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Ferramentas biotecnológicas para a remediação de lixo plástico, utilizando fungos marinhos

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biotecnologia, realizada sob a orientação científica da Doutora Teresa Alexandra Peixoto da Rocha Santos, Investigadora Principal com Agregação do Departamento de Química da Universidade de Aveiro e do Centro de Estudos do Ambiente e do Mar (CESAM), e do Doutor Artur Jorge da Costa Peixoto Alves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro.

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A vida é feita de rascunhos e de ideias desenhadas...
cada traço mil histórias, a vida toda para as contar...
Rui Santiago, cssr

o júri

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palavras-chave

Zalerion maritimum, *Penicillium brevicompactum*, polietileno, biodegradação, genómica, proteómica, máscaras, bioplástico de agricultura, planeamento experimental

Resumo

A presença de plásticos no ambiente e a sua contínua e crescente produção, torna o plástico uma questão da atualidade e que requer soluções urgentes. Este projeto de doutoramento teve como objetivo aumentar o conhecimento relativamente à capacidade de fungos em biodegradar plásticos, para desenvolver um processo biotecnológico para o tratamento de resíduos plásticos. *Zalerion maritimum*, um fungo marinho, e *Penicillium brevicompactum*, um fungo muito comum e amplamente distribuído, foram estudados e aplicados no tratamento de plásticos. Para desvendar as proteínas envolvidas nesse processo, foram utilizadas técnicas Ómicas, genómica e proteómica. Embora os genomas de ambos os fungos contenham genes que codificam enzimas associadas à biodegradação, como lacases e citocromo P450, essas enzimas não foram identificadas nas análises proteómicas. O estudo revela que as proteínas intracelulares envolvidas são produzidas constitutivamente, sugerindo que ambos os fungos utilizam as suas vias metabólicas normais para converter os monómeros de microplásticos em energia. Para otimizar as condições e obter maiores percentagens de remoção de microplásticos, foi utilizado o "uniform design" para encontrar o meio ótimo para a biorremediação de microplásticos de polietileno por *Z. maritimum*. Adicionalmente, foi determinada a capacidade de *P. brevicompactum* para biodegradar diferentes polímeros, incluindo amostras da indústria alimentar, um "mulch biofilme" e fibras de máscaras descartáveis. A resposta de *Z. maritimum* em contato com as fibras de máscaras faciais também foi estudada. *Penicillium brevicompactum* demonstrou afinidade com todos os polímeros testados, tendo demonstrado elevadas percentagens de remoção para o polietileno de baixa densidade. Além disso, a toxicidade dos polímeros também foi avaliada no solo, usando *Eisenia andrei* como animal modelo, na água doce, usando *Chironomus riparius* como animal modelo, e na água salgada, usando *Venerupis corrugata* como animal modelo. Segundo os resultados todos os polímeros apresentam algum nível de toxicidade, destacando a importância do desenvolvimento de um processo de biorremediação de (micro)plásticos. Em última análise, os resultados deste trabalho não apenas contribuem para nossa compreensão da biodegradação de plásticos por fungos, mas também apresentam um genoma completo de um fungo marinho e o primeiro estudo proteómico de fungos filamentosos cultivados na presença de (micro)plásticos. Enfatizam ainda, a importância global de aproveitar a capacidade de biodegradação destes fungos.

keywords

Zalerion maritimum, *Penicillium brevicompactum*, polyethylene, biodegradation, genomics, proteomics, facemasks, mulch biofilm, experimental design.

abstract

The presence of plastics in the environment, coupled with their continually increasing production, makes plastic a significant cotemporary issue requiring urgent solutions. This PhD project aimed to enhance the understanding of fungi's capacity to biodegrade plastics and develop a biotechnological process for plastic waste treatment. *Zalerion maritimum*, a marine fungus, and *Penicillium brevicompactum*, a widely distributed and adaptable fungus, were studied and applied to the treatment of plastic polymers. In this work, OMICs approaches, both genomic and proteomic, were employed to unravel the proteins involved in this process. Although the genomes of both fungi contained genes encoding enzymes associated with biodegradation, such as laccases and cytochrome P450, these enzymes were not identified in the proteomics analyses. It appears that the intracellular proteins involved are constitutively produced, suggesting that both fungi use their normal metabolic pathways to convert the microplastics monomers into energy. To optimize conditions and obtain higher percentages of removal of microplastics, Uniform design was used to find the optimal medium for the bioremediation of polyethylene microplastics by *Z. maritimum*. Additionally, the study explored *P. brevicompactum*'s ability to biodegrade different polymers, including samples from the food industry, a mulch biofilm, and fibers from facemasks. The response of *Z. maritimum* in contact with facemasks fibers was also assessed. *Penicillium brevicompactum* demonstrated an affinity for all tested polymers, particularly exhibiting high removal percentages for low-density polyethylene. Furthermore, the toxicity of these polymers was assessed, on soil, using *Eisenia andrei* as model organism, in freshwater using *Chironomus riparius* as model organism, and in saltwater using *Venerupis corrugata* as model organism. The results indicated that all polymers exhibited some level of toxicity, underscoring the importance of developing a bioremediation process for (micro)plastics. Ultimately, the outcomes of this research not only contribute to our understanding of plastic biodegradation by fungi, but also feature a complete genome of a marine fungus as well as the first proteomic study of filamentous fungi grown in the presence of (micro)plastics. Finally, the results also emphasize the global significance of harnessing the ability of fungi to biodegrade plastics.

Table of Contents

Thesis outline

This PhD thesis, entitled "Biotechnological tools for plastic waste remediation using marine fungi" focuses on studying and exploring the ability of fungi, namely *Penicillium brevicompactum* and *Zalerion maritimum*, to biodegrade plastics. The thesis comprises six chapters and present the different approaches that were used to gather that information. This was obtained by genomics and proteomics studies, as well as performing biodegradation experiments as presented in each chapter:

I. Introduction

It presents the problematic behind the aim of this thesis, reviews knowledge regarding the presence of plastics in the environment, how biodegradation has been studied as a promising and natural solution, and finally reviews why fungi are good candidates for this biotechnological application.

II. Complete genome sequence of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329, two fungi with potential for biodegradation of microplastics

The genomes of *P. brevicompactum* and *Z. maritimum* studied in this thesis, for the biodegradation of plastics, were sequenced and analysed, to identify genes encoding for enzymes potentially relevant in the biodegradation process and to assist further OMICs studies, namely proteomics.

III. Optimization of culture medium for polyethylene microplastics removal by *Zalerion maritimum* using Uniform Design

The chemical composition of the culture medium influences the behaviour of microorganisms, so in this chapter it is presented how experimental design can be used to optimize the chemical composition of culture medium to increase the removal of polyethylene microplastics.

IV. Proteome analysis of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329, two fungi with potential for biodegradation of microplastics

This chapter reports the cellular proteome profiles of *P. brevicompactum* and of *Z. maritimum* grown in the presence and absence of polyethylene microplastics in the optimized medium.

V. The use of *Penicillium brevicompactum* and *Zalerion maritimum* for the biodegradation of plastics in environmentally relevant conditions, and ecotoxicity studies of polymers in different habitats

In this chapter, the ability of *P. brevicompactum* and of *Z. maritimum* to degrade different polymers were tested. In addition, ecotoxicity tests regarding the polymers in different habitats are also present, highlighting the importance of the central aim of this thesis.

VI. General discussion

Overall discussion, with a summary of the main results achieved as well as what would be future lines of work.

Each chapter can be read individually, and some were published in peer-reviewed journals.

Chapter II:

- **Paço, A**.; Gonçalves, M. F. M.; da Costa, J. P.; Rocha-Santos, T. A. P. & Alves, A. Draft genome sequence of *Zalerion maritima* ATCC 34329, a (micro)plastic-degrading marine fungus. *Microbiology Resource Announcements* **12**, 5–7 (2023). doi:10.1128/mra.00017-23

Chapter V:

- Ferreira-Filipe, D. A.; **Paço, A.**; Natal-da-Luz, T.; Sousa, J. P.; Saraiva, J. A.; Duarte, A. C.; Rocha-Santos, T. & Patrício Silva, A. L. Are mulch biofilms used in agriculture an environmentally friendly solution? - An insight into their biodegradability and ecotoxicity using key organisms in soil ecosystems. *Science of The Total Environment* **828**, 154269 (2022). doi:10.1016/j.scitotenv.2022.154269

- Ferreira-Filipe, D. A.; **Paço, A.**; Pinho, B.; Silva, R.; Silva, S. A. M.; Jesus, F.; Pereira, J. L., Duarte, A. C.; Rocha-Santos, T. & Patrício Silva, A. L. Microplastics from agricultural mulch films: biodegradation and ecotoxicity in freshwater systems. *Science of The Total Environment* **912**, 169287 (2024). doi.org:10.1016/j.scitotenv.2023.169287

- **Paço, A**.; Oliveira, A. M.; Ferreira-Filipe, D. A.; Rodrigues, A. C. M.; Rocha, R. J. M.; Soares, A. M. V. M.; Duarte, A. C.; Patrício Silva, A. L. & Rocha-Santos, T. Facemasks: An insight into their abundance in wetlands, degradation, and potential ecotoxicity. *Science of the Total Environment* **904**, 166232 (2023). doi:10.1016/j.scitotenv.2023.166232

I. Introduction

1. (Micro)plastics in the environment

Plastic materials and plastic products have become one of the most important inventions from the last century, ensuring even the 1963 Nobel prize in Chemistry to Karl Ziegler and Guilio Natta[1]. There are a variety of types of plastic polymers, which are produced from different hydrocarbons and petroleum derivatives[2]. Over the last century, plastics became widely used in our daily life, in our care products, agriculture, clothes, cars, among many other examples, given that manufacturing with this type of material is simple and has low costs associated[3,4]. Plastic materials have helped to improve life quality and provided countless benefits to modern life, thanks to their cheap price, but also thanks to their light weight, usefulness, durability, and resistance to light, temperature, water, and chemicals[5]. Unfortunately, this last quality became, through the years, one of their biggest problems, as it makes plastic materials persistent and with low biodegradability in nature, allowing them to remain in the environment for long periods of time[6].

According to Plastics Europe[7], since the mass production of plastic started it continued to increase, reaching 391 million tons, in 2021, and most of this consists of single-use products. After being used, plastic material has three main disposable options, incineration, landfill, and recycling. Recycling in Europe has been increasing and in 2020 it reached 35%, exceeding the quantity of plastics that went to landfills[7]. Unfortunately, the amount destined to landfills is still considerable[7] and the recycling process is yet to be perfect. Consumers have problems identifying the different types of plastics or in some countries, like Portugal, there is only one type of recycling container for all types of plastics, so the separation, required for the recycling process, is difficult and inefficient. Other problems associated with recycling are the fact that some food contaminated plastics cannot be recycled, going to power generation, and that there is a limit on the recycling cycles due to degradation during use[8]. A small percentage goes to incineration, a process that has the positive side of producing energy but is also known to have some negative health and environmental impacts[9], including the

formation of ashes, made of small plastic particles, that remain in the bottom of the incinerators[2].

The high demand of our society for plastic materials allied to their incorrect disposal and/or poor waste management is leading to an accumulation of plastics in the environment and making plastics a global concern[10]. Reports show that, worldwide, there is a massive presence of plastics debris[11], with most of them falling in the microplastics definition[12]. Microplastics are defined as plastic particles with less than 5mm in their largest dimension, by the National Oceanic and Atmospheric Administration (NOAA)[13,14], and can be defined as primary or secondary. Primary microplastics are produced in this size and can be found for example in cosmetics and personal care products, or in drug delivery systems. In toothpaste or scrubs, these microplastics are marketed as "micro-beads" or "micro-exfoliates". Secondary microplastics are derived from the fragmentation of large plastics, or from plastics wrongly disposed in nature[14].

As early as the 1970s, the word microplastics was mentioned in a paper by Carpenter and Smith[15] who found small plastic debris inside animals and discussed its potential impact on them. For some years there was little or no mention of this word, but in the last two decades they have become the focus of intense research. In fact, according to Davtalab et al.[16], from 2016 till now the amount of papers on the microplastics topic has drastically increased, which reflects a rapid and exponential development in microplastics research.

Within the (micro)plastics hot topic, some research trends can be found. Papers can be divided, based on their research focus, into four principal categories[17]: Occurrence, abundance and distribution; Impacts, uptake by organisms and adsorption of chemicals; Fate of plastics and mitigation strategies; New solutions and alternatives.

Although being present in all environments and all around the globe, the marine environment is the most impacted, with (micro)plastics reported in all ocean extent[12,18], from the Arctic polar waters[19] to the Antarctic waters[20]. Reports show that around 4.8-12.8 million metric tons of plastic debris from land-based activities are disposed of in the ocean without any proper management strategy. These debris account for 80% of

the plastics that end up in the ocean[21] and enter through various ways spreading later until the most remote place, transported by winds and currents[22]. These issues make the marine environment the focus of intensive research, as evidenced by Davtalab et al.[16], that identifies the word “marine-environment” as one of the most used keywords in microplastic research papers.

A study demonstrated that marine organisms, ranging from zooplankton to turtles, fish of different sizes and birds, commonly are found with plastic debris in their gut[23]. This happens as (micro)plastics are mistaken for food and end up ingested by marine organisms, which could lead to bioaccumulation and bioaugmentation effects throughout the food web. The ingestion of these polymers may lead to physical and (bio)chemical impacts on the organisms, like problems in fertility and reproductive ability. Other problems that come from the presence of (micro)plastics are physical, like entanglement and suffocation[23]. For example, Baudrimont et al.[24] studied the impacts of polyethylene (nano)plastics in two species of microalgae and a bivalve. Results showed that the polyethylene caused growth inhibition of *Scenedesmus subspicatus* (freshwater green algae) and little or no effect in *Thalassiosira weissiflogii* (marine diatom). *Corbicula fluminea*, a freshwater clam, on the other hand, had an increase in fecal and pseudo-fecal production which suggests a cleaning mechanism. Mak et al.[25] aimed to identify the physical effects, behavioral changes and the gene expression profiles of two genes, *cyp1a* and *vtg1*, induced by microplastics, for that they exposed zebrafish to polyethylene microplastics. They concluded that microplastics tend to remain in the intestine of the organisms, which reduced the space for food. They found the genes, *cyp1a* and *vtg1,* to be up regulated and abnormal behavior, e.g. seizures, was observed. Effects on metabolic pathways and neurotoxicity were also found with acute exposure. Ecotoxicity effects were also studied in soil organisms like *Eisenia fetida*, Li et al. [26] investigated the ingestion and egestion of polyethylene and polypropylene in soil. The results suggested that microplastics induced oxidative stress and disturbed metabolic pathways related with neurodegeneration and inflammatory responses. The toxicity effects are caused not only by the inherent toxicity of (micro)plastics but also by chemical contaminants that are adsorbed onto their surface[27–29]. As demonstrated by Zhang et al.[30], their study revealed the combined effect of polyethylene and 9-

nitroanthracene in zebrafish. According to their results, the combined exposure led to neurotoxicity and disturbances in the energy metabolism of zebrafish, along with alterations in the intestinal microbial community related with inflammation.

The massive presence of plastic debris has environmental, economic and health impacts, as an example, there are reports describing the presence of microplastics in the human body and possible impacts on human health[31]. Therefore, most organizations have proposed legislation or policies to help reduce the production and/or utilization of plastics. Global and regional agreements have also been made to promote recycling and develop cleaning strategies[32]. For example, in the European Union, since July 2021, single-use plastics have been banned from the markets, the use of plastic bags is discouraged, and clear labelling of plastic products is incentivized[33]. Other measures include changing to degradable plastics or taking steps in product design to minimize the use of plastics. The development of initiatives to induce behavior change and sensitize the populations to this global problem is also crucial, as it encourages people to get involved and work to find a solution[34].

Unfortunately, despite all these efforts and legislations, during the Covid-19 pandemic, we, as society, completely relied on single use plastics. To prevent the transmission of the virus, medical staff and health workers relied on the use of single use medical facemasks, gloves and other protective equipment, all made of plastic polymers[35]. Some of the governmental measures to prevent spread of the virus involved the discourage of eating in crowed restaurants, which led to a higher use of “food to go”, that in most cases came in plastic containers[35]. Besides the higher quantity of plastics used during the pandemic, an additional concern was the disposal and treatment of these plastic items, as some governments recommended that all plastics potentially contaminated should be placed in a sealed and leak-proof garbage bag and put in mixed waste to go to landfills or, preferably, to incineration, which intended to prevent workers to get in contact with contaminated items, but causing a reduce in recycling[35]. Governments also adopted measures that involved the use of facemasks, and in some cases, it was mandatory that disposable facemasks should be used instead of fabric facemasks. Disposable facemasks are frequently made of plastic nanofibers, most commonly

polypropylene, and they are recommended to be exchanged every three hours, which makes these a major source of plastic waste and a great concern[36].

Bioplastics have been present as a substitute for normal plastic polymers[37]. Bioplastics comprehend bio-based plastics, plastics that are (at least partially) produced from organic material, like corn, sugarcane, cellulose or shrimps' shells, instead of fossil material[37–39]. However, the use of bio-based plastics are not problem-free, some are non-biodegradable, and some appear to be toxic to organisms[40,41]. Biodegradable plastics, are also comprehended in the bioplastics definition, and are degraded via microbial action and converted to natural compounds, like carbon dioxide, methane, and water[37,38]. Unfortunately, most of these plastics can be only degraded in specific conditions, that are not easily met in nature[4,42].

Biodegradation has been proposed as a promising solution, a feasible and natural way to help to reduce the plastics present in the environment, but despite being a promising process, until now microorganisms have not been applied to the treatment of plastic mixtures[43,44]. In addition, little is known about biodegradation, and more information regarding the mechanisms and enzymes involved are necessary.

2. Biodegradation of (micro)plastics

In the environment, (micro)plastics undergo abiotic and biotic degradation processes. Abiotic degradation is caused by natural factors, like light or wind. Biotic degradation, also referred to as biodegradation, is mediated by microorganisms[45]. Biodegradation is defined 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"[46].

The application of biodegradation to remove hazardous components and wastes from the environment is defined as bioremediation[47]. Bioremediation has been widely applied in the treatment of soils and waters contaminated with heavy metals, pharmaceuticals and pesticides, among others. In comparison to other physicochemical

methods, this biotechnological method is less expensive and has higher efficiency in removing contaminations[48].

In recent years, the possibility of applying biodegradation to reduce the presence and plastic accumulation in nature has become the focus of intense research[44]. In our daily life we use a large variety of plastic polymers, like polyethylene (PE), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET), with or without additives or with other chemical changes that alter their backbone. As each type of plastic has a different chemical formula and therefore different monomers and bonds, it is impossible to find just one microorganism, or an enzyme, to work as a mediator in the bioremediation of all plastic waste. Fortunately, since the beginning of the studies on the bioremediation of plastics, several microorganisms (fungi, bacteria and algae) have been studied for their ability to biodegrade different kinds of plastics, as reviewed by Ahmed et al.[43], Pathak and Navneet[49] and Kumar Sen and Raut[50].

Among the studies reported in literature and conducted on the biodegradation of plastics, some utilized microorganisms isolated from plastic particles[51,52], while other utilized microorganisms selected based on their proven ability to degrade other materials[53,54]. An example of the former is the use of microorganisms isolated from the platisphere, a term given to the communities that form biofilms on the multitude of plastics present in the aquatic environments[55]. A good example of the latter are white-rot fungi, which can degrade lignin[53,54].

According to these papers, polyethylene is the most used plastic in biodegradation studies[56], as it is also the most common plastic, used in a wide variety of products thanks to the possibility of being produced with different densities. For a consequence, it is also the principal component of plastic waste found in nature[2, 53,57–59].

According to the review made by Chia et al.[60], there are several studies where a variety of microalgae species have been isolated from PE from water bodies. For example, Kumar et al.[61] isolated a variety of species of microalgae from PE bags found in freshwater bodies in India. They later tested the growth of three of these species in contact with PE and concluded that the cyanobacterium (also called blue-green algae), *Anabaena spiroides*, is effective in the degradation of PE. This cyanobacterium was the

one that more efficiently colonized the low-density polyethylene (LDPE) sheets, and it showed an 8.18% of degradation of LDPE after 1 month of colonization. Scanning electron microscopic analysis showed that the LDPE sheets in contact with the cyanobacteria had a hole, proving the degradation of this material.

Sangeetha Devi et al.[62] isolated bacteria from plastic waste dumpsites in India and found that *Bacillus* spp. and *Pseudomonas* spp. can degrade plastic, being powerful PE degraders, achieving percentages of degradation over 40% in three months. This percentages were obtained in soils samples containing synthetic medium and with partially degraded polyethylene samples. The isolates were later grown in the same synthetic medium with HDPE as the only carbon source, for one month and in this case the highest percentage of degradation was around 23%. Hadad et al.[63], in turn, isolated a strain of *Brevibacillus borstelensis* from contaminated soils, near a petrochemical factory able to degrade PE at high temperatures (50-60 ^{o}C). *Brevibacillus borstelensis* was able to degrade 34% of UV-treated PE in 90 days of incubation. In one-month incubation in a medium with only UV-treated PE as a carbon source, *B. borstelensis* achieved 30% of degradation. Another example is *Alcanivorax borkumensis*, isolated by Delacuvellerie et al.[64] from floating plastics found in the Mediterranean Sea. This strain showed potential to form biofilms and degrade PE. *Alcanivorax borkumensis* expresses enzymes associated with the degradation of PE, and when in contact with this polymer for 80 days the bacteria formed a biofilm in the surface, showing high affinity with PE and 3.4% of degradation.

Sowmya et al. [65] isolated a fungus *Penicillium simplicissimum*, from soil samples of a local dumpsite, that was able to degrade 38% of UV-treated PE, in three months. *Penicillium simplicissimum* also showed to induce a decrease in the molecular weight of previously oxidized PE[66]. Paço et al.[67] studied *Zalerion maritimum*, a marine fungus, that in contact with PE was able to degrade over 60% of the microplastics in 3 weeks. Other authors, like Orhan et al.[68] tested the terrestrial fungus *Phanerochaete chrysosporium* in contaminated soils, demonstrating its ability to degrade PE. In this work, the authors evaluated other parameters rather than mass loss, like the percentage elongation of PE, noticing a reduction of 56% in three months.

In the bioremediation of plastics, fungi are promising candidates, as besides secreting enzymes, like laccases, manganese peroxidases and lignin peroxidases, their mycelia is also able to penetrate hydrophobic surfaces allowing a deeper attack to deteriorate the substrate[69].

Several factors influence the biodegradation of plastics, including polymers properties and environmental factors[45,69]. Among polymer properties, the shape and size play a crucial role, as plastics with a large surface area are more susceptible to microorganism attachment. Molecular weight and crystallinity also influence degradation as plastics with a low values for both are more easily attacked by microorganisms' enzymes[45,69]. Biodegradation is also influenced by various others polymer characteristics, like flexibility, functionality, morphology, chain length and backbone. Environmental factors such as temperature, pH, moisture and water content, influence biodegradation by affecting the behaviour of microorganisms[45,69].

It is also evident, that most microorganisms are not able to rely solely on plastics as nutrient source. So, to improve the bioremediation process and maximise the removal of plastics from the medium, an important and interesting step is the optimization of culture conditions. This would help engineer a more effective and sustainable bioremediation process, where only the necessary components are added to the medium, theoretically achieving the maximum plastic removal possible. In the case of the marine environment, marine fungi would be more indicated. Marine fungi produce useful enzymes for bioremediation and release them in higher amount when compared to bacteria[69].

Recent studies have also focused the biological processes involved in the biodegradation of plastic, not yet fully understood. These biological processes seem to change based on the plastic polymer and the microorganisms involved. Some authors, divide the process into four different phases, biodeterioration, biofragmentation, mineralization and assimilation[2].

The biodeterioration step is defined as the process that changes the surface and properties of the polymers and it is characterized by polymer initial breakdown in monomers. This phase can be mechanical/physical, chemical, or enzymatic, and it is

caused by the growth of the microorganisms on the surface and/or inside the polymers. Microorganisms can produce and secrete a complex matrix that will change and weaken the material, simultaneously favouring microbial growth. This normally creates an acidic environment that will increase erosion and favour oxidation reactions[2,70,71].

The biofragmentation step is characterized by the cleavage and fragmentation of the monomers through the action of enzymes. Besides the components that form the matrix referred to before, microorganisms can also produce enzymes that are able to bind and break the specific bonds, e.g., hydrolases and oxidoreductases[2,70,71].

The mineralization step occurs when the fragments, formed in the previous steps, are small enough to pass the cell membranes and enter the microbial cells. Inside the cells they will be oxidized and used as energy source and biomass growth[2,70,71].

The assimilation step is the final step and is the process by which the atoms from the fragments are integrated into the cells. During this, secondary metabolites are produced and later transported outside the cells. CO_2 and H_2O among others are also released[2,70,71].

Despite this division and all this information, there is a lot about the biodegradation process that is unknown. As mentioned before, it is known that hydrolases and oxidoreductases are involved, but which ones? It is already clear that are not just enzymes, or at least not the known enzymes, that are involved. Studies of plastic enzymatic degradation, show that it is difficult to achieve high removal rates, using only enzymes[2]. It is known that secondary metabolites, CO_2, and H_2O are produced, but by which mechanisms? Which are the metabolites being secreted? Are they toxic? How do microorganisms use plastic as a carbon source?

A deeper knowledge in this field will certainly lead to advances in waste treatment, as understanding the physiology of the microorganisms able to biodegrade plastics, and how their growth is influenced, would help to better apply it in the bioremediation process[72]. The advances in OMICs approaches, in the last decade, like genomics and proteomics, provide great opportunities to obtain information and answer some of these questions, in order to understand more about the process[73].

Whole genome sequencing of microorganisms able to degrade plastic can provide information regarding the genes involved in the ability to use plastics as a carbon source. In addition, these genes could be used in screenings of microorganisms, helping to find new organisms with this ability.

In turn, postgenomic technologies, such as proteomics and metabolomics, can give insights into the proteins and metabolites that are produced by the microorganisms when in contact with plastics. It would also allow the proposal of metabolic pathways involved and to understand how microbial metabolism changes with the presence of the plastics[73–75].

3. Fungi

Fungi are a diverse group, which includes a wide variety of species that can be found in a great variety of conditions and environments[73]. They can be found in complex soil matrix, in freshwater, in marine habitats and even in air samples[73]. Several studies have also shown that fungi can thrive in different climatic conditions, normally from 5 ^{o}C to 35 ^{o}C, but there are also species that grow very well near or below 0 ^{o}C and others near 60 ^{o}C[76].

Fungi play a crucial role in maintaining the balance of ecosystems, forming close relations with other organisms. For example, saprotrophic fungi, known as decomposers, significantly contribute to the recycling of energy and nutrients, by decomposing waste or dead tissue of plants and animals[77]. Mycorrhizal fungi exert a substantial influence on the plant communities as their symbiotic relationships assist plants in accessing nutrients that would otherwise be unavailable[77].

As natural decomposers that survive in different habitats, fungi produce a large variety of enzymes, that are incredibly versatile[78,79]. These enzymes catalyse different chemical reactions that aid in the bioconversion of complex substrates[78,79]. Thanks to the multitude of fungal enzymes, fungi are great agents in biotechnological products and processes, like bioremediation[73,79]. Some fungi species are used, for example, in the production of foods, e.g. cheeses and bread, and others in the pharmaceutical industry,

for example, for the production of antibiotics[78]. In other industries, fungal enzymes are extracted and used in the process, like in the textile industry or biorefinery[78].

White-rot fungi, for example, are a group of fungi able to degrade ligneous material. The key enzymes involved in that process are extracellular, which assists in the oxidization of low solubility substrates, that wouldn't enter in the cell. The enzymatic machinery of white-rot fungi not only give them the ability to break down complex organic compounds, but also enhances their tolerance to the contaminants. As a result, white-rot fungi have been widely studied for their application in the bioremediation of contaminated environments[80]. The ability of these organisms to degrade chemical compounds, pharmaceuticals, and some polymers has been demonstrated[73]. For example, a number of white-rot fungi species have been reported for their ability to reduce total phenolic and colour in olive-mill wastewaterd[73]. *Pleurotus sajor caju*, *Pleurotus ostreatus*, *Trametes versicolor* and *Phanerochaete chrysosporium* are examples of white-rot fungi widely studied for their bioremediation capacity, in different scenarios. For example, Freitas et al.[81] and Rocha-Santos et al.[82] tested the ability of *Pleurotus sajor caju*, *Trametes versicolor* and *Phanerochaete chrysosporium* to be applied in the treatment of effluents from pulp mills and showed their efficiency in removing organic compounds. *Trametes versicolor* and *P. ostreatus* were applied to the bioremediation of phenanthrene and pyrene[83].

Marine fungi are a diverse group with large biotechnological potential, especially in bioremediation thanks to their ability to adapt to harsh environmental conditions. Several studies showed that marine fungi can tolerate high levels of contamination, for example, plastics or metals[73]. Maamar et al.[84] isolated fungi from seawater samples in Oran, Algeria, and conclude that four marine species of *Penicillium* were able to metabolize hydrocarbons, serving as good candidates in the bioremediation of contaminated areas. Their great potential is also evidenced by their ability to produce unique and novel bioactive natural compounds, that can be used in cosmetic and pharmaceutical industry, in food industry and in agriculture[73,85]. Gonçalves et al.[85] showed how extracts obtained from marine species have antibacterial activity, especially the extract of *Aspergillus affinis*, and also anti-fungal activity, especially the extract of *Penicillium lusitanum*.

Extremophilic fungi are also very interesting for bioremediation purposes, as they normally survive in harsh conditions and have, for example, thermotolerance and pH tolerance. Studies have also shown that extremophilic fungi have tolerance to high levels of pollutants from industrial effluents, metals and insecticides[73]. In the same way, extremophilic fungi produce bioactive compounds that can be applied in different areas, for example, they produce anti-fungal compounds that act against soil-borne fungi and can be used as an alternative to pest control[86].

Despite the recognized importance of fungi, there is a lot that we still do not know about them, regarding diversity, physiology and ecological roles. More information on their function and on their enzymes is needed[77]. So, a better understanding and exploitation of fungi's full potential can be achieved through OMICs technologies[73]. For example, sequenced fungal genomes helped confirm the enormous potential of fungi for the production of secondary metabolites with biotechnological application[87,88].

Genomes sequences, draft or complete, are essential for other OMICs studies[75]. The author's Tyers and Mann[89] call it the "blueprint". Genomic studies of fungi are increasing, year by year, but the numbers of fully sequenced annotated genomes are still scarce when compared to the diversity of species existing of Fungi kingdom[90,91]. By 26th January of 2023, in the NCBI's Genome database included 16976 genomes of fungi. There are around 150000 fungal species described to date, and an estimation of the real number of species being over 3 million[92]. This large difference can be explained partially by the challenge presented by the "unculturable species", since insolation into pure cultures is always the gold standard for identifying, describing, and sequencing new species.

Other OMICs studies can help to unravel which enzymes are being excreted or which proteins are being produced in the different environments or situations where fungi survive, defined as proteomic studies. OMICs studies would also help to associate metabolites to the biosynthetic gene clusters, that in fungi are still understudied as well, these are defined as metabolomic studies.

4. Objectives of this PhD

Nowadays there is a search for sustainable approaches to reduce the amount of (micro)plastics present in the environment, especially in the marine one, and biodegradation by microorganisms has been pointed as an interesting solution.

Considering plastic biodegradation topic, fungi appear to be good candidates. Also, OMICs technologies seem useful to better understand (micro)plastics biodegradation and how it can be applied in the environment.

So, the work presented here had the following aims:

- To uncover genes encoding for proteins associated with biodegradation of plastics and to understand the biotechnological potential of *Penicillium brevicompactum* and *Zalerion maritimum*, through the sequencing and annotation of their genomes;
- To optimize the chemical composition of the culture medium for the biodegradation of (micro)plastics using *Z. maritimum*, through the application of experimental design methods;
- To unravel the proteins involved in the biodegradation of (micro)plastics, through the study of fungal proteome profile grown in contact with (micro)plastics;
- To study the application of these fungi and their ability to degrade (micro)plastics in environmentally relevant conditions. As well as to understand the toxicity of (micro)plastics, using model organisms.

5. References

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II. Complete genome sequence of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329, two fungi with potential for biodegradation of microplastics

Abstract

Penicillium brevicompactum CMG 72 and *Zalerion maritimum* ATCC 34329 have shown potential to use plastic as a carbon source. Genomic sequencing (Illumina NovaSeq) of both these species was made, as they appear to be great candidates to reduce the amount plastics in the environment. These genomes annotations will serve as important references for further OMICs studies regarding the ability of both fungi to biodegrade plastics and for further studies in biodegradation of plastics. *Penicillium brevicompactum* CMG 72 genome has a size of 31.1 Mb, a G+C content of 49.22% and 10917 predicted genes. Analyses found 332 genes encoding for carbohydrate-active enzymes (CAZymes) and 38 regions predicted as secondary metabolite biosynthetic gene clusters (BGCs). *Penicillium brevicompactum* CMG 72 genome is slightly smaller than other known genomes of *P. brevicompactum* CMG 72, but in general the statistics are very similar. In the case of *Z. maritimum* ATCC 34329 the genome has a size of 58.4 Mb, a G+C content of 44.39% and 10802 predicted genes[1]. In addition, 491 genes encoding for CAZymes were identified and 16 regions were matched with BGCs. Both genomes have genomic features that indicate the potential of these fungi for bioremediation processes, such as genes encoding for laccases, cutinases, monooxygenases, among others encoding for proteins involved in pathways responsible for the degradation of chemical compounds.

1. Introduction

Genomic and functional information provide insights into the biodegradation potential of microorganisms, serving also as a basis for future OMICs' analysis[2].

1.1. *Penicillium brevicompactum*

Penicillium species are very common, being found in a large range of environments, from water to soil, from indoor environments to food[3,4].

Thanks to the vast diversity of their secondary metabolites, the species of this ascomycete genus have applications in different economic areas, such as in the pharmaceutical industry thanks to the production of penicillin[4], and in the food industry where they are used to produce of diverse kinds of cheese and fermented sausages[3].

Despite this, the main function in nature of *Penicillium* species is the decomposition of organic materials. As natural decomposers and due to the production of a wide range of mycotoxins, they may also cause negative economic impacts, by affecting food[3].

Several *Penicillium* species have already been characterized considering their potential for the degradation of plastics[5]. For some, their degradability capacity was shown. Some authors showed that *Penicillium brevicompactum* is able to adhere to polyethylene plastics[6] and others have shown the propensity of this fungus to biodegrade polyvinyl alcohol[7]. *Penicillium brevicompactum* CMG 72 has been studied in our lab for the degradation of a mulch biofilm, showing the possibility of degrading plastics in soil[8] and in freshwater, it has also been studied for the biodegradation of PE and fiber from facemasks, as presented in this thesis, Chapter V.

Penicillium brevicompactum is also interesting as a producer of bioactive compounds, such as mycophenolic acid[9]. So, this would be an excellent species to be applied in a system of plastics removal, given that it grows in different situations and environments, it has an affinity with plastics, and it may produce bioactive compounds after the biodegradation.

1.2. *Zalerion maritimum*

The marine fungus *Zalerion maritimum* (or *Zalerion maritima*), was defined in 1963 by Anastasiou[10] and first described as *Helicoma maritimum* by Linder in 1944[11]. It is characterized by its beige to black ostiolate ascomata, depending on the pH and nutrient source of the medium[12,13].

Different studies since the 1970s, have shown that this marine fungus has the ability to degrade different compounds. For example, as a naturally occurring fungus on floating wood, *Z. maritimum* can degrade lignin, from hardwood or softwood, to CO_2 and other water-soluble products[14]. Sguros and Quevedo[15] also demonstrated the predisposition of *Z. maritimum* to use Aldrin and Dieldrin (two water insoluble pesticides) as substrate. Jones and Le Campion-Alsumard[16], in 1968, found submerged in the sea, polyurethane colonized by *Z. maritimum*, showing the capacity of this fungus to use plastic as a nutrient source. Later, in 2016, Paço et al.[17] studied *Z. maritimum* in contact with polyethylene and demonstrated that this fungus can remove, in one month, up to 60% of the microplastics from the medium. The ability of *Z. maritimum* to degrade fiber from facemasks was also studied and it is presented in this thesis, Chapter V. Based on these studies, it is possible to assume that *Z. maritimum* may be a promising biodegradation agent in the removal of plastics present in the environment, especially in the marine one.

