relevant institutions and it is contemplated in several frameworks such as the United Nations Sustainable Development Goals, the EU strategy of Circular Packaging and the EU roadmap dedicated to plastics strategy, launched in January, 2017 [42]. But how efficient are the existing conventional solutions for plastic waste management?

Reports from the EU state that a third of plastic waste goes to landfills and that the recycling and reuse of end-of-life plastics has a low rate [42]. As an example, in 2014, in the EU alone, from 25 million tonnes of plastic waste, only 30% was recycled. Landfilling (31% in 2014) and incineration (39% in 2014) are options with high using rates. These percentages may have economic reasons, such recycling being more expensive or the lack of incentives to use secondary plastics [43]. Therefore, the existing conventional solutions present some drawbacks.

Landfill approaches require space and environmental contamination of groundwater by landfill leachate migration may occur generated in wet-cell landfill approaches; regarding incineration approaches, they present the advantage of having the possibility to use the released energy for electric power generation, while also constituting an effective solution for mixed plastic wastes, without the need of a pre-separation step. However, the disadvantage is the generated environmental pollution caused by polychlorinated biphenyls and dioxins arising from the remaining ash and combustion gases [44]. Recycling rates are not as high as one would expect because it requires proper collection and separation stages. Moreover, only certain types of plastic are suited for mechanical reprocessing and some types have low recyclability. Recyclability of plastics is variable. For example, PET is easily recycled due to its thermoplastic nature; however, its recycling rate in Europe is less than 30% as the recycled products do not attend the price and properties standards of conventional ones [45].

As these are one of the most produced plastics and applied in a wide range of applications, the lack of sustainable and economical feasible end-of-life solutions aggravates its environmental impact [45].

2.1 Focus on PS and EPS waste management

Conventional solutions exist for PS and EPS waste management. However, despite these efforts, they present some drawbacks and limitations. Regarding EPS, although it is recyclable and it is done in some recycling centres, for example in the USA, in several parts of the world recycling does not take place [46, 47]. There are some constraints related to the properties of this form of the polymer that might explain why it is not recycled everywhere. Its collection and transportation costs are a major drawback for its recycling owning it to its low density relatively to its volume. Furthermore, proper prior handling while collecting is needed. EPS waste should be segregated before moving to the waste stream [48]. Recycling of EPS includes its granulation, compression in continue lengths, breaking it for generating pellets and then extrusion for using it as GPPS. As for other solutions such as landfilling, the major drawback is that EPS requires a large amount of space in landfills [47-49]. In the case of PS, this material is usually not accepted in recycling programs, consequently the majority of PS products are not separated and recycled [8].

EPS products, due to their frequent use in single use food packaging, have a shorter life span than PS products and therefore they turn into large volume of waste in a short period of time [6]. This is aggravated by the nature of EPS volume which is a focus in its recycling. It is relevant to reduce its volume for reducing the transportation costs, which can be attained by compressing the material, turning it into a high value product for producers of recycled plastic pellets. There is increasing interest in avoiding landfilling and incineration as a solution for EPS waste management because of the environmental impact of these solutions [48].

Chapter 3. State of art of Biotechnology-based solutions for plastic waste management

Solid waste management includes the steps of collection, transportation, processing and disposal. The problems arise when waste is mismanaged and not integrated in the management system. For example, in 2010 between 4.8 and 12.7 million tonnes of mismanaged plastic waste ended up in oceans [6]. Extrapolating these numbers to the year of 2025, between 100 and 250 million tonnes of plastic will have entered into oceans.

If the existing conventional solutions for plastic waste management such as recycling, landfilling and others present some drawbacks, therefore, sustainable,

innovating and efficient end-of-life solutions for plastics are needed. Nonetheless, encountering new solutions for plastic waste is not enough. To address the issue of plastic waste management, it is necessary to consider the complete life cycle of a plastic product. It is necessary to envision the whole journey of the plastic item we are holding in the present moment. More precisely, what was its origin? Did it use fossil fuels as primary resources? Are there more sustainable alternatives? And then, in the present moment, does this item has a single-use nature? And what about its future, how long will it last in the environment and to where is it going? [6,12,44]

Biotechnology-based solutions (**Figure 4**) have emerged as potential answers to these questions, offering more sustainable alternatives for several moments of the life cycle of a plastic item. However, in order for a biotechnological solution to be implemented it has to be cost competitive, scalable and efficient. Therefore, the on-development biotechnological approaches present themselves as integrated or complementary possible solutions to the already existent conventional solutions. As a multifaceted approach, there are, on one hand, proposed solutions for reducing the plastic waste, by means of employing microbial systems and multicellular organisms in biodegradation strategies; on the other hand, from a perspective of replacing, there is the possibility of introducing biodegradable segments in petroleum-based polymers and also its substitution for biopolymers or bio-compositers. From a perspective of conversion, there is the creation of add-value products from plastic waste. [44] These approaches will be further discussed.

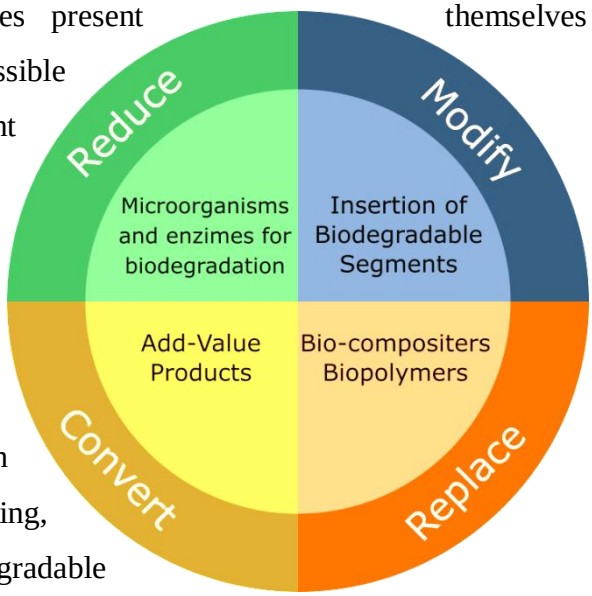


Figure 4 – Biotechnology approaches for plastic waste management, adapted from [44]

3.1 Biodegradation-based strategies

The concept of polymer degradation refers to any physical or chemical changes caused by abiotic factors, such as light, heat, chemical conditions or biotic factors. These induced changes in the material mechanical, optical or electrical properties include bond scission and formation of new functional groups. Polymer degradation may take place in the environment as photo, thermal, mechanical, chemical or biological degradation. Starting

with abiotic degradation routes, photodegradation occurs when the polymer is able to absorb tropospheric solar radiation, being the more common case the absorption of higher energy radiation, such as UV-B and UV-A. Thermal degradation is caused by high temperatures, when polymers suffer molecular scission and other chemical reactions leading to changes in its optical and physical properties [13]. Atmospheric pollutants, agrochemicals and particularly oxygen are major agents of chemical degradation [15].

Biodegradation is defined as any physical or chemical alteration in a material caused by biological activity. However, this definition is very simplistic as this phenomenon includes multiple steps. Despite giving focus to the "bio" contribution, actually in nature, both biotic and environmental agents, previously described, act synergistically [15].

Taking advantage of the naturally occurring process of biodegradation, biotechnological solutions have been tested by means of exploiting several biological agents, such as bacteria, fungi, algae and their enzymes for polymer degradation. This phenomenon has a fundamental role in the environment, since there are polymers entering water streams. In order to optimize laboratorial conditions and optimize the proposed solutions based on biodegradation it is important to understand how it occurs in nature [50-56].

Several organisms have been reported for their potential in biodegrading polymers. Most relevant bacteria associated with polymer degradation are *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Streptomyces badius*, *Streptomyces setonii*, *Rhodococcus ruber*, *Comamonas acidovorans*, *Clostridium thermocellumand Butyrivibrio fibrisolvens* and the fungi *Aspergillus niger*, *Aspergillus flavus*, *Fusarium lini*, *Pycnoporus cinnabarinusand* and *Mucor rouxii* [51]. They have been isolated from multiple sites, such as the rhizosphere soil of mangroves, marine water, dumping sites, municipal landfill areas and plastic surfaces buried in soil [54].

3.1.1 Overview of the biochemical routes

In the environment, plastics are either biodegraded aerobically, anaerobically in sediments and landfills or partly anaerobically in soil. The products obtained following aerobic biodegradation are, besides biomass, water and CO_2; after anaerobic biodegradation, CH_4 is produced [13].

Biodegradation involves several steps starting with the attachment of the microorganism to the polymer's surface, followed by its growth using the polymer as a carbon source. During the process, extracellular enzymes are secreted to cleave the polymer's chain [54]. The action of these enzymes is needed as some polymers are too

large to be absorbed through microbial membranes. Therefore, large polymers are first converted to their monomers and then these are mineralized. By breaking down first the polymer into oligomers, dimers and monomers, these become suitable to pass the semi-permeable microbial membrane and to be converted into metabolic intermediates used as energy and carbon sources by microorganisms [13].

Figure 5 clarifies in more detail the processes involved in biodegradation. Starting with biodeterioration in which the polymer is first converted into tiny fractions by microbial communities and abiotic factors; after, through the secretion of catalytic agents, those tiny fractions are cleaved into progressively smaller units, in a process called depolymerisation. Then, molecules which are recognised by microbial cells receptors cross the plasmatic membrane and, when in the cytoplasm, are incorporated in storage vesicles and into the microbial metabolism, during the step of assimilation. Finally, in the mineralisation step, organic acids, terpenes and others are released and intracellular metabolites are completely oxidised [15].

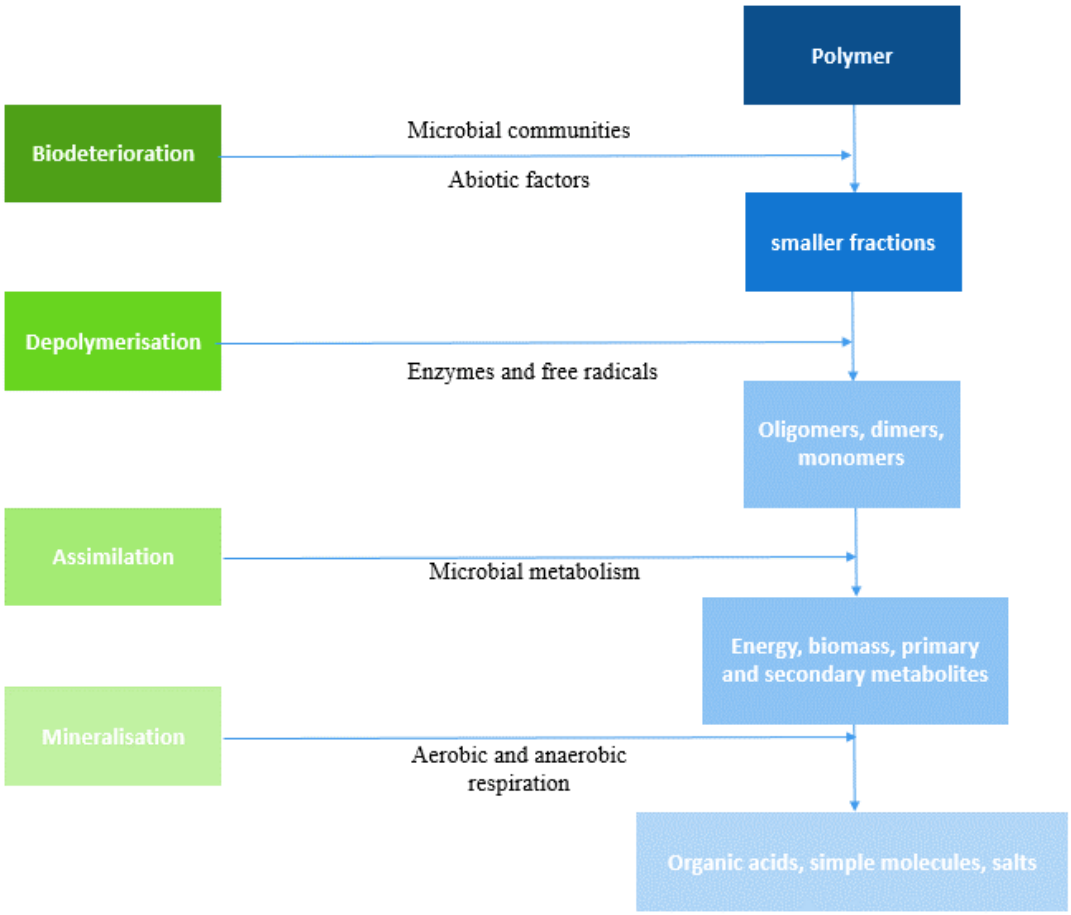


Figure 5 – Steps involved in the biodegradation process, adapted from [15]

In nature, the first breakdown of the polymer is not only done by microbial enzymatic activity. Abiotic hydrolysis is crucial and often precedes microbial degradation.

Furthermore, usually the process of mineralisation is not only done by a single microorganism, instead it arises from a microbial consortium effort composed by several organisms: some break down the polymer into its constituent monomers, some use the excreted less complex by-products. The dominant active groups of microorganisms depend on the environmental conditions available, if there is O_2, aerobic microorganisms prevail, while under anoxic conditions, an anaerobic consortium is mostly responsible for polymer degradation [13].

