Universidade de Aveiro Departamento de Biologia

2018

Ana Maria Simões do Paço

Biodegradation of microplastics: Optimization and Scale up

Biodegradação de microplásticos: Otimização e Aumento da escala

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

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presidente

Professora Doutora Maria Adelaide de Pinho Almeida
professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro

Doutora Ana Cristina Cardoso Freitas Lopes de Freitas
investigadora principal do Centro de Biotecnologia e Química Fina, Universidade Católica Portuguesa do Porto

Doutora Teresa Alexandra Peixoto da Rocha Santos
investigadora principal do Departamento de Química e do Centro de Estudos do Ambiente e do Mar (CESAM), Universidade de Aveiro

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palavras-chave

Zalerion maritimum, Nia vibrissa, fungo, biodegradação, microplástico, polietileno, poli(2,5- furanodicarboxilato de etileno), bioplástico, FTIR-ATR, SEM

resumo

O lixo marinho, em especial, os plásticos de pequenas dimensões, é uma das maiores ameaças ao ecossistema marinho. A presença destes microplásticos tem vindo a aumentar nos últimos tempos, devido ao uso indiscriminado de plástico pela população humana e à falta de políticas para a sua gestão.

Assim, é necessário encontrar maneiras de mitigar os seus impactos ou mesmo reduzir a sua presença. A biodegradação surge como uma solução promissora para este problema.

Neste trabalho, com o objetivo de desenvolver um processo de biorremediação, o potencial de biodegradação, de microplásticos de polietileno, do fungo Zalerion maritimum é explorado. Através da otimização do meio de cultura, por "Central composite design" e por "Uniform design", foi possível obter maiores taxas de remoção de microplásticos e verificar que o extrato de malte é o constituinte do meio mais relevante neste processo. Pelo aumento da escala foi possível verificar que mesmo num ambiente menos controlado o fungo biodegrada microplásticos.

Além disso, foi também testada a resposta do fungo Zalerion maritimum e do fungo Nia vibrissa, quando expostos ao poli(2,5-furanodicarboxilato de etileno) e foi possível verificar que ambos os fungos parecem ter a capacidade de biodegradar este micro(bio)plástico.

keywords

Zalerion maritimum, *Nia vibrissa*, fungus, biodegradation, microplastics, polyethylene, poly(ethylene2,5-furandicarboxylate), bioplastic, FTIR-ATR, SEM

abstract

Marine litter, specifically small plastic particles, is one of the major threats to the marine ecosystem. The presence of these microplastics has been increasing over the last few years due to their indiscriminate use and lack of polices for their management.

Thereby, it is necessary to encounter new ways to mitigate their impacts or even reduce their presence. Biodegradations is a promising solution to this problem.

In this work, with the objective of developing a bioremediation process the potential of *Zalerion maritimum*, to biodegrade polyethylene microplastics, is exploited.

Through optimization of the biodegradation medium, by Central composite design and by Uniform design, was possible to obtain higher percentages of microplastics removal and verify that malt extract was the most relevant compost medium to the process. By scale up it was possible to verify that in a less controlled medium the biodegradation of microplastics still occur.

In addition, the response of the fungi *Zalerion maritimum* and *Nia vibrissa* to exposure to poly(ethylene2,5-furandicarboxylate) has also been studied and it was possible to conclude that these fungi seems to have the capacity of biodegrade this micro(bio)plastic.

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ASTM	American Society for Testing and Materials
CCD	Central Composite Design
FDCA	2,5-furandicarboxylic acid
FTIR-ATR	Fourier transform infra-red spectroscopy
HDPE	High density polyethylene
LDPE	Low density polyethylene
LLDPE	Linear low-density polyethylene
LMDPE	Linear medium density polyethylene
NOAA	National Oceanic and Atmospheric Administration
PE	Polyethylene
PEF	Poly(ethylene2,5-furandicarboxylate)
PET	Poly(ethylene terephthalate)
PHA	Poly-3-hydroxyalkanoates
PHB	Poly[(R)-3-hydroxybutyrate]
SEM	Scanning electron microscopy
SPI	Society of the Plastic Industry
UD	Uniform Design

1. Introduction

1.1. Plastics in the marine environment

Marine litter is a major concern, since it is present in large quantities and spread throughout all oceans on Earth, having environmental, health and economic impacts[1–3]. The term marine litter encompasses different materials that were lost unintentionally or discarded in beaches, that were transported by rivers and winds from land to the seas, or that were disposed of directly in the oceans[1].

One of the most common material found in marine litter is plastic, it is present in various sizes and shapes and has great impacts in the marine ecosystems[1,3]. For this reason, there is a growing demand for new ways to reduce them.

Plastics are a sub-category of polymeric material, produce by the polymerisation of units (monomers), making an organic synthetic long chain-like molecule, normally petroleum-based. It is possible to produce different types of plastics, depending on the monomers or on the additives add to the chain, and they can be divided in thermoset and in thermoplastics[4,5]. Thermosetting plastics are formed from a resin or soft solid or viscous liquid prepolymer, and they are resistible to heat, not losing their form with high temperatures[6]. Thermoplastics normally are produced in the form of beads, and them heated and moulded in the intended shape, and can be remoulded.

This kind of material is highly used, in different areas and their mass production start in 1950s[5]. Since then, they have helped to improve life quality, due to their properties, as their light, temperature, water and chemical resistant, but also because the manufacturing of plastic products is easy and has a low cost associated[7].

Every year the production of plastics increases, in 2016 their production reached ≈335 million tonnes, and this large production creates big environmental problems[5]. As plastic are so resistant, they are also resistant to degradation in the nature, and the best way to reduce the plastic would be through recycling, but only 31.1% of plastics are recycled in Europe and 27.3% still go to the landfill[1,5].

Microplastics were firstly reported in the 1970s, in a publication describing the potential impact of plastic on marine animals, where Carpenter and Smith[8] refer the presence of small plastic particles inside the animals. Depending on the author, this term can have different definitions, which makes it difficult to analyse and compare different studies. Nowadays, some authors use the definition from the National Oceanic and Atmospheric Administration (NOAA). According to this organization, the term microplastics defines plastic debris with a size between 5mm and 1mm in size, and debris with less than 100 nm are called nanoplastics [9,10].

Microplastics, as represented in Figure 1, can be classified as primary microplastics or secondary microplastics, according to their origins. Primary microplastics are particles produced in this size, in the form of pellets, plastic-based granulates used in the cosmetics industry or in the form of a vector for drugs in medicine. Secondary microplastics are plastics debris, that result from the fragmentation of macro plastics, such as bottles or shopping bags. This fragmentation can be caused by different mechanisms, such as chemical and physical aging or degradation[9,10].

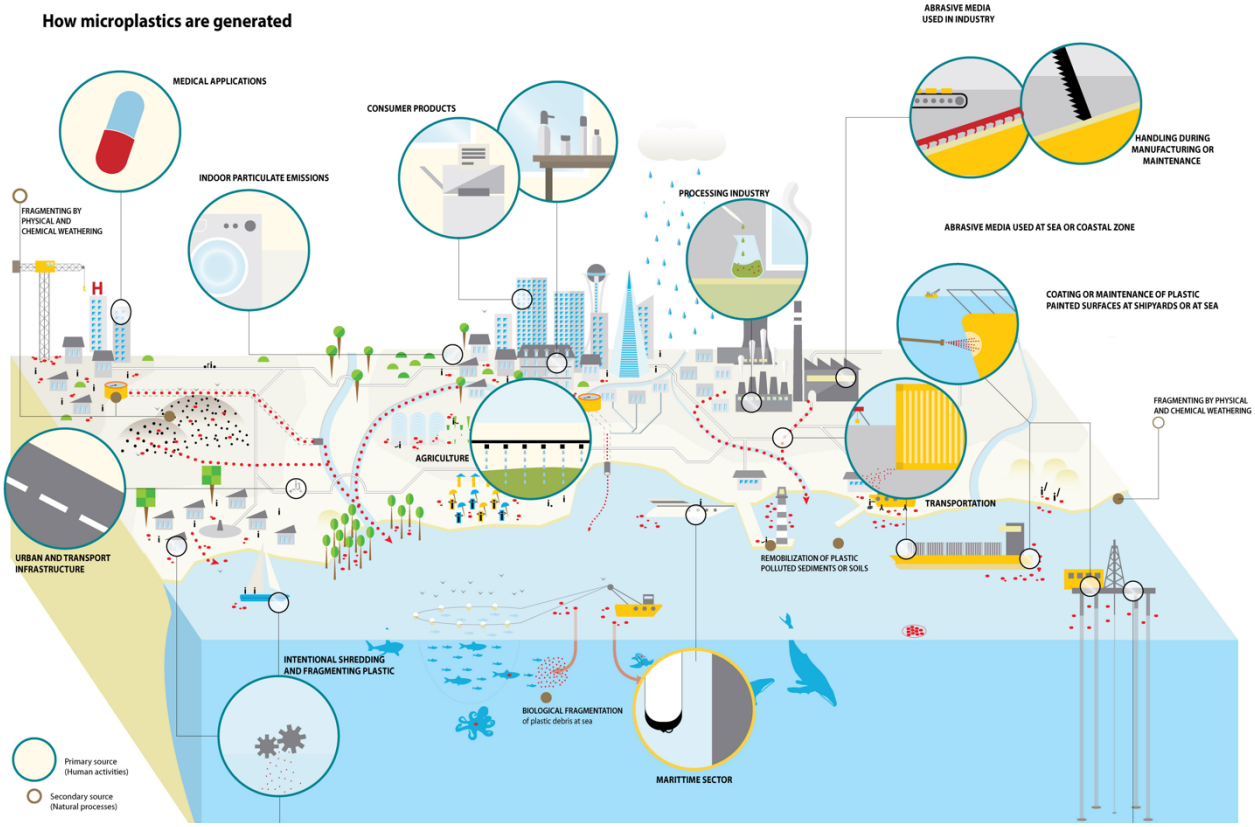


Figure 1 – Schematic presentation of the sources of microplastics[1] from Maphoto/Riccardo Pravettoni, available at http://www.grida.no/resources/6929.

Carpenter and Smith[8], in their paper also warn, for the first time, about the alarming presence of plastic pellets on the surface of the North Atlantic Ocean, in areas where dumping does not occur. These authors were unable to identify what kind of plastic the pellets were, but most of the samples would probably be polyethylene[11]. Later, in the same year, Carpenter et al[12] reported the presence of two types of polystyrene pellets, a crystalline and an opaque form, in the coastal waters of southern New England. It is also referred that the opaque pellets were selectively consumed by the fish on that area.

Since then the presence of plastic particles with every size has been reported, and in the past few years the interest in understanding how they end up in the ocean, how they suffer degradation and fragmentation, which are the impacts of their presence and how it would be possible to reduce them, has grown, being the topic of several papers[11,13–15].

In 2015, Jambeck et al[16] reported as estimate of the mass land-based plastic entering the ocean, based on data from solid waste and population density worldwide, and on the sources and main pathways of plastic into the oceans. The data was later resumed in Figure 2 by the Association GRID-Arendal[1], to help better understand the problem itself.

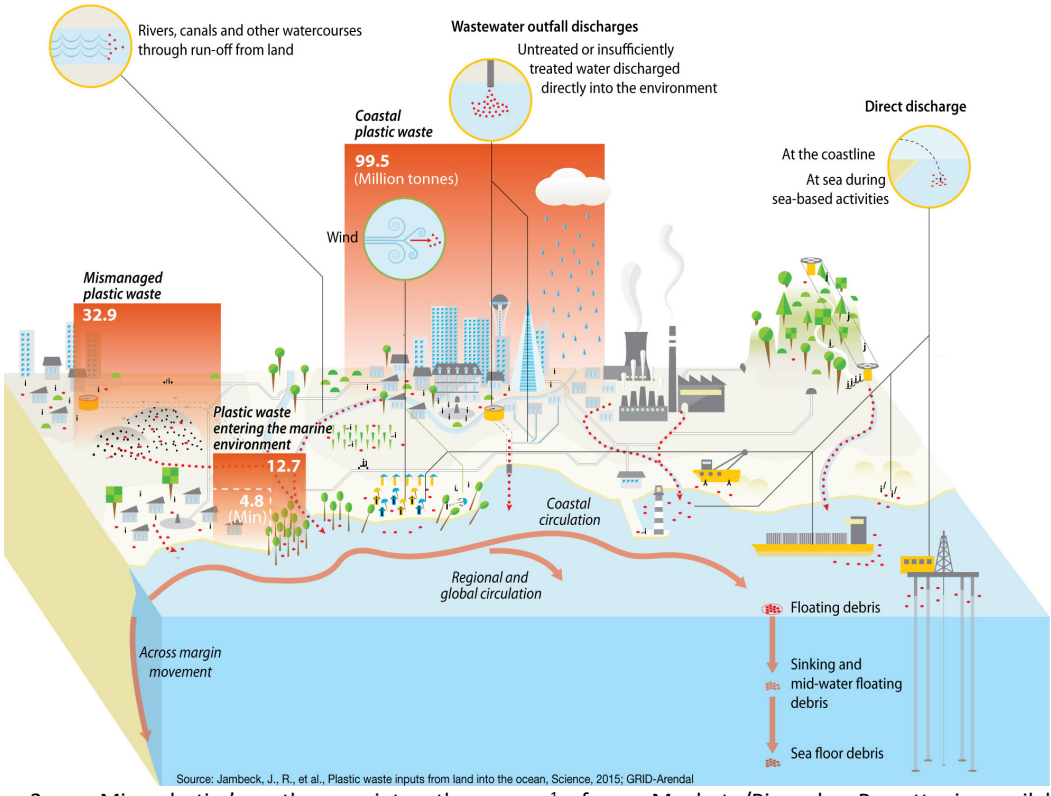


Figure 2 – Microplastics' pathways into the ocean[1] from Maphoto/Riccardo Pravettoni, available at http://www.grida.no/resources/6921.

As seen in Figure 2, plastic may enter the marine environment in multiple ways, by direct discharge in sea-based activities or at coastline, by the discharge of wastewater poorly treated or the discharged made by the industry, by rivers, canals or other sources that carry the mismanaged plastic waste. Due to their density and low weight the plastic will be carried by marine currents, as seen in Figure 3. That is why we may find plastic particles all around the globe, even in places with small population density like the Artic polar waters[13,17] or in the Antarctic waters and sediments [18–20].

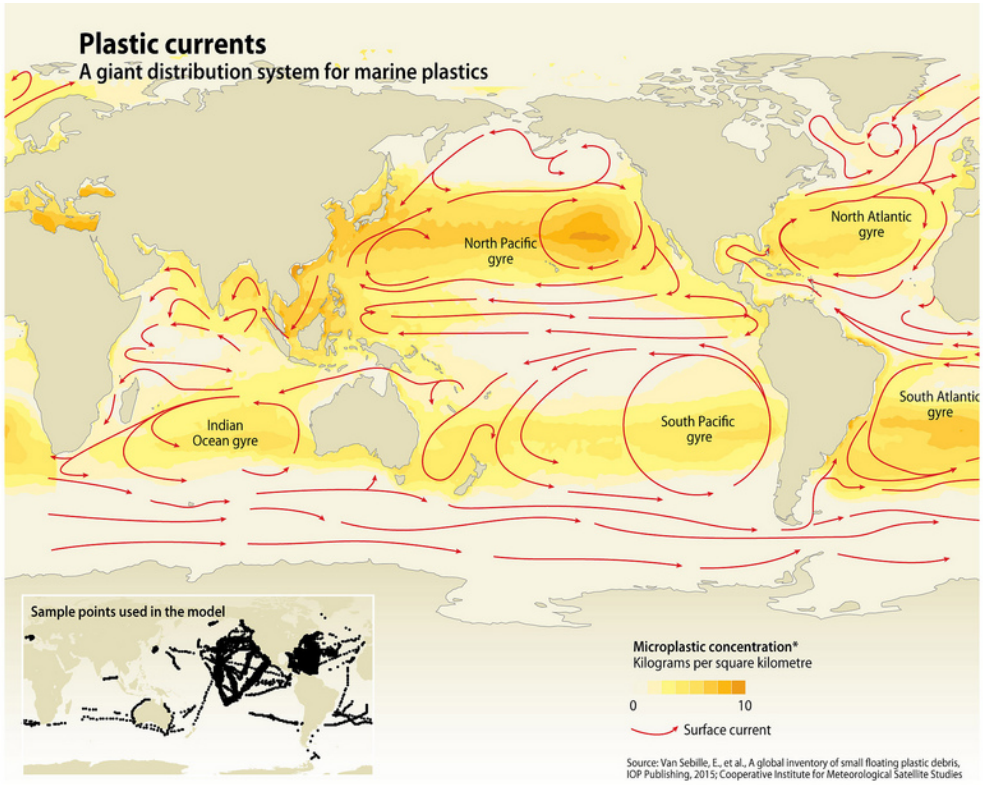


Figure 3 – Representation of the marine currents and the marine gyres[1] from Maphoto/Riccardo Pravettoni, available at http://www.grida.no/resources/6913.

Most of plastic particles are concentrated in mid-ocean gyres, as can be observed in Figure 3, and, more clearly, in Figure 4, with the help of the colours. This was described by Eriksen et al[21]. In this paper the authors, through sampling over the years and in different places, where able to estimate the number of particles and the total weigh of plastics in the ocean. They concluded that in the Northern Hemisphere the ocean regions contain 56.8% of plastic mass and 55.6% of particles, being the North Pacific the ocean with most plastic particles and contributes for 35.8% of the mass total. In the order hand, the Indian Ocean

is the region of the Southern Hemisphere with the higher number of particles and weight of plastics. These authors make this difference between mass and number, as microplastics normally contribute for the high number, but in mass terms are negligible, like evidenced by Lebreton et al[22], that find that microplastics accounted for 94% of the particles plastic founded by them, but in mass represents only 8% of the total.

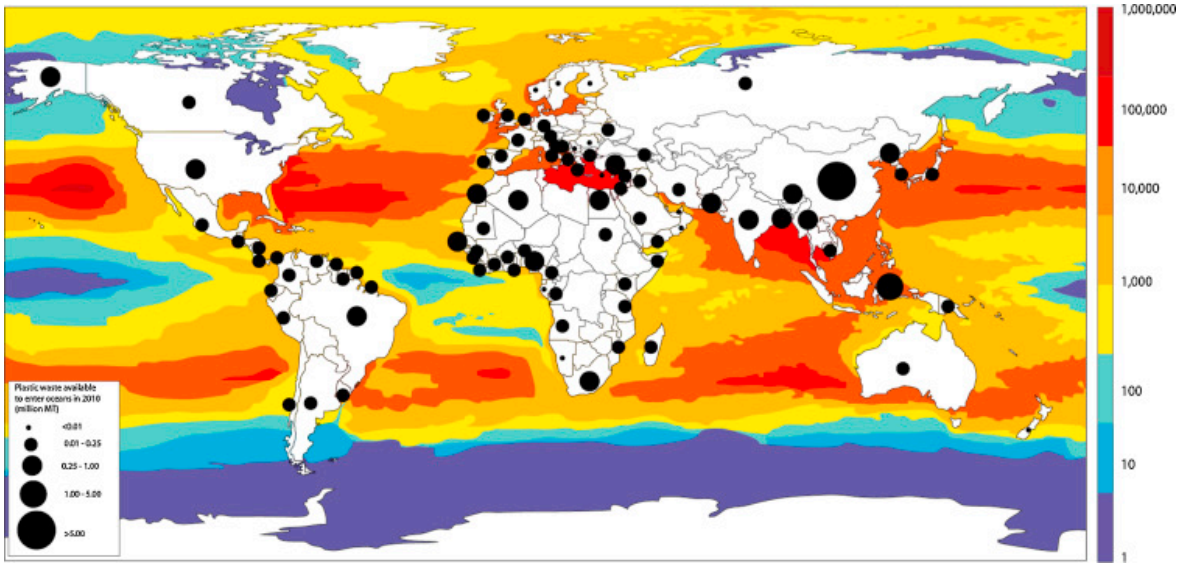


Figure 4 – Model of the amount of microplastics present in the marine environment and the mismanaged plastic particles available to enter in the oceans, retrieved from Zalasiewicz et al.[15] with the permission of Elsevier.