1.3. Objectives

To better understand the biotechnological potential of *P. brevicompactum*, the genome of *P. brevicompactum* CMG 72 was sequenced and analyzed. Moreover, a comparative analysis with the annotations already reported and available for other *P. brevicompactum* strains, in JGI Genome Portal database, and the annotation from our strain were made.

Despite the interesting properties of *Z. maritimum*, there is no genomic information for this fungus, or from other species of the genus *Zalerion*. So, here, it is reported the first whole genome sequencing and gene annotation of *Z. maritimum* ATCC 34329.

2. Material and methods

2.1. Fungal strain

Penicillium brevicompactum CMG 72 was isolated in our lab, from a contaminated culture of *Zalerion maritimum*. The identification of the strain was confirmed through a phylogenetic analysis of sequences of the internal transcribed spacer region (ITS) of the ribosomal RNA gene cluster. Briefly, the PCR amplification conditions of the ITS region were as described by Gonçalves et al.[18]. ITS sequence from *P. brevicompactum* CMG 72 was aligned with sequences from several *P. brevicompactum* species retrieved from GenBank using ClustalX version 2.1[19] and a Maximum Likelihood (ML) analysis was done using MEGA7[20]. ML analysis was performed on a neighbour-joining (NJ) starting tree automatically generated by the software. Nearest-neighbour-interchange was used as the heuristic method for tree inference with 1000 bootstrap replicates.

Zalerion maritimum ATCC 34329 was obtained in 2017 from the ATCC culture collection and maintained in our lab since then.

2.2. Culture conditions and DNA extraction

The fungi were cultured in Erlenmeyer flasks containing 50 mL of Potato Dextrose Broth (Merck, Darmstadt, Germany) at 25 ^{o}C, without agitation for 4 days, in the dark. Two mycelium-colonized agar plugs were used to inoculate each Erlenmeyer flask. Mycelia was filtered through sterile paper and ground in liquid nitrogen. Afterwards genomic DNA was extracted using the Guanidinium thiocyanate method[21].

DNA's quality was checked on 0.8% of agarose gel and its purity was analyzed, based on the 260/280 nm ratio, with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3. Genome sequencing and assembly

Genomic DNA, 100ng, was sequenced using the Illumina NovaSeq 6000 XP system in paired-end 150-bp (PE150) format with an S2 flow cell; the libraries were constructed by Eurofins (Brussels, Belgium) by "combining 3–4 paired-end shot gun libraries with 2 long jumping distance library."[22]

The quality of the raw sequenced data generated was analyzed using the program FastQC[23] and the assembly was conducted using SPAdes[24] (v.3.14) with default parameters. After assembly, the nuclear genome was analyzed with the web-application Quast[25], to assess the quality of the assembled genome and to obtain information about the size of the genome.

2.4. Gene prediction, annotation and functional analysis

Augustus web[26] was used for gene prediction, and it was trained[27] on the optimized algorithm for *Penicillium nalgiovense* in the case of P*. brevicompactum* and on the optimized algorithm for *Xylaria grammica* in the case of *Z. maritimum*.

Functional analyses were made using the web-based application dbCAN[28], that allowed the prediction of carbohydrate-degrading enzymes (CAZymes) using default settings. Using the web-based application antiSMASH version 5.0[29], the presence of Biosynthetic Gene Clusters (BGC) involved in secondary metabolism was screened, using a strictness "relaxed" option for the detection of well-defined and partials clusters containing the functional parts. Finally, the software OmicsBOx (1.4.12) was used to annotate genes using Blast2GO against different databases, using a blast alignment set to $1e^{-3}$. Blast2Go used NCBI's nonredundant protein (Nr), InterPro, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggnog), where there is also the information from the original COG/KOG database (eukaryotic cluster of Orthologous Groups of proteins), databases and Gene Ontology (GO) analysis.

2.5. Repetitive elements

Dispersed Repeat sequences (DR) were identified using OmicsBox (1.4.12) with the repeat masking option. Tandem Repeat sequences (TR) were identified using the web-application Tandem Repeats Finder[30]. The tRNAscan-SE tool was used, with default parameters for the identification of tRNAs[31].

2.6. Comparative analysis

The genome of *P. brevicompactum* CMG 72 was compared to the genomes of *P. brevicompactum* 1011305 and *P. brevicompactum* AgRF18, to evaluate possible genetic and metabolic diversity between strains. The information used was available in JGI Genome Portal database, such as statistics for the genome assembly, e.g., genome size and G+C content. The analysis were performed using the web-application Quast[25] and Icarus[32], a genome visualizer based on the Quast tool. Gene arrangement and variations between the three genomes were further analyzed by sequence alignment in Geneious Prime (2023.1.2) using the progressive Mauve[33] algorithm in the Mauve plugin[34] (1.1.3). Clinker[35] was used to generate the gene cluster comparison figures.

3. Results and discussion

3.1. Fungal strain identification

Phylogenetic analysis of ITS region showed that strain CMG 72 belongs to the *Penicillium brevicompactum* species. As can be seen in Figure 1, CMG 72 groups in the same clade as strain NRRL 2011 which is the ex-type strain of *P. brevicompactum*.

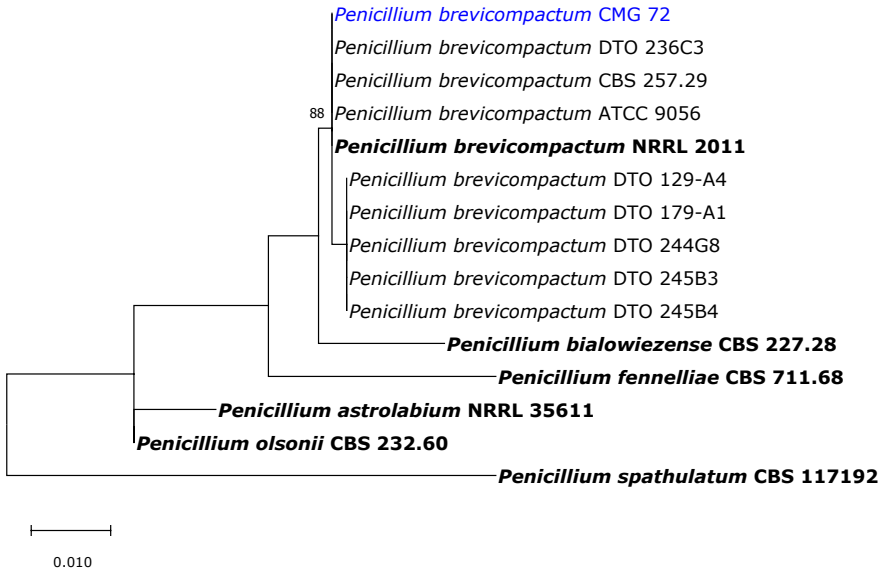

Figure 1 - Phylogenetic analysis of strain CMG 72. ITS sequences of CMG 72, and of several *Penicillium* species. Tree was constructed using the Maximum Likelihood method the Kimura 2-parameter model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site and rooted to *Penicillium spathulatum* (CBS 117192). Bootstrap values (≥ 85%) are shown at the nodes. Ex-type strains are in bold and the sequence used in this study is in blue.

3.2. Genome sequencing, assembly data and genomic characteristics

3.2.1. *Penicillium brevicompactum*

Table 1 presents the general data from the draft genome of *P. brevicompactum* CMG 72. It is a genome with a total length of 31.1 Mb, assembled in 240 contigs, with a size >=500 bp being the largest one 1505776 bp. The G+C content of the assembly is 49.22%, an expected value for a fungal genome, as it can commonly vary from 30 to 63%[36].

The assembly quality can be assessed based on three aspects: contiguity, completeness and correctness[37]. In terms of contiguity, that characterizes the effectiveness of the assembly, we can use the N50 as a measure, unfortunately, this will only be comparable with other genomes of similar size[25,37,38]. In this case, the N50 is 593622 bp, which means that when we align all contigs from the smallest to the one with 593622 bp, they will contain 50% of the total assembly length. For completeness, which gives an idea of the presence or absence of highly conserved genes, the most used measure is BUSCO (Benchmarking Universal Single-Copy Orthologs), where a higher score means that the genome contains a high number of highly conserved genes[25,37,38]. For the assembly of *P. brevicompactum* CMG 72 genome, we have obtained a BUSCO score of 99.01%. Correctness it is an aspect difficult to measure when sequencing new species, since it is normally defined as the concordance between the assembly and a reference.

A total of 10917 coding sequences were predicted, that represents a total length of 16.9 Mb. These are normal values for fungi, as some studies indicate that species from this kingdom may have between 9511 to 39074 protein-coding genes[39]. Furthermore, it appears to be an expected value for *Penicillium* species, where the number of protein-coding genes varies between 9000 and 12000[40,41].

Table 1 – Characterization of *Penicillium brevicompactum* CMG 72 genome assembly. Data were generated using the web-application Quast.

Characteristic	Value
Total assembly lenght (bp)	31099894
Number of contigs (>=500 bp)	240
Coverage (x)	220
N50 (bp)	593622
N90 (bp)	132764
L50 (bp)	19
L90 (bp)	57
G+C content (%)	49.22
BUSCO completeness (%)	99.01
Number of genes predicted	10917
Total length of predicted genes (bp)	16920327
Average length of predicted genes (bp)	1550
Predicted genes (%)	54.41

3.2.2. *Zalerion maritimum*

The genome statistics regarding the genome of *Z. maritimum* ATCC 34329 are presented in Table 2. The genome has a total length of 58.4 Mb, assembled in 2208 contigs, with a size >=500 bp, the largest of which comprises 455379 bp. The G+C content of the assembly is of 44.39%, a value considered normal for a fungal genome, as it normally varies between 30 and 63%. The quality of an assembly can be assessed based on three aspects: contiguity, completeness and correctness[37]. The N50 is used as a measure for contiguity, which characterizes the effectiveness of the assembly, unfortunately this value is only comparable with genomes that present the same size[25,37,38]. In our assembly, the N50 is 66184 bp, which means that when we aligned all contigs from the smallest to the one with 66184 bp, they will contain 50% of the total assembly length. BUSCO is commonly used for completeness, which gives an idea of the presence or absence of highly conserved genes, so a higher score means that the genome contains a high number of highly conserved genes[25,37,38]. In this case the BUSCO score obtained was 98.35%. Furthermore, a total of 10802 gene-coding sequences were predicted, and they present a total length of 16.6 Mb. These are normal values for fungi, as some studies indicate that species from this kingdom may have between 9511 to 39074 protein-coding genes[39].

Table 2 - Characterization of *Zalerion maritimum* ATCC 34329 genome assembly. Data were generated using the web-application Quast.

Characteristic	Value
Total assembly lenght (bp)	58434198
Number of contigs (>=500 bp)	2208
Coverage (x)	126
N50 (bp)	66184
N90 (bp)	14653
L50 (bp)	242
L90 (bp)	962
G+C content (%)	44.39
BUSCO completeness (%)	98.35
Number of genes predicted	10802
Total lenght of predicted genes (bp)	16598638
Average length of predicted gene (bp)	1537
Predicted genes (%)	28.41

3.3. Repetitive sequences and tRNAs prediction

In a genome the repetitive sequences can be divided in two different categories: Tandem repeats (TRs), when they come right after one another, and Interspersed Repeats (IRs), when they are dispersed throughout the genome and not near each other. Table 3 presents characterization of repetitive sequences and noncoding RNA, for *P. brevicompactum* CMG 72. In this genome there are 3302 TRs and 5064 IRs, other *Penicillium* species have a bigger representation of repetitive sequences in their genome[41]. The low number of repetitive sequences in this genome may be because our assemble software assumed similar repetitive motifs and assembled then together as an only contig[42].

Regarding tRNAs, 218 were predicted, which correspond to the 20 common amino acid codons, and of the 218, 15 appear to be pseudogenes. Other *Penicillium* species are said to have about 200 tRNAs[40,41] encoded in their genome, making this a normal amount.

Table 3 - Characterization of repetitive sequences and noncoding RNAs of *Penicillium brevicompactum* CMG 72 genome. Data were generated using the tRNAsacn-SE and Tandem Repeats Finder software.

Type	Number	Total Length (bp)	Percentage in Genome (%)
Interspersed Repeat (total)	5064	228330	0.7342
Unclassified	4	355	0.0011
Simples Repeats	4131	174835	0.5622
Low Complexity	748	34970	0.1124
Satellites	34	2934	0.0094
SINEs	12	692	0.0022
LINEs	15	785	0.0025
LTRs	64	5387	0.0173
DNA transposons	31	1874	0.0060
Rolling-circles	2	79	0.0003
Small RNA (snRNA, rRNA)	23	6419	0.0206
tRNAs (15 pseudo)	218	19322	0.0621
Tandem Repeat	3302	234284	0.7533

In the genome of *Z. maritimum* ATCC 34329, as seen in Table 4, there are 17108 TRs and 38079 IRs. Regarding tRNAs, 76 were predicted, corresponding to the 20 amino acid codons, and 3 of the 76 tRNAs predicted, appear to be pseudogenes. This number seems low and unexpected, based on the information for *Xylaria grammica*[43], but studies indicate that this may range from 24 to over 4000[44].

Table 4 – Characterization of repetitive sequences and noncoding RNAs for the *Zalerion maritimum* ATCC 34329 genome. Daat were generated using the tRNAsan-SE and Tandem Repeats Finder software.

Type	Number	Total Lenght (bp)	Percentage in Genome (%)
Interpersed Repeat (total)	38079	1904098	3.2585
Unclassified	10	869	0.0015
Simples Repeats	32571	1370361	2.3451
Low Complexity	4227	207968	0.3559
Satellites	31	1766	0.0030
SINEs	4	224	0.0004
LINEs	76	3789	0.0065
LTRs	562	85509	0.1463
DNA transposons	309	15932	0.0273
Rolling-circles	5	366	0.0006
Small RNA (snRNA, rRNA)	284	217314	0.3719
tRNAs (3 pseudo)	76	6831	0.0117
Tandem Repeat	17108	1563155	2.6751

3.4. Genome annotation

3.4.1. *Penicillium brevicompactum*

The genome of *P. brevicompactum* CMG 72 has 10609 genes according to the NCBI's nonredundant protein (Nr) database, meaning that only 2.82% of the protein coding sequences had no assigned name (Table S1). Against InterPro database, 8940 genes were associated with specific domains or families. The analysis with Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed 125 proteins involved in different pathways (Table S2). Using Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggnog), where there is also the information from the original COG/KOG database (eukaryotic cluster of Orthologous Groups of proteins), 8548 contigs were predicted and had terms assigned (Table S3).

These databases identified proteins already associated to *P. brevicompactum* and their bioactivity potential. For example, L-asparaginase[45] an enzyme used in pharmaceutical and food industry[46]. It belongs to an important group of therapeutic enzymes applied to the treatment of patients with lymphoblastic leukemia[47] and lymphoblastic lymphoma[48], since it reduces the amount of asparagine in the patient body, what in turn affect negatively the neoplastic cells. In the food industry, this enzyme is used, especially in deep fried recipes, to reduce a number of precursors for Millard reaction and consequently reduce the formation of acrylamides, carcinogenic compounds[46]. Proteins involved in the production of Mycophenolic acid[49] and in the production of Adenophostin[50], were also identified. Adephostin is an inositol trisphosphate receptor agonist, because despite being structurally different from inositol triphosphate is more potent and can control calcium channels[51].

Furthermore, enzymes and proteins that have been proposed to be involved in plastic biodegradation were also identified and annotated through these databases. Examples are laccase, monooxygenases, cytochrome P450s, cutinase, among others[52–54]. Laccases are multi-copper oxidases that can oxidase a large number of phenolic and non-phenolic compounds and have low substrate specificity[55] and it is the first enzyme associated with polyethylene biodegradation by fungi[56]. Monooxygenases alter organic substrates by catalyzing the insertion of one oxygen atom[57], the styrene monooxygenase has been associated to the degradation of polystyrene and the alkane monooxygenase to the

degradation of polyethylene by fungi[58]. Cytochrome P450s are a superfamily of important enzymes that can function as monooxygenases, but also as other types of enzymes, they act as "cleaners" of chemical compounds in the organisms[59], and they have been proposed to be involved in the metabolic pathways for the degradation of plastics, as they can break C-C bonds[60]. Cutinases are enzymes involved in the breakdown of cutin, also known as cutin hydrolases[61], that can hydrolyze carboxylate exters[62], and is believed to be involved in the degradation of polyethylene-terephthalate[63].

In turn, KEGG analysis revealed pathways involved in the degradation of chemical compounds, such as styrene and toluene, which also shows the potential for degradation of plastics by this fungus, especially polystyrene. Since the cleavage of this polymer can generate aromatic monomers like styrene and toluene[58]. KEGG analysis also identified the Citric acid cycle (TCA cycle) a potential final part of the metabolism involved in the biodegradation of plastics, where the final oxidized products are converted in to energy[53].

Gene Ontology (GO) analysis assigned GO terms to 6138 genes, which represents 56.22% of the genes present in the genome. This 6138 are distributed in three functional categories: molecular function, cellular components, and biological process. 3522 sequences were associated to biological process, 86.40% of that are related to cellular process, 25.44% to localization, 67.26% to metabolic process and 15.33% to biological regulation. 3186 sequences were linked to prediction of cellular component, 1334 sequences are associated to organelle, 1560 sequences to intracellular anatomical structure, 1906 sequences to membrane and 488 sequences to protein containing complex. 4398 sequences were associated to molecular function, this category can be divided in catalytic activity, and characterize 61.48% of these sequences, binding activity, accounts for 50.64% of these sequences, and finally transporter activity, 13.78% of these sequences.

3.4.2. *Zalerion maritimum*

The genome of *Z. maritimum* ATCC 34329 has 8858 protein coding genes according to NCBI's nonredundant protein (Nr) database corresponding to 82% of the protein coding

sequences (Table S4). Against InterPro database, only 927 gene-coding sequence had no match, all the other 9875 were associated with specific domains or families (Table S4). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed 115 proteins involved in different pathways (Table S5). With the Evolutionary Genealogy of Genes: Non-supervised Orthologous Groups (eggnog), where there is also the information from the original COG/KOG database (eukaryotic cluster of Orthologous Groups of proteins), was possible to predict and assign terms to 7563 of the contigs (Table S6).

By comparison against the NR database and using EggNOG, proteins already associated with biodegradation of plastics were identified and annotated, such as laccase and its precursor, monooxygenase, cutinase, and cytochrome P450, among others[52–54]. Laccases are multi-copper oxidases that can oxidase a large number of phenolic and non-phenolic compounds and have low substrate specificity[55] and it is the first enzyme associated with polyethylene biodegradation by fungi[56]. Monooxygenases alter organic substrates by catalyzing the insertion of one oxygen atom[57], the styrene monooxygenase has been associated to the degradation of polystyrene and the alkane monooxygenase to the degradation of polyethylene by fungi[58]. Cytochrome P450s are a superfamily of important enzymes that can function as monooxygenases, but also as other types of enzymes, they act as "cleaners" of chemical compounds in the organisms[59], and they have been proposed to be involved in the metabolic pathways for the degradation of plastics, as they can break C-C bonds[60]. Cutinases are enzymes involved in the breakdown of cutin, also known as cutin hydrolases[61], that can hydrolyze carboxylate exters[62], and is believed to be involved in the degradation of polyethylene-terephthalate[63].

In turn KEGG analysis revealed pathways involved in the degradation of chemical compounds, such as the degradation pathway of atrazine, a highly used pesticide, and the TCA cycle. The TCA cycle is a potential final part of the metabolism involved in the biodegradation of plastics, where the final oxidized products are converted in to energy[53].

Using Gene Ontology (GO) analysis assigned GO terms to 4932 genes, which accounted for 45.66% of the genome distributed in three functional categories: molecular function,

cellular components, and biological process. 1726 sequences were associated to biological process, of that 1531 are related to cellular process, 408 to localization, 1244 to metabolic process, 206 to biological regulation. 1621 sequences were linked to prediction of cellular component, 46.27% of that are associated to organelle, 56.32% to intracellular anatomical structure, 55.95% to membrane and 19.68% to protein containing complex. 2162 sequences were associated to molecular function, this can be divided in binding activity (1135 sequences) and in catalytic activity (1468 sequences).

3.5. Carbohydrate-active enzymes and Biosynthetic gene clusters

3.5.1. *Penicillium brevicompactum*

A total of 709 putative CAZymes were identified in the genome of *P. brevicompactum* CMG 72 (Table S7). This web-based application uses three annotation methods, HMMER, DIAMOND and Hotpep, and for 332 genes, the prediction was the same for the three of them. Of them, 195 were matched with Glycoside Hydrolases (GH), 69 were identified as Glycosyl Transferases (GT), 46 genes were associated with Auxiliary Activities (AA), 12 encode for Carbohydrate Esterases (CE), 11 for Carbohydrate-binding Module (CBM), and 10 as Polysaccharide Lyases (PL).

GH families were the most abundant, and 59 different families were matched to the genes of *P. brevicompactum* CMG 72. GH3 family (β-glucosidades) was the most common, followed by GH13 family (amylases), GH18 family (chitinases), GH5 family (cellulases), among others. Regarding GT families, 26 different ones were identified, and the most abundant was GT2 characterize by cellulose/chitin synthase, followed by GT1 family (UDP-glucuronosyltransferase) and GT90 (xylanase).

In *P. brevicompactum*'s genome 8 different families from AA were identified, being the most common AA3 family, where is possible to find the cellobiose dehydrogenase, followed by AA1 family, an important family for the biodegradation of plastic, since here is included laccase, as explained before. In the case of CE families were also 8 identified, like CE8 that is characterized by pectin methylesterase, involved in the removal of methyl-groups, and for this reason can be associated with the degradation of some kinds of plastics, like polyhydroxyalkanoates (PHA) or polypropylene[64].

For CBM families, only 6 were identified and only 5 PL families.

AntiSMASH identified 185 regions containing clusters candidates, and of them a total of 38 were predicted as biosynthetic gene clusters (BGCs) involved in the secondary metabolism of *P. brevicompactum* CMG 72. BGCs are defined as a group of two or more genes clustered together in a genome, that encode for proteins involved in a specific biosynthetic pathway, of which the product is a specialized metabolite[65]. Besides the biosynthetic genes, a BGC also have genes for expression control, self-resistance and export the compound encoded by them[66].

The identified regions encode 19 t1PKs (type 1 polyketide synthases), 12 NRPSs (non-ribosomal peptide synthase), 2 betalactones and 2 are involved in the production of terpene. The remaining 3 gene clusters can be involved in the biosynthesis of compounds that are NRPS and indole or NRPS and T1PKS. From the BGCs identified, 5 BGCs have 100% similarity with known BGCs, such the BGC involved in the synthesis of Mycophenolic Acid[49], an immunosuppressant used to prevent rejection of transplant organs[67], the BGC involved in the synthesis of Pyranonigrin E, an antioxidative compound[68], the BGC involved in the synthesis of Alternariol, a mycotoxin that have shown potential pharmacological effects[69,70], the BGC involved in the synthesis of Dimethyl coprogen, a siderophore for ferric iron[71], and the BGC involved in the synthesis of Naphthopyrone, a class of pigments that have several applications, especially in the pharmaceutical industry[72].

3.5.2. *Zalerion maritimum*

A total of 491 putative CAZymes were identified in the genome of *Z. maritimum* ATCC 34329 (Table S8). The web-based dbCan uses three different annotation methods, HMMER, DIAMOND and Hotpep and for 225 genes, the predictions obtained in the three databases were the same. For the 225, 122 were matched with Glycoside Hydrolases (GH), 57 were identified as Glycosyl Transferases (GT), 25 genes were associated with Auxiliary Activities (AA), 12 were matched as Carbohydrate Esterases (CE), 8 as Polysaccharide Lyases (PL) and 1 was associated as Carbohydrate-binding Module (CBM).

Among the CAZymes, the GH families were the most abundant and 49 different ones were associated with genes of *Z. maritimum*. The most common was GH3 family (β-glucosidades), followed by the GH47 family (α-mannosidase) and GH5 family (cellulases). 26 different GT families were identified, the most abundant were GT2 family (cellulose/chitin synthase) and GT90 (xylanase). 7 AA families were identified, and the most common were AA3 family (cellobiose dehydrogenase) and AA1 family, a family worth mentioning in the study of biodegradation of plastic, since here is included laccase that is related, as explained before. In the case of CE families 8 were identified, among them is possible to find CE8 that is characterized by pectin methylesterase, involved in the removal of methyl-groups, and for this reason can be associated with the degradation of some kinds of plastics, like polyhydroxyalkanoates (PHA) or polypropylene[64]. Finally, the genes were matched to 4 PL families and 1 CBM family.

Regarding secondary metabolism, antiSMASH identified 16 regions as biosynthetic gene clusters (BGCs). A group of two or more genes clustered together in a genome forms a BGC. They normally encode proteins involved in a specific biosynthetic pathway, of which the product is a specialized metabolite[65]. A BGC contains genes for expression control, self-resistance and export, besides the gene encoding for compound[66]. The identified regions encode for 1 T3PKS (type 3 polyketide synthases), 3 T1PKS (type 1 polyketide synthases), 5 NRPS (non-ribosomal peptide synthase), 2 terpenes, 2 fungal RiPPs (fungal ribosomally synthesised and post-translationally modified peptides). Furthermore, 1 BGC can be involved in the biosynthesis of compounds that are NRPS and indole and 2 in the production of compounds that are NRPS and T1PKS.

Some BGCs were possible to associate with already described BGCs, for example a BGC from *Z. maritimum* genome was matched with 100% similarity with the BGC involved in the synthesis of Choline. Choline is an essential nutrient for us humans, as it is needed for neurotransmitter synthesis, lipid transport, cell-membrane signaling, and methyl-group metabolism[73]. Another BGC identified in *Z. maritimum* genome was matched with 100% similarity with the BGC involved in the synthesis of the 1,3,6,8-tetrahydroxynaphthalene. 1,3,6,8-tetrahydroxynaphthalene is an indispensable precursor to an integral component of the conidial cell wall surface[74].

3.6. Comparative analyses of *Penicillium brevicompactum* genomes

3.6.1. General features

The JGI Genome Portal has the sequence of the genomes *P. brevicompactum* 1011305 and *P. brevicompactum* AgRF18. The genome assembly and characterization of both of these strains are presented in Table 5. *Penicillium brevicompactum* 1011305 and *P. brevicompactum* AgRF18 genomes are slightly bigger than the genome of *P. brevicompactum* CMG 72, having 32.1 Mb and 31.6 Mb, respectively.

All three strains similar G+C content, ranging from 48.97% to 49.22%. *Penicillium brevicompactum* AgRF18 has more protein coding genes (12343), than the remaining strains, it has 6.5% more coding genes than *P. brevicompactum* 1011305 and 11.6% more than *P. brevicompactum* CMG 72. On the other hand, *P. brevicompactum* CMG 72, the strain sequenced in this work is the one with the most annotated sequences.

Using Quast tool and the genome of *P. brevicompactum* CMG 72 as a reference, multiple comparative analyses were made between the genomes of the three strains (Table 5). The genome of *P. brevicompactum* 1011305 has a percentage of similarity with the genome of *P. brevicompactum* CMG 72 of 94.1% and a duplication ratio of 1.003, which indicates high resemblance between the genomes, with high sequence conservation and absence of major duplication events[25]. Between the genomes of *P. brevicompactum* CMG 72 and *P. brevicompactum* AgRF18 the percentage of the genome similarity was 94.5% and the duplication ratio 1.003, also showing that the genomes of these two strains are similar.

Orthologous regions between the genomes were identified (Figure 2). These regions are called locally colinear blocks (LCBs) and they are conserved segments that appear to be internally free of genome rearrangements, but that can be in different order in the whole genome, and even inverted[34]. The alignment of *P. brevicompactum* AgRF18 and *P. brevicompactum* CMG 72 genomes, also shows the high similarity, with several homologous regions between the two, a total of 290 LCBs. Most of the LCBs are translocated, which mean present in a different order in the genomes, and fifteen of them are inverted. In the case of *P. brevicompactum* 1011305 and *P. brevicompactum* CMG 72, the alignment of these two genomes also shows high similarity between them, with 235 LCBs, and nineteen inversions. In both cases, when analyzing each LCBs, it is

possible to see that they have a similarity always higher than 90%. The alignments are presented in Figure 2.

Table 5 – Characterization of three different strains of *Penicillium brevicompactum*. Strains 1011305, AgRF18, CMG 72 were analyzed using the web-application Quast.

Characteristic	1011305	AgRF18	CMG 72
Total assembly lenght (bp)	32108205	31630201	31099894
G+C content (%)	48.97	49.21	49.22
Number of coding genes	11536	12343	10917
Total length of predicted genes (bp)	18574246	18780704	16920327
Percentage in genome of predicted genes (%)	57.85	59.38	54.41
Number of annotated sequences	5.662	7.914	10.609

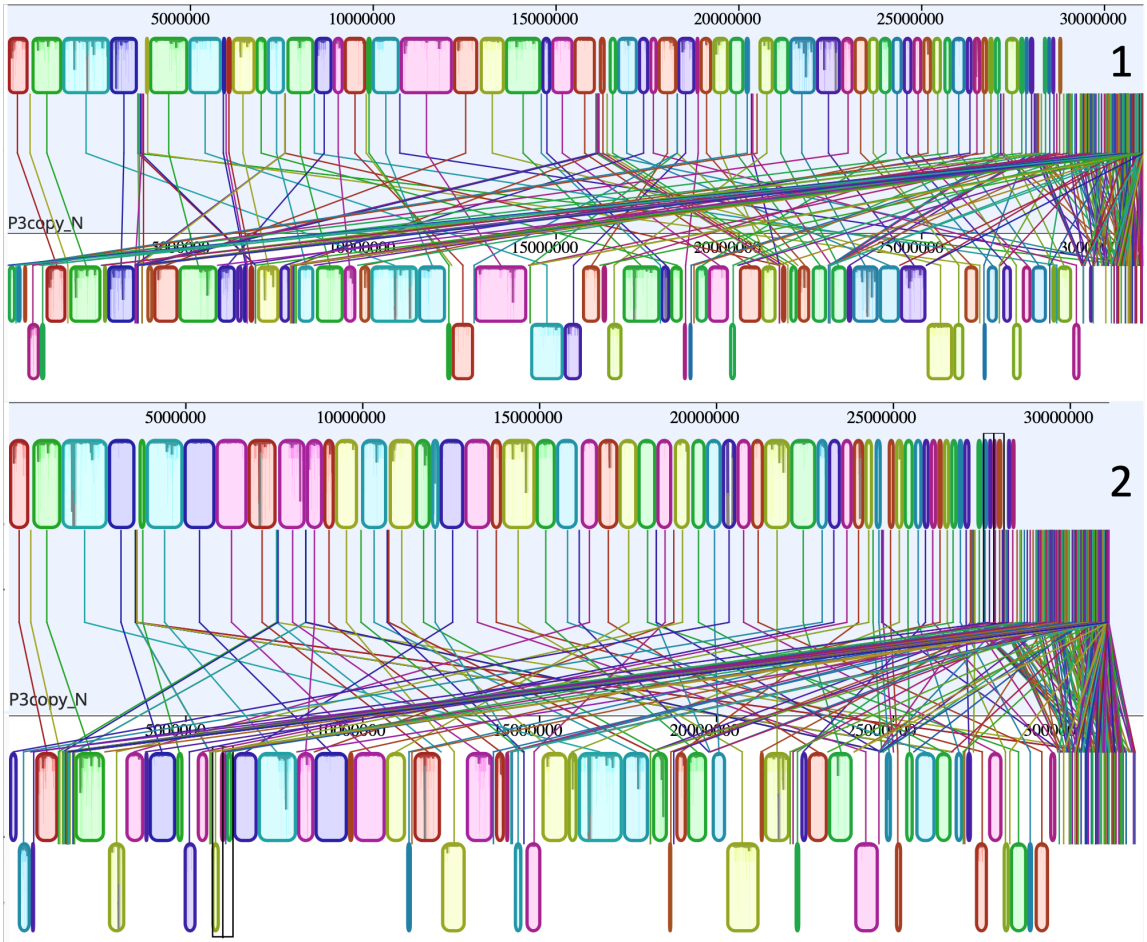

Figure 2 - Mauve alignment of the genomes *P. brevicompactum* AgRF18 and *P. brevicompactum* CMG 72 (1) and the genomes of *P. brevicompactum* 1011305 and *P. brevicompactum* CMG 72 (2). Performed with Geneious Prime.

3.6.2. Biosynthetic gene clusters

Strains, *P. brevicompactum* AgRF18 and *P. brevicompactum* 1011305 have more BGCs than strain CMG 72, forty-nine and forty-eight, respectively. Different from *P. brevicompactum* CMG 72 in the genomes from *P. brevicompactum* 1011305 and *P. brevicompactum* AgRF18, the most abundant gene are NRPS followed by T1PKS.

Figure 3 shows the similarity of known secondary metabolites BGCs from the three strains. It is possible to find similarities between the three genomes, for example clusters involved in the production of mycophenolic acid, alternariol, ankaflavin, aspercryptins, asperphenamate, endocrocin, Nidulanin a, notoamide A, Squalestatin s1 and patulin were identified in all three. Of these ten BGCs, some were detected with 100% similarity, others with lower similarity percentages. The low percentages may indicate genes partially incomplete or lost, or that they are BGCs yet to be defined.

In the case of Aspercryptins, the BGCs were identified with 26%, 33% and 40% of similarity with the known BGC involved in the synthesis of this compound (Figure 4). However, between the three we see that they are alike. Aspercryptins are linear lipopeptides defined by Henke et al.[75] as a product of a BGCs from *Aspergillus nidulans* and are the first example of peptide natural products with two lipid groups. In all three genomes, the BGCs encoding for Squalestatin S1 were identified with 60% of similarity, with the described BGC involved in the synthesis of this compound (Figure 4). This bioactive compound is an inhibitor of squalene synthase, and it is involved in the reduction of cholesterol content of cells. It has interesting applications in neuro diseases, as some studies indicate that this bioactive compound may act as a cure for prion-infected neurons and can protect against prion neurotoxicity. As mentioned before, the species *P. brevicompactum* is known to produce Mycophenolic Acid, and in the three strains this BGC can be found, but interesting enough this cluster has small differences between the three and the cluster present in GenBank, as seen in Figure 4. Mycophenolic acid act as an inhibitor of human inosine 5'-monophosphate dehydrogenase, a necessary target in immunosuppressive chemotherapy. This bioactive compound has been used since the 1900s, in immunosuppressant drugs for auto-immune disorders[49], and as referred before it is also used to prevent transplant organ rejection[76].

There was also BGCs that were detected only in one of the strains. For example, the dimethylcoprogen, the naphthopyrone and the pyranonigrin BGCs were only detected in the genome of *P. brevicompactum* CMG 72. The actinopolymorphol, the clapurines, the eupenifeldin, the penifulvin and the tryptoquialanine BGCs were only detected *P. brevicompactum* 1011305. And finally, the FR901483, the prolipyrone B, the shearinine D and the xenoacremone BGCs were only detected in *P. brevicompactum* AgRF18. This can show slightly differences in the genome, that prevent the BGCs to be detected by the AntiSmash algorithm or show how the secondary metabolite vary between strains.