In the biodegradation process there are two categories of enzymes involved: the extracellular and intracellular. However, generally, as chemo-organotrophic organisms, fungi obtain energy from the extracellular breakdown of organic compounds by means of secreting, usually large enzymes (20-60KDa) from sites of cell growth such as hyphal tips. Fungal enzymes may act as wall-bound enzymes or spread in the local environment [57].

Insights into which specific enzymes are associated with plastic degradation have been provided by several reports, with lipase, proteinase K, and dehydrogenases being the most commonly referred ones [58].

Manganese peroxidase and laccases have been pointed out as involved in the process of polyethylene biodegradation. Laccases are able to act on both polyaromatic and non-aromatic substrates and are, predominantly, secreted by lignin- degrading fungi. Therefore, white rot fungi have been used for plastic biodegradation studies since they are effective in the degradation of the recalcitrant natural polymer lignin. The susceptibility of polyurethane, polyvinylchloride and polyamide to microbial attack has been related to the biosynthesis of lipases, esterases, ureases and proteases [59]. **Table** 2 presents the bacterial and fungal enzymes reported as associated with the biodegradation of the discriminated types of plastic.

Table 2 – Overview of the reported enzymes associated with plastic degradation adapted from [59]

Source	Type of plastic as substrate	Microorganism	Enzyme
Bacterial	PCL	*Rhizopus delemar*	Lipase
	PHA	*Pseudomonas stutzeri*	Serine hydrolase
Fungal	PCL	*Aspergillus flavus*	Glucosidases
		Aspergillus Niger	Catalase, protease
		Fusarium	Cutinase
	Polyurethane	*Trichoderma sp.*	Urease
		Pestalotiopsis microspora	Serine hydrolase
	Polyethylene	*Phanerochaete chrysosporium*	Manganese peroxidase

3.1.2 Factors influencing biodegradation of plastics

Environmental conditions, such as temperature, humidity, pH, salinity and sunlight influence polymer degradation, the microbial population and enzyme activity [54].

Furthermore, the polymer's intrinsic properties, namely molecular weight, melting point, density and structure also determine its biodegradability [13, 52]. Lower molecular weight polymers are more easily biodegraded while a higher melting point is less favourable to biodegradation. Polymers possessing more crystalline regions are less prone to biodegradation, since amorphous regions are degraded faster. Moreover, the type of bonds, if they are more susceptible to be broken or not, its linearity and the presence of branching in the polymer's structure influences its propensity to biodegradation. The availability of functional groups is also relevant, and those with a hydrophilic nature are more prone to biodegradation [54, 56]. The additives on the polymer's constitution can slow down the biodegradation because they might be toxic for microorganisms. The physical form of the polymer - i.e., pellets, powder or fibres - has to be considered as well [54].

Although biodegradation plays an important role in the fate of plastics, in oceans it is considered to be a slow process [12], and at the benthic level its rates are reduced compared with less deep waters where there is a more diverse microbial community. These communities, constituted by autotrophs, heterotrophs and symbionts, have been found at the surface of debris, and contribute actively to the biodegradation of plastics. [7] Additionally, at the benthic level, oxygen and sunlight are less available, making the abiotic degradation process less preponderant, and, consequently, affecting the subsequent rates of biodegradation.

3.1.3 Products from microbial degradation of plastics

From a perspective of the feasibility and real application, in order for a biodegradation-based strategy to be implemented it has not only to be efficient, but also the toxicity of the produced by-products has to be considered. Reports show that, when using polyethylene as carbon source, CO_2 is a major product. Also, it has been reported the production of polysaccharides and proteins by *Rhodococcus rubber* (C208), *Rhodococcus rhodochrous* ATCC29672 and *Cladosporium cladosporoide* ATCC 20251 when using polyethylene as carbon source. [54] Mahalaksmi *et al*., (2012) [60] described the formation of octadecadienoic acid, octadecatrienoic acid, benzene dicarboxylic acid and cyclopropanebutanoic acid as products from microbial degradation of plastics from *Bacillus, Pseudomonas Aspergillus* and *Penicillium* species. Sowmya *et al.,* (2014) [61] reported carboxylic acids, aldehydes, alcohols, phenols, esters, ethers, alkyl halides and alkenes as products from *Chaetomium globosum.*

3.1.4 Strategies for following the biodegradation process

As first indicator of the occurrence of degradation, visual changes such as the roughening of the polymer surface, the formation of holes or cracks, de-fragmentation and changes in colour or formation of biofilms on its surface can be used. However, it does not give insights about the biodegradation metabolism which took place. These observations might be further analysed by SEM [13,52,54].

Weight loss is commonly used, although changes in other physical properties, such as tensile strength are stronger evidences [52]. To spot the formation and disappearing of new functional groups, Fourier Transformed Infrared Spectroscopy (FTIR) may be used [15, 54].

3.1.5 Combination approaches

Combinations of conventional solutions such as thermochemical and photochemical steps followed by biotechnology solutions have been proposed to enhance the biodegradation rates. These may be used as pre-treatments. More strategies have been presented, such as mixing the target polymers with prooxidants, genetic engineering to improve the microorganism efficiency and the addition of surface active agents or the stimulation of microorganisms to produce surfactants which allow them to attach to polymers surfaces [44].

3.1.6 Literature review of potential microorganisms to degrade plastics

Biodegradation of plastics by employing microorganisms or their enzymes has been gathering more attention in the last decades. Several bacteria and fungi have been reported for their potential for biodegradation, however with no significant practical application. Therefore, the screening of efficient microorganisms and further elucidation of how it occurs in order to better control this process and shape it to our aims is still lacking [54].

Different approaches have been reported, either some authors chosen to use only one type of microorganism and incubate it with the target polymer or work the other way around and from the target polymer isolate the microorganism communities which are associated with its degradation. It was also reported the isolation of one specie from an environment rich in microplastics, it is the example of *Idionella sakaiensis.* The first methodology has the advantage of allowing to study the molecular or metabolic processes involved and directly link one microorganism to the effects observed in the polymer [50-56]. Nonetheless, in the environment, microorganisms exist in communities and by this method, the community effect might be lost. Furthermore, some microorganisms are not cultivable in laboratory conditions, and their potential might be ignored.

Pseudomonas aeruginosa, Pseudomonas stutzeri, Streptomyces badius, Streptomyces setonii, Rhodococcus ruber, Comamonas acidovorans, Clostridium thermocellumand, and *Butyrivibrio fibrisolvens* are the dominant bacterial species reported for being associated with polymer biodegradation, while *Aspergillus niger, Aspergillus flavus, Fusarium lini, Pycnoporus cinnabarinusand* and *Mucor rouxiiare* are the prevalent fungal species. *Pseudomonas, Streptomyces, Corynebacterium* sp, Arthrobacter, Micrococcus and Rhodococcus have been more exploited for bioremediation, being *P. aeruginosa* broadly reported for polymer degradation via

biofilm formation [51]. **Table 3** presents the reported microorganisms associated with the biodegradation of several types of polymers, as well as some relevant findings.

An alternative to the use of microorganisms is to employ only their enzymes instead of the whole microbial cells. For example, studies reported the potential of the Cutinase enzyme family for the modification and degradation of PET and biodegradable plastics [44].

3.2 The biodegradable plastics approach

The term Bio-based plastic refers to plastics which are completely or partially obtained from biological resources. Examples are polyhydroxybutyrate (PHB) and polyhydroxyalkanoate (PHA), obtained via microorganisms, and Polylactic acid (PLA), polymerized from lactic acid monomers obtained via microbial fermentation of plant-derived sugars and starches. These alternatives do not represent a significant part of the plastic market due to its high production costs. Nonetheless, due to environmental increasing concerns, strategies are being found to reduce its costs and turn this biotechnology solution more viable in the future [8,44].

Bio-based plastics are mostly applied for packaging but PLA is also used in the textile sector for example, bio-based copolyester is used in high tech; bio-based polyamides are used for electronics, furniture, automotive and sports industries [8].

Bio-based polymers may be synthesised via microorganisms in the polymer final form of application without the need for chemical synthesis or they may be formed through chemical synthesis from monomers obtained from renewable sources. PLA is an example of the last type, as its monomers are obtained from renewable sources and its synthesis is chemical [8].

The term “biodegradable” refers to plastic items which can be degraded by living organisms, but not all bio-based plastics are biodegradable for example bio-PE and bio-PTT. Also, there are petroleum based products which can be biodegradable, for example PBAT (Polybutylene adipate terephthalate).

Biodegradable plastics found application in the agriculture sector, food packaging and organic waste collection. Although biodegradable, these plastics only degrade under defined circumstances, for example just like conventional plastic, biodegradable plastics won’t degrade efficiently in landfills.

Therefore, they should not be disposed into the environment under the premise that they are biodegradable. Certified biodegradable waste products are prone to be managed in composting plants or anaerobic digestors [8,52].

From a perspective of replacing conventional synthetic polymers for more sustainable alternatives there is the possibility of introducing biodegradable segments in recalcitrant polymer's chains. These segments include monomers, such as lactic and itaconic acid, that can be obtained from microbial fermentations using wastes as cheap resources source. This approach reflects the concept of circular economy, in which wastes are used to produce more valuable products. Replacement of conventional plastics might be done by two alternatives, either with natural polymers or with composites that result from merging synthetic polymers with natural biopolymers [44].

Bioplastics, according to European Bioplastics, are either bio-based, biodegradable, or feature both properties. Bioplastics can be obtained both from microorganisms and from plants. Examples of commonly used bioplastics are PHA, polyhydroxybutyrate, which has variations, namely poly-4-hydroxybutyrate, polyhydroxyvalerate, polyhydroxyhexanoate, polyhydroxyoctanoate, and copolymers. There are also starch-based bioplastics, suitable for mixing with biodegradable polyesters producing polycaprolactone, Polylactic acid is used in the form of films, fibers, cups, and bottles [8,44, 56].

However, these approaches might compromise and lead to loss of the polymers desired properties and the introduction of those type of monomers reduces the polymer's hydrophobicity [44]. Another limitation is that some replacement options are only partially biodegradable and therefore the problem persists at the end-of-life of these polymers. Furthermore, biodegradable plastics in marine environments persist for long periods although they are design to degrade under controlled circumstances [6].

Table 3. Overview of the several microorganisms reported for plastic biodegradation

Plastic	Microorganism/Microbial consortia	Relevant findings	Ref
PE	*Actinomycetes, Aspergillus, Penicillium, Zalerion Maritimum, Bacillus, Lysinibacillus, Pseudomonas, Staphylococcus, Streptococcus, Micrococcus, Streptomyces, Rhodococcus, Proteus, Listeria, Vibrio, Bravibacillus, Serratia, Nocardia, Diplococcus, Moraxella, Arthrobacter, Phanerochaete, Chaetomium, Gliocladium*	Pseudomonas species reported for biofilm formation with LDPE; *A. niger* is effective in PE degradation, *Rhodococcus ruber* colonizes and degrades PE by biofilm formation and hydrolysing enzymes; *Zalerion Maritimum* was the first marine fungus to be employed for microplastic degradation achieving high degradation rates.	[50-56; 60-65]
PP	*Aspergillus niger, Pseudomonas, Vibrio*	Several studies have been conducted on the biodegradation of this polymer trough soil burial tests and composting environments. Fungal species have shown ability to biodegrade it. A decrease in viscosity and the formation of new groups, were reported.	[52,66]
PU	*Fusarium solani, Aureobasidium pullulans sp., Pseudomonas* *Chlororaph*is, *C. acidovorans* TB-35	PU can be degraded by several fungal species however, its biodegradation is often incomplete.	[50-56]
Natural polymers	*Schlegelella thermodepolymerans, Pseudomonas* *, Streptomyces sp. SNG9, Ralstonia pikettiiT1, Acidovorax sp. TP4, Alcaligenes faecalis, Comamonas acidovorans, Alcaligenes faecalis, Schlegelella thermodepolymerans, Caenibacterium thermophilum, Clostridium botulinum, Clostridium acetobutylicum, Fusarium solani Fusarium moniliforme, Penicillium Roquefort, Amycolatopsis sp., Bacillus brevis*	PHA-degrading microorganisms have been reported to belong mostly to Basidiomycetes, Deuteromycetes and Ascomycetes; furthermore, they have been isolated from several sources, such as soil and marine environments	[50-56;58]

3.3 State of art of alternative solutions for PS and EPS waste management

As a response to the problem of EPS waste management, the solution adopted by several cities to handle it was banning its use. As an example, in New York, single-use EPS containers, identified as the most problematic sources of EPS waste, cannot be possessed, sold, or offered [49]. In San Francisco, a similar approach was implemented, prohibiting the use of EPS foam in takeout containers and shipping materials [67, 68]. Arguments for taking these restriction actions are the difficulties associated with recycling this material and the unsustainable disposal of large amounts of it, motivated by the single-use nature of the manufactured products made from EPS.

Are these overly extreme actions? From one side, the EPS industry argues for the advantages of this material in terms of ecological footprint, for example, if you replace it by other material which is heavier it increases the transportation efforts and the CO_2 emissions [29]. But on the other hand, some of the properties that make EPS so desirable are also environmental threats. However, meanwhile solutions are being developed for its replacement and waste management, action has to be taken also to solve the PS and EPS waste, which is already damaging the environment. Bioremediation strategies based on biodegradation fit in this last approach.

Although PS's high molecular weight prevents the attack of microbial enzymes, it has been recently established that it can be used as carbon source by several microorganisms. This last method has been done using soil and activated sludge. Furthermore, the addition of prooxidants, such as trace metals, has been investigated and authors reported it increased PS biodegradability. **Table 4** shows a brief resume of the PS forms studied and the respective microorganisms employed in those studies [69-76].