Normally, the most abundant type of plastic found is polyethylene (PE), as reported by different authors, such as Sadri and Thompson[23] that in a sampling on the Southwest of England found that 40% of the sampled plastics were PE , 25% were polystyrene (PS) and 19% were polypropylene. Zettler et al[24] and Rios et al[25] also found that PE and PS were the most abundant in their samples.

Since plastic particles are widely distributed in different areas of the marine environment, and have special characteristic, such as food smell[26], they are commonly mistaken by food. For consequence, as it is illustrated in Figure 5, marine animals, like fishes, turtles, seagulls[2] and others end up feeding on them.

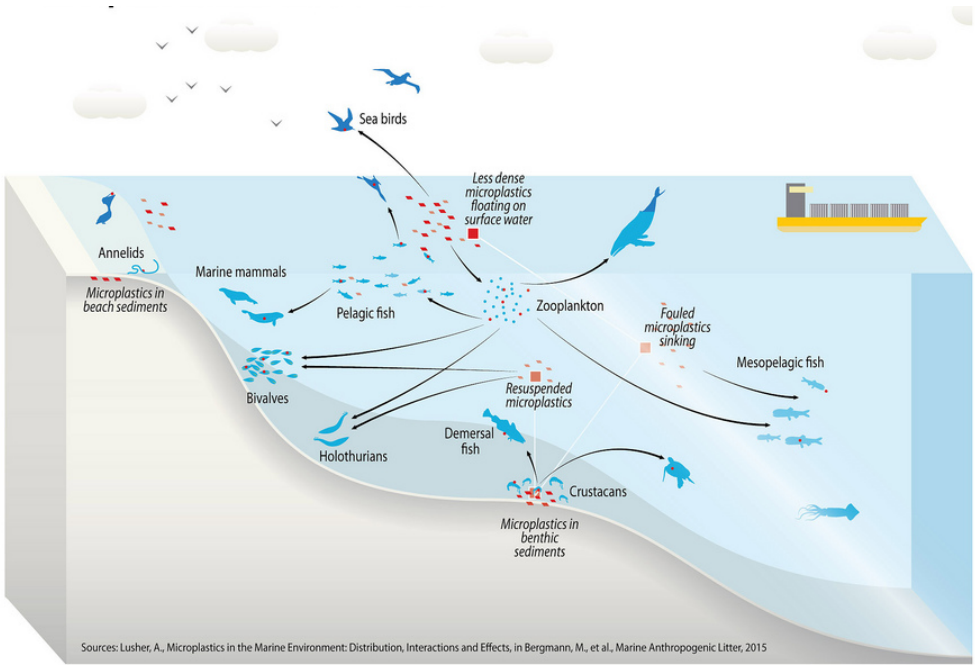


Figure 5 – Schematic representation of the relation between microplastics and the marine animals[1] from Maphoto/Riccardo Pravettoni, available at http://www.grida.no/resources/6904.

Microplastics, in specific, due to their small size, end up been ingested by all the animals in the food chain. In Figure 6 it is depicted the bioaccumulation effect, this occur because, as microplastics serve as food from zooplankton to big fishes, when a bigger animal feeds from a smaller animal with microplastics on the interior, it ends up ingesting more microplastics.

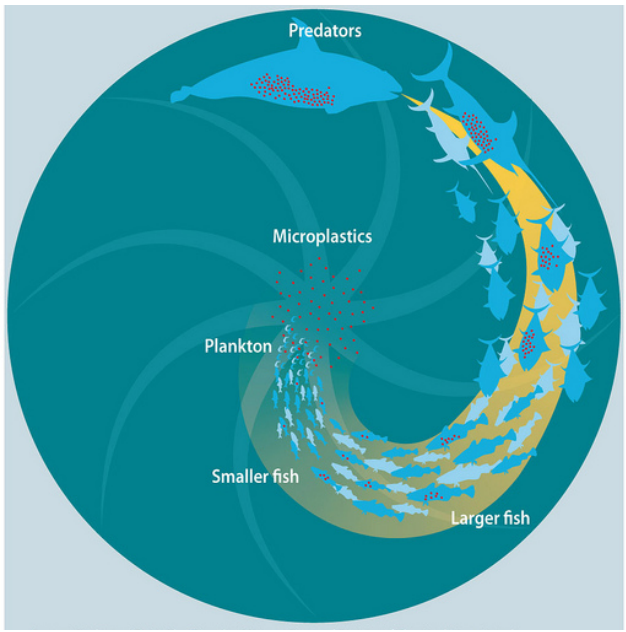


Figure 6 - Representation of the bioaccumulation effect of the microplastics[1] from Maphoto/Riccardo Pravettoni, available at http://www.grida.no/resources/6917.

Plastics, in general, when ingested by the animals, may have different physical or chemical impacts. Physical impacts are more associated with macroplastics, which can cause blockage of the digestive tract, which will lead to a reduction in food intake, starvation and loss of energy. However, microplastics can also cause blocking in the gut and changes in enzyme production, difficulty in breathing, reduce of vigour and mobility problems. Chemical impacts may be sublethal, when they alter animal behaviour, cause morphological changes and/or negative reproductive effects, or they may be lethal, when cause damage in central nervous system, cancer or death. Chemicals impacts are caused by the chemical composition of the plastics, but may also be caused by chemical contaminants adsorbed by plastics[7,27].

As microplastics are found in some species intended for human consumption[28,29], it is necessary to understand the possible impacts of their ingestion in human health. Some authors refer that, since microplastics are found in the digestive tract of the marine animals, parts not normally used in human diet, it is unlikely that their ingestion occur[4], so the concern is unnecessary.

However, recent findings indicate that microplastics can also enter in the marine organisms without ingestion, when they are taken up by the gills, or when they transfer from the gastrointestinal tract to the circulatory system[30–32]. Additionally, microplastics have been found in other natural products like honey[33] and sea salt[34], as it is extracted from polluted waters, but also in processed products like beer[35].

These evidences lead to the need for further investigation into the consequences of the consumption of microplastics on human health, but also ways of reducing microplastics in the marine environment[3,29].

1.2. Degradation of plastics – types and definition

Degradation of plastics occur naturally, due to biotic and abiotic agents, or by their combination. This process is influenced by the characteristics of the polymers and it takes a long time, some studies indicate between 20 to 450 years, but it can also take more[36].

Unfortunately, it is still unclear whether, after the process of degradation, the polymers actually disappear, became too small to be seen or became other toxic components.

1.2.1. Abiotic Degradation

1.2.1.1. Chemical

Chemical degradation occurs when the properties of the plastic polymers are altered. These alterations can be caused by the oxygen, oxidative degradation, that breaks covalent bonds and produces free radicals. Can also be caused by the water, degradation by hydrolysis, that acts in specific groups, like esters, ethers, amides, anhydrides and ester amides, breaking their covalent bonds. This kind of degradation is influenced by the polymer's structure, since an organised framework prevent diffusion of O_2 and H_2O, it occurs more easily in amorphous domains[37].

1.2.1.2. Mechanical

Mechanical degradation is caused when a pressure is applied to the polymer and leads to a break, to a damage in the polymer chains. The compression or shear forces can be caused by air or water turbulence, by snow pressure, bird damage or ageing due to load[37,38]. This type of degradation normally, can only be seen at the molecular level.

1.2.1.3. Thermal

Thermal degradation is caused by oxidative reactions when plastics are overheated, resulting in its fusion, and leads to changes in the properties of the polymers, like reduction of weight and ductility or colour changes. In this kind of degradation two different reactions

occurs, random molecular scission of the long chain backbone and scission of C-C bonds ate the chain-end[39].

This degradation hardly ever occurs in the nature, since the melting point of plastics are considerably higher than those observed in environmental conditions, but some plastics were developed with modifications in the composition, that make their melting point close to the environmental temperature[37].

1.2.1.4. Photodegradation

Photodegradation changes the physical and optical properties of plastic materials and occurs due to oxidative reactions, caused by UV radiation and visible light. It acts mostly in the ether groups of soft elements, originating ester, aldehyde, formate and propyl end-groups, and if the UV radiation has sufficient energy, C-C bond cleavage can occur[37,39].

This degradation has been described as the most efficient in the nature, but some plastics were also developed, with specific groups to improve this type of degradation[37].

1.2.2. Biodegradation

Biodegradation is defined in the ASTM standard D-5488-94d, (later replaced by the D-996-10a standard), as a "process which is capable of decomposition of materials into carbon dioxide, methane, water, inorganic compounds, or biomass in which the predominant mechanism is the enzymatic action of microorganisms, that can be measured by standard tests, in a specified period of time, reflecting available disposal conditions"[40]. This type of degradation will cause different changes on the plastic polymer, depending on the polymer and its previous biotic degradation, on the microorganism and on the environmental conditions[37,38,41].

The biodegradation process, in biologic terms is yet to be fully understand, is still unclear all the enzymes involved, and the biological process involved when a microorganism uses a plastic polymer as subtract to grow. For now, some authors divide the process into three

different phases, first biodeterioration, followed by biofragmentation and finally assimilation. A schematic representation of biodegradation is pictured in Figure 7.

- Biodeterioration

This phase can be mechanical/physical, chemical or enzymatic, and it is characterized by the initial breakdown of the polymers in monomers. It occurs due to growth of microorganisms on the surface and/or inside the plastic material. Biodeterioration causes macroscopic alterations in the polymer, so is possible to estimate by appearance of holes and cracks and changes in colour[37].

The physical way is based on the ability of microorganisms to secrete a complex matrix of polymers, which seep into the pores of the material and alter its moisture, heat transfer rates, pore size and distribution, which cause cracking, weakening the material. This matrix will also favour the penetration and development if the microorganisms, acting similarly to a surfactant, facilitating the exchanges between the hydrophobic and the hydrophilic phases[37,38].

The biochemical biodeterioration is caused by the increase of microorganisms, which leads to an increase in the chemicals produced by their metabolism. Some of the acids released can react with the polymers' components and increase erosion or can remove cations of the material, through oxidation reactions[37].

The enzymatic process depends on the capacity of the microorganisms to produce enzymes, such as lipases, ureases or proteases. These enzymes bind to some type of polymers and catalyse the hydrolysis of specific bonds[37,41].

- Biofragmentation

This phase is characterized by the cleavage and fragmentation of the monomers obtain previously, reducing their size. It is caused by enzymes, hydrolases (enzymatic hydrolysis) and oxidoreductases (enzymatic oxidation) or by radicular oxidation[37,38].

Biofragmentation can be estimate by studying the presence of low molecular weight molecules, or by separating the oligomers obtained and analyse them[37].

- Assimilation

This is the final phase, after the fragmentation the polymer´s fragments are small enough to be assimilated by the microorganisms, to pass through the membrane. They use the fragments as source of energy and elements, it works specially as their carbon source, to grow and reproduce. Some fragments are easily transported through the membrane, thanks to specific membrane carriers, but others need to undergo biotransformation into products that can directly assimilated[37,38].

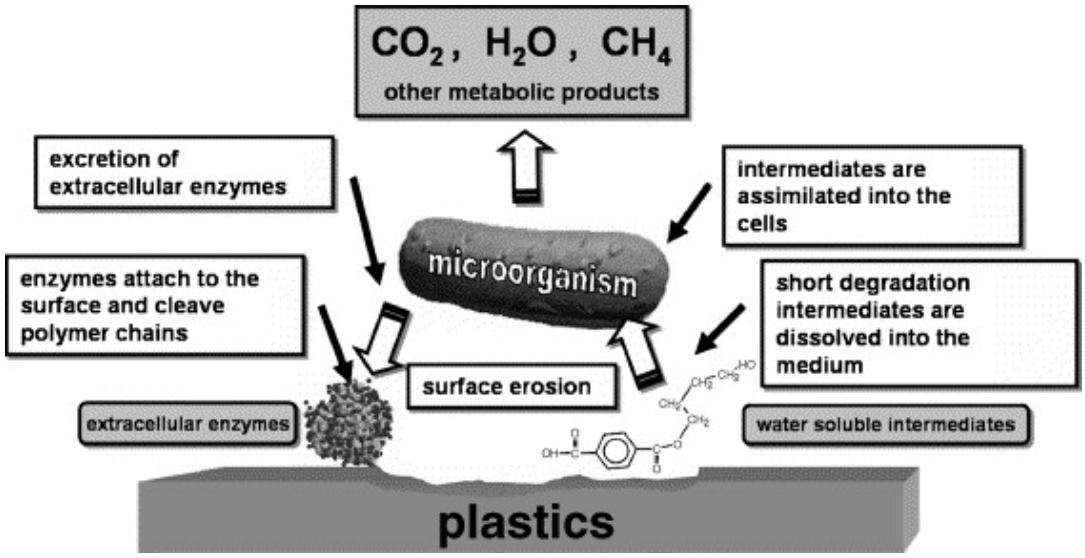


Figure 7 - Schematic presentation of the biodegradation's mechanisms, retrieved from Mueller et al.[42] with the permission of Elsevier

1.2.2.1. Microorganisms involved in biodegradation

Different authors have already studied biodegradation, and demonstrated that various microorganisms, fungi and bacteria, have the capacity to degrade different types of plastics. Some of the microorganisms that were studied were found in environments with high proportion of plastic. In Table 1, some microorganisms already identified for the predisposition to biodegrade PE are listed. Their biodegradation capacity was, for some authors, studied in virgin plastic particles, i.e., plastics, that had not been subject to any environmental exposure, others in plastics that suffered a previous abiotic degradation, like photodegradation.

Table 1 - Known microorganisms with the capacity of biodegrade polyethylene.

	MICROORGANISM	TYPE OF POLYETHYLENE	REFERENCE
BACTERIA	*Bacillus sp.*	PE	43,44
	Bacillus pumilus	PE	45,46
	Bacillus pumilus	LDPE	47
	Bacillus mycoides	PE	46
	Bacillus amyloliquefaciens	PE	46,48
	Bacillus subitilis	LDPE	47,49
	Bacillus halodenitrificans	LDPE	45
	Bacillus circulans	LDPE	50
	Bacillus brevies	LDPE	50
	Bacillus sphericus	LDPE	50
	Staphylococcus xylosus	PE	46
	Staphylococcus epidermis	LDPE	51
	Rhodococcus rhodochrous	PE	52
	Rhodococcus ruber	PE	53
	Pseudomonas fluorescens	PE	46
	Pseudomonas aeruginosa	LDPE	54
	Bacillus cereus	PE	45,46
	Paenibacillus macerans	PE	46
	Micrococcus lylae	PE	46
	Nocardia asteroides	PE	52
	Enterobacter asburiae	PE	43,44
	Burkholderia seminalis	LDPE	54
	Stenotrophomonas pavanii	LDPE	54
	Brevibaccillus borstelensis	LDPE	55
	Lysinibacillus xylanilyticus	LDPE	56
	Kocuria palustris	LDPE	47
	Arthobacter paraffineus	LDPE	57
FUNGI	*Aspergillus flavus*	LDPE	58
	Aspergillus flavus	HDPE	59
	Aspergillus awamori	PE	46
	Aspergillus versicolor	PE	60
	Aspergillus niger	LDPE	56,61
	Aspergillus tubingensis	HDPE	59
	Cunning-hamella sp.	PE	46
	Mucor sp.	PE	46
	Penicillum sp.	PE	46
	Penicillium simplicissimum	PE	62
	Penicillium pinophilum	LDPE	61,63
	Gliocladium viride	PE	46
	Mortierella subtlissima	PE	46
	Cladosporium cladosporoides	PE	52
	Zalerion maritimum	PE	64
	Acremonium kiliense	PE	60
	Verticillium lecanii	PE	60
	Gliocladium virens	LDPE	61
	Phanerochaete chrysosporium	LDPE	61,65
	Mucor circinilloides	LDPE	58

Considering the wide presence of microplastics and their impacts, and the biodegradation capacity of this microorganisms, this study aims to:

- Optimize a culture medium in order to maximize the response in terms of removal of microplastics (Chapter 2)
- Enhance the volume of experience in order to ascertain if under non-sterilized conditions and environmental exposure the biodegradation of microplastics still occur (Chapter 3)
- Assess the capacity of fungi (*Zalerion maritimum* and *Nia vibrissa*) to biodegrade a bioplastic (Chapter 4)

2. Optimization of the experimental conditions for the biodegradation of polyethylene microplastics by marine fungi

2.1. Introduction

In a previous work, it has been demonstrated that *Zalerion maritimum* was able to biodegrade PE microplastics[64], which shows that this can be a solution to the problem of (micro)plastics in the environment. In this work, it was also shown that biodegradation is influenced by the biomass grow and therefore by the culture medium conditions. Thus, the need of optimization of the experimental conditions was evident after an initial experiment (see Appendix A) where *Z. maritimum* was unable to grow and to biodegrade PE microplastics in a culture medium with a reduced nutrient content. Our biodegradation experiment is based on the idea that *Z. maritimum* can use microplastics as carbon source, being necessary to supplement, in the culture medium, the rest of the nutrients required for the growth of the fungus. Therefore, this study aims at establishing the optimum experimental conditions for the biodegradation of PE microplastics by *Z. maritimum* using statistical design of experiments.

The medium optimization involves five steps, (1) statistic design of the experiments; (2) experimental procedure; (3) analyses of the results with a statistical software; (4) perform a regression to estimate the coefficients of a mathematical model, where all variables and correlation between them are considered, Equation (1) - Correlation between response (Y) and independent factors, where β_0 represent the intercept and β represent the coefficient values; (5) with the help of the mathematical model determine the optimal values[66,67].

$$Y = \beta_0 + \sum_{i=1}^{K} \beta_i x_i + \sum_{i=1}^{K} \beta_{ii} x_{ii}^2 + \sum_{i=1}^{K} \sum_{\neq j=1}^{K} \beta_{ij} x_i \, x_j + \varepsilon \qquad \text{Eq (1)}$$

Two different experimental design were used, Uniform Design and Central Composite design, in order to compare the results and understand which one would be better, would give a more appropriate model. The three medium components, glucose, malt extract and

peptone were considered as independent factors or variables and the degradation percentage of PE microplastics was considered as response.

2.1.1. Uniform Design (UD)

UD is an experimental design developed by Fang and Wang in 1994[68], based on a number theory, where the design points scatter uniformly on the experimental domain. This experimental design was chosen, since within a small number of experiences is possible to obtain a great amount of information, as well as explore the relationships between the factors and the response[68]. UD also performs correctly even when the regression model is unknown[68]. The number of experiments is influenced by the number of factors and levels for each factor and is given by tables developed with the theory.

This experimental design has been successfully used in different fields since the 1980s. In the microbiology, authors as Xu et al[69], Chen et al[70], Li et al[71] and Mu et al[72], have already used UD as experimental design, to optimize a culture medium, for growth, for degradation or production of a compounds.

2.1.2. Central Composite Design (CCD)

CCD, also called Box-Wilson Design, was introduced by G.E.P. Box and K.B. Wilson in 1951[73]. This experimental design has been widely used in different areas for the optimization of experimental conditions, and in the case of microbiology, its use had already been reported by Sadhukhan et al.[74], Adinarayana et al.[75], Ooijkaas et al.[76] and Ibrahim et al.[77], for the optimization of conditions for the production or degradation of a specific compounds. For been widely used it was chosen as comparison term.

The number of experiments is given by the Equation 2, where 2^n represents the number of two-level factorial or fractional factorial design points, all the possible combinations pf +1 and -1 levels of factor; and $2n$ represents the number of axial points (star points), points with a fixed distance (α) from the center; and n is be the number of center points, and represents the replicate terms, which provide an estimation of the experimental error.

$$N = 2^n + 2n + n \qquad \text{Eq (2)}$$

2.1.3. *Zalerion maritimum*

Zalerion maritimum (or *Zalerion maritima*), the studied fungi, is a marine fungus belonging to the Ascomycota phylum[78,79], defined in 1963 by Anastasiou [80], but first described in 1944, as *Helicoma maritimum* by Linder [81]. This change was due to the fact that Anastasiou noticed that the species *Helicoma salinum*, also described by Linder in 1944, and the species *Zalerion nepura, Zalerion eistla, Zalerion xylestrix, Zalerion raptor*, described by Moore and Meyers in 1962, belonged to the same species being strains of *Zalerion maritimum* [80].