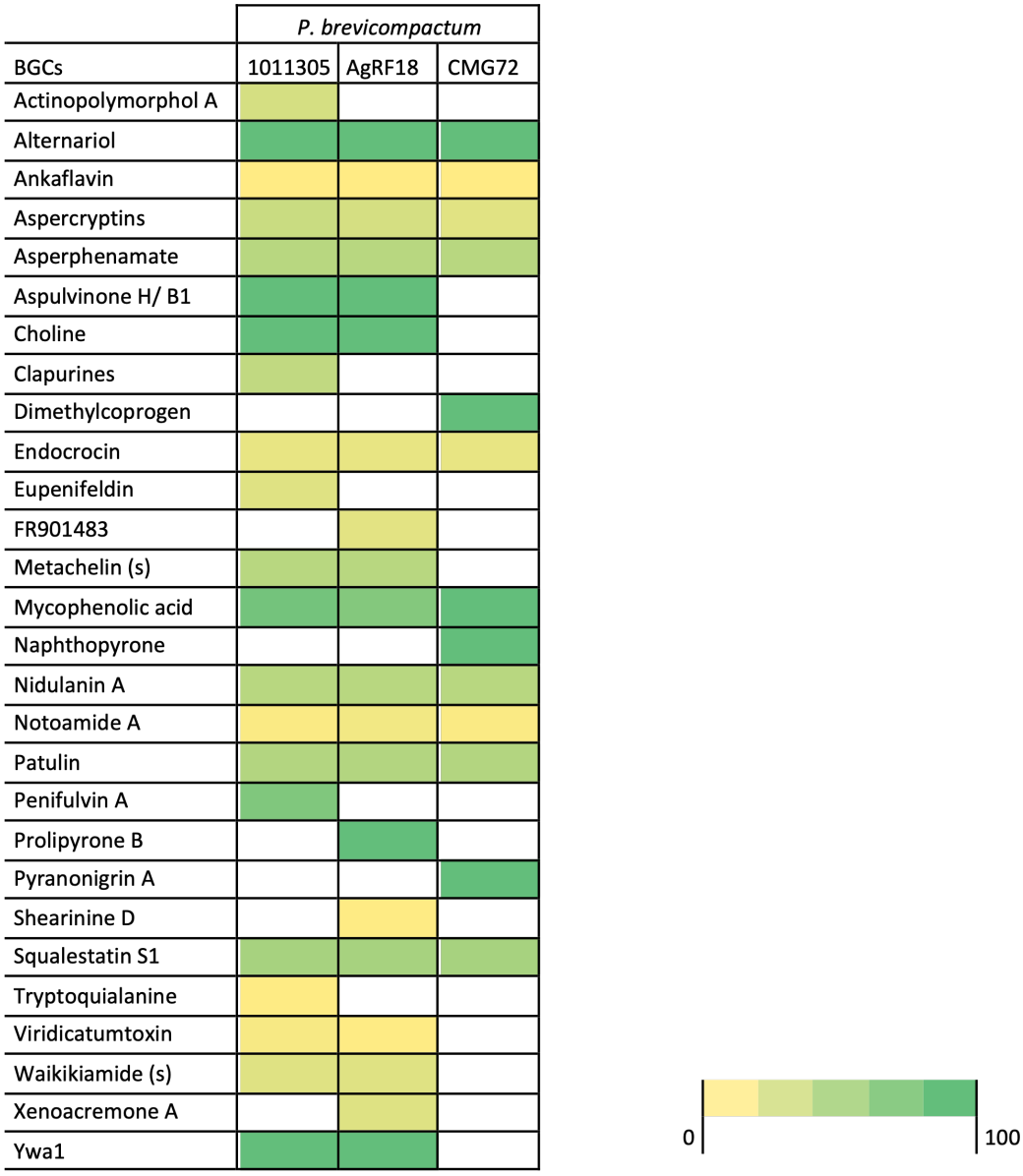

Figure 3 - Matrix indicating the similarity of secondary metabolite gene clusters of *P. brevicompactum* in relation to known clusters from the antiSMASH. The color key is given in percentage.

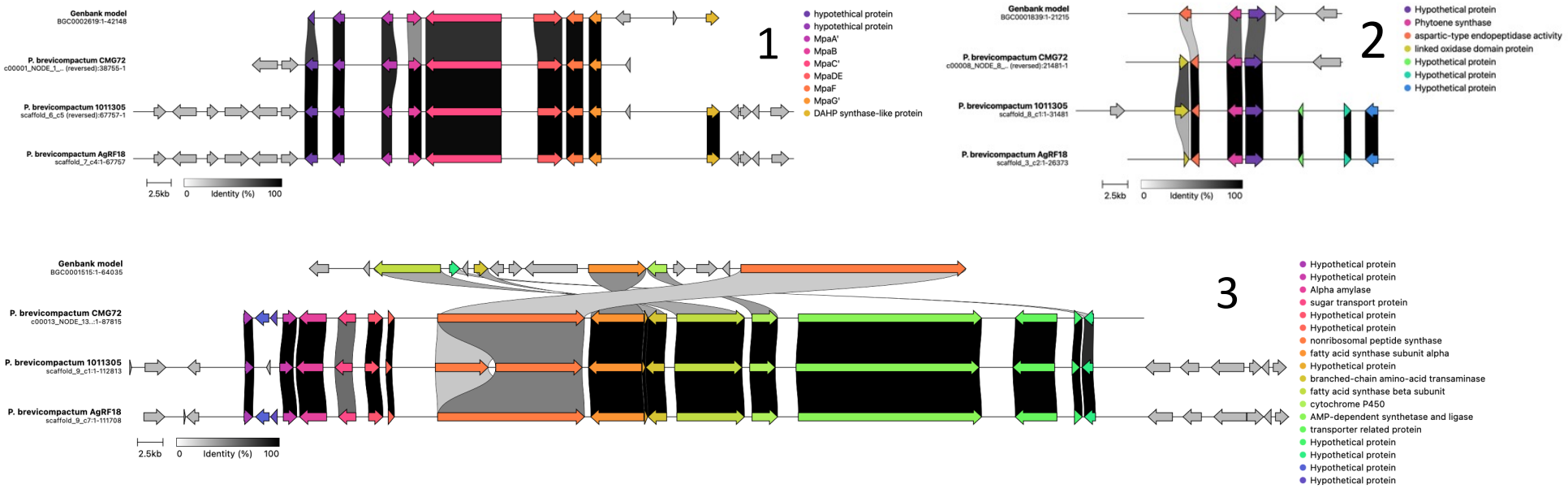

Figure 4 - Comparison of three biosynthetic gene clusters in *P. brevicompactum* strains, performed with Clinker. Image 1 represents the Mycophenolic acid BGCs, image 2 the Aspercryptins BGCs and Image 3 Squalestatin S1 BGCs.

4. Conclusions

This work characterizes the genome of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329. Both genomes include a great number of hypothetical proteins which shows the lack of sequencing data when working with fungi. Genes encoding for enzymes already associated with biodegradation of plastics were found in *P. brevicompactum* and in *Z.* maritimum. Metabolic pathways associated with bioremediation processes were also identified in both.

Moreover, these genomic annotations will serve as important references for further OMICs studies and for further studies on fungi being applied in the biodegradation of plastics. The genomic characterization of *Z. maritimum*, a marine fungus, also provides insights for future genomic analyses of marine fungi.

The genome of *Z. maritimum* and the genome of *P. brevicompactum* present biosynthetic clusters and CAZymes that demonstrate the biotechnological potential of both, as they seem to be able to produce a variety of secondary metabolites and bioactive compounds. Future work, regarding their ability to produce these compounds when in contact or after being in contact with microplastics, would be important to confirm that both are good candidates to be applied in a bioremediation process for microplastics.

Data availability

The whole-genome shotgun project for *Z. maritimum* ATCC 34329 has been deposited at DDBJ/ENA/GenBank under the accession number JAKWBI000000000. The version described in this chapter is version JAKWBI020000000. The raw sequence reads were deposited in the SRA under the accession numbers SRR18275426 and SRR18275427. The genome raw sequencing data and assembly are associated with the NCBI BioProject PRJNA810365 and BioSample SAMN26251155 within GenBank.

The whole-genome shotgun project for *P. brevicompactum* CMG 72 has been deposited at DDBJ/ENA/GenBank under the accession number JAVTHO000000000. The genome raw sequencing data and assembly are associated with the NCBI BioProject PRJNA827768 and BioSample SAMN27620207 within GenBank.

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III. Optimization of culture medium for polyethylene microplastics removal by *Zalerion maritimum* using Uniform Design

Abstract

The presence of plastics, especially microplastics, in the environment is a major problem impacting the environment. Recently, biodegradation has been suggested as a possible solution and different microorganisms have been studied for their potential capacity to biodegrade these particles. In this work, the use of marine fungi to remove microparticles of polyethylene has been improved by applying Uniform Design to optimize the concentration of three medium components (glucose, malt extract and peptone). The optimization showed that malt extract is the medium component which most influences the removal process. Medium optimal concentrations obtained were 4.47 g/L of glucose, 11.5 g/L of malt extract and 0.458 g/L of peptone. Data obtained in this work, can be applied in the development of a bioremediation process for the mitigation of plastics present in the environment.

1. Introduction

Plastic materials are highly used in our daily lives and in different areas, including healthcare, clothes, technology, food and drinking packaging, among others. They have become essential in modern life since their mass production started in 1950s[1]. Unfortunately, one of the characteristics that makes them so interesting is also the characteristic that makes them persistent in the environment, as they are extremely resistant to degradation[2]. Most of the plastics found in the environment tend to have small sizes, being characterized as microplastics when they have less than 5 mm[3]. The presence of (micro)plastic materials in the environment has several deleterious effects, and marine life is said to be the most impacted one, as it seems all plastics present in the environment tend to go to the oceans, through several mechanisms, e.g. winds and water currents[4].

There is a need to find new solutions to this problem, solutions that would help to reduce the (micro)plastics in the environment. In the context of "grey biotechnology", an interesting branch of biotechnology, that uses microorganisms or enzymes for different purposes, we have a possible solution, that is bioremediation[5]. Fungi are widely used in bioremediation processes thanks to their versatile enzymes, and their ability to biodegrade a variety of chemical compounds, including (micro)plastics[6,7]. Unfortunately, these processes are yet to be fully understood, for example it is not yet clear all the degradation pathways involved or how external conditions influence the efficacy[6]. Nevertheless, several studies already have pointed out that microbial culture medium composition has a strong influence in the bioremediation process, and in the microorganism behavior, as most of microorganisms need extra nutrients besides the contaminants to thrive[8]. *Zalerion maritimum*, has been studied for its ability to biodegrade polyethylene microplastics[7], among other contaminants[9,10]. Paço et al.[7] observed that the biodegradation by this fungus was influenced by its growth, which in turn was influenced by the culture medium composition. Therefore, to improve the bioremediation process, maximize the removal of contaminants and ensure that only essential nutrients would be added, a crucial step is to optimize the chemical composition of the culture medium.

As such, in the present work, we aimed to determine the optimum medium composition towards maximizing the biodegradation of PE microplastics by *Z. maritimum*, a marine fungus using statistical design of experiments. This will work as a first step in the development of a bioremediation process for the removal of microplastics.

2. Material and methods

2.1. Microplastics

The microplastics used were polyethylene (PE) microplastics obtained from PE pellets, acquired from Sigma-Aldrich (USA), that had spheroid morphology and approximately 2-4 mm, and were mechanically cut. To obtain microplastics with a defined size range, 1000 µm < MP < 250 µm, stainless still sieves were used.

2.2. Fungal strain and culture conditions

Zalerion maritimum ATCC 34329 was obtained in 2017 from the ATCC. The fungus was maintained, since then in our lab, in a culture medium with the composition of 35 g/L of sea salt (Sigma-Aldrich, Germany), 20 g/L of glucose (Labbox, Spain), 20 g/L of malt extract (Oxoid, United Kingdom) and 1 g/L of peptone (Sigma-Aldrich, Germany). Prior to experiments, the fungus was grown for two weeks, in the same medium, at 20 ^{o}C under stirring.

2.3. Experimental conditions

The experiments were performed in batch, using Erlenmeyer flasks with 50 mL of the culture medium defined by the experimental design, 35 g/L of salt and, approximately, 0.015 g of microplastics. Each Erlenmeyer was sterilized by autoclave and afterwards media was inoculated with, approximately, 0.50 g of fungus mycelium.

After inoculation, all the Erlenmeyer flasks were kept in a shaker at room temperature, at 120 rpm (Orbital MaxiHD OL30-HE, OVAN). After 30 days, the Erlenmeyer flasks were retrieved from the shaker and the fungus and microplastics were separated from the medium by filtration using a filter paper of 90 mm and 200 µm pore (Prat dumas, France). The recovered biomass was frozen and posteriorly lyophilized. Microplastics were kept for weighting and further analysis.

2.4. Design of experiments

The experimental design can be divided into five steps, (1) statistical design of the experiments; (2) experimental procedure; (3) analysis of the results to obtain the statistical significance of each variable; (4) performing a regression to estimate the coefficients of a mathematical model, where all variables and correlation between them are considered; (5) with the help of the mathematical model determine the optimal values[11,12].

Equation (1) presents the correlation between the response (Y) and the independent factors (x). K is the total number of independent factors, β_0 represent the intercept , i, ii, ij with β represent the coefficient values for linear, quadratic and interaction effects, respectively, x_i and x_j represent the coded levels and ε is the random error[11,12].

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

In this case, the removal percentage of PE microplastics was considered as the response, and the three medium components, glucose, malt extract and peptone were considered as independent factors or variables.

2.4.1. Optimization using uniform design

Uniform design is an experimental design developed by Fang and Wang in 1980. It is based on a number theory, where the design points scatter uniformly on the experimental domain. The number of factors and levels influence the number of experiments, and based on that the tables, developed with the theory[13], are chosen.

For this experimental design, a $U_{12}(4^3)$ matrix was used, as there were defined four levels for each factor and twelve runs. Table 1 presents the matrix with coded and non-coded values for three experimental variables, and for consequence, this table, defines the twelve experimental trials performed.

Table 1 - Uniform Design $U_{12}(4^3)$ matrix and percentage of microplastics removal for each experimental trial.

Run order	X 1 – Glucose (g/L)		X 2 - Malt Extract (g/L)		X 3 – Peptone (g/L)		Microplastics removal (%)
	Coded values	Actual values	Coded values	Actual values	Coded values	Actual values	
1	3	10	2	2	2	0.1	19.33
2	3	10	4	20	4	1	33.56
3	4	20	4	20	2	0.1	27.67
4	4	20	1	0	4	1	16.89
5	2	2	2	2	3	0.5	51.35
6	1	0	3	10	4	1	60.13
7	2	2	1	0	1	0	24.85
8	4	20	2	2	1	0	32.05
9	1	0	1	0	3	0.5	2.581
10	3	10	3	10	3	0.5	67.11
11	2	2	3	10	2	0.1	75.84
12	1	0	4	20	1	0	29.14

2.5. Software used for plots and statistical analysis

SPSS software was used for assessing the statistical significance of each variable in the data generated by the experimental model. Regression analysis was also performed in the same software, in order to obtain a second-order polynomial equation.

The 4D plots were obtained with Mathematica software.

WolframAlph website was used to solve the equation and obtain the optimal values.

3. Results and discussion

The percentages of PE microplastics removal by *Z. maritimum* obtained in each experimental trial of this experiment are presented in Table 1. These results, once again, highlight the ability of this species to biodegrade PE microplastics, as high percentages were achieved, across multiple medium compositions. Biodegradation is different depending on media composition, demonstrated by the percentages of microplastics removal ranging between 2.581% and 75.84%. Both these observations underscore the clear relevance and the necessity for a medium optimization step to enhance efficiencies in biodegradation studies.

The selected experimental design, for the medium optimization, was uniform design, chosen for its ability to provide a substantial amount of information within a small number of trials, and it also allows the exploration of relationships between the factors and the response[13]. This experimental design is also recognized for its effectiveness even when the regression model is unknown, as is the case in this work. Other authors, as Xu et al[14]., Chen et al.[15], Li et al.[16] and Mu et al.[17], have also employed uniform design to optimize the chemical composition of culture media for microorganisms, with some focusing on their application in the degradation of contaminants, and others in the production of compounds. In a previous study[18], the use of two experimental designs, uniform design and central composite design, for optimizing medium chemical composition, specifically for the removal of microplastics by a fungi, was compared. In that work, uniform design proved to be a robust and a more economical option for this type of application. It required fewer trials to achieve similar results and the same maximal value of microplastics' removal.

To explore the relation between the different medium components and the response (percentage of microplastics PE removal) statistical analyzes were performed. The results from the analysis of covariance, if a linear model is considered, are presented in Table 2. Accordingly, to the data, "glucose" is the most significant factor, as it is the one with lowest *p*-value. Indicating that, if a linear model is considered, "glucose" would be the medium compound with a bigger impact and influence on the percentages of PE microplastics removal by *Z. maritimum*. However, based on Table 2, this is not the most suitable model for the data of this experiment, since its adjusted R^2 is -0.069, a negative and low value.

Table 2 -Tests of between-subjects' effects when a linear model is considered. This model presents a R^2 of 0.252 and as adjusted R^2 of -0.069.

Source	DF	Type III Sum of Squares	Mean Square	F-value	*p*-value
Corrected Model	3	1036.867	345.622	0.785	0.539
Intercept	1	5148.593	5148.593	11.695	0.011
Glucose	1	905.824	905.824	2.058	0.195
Malt extract	1	32.098	32.098	0.073	0.795
Peptone	1	83.901	83.901	0.191	0.676
Error	7	3081.611	440.230	-	-
Total	11	21553.486	-	-	-
Corrected Total	10	4118.478	-	-	-

The best suitable model was found through regression analysis, with the help of a stepwise method. This method is characterized by first performing a test of between-subjects effects considering all interactions between the factors, followed by a successive removal of the variables with higher *p*-value, and the application of multiple regression. At the end, the model that best represents the data of this experiment was a second-order polynomial model, and it is presented in Equation (2).

In Equation (2), % of removal represents the percentage of PE microplastics removal achieved, "glucose", "peptone" and "malt extract" represent the concentration (g/L) of each medium component.

Equation (2) $\%\ of\ removal = 40.673 - 162.140 * glucose + 161.140 * malt\ extract + 1429.453 * peptone - 171.655 * malt\ extract^2 + 139.901 * glucose^2 - 51431.478 * peptone^2 + 1111.901 * glucose * peptone + 1568.252 * peptone * maltextract$

In this case, based on Equation (2), the best suitable model is a quadratic one, where only the interaction "malt extract*glucose" was removed. This implies that this interaction does not influence the removal of microplastics.

Table 3 present the test between-subjects effects when all the factors and interactions presented in Equation (2) are considered. The statistical results (Table 3) highlight the significance of the model found to be the best suitable for the data of this experiment (Equation (2)). Other information that can be obtained from Table 3 is that, despite being the most significant factor in a linear model, the factor "glucose" is not the one with lowest *p*-value, in this quadratic model.

Table 3 – Tests of between-subjects' effects when the model presented in Equation (2) is considered. This model presents a R^2 of 0.997 and an adjusted R^2 of 0.984.

Source	DF	Type III Sum of Squares	Mean Square	F-value	*p*-value
Corrected Model	8	4105.063	513.133	76.501	0.013
Intercept	1	1207.211	1207.211	179.978	0.006
Glucose	1	500.630	500.630	74.637	0.013
Malt extract	1	1511.858	1511.858	225.396	0.004
Peptone	1	264.725	264.725	39.467	0.024
Glucose*Glucose	1	442.299	422.299	65.940	0.015
Malt extract*Malt extract	1	1772.930	1772.930	264.319	0.004
Peptone*Peptone	1	540.947	540.947	80.647	0.015
Malt extract*Peptone	1	384.581	384.581	57.336	0.017
Glucose*Peptone	1	318.218	318.218	47.442	0.020
Error	2	13.415	6.708	-	-
Total	11	21553.486	-	-	-
Corrected Total	10	4118.478	-	-	-

The obtained model has an adjusted R^2 of 0.984, indicating that the model explains 98.4% of the experimental data, which is a good fit. With a *p*-value of 0.013, it proves its statistical significance, meaning that there is only a 1.3% chance that the obtained model occurred due to noise. Consequently, Equation (2) can be reliably used for predicting responses with any combination of the three variables, within the experimental range studied.

Equation (2) was used to encounter the o optimal concentrations for each medium component that would lead to a maximal percentage of microplastics removal, 100%. This was mathematically accomplished by substituting the term "% of removal" in Equation (2) with 100, resulting Equation (2) = 100 and subsequently solving it. The solution found for each variable was, approximately, 4.47 g/L of glucose, 11.5 g/L of malt extract and 0.458 g/L of peptone.

According to statistical analysis (Table 3), "malt extract" stands out as the most significant medium component, presenting one of the lowest *p*-values of the table, 0.004. The other factor with lowest *p*-value is the quadratic interaction of this same medium component, "malt extract * malt extract", emphasizing the substantial impact of malt extract. Based on Equation (2), the concentration of malt extract has a positive effect, meaning that higher concentrations of this medium component lead to higher percentages of plastic removal. This observation aligns with what was expected, as malt extract is a complete medium component, serving as a source of amino acids, peptides, proteins, nutrients, minerals, vitamins and carbohydrates, making it a source of nitrogen and carbon[19]. Malt extract is also described as an essential compound for the growth and metabolism of fungi[20]. Therefore, it can be concluded that the depletion of this component results in a lack of growth, consequently leading to a decrease in removal efficiency. Another supporting factor for this conclusion is that the optimal concentration for malt extract was 11.5 g/L, the highest concentration obtained, and this is also the one closest to the concentration in the optimal growth medium, which is 20 g/L.

In the case of "peptone", it was the factor with higher *p*-value, which was 0.024. Nevertheless, similarly to the concentration of malt extract, the concentration of

peptone has a positive impact. The concentration of peptone is statistically less significant compared to the concentration of malt extract, but it serves as a source of nitrogen like malt extract[19], although it is less complex. As mentioned earlier, malt extract contains vitamins and carbon sources and provides a slightly acidic environment favorable the growth and metabolism of fungi[21]. The difference in statistical significance may also be due to the tested concentrations, with peptone concentrations considerably lower than the interval used for the concentrations of malt extract. Alternatively, we can infer that the type of nitrogen source plays a role in the differentiation and formation of secondary metabolites[22].

Finally, the factor "glucose", has a *p*-value of 0.013 in the obtained model. Glucose, which is the typical source of carbon in fungi culture medium, exhibited a negative impact, on plastic removal. This means that the lower the concentration of glucose, a more accessible carbon source, the higher the percentages of removal would be obtained. This findings supports the idea that plastics serve as the carbon source, substituting for glucose[23]. The obtained concentration was 4.47 g/L, which is approximately one quarter of the concentration in the optimal growth medium (20 g/L).

Figure 1 illustrates the optimal region determined by graphing Equation (2). This allows to visually observe how the percentages of removal (color response) vary with the concentrations of the three variables, the medium components (x, y, z).

The optimal region, represented by colors, should be limited within the range 0 and 100. This constraint would ensure a better visualization of the model applied to this work, the percentage of removal of PE microplastics by *Z. maritimum*. Nonetheless, it is still possible to recognize the individual importance of each component, and as they play a significant role in influencing the percentage removal.

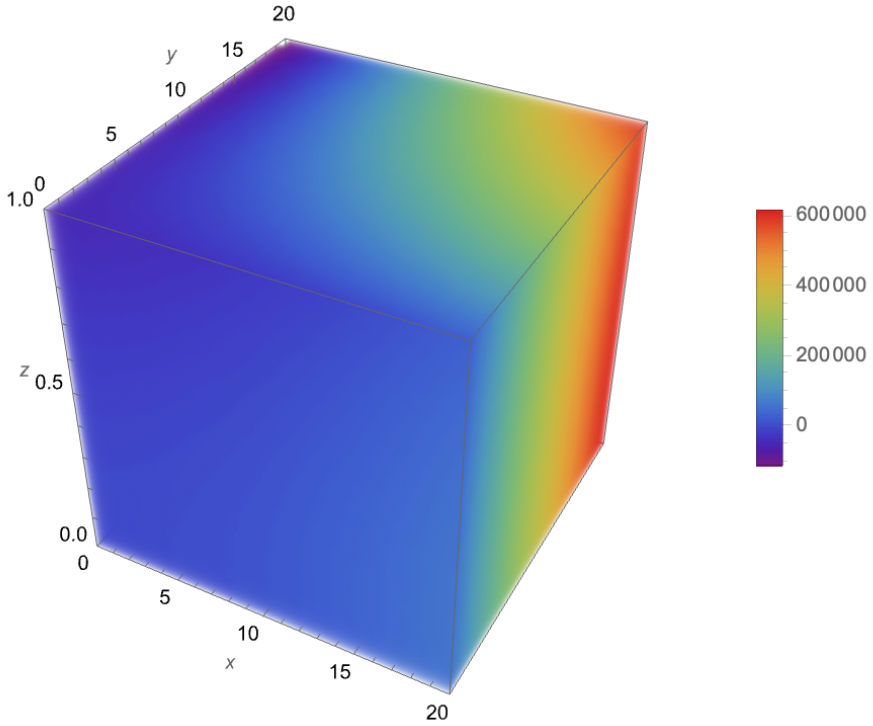

Figure 1 – 4D response for microplastic removal as a function of the concentrations of the medium components. The colors show the value of Equation (2) (microplastics removal) based on the variation of the concentrations of glucose (x), malt extract (y) and peptone (z).

The results obtained suggest that despite *Z. maritimum* being a fungus with bioremediation abilities, it cannot thrive solely on microplastics, supplementation of the culture medium is always necessary. Similar findings had been previously obtained for the optimal medium for the removal of PE microplastics by *P. brevicompactum*[18]. In that study, the optimal medium consisted of 4.6 g/L of glucose, 16.3 g/L of malt extract and 0.56 g/L of peptone[18]. These concentrations were remarkably close to those found for the optimal medium for the removal of PE microplastics by *Z. maritimum*, except for malt extract, which was 41.7% higher. This suggests that the metabolic pathways involved in plastic degradation by these two fungi are likely different. Nevertheless, for both fungi, malt extract concentration held the most statistical significance, as indicated by the lowest *p*-value. These findings align with previous results on the bioremediation of n-alkenes and polycyclic aromatic hydrocarbons by marine fungi, where an increase in malt extract concentration correlated with an increase in fungal cells and had a positive impact on the removal of the contaminants[24,25].

Additionally, it is noteworthy that both fungi required glucose and could not survive solely on microplastics. Other studies also support this idea, as few microorganisms can grow with only microplastics as carbon source[26,27].

While there are limited studies on the optimization of mediums for the removal of contaminants (bioremediation), Sowmya et al.[28] and Yamada-Onodera et al.[24] both reported that *Penicillium simplicissimum* are able to use PE films as carbon source, but still requires medium supplemented with other nutrients to thrive. Ravanipour et al.[29] also reported that, in the optimization of the bioremediation of polycyclic aromatic hydrocarbons by microorganisms, the concentration of nutrients in the medium was the factor with most significant impact.

4. Conclusions

The high potential of *Z. maritimum* for the removal of PE microplastics is demonstrated in this study. The relationship between the dependent variables and maximum percentage of microplastics removal were studied. Malt extract is the main regulatory factor to the microplastics removal. However, the growth medium had also to be supplemented with glucose and peptone, as the fungi could not strive only on microplastics. The obtained optimum chemical composition for the culture medium, serves as basis for further developments of a bioremediation process, for the removal of (micro)plastics present in the marine environment.

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IV. Proteome analysis of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329, two fungi with potential for biodegradation of microplastics

Abstract

The fungi *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329 have been studied as possible agents for the bioremediation of plastic contaminated sites, as these organisms show the potential to use plastic as a carbon source. A proteome analysis of both fungi was made to unveil which proteins are differently expressed by the exposure to polyethylene (PE) microplastics. The effect of PE microplastics on the cellular proteome of each fungus was assessed by LC-MS. Two-time points were used, with the intent to disclose if the cellular proteome profile changed throughout the biodegradation process. 847 different proteins were identified in *Z. maritimum* samples, and 832 proteins were identified in *P. brevicompactum* samples. Results indicate that there are changes in the abundance of some proteins and a good separation between the two groups (fungi grown in the absence and fungi grown in the presence of PE microplastics) was also evidenced. All proteins were found in both conditions, suggesting a constitutive expression of PE microplastics degradation enzymes by *P. brevicompactum* and *Z. maritimum*. Although, the proteins differentially expressed were not the same in both species they indicate that the metabolic pathways involved are the same when the carbon source is PE microplastics or when it is glucose.

1. Introduction

Wilkins et al.[1] were the first authors to use the term “proteome” and “proteomics”. Proteomics is defined as the study of all proteins or proteome in a given biological sample[2,3]. These samples can be an organism, organ, tissue, or cell at a specific time[4,5]. Proteomics technologies allow determining posttranslational modifications of proteins and, coupled with the adequate bioinformatic tools, proteomics allows to understand interactions and the localization of a protein[6]. Proteomics is closely influenced by genomics, but it gives a better understanding of the structure and function of an organism or cell, as it gives the information of which proteins are being produced at a given time point, under certain conditions[4]. Some proteins may be encoded on the genome, but may never be expressed, or be expressed only under certain conditions (age, environmental biotic and abiotic factors, among others). Unlike a genomic study, a proteomic analysis gives a “snapshot” of the proteins produced by the organism or cell in that moment[4]. The proteome is incredibly dynamic and reflects the environment and each change that occurs[5].

The first proteomics’ study date to 1975, thanks to the development of two-dimensional gel electrophoresis. Since then, new techniques were developed allowing an important growth of proteomics, especially the development of Mass Spectrometry (MS) technologies. Of course, the development of large-scale nucleotide sequencing and the advancements in genomics also had a huge impact, as the data obtained are used by MS protein identification software[5]. For example, in the case of fungal proteomics, research grew exponentially from 2005 onwards, thanks to the advances in genomics that led to the availability of a multitude of fungal genome sequences[7] in databases.

Fungal proteomics, especially of filamentous fungi, is an emerging and extremely important area. Studies have focused on the identification of proteins produced, for example, by plant pathogenic or human opportunistic fungi, which can help to further understand how to treat these infections, as well as to better understand their clinical consequences[7]. Filamentous fungi are a huge reservoir of valuable proteins and enzymes, and proteomics has also been used in their investigation as they are relevant for biotechnological and biomedical applications. Furthermore, proteomics is useful in the study of biochemical mechanisms used by fungi to survive in extreme environments

or in the presence of contaminants[8], as is the case of microplastics. Understanding how the proteome changes upon challenge with microplastics, can be a crucial step in plastic biodegradation studies and in the development of biotechnological approaches to reduce the wide presence of microplastics in the environment. Understanding how an organism's proteome changes in response to the presence of plastic may help to identify important degradative enzymes and to understand how these organisms use plastics as a carbon source[9–11]. Proteomics' studies of filamentous fungi are not abundant[12], and as far as we know there are no studies on the effect on the proteome of the biodegradation of any kind of plastics. The proteome profiles of some biodegradation processes of other chemical compounds, like the removal of 4-n-nonylphenol by *Metarhizium robertsii*[12] and the removal of tetrabromobisphenol A by *Phanerochaete chrysosporium*[13], have already been described.

Penicillium brevicompactum CMG 72 and *Zalerion maritimum* ATCC 34329 have shown the ability to biodegrade and use polyethylene (PE) microplastics as carbon sources, as presented in this thesis and in previous works[14,15]. Until now, there is no proteomic data available for *Zalerion maritimum* nor for any species of the genus *Zalerion*. There is only one genome of *Z. maritimum* in databases[16]. In the case of *Penicillium brevicompactum*, there is also no proteome profile published, but there are some proteomic studies of *Penicillium* species[17–20]. For *P. brevicompactum*, there are a few genomes already sequenced and annotated in databases, as presented in Chapter II of this thesis.

So, in this work, with the intent of better understanding the biotechnological potential of two fungi for the bioremediation of plastics and to understand how the proteomic profile changes when they are exposed to this material, the proteomic profiles of *P. brevicompactum* CMG 72 and of *Z. maritimum* ATCC 34329 were determined. This is the first report of a proteomic profile of *P. brevicompactum* CMG 72 and *Z. maritimum* ATCC 34329.

2. Material and methods

2.1. Microplastics

Polyethylene (PE) microplastics were obtained from PE pellets acquired from Sigma-Aldrich (USA) mechanically cut to obtain the intended sizes. The size, 1000 µm < MP < 250 µm, was defined with the help of stainless still sieves.

2.2. Fungal strain

Penicillium brevicompactum CMG 72 was isolated in our lab, as presented in Chapter II of this thesis, and has been maintained in our lab since its identification. *Zalerion maritimum* ATCC 34329 was obtained in 2017 from the ATCC culture collection and has been maintained in our lab since then.

Both fungi were kept in recommended conditions for growth, in a culture medium with the composition of 35 g/L of NaCl (Labbox, Spain), 20 g/L of glucose (Labbox, Spain), 20 g/L of malt extract (Oxoid, United Kingdom) and 1 g/L of peptone (Sigma-Aldrich, Germany). Prior to experiments, fungi were grown for two weeks, in the same medium, at room temperature in a shaker at 120 rpm.

2.3. Experimental conditions

For assessing the changes caused by PE microplastics in the proteome of *Z. maritimum* ATCC 34329 and of *P. brevicompactum* CMG 72, a batch experiment was performed using Erlenmeyer flasks (100 mL) with an optimized medium for microplastics removal. For *Z. maritimum* the optimized medium was composed of 4.47 g/L of glucose (Labbox, Spain), 11.5 g/L of malt extract (Oxoid, United Kingdom), 0.458 g/L of peptone (Sigma-Aldrich, Germany) and 35 g/L of sea salts (Sigma-Aldrich, Germany) (as described in Chapter III of this thesis). For *P. brevicompactum* the optimized medium was composed of 4.6 g/L of glucose (Labbox, Spain), 16.3 g/L of malt extract (Oxoid, United Kingdom), 0.6 g/L of peptone (Sigma-Aldrich, Germany) and 35 g/L of NaCl (Labbox, Spain)[15].

To each Erlenmeyer flask, approximately, 0.015 g of PE microplastics were added to the medium, sterilized by autoclave, and afterwards inoculated with, approximately, 0.50 g of the corresponding fungus mycelium. A control condition was carried out in the same conditions but without the addition of PE. Six replicates of each condition were

performed. After inoculation, all the Erlenmeyer flasks were incubated at 25 ^{o}C with agitation (120 rpm, Optic Ivymen System, Spain). At each time point (14 and 28 days for *Z. maritimum* or 21 and 28 days for *P. brevicompactum*), mycelia and microplastics were separated from the medium by filtration using a filter paper of 90 mm diameter and 200 µm pore (Prat dumas, France). Mycelia was carefully washed with ultrapure water and frozen at -80 ^{o}C.

Three extra replicates for each condition, fungi grown in the presence of PE and control, from each time point were also kept in the shaker to assess the growth of the fungi.

2.4. Protein extraction

Proteins were extracted as described before, with slight modifications[21]. Frozen mycelia were ground to a fine powder in a mortar with liquid nitrogen. Then 10 mL of 10 mM potassium-phosphate buffer (pH 7.4) containing 0.07% DTT and a protease inhibitor cocktail (Cytiva, USA) was added to the mortar, and later the solution was placed in a 15 mL tube. All the samples were sonicated for 1 min in ice, followed by 2 min of pause, three times. Later, the samples were agitated using an orbital agitator at 20 rpm, for 2 hours at 4 ^{o}C. Afterwards, the samples were centrifugated at 15000 g for 30 min, at 4 ^{o}C, and the supernatants were recovered. These supernatants were ultra-centrifuged at 48400g for 1 hour at 4 ^{o}C. To the obtained supernatants an equal volume of TCA/Acetone [20%/80% (w/v)] with 0.14% (w/v) DTT was added and then incubated at -20 ^{o}C overnight. Afterwards, the samples were centrifuged at 15000 g for 20 min, at 4 ^{o}C. The pellet was washed twice with 10 mL of ice-cold acetone: ice-cold acetone was added to the tube, followed by a centrifugation of 15000 g for 20 min, at 4 ^{o}C, the last wash step was with 10 mL of cold 80% acetone (v/v) to eliminate TCA traces. Finally, the 80% acetone was removed, and the residual acetone was air-dried.