Table 4 – Overview of the reported microorganisms associated with PS and EPS degradation, adapted from [76]

Type of PS	Microorganism investigated
HIPS	*Enterobacter sp., Citrobacter sedlakii, Alcaligenessp. Brevundimonas diminuta, Bacillus spp. Pseudomonas spp*

Styrofoam, EPS	*larvae of Tenebrio molitor Linnaeus, Microbacteriumsp. NA23, Paenibacillus urinalis NA26, Bacillus sp. NB6, pseudomonas aeruginosa NB26*
Modified PS (PS -graft-starch and corn copolymers, PS/CaSO4, PS/PLA nanocomposites)	*Rhodococcus pyridinivorans NT, pseudomonas aeruginosa*
Pure PS (disposable plate, standard PS flakes, powder,)	*Pseudomonas aeruginosa, Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, and Aspergillus niger, Rhodococcus ruber*

Early attempts on investigating the capability of microorganisms to biodegrade PS done by Kaplan *et al.* (1979) showed, in different microbial systems, the decomposition of 14C-PS, over 5 or 11 weeks ranging from 0.04 to 0.57% [70].
Illustrating the approach of employing a pure culture, Roi Mor *et al.* (2008) [70] studied the kinetics of biofilm formation by a biofilm-producing strain (C208) of the actinomycete *Rhodococcus ruber* and evaluated its capacity in the degradation PS. The authors demonstrated the affinity between the strain and PS pointing to the possibly to be the cause of PS degradation, since, when cultured on PS flakes, bacterial cells adhered to the PS surface. Results showed that, in 8 weeks, a small reduction in weight (0.8% of gravimetric weight loss) was observed. The authors also refer that, to the best of their knowledge, there were no reports in literature on the effective biodegradation of pure PS until then. For the biodegradation assays pure standard flakes were used, PS in powder and ELISA 96-well microtiter plates manufactured from pure PS. Regarding the assay's conditions, approximately 1.0 g of PS per 50mL of medium was inoculated with 2 mL of a mid-exponential phase culture. More recently, mineralisation of 14 C-labelled polystyrene, either labelled on the ring ([U-ring-14 C]-PS) or labelled at the β-carbon position of the alkyl chain ([b-14C]-PS), by *Penicillium variabile* CCF3219 was

investigated by Lili T *et al.* (2017) [71]. Furthermore, the effect of ozonation as a physico-chemical pre-treatment was also evaluated. The assays were conducted for 16 weeks, with the mineralisation rate being higher in the first week. The authors observed that the mineralisation of PS with lower molecular weight was higher, and pointed as an explanation the fact that the polymer with a lower molecular weight is more accessible to microorganisms. For the assays, 4 mg of biomass were used for a volume of 6mL containing PS films. Regarding the effect of the employed pre-treatment, the authors observed that after exposing PS films to O_3, the mineralisation was higher. The efficacies were of 0.15± 0.03% with pre-treatment and 0.010± 0.003% without any pretreatment. Castiglia *et al. (*2015) [72] employed the fungus *Aureobasidium pullullans* var. melanogenum, for EPS degradation; however, EPS beads resisted. Mohan *et al.* (2016) [73] reported the achievement of a weight loss of 23% (w/w) of HIPS films after 30 days with *Bacillus* sp. In a different approach, You Yang *et al.* (2015) [74] investigated the role of the gut bacteria from the larvae *Tenebrio molitor Linnaeus* in 13 C-labelled PS biodegradation. *Exiguobacterium* sp. strain YT2A was isolated from the guts of the mealworms. After 28 days of incubation, the authors observed pits and cavities on the surface of PS films, detected by SEM observations. These 0.02 mm films were obtained by dissolving styrofoam in xylene. Regarding the assay conditions, 100 mg of PS and 10 mL of cell suspension were added to a 40 mL culture suspension with approximately 10^8 cells/ml, achieving the degradation of 7.4±0.4%, of the PS particles over 60 days. Furthermore, water-soluble degradation products were detected. Authors have already described before, the isolation of PE-degrader bacterial strains, *Bacillus* sp. YP1 and *Enterobacter asburiae YT1*, from a plastic-degrader waxworm gut.

To illustrate the approach of evaluating modified PS biodegradation, some authors aimed to improve its biodegradability by the insertion of degradable monomers in its hydrocarbon backbone. One example is the incubation of polystyrene–starch copolymer with *Bacillus coagulans* and polystyrene–lignin copolymers with fungi. Also, it was reported that the insertion of various mono- or disaccharides into the carbon backbone of PS increased its biodegradability. [70]

Shimpi *et al.* (2012) [75] evaluated the potential of *Pseudomonas aeruginosa* for the biodegradation of modified PS composites, namely PS:PLA and PS:PLA:OMMT (organically modified montmorillonite). An active culture of *Pseudomonas aeruginosa* cells of 1%, 0.1 O.D (absorbance at 600 nm) was used, and inoculated with polymer composites in a shaking incubator, at room temperature, during 28 days. Changes in

turbidity were observed for all polymer compositions; however, 21 days was the optimum growth period and at 20% and 25% of PS: PLA polymer composites and 5 phr (Parts per Hundred Rubber) PS: PLA: OMMT nanocomposite. The maximum degradation percentages reported at 10% and 25% PS: PLA composites and 2 phr PS: PLA: OMMT nanocomposite were 9.9% and 5.7%), respectively. Nikolik *et al.* (2013) [76] evaluated the biodegradation of copolymers of corn starch and PS and cornstarch and poly(methacrylic acid) in a model of river water, reporting that complete degradation was achieved after 27 days for the starch–graft–polystyrene. 2 L of river water were mixed with artificial wastewater and used in these experiments. Polymers were used in the form of 1 cm thickness discs with 0.2 cm of diameter. *Escherichia coli, Proteus sp., Serratia marcescens, Klebsiella sp., Pseudomonas* aeruginosa and *Enterococcus faecalis* were isolated from the river water.

Regarding the metabolic pathways involved in PS biodegradation, the mechanisms are not fully understood, it was reported that several microorganisms are capable of biodegrading its monomer, styrene. The main pathway involves the oxidation of styrene to phenylacetate that enters the tricarboxylic acid cycle (TCA). However, there are few studies reporting the identification of which enzymes are involved in biodegradation of PS. [69]

Chapter 4. Focus on fungi for biotechnological approaches to plastic waste management

4.1 Work aims and objectives

From a critical view of what is already described in the scientific literature, it stands out the need for screening new efficient microorganisms capable of degrading EPS, as biodegradation studies with this form of the EPS polymer are scarce. Moreover, in most cases, research had only a descriptive nature and no solution was demonstrated to be viable for real-world applications. Another aspect is that there are no studies focused on the utilization of a marine organism for its biodegradation, which is relevant since the ocean is the ultimate place where microplastics end up.

Therefore, the aims of this work were to evaluate the ability of the marine fungi *Zalerion maritimum (Z. maritimum) and Nia vibrissa (N. vibrissa)* to biodegrade EPS. *Zalerion maritimum* was previously reported by Paço *et al.* (2017) [62] for its potential

in biodegrading polyethylene with high efficacy, when compared to other strategies reported in literature that, in general, achieved low biodegradation rates and took longer times. Later on, during the experimental work, another fungus was tested, *Nia vibrissa*. To the best of our knowledge, this fungus had not yet been tested for its ability to biodegrade EPS, although it has been described as capable of depolymerising pure PHA homopolymers [77].

4.2 Biological characterization of *Z. Maritimum* and *N. vibrissa*

Fungal biomass distinguishes from other organisms by having ergoesterol as the major sterol found its composition. Fungal cell walls are thick and composed by a network of polysaccharides which differ between taxonomic groups. Cell walls are a dynamic structure composed by polysaccharides, mannoproteins, chitosan, glucans, chitin, polyglucuronic acid, small quantity of proteins and glycoproteins [78].

The term marine fungi refers to species which grow and sporulate either in marine, intertidal or estuarine environments, being a heterogeneous group more defined in terms of ecology rather than physiology. They are part of several symbiotic relationships and are also relevant pathogens of marine animals and plants. They take a role in the biochemical and nutrient dynamic cycles of the oceans, as decomposers of several substrates as woody, herbaceous and animal parts. However, the majority thrives on lignocellulosic substrates. As they inhabit marine environments, they developed different metabolic pathways from terrestrial fungi which have been extensively studied for drug screening and bioactive compounds for cosmetic and pharmaceutical industries [79, 80].

Z. Maritimum is a marine fungus which belongs to the Ascomycota phylum, Pezizomycotina subphyla, Dothideomycetes class, Tubeufiales order and Tubeuficieae family. Fungi belonging to the Ascomycota phylum produce ascospores enclosed in the ascus. As a filamentous fungus with a fruiting body, it is therefore included in the Pezizomycotina subphyla [81-83].

N. vibrissa is a widespread marine fungus belonging to the Basidiomycota phylum, Agaricomycotina subphyla, Agaricomycetes class, Niaceae family which colonizes a variety of submerged drift or intertidal woody substrates. A morphological and phylogenetic analysis of *N. Vibrissa* collected in Portuguese waters was conducted by Egídia Azevedo *et al.* (2018) [84,85].

Chapter 5. Materials and methods

5.1 Microplastic obtainment and characterization

EPS pellets (linear formula (C_8H_8) n), with a melt index of 12.0-16.0 g/10 min (200°C/5kg) [86] were purchased from Normax. These were first acquired in the form of EPS pieces used for the transportation of electronic equipment. In order to obtain microplastic particles with the adequate size range, (from 1.0 – 4.0 mm – **Figure 6 –** 1-1.4mm) these larger pieces were mechanically greased with the help of a grinder. After, they were separated by size with adequate meshes of different pore size for each assay. The specific size of the utilized microplastics in each assay are further detailed in **Table 5**. The obtained fragments were analysed by optical (**Figure 7**) and electron microscopies (**Figure 8**) and FTIR-ATR (**Figure 9**) spectroscopy. The obtained microplastic pellets exhibit an irregular surface resulting from the grinding process as can be observed in **Figures 6**, **7** and **8**.

Figure 6 – EPS pellets obtained after grinding procedure (ranging between 1-4mm)

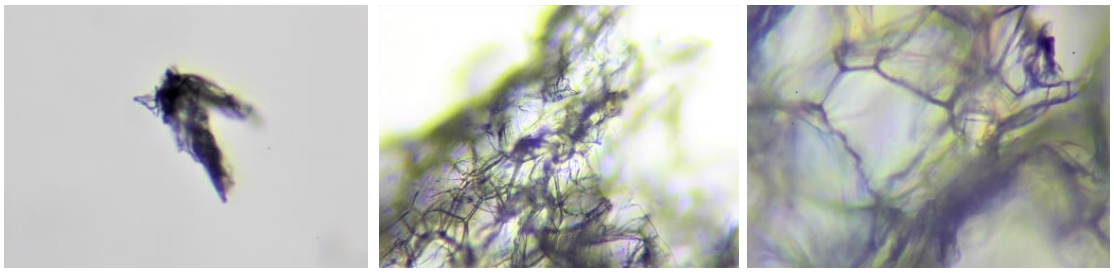


Figure 7 – EPS pellets under optic microscope observation

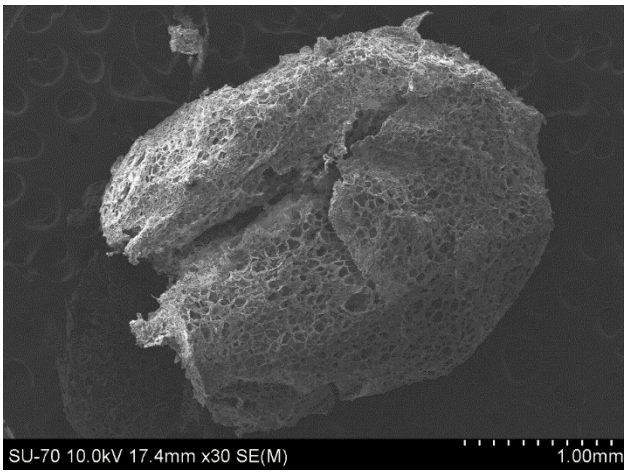


Figure 8 – EPS pellet under electronic microscope observation

As Fourier transform infrared (FTIR) spectroscopy is a selective analytical technique for evaluating inter and intra-molecular interactions in polymers it was used for identifying the main regions of EPS FTIR spectrum prior to biodegradation: region I – out-of-the-plane CH bonds, region II – in plane vibrations, region III stretching vibrations of aromatic and aliphatic C–H bonds. **Figure 9** presents the FTIR spectrum of the utilized EPS pellets. Ranging from 3200 to 2800 cm^{-1} is the part of the spectrum corresponding to the region of the C–H stretching modes. It is described in literature [87] peaks at 3082 cm^{-1} corresponding to absorptions from the aromatic C–H stretching vibrations, in this case it was found a peak at 3024.3 cm^{-1}, corresponding then to C-H stretching mode. Absorption bands at 2923 and 2848 cm^{-1} are described, respectively, as asymmetric and symmetric stretching vibrations of methylene groups (–CH2), in this case peaks were found at 2916.9 and 2336.1 cm^{-1}. Absorption band at 1452 cm^{-1} arises from C–C stretching vibrations in the aromatic ring. From 1300 cm^{-1} to 900 cm^{-1} is the fingerprint region where in-plane bending bands appear. The in-plane C–H bending of the phenyl ring is observed at 1028 cm^{-1}. From 900 to 675cm^{-1} is the pattern of the out-of-plane C–H bending bands where intense bands are found at 697 and 757 cm^{-1}, in this case a peak was found at 696.87 cm^{-1} [87-89]