This fungus is a member of the subphyla Pezizomycotina[78,79], that includes all filamentous fungi with a fruiting body, visible to the naked eye, it belongs to the Sordariomycetes[78,79] class characterized by their perithecial ascomata and inoperculate unitunicate asci, where the ascospores are contained[82–84]. It also belongs to the Lulworthiales order and to the Lulworthiaceae family[78,79] being a marine fungus that grows on submerge wood which can be characterized by its brown to black ostiolate ascomata, hyaline and filamentous ascospores and thin-walled asci[85,86].

Zalerion maritimum is one of the only reported anamorphs, name of the species at the asexual form in the Lulworthiales order being a hyphomycete and present a coiled conidia, his teleomorph is *Lulwoana uniseptata* (or *Lulworthia uniseptata*), the name of the species in the sexual reproductive stage[87–89].

In various studies from the 1970s and from the 1980s, this fungus showed ability to degrade different components. Henningsson and later Sutherland and Crawford, demonstrated that *Z. maritimum* is able to degrade lignin, to CO_2 and water-soluble products, in both hardwood and softwood lignocelluloses[90].

Sguros and Quevedo, studied the predisposition of this fungus to use Aldrin and Dieldrin as subtract, concluding that *Z. maritimum* can degraded both compounds under marine conditions and used it to grow[91]. Aldrin and Dieldrin are two pesticides highly used between the 1950s and 1990s, developed as an alternative to DDT (Dichlorodiphenyltrichloroethane), they were highly resistant in the environment and

despite not been hydrophobic and don't dissolve easily in water, they were the pesticides most detected in rivers and dieldrin, for instance was presented in watersheds. As any chemical pollutant, they have negative consequences in the organisms, been biomagnified along the food chain and they were linked to health problems in humans[92,93].

Jones and Le Campion-Alsumard, found polyurethane coatings submerged in the sea were colonized with four different fungi, *Z. maritimum* was one of them[94]. This article from 1968, already show the predisposition of this fungus to biodegrade plastics, as *Z. maritimum* was able to form a biofilm on this plastic polymer. Some studies support this, as showed that polyester based on polyurethane were vulnerable to fungus attack.

Other important function of *Z. maritimum* was discovered in 1973, by Catalfomo et al, they reported *Z. maritimum* as one of the marine fungi able to produce Choline sulfate[95].

Molina and Hughes[96] showed that *Z. maritimum* is able to live in a wide range of temperatures, from 5ºC to 40ºC, and in different salinity conditions, from 0 to 99.9‰. According to their study the fungus can live in conditions of extremes temperatures, if the salinity is higher. Previous studies corroborate this idea, and also the optimal temperature found buy the authors, between 20ºC and 25ºC[96,97]. Other important thing, described by these authors, it is that the fungus is able to adapt to the different conditions, but with repeated transfers in a same medium type a selection of physiological races can occur[96,97].

The medium pH is optimum at 7.5, but the fungus can show growth in a great range of pH, according to Churchland and McClaren's[98] findings, the fungus' mycelium changes color depending on the pH of the medium. In their experiments, the mycelium stayed beige in low-pH medium and became black in all other solutions[96–98], this also happens depending on the nutrients source.

Due to its great adaptability to the medium is possible to encounter this species in different locations. Studies report their present along all European coast[99,100], in the Canadian[98] coast and Malaysian coast[101]. Their anamorph has been reported in the Japan coast[88].

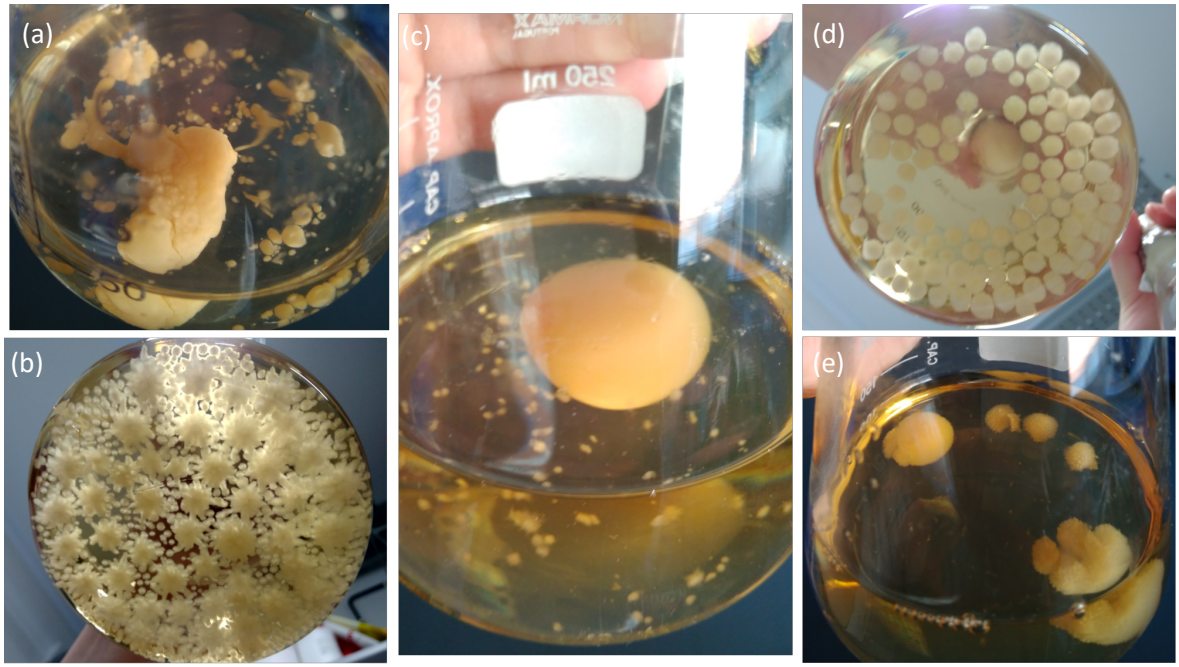


Figure 8 – Photos from the different forms that *Zalerion maritimum* presented in our laboratory. In (a) and (e) *Z. maritimum* presents an irregular form; figure (b) presents a form that resembles a star, due to sporulation; (c) and (d) present the fungus in its globular form.

2.1.4. Polyethylene (PE)

As the most widely produced polymer in the world, PE, seems to be also the most found polymer worldwide in the marine environments.

This polymer was accidentally discovered in 1933 by Eric Fawcett and Reginal Gibson as they tried to condense at high pressure and temperature, ethylene with benzaldehyde. In this "accident" they obtained a residue that was PE, but later failed to repeat the experiment successfully, so it was not until 1935 that chemist Michael Perrin was able to obtain large amounts of PE, using ethylene with traces of oxygen[102–104].

In 1939, the commercial production of high-pressure polyethylene, now known as low-density polyethylene, began and was widely used during the World War II. In the following years, different advances in production were made, in the manufacturing or in base products, until we reached the parameters used today[102–104]. It has become the most globally produced and widely used synthetic polymer, since as a thermoplastic, it can be melted and shaped into primary form and later reshaped into various forms and devices,

and also because it has excellent chemical resistant and it is the cheapest plastic polymer to manufacture[5,105].

This plastic have a linear formula ($H(CH_2CH_2)nH$), Figure 9, and a melt index of 1.0g/10 min (190°C/2.16kg). It is obtained by the polymerization of Ethylene (CH2=CH2), a simplest olefin, through the action of initiators and catalysts. The conditions for polymerization, can vary and will influence the composition, structure and properties of the polymer, existing a wide range of PE available in the industry[102–104].

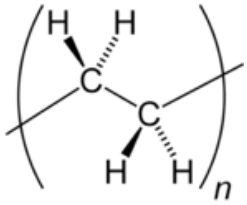
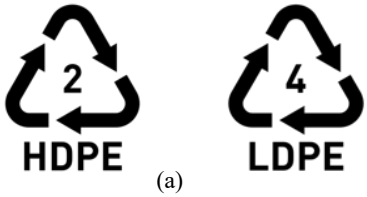


Figure 9 – Molecular representation of PE monomers.

This leads to the need for a grading system that differentiates the different types of PE. Based on the crystallinity, and consequently in the different densities, Society of the Plastic Industry (SPI) identified three main categories to define this plastic: Low density 0.910-0.925 g/cm3; Medium density 0.926-0.940 g/cm3; High density 0.941-0.965 g/cm3. The American Society for Testing and Materials (ASTM), has also defined types of PE, but more stringently, with five categories: High density polyethylene (HDPE) >0.941 g/cm3; Linear medium density polyethylene (LMDPE) 0.926-0.940 g/cm3; Medium density polyethylene (MDPE) 0.926-0.940 g/cm3; Linear low-density polyethylene (LLDPE) 0.919-0.925 g/cm3; Low density polyethylene (LDPE) 0.910-0.925 g/cm3. Some manufactures have their own classifications and nomenclatures, since classifications can't only be based on their density, is necessary classifications based on molecular weight or comonomer employed[102,105].



(a) (b)

Figure 10 – ASTM identification code for (a) High density polyethylene and for (b) Low density polyethylene.

2.2. Materials and Methods

2.2.1. Microplastics

Polyethylene pellets were approximately 2-4mm in size and exhibited spheroid morphology. The pellets were acquired from Sigma-Aldrich (USA) and mechanically cut to obtain microplastics with a size range 1000 µm < MP < 250 µm, defined with the help of sieves. Microplastics were characterized both by Optical, Figure 11(a), and Scanning electron microscopy, Figure 11(b) e (c), and also by Fourier transform infra-red spectroscopy, Figure 12. PE have characteristic peaks, as seen in Figure 12, between 2980-2800 cm^{-1}, 1500-1400 cm^{-1} and 750-650 cm^{-1}, caused by CH_2 asymmetric and symmetric stretching, bending and rocking deformations[106].

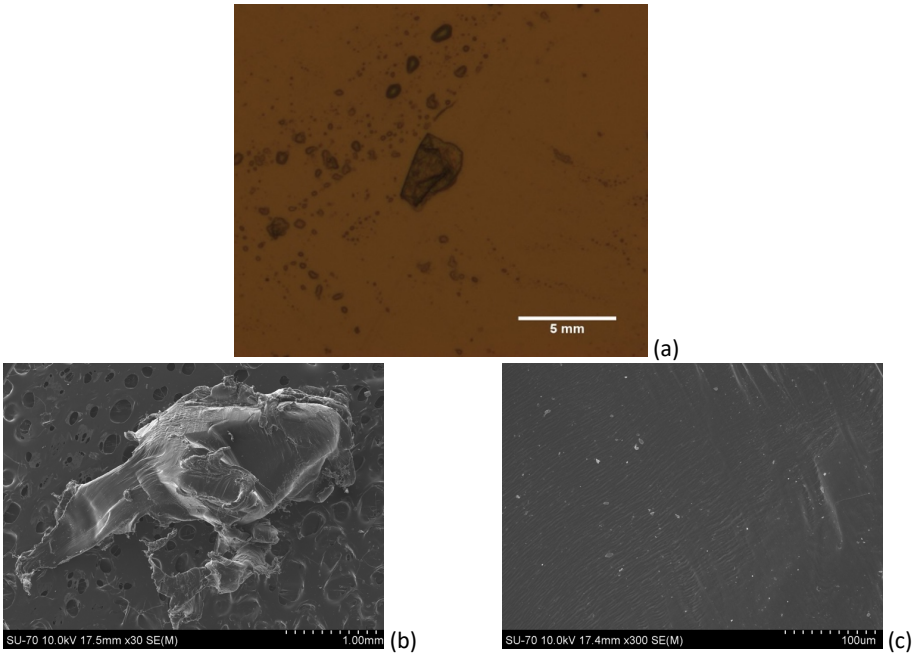


Figure 11 - Optical (a) and electron microscopy (b) and (c) images of the microplastic and its surface

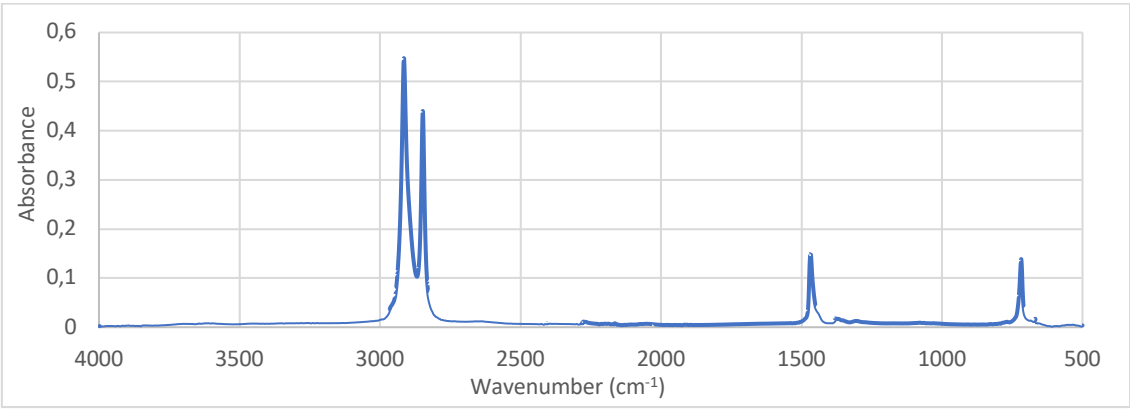


Figure 12 – FTIR spectra characteristic for a PE microplastic.

2.2.2. Microorganism

Zalerion maritimum (ATTC 34329, American type culture collection), was maintained in culture in our laboratory, in recommended conditions for growth[107], 2/3 weeks in a culture medium with the composition of 35 g/L of salt, 20 g/L of glucose, 20 g/L of malt extract and 1 g/L of peptone and temperatures around 20^{o}C.

2.2.3. Design of experiments and Statistical analysis

MINITAB software was used to obtain the tables for the CCD experiments, and to analyse the data generated by that experiments, through linear regression and maximization analysis.

SPSS software was used to do statistical analyses, like the study of statistically significance of each variable, on the data from both experimental designs.

A second-order polynomial regression equation was also obtained through SPSS software analysis for UD experimental data. The optimal conditions were obtained by solving the equation the help of WolframAlph website.

2.2.3.1. Uniform Design

For this experimental design were defined four levels for each factor, and twelve runs, obtaining a $U_{12}(4^3)$ matrix, as seen in Table 2.

Table 2 – Uniform Design $U_{12}(4^3)$ matrix.

RUN ORDER	X 1	X 2	X 3
1	3	2	2
2	3	4	4
3	4	4	2
4	4	1	4
5	2	2	3
6	1	3	4
7	2	1	1
8	4	2	1
9	1	1	3
10	3	3	3
11	2	3	2
12	1	4	1

2.2.3.2. Central Composite Design

In this experimental design, five levels for each factor were considered: -α, -1, 0, +1, +α, the design generated, can be seen in Table 3.

Table 3 – Matrix for a central composite design for three variables and a α=1.63.

STDORDER	RUNORDER	PTTYPE	BLOCKS	X 1	X 2	X 3
15	1	0	1	0	0	0
6	2	1	1	1	-1	1
18	3	0	1	0	0	0
1	4	1	1	-1	-1	-1
4	5	1	1	1	1	-1
3	6	1	1	-1	1	-1
20	7	0	1	0	0	0
11	8	-1	1	0	-1.63	0
14	9	-1	1	0	0	1.63
17	10	0	1	0	0	0
2	11	1	1	1	-1	-1
5	12	1	1	-1	-1	1
12	13	-1	1	0	1.63	0
16	14	0	1	0	0	0
9	15	-1	1	-1.63	0	0
19	16	0	1	0	0	0
13	17	-1	1	0	0	-1.63
7	18	1	1	-1	1	1
8	19	1	1	1	1	1
10	20	-1	1	1.63	0	0

2.2.4. Culture medium

The culture medium was composed by 35 g/L of salt[108], this was maintained fixed as it is necessary to keep the necessary salinity for the fungi, and simulate the salt water.

It was also composed by the factors to be optimized, glucose[109], malt extract[110] and peptone[111], and their concentration varied accordingly to the experimental designs characterized previously, as seen in Table 4 and Table 5.

Table 4 - Culture medium composition for the Uniform Design.

RUN ORDER	GLUCOSE (G/L)	MALT EXTRACT (G/L)	PEPTONE (G/L)
1	10	2	0.1
2	10	20	1
3	20	20	0.1
4	20	0	1
5	2	2	0.5
6	0	10	1
7	2	0	0
8	20	2	0
9	0	0	0.5
10	10	10	0.5
11	2	10	0.1
12	0	20	0

Table 5 - Culture medium for Central Composite Design.

RUN ORDER	GLUCOSE (G/L)	MALT EXTRACT (G/L)	PEPTONE (G/L)
1	12.5	12.5	0.7
2	20	5	1
3	12.5	12.5	0.7
4	5	5	0.4
5	20	20	0.4
6	5	20	0.4
7	12.5	12.5	0.7
8	12.5	0	0.7
9	12.5	12.5	1.2
10	12.5	12.5	0.7
11	20	5	0.4
12	5	5	1
13	12.5	25.1	0.7
14	12.5	12.5	0.7
15	0	12.5	0.7
16	12.5	12.5	0.7
17	12.5	12.5	0.2
18	5	20	1
19	20	20	1
20	25.1	12.5	0.7

2.2.5. Experimental conditions

The first experiment, based on Uniform Design, was performed using twelve batch reactors (50ml Erlenmeyer flask) with 30 mL of culture medium and approximately 0.010 g of microplastics. All batch reactors were autoclaved and later inoculated with approximately 0.30 g of fungus mycelium.

A second experiment, based on Central Composite Design, was performed using twenty batch reactors (this time 100mL Erlenmeyer flask) with 60 mL of culture medium and 0.020g of microplastics. The batch reactors were autoclaved and then inoculated with 0.60g of fungus mycelium.

A third experiment was realized, where thirty-two batch reactors (also 100 mL Erlenmeyer flask) were utilized, twelve of then had their culture medium based on Uniform Design and twenty of them had their culture medium based on Central Composite Design, so the experiments could be performed at the same time, and easier compared. Each batch reactor had 50 mL of a specified medium and 0.015g of microplastics, and was autoclaved and afterwards inoculated with 0.50g of fungus mycelium.

After inoculation, in all three experiments, the batch reactors were maintained for 30 days in a shaker, at room temperature and with stirring at 120 rpm. At the end the fungus and the microplastics were separated from the medium by filtration. The fungus biomass was retrieved and posteriorly frozen and lyophilized. The microplastics were kept for weighing and further analysis. The lyophilized biomass was also examined for the presence of microplastics, which may have not been completely degraded.

Figure 13 – Image obtained from the twelve batch reactors utilized in the first Experiment.

2.3. Results and discussion

Table 6 and Table 7 present the percentages of microplastics removed obtained in the experiments based on Uniform design and Central composite design, respectively. In Table 6, some data are missing, because due to contaminations it was not possible to recover that data and therefore were disregarded. Table 7 present all values, but some Erlenmeyer also had contaminations that may have slightly altered the fungus behavior, which may explain the variation between the value obtained in the first experiment and those from the second experiment, for example, in the case of sample 4 or sample 12.

According to both tables, in each experiment, the percentages of microplastics removed varied, approximately, from 0% to 94%. This wide variation proves the importance of a medium optimization to achieve a higher degradation.

In both cases, it was possible to observe that the results are not the same in the first and second experiments. This variation can be explained by different situations beside contaminations, like the fact that the fungi may had behave differently, as they are biological replicas and also by the fact that the experiments did not occur at the same time.

Table 6 - Bioegradation results from UD.

UNIFORM DESIGN	**% MICROPLASTICS REMOVED**	
RUN ORDER	First Exp.	Second Exp.
1	7.20	27.27
2	72.73	75.32
3	86.67	94.81
4	37.30	74.51
5	40.78	35.48
6	73.83	31.61
7	-	28.00
8	0.00	34.21
9	36.67	14.10
10	87.50	57.05
11	71.65	92.26
12	72.36	-

Table 7 - Bioegradation results with CCD.