The pellet was then resuspended with 200 µL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris), transferred to tubes, and stored at -80 ^{o}C.

2.5. Protein quantification and quality analysis

The quantification of proteins was made with the Pierce™ 660nm Protein Assay Reagent (Thermo Scientific, USA) according to the manufacturer's instructions, using Bovine Serum Albumin (BSA) as standard. All samples were quantified in triplicate.

Samples were denaturated by dilution (1:1) with denaturation solution (2% β-mercaptoethanol (v/v), 2% SDS (w/v), 8 M Urea, 100 mM Tris, 100 mM Bicine) and incubation for 5 min at 100 °C. The protein patterns were analyzed by SDS-polyacrylamide gel electrophoresis, 4-20% MP TGX Gel 10W 30 µL, (Bio-rad, USA), for quality assessment.

2.6. Sample preparation

Fifteen µg of each sample were diluted in 50 mM NH_4HCO_3 containing 1% SDS and 10 mM DTT (total volume of approximately 40 µL). After incubation at 80 °C for 10 min, the samples were further diluted with 350 µL of 50M NH_4HCO_3 and 8M urea. Removal of detergents, alkylation, and digestion of the samples followed the procedure of Filter Aided Sample Preparation (FASP) technique[22].

Afterward, the dried samples were re-dissolved with 1% formic acid and the peptide purification step was performed using C18 Omix tips (Agilent). The peptides were dried in a vacuum concentrator (SpeedVac, ThermoFisher Scientific, Waltham, USA) and stored at −20 °C until analysis.

2.7. LC-MS/MS analysis

Each sample was solubilized in 20 µL loading solvent A (0.1% TFA in water:ACN (99.5:0.5, v:v)) moments before analysis. Five µL of each sample was injected on an Ultimate 3000 RSLC nano system in-line connected to an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific, USA). Trapping was performed at 20 µl/min for 2 min in loading solvent A on a 20 mm trapping column, 300 µm internal diameter (I.D.), 5 µm beads (Thermo scientific, USA). The peptides were separated on a 50 cm µPAC™ Neo column (Thermo scientific, USA). Temperature was kept constant at 50 °C. Peptides were eluted by a linear gradient starting at 0.5% MS solvent B (0.1% FA in acetonitrile), reaching 4% MS solvent B after 2.1 min, 7% MS solvent B at 8 min, 26.4% MS solvent B at 90 min,

44% MS solvent at 110 min, 56% MS solvent B at 111 min, followed by a 3-minutes wash at 56% MS solvent B and re-equilibration with MS solvent A (0.1% FA In water). The first 8 min the flow rate was set to 750 nL/min after which it was kept constant at 250 nL/min until 90 min, after which it was linearly increased to 750 nL at 110 min and kept constant. The mass spectrometer was operated in data-dependent mode. Full-scan MS spectra (350-1500 m/z) were acquired at a resolution of 120000 in the Orbitrap analyzer after accumulation to a target AGC value of 100000 with a maximum injection time of 50 ms. The precursor ions were selected at Top Speed with a cycle time of 1s, after being filtered for charge states (2-7 included) and intensity (minimal intensity of 5E3) with a dynamic exclusion of 60 s (+/- 5 ppm window). The precursor ions were isolated in the quadrupole with an isolation window of 1.6 Da, accumulated to an AGC target of $1E^4$ or a maximum injection time of 45 ms and activated using HCD fragmentation (34% NCE). The fragments were analyzed In the Ion Trap Analyzer at rapid scan rate.

2.8. Data treatment

The raw data generated from liquid chromatography-MS was further inputted in Max-Quant (version 2.4.4.0), a quantitative proteomics software developed by Cox and Mann[23]. MS1 spectra were searched with the Andromeda peptide database engine[24] against a FASTA database of proteins from *Penicillium brevicompactum* downloaded from Uniprot (August 2023) and from *Zalerion maritimum* (September 2023) and analyzed for label-free quantification of the peptides present in the samples. The peptide database was constructed from in-silico prediction of tryptic peptides with up to two missed cleavages, carbamidomethylation of cysteines as fixed modifications, and oxidation of methionines and N-terminal acetylation as variable modifications. Peptide spectral matches were validated using a percolator based on q-values at a 1% false discovery rate (FDR). Identified peptides were assembled into protein groups according to the law of parsimony and filtered to 1% FDR. Perseus software (version 2.0.11)[25] enabled the affiliation of the protein groups into identified proteins. Identified proteins were filtered and only considered for analysis if present in 3 samples and using at least 3 peptides for identification. Reverse proteins and proteins identified only by site were filtered out. A multi-scatter plot and hierarchical clustering were performed to assess

the quality of the experiment. A two-sample t-test between control and plastic treated samples was performed with minimal fold change (s0) of 1.5 and 1% FDR.

MetaboAnalyst (version 5.0)[26] was used for statistical analysis, to obtain the Volcano plot and to perform a partial least squares-discriminant analysis (PLS-DA).

3. Results and discussion

3.1. Growth

As seen in Table 1, both *Z. maritimum* and *P. brevicompactum* grew more in contact with PE when compared to fungi grown without PE, in all time points, although the differences are not statistically different (t-test).

It is also possible to see that *P. brevicompactum* appears to lose weight from 21 to 28 days, indicating a loss in biomass after 28 days of growth when compared to 21 days. This is in accordance with what was also described in this thesis, Chapter IV.a, that *P. brevicompactum* growing in contact with high-density PE and low-density PE, had a decrease in biomass during the 28 days of experiment.

Table 1 – Growth of *Zalerion maritimum* and *Penicillium brevicompactum*. Data are presented as mean (n=3) ± standard deviation of dry weight (g).

	Z. maritimum		*P. brevicompactum*	
	14 days	28 days	21 days	28 days
With PE	0.72±0.05 g	0.86±0.03 g	0.927±0.007 g	0.9±0.1 g
Control	0.63±0.06 g	0.82±0.01 g	0.89±0.02 g	0.8±0.2 g

3.2. Quality assessment of protein profiles

Samples were quantified and the obtained concentrations are presented in Tables 2 and 3. When comparing both tables, *Z. maritimum* samples had always higher concentrations than the *P. brevicompactum* ones.

Table 2 – Protein concentration of *Zalerion maritimum* (cellular proteins). Protein concentration was determined according to the protocol of Pierce™ 660nm Protein Assay Reagent.

		Samples	Concentration (mean±SD) (μg/mL)
Fungi grown with PE	14 days	1	2034±106
		2	5592±372
		3	4743±262
		4	886.4±77.2
		5	3574±248
		6	2091±146
	28 days	1	2067±54
		2	3896±219
		3	2390±176
		4	4185±366
		5	4680±166
		6	4502±249
Control	14 days	1	4576±153
		2	6226±794
		3	3016±137
		4	3644±338
		5	5254±298
		6	1505±47
	28 days	1	6685±453
		2	3502±184
		3	6538±738
		4	4750±257
		5	3973±172
		6	5780±372

In Figure 1, four different SDS-polyacrylamide gels with the protein profile of *Z. maritimum* mycelia are depicted, each one corresponding to a time point, 14 or 28 days, and a condition, grown in the presence of PE microplastics or grown in control conditions.

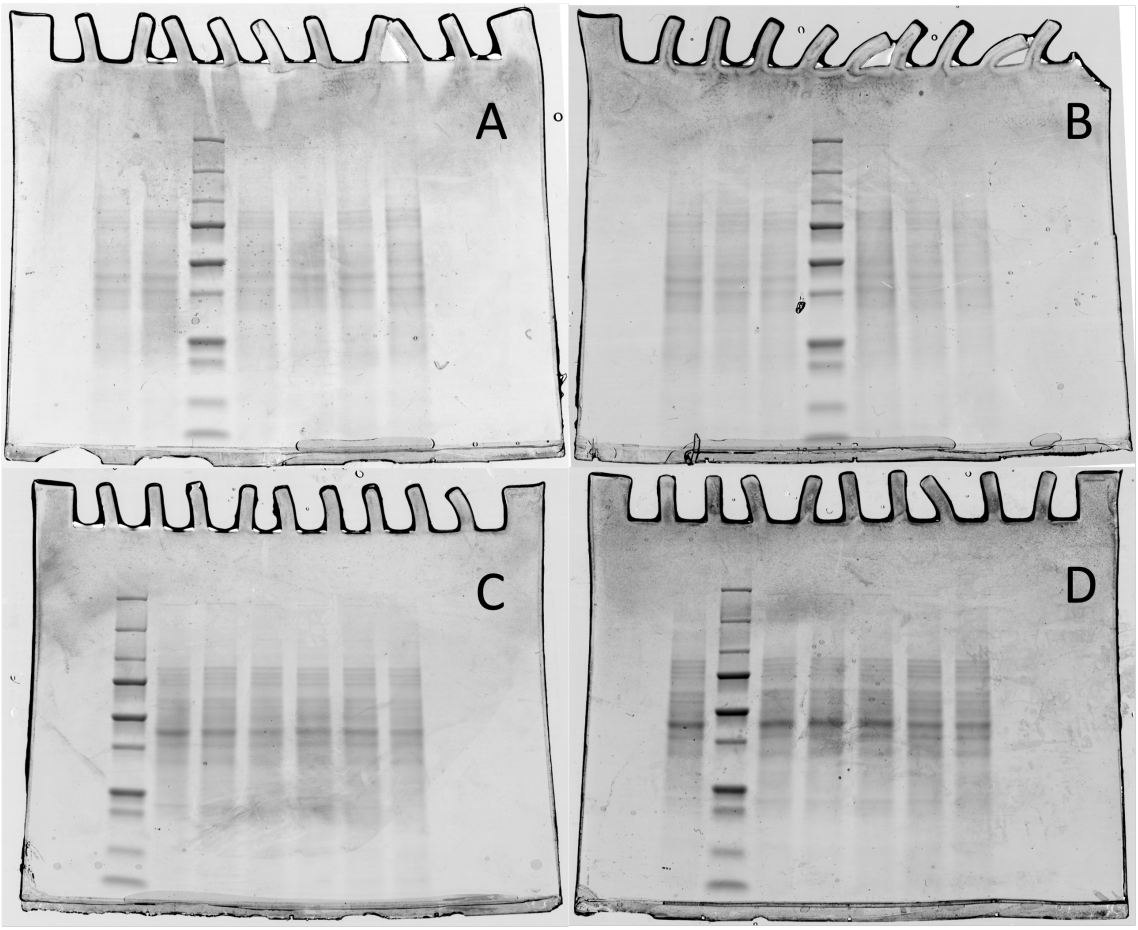

Figure 1 – Protein profiles of *Zalerion maritimum* mycelia. Proteins were separated by SDS-polyacrylamide gel. A/C – *Zalerion maritimum* grown in control conditions (optimal medium, 25°C) for 14 days (A) and 28 days (C). B/D – *Zalerion maritimum* grown in the presence with polyethylene microplastics (optimal medium, 25 °C) for 14 days (B) and 28 days(D). The protein ladder used has three high intensity reference bands of 75 kDa, 50 kDa and 25 kDa, the remaining protein bands are of 250 kDa, 150 kDa, 100 kDa, 37 kDa, 20 kDa, 15 kDa and 10 kDa.

Protein profiles of *Z. maritimum* are similar between samples for the same condition (fungus grown in the presence of PE or control condition) and for the same time point (e.g. Figure 1A). Moreover, based on the images in Figure 1, it is also possible to see that there are differences between the proteomic profile for the 14 days samples and the proteomic profile for the 28 days samples.

Table 3 - Protein concentration of *Penicillium brevicompactum* (cellular proteins). Protein concentration was determined according to the protocol of Pierce™ 660nm Protein Assay Reagent.

		Samples	Concentration (mean±SD) (µg/mL)
Fungi grown with PE	21 days	1	1457±309
		2	2130±419
		3	1267±181
		4	2390±163
		5	1377±450
		6	1267±24
	28 days	1	1378±157
		2	600±181
		3	1748±139
		4	2777±386
		5	1143±47
		6	2267±272
Control	21 days	1	1056±0
		2	1389±0
		3	1157±330
		4	2410±216
		5	1378±139
		6	2194±195
	28 days	1	2557±47
		2	2490±294
		3	643±47
		4	711±91
		5	982±52
		6	1267±181

Figure 2 shows the protein profiles of *P. brevicompactum* mycelia at 14 or 28 days of growth in the presence of PE microplastics and in control conditions.

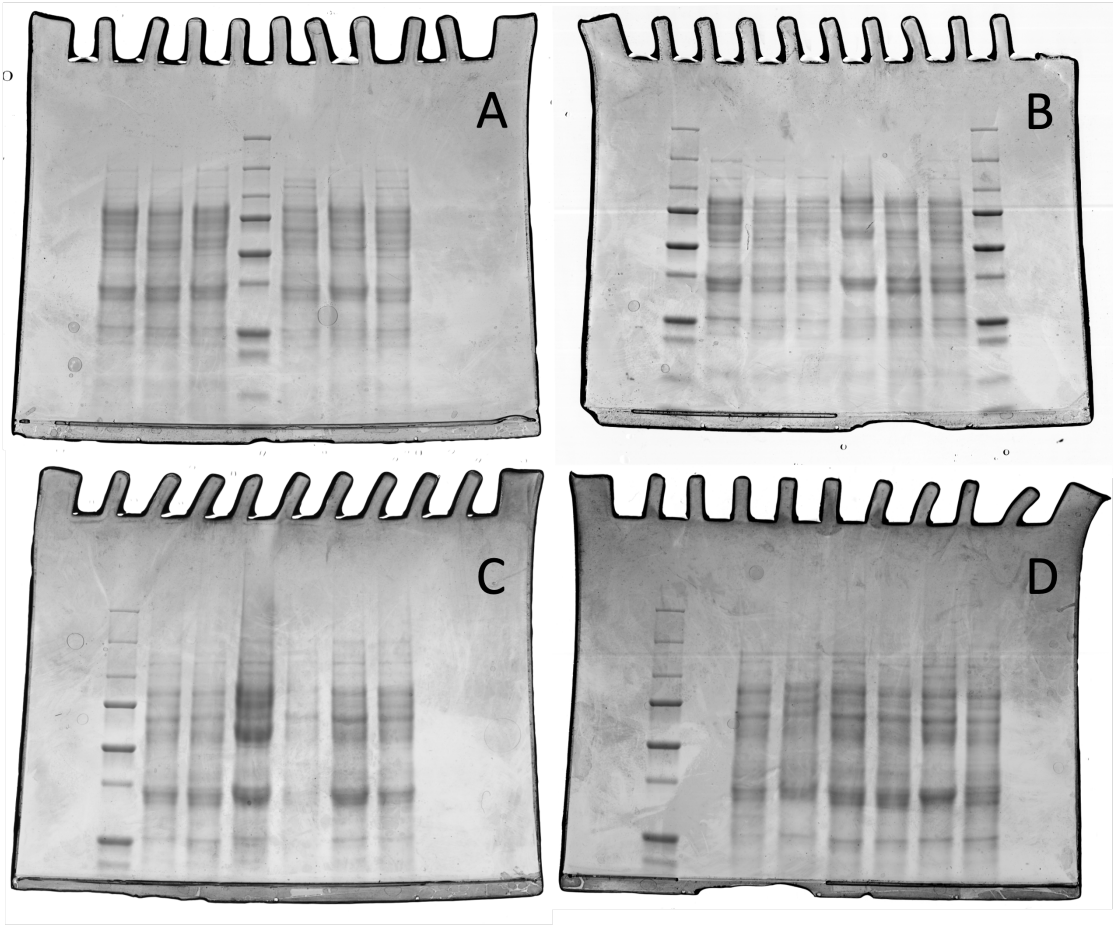

Figure 2 - Protein profiles of *Penicillium brevicompactum* mycelia. Proteins were separated by SDS-polyacrylamide gel. A/C – *Penicillium brevicompactum* grown in control conditions (optimal medium, 25°C) for 21 days (A) and 28 days (C). B/D – *Penicillium brevicompactum* grown in the presence with polyethylene microplastics (optimal medium, 25 °C) for 21 days (B) and 28 days (D). The protein ladder used has three high intensity reference bands of 75 kDa, 50 kDa and 25 kDa, the remaining protein bands are of 250 kDa, 150 kDa, 100 kDa, 37 kDa, 20 kDa, 15 kDa and 10 kDa.

For *P. brevicompactum* (Figure 2) it was possible to find differences between samples in the gels, for example in Gel B and C. Nonetheless, there were no visual differences between the protein profiles of control samples and those of the fungus grown in contact with PE.

3.3. Proteome profiles of mycelia

3.3.1. *Zalerion maritimum*

A total of 578 proteins were identified at 14 days and 805 proteins were identified at 28 days of growth. Most of the proteins identified at 14 days samples are present in the 28 days, as there are 536 proteins in common between the two time points (Table S1). Volcano plots were used to visualize and identify the proteins with significant fold changes (Figure 3 - A.1 and B.1). Based on the proteome profile and the quantification of the proteins on both time points samples for the two conditions, growing in the presence and the absence of PE, a Partial least squares-discriminant analysis was made (Figure 3 - A.2 and B.2).

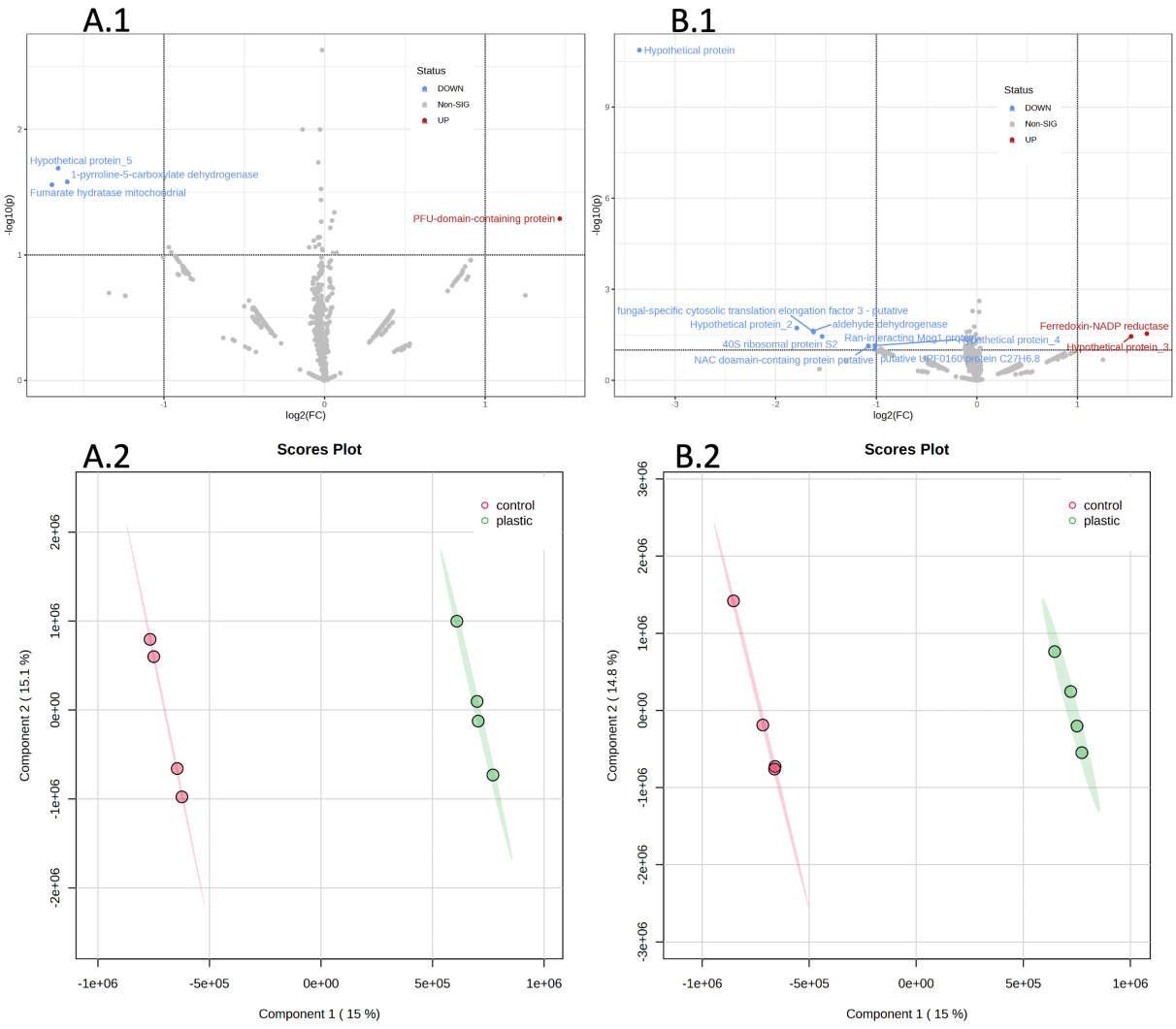

Figure 3 – Volcano plot (A.1 and B.1) and Partial least squares-discriminant analysis (PLS-DA, A.2 and B.2) of *Z. maritimum* proteome. Volcano plot showing log2 fold change plotted against log 10 adjusted p-value

for *Z. maritimum* samples grown in the presence of PE versus control, for 14 days samples (A.1) and 28 days samples (B.1). Data points in the upper right (ratio > 1.0) and upper left (ratio < -1.0) sections with $p<0.05$ represent proteins that are significantly dysregulated in *Z. maritimum* grown in the presence of PE. Cross-validated PLS-DA score plot for comparison of the protein profiles of samples of *Z. maritimum* grown in the presence of PE (green circles) versus control (red circles), for 14 days samples (A.2) and 28 days samples (B.2). Both analysis and graphics were generated using MetaboAnalyst (version 5.0).

As seen in Figure 3 there is a perfect separation between *Z. maritimum* growing in contact with PE and *Z. maritimum* growing in control conditions.

At 14 days (Figure 3 - A.1), only four proteins are significantly different in the proteome of *Z. maritimum* grown in the presence of PE (hypothetical protein, the 1-pyrroline-5-carboxylate dehydrogenase, the fumarate hydratase mitochondrial, and the PFU-domain containing protein). In the 28 days samples, (Figure 3 – B.1), eleven proteins are significantly different between the two conditions (4 hypothetical proteins, the fungal specific cytosolic translation elongation factor 3 – putative, an aldehyde dehydrogenase, the 40S ribosomal protein S2, the NAC domain-containing protein putative, the ran-interacting Mog 1 protein, the putative UPF0160 protein C27H6.8, and the ferrodoxin-NADP reductase).

To better understand the differences between the groups, the VIP scores, a measure of a variable's importance in the PLS-DA model, were analyzed.

The twenty proteins with higher VIP scores, correspond to those proteins with a higher impact in the separation of the two groups, for the 14 days samples, are presented in Figure 4.

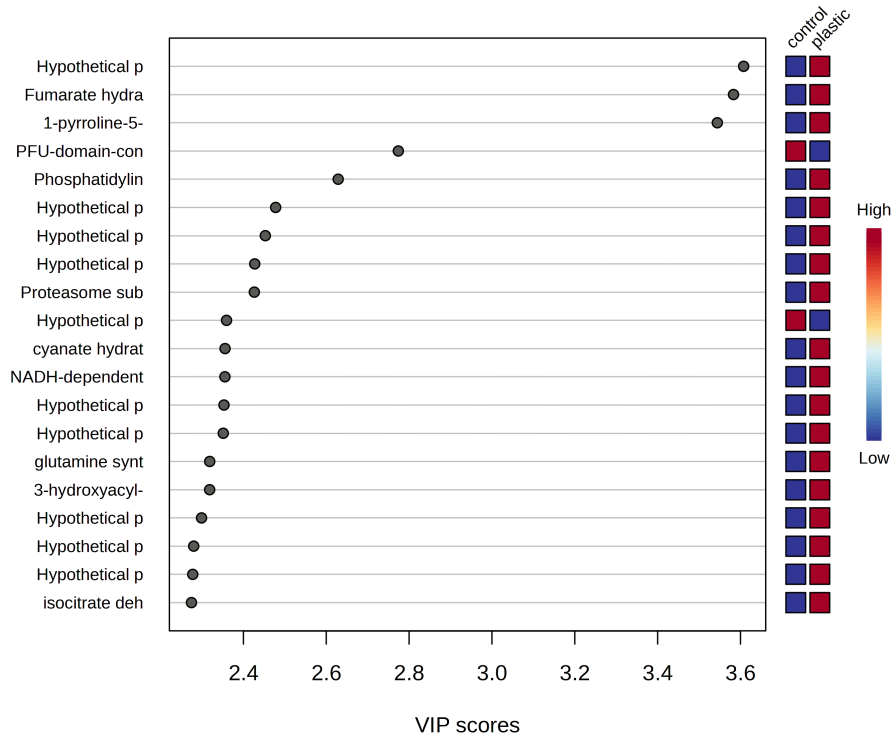

Figure 4 - Variable importance in projection (VIP) scores plot based on two components from the PLS-DA of proteins differentially expressed in control and and "plastic" group in the *Z. maritimum* grown for 14 days. Only the twenty proteins with higher VIP scores are presented (all have VIP>1). This analysis was performed and the graphic generated using MetaboAnalyst (version 5.0).

It should be noted that the lack of available annotation of the genome of *Z. maritimum*, hampers the identification of many proteins that contribute to group separation. In fact, of the twenty proteins with higher VIP score, nine are hypothetical proteins, for whose there is no functional information available, and therefore their biological role is not known, nor can be inferred.

The hypothetical protein (KAJ2901263.1) and PFU-domain-containing protein (KAJ2892233.1) are more abundant in control samples. The PFU-domain-containing protein, has a 44.1% similarity with Phospholipase A-2-activating protein (G9P257_HYPAI, Figure S1), which is a protein involved in ubiquitin homeostasis[27]. The ubiquitin degradation pathway plays an essential role in stress responses, such as nutrient limitation[28], which may indicate that the fungi growing in control conditions are in a nutrient limitation state more severe than the fungi growing with PE, which agrees

with the difference in biomass measured (Table 1). Both data support the hypothesis that plastic may serve as a carbon source for *Z. maritimum*.

The remaining eighteen proteins with higher VIP score (Figure 4) are more abundant in samples of fungi growing in the presence of PE. Some of these proteins are hypothetical proteins with no functional annotation associated. The proteins with functional data associated are fumarate hydratase mitochondrial (KAJ2894529.1), 1-pyrroline-5-carboxylate dehydrogenase (KAJ2905377.1), phosphatidylinositol transfer protein (KAJ2902851.1), proteasome subunit alpha type-2, cyanate hydratase (KAJ2895603.1), NADH-dependent flavin oxidoreductase-like protein (KAJ2898818.1), glutamine synthetase (KAJ2903273.1), 3-hydroxyacyl-CoA dehydrogenase (KAJ2898515.1) and isocitrate dehydrogenase putative (KAJ2898318.1).

Fumarate hydratase mitochondrial is an essential enzyme on the TCA cycle (Krebs cycle), that catalyzes the hydration of fumarate to L-malate. So, this enzyme has an essential role in central carbon metabolism[29]. Isocitrate dehydrogenase is also involved in the TCA cycle, catalyzing the oxidative decarboxylation of isocitrate to α-ketoglutarate while reducing $NADP^+$ to NADPH, therefore also playing important roles in cellular metabolism[30], affecting the flow of intracellular carbon sources and lipid biosynthesis[31]. The increased abundance of both these enzymes in the samples of fungi grown in contact with PE, suggests an activation of TCA cycle by PE due to the presence of more carbon sources. The final products of the metabolism in the biodegradation of plastics are proposed to enter the TCA cycle, where they are converted to energy[32]. 3-hydroxyacyl-CoA dehydrogenase is normally associated with lysine catabolism/metabolism and the production of Acetyl-CoA[33], which is also involved in the TCA cycle and other important cellular activities[32]. On the other hand, this enzyme may also be involved in lipid biosynthesis[33]. An increase quantity of it may indicate higher production of lipids, which in this case may indicate that when in contact with PE, fungi have more access to nutrients.

1-pyrroline-5-carboxylate dehydrogenase is involved in Proline metabolism, that has been associated with stress adaptation[34,35]. Phosphatidylinositol transfer protein has a potential role in regulating lipid and fatty acid metabolism in stress conditions, like heme-depleted conditions, and oxidative stress, and also to influence the resistance of

fungal pathogens[36–38]. NADH-dependent flavin oxidoreductase-like protein is associated with mitochondrial electron transport system[39] and is probably involved in responses to oxidative stress by maintaining the redox homeostasis[40]. Glutamine synthetase is a key central enzyme in the nitrogen metabolic pathway and it appears to have an important role in the homeostasis of ROS and oxidative stress[41]. These four enzymes appear to be involved in the response to stress, which may indicate that despite being able to use microplastics as nutrient source, the fungus is under stress. This may happen as the microplastics are an unusual source of nutrients to fungi, but also if during the metabolism involved in the biodegradation of microplastics, some toxic compounds are produced.

The cyanate hydratase, or cyanase, has not often been characterized in eukaryotic cells and appears to be involved in hyphae color. Cyanase has also been proposed as an enzyme useful for bioremediation[28,42]. *Zalerion maritimum* tends to change its color based on the carbon source[43], so the high presence of this enzyme may indicate that the fungi growing in contact with PE, changes its phenotype. In fact, it was possible to observe that in contact with PE, *Z. maritimum* changes its color from brown to black color.

The proteasome is a protease complex responsible for degradation of intracellular proteins. An increase of proteasome subunit alpha type-2 in mycelium grown in the presence of PE indicates an alteration in the metabolism toward an increase of proteolytic activity[44]. Some proteases (e.g. neprilysin and a cutinase-like enzyme) have also been associated with plastic degradation[45], so this one may also be able to degrade a bond found in PE.

The twenty proteins with the higher VIP scores, corresponding to the twenty with the higher impact in the separation of the two groups for the 28 days samples are identified in Figure 5. As before, the lack of annotation data hampers the identification of these proteins. There are 30% of “hypothetical proteins” with no function associated. Nonetheless, these proteins are involved directly or indirectly in substrate adaptation.

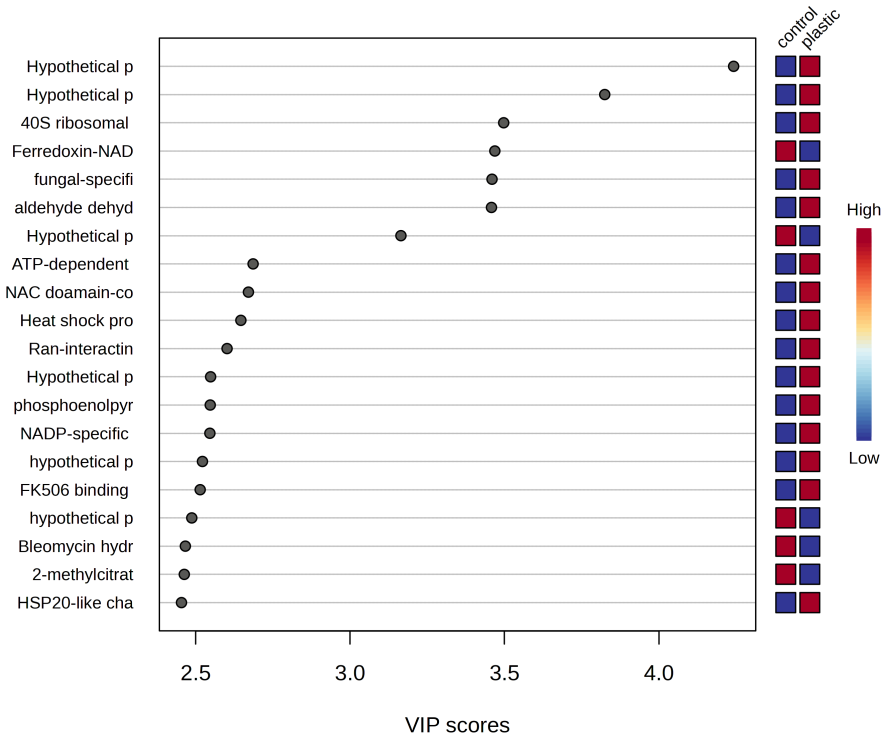

Figure 5 - Variable importance in projection (VIP) scores plot based on two components from the PLS-DA of proteins differentially expressed in control and and "plastic" group in the *Z. maritimum* grown for 28 days. Only the twenty proteins with higher VIP scores are presented (all have VIP>1). This analysis was performed and the graphic generated using MetaboAnalyst (version 5.0).

Ferredoxin-NADP reductase (KAJ2897813.1), bleomycin hydrolase protein putative (KAJ2894530.1) and 2-methylcitrate synthase mitochondrial (KAJ2903268.1) and two hypothetical proteins (KAJ2907290.1, KAJ2903247.1) are more abundant in the samples of *Z. maritimum* grown in control conditions (Figure 5). Ferredoxin-NADP reductase is a domain that may be present in different proteins and are non-ribosomal peptide synthase (NRPS) multidomain enzymes[46]. This protein has 80.6% similarity to UDP-galactopyranose mutase (Q1K582_NEUCR, Figure S2), a flavoenzyme that normally is involved in the cell wall thickness of fungi[47]. Bleomycin hydrolase protein putative is a cysteine protease that inactivates bleomycin, and cleaves small peptides[48]. 2-methylcitrate synthase mitochondrial is an essential enzyme for the 2-methylcitrate cycle, that has been associated with the cellular redox state, nitric oxide production, and growth of fungi[49].

The remaining fifteen proteins with higher VIP score (Figure 5) are more abundant in samples from fungi grown in the presence of PE. Four of them are hypothetical proteins (KAJ2902820.1, KAJ2906721.1, KAJ2895894.1, KAJ2893887.1). Remaining proteins are an aldehyde dehydrogenase (KAJ2896354.1), a phosphoenolpyruvate carboxykinase (KAJ2898276.1), the 40S ribosomal protein S2 (KAJ2892603.1), a fungal-specific cytosolic translation elongation factor 3 – putative (KAJ2903061.1), an ATP-dependent RNA helicase elF4A (KAJ2904713.1), a NAC domain containing protein (KAJ2906528.1), a heat shock protein sti1 like protein (KAJ2894431.1), the ran-interacting Mog1 protein (KAJ2896590.1), the NADP-specific glutamate dehydrogenase (KAJ2904842.1), a FK506 binding protein proline rotamase rapamycin-binding protein (KAJ2904462.1), and a HSP20-like chaperone (KAJ2904351.1).

Aldehyde dehydrogenase is involved in the oxidative degradation of lipids and appears to be important to enforce the cell wall integrity[50]. It is also said to be involved in ethanol stress tolerance[51].

Phosphoenolpyruvate carboxykinase is a key enzyme for microorganisms growing on carbon sources metabolized via the TCA cycle[52], as it links glycolysis and the TCA cycle, trough the production of oxaloacetate. The presence of this enzyme suggests that the fungi are growing with a carbon source other than glucose, e.g. using the PE microplastics as a carbon source.

NADP-specific glutamate dehydrogenase, similar to the glutamine synthetase that was more abundant in the samples of *Z. maritimum* grown in the presence of PE for 14 days, is involved in a metabolic pathway responsible for the assimilation of ammonia and N assimilation[53].

Heat shock protein are normally involved in proteostasis, the process involved in the regulation of proteins to maintain health and protect cells from stress[54]. HSP sti1 is part of a larger complex working as an HSP70 and HSP90-binding protein. HSP20-like appears to be involved in thermotolerance, acting as chaperone during stress response[55]. Like enzymes more abundant in the 14 days samples, this indicate that during the metabolism involved in the degradation of microplastics, fungi cells are under stress.

40S ribosomal protein S2, fungal-specific cytosolic translation elongation factor 3 – putative, ATP-dependent RNA helicase elF4A are more abundant in fungi grown in the

presence of PE and indicate that the fungi are extremely active in the production of new proteins. The presence of heat shot proteins supports this finding, as HSPs are related to protein homeostasis and turnover. The NAC domain containing protein is probably the nascent polypeptide-associated complex subunit alpha. Its presence also demonstrates a high active cell, as it is the first cytosolic protein to contact with the proteins emerging from ribosomes[56]. Ran-interacting Mog1 protein is involved in nuclear protein import[57]. FK506 binding protein proline rotamase rapamycin-binding protein is a peptidylprolyl isomerase protein involved in the regulation of a variety of cellular processes, like protein folding and trafficking, transcription, and chaperone activity[58].