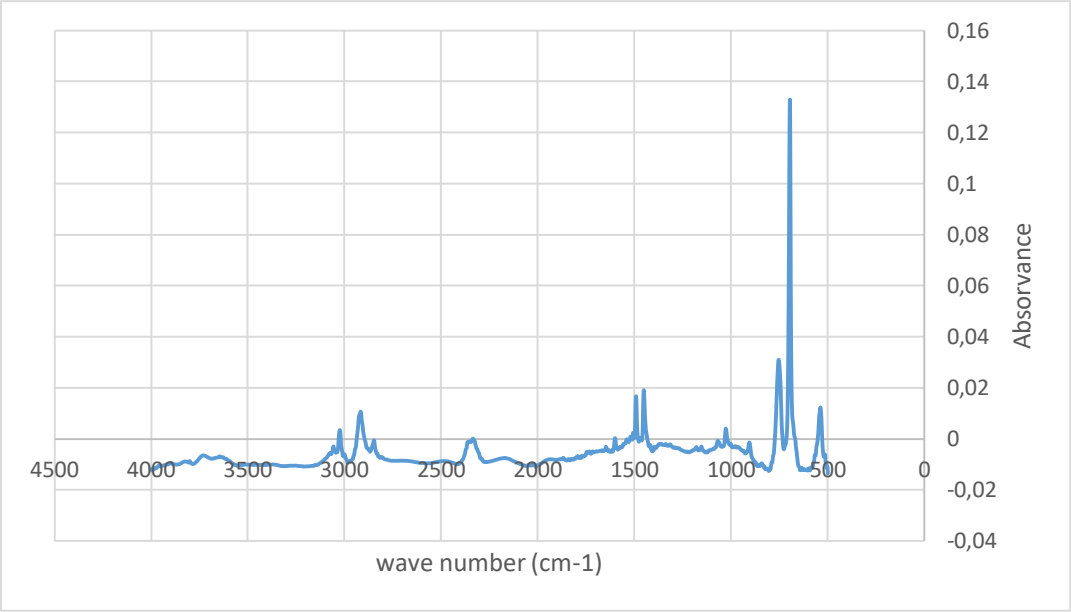


Figure 9 – EPS FTIR spectrum from a vulgar commercial supplier before the biodegradation assays

5.2 Preparation of the biological material and culture conditions of biomass growth

Prior to the realization of the biodegradation assays, it was necessary to obtain the marine fungus biomass in adequate amounts. These growing conditions parameters were previously optimized [62]. Therefore, *Z. maritimum*, sometimes also referred to as *Z. varium* (ATTC 34329, American type culture collection) was grown at 25 °C in a growth medium containing 20 g/L of glucose [90], 20 g/L of malt extract [91], 1 g/L of peptone [92], supplemented with 35 g/L of sea salts [93]. The marine fungus was incubated (HWY-200D, Lan Technics, USA) under stirring conditions (120 rpm) for 5 days prior to the assays. *N. vibrissa (*ATTC 34329, American type culture collection) biomass was obtained with the same procedure used for *Z. maritimum.*

5.3 Culture conditions of the biodegradation assays

Batch reactors (100 mL Erlenmeyer flasks) with a defined quantity of microplastics and a defined medium volume 10 times diluted minimum growth medium (2 g/L of glucose, 2 g/L of malt extract and 0.1 g/L of peptone with 35 g/L sea salts) were inoculated with a defined fungus biomass of filtered *Z. Maritimum* mycelium. Batch reactors were incubated (HWY-200D, Lan Technics, USA) in the dark and stirring was

maintained at 120 rpm for a maximum of 28 days. Temperature was kept at a constant 25 °C. [62] The same procedure was used for the biodegradation assays with *N. vibrissa*. These culture conditions were the standard for the basis of the biodegradation assays; however, during the assays, these were adjusted to the needs. The altered parameters in each assay are further described and their real values (for the inoculated biomass in each assay, the final biomass as well as for microplastic weight measurements) are in Appendix A.

Regarding the experimental design, four batch reactors (replicas) were sampled after incubation periods of 7, 14, 21 and 28 days. Three or four (depending on the assay) additional batch reactors were kept throughout the experience time, 28 days, as controls. Four contained only microplastics in the diluted minimal growth medium, in order to evaluate any potential effects derived from their presence in the growth medium and other four additional batches contained only fungi in the diluted minimal growth medium, in order to evaluate the growth of the fungi in the medium without the microplastics.

At the end of each assay established time, samples were retrieved from the shaker by collecting the fungus biomass and the remaining microplastics. Both were separated from the medium by filtration, using 47 mm diameter glass fibre filters (Whatman plc, UK). **Table 5** presents for each performed biodegradation assay, the respective experimental conditions – biomass inoculated (g) (A), microplastic dimension (mm) (B) and added quantity (g/L) (C), medium culture conditions (g/L) (D – glucose; E – malt extract; F – peptone; culture medium volume (mL) (G), stirring conditions (rpm) (H) and days of assay (I). (1) Preliminary evaluation of *Z. Maritimum* ability do biodegrade EPS (2) Biodegradation assay with EPS and *Z. Maritimum* (3) Optimization assay with *Z. Maritimum* and EPS (4) Preliminary evaluation of *N. vibrissa* ability do biodegrade EPS (5) Biodegradation assay with EPS and *N. vibrissa* in optimized medium.

Table 5 – Experimental parameters (A – biomass inoculated (g), B-microplastic dimension (mm), C- added pellet quantity (g/L), D -glucose (g/L), E-malt extract (g/L) F- peptone (g/L), G - medium culture volume (mL), H – stirring conditions (rpm) I – days of the assay), for each biodegradation assay 1,2,3,4 and 5.

Assay	A	B	C	D	E	F	G	H	I
1	0.50	<5	0.13	2	2	0.1	50	120	28
2	0.50	<5	0.13	2	2	0.1	50	120	28
3	0.25	1,2,3*	0.13-0.26	10	3-10	0.5	25	150	28

4	0.15	1,4 - 2	0.13	2	2	0.1	15	150	15
5	0.25	1 -1,4	0.1458	10	20	0.50	25	150	28

*1: (1.0-1.40 mm); 2: (1.4-2.0 mm); 3: (2.0-4.0 mm).

5.4 Biomass and microplastic analysis after the assay

After the collection of the fungus biomass and its separation from the remaining microplastics, its wet weight was evaluated. Then, after subjected to an overnight drying process, at 100 °C (Binder, Germany) the dry weight was also measured. The collected biomass at 7, 14, 21 and 28 days of experiment as well as a sample of the initial fungus (0 days) were frozen and lyophilized for further analysis by FTIR-ATR spectroscopy. The remaining microplastic particles separated from the fungus were also subjected to an overnight drying process, at 50ºC (Binder, Germany) in order to measure its dry weight and then analysed by FTIR-ATR spectroscopy.

FTIR-ATR analyses were performed using a Perkin Elmer (USA) Spectrum BX FTIR instrument. Samples were analysed at a 4 cm^{-1} resolution within the 4000 – 550 nm range. This analysis allows the detection of changes in the chemical composition of the lyophilized fungus after exposure to microplastics and direct comparison with the control samples. Regarding the microplastics recovered at 7, 14, 21 and 28 days of assays these were analysed for the detection of any change in their chemical composition due to biodegradation.

For the determination of the percentage degradation (calculated in dry weight), equation (1) was used, as reported by Shimpi *et al.* (2012) [75].

$$\% \text{ Degradation} = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100 \qquad (1)$$

For the determination of the biomass growth percentage (calculated in dry weight) equation (2) was used. To obtain the initial dry weight, the coefficient between dry and fresh weight was obtained as in the moment of the beginning of the assay. In order to obtain this coefficient four replicas of the fungus biomass were weighted and left in an overnight drying process, at 100 °C (Binder, Germany). Initial weigh was obtained by: dry weight inoculum – fresh weight × (Dry weight/ fresh weight).

$$\% \text{ Biomass growth} = \frac{Final\ weight - Initial\ weight}{Initial\ weight} \times 100 \quad (2)$$

From the FTIR spectra of microplastic pellets after biodegradation, the Carbonyl Content (COi) was calculated as showed in equation 3 as a way of evaluating the degradation of the polymeric samples. The COi was calculated by the ratio between the absorbance peak relative to the carbonyl groups and the reference absorbance peak for EPS (1452 cm^{-1}). [94]

$$COi(\%) = \frac{absorbance\ of\ carbonyl\ groups}{reference\ peak\ absorbance} \times 100 \quad (3)$$

5.5 Optimization assays

During the investigation, it was relevant to perform an optimization assay and the statistical model central composite design (CCD) was the chosen model. Central composite design has been extensively used in literature for medium culture optimizations with microorganisms. This model is the most commonly used response surface design method. [95-99] **Table 6** presents the value ranges for each tested continuous and categorical variable (malt concentration, EPS quantity and pellet dimensions). This table was obtained with Minitab software (version 17). A two-level full factorial design was implemented, with 39 base runs, 1 replicate, 1 base block, 2 continuous variables and 1 categorical variable resulting in a design with 12 cube points, 15 centre points in cube and 12 axial points, with α: 1.41421.

Table 6 – Experimental Design obtained with central composite design (CCD) in Minitab software version 17

Experimental design	Malt (g/L)	EPS Quantity (g/L)	EPS dimension*
1	2.9289	0.26000	1
2	20.000	0.19500	2
3	17.071	0.26000	1
4	2.9289	0.13000	1
5	10.000	0.10310	1
6	10.000	0.28690	1
7	10.000	0.19500	2
8	10.000	0.19500	3
9	2.9289	0.13000	3
10	10.000	0.10310	2
11	10.000	0.28690	2
12	10.000	0.19500	2
13	10.000	0.19500	1

14	10.000	0.19500	2
15	2.9289	0.26000	3
16	10.000	0.19500	1
17	17.071	0.13000	2
18	10.000	0.19500	1
19	10.000	0.28690	3
20	17.071	0.26000	3
21	17.071	0.26000	2
22	10.000	0.19500	3
23	0.0000	0.19500	1
24	10.000	0.19500	2
25	2.9289	0.13000	2
26	10.000	0.19500	1
27	20.000	0.19500	3
28	10.000	0.19500	2
29	17.071	0.13000	1
30	10.000	0.19500	3
31	10.000	0.10310	3
32	10.000	0.19500	1
33	0.0000	0.19500	2
34	2,9289	0.26000	2
35	17.071	0.13000	3
36	10.000	0.19500	3
37	0.0000	0.19500	3
38	10.000	0.19500	3
39	20.000	0.19500	1

**1: (1.0-1.40 mm); 2: (1.4-2.0 mm); 3: (2.0 -4.0 mm)

Chapter 6. Results and discussion

6.1 Preliminary evaluation of *Z. maritimum* ability do biodegrade EPS (Assay 1)

Paço *et al.* (2017) [62] previously demonstrated the ability of *Z. maritimum* to biodegrade polyethylene. Results showed that the fungus was capable of utilizing PE pellets causing its decrease in both mass and size, in a medium with minimum nutrients. The authors reported that for 14 days, a biomass variation of 82 ± 2% was accompanied by a mass variation of the polymeric materials of 57 ± 3% with the removal exceeding 43%. As this fungus showed the ability to biodegrade polyethylene, it was the first to be investigated for its ability to biodegrade polystyrene. This ability to biodegrade PE may

be related to the capability of these fungi to decompose lignocellulose polymers, suggesting that they may also be able to biodegrade other complex polymers, such as those present in plastics.

The preliminary evaluation of *Z. maritimum* was conducted for 4 weeks and allowed to assess if there was potential for using this fungus for EPS biodegradation. Furthermore, it allowed to screen the need of adapting some parameters such as EPS dimensions and quantity to the experimental procedures, since it was the first time that this plastic type was tested with the fungus. This preliminary study also helped to point out the possible practical challenges of working with this kind of plastic that might interfere with the efficacy of the biodegradation process. Namely, polystyrene's static electricity caused the particles to adhere to the glass walls of the batch reactor, thus reducing the contact rate with the fungal biomass of the suspension.

In this preliminary analysis, samples were only took after 3 and 4 weeks in order to understand the maximum ability of the fungus to biodegrade and its reaction to possible products of the polymer's decomposition.

Table 7 shows the percentage of biomass variation in 21 and 28 days of experiment. In 28 days, a percentage of 593 ± 107% was achieved. In 21 days a percentage of 711 ± 300% was observed. In the control replicas, for 28 days a percentage of 547 ± 89% was achieved and at 21 days of experiment a percentage of 759 ± 162% was noted. Results indicate that there was no statistically difference between the biomass growth percentage of replicas and controls (t-student test with Minitab software version 17, p-value (28 days) = 0.533137; p-value (21 days) = 0.78802. These observations pointed towards the non-toxicity of EPS for *Z. maritimum*, thus enabling further biodegradation assays to be performed. However, these results should be viewed only as indicative, as full evaluation of the toxicity of EPS towards these organisms requires specific toxicological assays, in order to accurately determine if EPS is indeed toxic for the fungus. If the fungus showed a negative acceptance of the polymer presence it could indicate that the proposed solution would not be viable, because biodegradation depends strongly on the biomass viability.