CENTRAL COMPOSITE DESIGN	% MICROPLASTICS REMOVED	
RUN ORDER	First Exp.	Second Exp.
1	88.00	69.62
2	70.48	69.48
3	86.54	78.98
4	6.67	44.97
5	72.73	73.68
6	76.00	91.77
7	85.24	78.00
8	34.55	19.33
9	85.83	60.53
10	72.11	50.00
11	94.07	76.10
12	40.00	83.55
13	93.16	74.50
14	81.85	66.88
15	52.27	37.82
16	88.57	78.06
17	87.78	71.14
18	73.33	87.10
19	67.00	36.67
20	79.47	78.95

2.3.1. Effect of different medium compounds on polyethylene's biodegradation

2.3.1.1. Uniform Design

In Table 8 is presented the results from the analysis of covariance, if a linear model is considered, based on that table is possible to conclude that "malt extract" is the most significant factor, the compost that more influence the percentages in the biodegradation of PE microplastics. However, based on this table, is also possible to understand that a linear model is not the best suitable, since adjusted R^2 is just 0.577.

Table 9 present the test between subjects when all interactions between the factors are considered. In this case, the adjusted R^2 is 0.961 which indicates a high significance of the model, but none of the factor seen to be important, since all "sig" values are higher than 0.05. This means that a regression analysis is necessary in order to find a model with a

better fit. Despite that, “malt extract” stays the most important factor, as its “sig” value is the smaller and closer to 0.05.

Table 8 - Tests of between subjects’ effects, when a linear model is considered for the first experiment with UD, where “df" stands for degree of liberty, “F” stands for F-value and “sig” for P-value.

DEPENDENT VARIABLE: DEGRADATION			**A R SQUARED = 0.704 (ADJUSTED R SQUARED = 0.577)**		
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	6644.981a	3	2214.994	5.552	0.029
INTERCEPT	1355.062	1	1355.062	3.397	0.108
GLUCOSE	342.358	1	342.358	0.858	0.385
MALTEXTRACT	5693.872	1	5693.872	14.272	0.007
PEPTONE	658.008	1	658.008	1.649	0.240
ERROR	2792.621	7	398.946	-	-
TOTAL	40728.98	11	-	-	-
CORRECTED TOTAL	9437.602	10	-	-	-

Table 9 - Tests of between subjects’ effects for the first UD experiment, when a quadratic model is considered, where “df" stands for degree of liberty, “F” stands for F-value and “sig” for P-value.

DEPENDENT VARIABLE: DEGRADATION		**A R SQUARED = 0.996 (ADJUSTED R SQUARED = 0.961)**			
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	9400.633a	9	1044.515	28.253	0.145
INTERCEPT	0.134	1	0.134	0.004	0.962
GLUCOSE * GLUCOSE	99.209	1	99.209	2.684	0.349
PEPTONE * PEPTONE	243.335	1	243.335	6.582	0.237
MALTEXTRACT * MALTEXTRACT	1020.319	1	1020.319	27.599	0.120
GLUCOSE * MALTEXTRACT	45.414	1	45.414	1.228	0.467
GLUCOSE * PEPTONE	130.221	1	130.221	3.522	0.312
MALTEXTRACT * PEPTONE	27.459	1	27.459	0.743	0.547
GLUCOSE	131.89	1	131.890	3.568	0.310
MALTEXTRACT	2782.518	1	2782.518	75.265	0.073
PEPTONE	289.803	1	289.803	7.839	0.218
ERROR	36.969	1	36.969	-	-
TOTAL	40728.98	11	-	-	-
CORRECTED TOTAL	9437.602	10	-	-	-

The regression analysis was done by successively removing the factors with the high “sig” value. For example, based on Table 9, the “malt extract*peptone” interaction and “glucose*malt extract” interaction, can be removed obtaining the Equation (3). As it can be

removed, it implies that these two interactions do not influence, as much as the others, the degradation. According to the coefficients used in the equation, the concentrations of peptone and malt extract influence positively the degradation, which means that with a higher concentration of this compound, high percentages of degradation of PE microplastics occur. On the other hand, the glucose concentration negatively affects the degradation, so a lower concentration of glucose is necessary to obtain higher degradation percentages.

According to the characterization, presented in Table 10, of Eq. (3), it is a model with good fit to the experimental data and a high significance, as its "sig" value is 0.007, and R adjusted is 0.960, meaning that 96% of the data is explained by this model.

$$\%degradation = \\ = -5.179 - 5.029 * glucose + 149.773 * peptone + 10.236 \\ * malt\ extract + 0.0229 * glucose^2 - 143.752 * peptone^2 \\ - 0.314 * malt\ extract^2 + 2.224 * glucose * peptone \quad \text{Eq. (3)}$$

Table 10 – Characterization for the model presented in Equation 3, where "df" stands for degree of liberty, "F" stands for F-value, "sig" for P-value, and "R" for correlation coefficient.

MODEL		SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.	R	R^2	ADJUSTED R
1	Regression	9325.138	7	1332.163	35.536	0.007	0.994	0.988	0.960
	Residual	112.464	3	37.488	-	-	-	-	-
	Total	9437.602	10	-	-	-	-	-	-

Table 11 present the results from an analysis of covariance to the results from the second experiment with UD, if a linear model is considered. Based on that table is possible to conclude that the "malt extract" is the most significant factor, "sig"<0.05, which is in agreement with the findings in the previous experiment. Also, as in the previous experiment, the linear model is not suitable, since adjusted R^2 is only 0.382.

A test between subjects considering all interactions between the factors is presented in Table 12. In this case, the adjusted R^2 is 0.865 which indicates a significance of the model, but similar to the other case, none of the factor seen to be important, since all "sig" values are higher than 0.05 and so a regression analysis is necessary. The "malt extract" factor also, remains as the most important, as its "sig" value is the smaller and closer to 0.05.

Table 11 - Tests of between subjects' effects, when a linear model is considered for the second experiment with UD, where "df" stands for degree of liberty, "F" stands for F-value and "sig" for P-value.

DEPENDENT VARIABLE: DEGRADATION			**A R SQUARED = 0.567 (ADJUSTED R SQUARED = 0.382)**		
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	4851.360a	3	1617.120	3.060	0.101
INTERCEPT	1626.773	1	1626.773	3.078	0.123
GLUCOSE	801.26	1	801.260	1.516	0.258
MALTEXT	3081.478	1	3081.478	5.831	0.046
PEPTONA	0.404	1	0.404	0.001	0.979
ERROR	3699.088	7	528.441	-	-
TOTAL	36790.427	11	-	-	-
CORRECTED TOTAL	8550.448	10	-	-	-

Table 12 - Tests of between subjects' effects for the second UD experiment, when a quadratic model is considered, where "df" stands for degree of liberty, "F" stands for F-value and "sig" for P-value.

DEPENDENT VARIABLE: DEGRADATION	**A R SQUARED = 0.987 (ADJUSTED R SQUARED = 0.865)**				
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	8435.367	9	937.263	8.144	0.266
INTERCEPT	608.944	1	608.944	5.291	0.261
GLUCOSE * GLUCOSE	202.109	1	202.109	1.756	0.412
MALTEXTRACT * MALTEXTRACT	2.403	1	2.403	0.021	0.909
PEPTONE * PEPTONE	107.053	1	107.053	0.930	0.512
GLUCOSE	163.214	1	163.214	1.418	0.445
MALTEXTRACT	1120.758	1	1120.758	9.739	0.197
PEPTONE	1.687	1	1.687	0.015	0.923
GLUCOSE * MALTEXTRACT	123.129	1	123.129	1.070	0.489
GLUCOSE * PEPTONE	688.249	1	688.249	5.981	0.247
MALTEXTRACT * PEPTONE	24.161	1	24.161	0.210	0.726
ERROR	115.081	1	115.081	-	-
TOTAL	36790.427	11	-	-	-
CORRECTED TOTAL	8550.448	10	-	-	-

The regression analysis was done by removing the factors with the highest "sig" value, for example, based on table 12, it was removed the "malt extract^2", "peptone" and "peptone*malt extract" interaction, obtaining the Equation (4), characterized in table 13.

In agreement with the first experiment based on UD, the interaction "peptone*malt extract" does not seem to be relevant, the concentration of glucose has a negative effect

on the degradation percentage and the concentration of malt extract has a positive effect. This means that, although the equation is different, the conclusions about the principal factors affecting the percentages of degradation are similar.

Table 13 shows that Eq. (4) represents a model with a high significance and good fit to the experimental data, since it has a "sig" value of 0.002 and a R adjusted of 0.955, which indicate that 95.5% of the data is described by the model.

$$\%degradation = \\ = 41.194 - 7.083 * glucose + 6.728 * malt\ extract + 0.330 \\ * glucose^2 - 78.440 * peptone^2 - 0.211 * glucose \\ * maltextract + 6.017 * glucose * peptone \quad \text{Eq. (4)}$$

Table 13 – Characterization for the model presented in Equation 4, where "df" stands for degree of liberty, "F" stands for F-value, "sig" for P-value, and "R" for correlation coefficient.

MODEL		SUM OF SQUARES	DF	MEAN SQUARE	F	SIG.	R	R^2	ADJUSTED R
1	Regression	8397.627	6	1399.604	36.634	0.002	0.991	0.982	0.955
	Residual	152.821	4	38.205	-	-	-	-	-
	Total	8550.448	10	-	-	-	-	-	-

2.3.1.2. Central composite design

The results from a covariance analysis, if a linear model is considered, in the results from the first experiment with CCD, can be observed in Table 14. According to Table 14, "malt extract" is the most significant factor, which is in agreement with the findings of the experiments with UD, but it seems that "glucose" can also be significant, as its "sig" value is also <0.05. The linear model is not suitable, so is necessary a test where all interactions are considered, as seen in Table 15.

Table 15 indicates that, according to these data, the concentration of peptone does not influence the degradation of the PE microplastics, as its "sig" value isolated or in interactions are higher than 0.05 and they are the highest "sig" values on the table. This conclusion differs from those based on the analysis of UD experiments, where peptone appears to positively influence in both of them, but it is in agreement with the disregard of the "malt extract*peptone" interaction.

Table 14 - Tests of between subjects' effects, when a linear model is considered for the first experiment with CCD, where "df" stands for degree of liberty, "F" stands for F-value and "sig" for P-value.

DEPENDENT VARIABLE: DEGRADATION			R SQUARED = 0.419 (ADJUSTED R SQUARED = 0.310)		
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	4013.789a	3	1337.930	3.85	0.030
INTERCEPT	1162.332	1	1162.332	3.345	0.086
GLUCOSE	1740.766	1	1740.766	5.010	0.040
MALTEXTRACT	2272.754	1	2272.754	6.540	0.021
PEPTONE	0.269	1	0.269	0.001	0.978
ERROR	5559.878	16	347.492	-	-
TOTAL	112628.214	20	-	-	-
CORRECTED TOTAL	9573.668	19	-	-	-

Table 15 - Tests of between subjects' effects for the first CCD experiment, when a quadratic model is considered, where "df" stands for degree of liberty, "F" stands for F-value and "sig" for P-value.

DEPENDENT VARIABLE: DEGRADATION			R SQUARED = .886 (ADJUSTED R SQUARED = .784)		
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	8484.704a	9	942.745	8.657	0.001
INTERCEPT	1008.589	1	1008.589	9.262	0.012
GLUCOSE * GLUCOSE	970.816	1	970.816	8.915	0.014
MALTEXTRACT* MALTEXTRACT	1147.507	1	1147.507	10.538	0.009
PEPETONE * PEPTONE	8.585	1	8.585	0.079	0.785
GLUCOSE	3648.077	1	3648.077	33.500	0.000
MALTEXTRACT	2966.203	1	2966.203	27.239	0.000
PEPTONE	168.677	1	168.677	1.549	0.242
GLUCOSE * MALTEXTRACT	2031.394	1	2031.394	18.654	0.002
GLUCOSE * PEPTONE	449.7	1	449.700	4.130	0.070
MALTEXTRACT * PEPETONE	41.132	1	41.132	0.378	0.553
ERROR	1088.964	10	108.896	-	-
TOTAL	112628.214	20	-	-	-
CORRECTED TOTAL	9573.668	19	-	-	-

The equation presented, Equation (5), according to the software MINITAB explain the data obtained in this experiment with CCD. Based on that equation, “glucose”, with this data, appears to have a positive effect like “mal extract” and “peptone”, and all the interactions and quadratic effect of the factors seem to have a negative effect.

$$\begin{aligned} \%degradation = \\ &= -90.50 + 11.07 * glucose + 9.97 * malt\ extract + 65.50 \\ &* peptone - 0.147 * glucose^2 - 0.160 * malt\ extract^2 \\ &- 8.30 * peptone^2 - 0.283 * glucose * maltextract - 3.33 \\ &* glucose * peptone - 1.01 * maltextract * peptone \end{aligned} \quad \text{Eq. (5)}$$

In Table 16 the results from covariance analysis in the case of a linear model was considered, for the results from the second experiment with CCD, are presented, and Table 17 presents the values when all interactions are considered. Although in Table 16, the “sig” value from “malt extract” is higher than 0.05, it is the lowest “sig” value on the table, so it is still possible to conclude that it is a significant factor.

Table 17 corroborate that “malt extract” is the most significant factor as it is the factor with “sig” value lower than 0.05. The quadratic effect of “glucose” (“glucose^2”) and “malt extract” (“malt extract^2”) appears to be insignificant in this statistical analysis, as their “sig” values are the highest and bigger than their “F” value.

Table 16 - Tests of between subjects’ effects, when a linear model is considered for the second experiment with CCD. where “df" stands for degree of liberty, “F” stands for F-value and “sig” for P-value.

DEPENDENT VARIABLE: DEGRADATION			**R SQUARED = 0.134 (ADJUSTED R SQUARED = -0.028)**		
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	922.520a	3	307.507	0.825	0.499
INTERCEPT	3615.887	1	3615.887	9.700	0.007
GLUCOSE	21.543	1	21.543	0.058	0.813
MALTEXTRACT	845.521	1	845.521	2.268	0.152
PEPTONE	55.456	1	55.456	0.149	0.705
ERROR	5964.368	16	372.773	-	-
TOTAL	94951.11	20	-	-	-
CORRECTED TOTAL	6886.888	19	-	-	-

Table 17 - Tests of between subjects' effects for the second CCD experiment, when a quadratic model is considered, where "df" stands for degree of liberty, "F" stands for F-value and "sig" for P-value.

DEPENDENT VARIABLE: DEG2			**R SQUARED = 0.541 (ADJUSTED R SQUARED = 0.128)**		
SOURCE	Type III Sum of Squares	df	Mean Square	F	Sig.
CORRECTED MODEL	3725.095a	9	413.899	1.309	0.339
INTERCEPT	350.596	1	350.596	1.109	0.317
GLUCOSE * GLUCOSE	18.27	1	18.270	0.058	0.815
MALTEXTRACT * MALTEXTRACT	389.613	1	389.613	1.232	0.293
PEPTONE * PEPTONE	33.299	1	33.299	0.105	0.752
GLUCOSE	1093.876	1	1093.876	3.460	0.093
MALTEXTRACT	2215.964	1	2215.964	7.009	0.024
PEPTONE	214.081	1	214.081	0.677	0.430
GLUCOSE * MALTEXTRACT	915.495	1	915.495	2.895	0.120
GLUCOSE* PEPTONE	751.721	1	751.721	2.378	0.154
MALTEXTRACT* PEPTONE	678.206	1	678.206	2.145	0.174
ERROR	3161.793	10	316.179	-	-
TOTAL	94951.11	20	-	-	-
CORRECTED TOTAL	6886.888	19	-	-	-

Equation (6) was given by MINITAB software, as representative for the data from the second experiment with CCD. This equation appears to be in agreement with the Eq. (5), as "glucose", "malt extract" and "peptone" have a positive effect on degradation, and the interactions and quadratic effect have a negative effect.

$$\begin{aligned} \%degradation = \\ &= -53.80 + 6.05 * glucose + 8.62 * malt\ extract + 75.60 \\ &* peptone - 0.0196 * glucose^2 - 0.093 * malt\ extract^2 \\ &+ 16.2 * peptone^2 - 0.190 * glucosemaltextract - 4.31 \\ &* glucosepeptone - 4.09 * maltextractpeptone \end{aligned} \qquad \text{Eq. (6)}$$

2.3.2. Optimization of the culture medium composition

Based on Eq. (3) and Eq. (4), obtained with the experiments of Uniform design, is possible to encounter the optimal concentrations for each component in order to maximize the degradation of PE microplastics. The solution find in both equations was, approximately, 4.6 g/L of glucose, 16.3 g/L of malt extract and 0.56 g/L of peptone.

In the case of the experiments of Central Composite Design, the values were not obtained by solving the equations, but through a maximization program using the experimental data. Some values obtained, can be seen in Table 18. The variations between the first and the second experiment can be explained by the small variations in the degradation values. With the help of this table, the values chosen to be utilized were 11g/L of glucose, 20 g/L of malt extract and 0.20 g/L of peptone.

Table 18 - Medium composition and %degradation obtained through maximization for the CCD data.

EXPERIMENT	GLUCOSE (G/L)	MALT EXTRACT (G/L)	PEPTONE (G/L)	%DEGRADATION
1º CCD	0	25	1.20	95
1º CCD	12.5	20	0.20	90
2º CCD	11	25	0.20	102
2º CCD	12.5	23	0.20	100
2º CCD	0	20	1.20	97

2.5. Conclusion

Malt extract proved to be the most significant factor for the degradation of PE microplastics. In all the experiments, with UD and CCD, this was the factor with lower "sig" value, and always had a positive influence, the higher the concentration of malt extract the higher the degradation. This was expected, as this medium compound is the source of amino acids, peptides, proteins, nutrients, minerals, vitamins and carbohydrates, being therefore a source of nitrogen and carbon[112]. This component is described as essential for the growth and metabolism of fungi[113], and when they run out of this key source of nutrients they cannot survive, and for consequence cannot biodegrade the PE microplastics.

Peptone, on the other hand, is also an important source for nitrogen[112], so in most of the experiments had, as malt extract, a positive effect, the higher the concentration of peptone, the higher the degradation percentages. However, it was not found to have a high significance, which may have happened as the concentrations tested for this component were considerably lower than the concentrations tested for malt extract concentration.

Glucose, in most of the experiments, did not have significance in the degradation percentages, but always had a negative effect. This prove the assumption that microplastics work as substitute for the carbon source[41], since a lower concentration of glucose, a more accessible carbon source, is required to achieve high percentages of degradation of PE microplastics. According to the results it is always necessary to supplement the medium with glucose, *Z. maritimum* it is not able to survive with only PE as carbon source. Other works support this idea, as some microorganisms can grow only with plastics as carbon source, but others need something more, beside the plastics[114].

The optimum concentrations found with the two different experimental designs are slightly different, but, based on the analytical analysis, the results of UD appear to be more accurate than those obtained with the CCD, as their R^2 and F value, show greater adequacy to predict the experimental data.

3. Biodegradation of polyethylene microplastics by marine fungi: Scale up

3.1. Introduction

Beside the optimization of the medium, it is also necessary to understand if the fungus is still capable of biodegrading the microplastics on a larger scale and in a less controlled environment, in order to start developing a treatment plant and help with the plastic environmental issues. Therefore, this study aims to validate the medium optimization, described in Chapter 2, and to understand how some factors could influence this bioremediation process.

3.2. Materials and methods

3.2.1. Microorganism and microplastics

The microorganism used was the same from the previous chapter, the microplastics were also the same, only varied in size, in the second experiment, where some were mechanically cut to be in the size range of 2000 μm < MP < 1000 μm.

3.2.2. Culture medium

The culture medium composition in the first experiment, was 35 g/L of salt, 2 g/L of glucose, 2 g/L of malt extract and 0.1 g/L of peptone.

In others experiments the culture medium compositions were based the on preliminary analysis of the optimization experiment with Uniform Design, where the optimized concentrations obtained where, 35 g/L of salt, 1 g/L of glucose, 17 g/L of malt extract and 0.22g/L of peptone. These concentrations, as seen in the previous chapter suffered some changes, due to modifications in the statistical analysis.