When comparing the 14 days and 28 days growth samples, among the twenty proteins with more impact in the separation of groups for the 28 days samples (higher VIP score) there are seven proteins: two hypothetical proteins, the Ferredoxin-NADP reductase, the fungal-specific cytosolic translation elongation factor 3 – putative, the NAC domain containing protein, and the two heat shock proteins sti1 and HSP20, that were not identified at 14 days growth.

In contrast, in the samples of 14 days, only two hypothetical proteins of the twenty were not identified on the 28 days samples. This difference may indicate that at 28 days, *Z. maritimum* is more active and in a recycling phase, producing new proteins, and simultaneously under more stress. Stress might be due to the reduction of nutrients in the medium or/and due to the increase of toxic metabolites produced during PE biodegradation[59].

Nonetheless, data suggests that the presence of PE leads to an activation of the TCA cycle (Figure 6), which has already been described by other authors[60]. This central metabolic pathway has been associated with the degradation of polymers, and it is involved in the downstream steps of the biodegradation process, the transformation of the monomers in energy, after the extracellular steps.

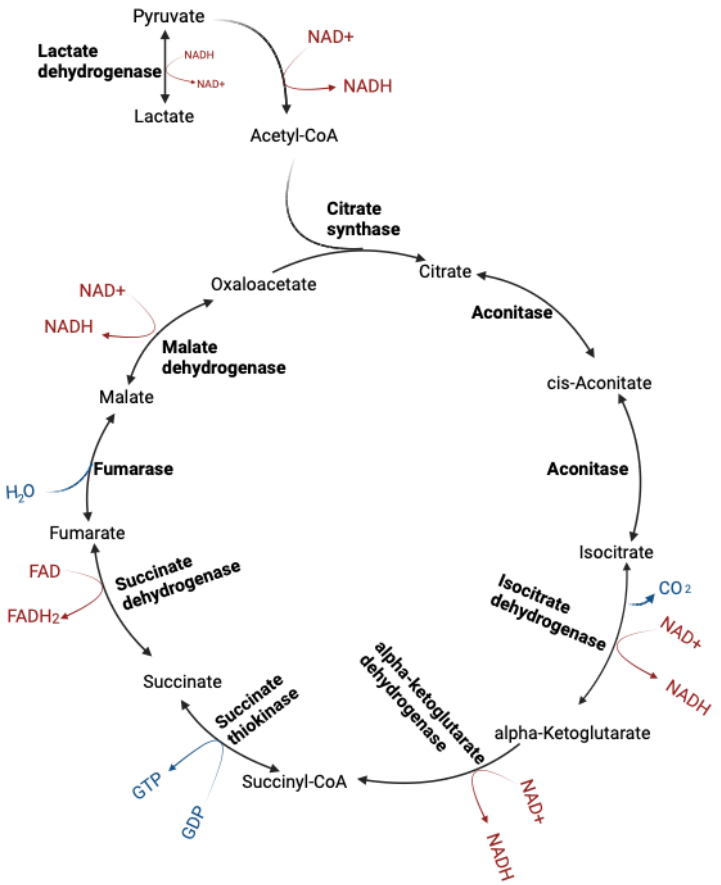

Figure 6 – Schematic representation of the TCA cycle, created in BioRender.com.

In *Z. maritimum*, fumarate hydratase (or fumarase) and isocitrate dehydrogenase were more abundant in the samples from fungi growing in contact with PE. In addition, 3-hydroxyacyl-CoA dehydrogenase, phosphoenolpyruvate carboxykinase, enzymes involved in the production of intermediates for the TCA cycle were also more abundant in the samples from fungi growing in contact with PE. When studying intracellular proteomics of a *Rhodococcous* strain in the presence of PE, Tao et al.[61] also found that it produces more enzymes involved in the TCA and pyruvate cycle, as well as enzymes involved in the conversion and biosynthesis of fatty acids, as is the case of our investigation.

Additionally, proteins such as 1-pyrroline-5-carboxylate dehydrogenase, Phosphatidylinositol transfer protein, NADH-dependent flavin oxidoreductase-like protein, Glutamine synthetase, HSP sti1 like protein, HSP20-like chaperone are also more abundant in samples of *Z. maritimum* growing in contact with PE and may be

related to the response of *Z. maritimum* to the products of degradation or to the stress of using a different, certainly more recalcitrant carbon source. Further studies are necessary to confirm these hypothesis. There is still a lack of information regarding which products are being produced during the biodegradation, specifically their toxicity. Most of the enzymes that have been directly associated with PE's biodegradation are extracellular, as reviewed by Zhang et al.[60]. The genome of *Z. maritimum*, includes genes encoding for enzymes already associated with biodegradation, laccases, cutinases, monooxygenases, cytochrome P450, and alcohol dehydrogenases, as described previously in Chapter II. In this proteomic analysis, none of these proteins were identified, which is certainly linked to the fact that only the cellular proteome was analyzed. SignalP-6.0 was used to confirm if these genes encode extracellular proteins (as described in Supplementary Information). Most laccases and cutinases were shown to have a signal peptide, directing the proteins for the extracellular medium, a possible reason for not being identified in the samples. On the other hand, most monooxygenases, cytochrome P450, and alcohol dehydrogenase did not present a secretory signal peptide, nonetheless were not identified in any of the samples.

3.3.2. *Penicillium brevicompactum*

After 21 days of growth, 441 cellular proteins were identified, and of them approximately 67% (295) were identified as hypothetical proteins. At 28 days of growth, 665 proteins were identified, of them, 65% (434) were identified as hypothetical proteins[62].

In total, there were 274 proteins in common between the two time points (Table S2). Proteins associated with the Mycophenolic acid biosynthesis cluster were identified at both time points, indicating that even in contact with plastics, this fungus may produce this bioactive compound (Table S2).

Proteins with statistical different abundance in both time points (Figure 7 - A.1 and B.1). and PE vs control condition (Figure 7 - A.2 and B.2) were analyzed.

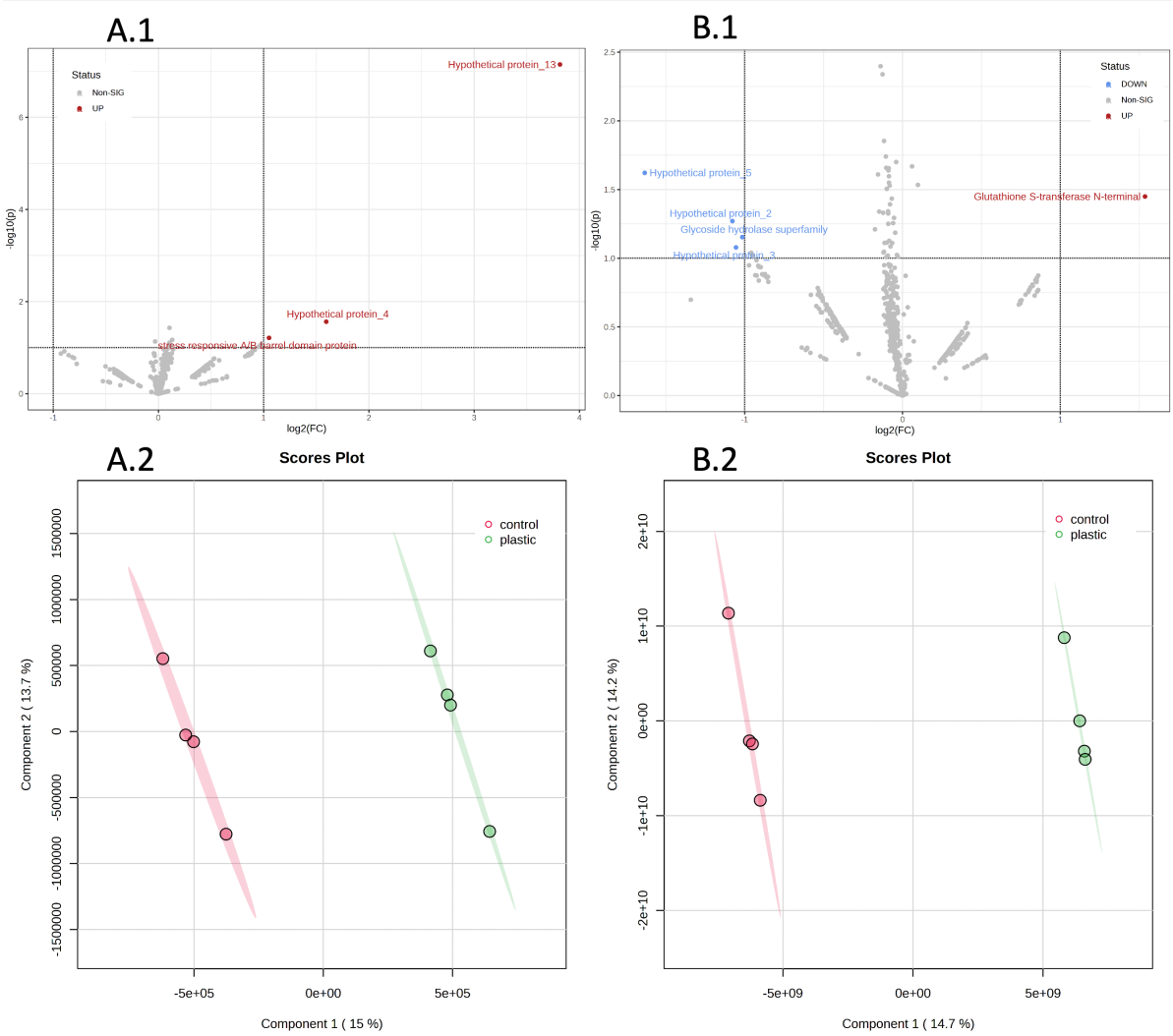

Figure 7 - Volcano plot (A.1 and B.1) and Partial least squares-discriminant analysis (PLS-DA, A.2 and B.2) of *P. brevicompactum* proteome. Volcano plot showing log2 fold change plotted against log 10 adjusted p-value for *P. brevicompactum* samples grown in the presence of PE versus control, for 14 days samples (A.1) and 28 days samples (B.1). Data points in the upper right (ratio > 1.0) and upper left (ratio < -1.0) sections with p<0.05 represent proteins that are significantly dysregulated in *P. brevicompactum* grown in the presence of PE. Cross-validated PLS-DA score plot for comparison of the protein profiles of samples of *P. brevicompactum* grown in the presence of PE (green circles) versus control (red circles), for 14 days samples (A.2) and 28 days samples (B.2). Both analysis and graphics were generated using MetaboAnalyst (version 5.0).

There is a good separation between the data from *P. brevicompactum* grown in the presence of PE and *P. brevicompactum* growing in control conditions in both 21 days and 28 days samples (Figure 7 – A.1 and B.1), confirming alteration of these two proteomes.

At 14 days (Figure 7 – A.2), three enzymes, two hypothetical proteins, and a Stress-responsive A/B barrel domain are significantly different between the two conditions, at 21 days samples. In the case of 28 days samples (Figure 7 – B.2), there are five proteins significantly different between the samples obtained from *P. brevicompactum* grown in the presence of PE and *P. brevicompactum* grown in control conditions. Glutathione S-transferase is significantly less abundant in the samples from *P. brevicompactum* grown in the presence of PE, and on the other hand, three hypothetical proteins and a protein from the glycoside hydrolase superfamily are significantly more abundant in the samples from *P. brevicompactum* grown in the presence of PE.

The VIP scores were also analyzed for the samples of *P. brevicompactum*, to comprehend the differences between the two conditions. Figure 8 presents the VIP scores of the twenty proteins, with higher VIP scores, from the 21 days samples.

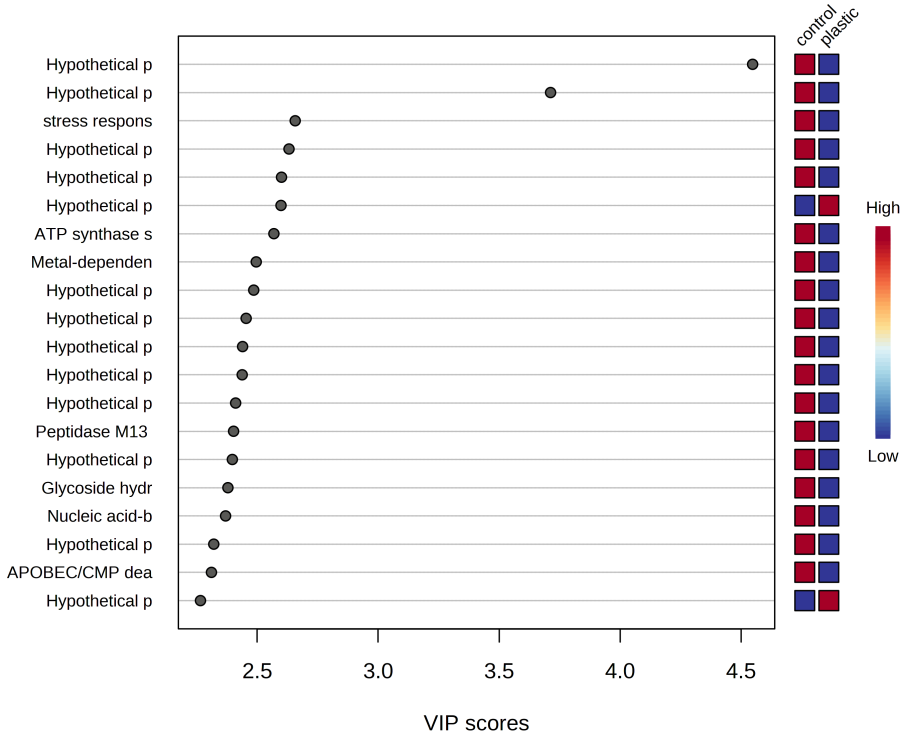

Figure 8 - Variable importance in projection (VIP) scores plot based on two components from the PLS-DA of proteins differentially expressed in control and and "plastic" group in the *P. brevicompactum* grown for 21 days. Only the twenty proteins with higher VIP scores are presented (all have VIP>1). This analysis was performed and the graphic generated using MetaboAnalyst (version 5.0).

Most of the proteins (18 out of 20) presented in the graphic are less abundant in the samples from *P. brevicompactum* grown in the presence of PE. Only two hypothetical proteins (KAJ5357897.1, XP_056812512.1) are more abundant in samples from the fungi grown in the presence of PE.

Stress-responsive A/B barrel domain (XP_056814377.1) is normally associated with plant proteins and it is produced when plants are in contact with pathogens, including fungi[63]. It has also been associated with bacterial fructose-bisphosphate aldolase. So, it may be produced when the fungus is under stress for being in a medium with a low concentration of nutrients, as this protein is more abundant in control samples.

Metal-dependent protein hydrolase (XP_056814638.1) is associated with the hydrolysis of different substrates. It can be involved in the hydrolysis of proteins and carbohydrates, so the one identified in this proteomic study may be also involved in the stress response for being in a reduced medium[64].

Glycoside hydrolase superfamily protein (XP_056806292.1), belongs to a superfamily composed of 168 families of CAZymes[65], and it has 80.2% similarity with chitinase (A0A1V6UY11_9EURO, Figure S3). Chitinase can be involved in autolysis, the natural process of self-digestion, showing that the fungi are under nutrient stress, for being in a reduced medium. Peptidase M13 neprilysin (XP_056809372.1), the peptidases are enzymes that breakdown peptide bonds and cleave long chain proteins, the M13 family in specific and neprilysin are involved in the inactivation of signaling peptides[66], and neprilysin have been associated to the degradation of polyethylene terephthalate[45].

The protein nucleic acid-binding OB-fold (XP_056805720.1) is also associated with stress response, as well as the ATP synthase subunit beta (XP_056807502.1) and APOBEC/CMP deaminase zinc-binding (XP_056816657.1), this last may represent two enzymes, the cytidine deaminase and the deoxycytidylate deaminase.

Based on all these enzymes, which are more abundant in the control samples, and most are associated with the degradation of cellular components to obtain energy, it is possible to infer that the fungi grown in the presence of PE have more nutrients in the medium, and they are using the plastic as carbon source.

Figure 9 presents the VIP scores of the twenty proteins with higher VIP scores, that contribute to a better separation of groups, in the case of the 28 days samples.

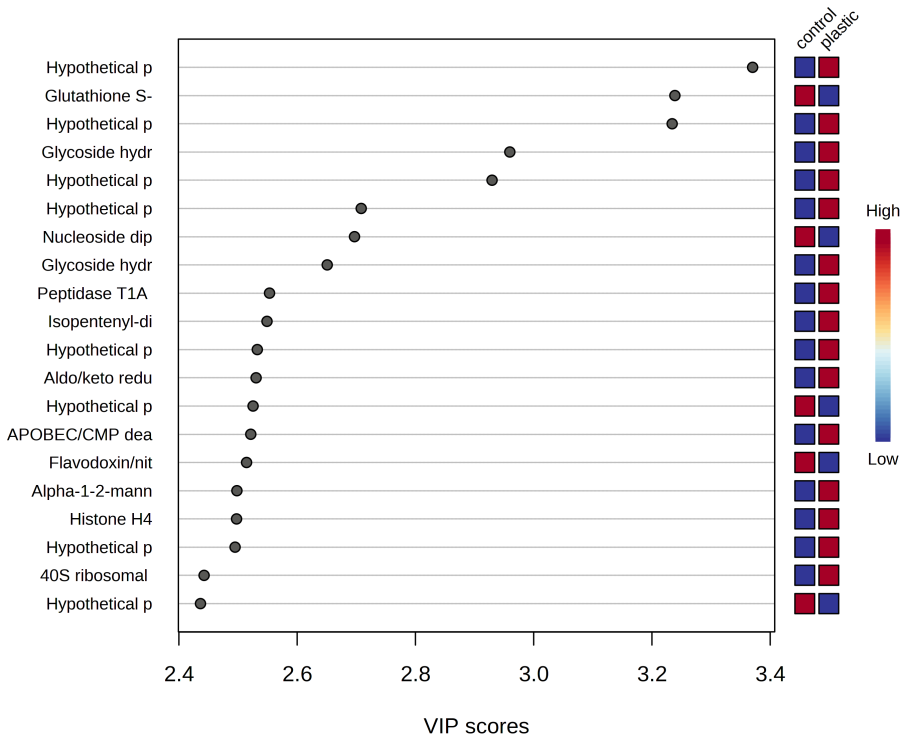

Figure 9 - Variable importance in projection (VIP) scores plot based on two components from the PLS-DA of proteins differentially expressed in control and and "plastic" group in the *P. brevicompactum* grown for 28 days. Only the twenty proteins with higher VIP scores are presented (all have VIP>1). This analysis was performed and the graphic generated using MetaboAnalyst (version 5.0).

Glutathione S-transferase (KAJ5362848.1), nucleoside diphosphate kinase (XP_056811242.1), flavodoxin/nitric oxide synthase (KAJ5342733.1), and two hypothetical proteins (XP_056810486.1 and XP_056808795.1) are less abundant in samples of fungi grown in the presence of PE.

Glutathione S-transferase, like in the 21 days samples, can be associated with stress response. Nucleoside diphosphate kinase is an important enzyme to maintain normal cellular function and survival of fungi[67], and it is a multifunctional enzyme that regulates various biological processes, including stress responses[68], also showing that in a medium without PE, the fungi are in stress, probably for lack of nutrients.

At 28 days samples, a protein from the glycoside hydrolase superfamily (KAJ5363048.1) is more abundant in the samples of fungi grown in the presence of PE. This family

includes several proteins, this in one has a similarity of 78.8% with glucan endo-1,3-beta-glucosidase (A0A0A2LMY4_PENIT, Figure S4). A involved in the degradation of beta-glucan and other substrates[69], so it can be involved in degradation of PE as well, as it is able to break complex bonds.

Other protein from the glycoside hydrolase family 2 (XP_056815070.1) is also more abundant in the samples of *P. brevicompactum* grown in the presence of PE, it has 83.9% similarity with beta-mannosidase (A0A1V6PWH8_9EURO, Figure S5). Beta-mannosidase is involved in the hydrolysis of complex plant saccharides, so it may be also involved in the degradation of PE. The enzyme alpha-1,2-mannosidase (KAJ5349466.1) is also more abundant in the samples of fungi grown in the presence of PE.

Peptidase T1A proteasome beta-subunit (XP_056807437.1) is associated with proteolysis, the breakdown of proteins, and it is a crucial protein for several biological processes, including protein turnover[70]. Peptidase T1A proteasome beta-subunit is more abundant in the *P. brevicompactum* grown in the presence of PE, so this protein may be involved in the degradation of PE.

A protein from the aldo-keto reductase family (XP_056805678.1) was also identified as more abundant in *P. brevicompactum* grown in the presence of PE. This family encompasses several enzymes that catalyze redox transformations, for example, involved in the detoxification of xenobiotics compounds[71]. Plastics are considered as a xenobiotic material since they are not naturally produced and are not expected to be present within organisms[72]. The enzyme identified has a similarity of 88.5 % with D-Xylose reductase (A0A1F5LUS4_9EURO, Figure S6).

Isopentenyl-diphosphate delta-isomerase (XP_056812392.1), an enzyme involved in the isoprenoid biosynthesis pathway, APOBEC/CMP deaminase zinc-binding (XP_056816657.1), Histone H4 (XP_056809062.1) and 40s ribosomal protein S22 (XP_056810109.1) are also more abundant in *P. brevicompactum* grown in the presence of PE. The APOBEC/CMP deaminase zinc-binding protein (XP_056816657.1) has a high VIP score in both time points, however, at 21 days it is more abundant in *P. brevicompactum* from control samples, and in 28 days samples it is more abundant in *P. brevicompactum* grown in the presence of PE. APOBEC/CMP deaminase zinc-binding protein belongs to the cytidine deaminases family, important enzymes involved in the

pyrimidine salvage pathway[73]. These enzymes allow the cell to recycle pre-existing pyrimidine bases and nucleosides. This indicates a difference in the metabolism between the two conditions, fungi grown in the presence of PE and control. Also, probably at 21 days the fungus from control is more active in the turnover of proteins, and that this happens more intensely to the *P. brevicompactum* grown in the presence of PE at 28 days.

In contrast with the 21 days samples, in the 28 days samples, most of the proteins that contribute to a good separation of groups are more abundant in *P. brevicompactum* grown in the presence of PE, instead of control samples. Nonetheless, in general, it was not possible to find large differences between these two conditions, suggesting that *P. brevicompactum* can degrade plastics and use them as a carbon source using enzymes that are constitutively expressed[74]. An example of this is the enzymes of the TCA cycle, a suggested step on the degradation of plastics that is commonly used by fungi in the production of energy.

As mentioned before, the enzymes associated with the biodegradation of PE are extracellular, and the genome of *P. brevicompactum* encodes for the same previously mentioned enzymes, laccases, cutinases, monooxygenases, cytochrome P450, and alcohol dehydrogenase. Similarly, to *Z. maritimum*, none of these enzymes were identified by proteomics.

Finally, in the samples from *Z. maritimum,* more proteins were quantified than in the samples from *P. brevicompactum*. However, there are also biological reasons, as it was only studied the cellular proteome and *P. brevicompactum* may secrete more proteins to the extracellular medium, than *Z. maritimum*. The proteins identified in the cellular proteome of *P. brevicompactum* and *Z. maritimum* suggest that the presence of PE in the medium affects the metabolism of both fungi, but it appears to activate different metabolic pathways in each one.

Different time points were used in the two fungi, with the intent of analyzing in which greater changes would be found if between 21 and 28 days or between 14 and 28 days. It was also based on the previous works with both fungi[14,15]. According to the obtained results, the metabolism of both changed between the two time points, and *P.*

brevicompactum has fewer proteins in common between the two time points, then *Z. maritimum*. *Penicillium brevicompactum* metabolism appears to change more, but it is necessary to state that these samples had some problems and may be considered as only preliminaries, more analysis should be done. Additionally to the cellular proteome and considering that the literature describes that most of the proteins associated with the degradation of PE are extracellular[61], the secretome (or the secreted proteome) should also be characterized. Nonetheless, as far as we know, this is the first characterization of the cellular proteome of filamentous fungi in contact with PE. So, it was expected that most of the enzymes we identified are being associated with the degradation of PE for the first time.

4. Conclusions

This study shows once again the potential of *Z. maritimum* and *P. brevicompactum* to degrade polyethylene and shows that this polymer affects their metabolism and how it changes. It is the first proteomic study for filamentous fungi grown in the presence of polyethylene, which gives new information, and most enzymes are associated with the biodegradation process for the first time. Contrary to what was expected, no proteins were identified in only one of the conditions, all proteins could be found in fungi grown in the presence of PE and in fungi grown in control conditions. So, our data supports that most of proteins involved in the degradation of PE are constitutively expressed.

A secretome characterization is still necessary to unveil more proteins and better understand the metabolism behind the biodegradation of PE. It would also be important to analyze the products that are being produce during the biodegradation process, to disclose if some of them may be toxic to the fungi.

5. References

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V. The use of *Penicillium brevicompactum* and *Zalerion maritimum* for the biodegradation of plastics in environmentally relevant conditions, and ecotoxicity studies of polymers in different habitats

a. Biodegradation of polyethylene samples from the food industry: a study using polyethylene obtained from a yogurt bottle and plastics bags, an optimized medium and *Penicillium brevicompactum*

Abstract

Plastic polymers are widely used in the food industry, thanks to their great advantages, and in some cases, there has been difficulty in finding new options that may replace these polymers. Although plastic waste can be recycled, not all reach recycling facilities and some, from the food industry, cannot be recycled. Therefore, in recent years a new solution has been needed for all the used plastic. The ability of some microorganisms, like fungi, to biodegrade and used plastic as a carbon source has been studied as a natural solution. *Penicillium brevicompactum* has already been studied for the degradation of polyethylene (PE), so in this work its ability to degrade samples of PE from the food industry was put to the test. Results showed that this fungus has great affinity with low-density PE and high-density PE, growing in their surface and despite not achieving with neither the optimal percentages of removal (100%), it achieved reasonable results, around 20% and 40% for each type of PE.

1. Introduction

Polyethylene (PE) is the most widely used plastic and for consequence it is the most globally produce synthetic polymer. As a thermoplastic, it can be melted and shaped to a primary form in the production factories and then reshaped into the various forms and devices[1,2]. It was discovered in 1933, by Eric Fawcett and Reginal Gibson, by accident, as they tried to condense at high pressure and temperature, ethylene, and benzaldehyde. However, only in 1935 the chemist Michael Perrin was able to repeat this and obtain large amounts of PE, using ethylene with traces of oxygen. Four years later, high-pressure polyethylene started to be commercially produced and was widely used during World War II. Driven by the war, several advances were made to improve their production, until the parameters that are still used nowadays were achieved[3,4].

The conditions and parameters used in the polymerization of PE will vary and influence its composition, structure, and properties, creating what can be considered different types of PE. American Society for Testing and Materials (ASTM) identifies five types of PE, based on theirs crystallinity: Low-density polyethylene (LDPE), Linear low-density polyethylene, Medium density polyethylene, Cross-linked polyethylene and High-density polyethylene (HDPE)[5]. Each type of PE has a multitude of applications in agriculture, construction, biomedical devices[6], toys, food industry, among many others[4].

In the food industry plastics are extremely important, as versatile materials they can be used as bottles for edible oils and sauces or beverages, as packaging films and trays for vegetables, fruits, and meats, as pots for yogurt, as tubs for margarine or ice cream.

A great advantage of using plastics is that these types of materials ensure that the natural taste is kept in the food. Furthermore, the barrier properties of the plastics play a crucial role in preserving the quality of food by safeguarding it from external contamination[7]. The protection of perishable food items extends their shelf life. Consequently, when analyzing global usage of plastics, it becomes evident that the major global application of plastics is in packaging[2]. Among the most common types of plastics used for packing are LDPE, HDPE, polyethylene terephthalate (PET) and polystyrene (PS), being the LDPE the most used film packing[8] and HDPE is used thanks to its chemical and mechanical resistance.

Unfortunately, the numbers also indicate that only 8.5% of all plastics used in packing were recycled in 2021[2], creating pressure and problems in the environment. As a result, most of the plastics found in the wild, coastal areas and oceans are PE, and a lot of them are related to the food industry, for example the six pack rings found in turtles or the straws that also affect this animal[9].

In the search for solutions for this problem, there have been proposed the use of new and biodegradable materials, that would cause less impact when reaching the environment[10], and also ways to remove the plastics already in the environment. In the last, biodegradation by fungi has been a subject of intense research as it seems to be a promising and natural way[11,12].

Actually, the biodegradation of polyethylene by the fungi *P. brevicompactum* has been optimized and values around the 100% were achieved[10]. Unfortunately, it is still necessary to understand how this fungus would behave in real settings. So, this work intends to take a first step in that direction by studying the biodegradation of PE samples, from plastics obtained from food packaging, by *P. brevicompactum*.

2. Material and methods

2.1. Biodegradation assay

2.1.1. Microorganisms culture conditions

Penicillium brevicompactum CMG 72 was grown at about 20 ^{o}C in 250 mL Erlenmeyer flasks in an orbital shaker at 120 rpm (Orbital MaxiHD OL30-HE, OVAN), with a liquid growth medium consisting of 35 g/L of salt (LabKem), 1 g/L of peptone (Sigma Aldrich), 20 g/L of glucose (LabKem), and 20 g/L of malt extract (Oxoid).

2.1.2. Microplastics preparation and characterization

Two types of polyethylene (PE) microplastics were used, high density polyethylene (HDPE) from a yogurt bottle and low-density polyethylene (LDPE) from a plastic bag used for conserving food. To reduce these plastics to microplastics size, they were manually cut using a scissors and a tweezer. As presented in Figure 1, HDPE microplastics presented a rectangles shape, Figure 1 a), and LDPE presented square shape, Figure 1 b).

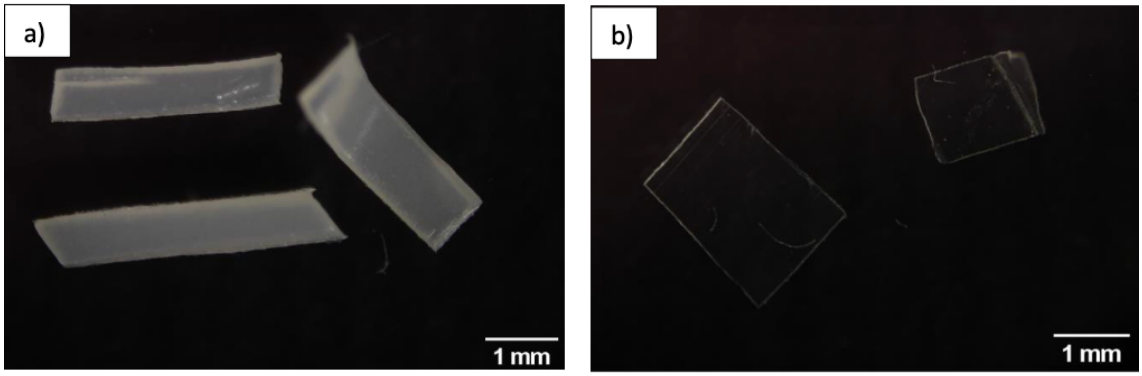

Figure 1 – Photos of microplastics obtained with an optic microscope, Olympus BX41, with an amplification of 4x, for the two types of PE: HDPE (a)) and LDPE (b)).

2.1.3. Experimental conditions

The experiment was performed in batch for 28 days, with time points at 7, 14, 21 and 28 days, using fifty-six mL Erlenmeyer flasks (100 mL), placed in an orbital shaker at room temperature, around 20 ^{o}C, with 120 rpm.

At each time point fourteen Erlenmeyer flasks were retrieved, two were control for fungi growth, so it only contained fungi in the optimized medium. Four were control for the effects of the medium in the two types of microplastics, so two contained only LDPE in the optimized medium and the other two contained HDPE in the optimized medium. The remain eight Erlenmeyer flasks, four of them contained LDPE and fungi in the optimized medium, and the other four HDPE and fungi in the optimized medium.

The used medium had been previously optimized to PE biodegradation, 35 g/L of salt, 4.6 g/L of glucose, 16.3 g/L of malt extract and 0.6 g/L of peptone[13].

All Erlenmeyer flasks containing the medium, and the ones containing medium and 0.015 g of microplastics were autoclaved for sterilization and afterwards they were inoculated with 0.5 g of fungi, except the ones that were control for the microplastics.

2.1.4. Separation of microplastics from fungi

On each sampling day (7, 14, 21, and 28), fourteen Erlenmeyer flasks (2 containing only fungus, 4 containing only microplastics, and 8 containing both microplastics and fungus) were removed from the orbital shaker and subjected to a filtration process using a filter of 90 mm diameter and 200 µm pore (Prat dumas, A009210). During filtration, and when

possible, microplastics were separated from fungi and placed in a previously weighted 2 mL glass flask; mycelium was also placed in a previously weighted glass flask, frozen and later lyophilised. The lyophilised mycelium was analysed and the remaining microplastics were retrieved. The flasks containing microplastics were placed in an oven at 60 ^{o}C for drying.

Some of the microplastics were not successfully detached from the fungi, so they were submitted to a digestion treatment, using 20 mL of nitric acid (70%) for 1h and afterwards they were filtered and washed with deionized water.

2.1.5. Analysis of microplastics after contact with fungi

Fourier-transform infrared spectroscopy – attenuated total reflectance (FTIR-ATR) analyses of lyophilized fungi and microplastics were carried out using a Perkin Elmer (USA) Spectrum BX FTIR instrument. The analyses were made with 64 scans at a 4 cm^{-1} resolution within the 4000–500 nm range.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) analyses of lyophilized fungi were performed using Bruker Avance-III 400 MHz spectrometer, operating at 9.4 T. The spectra were recorded at room temperature at a spinning rate of 12 kHz.

Scanning electron microscope (SEM) technique was performed in a SEM Hitachi S4100 Field emission gun, operated at 15 kV. The samples were placed directly onto the carbon tape and then coated with carbon evaporation.

The elemental analysis of lyophilized fungi was carried out using a Leco TruSpec 630-200-200. To acquire the data the combustion furnace temperature was 1075^{o}C and the afterburner temperature 850^{o}C. For carbon, hydrogen and sulfur detection it was used infrared absorption, for nitrogen detection it was used thermal conductivity.

3. Results and discussion

3.1. Biodegradation by *Penicillium brevicompactum*

Table 1 presents the inoculated biomass, as dry weight, at the beginning of the experiment, the dry weight of the recovered biomass at each time point and the growth of *P. brevicompactum* growing in contact with LDPE or HDPE, or growing in control conditions.

Table 1 – Inoculated dry biomass (wet biomass weighted at inoculation multiplied by 0.0553g dry biomass/wet biomass), dry biomass at the end of experiment (lyophilized biomass), and growth for the fungi throughout the experiment.