Table 7 - % of Biomass weight increase till 28 days of assay (dry weight)

Days of assay	Inoculated biomass (wet weight) (g)	Inoculated biomass (dry weight) (g)	Final biomass (dry weight) (g)	% of biomass weight increase (mean ± SD) (g)
21	0.5000	0.009826	0.0499	711 ± 300
	0.4300	0.008451	0.1044	
	0.4600	0.009040	0.07090	
	0.4800	0.009433	0.07690	
28	0.4900	0.009630	0.05290	593 ± 107
	0.5000	0.009826	0.07130	
	0.4800	0.009433	0.06530	
	0.5000	0.009826	0.07920	
21 (control)	0.4800	0.009433	0.09300	759 ± 162
	0.5200	0.010219	0.09000	
	0.4700	0.009237	0.08730	
	0.6100	0.011988	0.07480	
28 (control)	0.5200	0.010219	0.07290	547 ± 89
	0.4700	0.009237	0.05040	
	0.4600	0.009040	0.06170	
	0.4800	0.009433	*	

*replica not considered due to contamination

Focusing on the removal percentages, in 21 days it reached 60±9% and for 28 days 38±19%. The highest removal percentage obtained in 28 days was 66% and the lowest was 25%; in 21 days the highest value was 70% and the lowest was 48%.

When looking at these obtained removal percentages several aspects need to be taken into consideration since it was a preliminary evaluation. For example, due to the polymer's pellet dimensions, it was sometimes difficult to separate these from the biomass or some got lost during the recovery process. However, looking at the controls, the highest loss percentages were still inferior to the highest achieved removal percentages. **Table 8** shows the percentage losses and removal percentages obtained.

Table 8 - % of EPS removal percentages till 28 days of assay

Days of assay	Initial microplastic (dry weight) (g)	Final microplastic (dry weight) (g)	% of removal (replicas) / % of losses (control) (mean ± SD)
21	0.0130	0.00530	60±9
	0.0127	0.00380	
	0.0135	0.00700	
	0.0126	0.00470	
28	0.0133	0.0090	38±19
	0.0132	0.00990	
	0.0136	0.00460	
	0.0125	0.00880	
21 (control)	0.0129	0.0128	15 ± 26
	0.0127	0.0118	
	0.0125	0.00580	
	0.0125	0.0136	
28 (control)	0.0138	0.0120	14 ± 12
	0.0125	0.0105	
	0.0121	0.0164	
	0.0135	0.00970	

As these results pointed towards the ability of *Z. maritimum* to be used for EPS biodegradation, the experimental work proceed to assay 2, to better quantify its removal ability.

6.2 Biodegradation assay with EPS and *Z. maritimum* (Assay 2)

This time, in order to better elucidate and follow the process of biodegradation of EPS by *Z. maritimum*, samples were collected after 7, 14 and also 21 and 28 days. **Figure 10** shows the percentage of biomass variation during the experiment. In 7 days a percentage of 35 ± 16% was achieved, in 14 days a percentage of 48 ± 6%, in 21 days 80 ± 14%, and in 28 days 62 ± 25%. In the control replicas, for 7, 14, 21 and 28 days, respectively 29 ± 19%, 29 ± 33%, 81 ± 9% and 43 ± 0% (only one replica considered due to contaminations) were achieved. (T-student tests done with Minitab software version 17 indicate that there were no statistically differences between replicas and control regarding the % of weight increase during 28 days of assay: p-value (7 days) = 0.656836; p-value (14 days) = 0.323789; p-value (21 days) = 0.955665; p-value (28 days) = 0.176432).

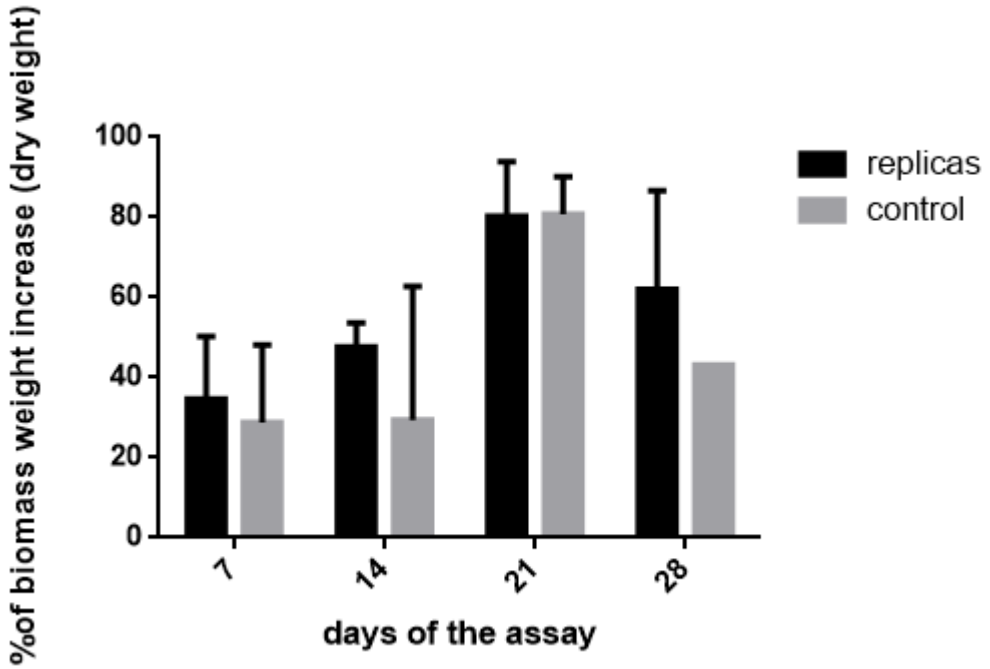


Figure 10 - % of biomass weight increase (dry weight) during 28 days of assay

There was a more accentuated weight increase until the third week, the most significant observed between 14 days and 21 days. Looking at the behaviour of the control samples, the majority of the biomass was also formed until 21 days. These behaviours of

biomass growth might be significant to understand to what extent it is worth to prolong the biodegradation process. In a study with the same time sampling periods by Shimpi *et al.* (2012) [75] after incubating an active bacterial culture in sterile basal mineral salt medium with different pre weighed polymer composites also for 7, 14, 21, and 28 days, the authors also reported the observation of culture density changes. The authors also described that after 21 days there was a reduction in bacterial growth when compared to 28 days and attributed this observation to lyses of bacterial cells for *P. aeruginosa*. They observed changes in bacterial growth visibly. Therefore, it may be possible that a similar process is taking place that results in this apparent loss of biomass.

Figure 11 shows the percentage of microplastic variation in 7, 14, 21 and 28 days of experiment. Respectively, the mean removal percentages obtained were of 13 ± 10%, 21 ± 10%, 7 ± 9%, and 11 ± 10%. (T-student tests done with Minitab software version 17 indicate that there were statistically differences between replicas and control for 7 and 14 days: p-value <0.05 (7 days) = 0.0437591; p-value (14 days) = 0.00777212; p-value (21 days) = 0.664499; p-value (28 days) = 0.211736).

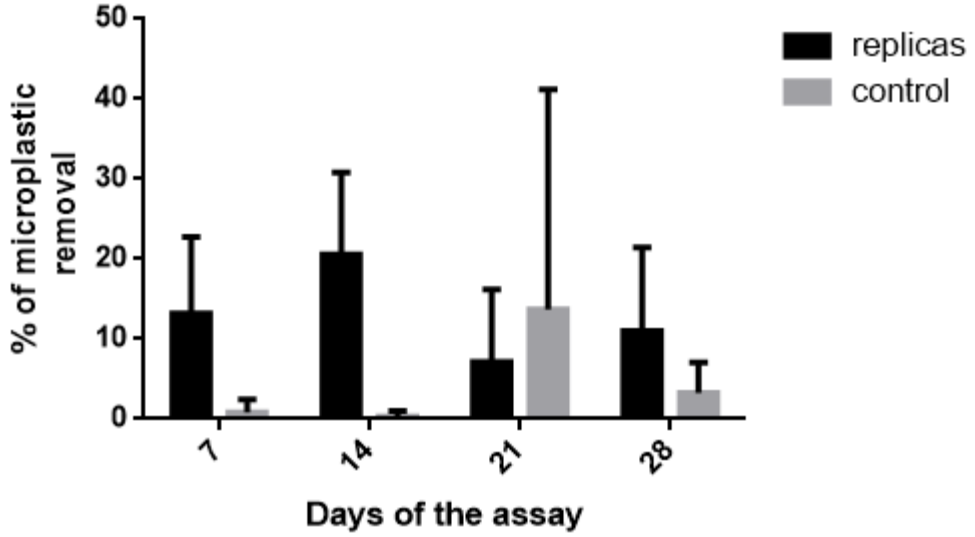


Figure 11 - % of microplastic removal during 28 days of assay

In comparison with the preliminary assays the removal percentages were much lower. This decrease in efficiency may be due to biomass intrinsic variation in performance, due to the assay conditions or intrinsic EPS pellets characteristics. Some described factors that might influence biodegradation rates are temperature, humidity, pH and salinity. The ones related with the pellet's properties are its dimension and quantity added to the medium, which in further assays were took into consideration.

Looking at the results of biomass weight increase % and now the performance of the fungus in EPS removal, it seems to indicate that the process of biodegradation occurs mainly in the first two weeks. When thinking from a cost-effective perspective, it is important to know for how long it is worth to extend the removal process, as previously said. Here, for the first two weeks the majority of the biomass is formed as well as the significant removal percentages.

Regarding the apparent less efficiency of *Z. maritimum* to biodegrade EPS when compared to PE, potential reasons may include the different crystalline nature of these polymers, the type of bonds presents in their structure, linearity, branching and different functional groups. All these factors must influence the different biodegradation rates between different polymers, since biodegradation starts with the polymer's cleavage converting it into tiny fractions. There are also physical restraints that may also contributed to this lower efficiency in the degradation of EPS, namely, the higher buoyancy of the latter, that greatly impaired an efficient contact between the suspended fungal biomass and the suspended polymer.

Even with these lower removal percentages, to the best of knowledge, it still is the first time that a marine fungus was used to biodegrade EPS, which presents some advantages over the reported organisms used in literature. As a marine organism, it can be easier to adapt this bioremediation strategy for real applications when treating saline waters. Also, the process of removing the fungus biomass from the treated effluent after the biodegradation process is easier then removing bacteria, for example. Moreover, the fungus' biomass can be valorised after the biodegradation process. For example, it can be exploited for the production of electricity or used as a source for bioactive compounds. When compared to other results obtained by other authors with different organism, the percentages of removal of the performed assay are in some cases higher or similar. Roi Mor *et al.* (2008) [70] achieved a reduction of 0.5% and 0.8% in PS gravimetric weight for 4 and 8 weeks. Authors attributed the limited growth along with the low degradation rate to the consumption of low molecular impurities on the polymer surface. In the case of this assay, a similar explanation can be extrapolated. Mohan, A.J. *et al.* (2016) [73] achieved a weight loss of 23% (w/w) of HIPS film in 30 days with *Bacillus* sps. In **Figure 12,** both microplastic removal and biomass weight percentages during the assay are plotted.

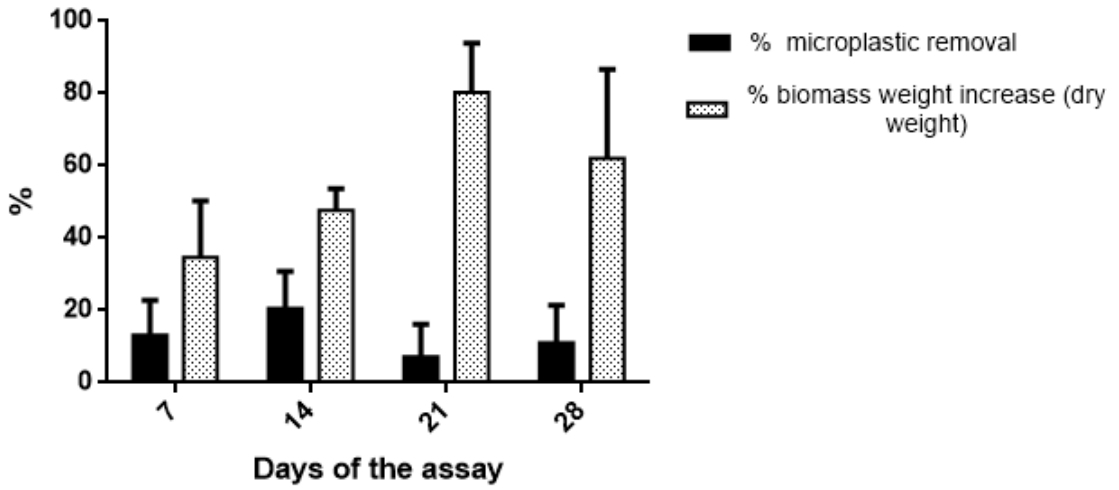


Figure 12 – % of microplastic removal during the 28 days of assay and biomass weight increase % of *Z. maritimum*

increase in biomass weight, although this behaviour does not occur for the rest of the assay. Paço *et al.* (2017) [62] described the behaviour of Z. *maritimum* growth in the presence of polyethylene. According to the authors, the significant biomass variation occurred in the first 7 days. The authors attributed the slightly higher mass increase of *Z. maritimum* after 7 days, when comparing to controls, to the use of the polymeric material as a nutrient source. In the case of this assay, the higher weight increase of the replicas exposed for 28 days when comparing to controls, might be explained by the same reason. However, in the case of EPS results seem to not always indicate a positive correlation between biomass weight increase and EPS removal. Paço *et al*. (2017) [62] observed a positive correlation between biomass variation and the percentage of removed plastics.