In the final experiment the culture medium composition was the one obtained with Central Composite Design, 35 g/L of salt, 11g/L of glucose, 20g/L of malt extract and 0.20 g/L f peptone.

3.2.3. Experimental conditions

In order to work in a larger scale, fish tanks with a capacity of 2L were used as batch reactors, and they were maintained in a water bath, to keep a constant temperature of 20ºC. The culture medium was autoclaved with the microplastics, and then poured to the batch reactors and inoculated with the fungus' mycelium. To improve agitation and contact of the fungus with the microplastics, aeration was used.

In the first experiment, "Impact of the microplastics on the growth rate", six batch reactors, with 1.5 L of medium, 15 g of fungus' biomass and 1.80g microplastics (tanks A_1, A_2, B_1 and B_2) or 3.60g of microplastics (tanks C and D) were used. Tanks A_1, A_2, B_1 and B_2 were maintained only for 15 days, tanks C and D were maintained throughout all the experiment, 30 days. Tanks A_2 and B_2 were inoculated with the biomass recovered from the tanks A_1 and B1, respectively. This experiment is depicted in Figure 14.

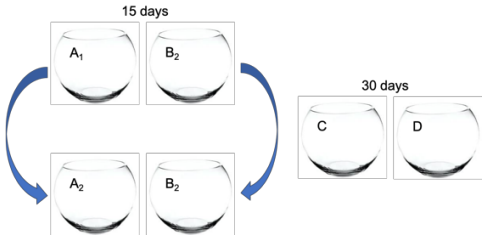


Figure 14 – Schematic representation of the experiment – Impact of the microplastics on the growth rate.

In the other experiment, "Changes in biodegradation with microplastics size", four batch reactors, with 1.3L of medium, 0.40g of microplastics and 13g of fungus' biomass were used. Two batches were maintained for 31 days and had microplastics in the range of 1000 µm < MP < 250 µm. The other two batches were maintained for 30 days and the microplastics were in the range of 2000 µm < MP < 1000 µm. Due to evaporation, was necessary to add, every other day, autoclaved deionized water or an autoclaved solution of salt water with 35g/L. This experiment is depicted in Figure 15.

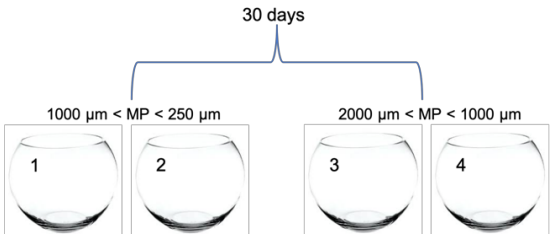


Figure 15 - Schematic representation of the experiment – Changes in biodegradation with microplastics size.

A final experiment, “Biodegradation in an optimized medium”, was performed using three batch reactors, with 1.3L of medium, 0.40g of microplastics and 13g of fungus’ biomass, were maintained for 30 days and every Friday and every Tuesday, throughout the experiment, 500mL of autoclaved deionized water was added to each batch.

At the end of each experiment the medium, microplastics and biomass were separated by filtration. The recovered biomass was frozen and later lyophilized and the microplastics were keep for further analysis.

3.2.4. Microplastics treatment

Some of the recovered microplastics had organic material attached and therefore a cleaning treatment was necessary. The microplastics were maintained, for 1 hour, in a 100mL beaker with approximately 20mL of 65% HNO_3. After the treatment the microplastics were removed, left to dry in an oven, for two hours, and weighted. The choice of treatment was based on a trial experiment developed in our laboratory seen in Appendix B.

3.2.5. Techniques used to analyze the microplastics

Fourier transform infra-red spectroscopy (FTIR-ATR) was used to evaluate the degradation occurred in the microplastics. FTIR-ATR analyses were carried out using a Perkin Elmer (USA) Spectrum BX FTIR instrument. The microplastics were analysed at $4cm^{-1}$ resolution within the 4000-550nm range.

Scanning electron microscopy (SEM) was used to characterize the microplastics and to verify the presence of spores of *Z. maritimum* in their surface. The analyses in SEM were performed using a Field Emission Gun (FEG) - SEM Hitachi S4100 microscope (Japan), operated at 15 kV. The samples were prepared by direct deposition onto the carbon tape and then coated by carbon evaporation.

3.3. Results and discussion

3.3.1. Impact of the microplastics on the growth rate of *Zalerion maritimum*

This experiment was intended to understand how the growth varies after the exposure to microplastics in scale up conditions, but as this was one of the firsts experiments some problems were also detected.

Table 19 presents the fungus' biomass in the beginning and at the end in each tank, it can be observed an increase in biomass along the time, but the growth rate varies.

According to Table 19 the lower growth rate was registered in tanks A_2 and B_2, which leads to the possible conclusion that after an initial exposure to microplastics the fungus grows less when exposed again, but this can also be explained by a different growth phase of this microorganism[115]. Based on the data, it is also possible to observe that the growth rate after the initial 15 days is similar to the ones register to 30 days, this may be explained by crystallization problems. Over the experiment, evaporation was noticed, this led to the loss of most of the medium, to the formation of salt crystals and as a consequence, an increase in salinity, interfering with the growth of the microorganisms. These problems do not alter the previous conclusion, since in tanks A_2 and B_2, started in a fresh medium, in the same conditions in which tanks A_1 and B_1 started.

Table 19 - Biomass and respective growth rate of Zalerion maritimum.

TANK (START DATE)	INOCULATED BIOMASS (WET) (G)	FINAL BIOMASS (WET) (G)	GROWTH RATE
A_1 (21/11/2017)	15.56	23.86	0.5334
A_2 (05/12/2017)	15.59	17.14	0.0994
B_1 (21/11/2017)	15.48	35.62	1.301
B_2 (05/12/2017)	15.66	19.98	0.2758
C (21/11/2017)	15.09	30.18	1.000
D (21/11/2017)	15.32	26.03	0.699

As higher concentrations of microplastics were used in this essay, the percentages of biodegradation were insignificant, but as seen in Figure 16 the fungi grew on the microplastics surface.

These microplastics were analyzed by FTIR-ATR spectroscopy, in order to assess the chemical changes caused by the biodegradation by *Z. maritimum*.

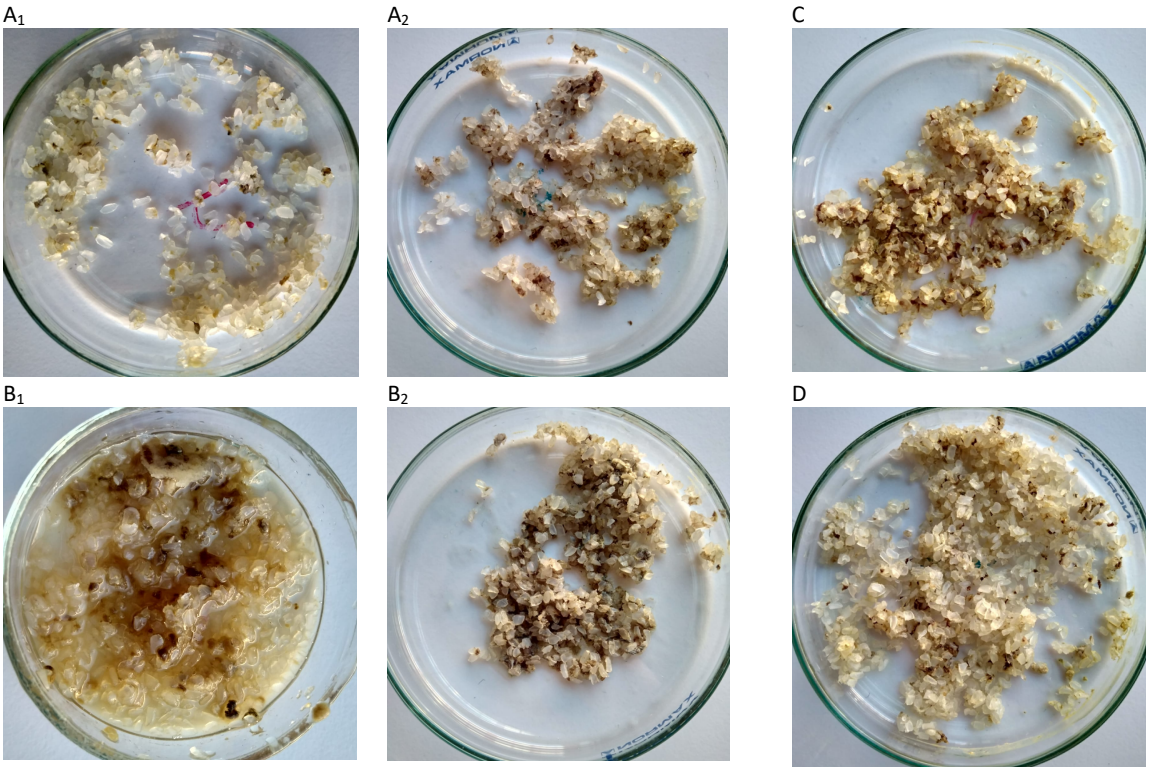


Figure 16 - Photos from microplastics covered with fungus biomass from the different tanks. The letters above each photo, identify from which tank the microplastics where recovered.

In Figure 17, SEM images of the PE microplastics when exposed to *Z. maritimum* in similar conditions to the ones of tank C and tank D, are shown. These highlight the growth of *Z. maritimum* on the surface of the microplastic particles. In Figure 17 (a) it is presented the microplastic, and it is possible to recognize some change on its surface. Figure 17 (b), shows that the changes are actually fungal spores growing on the surface. Figure 17 (c) and (d), a larger approximation to the microplastics surface, presents in greater detail the presence of the fungal spores, and allows the identification of the formation of a biofilm.

These pictures help to prove that at microscopic level it is even clearer that the fungus is able to grow on this polymer surface and use it as a subtract. Previous authors have also reported these findings, like Tribedi and Sill[116] and Mahalakshmi and Siddiq[117].

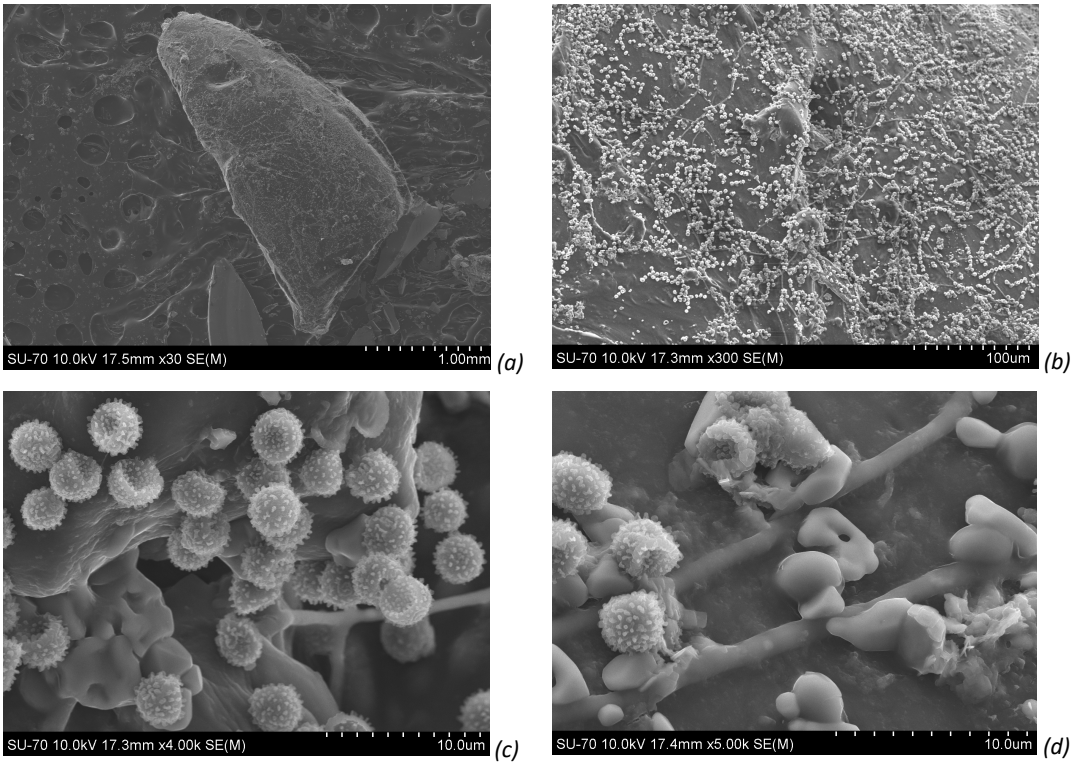


Figure 17 – Images obtained by SEM from a microplastic covered with fungus.

The FTIR spectra obtained for the samples recovered from each tank, and a FTIR spectra from a virgin PE microplastic, are presented in Figure 18.

It is possible to observe that degradation occurred since, like is evidenced in Table 20 with the relative areas, there was a decrease in the characteristic peaks from PE microplastics, and the appearance and increase in peaks in the regions 3700-3000 cm^{-1}, caused by the hydroperoxide and hydroxyl groups, 1700-1500 cm^{-1} due to carbonyl groups, and 1200-950 cm^{-1} caused by double bonds, these new groups result from reactions involved in the oxidation of the polymer. FTIR analyzes has been widely used to prove degradation[118–120], as it shows chemical alterations, and biodegradation as well[121–124].

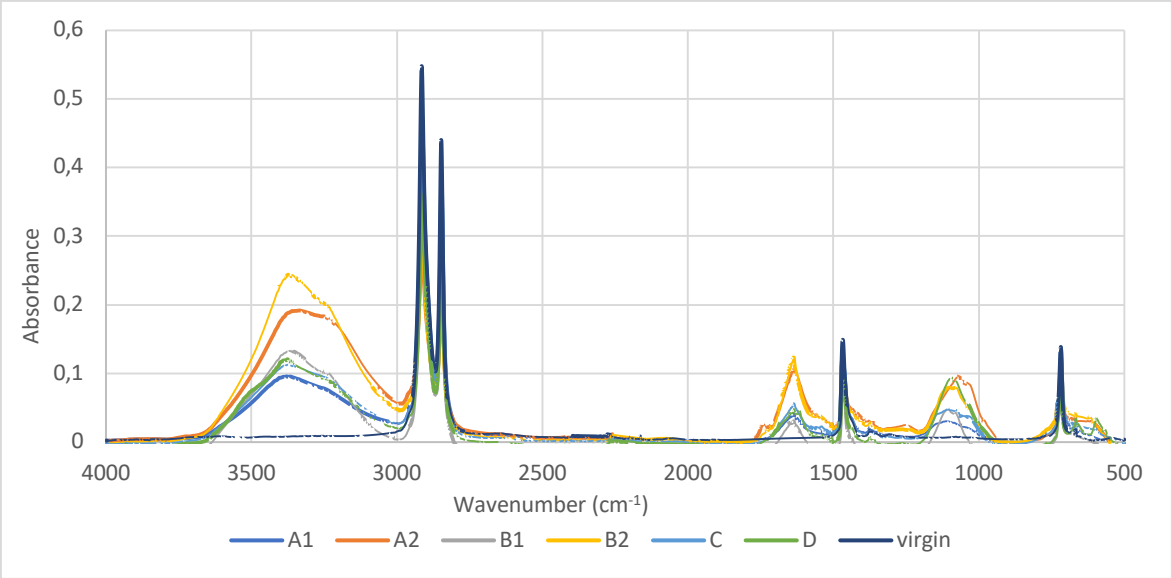


Figure 18 - FTIR-ATR spectra in the region 500 – 4000 cm^{-1} from samples of PE microplastics recovered in each tank, compared with a virgin PE microplastic.

Table 20 – Relative areas from the identified regions from the microplastics spectra.

REGIONS / SPECTRA	3700-3000 CM^{-1}	2980-2800 CM^{-1}	1700-1500 CM^{-1}	1500-1400 CM^{-1}	1200-950 CM^{-1}	750-650 CM^{-1}
A1	37.14	27.48	4.078	3.889	4.559	3.336
A2	78.97	20.95	11.28	4.525	13.82	3.747
B1	58.81	25.72	5.016	3.361	7.673	4.244
B2	91.03	21.54	11.8	4.527	10.83	4.889
C	46.75	25.6	6.081	5.075	7.726	4.275
D	48.61	22.67	5.811	3.194	13.21	3.487
VIRGIN	5.500	29.26	0.990	3.656	1.194	2.869

Besides the evaluation of the peaks present in the spectra, it is possible to evaluate the degradation degree of the microplastics through the calculation of the Carbonyl Index. As mentioned before, the carbonyl group is not present in the PE virgin, appearing after oxidation reactions, proving the occurrence of degradation[123,125].

Carbonyl index is calculated with the help of Equation (7), where Abs(1) is the absorbance at 1638 cm^{-1} and Abs(2) is the absorbance at 1468 cm^{-1}, a reference peak for PE.

$$CI = \frac{Abs(1)}{Abs(2)} \qquad \text{Eq. (7)}$$

Table 21 shows, as expected, that the microplastics exposed to the fungi present a higher Carbonyl Index, when compared to the virgin PE. This reinforces the idea that degradation occurred, contributes to prove that biological action occurred in the microplastics and for consequence the potential of *Z. maritimum* to degrade PE microplastics.

When the carbonyl index from microplastics recovered from A_1 and B_1 are compared, respectively, with the carbonyl index from the microplastics from A_2 and B_2, is possible to observe that the carbonyl index appears to be higher in the tanks A_2 and B_2, leading to a possible conclusion, that despite the growth rate decreases in the second contact with the microplastics, the degradation increases.

As expected, the samples recovered after 1 month of exposure, tanks C and D, have a higher carbonyl index, are more degraded, than the samples from the tanks exposed only for 15 days.

Table 21 - Carbonyl index for the analyzed samples.

SPECTRA	A1	A2	B1	B2	C	D	PE
ABS(1)	0.0153	0.1028	0.0173	0.0964	0.0419	0.0541	0.0051
	0.0238	0.0336	0.0506	0.0945	0.0574	0.0244	
	0.0380	0.0486	0.0215	0.1236	-	-	
ABS(2)	0.1351	0.0820	0.1318	0.1051	0.1166	0.1057	0.1489
	0.1343	0.1288	0.1173	0.1162	0.1222	0.1525	
	0.1176	0.0974	0.1303	0.0978	-	-	
CI	0.2048 ± 0.0787	0.2953 ±0.1546	0.2427 ±0.1343	1.0811 ±0.1421	0.4144 ±0.0549	0.3358 ±0.1760	0.0343

3.3.2. Changes in biodegradation with microplastics size

The biomass changes between the beginning and the end of the experiment can be seen in Table 22. Although increase in the biomass can be observed in Table 22 for the four tanks, this growth is not similar, even between the tanks with the same conditions.

This variation could potentially be explained by contaminations, which altered the growth environment, since in some tanks a pink bacterial contamination was observed.

Table 22 - Biomass and respective variation of *Zalerion maritimum*.

TANK	INOCULATED (WET) (G)	INOCULATED (DRY) (G)	FINAL (DRY) (G)	VARIATION	GROWTH
1 (04/04/2018)	13.174	1.172	4.182	3.010	2.567
2 (04/04/2018)	13.080	1.164	2.054	0.890	0.7640
3 (10/05/2018)	13.155	1.171	3.494	2.323	1.984
4 (10/05/2018)	13.382	1.191	6.796	5.605	4.706

Table 23 shows the variation between the microplastics in the beginning and at the end of the experiment, and for consequence the percentage of removed microplastics.

According to Table 23, a clear reduction of microplastics occurred in all four tanks, but the highest percentage of removed microplastics were observed in the tanks with smaller microplastics. This can be explained by the higher surface-to-volume ratio that allows a better contact between the fungi and the microplastics, but also by the fact that is more difficulty to separate the microplastics at the end of the experiment. Some papers have also reported this preference of microorganisms for plastic particles with small sizes, like Kawai et al[126].

Table 23 - Microplastics in the beginning and at the end in each tank.