		Inoculated dry biomass (g)	Dry biomass end (g)	Growth mean±SD
7 days	Control 1	0.0282	0.3330	0.34±0.03
	Control 2	0.0316	0.4035	
	LDPE 1	0.0289	0.4432	0.36±0.04
	LDPE 2	0.0324	0.3611	
	LDPE 3	0.0336	0.3997	
	LDPE 4	0.0311	0.3556	
	HDPE 1	0.0313	0.4179	0.38±0.04
	HDPE 2	0.0350	0.4053	
	HDPE 3	0.0338	0.4797	
	HDPE 4	0.0284	0.3610	
14 days	Control 1	0.0305	0.5316	0.49±0.01
	Control 2	0.0301	0.5160	
	LDPE 1	0.0357	0.5464	0.53±0.02
	LDPE 2	0.0279	0.5298	
	LDPE 3	0.0294	0.5722	
	LDPE 4	0.0282	0.5833	
	HDPE 1	0.0316	0.5847	0.50±0.04
	HDPE 2	0.0291	0.5225	
	HDPE 3	0.0343	0.5436	
	HDPE 4	0.0287	0.4753	
21 days	Control 1	0.0302	0.4829	0.51±0.06
	Control 2	0.0301	0.5943	
	LDPE 1	0.0379	0.4939	0.51±0.06
	LDPE 2	0.0340	0.4367	
	LDPE 3	0.0283	0.5586	
	LDPE 4	0.0368	0.4578	
	HDPE 1	0.0355	0.6080	0.43±0.08
	HDPE 2	0.0279	0.3938	
	HDPE 3	0.0310	0.4662	
	HDPE 4	0.0322	0.3973	
28 days	Control 1	0.0279	0.5130	0.48±0.01
	Control 2	0.0292	0.4947	
	LDPE 1	0.0305	0.2408	0.48±0.01
	LDPE 2	0.0296	0.3890	
	LDPE 3	0.0301	0.2547	
	LDPE 4	0.0326	0.2590	
	HDPE 1	0.0319	0.2549	0.21±0.05
	HDPE 2	*	*	
	HDPE 3	0.0291	0.2851	
	HDPE 4	0.0287	0.1672	

The growth of fungi in the control samples increased through the first twenty-one days, and slightly decreased in the last week, probably due to the scarcity of nutrients, fungi may have used all the available nutrients in the medium in the first twenty-one days.

In the case of the fungi in contact with both PE samples, HDPE and LDPE, the peak of growth was at 14 days. At 28 days the growth was much lower than expected, for the samples in the presence of HDPE, and when compared to the rest of the days the values were incredibly low. In the samples in contact with LDPE, the growth at 21 days and at 28 days, were similar. This difference may indicate that the metabolism involved in the degradation of HDPE is different from the one involved in the degradation of LDPE, which may cause a variation in the availability of nutrients, and in the other hand the production of different metabolites. It can also indicate that during the degradation of each type of PE, different chemical compounds are produced, as intermediates, and they may display different toxicity to the fungi.

Figure 2 presents the FTIR spectrum for a sample of fungi at the beginning of the experiment, this spectrum is characterized by some peaks. One peak in the region of 3700-3050cm^{-1} attributed to bond vibrations, carboxyl, hydroxyl or phenol groups and from amides' N-H vibrations. Two peaks at 3000-2800 cm^{-1} attributed to the functional groups CH_2 and CH_3 and a peak at 1800-1700 cm^{-1} attributed to C=O bonds, in this case this three are associated with lipids. Peaks at 1700-1500 cm^{-1} and at 1450-1250 cm^{-1} attributed to amides and a peak at 1500-1400 cm^{-1} attributed to the C-N bonds, and in this case they all are associated with the proteins from the fungi. Peaks at 1150-1000 cm^{-1} that correspond to C-O bonds, and are associated to polysaccharide in this case[12–14].

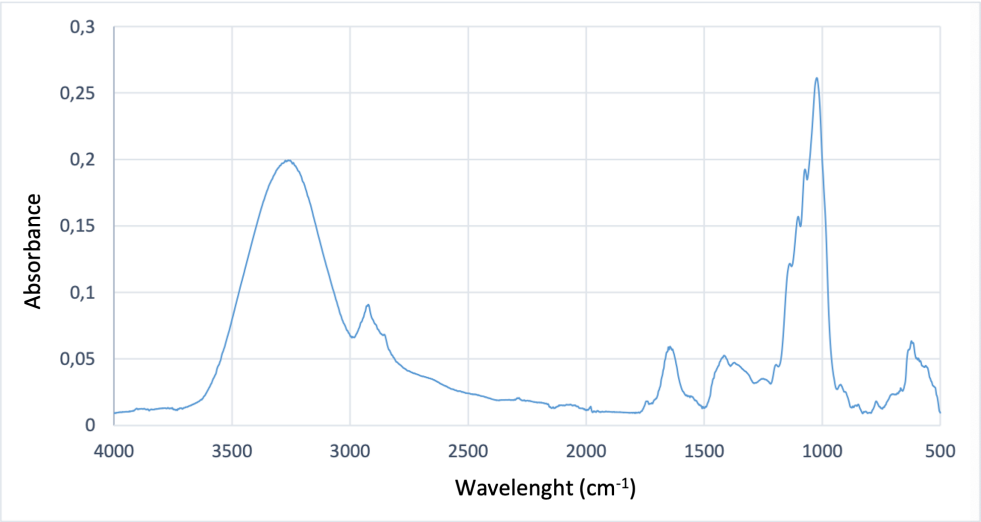

Figure 2 – FTIR spectrum for *P. brevicompactum* in the beginning of the experiment, before being in contact with microplastics.

Figures 3 and 4 present the spectra of fungi growing in contact with HDPE and LDPE, respectively, compared with the spectra of fungi growing without any contact with the microplastics, in the four time points.

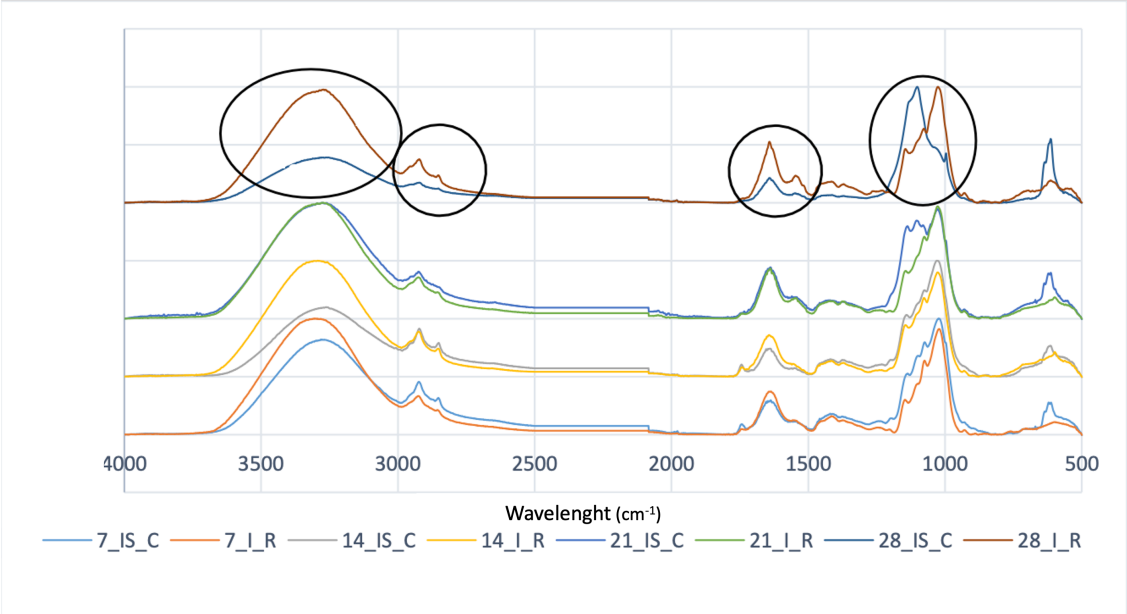

Figure 3 – FTIR spectra from *P. brevicompactum* growing in contact with HDPE (7_I_R; 14_I_R; 21_I_R; 28_I_R) and from *P. brevicompactum* growing without contact with microplastics (7_IS_C; 14_IS_C; 21_IS_C; 28_IS_C), throughout the experiment

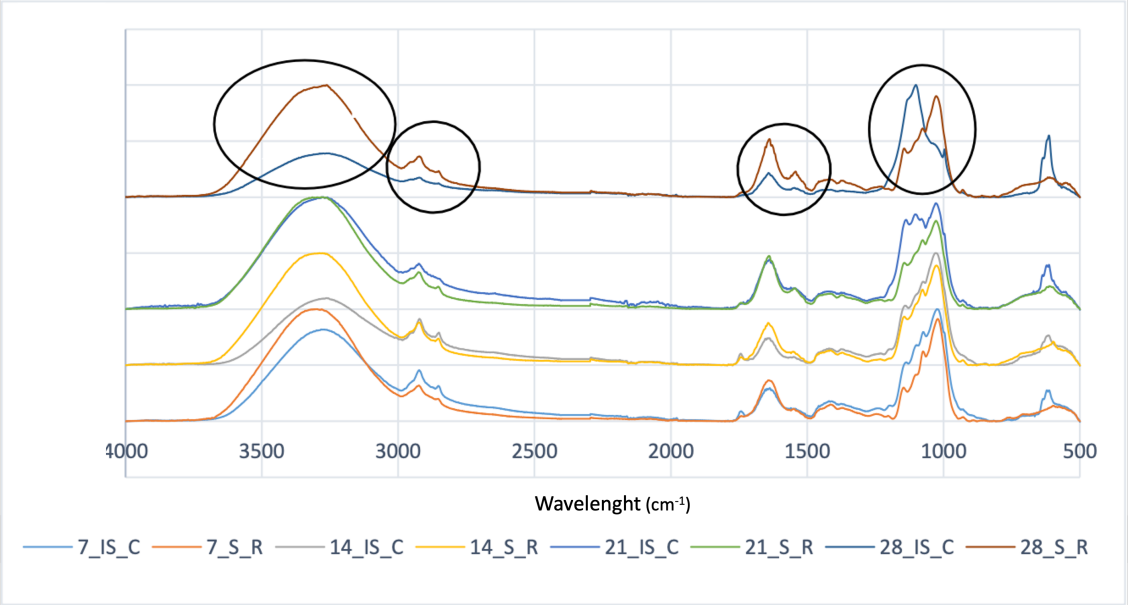

Figure 4 - FTIR spectra from *P. brevicompactum* growing in contact with LDPE (7_S_R; 14_S_R; 21_S_R; 28_S_R) and from *P. brevicompactum* growing without contact with microplastics (7_IS_C; 14_IS_C; 21_IS_C; 28_IS_C), throughout the experiment.

In all the spectra, it is possible to see the characteristic peaks explained before. The biggest differences are marked in both figures with black circles, and were found at 28 days, for the fungi in contact with both types of PE. An increase in the amplitude in the region of 3600-3000 cm^{-1} and 1700-1500 cm^{-1}, at the samples in contact with both PE when compared to the samples of control, which may indicate a possible rise in protein content in the fungi in contact with both types of PE. The peak at 3050-2800 cm^{-1} has become more evident in the samples of fungi in contact with both types of PE, which may evidence a rise in lipids.

The samples for control fungi changed over time in the region 1200-1000 cm^{-1}, which may indicate that in the case of fungi without contact with microplastics, there was an increase in polysaccharides over time. This alteration can be explained by the changes in the fungi metabolism for being in a medium with lack of nutrients, with a reduced carbon or nitrogen source. These normally induce the fungi to produce proteolytic enzymes and degrade the intracellular proteins to use the C skeleton, from amino acids, for energy production. Moreover, they also tend to produce enzymes for the liberation of the carbohydrates from the fungi cell wall and consequently there is an accumulation of polysaccharides.

The chemical composition for the culture medium was optimized to improve the removal of PE microplastics, and since it is proposed that the fungi use this material as carbon source, it is normal that the fungi growing in this medium but without contact with microplastics present signs of being in a reduced medium. This also proves, once again, that the fungi use both types of PE as a source of nutrients, and even there is an increase in the production of lipids and proteins in that case.

Since with the FTIR spectra of the fungi, growing in contact with both types of PE, was not possible to understand the reduced growth observed in Table 1 and it showed a lack of signs of being in a reduced medium, we decide to analyze the 28 days samples with NMR, the obtained spectra are presented in Figure 5.

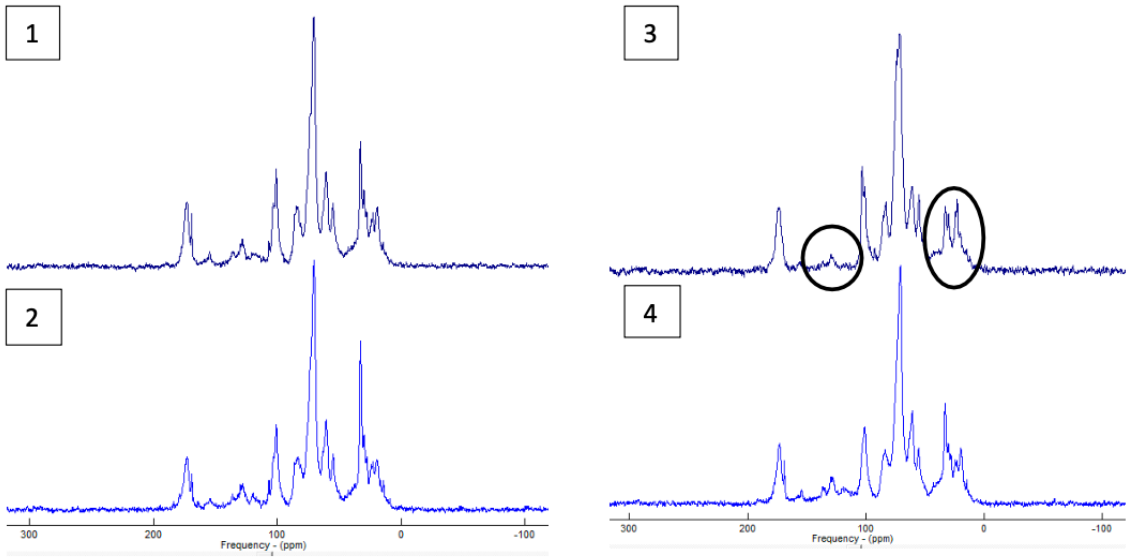

Figure 5 – ^{13}C NMR spectra of *P. brevicompactum* throughout the experiment. 1 – Fungi grown in contact with LDPE for 28 days; 2 – Fungi grown in contact with HDPE for 28 days; 3 – Sample of fungi at the begging of the experiment; 4 - Fungi grown only in an optimized medium for 28 days.

In the ^{13}C NMR spectra of the fungi in general, it is possible to find the following regions, based on the chemical shift assignments, 205-220 ppm, corresponding to C=O from ketones; 190-220 ppm, C=O from aldehydes; 170-185 ppm, C=O from acids and esters; 125-150 ppm, C from aromatic rings; 115-140 ppm, C=C from alkenes; 50-65 ppm, correspond to RCH_2OH; 40-45 ppm, RCH_2CL; 37-45 ppm, RCH_2NH_2; 25-35 ppm, R_3CH; 20-30 ppm, CH_3CO^-; 16-25 ppm, R_2CH_2; 10-15 ppm, RCH_3. Comparing the four spectra

presented in Figure 5, it is possible to conclude that 1 (fungi growing with contact with LDPE for 28 days), 2 (fungi growing with contact with HDPE for 28 days) and 4 (fungi growing for 28 days without contact with microplastics) are similar. The biggest differences are between these three spectra and the spectrum obtained for the sample of the fungi at the beginning of the experiment. The differences can be found in the region 0-50 ppm and 100-150 ppm, showing variations in the compounds with C-C and C=C bonds, respectively, which may correspond to proteins, polysaccharides, and lipids of these fungi. Both regions present a smaller chemical shift in the fungi from the beginning of the experiments suggesting an increase of proteins, polysaccharides, and lipids during the experiment. Even so, aligned with the FTIR results, the fungi from control sample, present in both regions also smaller chemical shift then fungi from samples in contact with both types of PE, possibly representing a smaller amount of lipids and proteins.

Furthermore, the same four samples, fungi at the beginning of the experiment, fungi growing for 28 days with or without contact of both types of PE microplastics, were analyzed by elemental analysis. The obtained results are presented in Table 2.

Table 2 – Percentage of carbon, hydrogen, nitrogen and sulfur for the *P. brevicompactum* throughout the experiment. 1 – Fungi grown in contact with LDPE for 28 days; 2 – Fungi grown in contact with HDPE for 28 days; 3 – Sample of fungi at the beginning of the experiment; 4 - Fungi grown only in an optimized medium for 28 days.

Sample	Weight (mg)	%C	%H	%N	%S
1	1.58	24.02	4.32	2.09	0.38
2	1.50	23.01	4.00	1.95	0.34
3	1.49	25.31	4.75	1.34	0.25
4	1.95	17.23	3.37	1.54	0.65

The fungi from samples growing in contact with LDPE and HDPE, samples 1 and 2, respectively, are very similar between them. Based on this, it is possible to say that the density of PE did not affect the elemental composition of the fungi, both had the same impact. Despite that, it is possible to say that the microplastics itself, HDPE and LDPE, both had some kind of impact, as when comparing the results with sample 4, there are changes in all elements. The percentage of carbon and hydrogen decreases, possible

due to a lack of nutrients, proving that microplastics may serve as a source of nutrients to the fungi. On the other hand, the percentage of nitrogen and sulfur slightly increase. The sample of fungi from the beginning of the experiment, sample 3, shows percentage of carbon and of hydrogen slightly superior then samples 1 and 2, and percentage of nitrogen and sulfur inferior then that same samples. These changes also indicate changes in the metabolism of the fungi, also indicating a rise in proteins similar to the results from FTIR and ^{13}C NMR.

Regarding the percentages of removal of both types of PE by *P. brevicompactum*, they are presented in Table 3.

Table 3 – Microplastics HDPE and LDPE in the beginning and at each time point, and removal percentages throughout the experiment.

		MP initial (g)	Mp final (g)	Removal (%) mean±SD
7 days	C. LDPE 1	0.0149	0	3±3
	C. LDPE 2	0.0155	0.0008	
	LDPE 1	0.0151	0.0085	38±11
	LDPE 2	0.0148	0.0054	
	LDPE 3	0.0152	0.0049	
	LDPE 4	0.0152	0.0041	
	C. HDPE 1	0.0150	0.0149	0.3±0.3
	C. HDPE 2	0.0150	0.0150	
	HDPE 1	0.0149	0.0123	28±10
	HDPE 2	0.0148	0.0113	
	HDPE 3	0.0149	0.0113	
	HDPE 4	0.0149	0.0082	
14 days	C. LDPE 1	0.0152	0.0010	4±3
	C. LDPE 2	0.0152	0.0001	
	LDPE 1	0.0155	0.0070	44±6
	LDPE 2	0.0148	0.0067	
	LDPE 3	0.0146	0.0049	
	LDPE 4	0.0148	0.0076	
	C. HDPE 1	0.0152	0.0150	2.0±0.6
	C. HDPE 2	0.0153	0.0149	
	HDPE 1	0.0150	0.0099	28±10
	HDPE 2	0.0149	0.0093	
	HDPE 3	0.0151	0.0107	
	HDPE 4	0.0147	0.0131	
21 days	C. LDPE 1	0.0148	0.0003	3±1
	C. LDPE 2	0.0149	0.0006	
	LDPE 1	0.0153	0.0062	40±5
	LDPE 2	0.0149	0.0067	
	LDPE 3	0.0155	0.0063	
	LDPE 4	0.0152	0.0049	
	C. HDPE 1	0.0150	0.0147	1.3±0.6
	C. HDPE 2	0.0148	0.0147	
	HDPE 1	0.0147	0.0123	15±1
	HDPE 2	0.0152	0.0129	
	HDPE 3	0.0150	0.0128	
	HDPE 4	0.0152	0.0130	
28 days	C. LDPE 1	0.0149	0.0001	1.0±0.3
	C. LDPE 2	0.0154	0.0002	
	LDPE 1	0.0148	0.0017	13±4
	LDPE 2	0.0152	0.0029	
	LDPE 3	0.0151	0.0013	
	LDPE 4	0.0154	0.0018	
	C. HDPE 1	0.0155	0.0155	2±2
	C. HDPE 2	0.0151	0.0145	
	HDPE 1	0.0155	0.0152	8±8
	HDPE 2	0.0155	0.0124	
	HDPE 3	0.0152	0.0139	
	HDPE 4	0.0149	0.0147	

The percentages in the different controls were low, and closer to 0 in most cases, the higher percentages were in control from LDPE, the microplastics form by small fragments of plastic bags. These values represent losses during the filtration and separation step, or small fragments that can stay in the Erlenmeyer flasks' walls, this last may explain why the values are higher in the case of LDPE, as they are transparent.

Despite this, the percentages obtained in the samples that were in contact with fungi were much higher. In general, the percentages of removal were higher in the LDPE in contact with *P. brevicompactum*, which may indicate that a plastic with lower density is easier to the be degrade by this fungus and used as a carbon source. LDPE is more flexible and has low crystallinity when compared to HDPE, it also has more accessible hydrolysable groups[14] which may improve degradation. These findings contrast with the conclusions of Ojha et al.[15], since these authors conclude that two species of *Penicillium*, *Penicillium oxalicum* and *Penicillium chrysogenum*, showed better ability to degrade HDPE, when compared to LDPE. They found higher weight losses in the HDPE sheets then in LDPE sheets for the same amount of time in contact with the fungi.

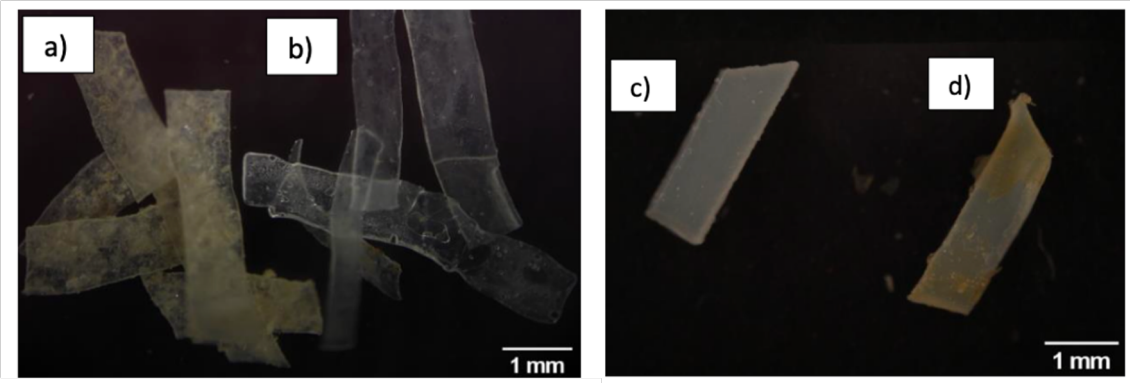

Figure 6 - Microplastics' images obtained with an optic microscope, Olympus BX41, with an amplification of 4x, for the two types of PE in the two different conditions: LDPE from samples in contact with *P. brevicompactum* (a)) and LDPE from control samples (b)), HDPE from samples in contact with *P. brevicompactum* (c)) and HDPE from control samples (d)).

In this work, the percentages for both types of PE were higher at the end of 14 days, and lower at the 28 days samples. This may be explained by Figure 6, where is possible to see how the microplastics have completely change color after 28 days in contact with *P. brevicompactum*, when compared to the control sample. The microplastics that were in contact with fungi, present a yellowish color caused by the presence of the fungi that grew in the surface, and that for consequence have a positive impact on the weight. This also evidence the affinity that *P. brevicompactum* have for PE in general, regardless the density. This high affinity or tendence of adhesion to PE by *P. brevicompactum*'s spores and biomass have already been reported by Fernandes et. al[17].

The same samples were also examined by SEM, and the results are presented in Figure 7. As seen in Figures 7.C/7.C.ii and 7.D/7.D.ii, both PE microplastics, that stay in contact with *P. brevicompactum* through 28 days, present fungal spores in their surface, marked with an arrow, and some cracks. In contrast, both PE microplastics that were kept in the medium trough the same amount of time, Figures 7.A/7.A.ii and 7.B/7.B.ii show some residues of the medium, salt and maybe some glucose, but it does not show cracks or rough surface.

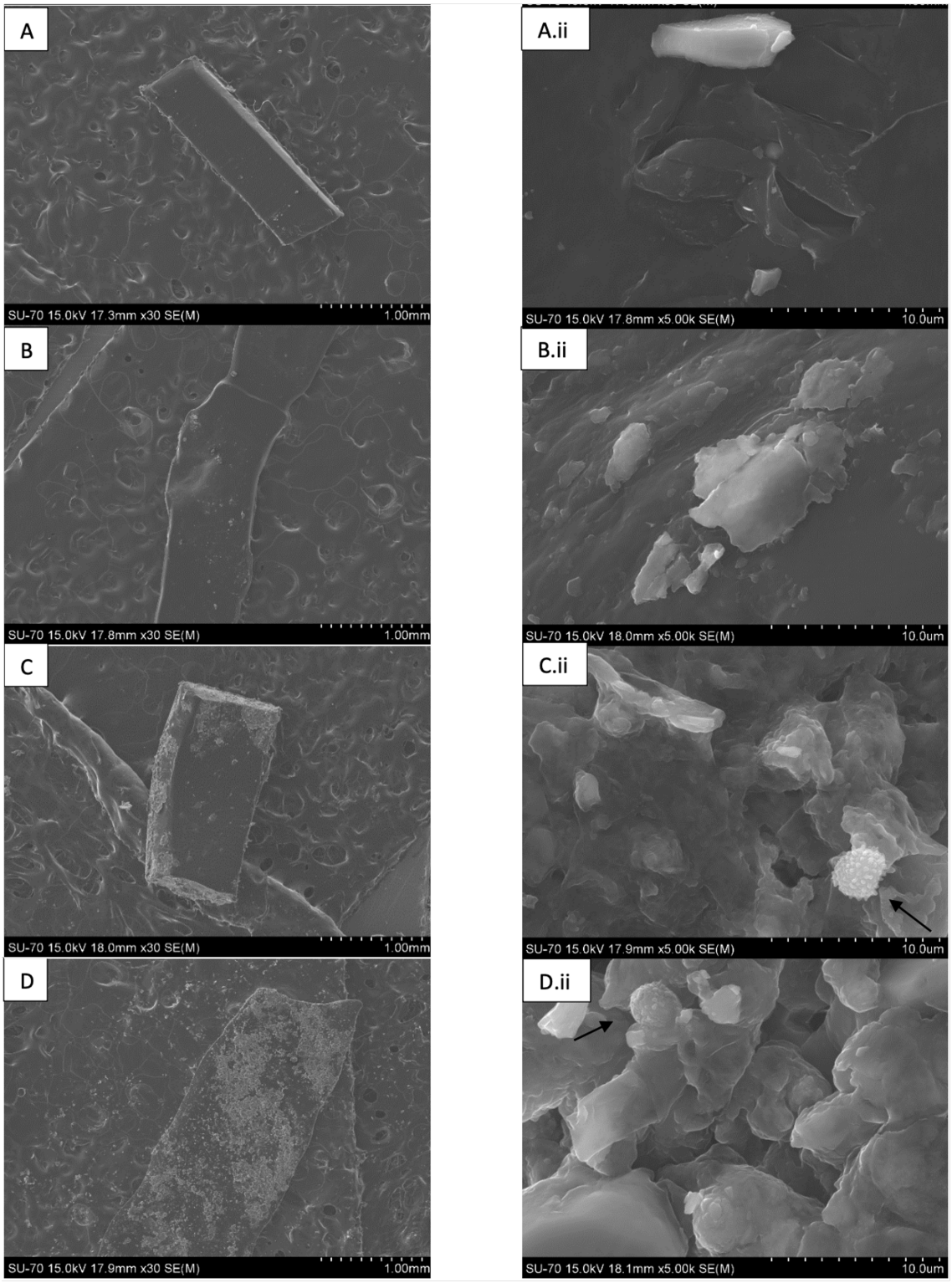

Figure 7 – SEM images of the two types of PE, in two different conditions, HDPE from control samples (A/A.ii) and LPDE from control samples (B/B.ii), HDPE from samples in contact with *P. brevicompactum* (C/C.ii) and LDPE from samples in contact with *P. brevicompactum* (D/D.ii).

FTIR spectra for each type of PE microplastic is presented in Figure 8. Both present the characteristic peaks of PE, namely one in 3000-2800 cm^{-1}, that correspond to the asymmetric stretching of CH_2; 1500-1450 cm^{-1}, that correspond to symmetric stretching of CH2; 750-700 cm^{-1}, corresponding to the deformation of CH_2. Between the two types of PE, LDPE and HDPE, it is possible to find small differences at 2919 cm^{-1}, 2851 cm^{-1}, 1473 cm^{-1} and 1463 cm^{-1} [17].

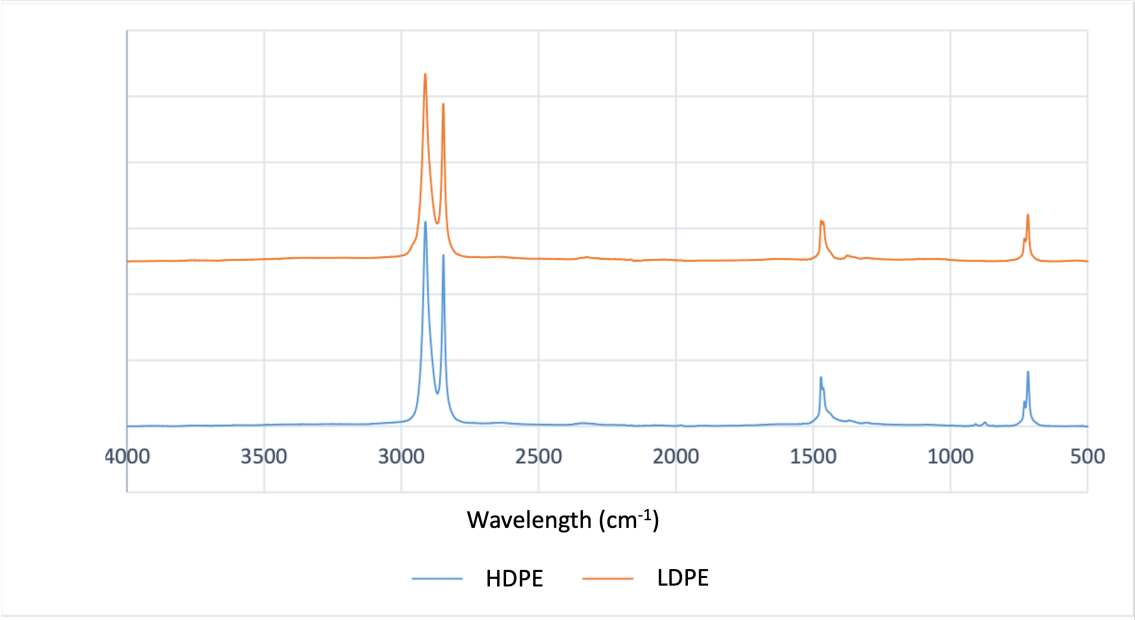

Figure 8 - FTIR spectra for HDPE and LDPE in the beginning of the experiment.

In Figures 9 and 10, it is possible to find the FTIR spectra for microplastics, HDPE and LDPE, respectively, kept in the optimized medium in comparison with microplastics, HDPE and LDPE, respectively, kept in contact with *P. brevicompactum*, throughout the experiment. All spectra show the same peaks referred before, characteristics from PE, and in the case of control samples, they are the only peaks found in the spectra, as expected. This means that the medium had no impact in the degradation of both types of PE microplastics. In the spectrum from HDPE and LDPE in contact with *P. brevicompactum* it is possible to find extra bands, that are normally associated with the degradation of this type of polymers. A band in the region 3600-3000 cm^{-1}, caused by the formation of OH groups, a peak at 1700-1500 cm^{-1}, caused by the vibrations of

carbonyl group, and a band at 1200-1000 cm^{-1} due to the ligations C-O-C[12]. For both types of PE kept in contact with *P. brevicompactum* for 21 and 28 days, it was difficult to have a good spectrum, as most had influence of the fungi on their surface, as shown before, so the spectrum presented in the figures, are the ones without this influence. For some reason, these spectra also present smaller bands on the regions that mark degradation, it could be inferred that microplastics with less fungi in their surface show less degradation due to a lack of contact with the fungi. Nonetheless, all the spectra presented show signs of degradation.

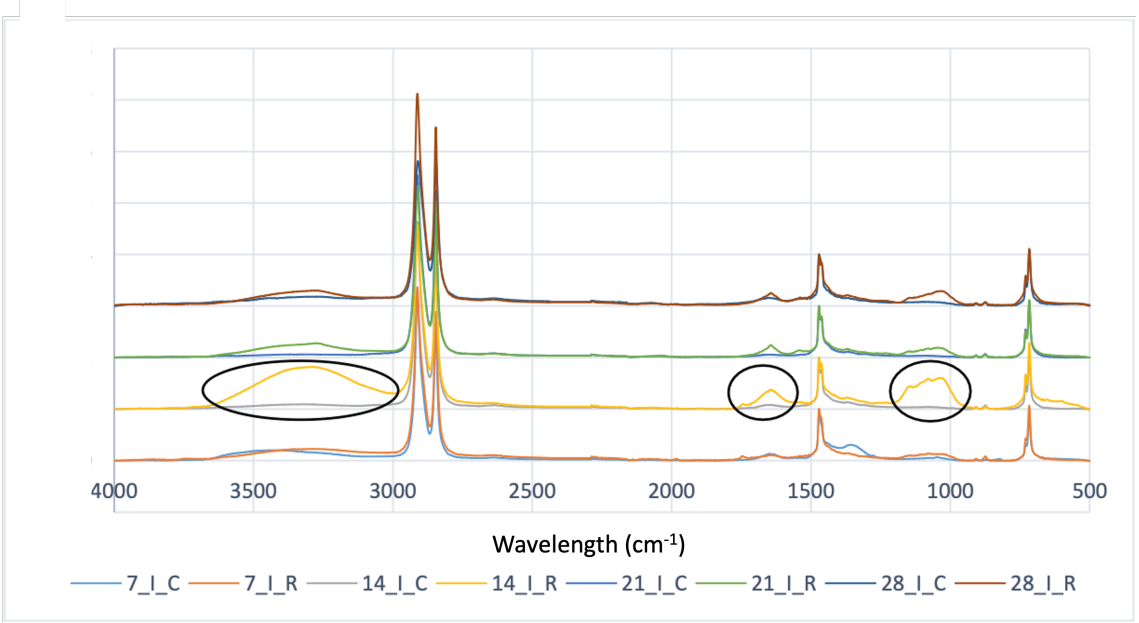

Figure 9 - FTIR spectra from HDPE in contact with *P. brevicompactum* (7_I_R; 14_I_R; 21_I_R; 28_I_R) and from the microplastics kept only in the medium (7_I_C; 14_I_C; 21_I_C; 28_I_C), throughout the experiment.

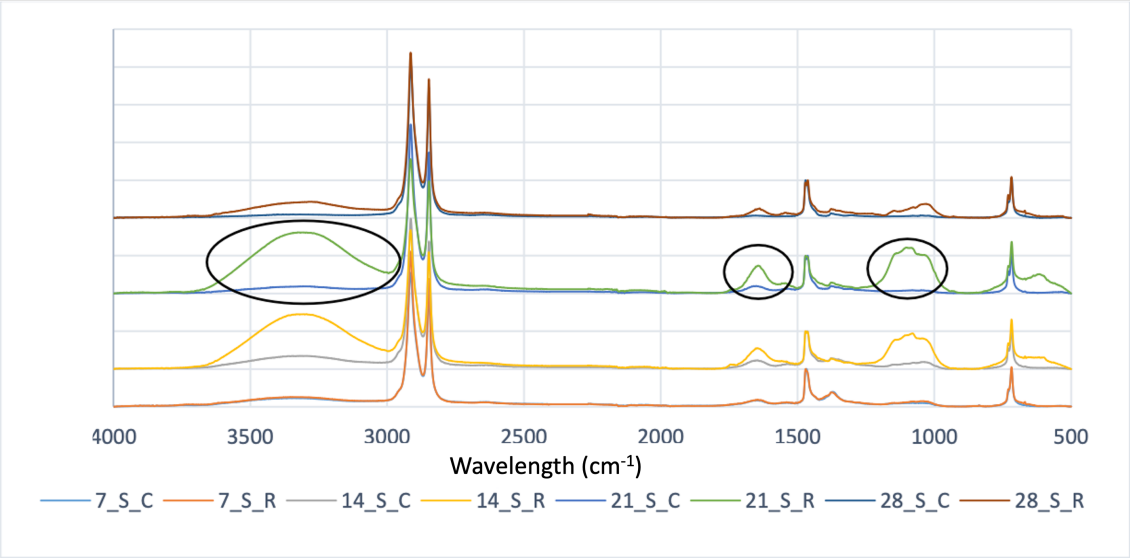

Figure 10 - FTIR spectra from LDPE in contact with *P. brevicompactum* (7_S_R; 14_S_R; 21_S_R; 28_S_R) and from the microplastics kept only in the medium (7_S_C; 14_S_C; 21_S_C; 28_S_C), throughout the experiment.