Regarding the obtained removal percentages, some options could be explored in order to improve the biodegradation rates. For example, the decrease of the solution volume where the biodegradation assay takes place. This could solve the static electricity obstacle of EPS that leads to a non-uniform homogenization, since some of the polymer particles were on the walls of the flask instead of being all the time in contact with the fungal biomass. Also, it could be possible to consider some kind of treatment to the polymer prior to biodegradation, since usually the first breakdown in nature is not due to biochemical processes, but rather physical ones, such as radiation/oxidation. For example, under oxidizing conditions (e.g., ozonation), could result in improved biodegradation performance, owing to the introduction of oxygen into the polymer matrix and the formation of functional groups, such as carbonyl and hydroxyl, more susceptible to biodegradation pathways. Other possibility would be to include biodegradable monomers

in the polymer chain as those molecules may help to enhance the polymer's accessibility to the microorganisms. This can be illustrated by an already mention approach done by for example, Nikolik *et al.* (2013) [76] where authors tested the biodegradation of starch–graft–polystyrene and starch–graft–poly (methacrylic acid) copolymers in model river water. They reported that the highest biodegradation percentages were achieved for those copolymers with the highest amount of starch in their composition. The authors reported that starch–graft–poly (methacrylic acid) copolymers were completely degraded after 21 days and the starch–graft–polystyrene copolymers were partially degraded (45.8–93.1 % mass loss) after 27 days.

Other approach that could be tested would be the utilization of more organisms working together as the process of mineralisation does not have to be done only by a single microorganism. As reported in literature it might happen from a microbial consortium effort composed by several organisms, where some break down the polymer into its constituent monomers, some use them excreting less complex by-products and some are able to use these. These could be also an alternative approach to test in further assays. Moreover, the addition of prooxidants, such as trace metals, has been investigated also and reported to increased PS biodegradability, so it could be tested in further essays.

Another aspect to take into account when explaining the degradation rates during EPS biodegradation is the formation of possible toxic compounds that might inhibit the biodegradation process. Furthermore, it might compromise the viability of the culture medium where the biodegradation process took place in terms of toxicity. Some authors reported the formation of intermediates as styrene oxide, phenyl ethanol, phenyl acetaldehyde,1-Phenyl-1,2-ethane diol in the presence of *Bacillus* sps and *Pseudomonas* sps (Phenyl ethanol). Further assays should be planned to see if some of the intermediate compounds influence the biodegradation process.

Table 9 presents the carbonyl index calculated for the microplastic samples after 7, 14, 21 and 28 days of assay. The carbonyl content is a measure of the proportion of C=O bonds present in the samples as this bonding is formed due to the oxidation of the materials. The increase of this index indicates a higher polymer backbone scission (as this is not a characteristic group found in EPS), and can be observed in the obtained results, showed in **Table 9**. Carbonyl index is increasing from samples of 7 days of essay to 28 days, while in the control microplastic samples such behaviour did not occur. This results seem to point towards the ability of the fungus biomass to cause the scission of the polymer's backbone structure. [100]

Table 9 – Carbonyl index calculated for microplastic samples after 7, 14, 21 and 28 days of assay

	7 days (mean ± SD)	14 days (mean ± SD)	21 days (mean ± SD)	28 days (mean ± SD)
Control	0.59±0.07	0.49±0.04	0.54±0.03	0.52±0.08
Replicas	0.56±0.01	0.62±0.07	0.62±0.01	0.81±0.03

Table 10 and **11** show the areas of several peaks present in *Z. maritimum* biomass FTIR spectra after 7, 14, 21 and 28 days of exposure, as well as the same peaks for the control samples (not exposed to microplastics) and an inoculum sample (fungus biomass at 0 days of essay not exposed to microplastics). Absorption bands at 3700 to 3500 cm^{-1} are attributed to bond vibrations of carboxyl, hydroxyl or phenol groups and to amides' N-H vibrations; at 3050–3000 cm^{-1} to C-H bonds from lipids; at 2996–2800 cm^{-1} to vibrations of CH_2 and CH_3 functional groups from lipids or proteins; at 1800–1700 cm^{-1} to C=O bonds, typically from lipids; between 1700 and 1500 cm^{-1} to amides in proteins; between 1200 and 1100 cm^{-1} to vibrations of C-O bonds, found in carbohydrates. [62,101,102]

Table 10 – Areas of several peaks present in *Z. maritimum* FTIR spectra after 7, 14, 21 and 28 days of exposure to microplastics during assay 2 (0 days – a biomass sample not exposed to microplastics and promptly analysed, without being subjected to the medium culture for any days)

Region (cm^{-1})	F7	F14	F21	F28	0 days
3700-3000	44±13	71 ±20	51± 11	38± 14	114± 5
3000-2800	11 ±2	16± 2	12± 1	9± 4	20± 2
1800-1700	2.2± 0.4	2.8± 0.5	2.6± 0.1	2.1± 0.3	1.6± 0.2
1700-1500	9± 3	13± 1	9± 1	7± 2	15.3± 0.9
1500-1250	9± 2	13.1± 0.4	9.1± 0.7	7± 2	14.0± 0.8
1200-1100	9± 3	10± 1	9± 1	7± 3	11.1± 0.6
1100-1000	12± 4	21± 3	14± 3	10± 5	28.8± 0.8

Table 11 - Areas of several peaks present in *Z. maritimum* FTIR spectra control after 7, 14, 21 and 28 days of assay 2 without being exposure to microplastics

Region (cm^{-1})	7C	14C	21C	28C
3700-3000	67±24	48 ±7	47± 7	68.3
3000-2800	16 ±3	11± 1	11± 2	15.4
1800-1700	2.2± 0.2	2.3± 0.1	1.99± 0.05	2.74
1700-1500	13± 3	9.1± 0.7	8± 2	12.2
1500-1250	12± 2	9.000± 0.008	8± 1	12.4
1200-1100	11± 2	9± 0.2	7± 3	9.23
1100-1000	20± 8	14± 2	12± 5	20.4

Variations in the profile of these peaks will be related to the fungus metabolism during the biodegradation process which is influenced by the availability of medium nutrients in relation with microplastics being a source of carbon.

Paço *et al*. (2017) [62] attributed the overall behaviour seen in the *Z. maritimum* FTIR spectrum to the lack of nutrients in the medium, which is related with the fungus search for endogenous sources of carbon. Authors pointed that in the case of the control fungus (*Z. maritimum* which was not exposed to microplastics) it was observed a more significant variation in the spectrum. This can be explained by the utilization of microplastics as source of carbon by the fungus biomass exposed to them in the essays.

In the case of this study, as seen in Table 11 and 12, the areas of the peaks from the samples present variations towards the inoculum which can be attributed to alterations in the lipidic and proteic content since the fungus when exposed to microplastics is in a reduced nutrient medium. It is likely that the fungus was induced to produce proteolytic enzymes to degrade intracellular proteins in search for endogenous energy and carbon sources. As the variations of the peak areas present some differences between the control samples and the ones exposed to microplastics it can be due to the presence of microplastics as a nutrient source. However further studies would have to be done to better evaluate this differences in the variation of the lipidic and proteic content between the control and the fungus exposed to microplastics.

6.3 Optimization assay with *Z. maritimum* and EPS (Assay 3)

Since there were several factors that could be influencing the biodegradation rates, some of them were chosen for further investigation to understand its significance through a response surface design. The chosen factors seen as variables were EPS quantity, pellet dimension and media composition, namely, malt extract concentration, as determined by previous studies (in-house obtained, unpublished data). Malt extract is a nitrogen source and provides the acidic environment and the nutrients needed for the metabolism and growth of the biomass [92] In-house obtained results for growth medium optimization (malt extract, peptone and glucose concentration where the variables) for the degradation of polyethylene by *Z. maritimum*, indicated that, the component malt extract was the most significant and that peptone and glucose had little influence on the overall performance of this fungus. Therefore, using the optimum values for glucose and peptone found in this first optimization, malt extract was selected to be a continuous variable for screening its significance also in the biodegradation performance of EPS (since the solution medium is the same and the fungus is the same). Glucose and peptone were set on 10 g/L and 0.5 g/L (optimum values found in the previous optimization for Z. *maritimum* PE biodegradation), respectively, while malt was screened between 2.9289 g/L and 17.0711 g/L.

The other continuous variable selected was the concentration of EPS pellets added to the medium, which was screened between 0.13 g/L and 0.26 g/L. Defined as a categorical variable, the dimension of the pellet particles were set into three categories, 1 mm – 1.40 mm; 1.40-2 mm and 2-4mm.

Central composite design was chosen to perform the optimization assay based on its well-established utilization by several authors, including in similar contexts for culture medium optimization for fungal strains, other microorganisms including marine ones.

Chosen factors and levels of composite design are presented in **Table 12** as well as the conditions for each experimental group, including the obtained response (removal percentages).

Table 12 – Obtained responses (% biomass growth -dry weight and % PS removal) in the optimization assay (*contaminated samples)

Experimental group	Malt (g/L)	EPS added quantity (g/L)	EPS pellet dimension	% removal
1	0.0730	0.00650	1	0.00
2	0.500	0.00490	2	*
3	0.427	0.00650	1	15.2
4	0.0730	0.00330	1	14.7
5	0.250	0.00260	1	0.00
6	0.250	0.00720	1	9.90
7	0.250	0.00490	2	0.00
8	0.250	0.00490	3	*
9	0.0730	0.00330	3	0.00
10	0.250	0.00260	2	0.00
11	0.250	0.00720	2	12.3
12	0.250	0.00490	2	0.00
13	0.250	0.00490	1	39.6
14	0.250	0.00490	2	*
15	0.0730	0.0065	3	0.00
16	0.250	0.00490	1	52.1
17	0.427	0.00330	2	34.4
18	0.250	0.00490	1	0.00
19	0.250	0.00720	3	0.00
20	0.427	0.00650	3	1.50
21	0.427	0.00650	2	0.00
22	0.250	0.00490	3	0.00
23	0.00	0.00490	1	0.00
24	0.250	0.00490	2	0.00
25	0.0730	0.00330	2	0.00

26	0.250	0.00490	1	0.00
27	0.500	0.00490	3	0.00
28	0.250	0.00490	2	*
29	0.427	0.00330	1	38.2
30	0.250	0.00490	3	0.00
31	0.250	0.00260	3	0.00
32	0.250	0.00490	1	61.2
33	0.00	0.00490	2	14.6
34	0.0730	0.00650	2	38.8
35	0.427	0.00330	3	0.00
36	0.250	0.00490	3	68.7
37	0.00	0.00490	3	0.00
38	0.250	0.00490	3	0.00
39	0.500	0.00490	1	80.9

The highest removal percentage achieved was 80.9% when malt extract was at the highest concentration, EPS quantity was at 0.0049 g and the smallest particle size was used. The optimum values obtained for malt, the EPS pellet dimension and its quantity were, respectively, 20 g/L, 0.1458 g/L and the smallest dimension (between 1.0 - 1,40 mm). **Figure 13** shows these optimum values plotted by Minitab software version 17, where C1 stands for malt extract concentration, C2 for EPS concentration and C3 for pellet dimensions (being C4 the response – microplastic % removal). It was expected that the smallest pellet size was more prone to be utilized by the fungus. Regarding malt extract, the optimum value corresponds to 10 times the concentration used in previous assays (the same concentration that is used for biomass growth; in biodegradation assays a minimum medium culture is used, 10 times diluted); the optimum polystyrene concentration was slightly higher than the previous concentrations tested. Hence, it may be inferred that malt extract was the most relevant variable for the biodegradation process of EPS, as previously found for PE, although its *p*-value model term indicates that is not significant for the response variance observed. However, when looking at the experimental results and the statistical analysis side by side, in fact the highest percentage removals were obtained at higher malt concentrations and small pellet size.

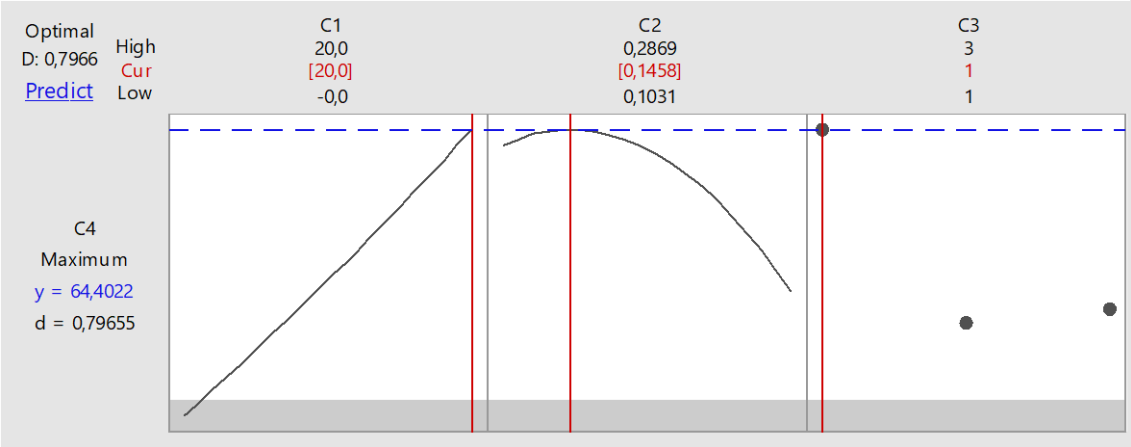


Figure 13 – Optimum values plotted by Minitab Software version 17 for malt extract (C1), EPS concentration (C2) and pellet dimensions (C3).