TANK	MICROPLASTICS BEGNING (G)	MICROPLASTCICS RECOVERED (G)	MICROPLASTICS REMOVED	%MICROPLASTICS REMOVED
1 (1000 MM < MP < 250 MM)	0.4020	0.0365	0.3655	90.92
2 (1000 MM < MP < 250 MM)	0.4010	0.0850	0.3160	78.80
3 (2000 MM < MP < 1000 MM)	0.4060	0.1419	0.2641	65.05
4 (2000 MM < MP < 1000 MM)	0.4070	0.2271	0.1799	44.20

According to Table 22 and Table 23, growth and reduction does not seem to be related, since when comparing tanks 1 and 2, the one with highest growth was also the one with highest percentage of microplastics removed, but when comparing tanks 3 and 4, this relation is not true.

As seen in the previously described experiment, in the Figure 16 and Figure 17, the mycelium grows in the surface of the microplastics, but the microplastics can also be aggregate by the fungi mycelium, when already have a smaller size.

For this reason, the lyophilized fungus was analyzed for the presence of microplastics. The recovered microplastics were analyzed by FTIR, and the spectra are presented in Figure 19, a spectrum from PE microplastic not exposed to fungus is also present, to help with the comparison.

As seen in Figure 19 and as it is confirmed in Table 24, by the relative areas, some peaks, the characteristic from PE decrease and the regions utilized to show degradation, as previously explained, like 3700-3000 cm^{-1}, 1700-1500 cm^{-1} and 1200-950 cm^{-1}, increase.

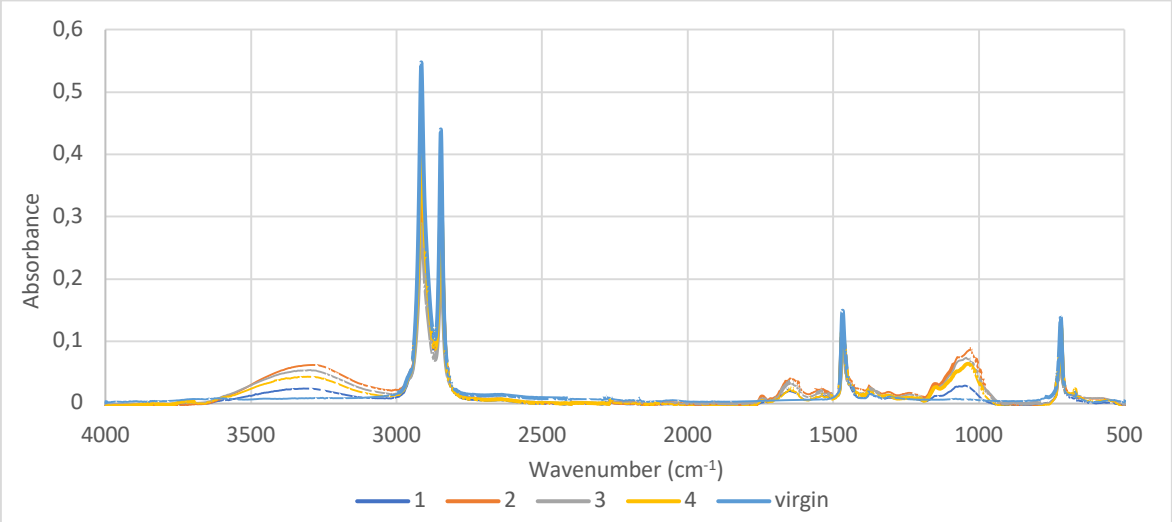


Figure 19 – FTIR-ATR spectra in the region 500 – 4000 cm^{-1} from samples of PE microplastics recovered in each tank, compared with a virgin PE microplastic.

Table 24 - Relative areas from the identified regions from the microplastics spectra.

REGIONS / SPECTRA	3700-3000 CM^{-1}	2980-2800 CM^{-1}	1700-1500 CM^{-1}	1500-1400 CM^{-1}	1200-950 CM^{-1}	750-650 CM^{-1}
1	11.62	25.05	3.105	3.669	4.582	2.807
2	27.79	24.09	5.652	4.400	12.62	3.104
3	22.7	19.05	4.133	3.282	10.12	2.625
4	21.42	25.72	3.864	4.231	9.58	3.604
VIRGIN	5.500	29.26	0.9897	3.656	1.194	2.869

The alterations in the FTIR spectra suggest that degradation have occurred, but this can be confirmed using, the Carbonyl index. The results for the carbonyl index for each sample are presented in Table 25. In this case the Abs(1) was considered the absorbance at 1647 cm^{-1} and Abs(2) the absorbance at 1469 cm^{-1}.

As expected, according to Table 25, the samples of microplastics exposed to fungi have a higher carbonyl index than the PE microplastic, substantiating the assertion that degradation has occurred. The sample from tank 2, despite being the one with higher carbonyl index, does not correspond to the tank where higher degradation percentage was achieved, this may happen as the samples are randomly chosen to be analyzed by FTIR, and possible the most oxidized would already been utilized by the microorganism[123]. As seen in the table the microplastics from the same tank do not present the same degree of degradation.

Table 25 – Carbonyl index of the analyzed samples.

SPECTRA	1	2	3	4	PE
ABS(1)	0.0245	0.0284	0.0261	0.0125	0.0066
	0.0112	0.0436	0.0168	0.0096	
	0.0170	0.0459	0.0356	0.0303	
ABS(2)	0.1128	0.1314	0.1152	0.1047	0.1489
	0.1261	0.1023	0.0748	0.1126	
	0.1224	0.1174	0.0857	0.1255	
CI	0.1482 ± 0.0527	0.3441 ± 0.0919	0.2885 ± 0.0895	0.1485 ± 0.0671	0.0444

3.3.3. Biodegradation in an optimized medium

The growth of *Zalerion maritimum* in an optimized medium for degradation of PE microplastics, is presented in Table 26, this growth is calculated based on the biomass at the beginning and at the end of the experiment. In the three tanks increase in biomass can be observed, and the growth are similar in all the tanks.

A contamination, this time by a green fungus occurred in all three tanks, but in tank 2 this contamination hardly grew, allowing a better development of *Z. maritimum*, when compared to the other tanks. In tank 1 and 3, in the other hand, the contamination grew filling up all the surface of the tank, and in tank 1 was even necessary to remove some of this contamination during the time of experiment.

Table 26 - Biomass and respective variation of *Zalerion maritimum*.

TANK	INOCULATED (WET) (G)	INOCULATED (DRY) (G)	FINAL (DRY) (G)	VARIATION	GROWTH
AQ1	14.00	1.120	7.890	6.770	6.045
AQ2	13.00	1.040	9.036	7.996	7.688
AQ3	13.30	1.064	8.181	7.117	6.689

The variation between the microplastics weight in the beginning and at the end of the experiment, and for consequence the percentage of removed microplastics are presented in Table 27.

Based on Table 27, a clear reduction of microplastics has occurred in all three tanks, since high percentages of removal were achieved. Unfortunately, however, such percentages of microplastics removed may have been influenced by more factors other than biodegradation, such as losses due to the formation of foam, due to losses in the removal of the contamination (referred before) and even due to losses during the filtration step. Nevertheless, the values present in Table 27, still demonstrates that, in an optimized medium, biodegradation happens more easily and efficiently. This experiment also serves as validation of the optimized medium, as the percentages of microplastics removed increase, as expected.

Table 27 - Microplastics in the beginning and at the end in each tank.

TANK	MICROPLASTICS BEGNING (G)	MICROPLASTCICS RECOVERED (G)	MICROPLASTICS REMOVED	%MICROPLASTICS REMOVED
AQ1	0,4347	0,0055	0,4292	98,73
AQ2	0,4161	0,0409	0,3752	90,17
AQ3	0,4400	0,0247	0,4153	94,39

Since, most likely, the microplastics were aggregated by the fungi mycelium, due to their small size, the lyophilized fungus was analyzed for the presence of microplastics. Figure 19 display the spectra obtained by the FTIR analysis of the recovered microplastics and spectrum from PE microplastic not exposed to fungus.

According to Figure 20 and Table 28, the relative areas, the characteristic peaks from PE decrease and some regions increase, leading to the conclusion that biodegradation occurred as previously explained.

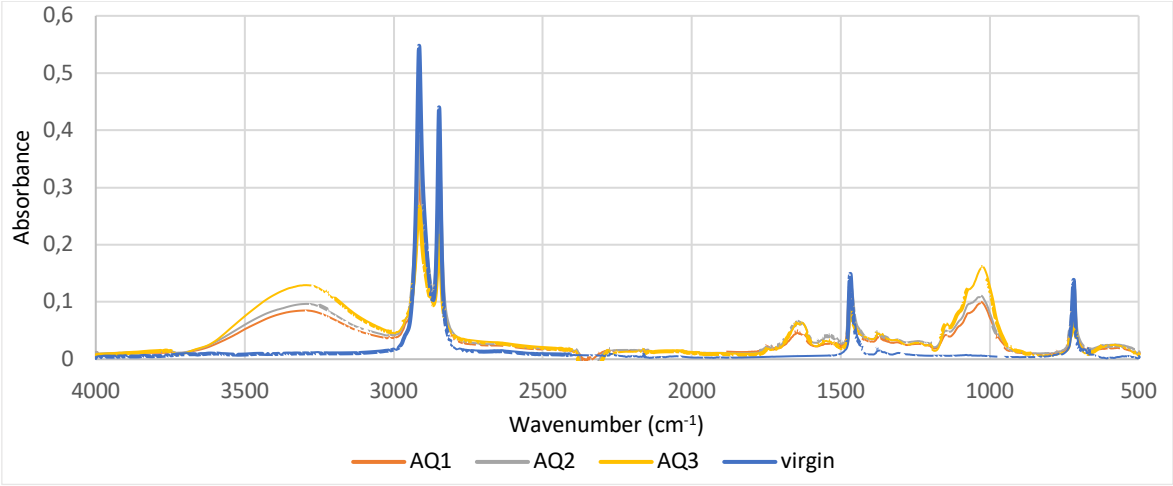


Figure 20 - FTIR-ATR spectra in the region 500 – 4000 cm^{-1} from samples of PE microplastics recovered in each tank, compared with a virgin PE microplastic.

Table 28 - Relative areas from the identified regions from the microplastics spectra.

REGIONS / SPECTRA	3700-3000 CM-1	2980-2800 CM-1	1700-1500 CM-1	1500-1400 CM-1	1200-950 CM-1	750-650 CM-1
AQ1	27.27	21.38	2.946	3.082	10.02	1.618
AQ2	35.02	25.51	5.862	4.230	13.08	2.390
AQ3	49.83	19.89	5.712	3.560	19.07	1.659
VIRGIN	5.500	29.26	0.9897	3.656	1.194	2.869

As before, degradation was confirmed by calculating the Carbonyl Index of each sample. Table 29 present the carbonyl index obtained for each sample, in this case the Abs(1) was considered the absorbance at 1648 cm^{-1} and Abs(2) the absorbance at 1464 cm^{-1}.

In table 29, it is possible to see that the samples of microplastics exposed to fungi have a higher carbonyl index than the PE microplastic, proving that degradation have occurred.

As seen in the table the microplastics from the same tank do not present the same degree of degradation.

Table 29 - Carbonyl index of the analyzed samples.

SPECTRA	AQ1	AQ2	AQ3	PE
ABS(1)	0.0379	0.0636	0.0890	0.0066
	0.0477	0.0532	0.0593	
	-	0.0376	0.0638	
ABS(2)	0.1285	0.1195	0.0734	0.1489
	0.1014	0.1135	0.0903	
	-	0.1277	0.0817	
CI	0.3827 ± 0.0874	0.4318 ± 0.1004	0.8837 ± 0.2382	0.0444

3.4. Conclusion

With these experiments it was possible to understand the problems encountered with scale up, and find solutions for them, like the evaporation, contaminations and formation of foam in the first days. These problems and the lack of time did not allow a continual Scale up, as we did not increase the size more than 1.3 L.

These essays suggested, once again, that *Z. maritimum* uses PE microplastics as substrate and that it is capable of actively grow in their presence even in larger scale. The experiments also helped to get more information about different and important aspects to have in consideration when planning an experiment in large scale.

It was possible to begin to understand how the fungus could behave after a first exposure to microplastics. Apparently, although growth of the fungus reduces, the microplastics present sings of further degradation, as present a higher carbonyl index and more signs of degradation on the FTIR spectra. This could show that the fungus adapts to the new subtract.

Furthermore, it was possible to conclude that the size of microplastics influence the degradation percentages, as expected. Smaller microplastics show to be more easily aggregate by the fungus and for consequence degraded.

With the last described experiment was possible to validate the optimization with CCD, described in Chapter 2. Additionally, it was possible to prove that even in a larger volume it is possible to obtain good degradation results, since the percentages of PE microplastics removed were high and, the recovered microplastics showed signs of degradation in the FTIR spectra.

4. Removal of bioplastics of Poly(ethylene2,5-furandicarboxylate) using marine fungi

4.1. Introduction

Due to environmental impacts of petroleum-based plastics, like PE, bioplastics emerged, in the last few years, as potential solution. This study aims to assess the capacity of both *Zalerion maritimum* and *Nia vibrissa* to biodegrade a bioplastic, poly(ethylene2,5-furandicarboxylate).

4.1.1. *Nia vibrissa*

Nia vibrissa (or *Nia vibris*), from submerge wood off the coast of Florida, was described first in 1959 by Moore and Meyers[127]. *Nia vibrissa* is one of the few marine fungi that belongs to the Basidiomycota phylum[128,129], characterized by fungi with ecological roles, for example as decomposers[130].

The Basidiomycota phyla includes a wide variety of species, with different characteristics, so over time their class and orders underwent changes[131] and as *Nia vibrissa* is a taxonomically enigmatic[132], its taxa is constantly changing. At the moment *Nia vibrissa* is part of the subphylum Agaricomycotina[128,129], a subphyla that includes fungi with varied macroscopic and microscopic features, the only unifying morphological characteristic is their dolipore septa associated with septal pore cap[133]. *Nia vibrissa* belongs to the Agaricomycetes class, Agaricomycetidae sub-class and more specifically to the Agaricales order and Niaceae family[128,129].

Nia vibrissa is characterized by a subglobose, gasteroid fruiting body[134], by its septal pore cap perforate[133], and by its mycelium or spore color that varies between pink and brown[135,136]. It has also be defined as a white rot fungus due to its capacity of degrade lignin and lignocellulose[137,138], different authors have studied this capacity[139] and uncovered the enzymes responsible for the degradation of lignin and lignocellulose[140,141].

Matavuly and Molitoris, chose this fungus, to test its capacity of biodegrade poly-3-hydroxyalkanoates (PHA), more specific Poly[(R)-3-hydroxybutyrate] (PHB) and BIOPOL^{TM}. They concluded that only one strain of *Nia vibrissa* was able to depolymerize PHB. In fact,

this was the only fungus of Basidiomycota with this ability and none was able to degrade BIOPOL[142,143].

According to Helmholz et al[144], *N. vibrissa* is a marine fungus able to produce biological extracts with potential for the development of new natural products. They studied better conditions to obtain the higher biological activity of the ethanolic extract[144].

As well as *Z. maritimum, N. vibrissa*, can also survive in a variety of conditions, and can live in extremes temperatures if the salinity is also high[145]. For this reason, its presence has been documented in various locations, including in Europe, on the coast of Italy[146], England[145], Portugal and Turkey[136], in Asia, on the coast of Japan and Singapore[136], in Oceania, on the Fiji coast[147] and in America on the North American Coast[145].

4.1.2. Bioplastics

The term bioplastics comprehend bio-based plastics and biodegradable plastics, both as both characteristics could help to reduce the negative impacts of plastics[148]. Biodegradable plastics are polymers with small changes in the polymeric chain, improving degradation by different microorganisms[149]. Bio-based plastics are polymers obtained from a natural source or renewable resource[149]. Bio-based is defined in the European standard EN 16575 as "derived from biomass" [150], and these plastics can be directly extracted from biomass (plants), polymerized from monomers obtain from biomass or produced by a wide range of microorganisms and later extracted[148,149].

Poly(ethylene2,5-furandicarboxylate) (PEF) is an alipharomatic polyester with a linear formula $(C_8H_6O_5)_n$, depicted in Figure 21, with a melting point of 265°C and it is a bio-based plastic. In the recent years the interest in this bioplastic based on renewable resources[151] has grown, but it was first described in 1940's, in a patent from Celanese Corporation of America[152].

Several synthesis conditions have been described in the literature, where the reagents, catalysts, temperatures and pressure vary. The most adopted synthesis is a two-step reaction, first a transesterification reaction of 2,5-furandicarboxylic acid (FDCA) or its

dimethyl-(DMFDC) derivative with EG, carried out under nitrogen and a 215ºC, followed by a polytransesterification with increasing the temperature[153].

PEF is an analogue for poly(ethylene terephthalate) (PET), but offers attractive thermal and superior barriers properties, it can support higher temperature conditions[153,154]. It also have attractive properties in terms of oxygen and carbon dioxide permeability, as they are lower than the observed for PET[153].

Weinberger et al.[154] have studied the potential of PEF to be degraded by two different enzymes, one obtained from a fungi and other from a bacteria. The authors concluded that PEF is highly degraded by the fungal enzyme, showing their potential for fungal biodegradation. Austin et al.[155] reported similar findings for a different bacterial enzyme.

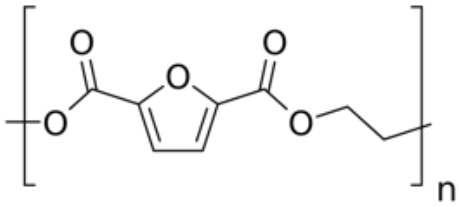


Figure 21 – Molecular structure of PEF.

4.2. Materials and methods

4.2.1. Microplastics

PEF fragments had different shapes and a size <2mm. They were synthesized as described on the paper of Araújo et al.[151] and kept in a desiccator shared from light and at room temperature in our laboratory. PEF microplastics were characterized by FTIR-ATR spectroscopy, as seen in Figure 22.

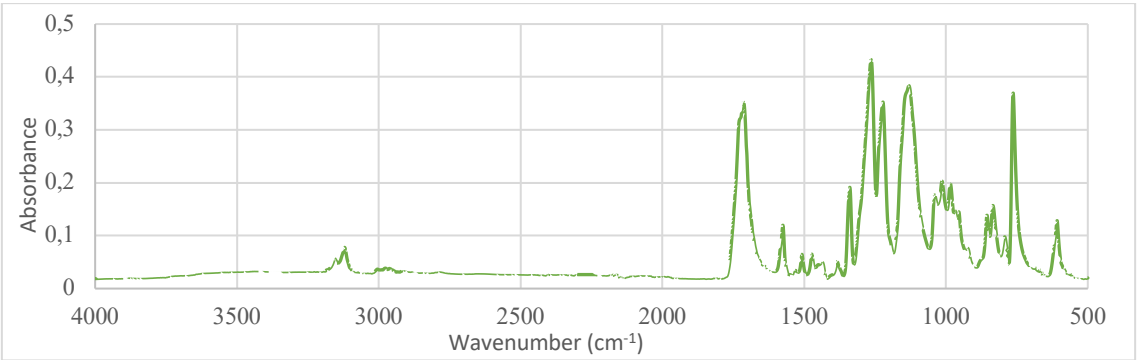


Figure 22 – Characteristic FTIR spectra of PEF.

4.2.2. Microorganisms

Z. maritimum was the same previously characterized.

Nia vibrissa (ATCC 34606) was grown for approximately 10 days in a culture medium with the composition of 35g/L of salt, 20g/L of glucose, 20 g/L of malt extract and 1g/L of peptone and temperatures around 20°C[156].

4.2.3. Culture medium

In the experiment to assess the biodegradation capacity of *Z. maritimum*, a medium with ten time less supplements was utilized, 2g/L of glucose, 2g/L of malt extract and 0.1 g/L of peptone.

Based on some problems, the other experiment to assess the biodegradation of PEF by *Z. maritimum* and by *N. vibrissa* was made using the first optimize medium composition, 35 g/L of salt, 2 g/L of glucose, 18 g/L of malt extract and 0.22g/L of peptone.