In general, *P. brevicompactum* showed good results for the removal and degradation of both types of PE, despite the low percentages calculated for 28 days. As these experiments were performed with an optimized medium, the expected values were to be closer to 100%, but the optimization was made with smaller particles of standard PE[10].

In this work, PE samples from the food industry were used, the LDPE was from a plastic bag and the HDPE was from a yogurt bottle, so they are polymers that were already used in the manufacturing of these items, which may have added some alterations and they have also been used before been put in contact with *P. brevicompactum*.

Nonetheless, the max values obtained, around 40% to LDPE and around 20% to HDPE were good values when compared to the ones present in the literature. For example, *Trichoderma harzianum* was able to degrade 23% of PE, but in 3 months[18]. Two different species of *Aspergillus*, *Aspergillus flavus* and *Aspergillus tubingensis* were able to degrade around 7% of HDPE in 30 days[19]. *Aspergillus glaucus* and *Aspergillus niger* were able to degrade 28.80% and 17.35%, respectively, within one month[20]. There is also *Zalerion maritimum*, that was reported to be able to degrade around 65% of PE microplastics within 28 days[12]. So, the values obtained in this work fall within the ones that have been reported in general for degradation of fungi[21].

4. Conclusions

This study focused on applying an optimized medium for the biodegradation of microplastics on samples from the food industry, for the first time. The capacity of *Penicillium brevicompactum* to biodegrade two different types of polyethylene, from a plastic bag, with low density, and the other from a yogurt bottle, with high density.

Despite using an optimized medium that have shown percentages close to 100%, in this case, the values obtained, were around 40% to LDPE and around 20% to HDPE. The results also showed how the density affects the ability of *P. brevicompactum* to use the microplastics as carbon source, since the percentages of removal obtained were differently. Nonetheless, it was possible to understand that the fungus has great affinity with both, growing in the surface and changing it, as seen in the SEM images.

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b. Are mulch biofilms used in agriculture an environmentally friendly solution? - An insight into their biodegradability and ecotoxicity using key organisms in soil ecosystems

Abstract

Biobased and biodegradable plastic mulch films (aka, mulch biofilm) have emerged as a sustainable alternative to conventional plastic mulch films in agriculture, promising to reduce soil contamination with plastic residues through in situ biodegradation. However, current standards certifying biodegradable plastics cannot predict biodegradability in natural settings. The scarce studies considering the possible biodegradation and ecotoxicity of mulch biofilms in soil systems question the environmental friendliness of these alternative options. This study assessed the biodegradation of a commercially available mulch biofilm by the soil-dwelling fungus *Penicillium brevicompactum* (in solid culture media and soil for 15 and 28 days, respectively), and the ecotoxicological effects of mulch biofilm microplastics on the earthworm *Eisenia andrei* (pristine or UV-weathered, at 0.125–0.250–0.500 g/kg).

Results (from microplastics' mass loss, microscopy, and FTIR spectroscopy) suggest that the presence of *P. brevicompactum* promotes mulch biofilm's biodegradation. Exposure to environmental concentrations of pristine biofilm microplastics (and its ingestion) increased earthworms' sensitivity to touch, induced physiological alterations, decreased energy reserves, and decreased their reproduction (>30%). Conversely, exposure to weathered biofilm microplastics slightly increased earthworms' sensitivity, as well as carbohydrate reserves, without affecting their reproduction.

The tested mulch biofilm seems to be, at first sight, an environmentally friendly alternative as it presented susceptibility for biodegradation by a widespread fungus, and the absence of ecotoxicological chronic effects on a key macroinvertebrate species in soil ecosystems when considering environmental relevant concentrations and plastics weathered conditions. Notwithstanding, the obtained results highlight the need to revise current standards, as they often neglect the role of, and their chronic effects on, naturally occurring organisms.

Keywords: Biofilms, soil ecotoxicology, plastics biodegradation, fungi, earthworms.

1. Introduction

The application of plastic films for agricultural mulching continues to increase significantly worldwide, as it can improve crop yield, decrease pesticides' inputs to the field, stabilise soil moisture, and thus tackle food demand for the growing world population[2,3]. However, most plastic mulching is polyethylene (PE) based, resulting in intense loads of PE residues on agricultural soils, contaminating soil and potentially adjacent ecosystems, and perpetuating the already massive plastic pollution.

Biodegradable and biobased plastics have emerged as a promising alternative to alleviate plastic pollution and environmental degradation[4], occupying more than 10% of the plastics market share (e.g., MarketWatch, 2021[5]). Several international standards specify the requirements for biodegradable plastics in several settings, such as composting, home composting, and soil or water compartments[6–8]. Typically, full biodegradation is assessed as the 1st tier of testing, and ecotoxicity is addressed as the 2nd tier. However, most of these standards have several restrictions limiting their reliability when predicting their environmental friendliness in realistic scenarios. Such limitations include, for instance, the use of unrealistic testing conditions, such as temperatures above 30 °C, the application of sludge in the biodegradation assays, and the assessment of acute - survival toxicity tests[9]. Thus, 'biodegradable plastics' that perform well in biodegradation tests might not necessarily degrade appropriately in the natural environment and be free of (eco)toxicological effects[10–13]. Many plastic materials certified as biodegradable/compostable in laboratory conditions revealed minimal biodegradation in home composting (sometimes even under industrial composting conditions) or agricultural environments and seemed to negatively affect both compost quality and biota[14,15]. Granted materials are designed to be biodegradable under specific conditions, and their discard in environments distinct from the ones where their biodegradation is intended, resulting in unsatisfying biodegradation performances. From a consumer perspective, biodegradable plastics are often (and mistakenly) interpreted as degradable in natural environments[16]. As such, the certification of these products requires, from the start, a solid educative component to avoid incorrect discarding of these supposedly environmentally friendly materials.

The biodegradation processes of plastics (particularly mulch biofilms) by naturally occurring soil organisms and their ecotoxicological effects are still poorly covered compared with research focusing on aquatic environments[17]. Furthermore, as reviewed by Büks and Kaupenjohann[18], the ecotoxicological effects of biofilms on soil biota have, for the most part, considered pristine microplastics, often disregarding their natural ageing processes through, for example, UV radiation, and this weathering process' effects on their ecotoxicity, thus losing some environmental relevance.

Considering such knowledge gaps, this study aimed at addressing the biodegradability of a commercially available mulch biofilm in solid medium and agricultural soil by the fungus *Penicillium brevicompactum*. It also aimed at evaluating the ecotoxicological effects of mulch biofilm microplastics on the soil earthworm *Eisenia andrei* considering environmentally relevant exposure scenarios (microplastics in pristine form and aged by UV radiation, at concentrations found in agroecosystems). The fungus *P. brevicompactum* (phylum: Ascomycota, order: Eurotiales, family: Aspergillaceae)[18] can be found in agricultural environments, particularly in areas with decaying vegetation[19]. In addition to their ecological role in the decomposition of organic matter and nutrient cycling[19], they can also bind and possibly biodegrade plastic materials, such as polyhydroxy butyrate (PHB) and PE[20], which lead us to hypothesise that the same might occur to the selected mulch biofilm. The earthworm *E. andrei* (phylum: Annelida; order: Opisthopora, family: Lumbricidae) is commonly found in vermicomposting and agroecosystems, where they play an essential role as engineers by improving, for instance, soil/compost aeration and nutrients cycling, and as a key-organism on terrestrial food webs[21,22]. Like any earthworm, *E. andrei* is a detritivore, i.e., it ingests large amounts of soil or specific fractions of soil (i.e., organic matter), thus involuntarily ingesting microplastics through feeding activity[23]. The ingestion of pristine fuel-based microplastics often seems to compromise their development, reproduction, and biochemical homeostasis, inducing various chemical and metabolic changes[24,25], which lead us to hypothesise that the same might occur in the presence of weathered microplastics resulting from the fragmentation of the selected mulch biofilm.

2. Material and methods

2.1. Test polymer and preparation

A certified and commercially available agricultural mulch biofilm (hereafter referred to as biofilm) was supplied by a local company, mechanically broken down through grating to form microplastics (see Fig. S1, Supplementary information), and sieved by hand (mesh size: 2, 1, 0.5, 0.065 mm). The fractions between 0.5 mm and 1 mm were used for the biodegradation assays, whereas the fractions between 0.065 and 0.5 mm were used for the ecotoxicity tests. Biofilm microplastics selected for the ecotoxicological tests were split into pristine (microplastics without ageing process) and weathered (microplastics subjected to UV type C radiation at 240 nm with a Uvitec LF-206.LS lamp for 21 days at room temperature (20 °C), to simulate plastic photodegradation as observed for PE after the same exposure period[26]. Biofilm microplastics under UV-C radiation were regularly revolved to guarantee a uniform rate of UV degradation. Radiation intensity was registered every 3–4 days using a VLX-3W Radiometer. Both treatments were analysed using Fourier Transformed Infrared Spectrometry with Attenuated Total Reflectance (ATR-FTIR) as detailed in Section 2.6.

2.2. Biological material and culture conditions

Penicillium brevicompactum was grown at 25 °C in ideal conditions in a liquid growth medium containing 20 g/L of glucose, 20 g/L of malt extract, and 1 g/L of peptone, under stirring before the application of the biological material in the tests (adapted from Paço et al.[27]). Additionally, for tests in solid culture media, an extra growth step was performed in agar-complemented media for a week, adding 10 g/L of agar to the previously described media composition.

Eisenia andrei was supplied by CloverStrategy Lda., and their culture followed methods described in international guidelines (OECD nr. 207[28]; OECD nr. 222[29]) and based on the requirements of Good Laboratory Practices (GPL). Briefly, earthworms were maintained in a medium constituted by: 1 kg of *Sphagnum* sp. peat (previously mixed with 12 g of CaCO3 to increase its pH), 1 kg of cow manure defaunated through two freeze-thaw cycles (48 h at −20 °C followed by 48 h at 25 °C[30]), and 1 L water. In total, 320 organisms were used for testing. Such organisms possessed well-developed clitellum, age under 12

months old and with under a month age difference, a length between 60 mm and 120 mm as well as a diameter of 3 mm to 6 mm (as described by Jänsch et al.[31]), and a fresh weight between 250 mg and 600 mg. The selected organisms were allowed to acclimate for 48 h in the test soil.

2.3. Test soil

The soil used for testing was a sandy loam soil collected in the Agrarian School of Coimbra (Coimbra, Central Portugal), already fully characterised (properties can be found in Table S1)[32]. The soil was defaunated from macrobiota (i.e., by removing all remaining observable biological material such as plant twigs and earthworm remains, by tweezers), sieved with a 5 mm mesh, and kept at −20 °C until use.

2.4. Biodegradation assays

The biodegradation assays occurred in solid culture medium and test soil. For the biodegradation assay that occurred in solid culture medium, a 10 times diluted medium was used (see Section 2.1 for specifications). Two controls for fungi (each containing 1 cm^2 section of the fungi grown solid medium), two controls for microplastics (each containing between 0.0030 and 0.0050 g of microplastics dispersed randomly), and four replicates containing the same amount of microplastics and fungus were prepared for each sampling day (here 5, 10, and 15 days of incubation).

The biodegradation assay setup that occurred on soil was based on a preliminary experiment (see Table S2 and Fig. S2 in the Supplementary information section). For this purpose, soil with nutritional supplements was used to favor fungal growth. Nutritional supplementation was provided to overcome the loss of organic matter and nutrients due to freezing and defaunation processes. For this purpose, each replicate of 50 g of soil was enriched with 0.0015 g of peptone, 0.03 g of D-glucose, and 0.03 g of malt extract added along with distilled water in quantity to achieve 50% of the soil water holding capacity (WHC). After thoroughly mixing, the soil portions were autoclaved (Hanshin Medical HS-2522SD) and distributed neatly in Petri dishes under a laminar flow cabinet. Biodegradation soil assays consisted of two controls for fungi (containing 5 small fragments of fungal biomass retrieved from the batch reactors, each with

approximately 2.5 mm in diameter), two controls for microplastics (between 0.0030 and 0.0050 g), and four replicates containing the same amount of microplastics and fungi, retrieved at each sampling day (here 7, 14, 21 and 28 days of incubation). All weightings were performed on a Sartorius Entris 2241-1S balance.

2.4.1. Extraction and analysis of fungi and microplastics

Fungal spreading areas were calculated before separating and extracting fungi or microplastics from solid medium and soil. For this purpose, all samples were photographed (13-megapixel F/2 camera) with a proper scale. Such photos were then imported to ImageJ version 1.53e software, where the diameter in centimeters was converted into a diameter in pixels using the "Set Scale" command. Visual artefacts were removed, and the area was calculated using the "Adjust Threshold" and "Analyse Particles" commands (see Fig. S3 in Supplementary material).

The separation of the fungi material and microplastics was performed in two ways, depending on the test medium. With solid culture medium, the entire content of each petri dish was transferred into beakers, where warmed water dissolved the solid matrix, allowing for the retrieval of the floating fungi with a spoon, after which it was left to dry for 2 h at room temperature. In soil, fungi were retrieved directly by eye with sterilised pincers (although its complete retrieval was limited by the fungi spreading within the soil matrix) and checked for the presence of biofilm microplastics. Collected biological material (in both biodegradation assays) was stored in small glass vials, frozen at −20 °C, and freeze-dried for further analysis. The separation of the biofilm microplastics in both experiments started with the retrieval, with pincers, of those with no medium or biomass attached. In the solid culture media assays, treatments containing biofilm microplastic particles were submitted to the same boiling water procedure as controls containing just fungi and then retrieved with a pincer from the water surface. In the soil assays, biofilm microplastics were retrieved with a density separation procedure using a saline solution (300 g NaCl per litre of distilled water). For this purpose, the soil contents of each petri dish were thoroughly mixed with the saline solution, allowed to settle for 20–30 min, filtered through cellulose filters, and observed (and photographed) under a 1600× 8 LED Zoom USB Microscope Digital Magnifier. Retrieved biofilm microplastics (from both solid medium and soil) were then transferred into glass bottles,

placed within a closed container to avoid contaminations or spills, and left to dry at least overnight. After this period, biofilm microplastics were weighted again (Radwag MYA 2.3Y microbalance). The percentage of microplastics removal was determined by subtracting the final weight to their initial weight, multiplied by 100. The Grubbs' test determined outliers at 95% confidence[33]. Biofilm microplastics (dried samples) were further analysed with ATR-FTIR as detailed in Section 2.6.

2.4.2. Biomass assessment

Biomass increase was assessed through weighting, and superficial area spread analysis. The former compared the final masses of fungi samples from each timepoint (0, 5, 10, 15 days in the solid medium; and 7, 14, 21, 28 days in soil) with the initial mass (0 days). Collected biological samples were stored in small glass vials, lyophilised, and analysed by FTIR-ATR spectroscopy, as detailed in Section 2.6.

2.5. Ecotoxicity bioassays

For each biofilm microplastic group (pristine or weathered), the tested concentrations were 0.125 g, 0.250 g, and 0.500 g of plastic per kilogram of soil plus a control condition (no microplastic added). Such concentrations are within the reported concentrations of microplastics in soils, including hotspots as agricultural fields, worldwide (e.g., as in Koin, Germany, MP concentrations in their lands can achieve 915 ± 63 mg/kg; as reviewed by Büks and Kaupenjohann[34]). The soil was spiked with pristine or weathered biofilm microplastics in a stainless-steel bowl and thoroughly mixed with a small stainless-steel rototiller to obtain the desired concentrations. The soil humidity was set at 50% of the WHC.

Ecotoxicity tests followed the OECD guideline N^{o} 207 and 222: Earthworm Reproduction Test (*Eisenia fetida*/*Eisenia andrei)*[28,29]. Briefly, each polymer treatment (pristine vs weathered) consisted of four replicates for control and each spiked soil. Each replicate consisted of 500 g (DW) of control or contaminated soil in a 1 L glass vial, along with 10 synchronised adult earthworms (i.e., with a well-developed clitellum). Tests ran in a $16h_L$:$8h_D$ light cycle at 19 °C. Defaunated (3 frost cycles at −20 °C) cow dung was humidified (2–4 mL distilled water per 100 g, depending on their hydration level) and used to feed earthworms every week for the first 28 days (15 g of humidified cow dung

was added to each glass vial). Soil humidity was checked (weight loss) every week. Flora sprouted in soils of each replicate was taken out before weight control. If needed, some additional water was sprinkled in to replenish soil humidity.

After 28 days of exposure, living adults were removed and gently rinsed with distilled water. At this stage, 3 adult earthworms per replicate were allowed to purge, individually, in glass Petri dishes containing a humidified cellulose filter paper for 24 h, in the dark, at room temperature (further details in Section 2.5.1). After purging, such earthworms were frozen at −20 °C, freeze-dried for 3 days, kept in the exicator whenever not under examination. Their dry weights were measured, and finally, random segments cut off from all earthworms were analysed using FTIR (Section 2.6).

The soil contents of each vial were then carefully put back to let all the laid cocoons hatch, and juveniles develop for the subsequent 28 days. After this period, the test vessels were placed in a water bath at 54 °C to force the juveniles to migrate to the soil surface. Juveniles were collected and stored in 70% ethanol for further quantification. Each vial was then double-checked for the presence of any remaining juveniles.

2.5.1. Examination of the earthworm purging

After 28 d of exposure, 3 randomised adult earthworms were placed, individually, in a petri dish containing moistened filter paper and allowed to purge their gut for 24 h. After removing the earthworms, each Petri dish and filter paper containing egestion residues were carefully observed under a dissection USB microscope (1600× 8 LED Zoom USB Microscope Digital Magnifier). Faeces suspected to contain biofilm microplastics from each earthworm were scrapped from the surfaces, collected with stainless-steel tweezers, and transferred into glass tubes. Organic matter was degraded using a Fenton reaction (Fe(II) + H2O2 1:1 solution), prepared by transferring 1 mL of both Fe(II) 0.01 M (pH = 6) and 1 mL of H2O2 30%, in that order, into the vials where the organic matter was collected and left reacting overnight in the oven at 50 °C. After this period, the reaction was stopped by adding 2 mL of a 300 g/L previously filtered NaCl solution, matching the total volume of the former reagents (2 mL). Samples were then mixed by shaking the tubes and immediately filtered into once burned glass microfiber filters (Whatman® glass microfiber filters Grade GF/C – 1.2 µm, 47 mm). Filters were then left

drying at room temperature overnight and then checked for the presence of microplastics using the same USB microscope.

2.5.2. Data analysis

One-Way Analysis of Variance (ANOVA) using Dunnett's post hoc test was used the reproduction and egestion endpoints to probe for significant differences between treatments (concentrations), at a 95% confidence level, after performing Kolmogorov-Smirnov and Levene's tests to analyse normality and variance homogeneity. All statistical analyses were performed with Sigma Plot 14.5 software (Systat Software Inc., San Jose, CA).

2.6. ATR-FTIR analysis

FTIR absorbance spectra were obtained for all biofilm and biological samples, using PerkinElmer Spectrum BX equipment and software, at a 4.0 cm^{-1} resolution, in a 4000–500 cm^{-1} or 2000–500 cm^{-1} range.

2.7. Quality control/quality assurance measures

All glass material was previously acid-washed, thoroughly rinsed with distilled water, covered with aluminium foil or with aluminium caps, and appropriately stored before using, being stainless material and glassware preferred over plastic. The working place (bench) was cleaned with 70% of ethanol, all the stainless-steel material (tweezers, scissors, among others) were sterilised under UV radiation inside the sterilised laminate flux chamber (Captair(R) Bio - Biocap DNA/RNA), while fungi incubation occurred under Bunsen flame.

3. Results and discussion

3.1. Biodegradation of the biofilm microplastics exposed to *Penicillium brevicompactum*

The percentage of removal of biofilm microplastics on solid media and soil matrix (which reflects a loss in microplastics mass) can be found in Table 1. Results showed a clear outlier in the microplastic-controls sampled on the 5th day in solid media, with a recorded average of microplastics removal of 33 ± 10%, considerably higher than in other replicas or controls, even after longer exposure periods.

Table 1 - The initial weight of biofilm microplastics added to each biodegradation treatment, and the percentage of removed biofilm microplastics after the incubation period (here corresponding to a loss in mass), in the absence (containing only microplastics, MP) or presence of *Penicillium brevicompactum* (microplastics plus fungi, MP + F), in solid culture medium and soil matrix. Data are presented as average mass (g) and percentage (%) ± standard deviation. [a]Denotes the absence of duplicate due to contamination.

Experiment	Sampling day	Treatment	initial MP(g)	MP loss (g)	MP removal (%)
Solid Culture medium	5	MP + F	0.0045 ± 0.0002	0.0003 ± 0.0001	6 ± 2
		MP	0.0046 ± 0.0001	0.0015 ± 0.0004	33 ± 10
	10	MP + F	0.0046 ± 0.0002	0.0006 ± 0.0005	12 ± 10
		MP	0.0045 ± 0.0000	0.0004 ± 0.0004	9 ± 10
	15	MP + F	0.0046 ± 0.0001	0.0010 ± 0.0004	21 ± 9
		MP	0.0046 ± 0.0001	0.0004 ± 0.004	9 ± 9
Soil	7	MP + F	0.0023 ± 0.0006	0.0008 ± 0.0007	30 ± 19
		MP	0.0020 ± 0.0001	0.0009 ± 0.0002	46 ± 13
	14	MP + F	0.0026 ± 0.0007	0.0010 ± 0.0009	36 ± 24
		MP	0.0023 ± 0.0001	0.0005 ± 0.0005	21 ± 23
	21	MP + F	0.0021 ± 0.0003	0.0005 ± 0.0002	24 ± 13
		MP	0.0020 ± 0.0002	0.0005[a]	25
	28	MP + F	0.0022 ± 0.0001	0.0007 ± 0.0008	34 ± 39
		MP	0.0018 ± 0.0000	0.0013[a]	26

Despite, and still, in solid media, an increased mass loss in the biofilm microplastics was observed in the absence of *P. brevicompactum* after 10 days, remaining similar after 15 days of exposure. In the presence of *P. brevicompactum*, biofilm microplastics mass loss increased to a greater extent compared to control groups, increasing with the time of exposure (6 ± 2 to 21 ± 9, from 5th to 15th day).

The biofilm microplastics mass loss observed in control conditions (i.e., only microplastics) could be related to the fact that this mulch biofilm is degradable in the presence of abiotic factors (e.g., water, UV radiation, among others) or due to limitations when retrieving the microplastics from the solid medium. Still, the higher biofilm microplastics mass loss observed in the presence of *P. brevicompactum* compared to those in control conditions with just biofilm microplastics suggests the fungi have a role in the biofilm's degradation process.

In the soil matrix, results indicate increased biofilm microplastics mass loss until the 14th day. From the 14th incubation day, results fluctuated with no defined pattern. These observations, paired with the unusually high values obtained for control conditions on the 7th day, might be related to the difficulty of retrieving the totality of the microplastics, especially considering how close the control removal values for the remaining time points are. When comparing with results from the solid culture media experiment, however, in similar timeframes (the 15th day of the solid culture media experiment and the 14th day of the soil experiment), biofilm microplastic removal rates are 21% and 36%, or 12% and 15% when subtracting controls, suggesting some relationship between these figures. Notably, *P. brevicompactum* has been found to adhere to the biofilm microplastics several times throughout the experiments, as shown in Fig. 1.

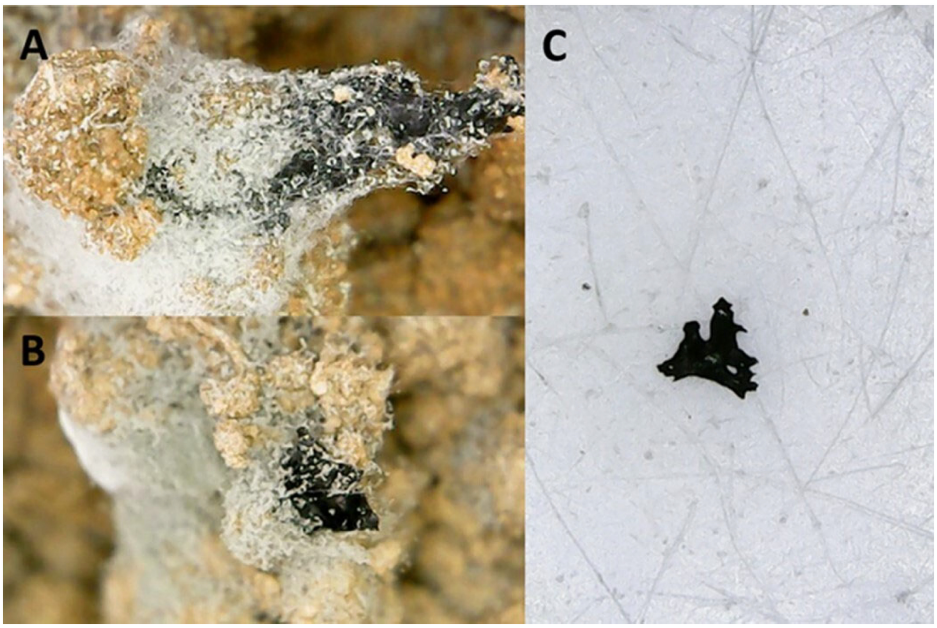

Figure 1 - Microscope images of *Penicillium brevicompactum* attached to the biofilm microplastics (1600×, 8 LED Zoom USB Microscope Digital Magnifier) after 28 days of incubation in soil (A, B), and of biofilm microplastics surface alterations (with visible holes) after removing the fungus (C).

The ability of *P. brevicompactum* to biodegrade polymers has been previously reported for polyvinyl alcohol (PVA), which removal levels were as high as 81% after 10 days of exposure in an agitated liquid medium[35]. The genus *Penicillium* has a wealth of fungi species and strains capable of inducing the biodegradation of various plastics, including PE mulch films, by species such as *P. simplicissimum*, *P. oxalicum*, and *P. P. chrysogenum*[20,36–38]. Also, reportedly able to induce PE degradation are fungi species from genera such as *Aspergillus*, *Cladosporium*, *Fusarium*, and *Phanerochaete*, although direct comparisons between findings remain challenging due to the lack of standardised methodologies[39,40]. Nonetheless, *P. brevicompactum* seems to be able to theoretically, just like many other fungi species with a variety of polymeric materials, act as a bioremediation agent by accelerating this mulch biofilm's inherent biodegradation process.

The development of *P. brevicompactum* seemed to depend on the test medium (Table 1, see also Tables S3 and S4 as Supplementary information). For example, in solid culture medium, fungal biomass increased (>500%) over the incubation period in the presence and absence of biofilm microplastics. Conversely, in the soil matrix, the assessment of

this evolution became more difficult due to the unreliable isolation of the totality of the fungal biomass.

To assess how the homeostasis of the fungi was affected and verify to which extent the biofilm microplastics were affected by the presence of this species, FTIR-ATR analysis was performed, and the results for both materials after each incubation period can be found in Fig. 2.

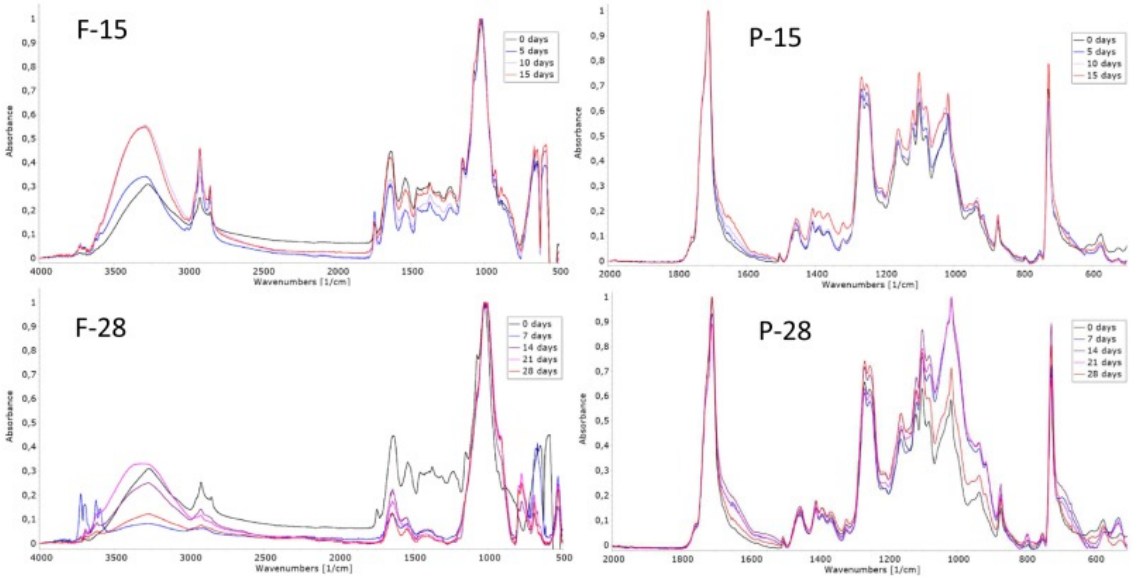

Figure 2 - Spectra from the FTIR-ATR analysis of the fungal biomass (F) and mulch biofilm (P) samples from the solid culture media (15) and soil (28) experiments after 15th and 28th incubation day, respectively.

Briefly, the spectra of these biological materials comprises[25,27,41]: i) a broad peak corresponding to OH bond vibrations from groups such as carboxyls, hydroxyls, and phenols, as well as NH vibrations from amides that can be found in the 3650–3000 cm^{-1} region; ii) bands with peaks at approximately 2918 and 2851 cm^{-1}, attributed to asymmetric stretching of methylene and CH_2 symmetric stretching, respectively, possibly indicative of the presence of lipids and proteins; iii) a peak in the 1745–1720 cm^{-1} region, consistent with CO elongations and OH deformations of carboxyl groups, as well as COO^- ions (as well as other small indentation bands at 1600 cm^{-1} and in the vicinity of 1575–1540 cm^{-1} and 1390–1375 cm^{-1} regions), whereas a peak observable at approximately 1230 cm^{-1} can also be indication of this functional group's presence; iv) peaks in the 1660–1620 cm^{-1} region that can be attributed to CC vibrations, as well as

quinine, ketone and conjugated carboxyl group; v) peaks at approximately 1630 and 1540 cm^{-1} commonly attributable to amide I and II functional groups, and thus this region can be used to monitor protein contents; vi) bands between 1450 and 1260 cm^{-1}, likely indicators of the functional groups CH_2, CH_3 and PO also indicative of protein and lipid contents; vii) a thin peak between 1462 and 1454 cm^{-1} possibly attributed to symmetric CH deformation from CH_2 groups, as well as OH deformation and CO elongation from phenolic groups; viii) a region observable at 1260–1180 cm^{-1} can be attributed to polysaccharides with COC and COP functional groups; ix) peaks at approximately 1150 and 1070 cm^{-1}, possibly attributed to CO stretching, as well as CH2 bending, for the former; x) a peak at approximately 810 cm^{-1} attributable to CH bending, and xi) a band at 750–600 cm^{-1} indicative of alkene (C=C) bending.

The fungal biomass showed different responses throughout several experiments. An apparent build-up of carbohydrates was observed in agar media, with protein and lipidic contents slightly decreasing with exposure compared to control conditions. A decrease in proteins and lipid reserves was observed on soil, particularly in peaks such as the ones at approximately 1745 and 1245 cm^{-1}, while carbohydrate contents fluctuated randomly. In addition to the mass and FTIR-ATR analysis of the mulch biofilm, these results suggest that *P. brevicompactum* might aid in the breakdown of the polymer and even use it for sustenance when considering the apparent increase in carbohydrate reserves. Results from the soil experiment, which could be interpreted as contrary to the ones from the agar media experiment, could have suffered from the lack of acclimation and the contamination of samples with soil particles, which could have contributed to a more skewed reading. However, initial declines in proteins and lipids have been previously reported in fungi in minimal media in contact with microplastics, as they acclimated towards their metabolization through an initial internal search for energy[27].

In the FTIR spectra of the mulch film microplastics, the following regions can be observed[42–44]: peaks at approximately 1721–1717 cm^{-1}, attributable to C=O, a broad carbonyl peak at 1850–1550 cm^{-1}, a band at about 1456 cm^{-1} that can be initiative of phenylene, followed by another peak around 1274 cm^{-1}, attributable to ester linkages and, finally, a sharp peak at approximately 732 cm^{-1}, which can be attributed to CH

planes from benzene groups. Peaks found in the 1118–1081 cm^{-1} region can be attributed to CO groups, and the region at approximately 1063 cm^{-1} can denote CH_2OH. It can be observed in Fig. 2 that the mulch film microplastics show some evidence of degradation throughout the experiments, especially when in contact with *P. brevicompactum*. Adding to the previously described percentage of mulch film microplastics removal, the FTIR-ATR spectra show a widening of the right side of the 1720 peak, formerly attributed to ester groups, which can be indicative of the increase of low molecular weight esters in the samples in line with what happens during the degradation of the PBAT (polybutylene adipate terephthalate) component of the mulch biofilm into PBA and PBT (polybutylene adipate and terephthalate, respectively). Although shifts in the rest of the above-focused regions remained limited, this change in specificity could be indicative that degradation may have occurred and that when in contact with the fungi biomass, the extent of the degradation would be higher, corroborating the apparent higher degrees of biofilm microplastic removal also when in contact with the fungal biomass.

3.2. Ecotoxicological effects in *Eisenia Andrei*

The ecotoxicological tests fulfilled the requirements from the OECD 207 and 222 guidelines. The pH remained similar throughout the tests, varying between 6.11 and 6.50, with no significant variation based on microplastic spiking, type, or concentration-wise. Humidity remained similar throughout the test (17.6 ± 0.3%). The adult earthworms' weights remained similar between all treatments, and overall survival was 98.8% in control conditions and 100% in all microplastics treatments.

The presence of biofilm microplastics in earthworm casts (faeces) was confirmed after the purging period of 24 h, with a correlation between the number of egested microplastics and their concentration in the soil (Fig. 3). These findings suggest that earthworms ingest microplastics and soil particles without strong selective feeding behaviour, and such ingestion/egestion might increase to some extent with the number (or concentration) of such particles in soils. Similarly, low-density polyethylene (LDPE), with 100–200 µm in size, were also reportedly found on the casts of *E. fetida*, also in a

clear concentration-dependent manner (e.g., <1 item at 0.1 g/kg to 0.8–1.2 items at 0.5 0.5 g/kg)[45].

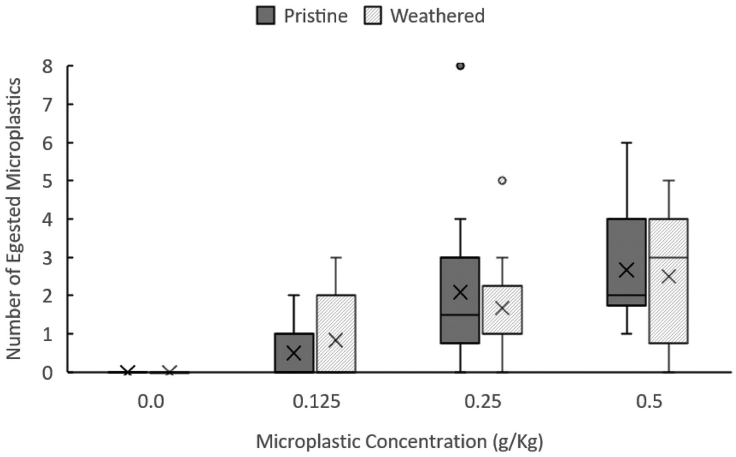

Figure 3 - The number of egested mulch biofilm microplastics (pristine or weathered) by *Eisenia andrei*, after 24 h purging in dark conditions, at room temperature. The "X" symbol within the boxes represents the average number of egested microplastics, whereas the quartiles correspond to the horizontal lines delineating the box plot. Outlying circles stand for outliers.