Table 13 shows the analysis of variance with *p*-values for the plotted variables. For linear, square and 2-way interactions it appears that none of these are significant to explain the variation in the response, since *p*-values are superior to 0.005. The obtained R^2 was 0.4287, which shows that the model explains 42.87% of the variance. Ideally, the model should better fit these response variances observed. However, the obtained *p*-value for lack of fit was 0.905 which is higher than alpha, indicating that the test does not detect any lack-of-fit.

Table 13 – Analysis of variance for the plotted model of C1, C2, C3 versus C4

Model		DF	Adj SS	Adj MS	F-value	p-value
Linear	C1	1	1022.4	1022.4	2.1700	0.15500
	C2	1	0.00	0.0100	0.00	0.997
	C3	2	2153.7	1076.8	2.2900	0.12600
Square	C1*C1	1	11.5	11.6	0.0200	0.877
	C2*C2	1	1231.7	1231.7	2.6200	0.12100
2-way interaction	C1*C2	1	533.9	533.92	1.130	0.2990
	C1*C3	2	1393.4	696.70	1.4800	0.25100
	C2*C3	2	131	65.6	0.140	0.871

From this optimization, optimum values were found to utilize in further assays and see if the biodegradation rates could improve. **Figure 14** shows the three-dimensional surface plot that describes the relationship between the fitted response and the two

continuous variables C1 – malt extract concentration and C2 – EPS concentration. The malt concentration region for a high removal percentage is narrow, suggesting that this medium component has influence on the removal performance. Another observation to take into consideration that arises from this optimization analysis is the fact that the process requires a high concentration of malt. Since this process aims to be utilized in real conditions, such a high concentration of this medium component is not very economically viable. It is then necessary to find a compromise between an economically feasible medium components concentration and a reasonable and sufficient removal performance.

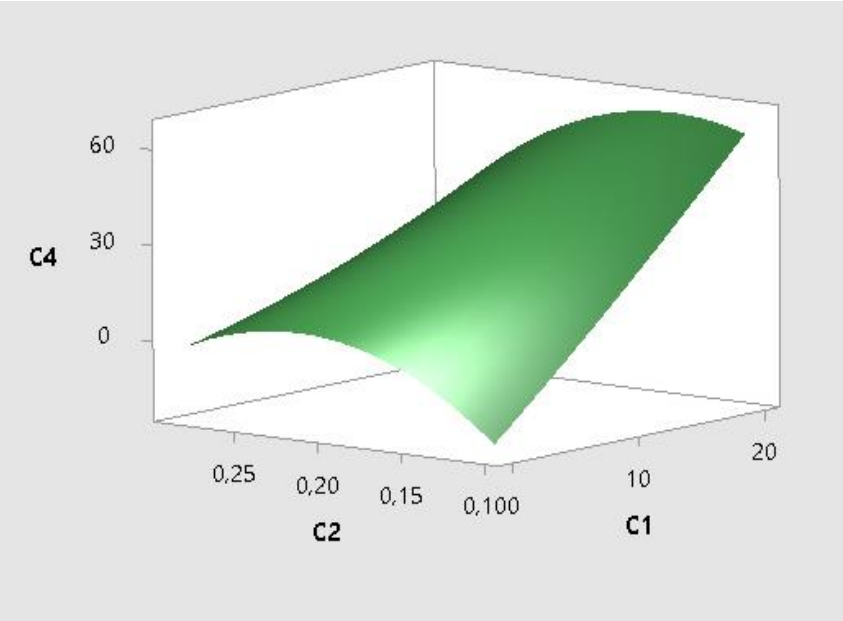


Figure 14 – Three dimensional Surface plot of the two continuous variables C1 – malt extract and C2- EPS concentration for response variance of C4 – microplastic removal percentage, fixing C3 to the smaller particle size tested.

Figure 15 presents the main effects plot to illustrate how different levels of the tested factors affect the response differently. As the presented lines are not horizontal, it suggests that there is a main effect and that different levels of malt extract concentration, EPS concentration and EPS pellet dimension affect differently the microplastic removal percentage.

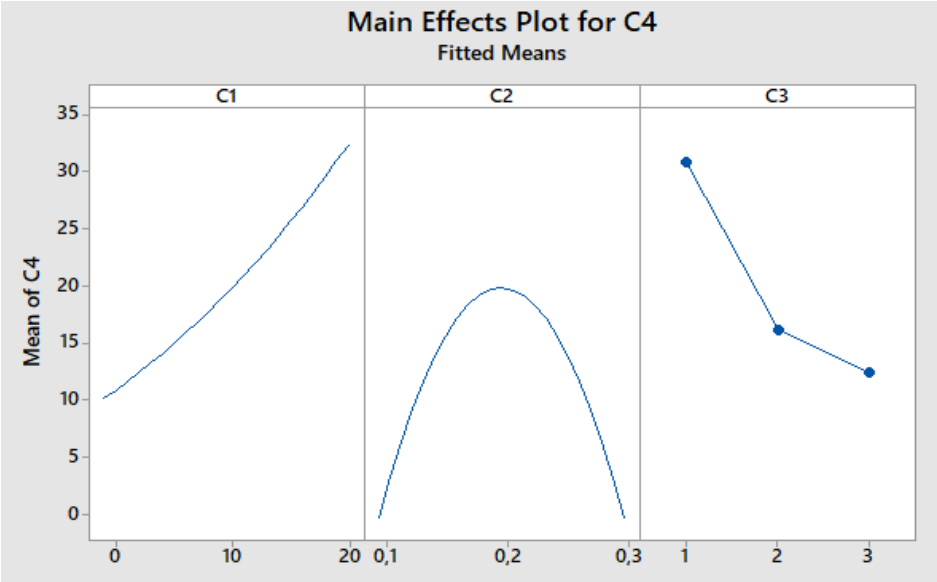


Figure 15 – Main effects plot for C1-malt extract concentration, C2- EPS concentration and C3- EPS pellet dimension and C4 microplastic removal %

6.4 Preliminary evaluation of *N. vibrissa* ability do biodegrade EPS (Assay 4)

Although *Z. maritimum* showed ability to degrade EPS, the process appears to be variable, since in some cases it achieved a high biodegradation percentage but in other assays these were low. Furthermore, the process seemed to require a high level of malt. As the purpose of this work was to evaluate potential solutions for EPS biodegradation, another marine fungus was tested. A preliminary evaluation of the ability of *N. vibrissa* to biodegrade EPS was conducted for 15 days. Some parameters such as the volume of the medium solution were adjusted with the previous knowledge from the already performed assays. This time, a lower volume was used in order to improve homogenization and allow a better contact between the fungus and the EPS pellets.

Table 14 shows the percentage of biomass variation during the days of the experiment. In 5 days a percentage of (181 ± 36%) was achieved, in 10 days a percentage of (271 ± 150%) and in 15 days (123 ± 45%). In the control replicas, for 15 days a percentage of 114 ± 36% was observed. These observations pointed towards the non-toxicity of EPS for *N. vibrissa*, enabling further biodegradation assays to be performed. Toxicological assays would have to be done in order to accurately determine if EPS is toxic, but this pointed towards the possibility of using this fungus for EPS degradation. When compared to the % of biomass weight increase achieved by *Z. maritimum* in the presence of EPS, *N. vibrissa* showed a higher percentage in the same time period, which can be an advantage since biodegradation depends on the biomass.

Table 14 - % of biomass weight variation during the days of the experiment

Days of assay	Inoculated biomass (wet weight) (g)	Inoculated biomass (dry weight) (g)	Final biomass (dry weight) (g)	% of biomass weight increase (mean ± SD) (g)
5	0.181	0.0142	0.0351	181 ± 36
	0.198	0.0156	0.0431	
	0.150	0.0118	0.0376	
10	0.178	0.0140	0.0369	271 ± 150
	0.189	0.0149	0.0455	
	0.150	0.0118	0.0640	
15	0.159	0.0125	0.0254	123 ± 45
	0.155	0.0122	0.0334	
	0.190	0.0149	0.0287	
15 (control)	0.189	0.0149	0.0281	114± 36
	0.180	0.0141	0.0339	
	0.150	0.0118	*	

*replica out due to contaminations

Focusing on the removal percentages, in 5 days, it reached 44±3%; in 10 days, 31±21% and in 15 days 49±3% (**Table 15**). When compared to the performance of *Z. maritimum* for the same time period in the preliminary evaluation of its ability to biodegrade EPS, *N. vibrissa* achieved higher percentages of microplastic removal. However, looking at standard deviation, replicas showed great variability between them.

Table 15 - % microplastic removal during the days of the assay

Days of assay	Initial microplastic (dry weight) (g)	Final microplastic (dry weight) (g)	% of removal (replicas) / % of losses (control) (mean ± SD)
5	0.00340	0.00200	44±3
	0.00360	0.00400	
	0.00370	0.00200	
10	0.00360	0.00400	31±20
	0.00370	0.00200	
	0.00360	0.00300	
15	0.00400	0.00200	50±3
	0.00420	0.00200	
	0.00370	0.00200	
15 (control)	0.00340	0.00300	10±2
	0.00390	0.00400	
	0.00440	0.00400	

6.5 Biodegradation assay with EPS and *N. vibrissa* in optimized medium (Assay 5)

Since *N. vibrissa* showed a higher biomass weight increase and higher removal percentages for the same time period in the preliminary evaluation, the assays were expanded. With the previous knowledge from the optimization assays performed with *Z. maritimum* concentrations of medium nutrients were fixed at optimum values. Malt was set at 20 g/L (optimum value obtained in the optimization – assay 4), glucose at 10g/L and peptone at 0.5 g/L (optimum values obtained in a previous optimization for Z-maritimum with PE). The optimum concentration for EPS pellets was found to be 0.1458 g/L in the previous assay (assay 3), so it was used for this one. The optimum dimension

for EPS pellets was also set based on the previous assay (assay 4), 1.0-1.40mm. In 7 days it reached 47±16%, in 14 days 32±4%, in 21 days 21±21% and in 28 days 27±20% (**Figure 16**). Controls showed maximum of losses of 14±27%.

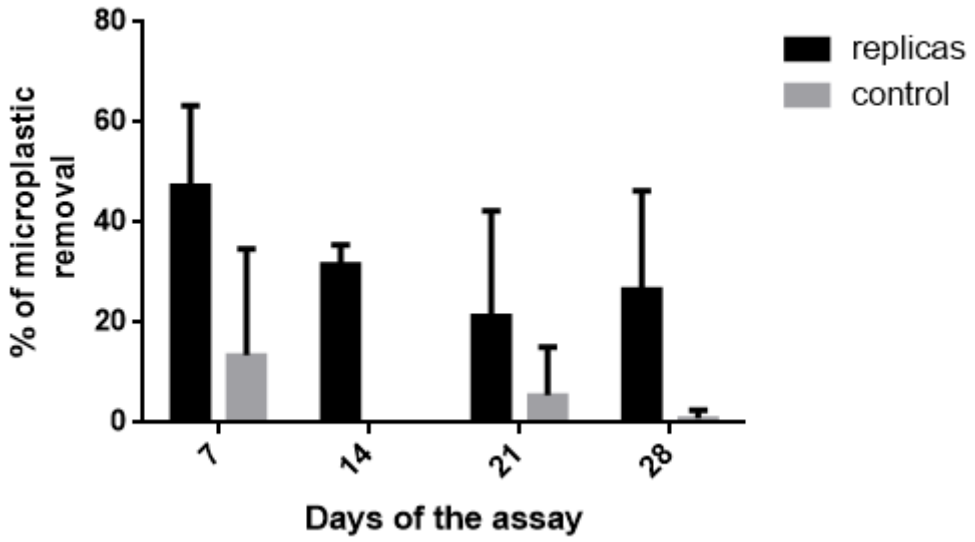


Fig.16 - microplastic removal percentages during the 28 days of assay with *N.vibrissa*

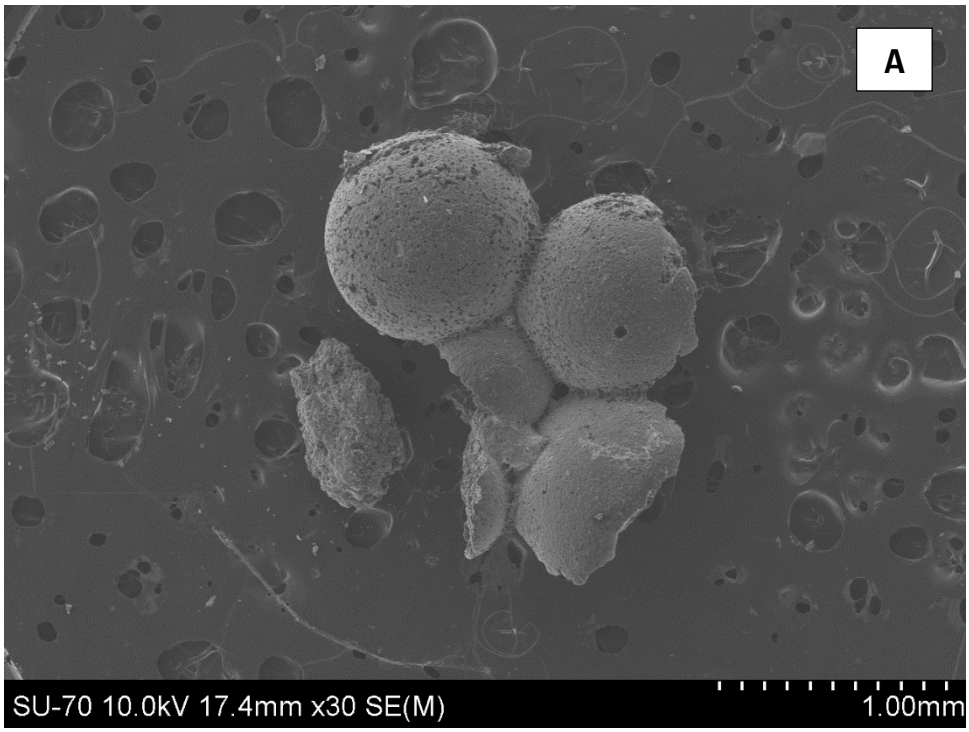
6.6 Searching for more biodegradation evidences

For assessing the degree of biodegradation, several authors used FTIR analysis of plastic pellets and the observation of changes in the polymers surface. For example, HIPS films without any microbial treatment have been shown to exhibit a plane and smooth surface, while those samples which were exposed to microorganisms showed incisions, pits and holes [73]. Other studies previously stated similar observations for different polymers, such as Zhanyong *et al.* (2012) for poly (3-hydroxybutyrate-co-3-hydroxyvalerate) and Kohei *et al.* (2000) for Poly Lactic Acid. When Valeria *et al*. (2015) [72] studied the deterioration of EPS caused by *Aureobasidium pullulans* var. melanogenum, they confirmed that this organism was responsible for the spots found in the polymer surface, although SEM images revealed that the fungus only grew on the surface of EPS beads. Therefore, the authors indicated that the colonization was restricted to the bead surface and found no evidences of hyphal penetration. Similar observations were found in this study and are further described.