4.2.4. Experimental conditions

Two different experiments were performed in order to analyse the biodegradation potential of PEF. A first experiment, using *Zalerion maritimum* was realized for 28 days, using thirty-two batch reactors (100mL Erlenmeyer flask) with 25 mL of culture medium. In twenty-four batch reactors, 0.0070g of PEF fragments were added. The thirty-two were later autoclaved, and to the eight batch reactors without microplastics and to sixteen of the ones with microplastics was inoculated 0.30g of fungus mycelium.

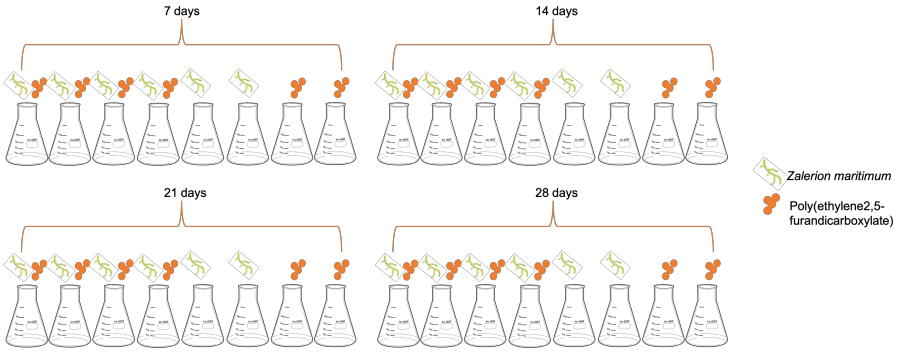


Figure 23 -Schematic representation of the experiment to assess the capacity of *Z. maritimum* to biodegrade PEF microplastics.

A second experiment, only lasted 14 days, and was done in order to compare the capacity of *Z. maritimum* and *N. vibrissa* of biodegrade this micro(bio)plastic. It was performed with twenty-eight batch reactors (100 mL Erlenmeyer flask) with 25mL of culture medium, to twenty of them was added 0.0070g of PEF fragments. All batch reactors were autoclaved and later half of the ones without fragments were inoculated with *Z. maritimum* and the other half with *N. vibrissa*. Eight of the batch reactors with microplastics were inoculated with *N. vibrissa* and other eight were inoculated with *Z. maritimum*.

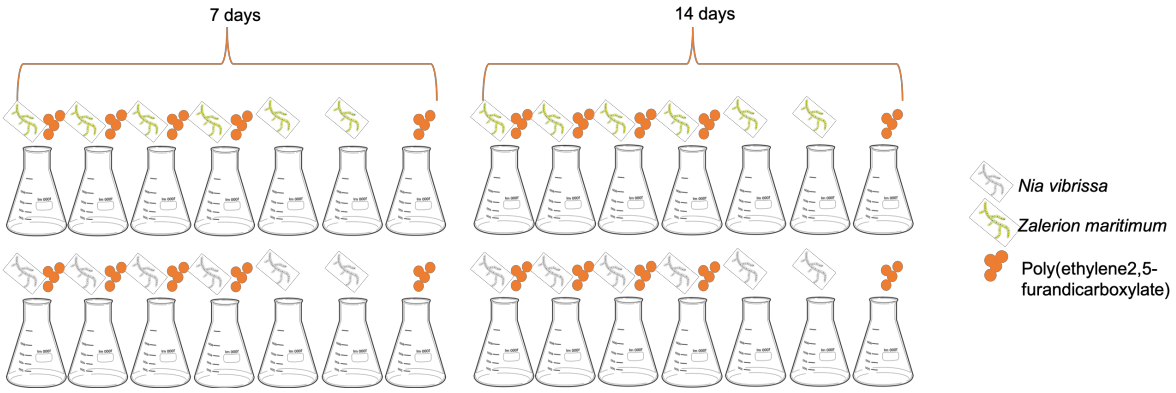


Figure 24 - Schematic representation of the experiment to compare the capacity of *Z. maritimum* and *N. vibrissa* to biodegrade PEF microplastics.

In both experiments, as described, some batch reactors only contained fungi (CF), to evaluate the growth of the fungi in the medium without the microplastics. Others batch only contained microplastics (CM), to evaluate the interaction between the microplastics and the medium and the possible problems in the filtration step.

All biological treatments were tested by maintaining, for the referred time, the batch reactors in a shaker, at room temperature and with stirring at 120 rpm. At each time line the batches were removed from the shaker and the fungus and microplastics were separated from the medium by filtration. The fungus biomass was retrieved and posteriorly frozen and lyophilized. The microplastics were saved for weighing and further analysis.

4.2.5. Techniques used to analyze the microplastics

As in the previous chapter microplastics were analysed by FTIR-ATR, the same instrument previously described.

4.1. Results and discussion

4.1.1. First experiment to assess the capacity of *Zalerion maritimum* to biodegrade PEF microplastics

Table 30 presents the weight of biomass of *Z. maritimum* before and after the exposures to PEF microplastics, and for the control samples. As seen in Table 30, fungus exposed to PEF grow considerably less than the fungus at the control samples, this can be explained by a possible negative effect of the PEF microplastics to the fungus. It can also be explained by some difficulties experienced with the contact between the fungus and the microplastics, as due to their size and density, the microplastics would remain at the bottom center of the batch reactor.

Table 30 - Variation of fungus biomass throughout the experiment.

BATCH REACTOR	INOCULATED BIOMASS (WET) (G)	INOCULATED BIOMASS (DRY) (G)	FINAL BIOMASS (DRY) (G)	VARIATION OF BIOMASS	GROWTH	GROWTH MEAN
7 DAYS						
R1	0.544	0.048	0.038	-0.010	-0.209	-0.012
R2	0.399	0.036	0.034	-0.002	-0.054	
R3	0.302	0.027	0.035	0.008	0.295	
R4	0.386	0.034	0.032	-0.003	-0.080	
CF1	0.302	0.027	0.046	0.019	0.715	0.888
CF2	0.308	0.027	0.056	0.029	1.061	
14 DAYS						
R1	0.404	0.036	0.035	0.000	-0.013	0.270
R2	0.297	0.026	0.040	0.014	0.517	
R3	0.286	0.025	0.036	0.011	0.430	
R4	0.306	0.027	0.031	0.004	0.146	
CF1	0.359	0.032	0.046	0.014	0.437	0.439
CF2	0.321	0.029	0.041	0.013	0.442	
21 DAYS						
R1	0.326	0.029	0.059	0.030	1.037	1.000
R2	0.328	0.029	0.066	0.037	1.254	
R3	0.324	0.029	0.063	0.034	1.181	
R4	0.360	0.032	0.049	0.017	0.526	
CF1	0.340	0.030	0.072	0.042	1.373	1.004
CF2	0.338	0.030	0.049	0.019	0.636	
28 DAYS						
R1	0.333	0.030	0.041	0.011	0.377	0.364
R2	0.356	0.032	0.041	0.009	0.285	
R3	0.297	0.026	0.045	0.019	0.706	
R4	0.354	0.032	0.034	0.003	0.089	
CF1	0.288	0.026	0.038	0.012	0.467	0.632
CF2	0.340	0.030	0.054	0.024	0.798	

The weight variation of the PEF microplastics before and after exposure to the fungus are shown in Table 31. Through a first eye, would be possible to assume that degradation occurred successfully, but looking more careful, we see that the percentages of removed microplastics exposed to *Z. maritimum* and the percentages of the PEF from control are incredibly similar. This finding means that the percentages of removed microplastics are influenced by problems in the separation method, such as adherence of the PEF microplastics to the batch reactors and filter used in the filtration step, the PEF particles, due to their small size, may also have remained in the medium.

Table 31 – PEF microplastics at the beginning and at through the experiment.

BATCH REACTOR	MICROPLASTICS BEGINNING	MICROPLASTICS RECOVERED	MICROPLASTICS REMOVED	% MICROPLASTICS REMOVED	% MICROPLASTICS REMOVED MEAN
7 DAYS					
R1	0.0075	0.0044	0.0031	41.3%	50.4%
R2	0.0075	0.0033	0.0042	56.0%	
R3	0.0071	0.0032	0.0039	54.9%	
R4	0.0069	0.0035	0.0034	49.3%	
CM1	0.0074	0.0040	0.0034	45.9%	45.6%
CM2	0.0075	0.0036	0.0034	45.3%	
14 DAYS					
R1	0.0072	0.0025	0.0047	65.3%	47.4%
R2	0.0070	0.0062	0.0008	11.4%	
R3	0.0070	0.0023	0.0047	67.1%	
R4	0.0068	0.0037	0.0031	45.6%	
CM1	0.0071	0.0033	0.0038	53.5%	44.5%
CM2	0.0076	0.0049	0.0027	35.5%	
21 DAYS					
R1	0.0075	0.0051	0.0024	32.0%	64.4%
R2	0.0072	0.0013	0.0059	81.9%	
R3	0.0070	0.0018	0.0052	74.3%	
R4	0.0072	0.0022	0.0050	69.4%	
CM1	0.0069	0.0019	0.0050	72.5%	65.4%
CM2	0.0072	0.0030	0.0042	58.3%	
28 DAYS					
R1	0.0067	0.0032	0.0035	52.2%	57.2%
R2	0.0074	0.0041	0.0033	44.6%	
R3	0.0072	0.0034	0.0038	52.8%	
R4	0.0072	0.0015	0.0057	79.2%	
CM1	0.0075	0.0039	0.0036	48.0%	49.7%
CM2	0.0070	0.0034	0.0036	51.4%	

The PEF microplastics recovered were analyzed by FTIR and the spectra obtained are presented in Figure 25, as well as the characteristic FTIR spectrum of PEF.

As seen in Figure 25, only the microplastics from 21 days samples shows possible signs of degradation, as it is possible to observe the appearance of two bands in regions of 3600-3200 cm^{-1} and 1660-1600 cm^{-1}, that are not characteristic of PEF. For the replica that presented this degradation signs, a control sample and the original PEF the relative areas were calculated and are presented in Table 32.

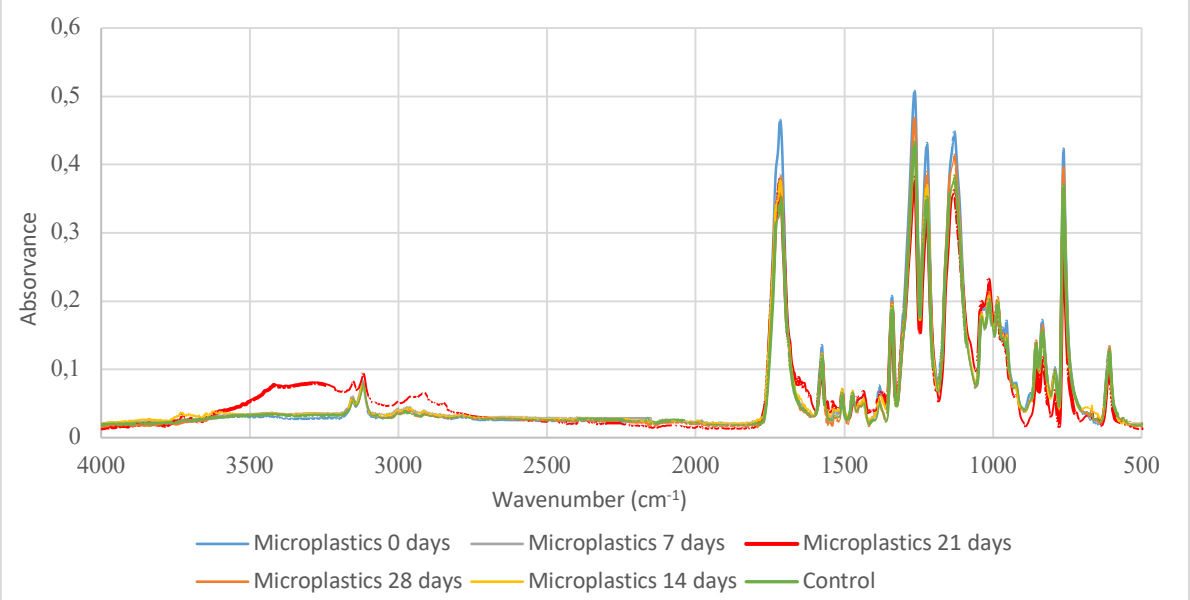


Figure 25 – Infrared spectra in the region 500-4000 cm^{-1} from the PEF microplastics throughout the experiment.

Table 32 - Relative areas from the identified regions from the microplastics spectra.

REGIONS / SPECTRA	3600-3200 CM-1	3100-2800 CM-1	1800-1660 CM-1	1660-1600 CM-1	1600-600 CM-1
21 DAYS	28.05	17.32	16.22	3.871	85.76
CONTROL	13.61	10.24	22.24	2.345	102.8
VIRGIN	11.68	9.759	25.88	2.381	132.3

4.1.2. Comparison between *Zalerion maritimum* and *Nia vibrissa* capacity of biodegrade PEF microplastics

Table 33 and Table 34, represent, respectively, the weigh at the beginning and at the end of exposure of *Z. maritimum* and *N. vibrissa*.

Analyzing the data from Table 33, it is possible to conclude that, this time, *Z. maritimum* was able to grow in the presence of the PEF microplastics. The changes in the experimental conditions allowed better contact, as the fungus grow the same or, after 14 days, slightly more when exposed to PEF microplastics than the fungus in the control samples.

According to Table 34, *N. vibrissa* appear to grow more than *Z. maritimum*, although it seems to grow less in 14 days than in 7 days. Despite that, the difference between the grow in the presence of PEF microplastics and the control samples seem to be insignificant, on the case of *N. vibrissa.*

Table 33 - Variation of Zalerion maritimum biomass throughout the experiment.

BATCH REACTOR	INOCULATED BIOMASS (WET) (G)	INOCULATED BIOMASS (DRY) (G)	FINAL BIOMASS (DRY) (G)	VARIATION OF BIOMASS	GROWTH	GROWTH MEAN
7 DAYS						
R1	0.29	0.026	0.13	0.104	4.037	2.165
R2	0.33	0.030	0.09	0.061	2.064	
R3	0.40	0.036	0.08	0.044	1.247	
R4	0.34	0.030	0.07	0.040	1.313	
CF1	0.32	0.028	0.11	0.081	2.862	2.803
CF2	0.42	0.037	0.14	0.103	2.745	
14 DAYS						
R1	0.33	0.029	0.14	0.111	3.767	4.953
R2	0.32	0.028	0.25	0.222	7.778	
R3	0.32	0.028	0.17	0.142	4.969	
R4	0.34	0.030	0.13	0.100	3.296	
CF1	0.31	0.028	0.13	0.102	3.712	3.924
CF2	0.35	0.031	0.16	0.129	4.136	

Table 34 - Variation of Nia vibrissa biomass throughout the experiment.

BATCH REACTOR	INOCULATED BIOMASS (WET) (G)	INOCULATED BIOMASS (DRY) (G)	FINAL BIOMASS (DRY) (G)	VARIATION OF BIOMASS	GROWTH	GROWT MEAN
7 DAYS						
R1	0.32	0.020	0.22	0.200	9.742	6.784
R2	0.41	0.026	0.12	0.094	3.573	
R3	0.34	0.022	0.15	0.128	5.893	
R4	0.28	0.018	0.16	0.142	7.929	
CF1	0.36	0.023	0.18	0.157	6.813	8.001
CF2	0.23	0.015	0.15	0.135	9.190	
14 DAYS						
R1	0.31	0.020	0.16	0.140	7.065	5.613
R2	0.29	0.019	0.13	0.111	6.004	
R3	0.44	0.028	0.14	0.112	3.972	
R4	0.39	0.025	0.16	0.135	5.410	
CF1	0.26	0.017	0.15	0.133	8.014	5.523
CF2	0.62	0.040	0.16	0.120	3.032	

Table 35 and Table 36 shows the percentage of microplastics removed, by the fungi *Z. maritimum* and *Nia vibrissa*, respectively. According to these tables, it would be possible to assume that high percentages of removal were achieved, but in the control samples, the microplastics were not all recovered, which means that this percentages may be influenced by the medium or problems in the filtration and separation, the same problem from the previously described experiment.

Table 35 – Variation of PEF microplastics before and after their exposure to Zalerion maritimum.

BATCH REACTOR	MICROPLASTICS BEGINNING	MICROPLASTICS RECOVERED	MICROPLASTICS REMOVED	% MICROPLASTICS REMOVED	% MICROPLASTICS REMOVED MEAN
7 DAYS					
R1	0.0075	0.0018	0.0057	76.0%	88.8%
R2	0.0077	0.0004	0.0073	94.8%	
R3	0.0077	0.0008	0.0069	89.6%	
R4	0.0076	0.0004	0.0072	94.7%	
CM1	0.0079	0.0032	0.0047	59.5%	71.0%
CM2	0.0074	0.0013	0.0061	82.4%	
14 DAYS					
R1	0.0075	0.0031	0.0044	58.7%	68.0%
R2	0.0077	0.001	0.0067	87.0%	
R3	0.0078	0.0016	0.0062	79.5%	
R4	0.0075	0.0040	0.0035	46.7%	
CM1	0.0079	0.0026	0.0053	67.1%	67.1%
CM2	0.0076	0.0025	0.0051	67.1%	

Table 36- Variation of PEF microplastics before and after their exposure to Nia vibrissa.

BATCH REACTOR	MICROPLASTICS BEGINNING	MICROPLASTICS RECOVERED	MICROPLASTICS REMOVED	% MICROPLASTICS REMOVED	% MICROPLASTICS REMOVED (MEAN)
7 DAYS					
R1	0.0078	0.0022	0.0056	71.8%	77.0%
R2	0.0074	0.0025	0.0049	66.2%	
R3	0.0073	0.0008	0.0065	89.0%	
R4	0.0079	0.0015	0.0064	81.0%	
CM1	0.0079	0.0032	0.0047	59.5%	71.0%
CM2	0.0074	0.0013	0.0061	82.4%	
14 DAYS					
R1	0.0072	0.0014	0.0058	80.6%	76.3%
R2	0.0076	0.0013	0.0063	82.9%	
R3	0.0073	0.0025	0.0048	65.8%	
R4	0.0079	0.0019	0.0060	75.9%	
CM1	0.0079	0.0026	0.0053	67.1%	67.1%
CM2	0.0076	0.0025	0.0051	67.1%	

On Figure 26 and Figure 27 is possible to see the FTIR spectra from the PEF exposed to *Z.maritimum* and *N. vibrissa*, respectively, the PEF from control samples, and the characteristic spectrum for PEF, to an easier comparison. According to both Figure 26 and Figure 27, we can only conclude that degradation occurred, on the sample exposed for 15 days to *N. vibrissa*. Since, the appearance of bands in the regions of 3600-3200 cm^{-1} and 1660-1600 cm^{-1} it is only evident in Figure 27 and proved with Table 37. This may happen, as *N. vibrissa* had already been described as possible degrader of poly-3-hydroxyalkanoates (PHA), biopolymer[142,143].

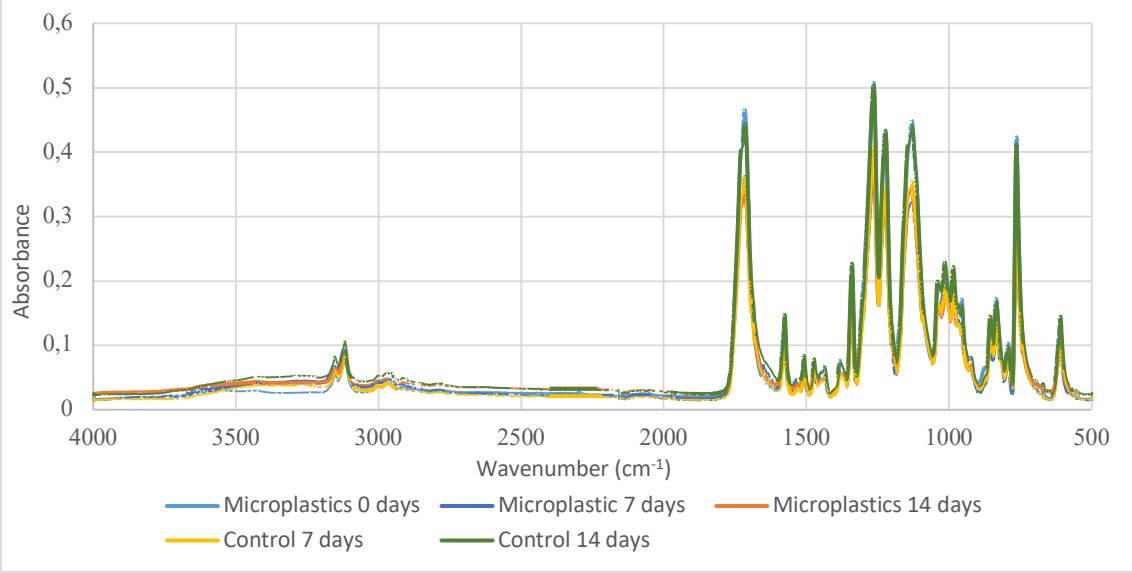


Figure 26 - Infrared spectra in the region 500-4000 cm^{-1} from the PEF microplastics exposed to *Zalerion maritimum*.

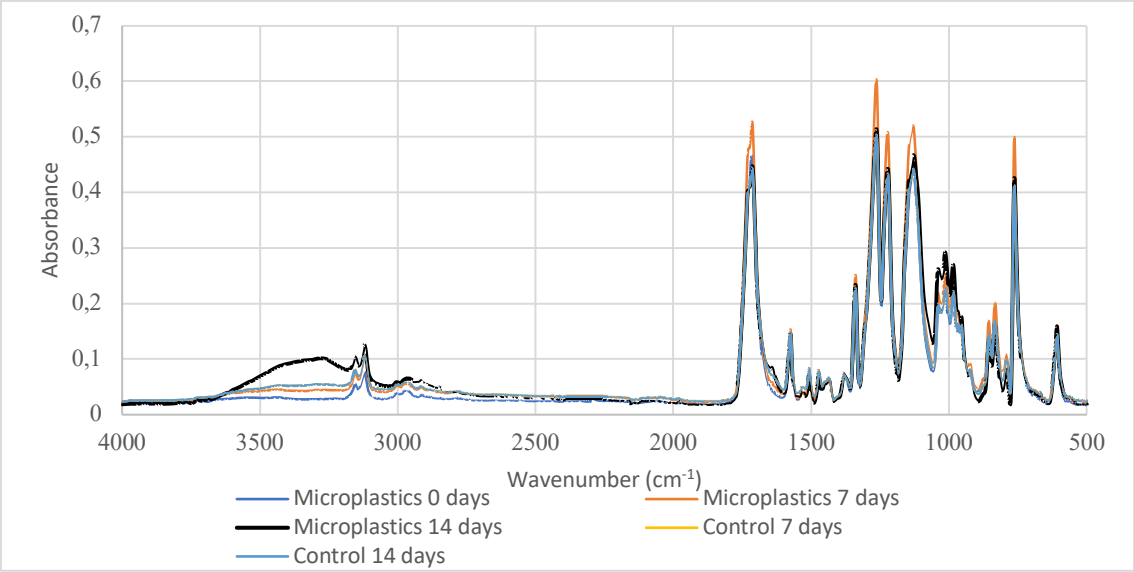


Figure 27 - Infrared spectra in the region 500-4000 cm^{-1} from the PEF microplastics exposed to *Nia vibrissa*.

Table 37 - Relative areas from the identified regions from the microplastics spectra of 14 days exposed to *N. vibrissa*, a control and a virgin sample.

REGIONS	3600-3200	3100-2800	1800-1660	1660-1600	1600-600
SPECTRA	CM-1	CM-1	CM-1	CM-1	CM-1
14 DAYS	32.92	16.47	26.64	3.898	141.4
CONTROL	17.67	13.93	25.38	3.299	131.9
VIRGIN	11.68	9.759	25.88	2.381	132.3

4.2. Conclusion

These preliminary essays demonstrated the potential of *N. vibrissa* to biodegrade PEF microplastics, as well the potential of *Z. maritimum* to biodegrade PEF microplastics, responding to the current need of alternatives ways of minimizing the presence of plastics in the marine environment.

In the experiments of the fungi exposed to PEF microplastics, was not possible to see meaningful weigh variations, due to problems in the filtration step, caused by the small size of PEF microplastics. However, some samples show signs of degradation in their FTIR spectra, leading to the possible conclusion that biodegradation occurred.

5. Conclusions and future perspectives

The main purpose of this dissertation is to exploit the biodegradation capacity of *Zalerion maritimum* with the possibility of developing a bioremediation process. With this intent was made the optimization of the biodegradation medium and Scale up of experiments. Preliminary tests of the biodegradation capacity of *Zalerion maritimum* and *Nia vibrissa* of a bioplastic were also performed.

The biodegradation medium was optimized with the aim of improving the process and obtain higher percentages of PE microplastics removal with *Zalerion maritimum*. The objective of the Scale up experiments was to understand how the fungus would behave in a bigger scale and in a less controlled environment.

With the medium optimization was possible to understand that malt extract is the main regulatory factor in the degradation of PE microplastics, and that peptone and glucose can be in low concentrations, since microplastics will serve as substitutes for the carbon source and peptone is necessary in low concentrations even in growth medium. In this dissertation, only the optimized medium obtained with CCD was validate, in Scale up experiments, thus, there is the need to validate the optimized medium by UD, as well.

In the Scale up approach, the first step was successively achieved, as high percentages of microplastics removal were obtained in a less controlled environment and in a 2L tank. The experiments also helped to get information about different and important aspects to have in consideration when planning a bioremediation process in large scale. Even so, future scale up approaches, such as successive increases in volume, are still required in order to establish an effective bioremediation treatment.

Other experiments are also needed to clarify certain aspects that were not fully understood or that were not addressed in this dissertation. For example, as different microplastics recovered in the experiments show different degrees of degradation, it is necessary in future work to analyze all recovered microplastics to understand the extent of degradation in all of them. It is also necessary to study *Zalerion maritimum*, its growth characteristic and how this fungus metabolic acts over plastic. For example, which are the metabolic

products of this biodegradation process, and what products remain in the culture medium. It could also be interesting to re-study *Zalerion maritimum* response to microplastics in a bigger size range, but for a longer exposure time. In order to see, if when the fungus grow on the surface of the microplastics, the extent of degradation is higher and if the microplastics would reduce in size over time.

In the preliminary tests to assess *Zalerion maritimum* and *Nia vibrissa* capacity of biodegrade PEF, both fungi showed to be promising biodegraders, as alterations in the FTIR spectra are observed. Due to problems in the contact between the fungi and the microplastics, it is necessary further research to clarify this capacity, it is necessary to do, for example, a volume medium to increase interaction between them. It could also be interesting to work with PEF microplastics in a bigger size rang, to reduce losses in the filtration step, or encounter new and efficient separation methods.

6. References

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7. Appendix A

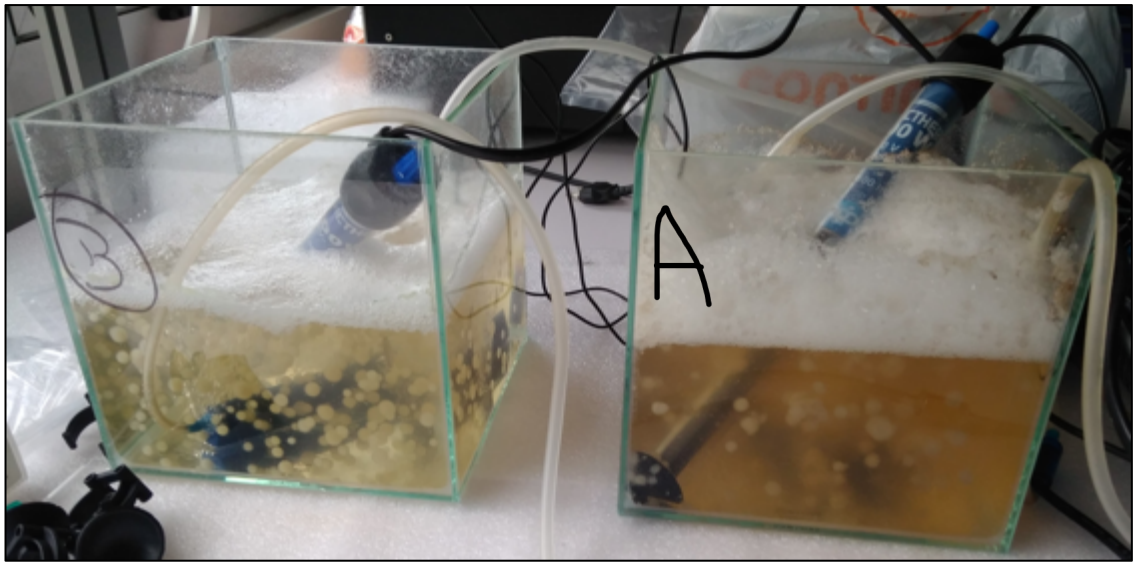

Figure 28 – Photo from the two tanks used in the experiment

A preliminary experience of Scale up was made, using two tanks of 5L as batch reactors, with 3L of culture medium, represented in the Figure 28.

One tank (A) had a medium composed by glucose, malt extract and peptone in concentrations 10x lower than the optimal concentrations for the normal growth. The other tank (B) had a medium composed by the same medium components but in a concentration only 100x less. To both were added approximately 6.50g of microplastics with a range size between 2000 µm < MP < 1000 µm, and approximately 30.00g of fungal biomass.

In tank A the fungus was able to grow and aggregate the microplastics, as seen in Figure 29, and the microplastics on the medium were covered with fungal biomass. In tank B, the fungus was unable to grow, the recovered mass at the end of the experiment was lower than the initial mass, and on the contrary the microplastics recovered had the same weigh and did not show any difference.

In Figure 30, the spectra of the fungus from tank B and tank A, are presented, as well as a spectrum from the fungus that grow in the normal culture medium. It is possible to observe a great difference between then.

Figure 29 – Photo of the interaction between *Zalerion maritimum* and PE microplastics

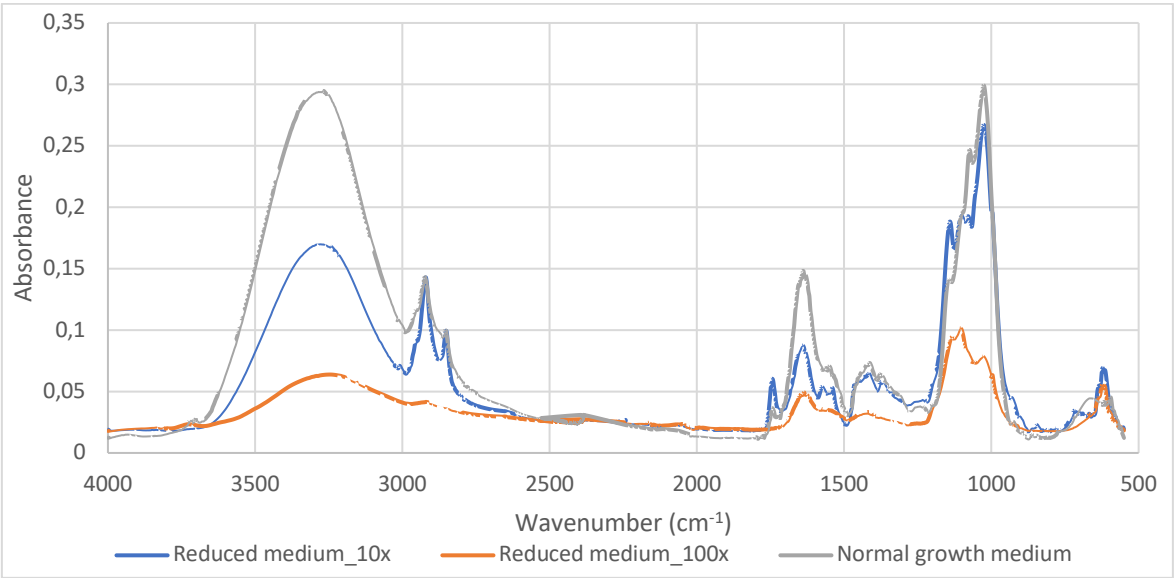

Figure 30 – FTIR-ATR spectra in the region 500 – 4000 cm^{-1} from samples of fungus from both tanks and a sample of a fungus not exposed to microplastics.

8. Appendix B

8.1. Context

As *Z. maritimum* grew on the surface of the microplastics, it was necessary to remove the biological matter in order to later weigh and analyze the microplastics by FTIR.

Due to the lack of standardized methods for that purpose, three solvents were chosen to be tested, Sodium Hydroxide (NaOH) in three different concentrations, Nitric Acid (HNO_3) in two different concentrations and Hydrogen peroxide (H_2O_2), alone and combined with an iron (III) solution (Fe III). Their effect on microplastics covered with fungal biomass and on virgin PE microplastics was compared, in order to see possible digestion consequences on the microplastics.

The reagents were chosen based on a literature search[157–161], the highest concentrations were defined based on the concentrations referred on the articles, and the lowers were defined, since the biological material in the microplastics surface was considerably lower than the biological material removed in the papers. The time for digestion was defined, based on the NOAA procedure[160].

8.2. Material and methods

8.2.1. Chemicals and microplastics

The utilized reagents were Sodium hydroxide, in the form of pellets and Nitric Acid (65%) from Sigma Aldrich, and Hydrogen peroxide (35%) from LabKem.

The solution of NaOH (1M) was prepared by dissolving the pellet in distilled water, the other solutions, NaOH (0.5M) and NaOH (0.1M) were prepared by diluting the solution of NaOH (1M) with distilled water. The solution of HNO_3 (35%) was also made by diluting the stock solution with distilled water.

The PE microplastics virgins were the same described previously on the experiments. The PE microplastics covered with fungal biomass were obtained from the experiment described in Appendix A.

8.2.2. Digestion treatment conditions

Approximately 0.0120 g of virgin microplastics were added to 100mL beakers. For each beaker was added 20 mL of a digestion solution, three replicates were considered. The same was done for the microplastics covered with biomass.

The microplastics exposed to the acid and basic treatment were maintained in the solutions for one hour at room temperature, and later separated by filtration.

On the other hand, in the H_2O_2 treatment, the beakers containing the microplastics and the solution were heated up, on a hotplate, for approximately 20 min until 75ºC. After that period, were maintained the remain time (until the end of 1 hour) on the lab bench, for posterior filtration.

The treatment with H_2O_2 associated with the Fe(III) solution, was similar to the previously described, the only difference was that 10 mL of H_2O_2 where add to the beaker and 10 ml of the Fe(III) solution, and then heated up, on a hotplate, for approximately 20 min until 75ºC. The following steps were the same.

8.2.3. Techniques to analyze the microplastics

To confirm that degradation was not caused by the solutions, some microplastics were analyzed by FTIR, on the equipment previously described.

8.3. Results and discussion

According to the tables was possible to see that the better treatments were H_2O_2 associated with the Fe(III) solution and HNO_3 65%, as the weigh variation on the virgins were insignificant and in the microplastics covered with fungal biomass, this weigh variation is significative and represent the loss of the biological matter.

Through visual inspection, was possible to reach the same conclusion, but as seen in the figure, the microplastics exposed to H_2O_2 associated with the Fe(III) solution present signs of degradation, and the exposed to HNO_3 65% do not present.

Based on these findings the treatment HNO_3 65% appears to the best, the one with better removal of fungal biomass and with less negative's effects on the microplastics surface.

Table 38 – Variation of weigh of the microplastics with NaOH treatment

NaOH 0.1M	Beginning	End	dif
F1	0,0128	0,0117	0,0011
F2	0,0148	0,0127	0,0021
F3	0,0124	0,0116	0,0008
V1	0,0117	0,0126	-0,0009
V2	0,0112	0,0136	-0,0024
V3	0,0115	0,0123	-0,0008
NaOH 0.5M			
F1	0,0127	0,0110	0,0017
F2	0,0134	0,0140	-0,0006
F3	0,0123	0,0126	-0,0003
V1	0,0113	0,0070	0,0043
V2	0,0133	0,0141	-0,0008
V3	0,0111	0,0122	-0,0011
NaOH 1M			
F1	0,0119	0,0128	-0,0009
F2	0,0135	0,0130	0,0005
F3	0,0116	0,0130	-0,0014
V1	0,0137	0,0151	-0,0014
V2	0,0130	0,0140	-0,0010
V3	0,0129	0,0143	-0,0014

Table 39 - Variation of weigh of the microplastics with HNO_3 treatment

HNO_3 35%	Beginning	End	dif
F1	0,0128	0,0108	0,0020
F2	0,0104	0,0099	0,0005
F3	0,0103	0,0069	0,0034
V1	0,0126	0,0088	0,0038
V2	0,0155	0,0152	0,0003
V3	0,0145	0,0144	0,0001
HNO_3 65%			
F1	0,0113	0,0092	0,0021
F2	0,0133	0,0092	0,0041
F3	0,0148	0,0125	0,0023
V1	0,0111	0,0057	0,0054
V2	0,0125	0,0119	0,0006
V3	0,0130	0,0139	-0,0009

Table 40 - Variation of weigh of the microplastics with H_2O_2 treatment

H_2O_2	Beginning	End	dif
F1	0,0134	0,0100	0,0034
F2	0,0110	0,0084	0,0026
F3	0,0108	0,0103	0,0005
V1	0,0125	0,0133	-0,0008
V2	0,0114	0,0121	-0,0007
V3	0,0132	0,0136	-0,0004
H_2O_2+Fe			
F1	0,0128	0,0118	0,0010
F2	0,0134	0,0124	0,0010
F3	0,0115	0,0102	0,0013
V1	0,0140	0,0143	-0,0003
V2	0,0137	0,0132	0,0005
V3	0,0133	0,0138	-0,0005

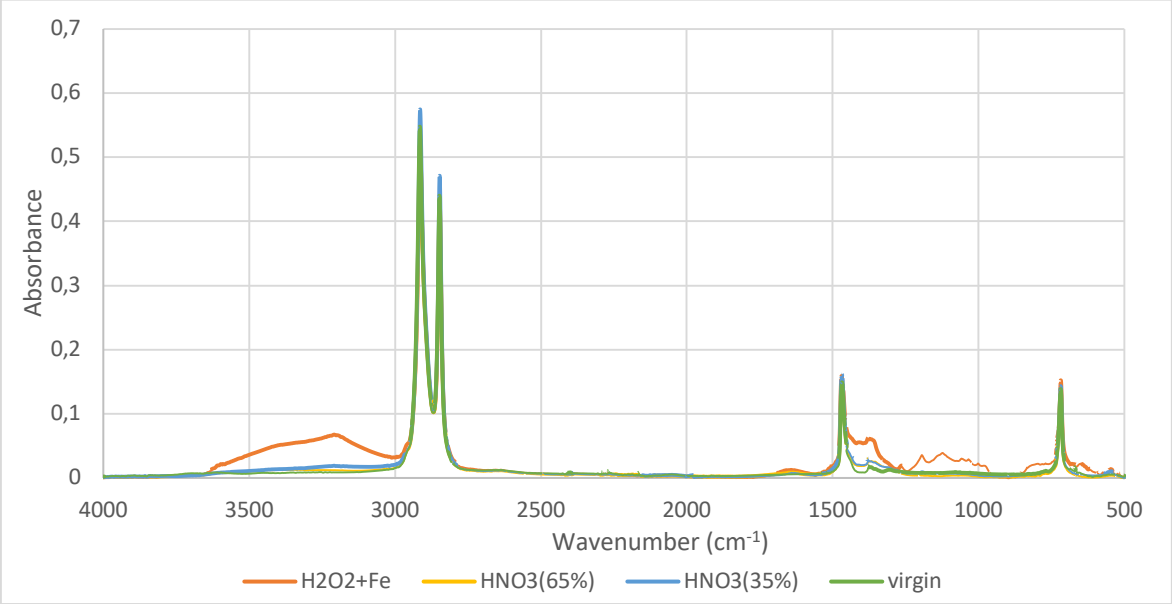

Figure 31 - FTIR-ATR spectra in the region 500 – 4000 cm^{-1} from samples of PE microplastics exposed to treatment with H_2O_2 and HNO_3

8.4. Conclusion

Based on this data, it was possible to conclude that HNO_3 65%, in the case of microplastics covered with fungus biomass, would be the best cleaning treatment.