In the case of this study, in some of the assays the fungus biomass (from *Z. maritimum*) adhered to EPS pellets as shown in **Figure 17**.

Figure 17- EPS pellets adhered to the biomass fungus (*Z. maritimum*)

Figure 18A and 18B shows SEM images of an EPS pellets after 28 days in contact with *Z. maritimum* (18A) and an EPS pellet of 28 days of control (18B) without being exposed to the fungus.



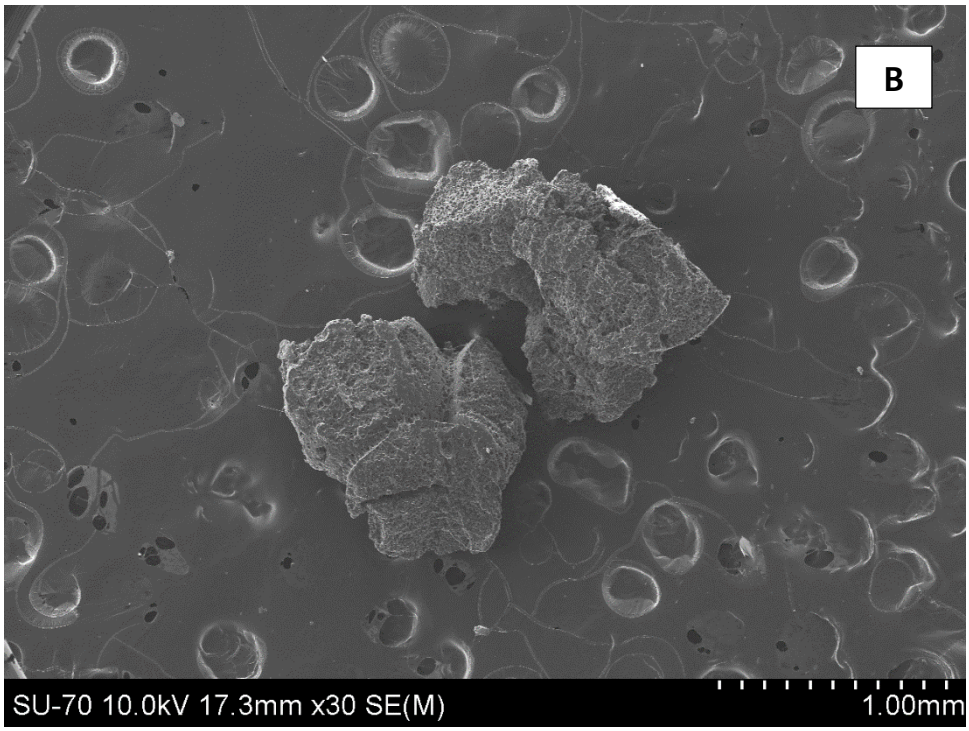


Figure 18 A and 18 B – SEM images of EPS pellets after 28 days of being exposed to fungus (A) and without being exposed (B)

SEM images showed no evidences of hyphal penetration and the EPS surface seems to not have significant morphological changes. However, more SEM observations would have to be obtained of more samples to evaluate if indeed there were surface alterations in the pellets exposed to fungus.

Chapter 7. Conclusions and future perspectives

After the performed assays with *Z. maritimum*, it can be concluded that this fungus shows potential for being used in EPS biodegradation. However, to what extent precisely is yet undetermined, as it varied from assay to assay. For example, during the optimization experiment (assay 3), in some replicas, there was no measurable biodegradation, while in others, it achieved values in the order of 60% and even 80% (in punctual cases). From the analysis of the variances of the optimization results (assay 3), optimum values for malt extract concentration, EPS concentration and pellet dimension were obtained. Although model term *p*-values showed that these variables were not significant for the response obtained, main plot effects graphic showed that different levels of these variables affected the response differently. Higher malt extract concentrations and smaller pellet size provided higher responses (expressed as microplastic removal percentages). Further

assays will have to be conducted to evaluate which variables of the assay most influence the removal performance.

When compared to values reported in the literature, the removal percentages obtained were similar or higher. Those cases where removal was more successful, authors incorporated biodegradable monomers, utilized a mixed culture approach or did some previous treatment to pellet samples. All these approaches could be investigated in further assays to ascertain whether the removal percentages improve.

Regarding *N. vibrissa* potential, initially, in the same time period, showed a higher biomass weight increase and higher removal percentages. However, even in optimized medium with the smallest pellet dimension the process appeared to be variable and further assays have to be conducted to evaluate to what extent the process can be optimized.

In this work, what to do after remediation process occurs was not exploited. One drawback of the multiple proposed bioremediation-based strategies is precisely what to do with the remaining biomass after the process. The potential of fungal biomass has already been exploited in Biotechnology for heavy metal removal, for the production of enzymes in food industry, the production of antibiotics and the production of recombinant proteins for health application purposes. The enzyme industry is particularly developed as half of the commercially available enzymes are provided by fungi [82]. Therefore, following bioremediation, the potential of the fungal biomass should be further explored and the final process could, in fact, result in a circular economy-based model. In essence, the products of the bioremediation process could be used for either electricity production or isolation of bioactive-compounds. Also, the intermediates which are formed during the biodegradation might be investigated for recovery and used in other applications.

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Appendix A

Table 1A – weight measurements of *Z. maritimum* biomass during assay 2

Growth percentage	Time of the essay (days)	Inoculated biomass-fresh weight (g)	Inoculated biomass-dry weight (g)	Final biomass-fresh weight (g)	Final biomass-dry weigh (g)
replicas	7	0.4070	0.03070	0.9790	0.04650
	7	0.5600	0.04224	1.1220	0.05280
	7	0.5240	0.03953	0.9780	0.03470
	7	0.6070	0.04579	1.222	0.05830
	14	0.4920	0.03711	1.334	0.05540
	14	0.5190	0.03915	1.218	0.06070
	14	0.5650	0.04262	1.031	0.05990
	14	0.4870	0.03674	1.219	0.05350
	21	0.5480	0.04134	1.481	0.07810
	21	0.5160	0.03892	1.656	0.07380
	21	0.5070	0.03824	1.323	0.06960
	21	0.5970	0.04503	1.212	0.07230
	28	0.5070	0.03824	0.9970	0.05590
	28	0.5090	0.03839	1.172	0.05750
	28	0.4960	0.03741	1.258	0.07120

Table 2A and 3A – weight measurements for calculating dry weight/fresh weight of *Z. maritimum* and *N. vibrissa* biomass samples

Replicas (0 days) 2A	biomass-fresh weight (g)	biomass-dry weight (g)	dry weight/fresh weight
R1	0.8020	0.05510	0.0687
R2	0.5560	0.05010	0.0901
R3	0.6950	0.04690	0.0675

Replicas (0 days) 3A	biomass-fresh weight (g)	biomass-dry weight (g)	dry weight/fresh weight
R1	0.8070	0.0640	0.07931
R2	0.3170	0.0240	0.07571
R3	0.4680	0.0350	0.07479
R4	0.3430	0.0290	0.08455

	28	0.5310	0.04005	1.236	*
control	7	0.5930	0.04473	1.132	0.04920
	7	0.5420	0.04088	1.093	0.04880
	7	0.6340	0.04782	1.151	0.07400
	7	0.5250	0.03960	0.7800	0.05190
	14	0.5010	0.03779	0.9740	0.01450
	14	0.4920	0.03711	1.152	0.04260
	14	0.5910	0.04458	1.509	0.0747
	14	0.5610	0.04232	1.381	0.0448
	21	0.5190	0.03915	1.632	0.07380
	21	0.5160	0.03892	1.897	0.08890
	21	0.5360	0.04043	1.669	0.07420
	21	0.5390	0.04066	1.469	0.06920
	28	0.5450	0.04111	1.353	*
	28	0.5060	0.03817	1.366	*
	28	0.5350	0.04036	0.9930	0.05780
	28	0.5300	0.03998	1.356	*

*replicas not used due to contamination

Table 4A – weight measurements of microplastic samples during assay 2

Microplastic measurement	Time of the essay (days)	Inoculated microplastic dry weight (g)	Final microplastic weigh (g)			
replicas	7	0.01350	0.01030	14	0.01380	0.01390
	7	0.01330	0.01190	14	0.01280	0.01290
	7	0.01380	0.01310	14	0.01460	0.01160
	7	0.01490	0.01500	14	0.01320	0.01520
control	7	0.01510	0.01560	14	0.01480	0.01460
	7	0.01300	0.01370	14	0.01260	0.01300
	7	0.01700	0.01950	14	0.01300	0.01500
	7	0.01220	0.01180	14	0.01420	0.01430
replicas	21	0.01410	0.01400	28	0.01400	0.01650
	21	0.01400	0.01210	28	0.01200	0.01190
	21	0.01500	0.01780	28	0.01200	0.00940
	21	0.01330	0.01760	28	0.01230	0.01100
control	21	0.01330	0.01420	28	0.01350	0.01360
	21	0.01170	0.01490	28	0.01430	0.01350
	21	0.01510	0.00680	28	0.01260	0.01290
	21	0.01540	0.01840	28	0.01340	0.01240

Table 5A – weight measurements of microplastic samples during assay 3

Microplastic measurement	sample	Inoculated microplastic dry weight (g)	Final microplastic weigh (g)			
replicas	1	0.0064	0.0075	12	0.0048	0.0051
	2	0.0048	*	13	0.0048	0.0029
	3	0.0066	0.0056	14	0.0049	*
	4	0.0034	0.0029	15	0.0065	0.0073
control	5	0.0027	0.0028	16	0.0048	0.0023
	6	0.0071	0.0064	17	0.0032	0.0021
	7	0.0050	0.0076	18	0.0049	0.2448
	8	0.0049	*	19	0.0072	0.0072
replicas	9	0.0032	0.0044	20	0.0066	0.0065
	10	0.0026	0.0035	21	0.0065	0.0075
	11	0.0073	0.0064	22	0.0050	0.0063

	23	0.0048	0.0049	32	0.0048	0.0041
control	24	0.0049	0.005	33	0.0067	0.0041
	25	0.0032	0.005	34	0.0032	0.0042
	26	0.0046	0.0055	35	0.0048	0.0015
	27	0.0048	0.4568	36	0.005	0.0070
	28	0.0049	*	37	0.005	0.0051
	29	0.0034	0.0021	38	0.0047	0.00090
	30	0.0049	0.0057	39	0.0048	0.0041
	31	0.0027	0.0028			

*replicas not used due to contamination

Table 6A – weight measurements of microplastic samples during assay 5

Microplastic measurement	Time of the essay (days)	Inoculated microplastic dry weight (g)	Final microplastic weigh (g)			
replicas	7	0.0038	0.00130	14	0.0035	0.00220
	7	0.0040	#VALOR!	14	0.0032	0.00220
	7	0.0033	0.00200	14	0.0036	0.00250
	7	0.0034	0.00214	14	0.0032	0.00230
control	7	0.0035	0.0016	14	0.0033	0.0044
	7	0.0033	0.0038	14	0.0032	0.0038
	7	0.0033	0.0034	14	0.0034	0.0043
replicas	21	0.0036	0.00170	28	0.0034	0.00230
	21	0.0032	0.00290	28	0.0035	0.00350
	21	0.0033	0.00290	28	0.0034	0.00180
	21	0.0035	0.00310	28	0.0033	0.00240
control	21	0.0036	0.003	28	0.0035	0.0034
	21	0.0046	0.0048	28	0.0034	0.0056
	21	0.0036	0.0049	28	0.0036	0.0039