The size of ingested/egested particles (see Fig. S4 Supplementary information) presented a size ranging from 100 µm up to 500 µm, similar to the original microplastics used to spike the soil. The ingestion of microplastics up to 500 µm in size is not surprising when considering the mouth apparatus size of earthworms from the temperate region, whose diameter averages 3000 µm[25].

These findings (related to the size of egested biofilm microplastics) contrast with previous studies on other earthworm species (*E. fetida*, *Lumbricus terrestris*). In these studies, microplastics found on earthworms' gut had tendentially lower dimensions than the original pool-sizes used to spike the soil (up to 4850 µm, depending on the study), possibly attributed to a selective ingestion behavior[46–49]. Other explanations put forward considering the presence of lower-sized microplastics in earthworms' gut or casts were the potential fragmentation of these particles inside the organisms' gastrointestinal tracts and/or potential degradation due to the possible presence of actino-bacteria and firmicutes isolated from the earthworm's gut[50]. In our case, biofilm microplastics as large as 500 µm in diameter were found among the egested particles, remaining unclear if any breakdown of the biofilm microplastics happened. To deepen

knowledge on this topic, full gut content (containing microplastics) should be carefully evaluated and characterized in terms of quantity, shape, or size of microplastics using stereoscopic or microscopic techniques and alteration on functional groups using micro-FTIR.

Along with the confirmed ingestion of biofilm microplastics, symptoms indicative of stress were observed in earthworms, particularly in the treatments involving pristine biofilm microplastics (see Fi. S5 and S6 in the Supplementary information). Some examples include coelomic fluid accumulation (yellowish colour in the derma and/or extremities), ring deformities, cleavage furrows, high sensitivity to touch (vigorous contouring movements, and not just enrolling as commonly observed in healthy worms), and in more extreme cases, bisections. Such physiological and behavioral alterations have been identified in the literature as clear evidence of stress to natural and/or chemical stressors[51–53].

Pristine biofilm microplastics significantly decreased the number of juveniles, even in the lowest concentration tested ($F[3,14] = 7.444$; $P = 0.005$) (see Fig. 4). Conversely, weathered biofilm microplastics did not induce significant changes in the number of juvenile earthworms ($F[3,15] = 0.861$; $P = 0.488$).

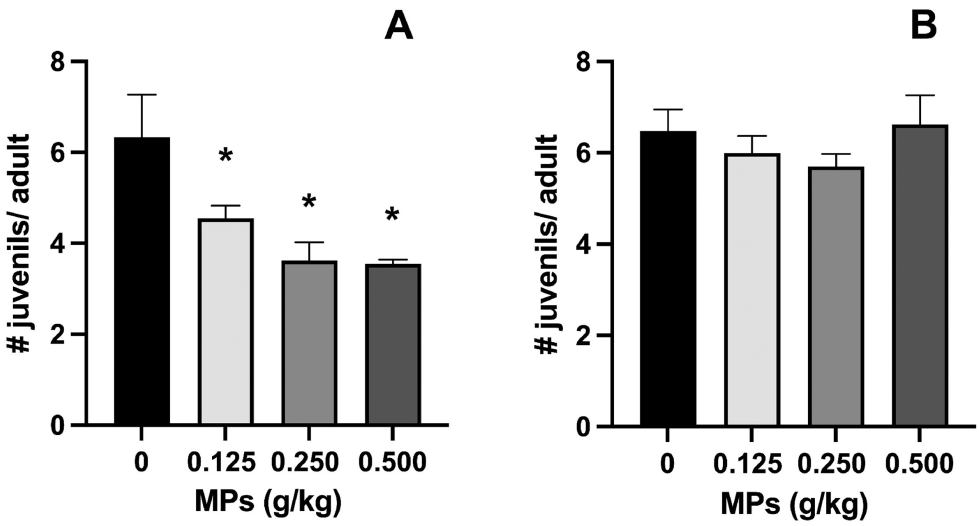

Figure 4 - Offspring numbers per adult earthworm exposed to pristine (A) and UV-C (B) weathered biofilm microplastics, with asterisks above columns denoting a statistically significant difference against control, as Dunnett's method in the one-way ANOVA analysis. Data are shown as the mean ± standard error of the mean.

Our findings considering pristine biofilm microplastics are congruent with previous studies that reported negative impacts of pristine microplastics of PE, PLA, and PVC in biomass and reproduction on the closely related earthworm *Eisenia fetida*, and in white worm *Enchytraeus crypticus*[54–56]. Such adverse effects, however, were found for concentrations considerably higher than the ones tested in our study (the concentrations tested in these referenced studies varied, as a whole, from 0.3 to 3 g per kg of dry soil, whereas the present study tested at most 0.5 g/kg). The effects of microplastics on earthworms are still far from consensual, with studies arguing for microplastics' innocuity even at higher concentrations, including some using Mater-Bi® formulations[57], as is also the case of our tested mulch biofilm (which reduced >30% the number of juveniles in concentrations ≥0.125 g/kg).

In this study, weathered mulch biofilm microplastics did not affect *E. andrei* reproduction. Considering that the ingestion/egestion of microplastics was similar on both pristine and weathered mulch biofilm microplastics treatments, the absence of chronic ecotoxicity of weathered plastic particles could be attributed to the potential degradation of the polymer by UV radiation (chemical desorption of plasticisers/additives during the ageing process). Comparing the ATR-FTIR spectra before and after said ageing by UV radiation (see Fig. S7, Supplementary information), an apparent decrease in intensity and band widening of previously more defined peaks can be seen. Previous studies refer to the occurrence of Norrish crosslinking and scission reactions consistent with the apparent degradation experienced after this ageing process[43,58]. The presence of ester groups in this material (in addition to aldehydes and ketones), as part of the polybutylene adipate terephthalate (PBAT) component of the formulation and corresponding breakdown products polybutyl acrylate (PBA) and polybutylene terephthalate (PBT), make this material susceptible to absorb this radiation, facilitating the occurrence of said reactions[43,58]. Thus, such chemical changes might be responsible for lessening the chronic ecotoxicological effects of weathered biofilm microplastics on *E. andrei*, indicating that, in natural settings (where UV radiation prevails), tested mulch biofilm might not impose an ecotoxicological risk to earthworms. The distinct effect between pristine and weathered biofilm microplastics on *E. andrei* reproduction can be partially explained by the chemical changes observed on adult

earthworms depicted from FTIR spectra, as observed in Fig. 5. Observable bands and peaks include the following: 3650–3000 cm^{-1}; 2918 and 2851 cm^{-1}; 1745–1720 cm^{-1}; 1575–1540 cm^{-1}; 1660–1620 cm^{-1}; 1630 and 1540 cm^{-1}; 1450 and 1260 cm^{-1}; 1260–1180 cm^{-1}; 1150 and 1070 cm^{-1}; 750–600 cm^{-1}; which attributions to functional groups were already described on fungi samples in the previous section (Section 3.1).

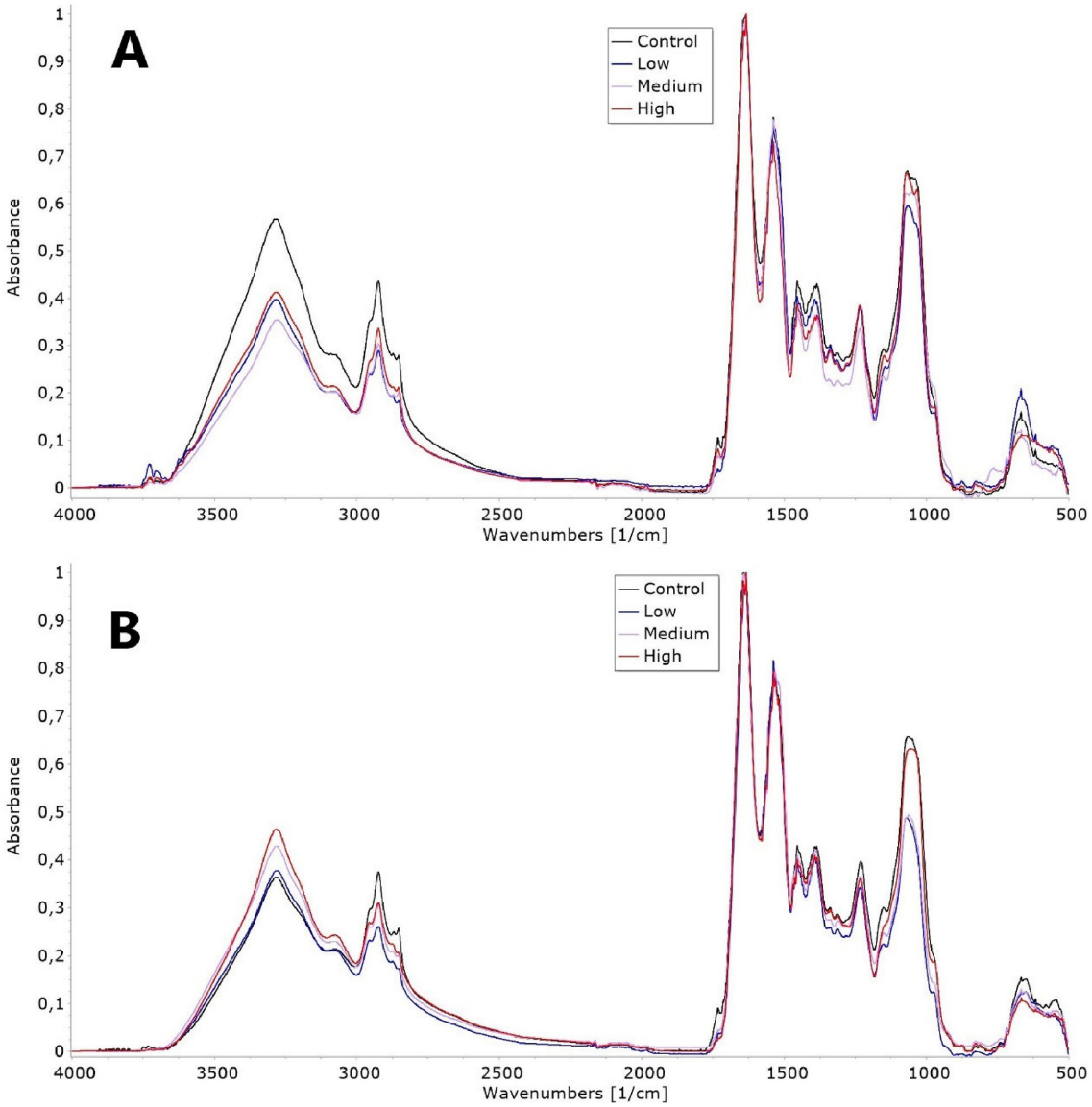

Figure 5 - Spectra from the FTIR-ATR analysis of the *E. andrei* samples after 21 days exposure to pristine (A) and weathered (B) microplastics. Tested conditions: Control - 0 g/kg, Low - 0.125 g/kg, Medium - 0.250 g/kg, High - 0.500 g/kg.

Briefly, earthworms exposed to pristine biofilm microplastics presented a generalised decrease in peak absorbances compared to organisms raised under control conditions,

particularly those potentially related to reserves of carbohydrates, lipids, and proteins. Alterations on energy reserves were not found as substantial in organisms exposed to weathered biofilm microplastics, with the exception found in the increased absorbance in the higher wavenumber regions interpreted as an indication of accumulation of carbohydrates.

Depletion of energy reserves was observed in detritivores invertebrates (Annelida) exposed to pristine microplastics. For example, *Arenicola marina* (a marine worm) exposed to unplasticized polyvinyl chloride (UPVC, up to 5% sediment weight) presented a compromised feeding activity and a decrease in total energy reserves[59]. A reduction in carbohydrates and sugars was observed in *Lumbriculus variegatus* (a freshwater worm) exposed to pristine PE (up to 20 g/kg sediment weight)[60]. Limited energy acquisition, allied with potential energy expenditure with detoxification processes or immune responses due to the presence of microplastics, diminishes the amount of energy available for other maintenance costs, such as those of reproduction[60], which could explain the compromised reproduction on earthworms exposed to pristine biofilm microplastics.

Conversely, FTIR spectra from *Eisenia andrei* exposed to pristine PE (which include, among others, the tested concentration of 0.125 and 0.500 g/kg) revealed a consistent trend in the increment on protein, lipids, and polysaccharides with increasing concentrations of these particles in the soil[25]. The increased content in energy reserves was attributed to the potential need to trigger multiple stress-response mechanisms of the earthworms' immune system, which involve a wide range of molecules/enzymes, along with the accumulation of coelomic fluid - as also observed in earthworms from our study, exposed to both pristine and weathered microplastics. A potential increase in carbohydrates (inferred from FTIR spectra) was observed in earthworms exposed to weathered biofilm microplastics but without a clear pattern. The inferences withdrawn from FTIR spectra are, however, merely qualitative; and should be complemented with the assessment of biomarkers (e.g., energy reserves, energy consumption, oxidative stress, immune response, among others) to better understand the mechanisms that might underpin the microplastics (biobased and/or biodegradable) ecotoxicity.

Recently have been reported adverse effects of pristine microplastics in nematodes and earthworms, at lower levels of biological organization, such as impairments on the gut system and microbiota (in 75% of the cases), sensory and neuromuscular functions (55% of the cases), immune system responses (50% affected), and metabolic activity (46% affected), as reviewed by Ji et al.[24]. Earthworms mainly presented deviations in DNA and carbohydrate metabolism and increased levels of oxidative stress on the metabolic end, behavioural changes, and drops in reproduction success, survival levels, and overall growth. Although over 90% of the plastics considered by Ji et al.[24] were non-biodegradable, biodegradable materials do not necessarily have fewer damaging effects, making the relative lack of studies designed around these materials much more concerning, despite their recent growth and future expectations.

Biobased and technically degradable materials also proved to cause a range of ecotoxicological effects on nematodes, snails, isopods, among others[24], proving that these materials' misuse can end up posing the same threats like the ones they were designed to suppress (i.e., the non-biodegradable ones) when their concentrations achieve high levels (in the order of g/kg).

4. Final remarks

Biobased and biodegradable plastics are currently regarded as powerful alternatives to combat the plastics economy's dependence on fossil fuels and the persistence of harmful and bioavailable plastic pollutants in the environment. However, the amount of literature available for these materials compared to their petrochemical alternatives is still limited in scope and number.

In this study, two main working hypotheses were tested. Firstly, we tested if the selected biofilm was susceptible to biodegradation in the presence of a widespread fungal species. *P. brevicompactum* seemed to be able to impact the biodegradation process of this material by attaching to plastic surface and inducing its mass losses and alterations in some functional groups. Secondly, we tested if the biofilm could, upon ingestion of their microplastics in pristine form or UV-weathered under environmentally relevant concentrations, induce chronic effects in the earthworm *Eisenia andrei*. The presence and ingestion of pristine biofilm microplastics negatively affected the earthworms'

sensitivity, reproduction, and energy reserves in all tested concentrations; conversely to UV-weathered, that did not affect the earthworms' reproduction nor (significantly) their energy reserves.

The results from this study seem to underline the potential environmental friendliness of the selected biofilm, as it presented susceptibility for biodegradation by a widely spread fungal species, with no observed ecotoxicological chronic effects on a key macroinvertebrate species in agroecosystems when considering environmental relevant concentrations and plastics weathered conditions. Results also highlight the narrow scope of the currently applied certification methodologies, as they often neglect the role of, and their chronic effects on, naturally occurring organisms.

Behavioural and physiological alterations and the evaluation of chronic effects on different target and non-target organisms (with different feeding guilds) should also be considered in future studies and considered in ecotoxicity tests for plastic products certification, since, as it stands, the currently applied certification process might be excessively permissive and incognizant of essential factors for soil species' health.

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c. Microplastics from agricultural mulch films: Biodegradation and ecotoxicity in freshwater systems

Abstract

The application of bio-based biodegradable mulch films in agriculture has raised environmental concerns regarding their potential impacts on adjacente freshwater ecosystems. This study investigated the biodegradation of microplastics derived from a bio-based biodegradable mulch (bio-MPs) and its acute and chronic ecotoxicity considering relevant scenarios (up to 200 and 250 mg/kg of sediment, using pristine and/or UV-aged particles), using the fungus *Penicillium brevicompactum* and the dipteran *Chironomus riparius* as model organisms, respectively, due to their ecological relevance in freshwater environments.

Fourier-transform infrared spectroscopy analysis suggested changes in the fungus's carbohydrate reserves and bio-MP degradation through the appearance of low molecular weight esters throughout a 28 day biodegradation test. In a short-term exposure (48 h), *C. riparius* larvae exposed to pristine or UV-aged bio-MPs had up to 2 particles in their gut. Exposure to pristine bio-MPs decreased larval aerobic metabolism (<20 %) and increased neurotransmission (>15 %), whereas exposure to UV-aged bio-MPs activated larval aerobic metabolism (>20 %) and increased antioxidant defences (catalase activity by >30 % and glutathione-s-transferase by >20 %) and neurotransmission (>30 %). Longer-term (28-d) exposure to UV-aged bio-MPs did not affect larval survival and growth nor the dipteran's emergence but increased male numbers (>30 %) at higher concentrations.

This study suggests that the selected agricultural bio-based mulch film is prone to biodegradation by a naturally occurring fungus. However, there is a potential for endocrine disruption in the case of prolonged exposures to UV-aged microplastics. This study emphasises the importance of further research to elucidate the potential ecological effects of these plastic products, to ensure effective management practices, and to establish new regulations governing their use.

Keywords: Bio-based plastics, microplastic pollution, fungi biodegradation, chironomids, biomarkers

1. Introduction

For decades, we have witnessed the continuous growth of plastic production and consumption, which started as a matter of convenience and is now pivotal in modern society. Despite the benefits of plastics to our societies, their rapid and escalating production and waste generation outpaced the development and implementation of effective management and mitigation strategies, resulting in a widespread plastic pollution crisis[1]. The development of bio-based solutions (i.e., polymers derived from renewable resources acquired from biomass, by-products, and organic residues) has been prioritised by several governments and international organisations to reduce the environmental burden and economic pressure caused by the overuse of petroleum-based plastic products[2,3]. In some sectors, such as agriculture, bio-based plastics should also present *in situ* biodegradability (e.g., mulch biofilms) to reduce plastic pollution in natural environments[4]. However, with increased use of bio-based plastics designed for *in situ* biodegradation, doubts remain about their environmental friendliness, particularly when considering their potential limited biodegradation in natural settings or leakage to adjacent (non-target) environments, the release of potentially harmful compounds during degradation processes, their increased bioavailability, and the potential increase of organic matter in the environment, which could have downstream adverse effects[5].

Several international standards specify the requirements for biodegradable plastics in soil or water (e.g., EN 13432:2019, OK Biodegradable Soil OK10-e/OK Biodegradable Water: OK11-e), where generally biodegradability is assessed as the 1st tier of testing, and, eventually, ecotoxicity is addressed as the 2nd tier of testing[6]. For example, a bio-based mulching film designed for *in situ* biodegradation in agricultural fields would be subjected to biodegradation tests in soils and ecotoxicity in representative soil biota (e.g., earthworms, plants) (e.g., OK Biodegradable Soil OK10-e), neglecting its effects in adjacent aquatic environments. In natural settings, agricultural bio-based mulching films deteriorate originating macro to microplastics[7], which, with the irrigation system, intensive rains and winds, can eventually find their way into adjacent aquatic environments such as rivers and streams. Once in freshwaters, the fate of agricultural biofilm microplastic, biodegradation, and ecotoxicity likely differ from soil-mimicked

conditions. The few available studies on freshwater environments have shown that biodegradable mulch films can form large amounts of microplastics due to their slow degradation (in some cases, this number can be further increased by exposure to UV light), eventually generating more microplastics than conventional (non-biodegradable) fuel-based plastics[8]. Concomitantly, exposure to environmental concentrations of microplastics from biodegradable plastics seemed to trigger behavioural alterations and oxidative stress-related biochemical responses in several freshwater biota, such as the fish *Danio rerio*[9,10], the bivalve *Dreissena polymorpha*[11], and the dragonfly larvae *Aphylla williamsoni*[10].

This study aimed to address the biodegradation and ecotoxicity of a bio-based biodegradable mulch film (Mater-bi® based), in the form of microplastics (here referred to as bio-MPs), in simulated freshwater environments. For this purpose, naturally occurring organisms were used as test species – the filamentous fungi *Penicillium brevicompactum* (for the biodegradation assay) and the dipteran *Chironomus riparius* (for the ecotoxicity assays). The fungus *P. brevicompactum* plays a crucial role in the decomposition of organic matter, plastics biodegradation (e.g., polyamide-based, polyvinyl alcohol (PVA), Mater-Bi®-based)[12–14], and can be found in freshwater environments, including drinking water[15]. The chironomid *C. riparius* (Diptera; Chironomidae), particularly in its larval stage, is a benthic macroinvertebrate that has a pivotal role in accelerating nutrient cycling and in freshwater food webs[16]. Being detritivores, *C. riparius* larvae may ingest other substrates while obtaining food, making them susceptible to unintentional ingestion of contaminants such as microplastics[17] and eventual accumulation in their gut. Microplastics ingestion by dipteran larvae may lead to the activation of the immune system, oxidative stress events, altered energy metabolism, and potentially/eventually compromising larval growth and imagoes emergence, as already reported for polyethylene – a petrochemical polymer also used for agriculture mulch plastics[17–19]. However, microplastic toxicity in dipterans has only been reported for their pristine form, although in natural environments, such particles are prone to UV-degradation along with other weathering processes, which seem to trigger distinct ecotoxicity[20–22]. This investigation addressed the acute and chronic

effects of *C. riparius* exposure to bio-based biodegradable MPs (bio-MPs) in their pristine and/or UV-aged form.

Considering our integrative study, we hypothesised that [1] bio-MPs do not present signs of biodegradation by *P. brevicompactum,* within a 28 day timeframe; [2] pristine and/or UV-aged bio-MPs induce ecotoxicity in *C. riparius,* with greater effects in the latter. With an holistic approach, this study provides the first evidence on both biodegradation and ecotoxicity of bio-based and biodegradable agricultural mulch films in an often-neglected environment – the freshwaters.

2. Material and methods

2.1. Test species and culture maintenance

Penicillium brevicompactum (CMG 72) grew in 100 mL liquid medium consisting of 35 g/L of NaCl (LabKem), 1 g/L of peptone (Sigma Aldrich), 20 g/L of glucose (LabKem), and 20 g/L of malt extract (Oxoid)[23] , previously autoclaved (Hanshin Medical HS-2522SD), in 250 mL Erlenmeyers, and kept in an orbital incubator (OVAN I10-OE) at 120 rpm and 20 °C. Before experiments, the fungus was transferred to 250 mL Erlenmeyers containing 100 mL of Artificial Pond Water (APW) consisting of 0.294 g/L CaCl2·2 H2O (calcium chloride dihydrate), 0.123 g/L MgSO4·7 H2O (magnesium sulphate heptahydrate), 0.065 g/L NaHCO3 (sodium bicarbonate), 0.006 g/L KCl (potassium chloride), 0.008 g/L K2HPO4 (dipotassium hydrogen phosphate), 0.085 g/L NaNO3 (sodium nitrate), and 0.028 g/L NaSiO3·9H2O (sodium metasilicate nonahydrate), previously autoclaved, and kept in the orbital incubator at 120 rpm and 20 °C.

Cultures of *C. riparius* were maintained according to the OECD guideline 233[24]. Briefly, *C. riparius* larvae were kept at 20 ± 1 ºC under a 16:8 h light-dark photoperiod. The larvae were grown in glass aquaria confined in an acrylic cage to contain the adults. The aquaria contained a layer (~3 cm) of inorganic sediment previously sterilised (500 °C for 4 h, LBX Instruments MUFU-020-001) and sieved (<1 mm), as well as hard water (ASTM, 1980[25]) iin 1:4 ratiom, with continuous aeration. Cultures were fed three times a week (*ad libitum*) with a macerated suspension of TetraMin® (Tetrawerke, Melle, Germany). The hard water and the sediment were renewed every two weeks. Fourth-instar larvae (~12 days post-hatching) were used in the short-term experiment following previous

investigations[18,19]). Larvae < 48 h post-hatching were used in the life-cycle experiment following OECD guidelines[24].

2.2. Test polymer

The tested polymer was a certified and commercially available agricultural mulch biofilm, gently provided by a local company requesting anonymity. Such agricultural mulch biofilm consists of a recent Mater-Bi® formulation. Once in the laboratory, mulch biofilm plastics were mechanically fragmented with a stainless-steel grater, which resulted in macro and microplastic fragments. These fragments were then manually sieved through stainless steel meshes (Filtra Vibración) of 2, 1, 0.5, and 0.125 mm, sequentially, under dry conditions to avoid bio-MP aggregation. The 1–2 mm fraction was used for the biodegradation assays, to facilitate their physical and chemical characterisation after the experiment, as well as the visual assessment of fungal attachment, whereas the smallest fraction (< 0.125 mm) was used in the ecotoxicity assays, owing to their bioavailability to the C. riparius larvae, whose mentum width can reach up to 130 µm[26].

2.3. Biodegradation assays

The biodegradation assays were performed in 50 mL batch reactors of APW and endured for 28 days, with a sampling moment every 7 days (i.e., on days 7th, 14th, 21st, and 28th of incubation) (Fig.1). The following experimental design was followed: 8 controls containing only *P. brevicompactum* (*N* = 2 per sampling day), 8 controls containing only bio-MPs (*N* = 2 per sampling day) and 16 batch reactors containing both microplastics and fungus (*N* = 4 per sampling day). The batch reactors containing bio-MPs were added to the test medium (~7 mg per reactor) before autoclaving. In the treatments that required the presence of *P. brevicompactum*, the inoculation was performed with ~0.04 g (fresh weight of pre-grown mycelium) per reactor after the autoclaving of the test medium (containing or not bio-MPs). The 32 batch reactors were incubated at 120 rpm and 20 ± 1 °C, with natural light (18 h:6 h light:dark photoperiod). A schematic representation of the biodegradation assay (and tested conditions) is presented in Fig.1.

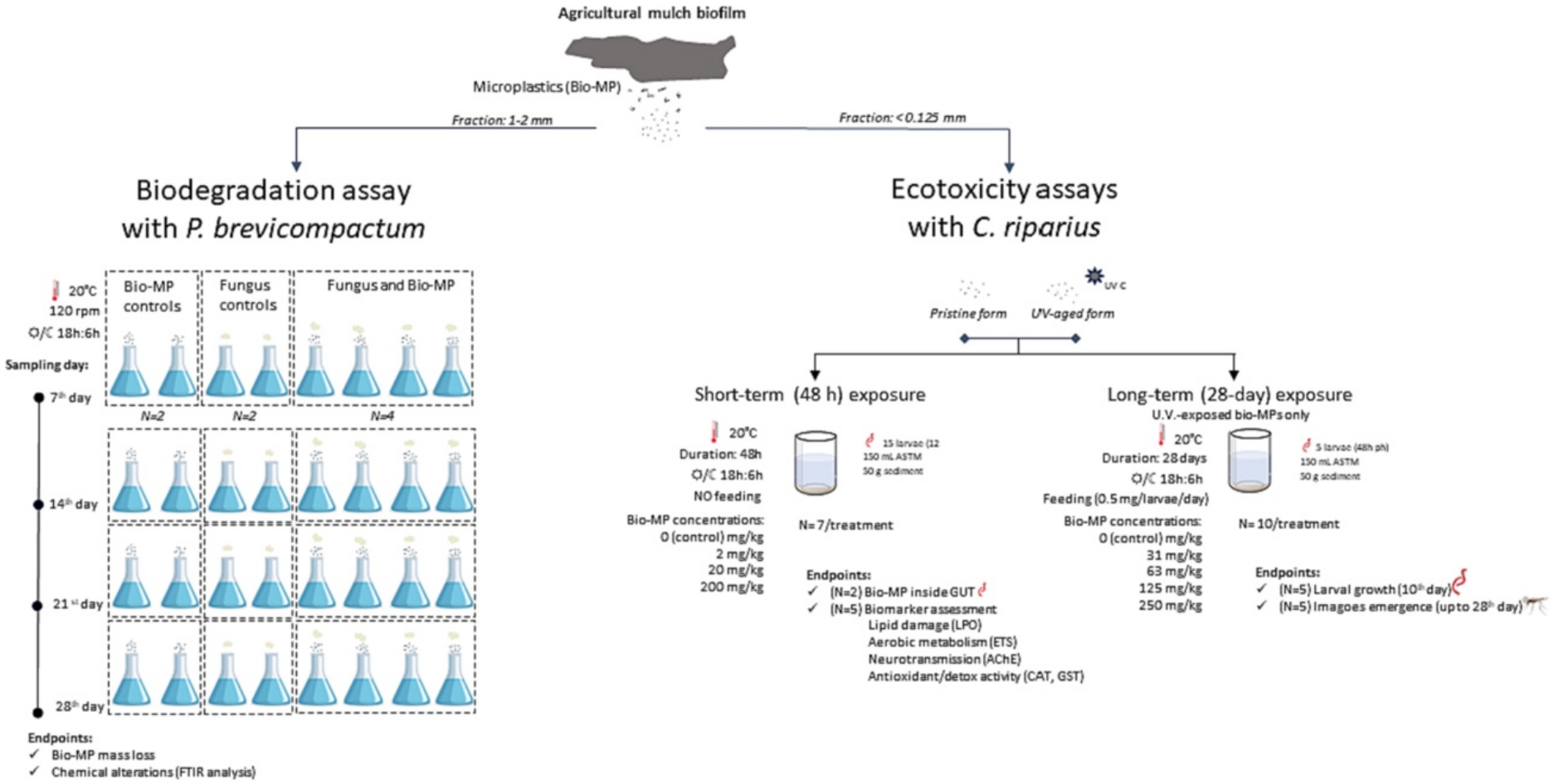

Figure 1 - Schematic representation of all bioassays performed in this study: a biodegradation assay with *Penicillium brevicompactum*; and two ecotoxicity assays performed with *Chironomus riparius.*

At each sampling day, 8 batch reactors (2 batch reactors containing only the fungus, 2 batch reactors containing only bio-MP, and 4 batch reactors containing both bio-MP and the fungus) were removed from the incubator. Each sample was filtered (qualitative analytical filter paper, 7–10 µm pore, Prat Dumas, A009106). During filtration, bio-MPs were detached from *P. brevicompactum* biomass with stainless steel tweezers, removed, and transferred to a previously weighted glass flask; the microplastics that were not successfully detached from the fungal biomass were further lyophilised, subsequently submitted to a 24-h digestion with 30 % hydrogen peroxide (H_2O_2, Sigma Aldrich), following a preliminary study exploring several reagent options towards the removal of fungal material without compromising the bio-MP physical and chemical integrity, as detailed in the supplementary information, Section II; and then filtered onto glass microfibre filters (0.7 µm pore size, Whatman®). Microplastics from each reactor were pooled, oven-dried at 50 °C, and then weighed. Fungal growth (%) and bio-MP mass loss (%) were calculated as referred to in supplementary information (Sections I and II, respectively).

Potential degradation of bio-MPs and biomass structural changes were assessed by FTIR-ATR with a Perkin Elmer (USA) Spectrum BX FT-IR System equipped with a Specap Golden Gate® ATR accessory to determine their eventual changes in functional groups. FTIR analysis of bio-MPs was only performed in particles that were successfully recovered and separated from any fungal material. Lyophilised fungal material was also analysed after being ground to powder and any remaining visible bio-MPs removed. The equipment was operated in the absorbance mode at wavenumbers between 400 and 4000 cm^{-1}, with 32 performed scans, a resolution of 4 cm^{-1}, and an interval of 2 cm^{-1}. First, the background spectra were acquired. Then, a small amount of dried sample (previously separated bio-MPs and fungi) was placed in the FTIR to cover the required surface 1 mm × 1 mm. After that, the spectra were acquired for each sample. The sample can be recovered after analysis as this is a non-destructive technique.

2.4. Ecotoxicity bioassays

A short-term test (48 h) was first performed in *C. riparius* larvae (4^{th} instar) to address early warning indicators of stress (i.e., biomarkers of aerobic metabolism, lipid

peroxidation, neurotoxicity, and energy reserves - proteins, lipids, and carbohydrates[27]). Because these biochemical responses play a critical role in organism growth and reproduction[28], a long-term test (up to 28 days) followed to assess larval growth and imagoes (midge) emergence. For this purpose, both pristine and UV-aged bio-MPs (bio-MPs exposed to UV-C light at 240 nm using the Uvitec LF- 206.LS lamp for 21 days at 20 °C)[29] were used for the short-term test, in a lower concentration range than the one applied in the long-term test. In the long-term test, only UV-aged particles were used due to their higher environmental relevance, as, *in situ*, agriculture mulch biofilms are exposed to UV radiation throughout their usage.

Bio-MPs aged by UV light for 21 days were used for these tests because chemical changes indicative of photodegradation were observed by Fourier transform infrared spectroscopy – attenuated total reflectance (FTIR-ATR) (Fig. S1, Section I, Supplementary Information), as also reported in other previous study[29].

- Short-term (48h) exposure

Short-term exposure to assess biochemical biomarkers followed Silva et al.[19]. Fourth instar larvae of *C. riparius* (−12 days post-hatching) were exposed to a range of concentrations of bio-MPs (0 (control), 2, 20, 200 mg per kilogram of sediment) both for pristine and UV-aged bio-MPs. To achieve these concentrations, the bulk sediment was spiked with the corresponding amount of bio-MPs in a glass vial and thoroughly mixed with a stainless-steel spatula[19]. Larval exposure was performed in 200 mL glass vials containing 50 g of spiked sediment (excluding the controls, which contained no bio-MPs) and 150 mL of ASTM hard water[25] that was gently added to the sediment to avoid resuspension of the bio-MPs. Test vials were allowed to equilibrate for 24 h. Then, 15 larvae were added to each vial, and a 48 h exposure period was allowed without food, at 20 °C, under a 16:8 light:dark photoperiod. Seven replicates per treatment were used (for both bio-MPs categories). Two replicates of each condition were used to prove bio-MP ingestion, and the remaining ones were used to evaluate biochemical biomarkers. At the end of the exposure period, larvae from each vial were collected, rinsed with ultra-pure water to remove possible bio-MPs that might have adhered to the body, and dried on filter paper to remove moisture without harming the organisms. This step also allowed for the detachment of any other potential bio-MPs that may have been present

on the larvae's surface after the rinsing into the filters. The larvae were weighed, frozen using liquid nitrogen, and stored at −80 °C. A schematic representation of the short-term test with *C. riparius* (and tested conditions) is presented in Fig. 1.

- Long-term (28-d) exposure

A long-term assay was performed for the UV-aged bio-MPs following international guidelines for testing[24]. Briefly, ten replicates of 5 larvae in their 1st instar (>48 h post-hatching) were exposed to control (absence of MPs) and UV-aged bio-MP at 0, 31, 63, 125, 250 mg/kg of dried sediment, for 28-days. During the experiment, larvae were fed every two days (0.5 mg of macerated TetraMin® per organism per day), and the test conditions were the same as described for culturing. After ten days, larvae from five replicates of each treatment were rinsed with ultrapure water, transferred to a glass Petri dish, preserved in 70 % ethanol, counted, and measured (total length) under a USB microscope (NORTHIX 1600×) fitted with a calibrated micrometre scale. The remaining five replicates (of the initial ten) were used to follow the emergence of imagoes (midges) until the end of the test. Imagoes were collected daily from emergence traps and placed in 5 mL tubes with ethanol 70 % for identification of the sex (male/female). Water quality parameters (pH, dissolved oxygen, and conductivity) were evaluated every three days. A schematic representation of the long-term test with *C. riparius* (and tested conditions) is presented in Fig.1.

2.4.1. Biomarkers assessment

Biomarkers related with oxidative stress and aerobic metabolism were assessed following the optimised procedure described by Silva et al.[19]. Namely, the tested biomarkers were Lipid Peroxidation (LPO), Acetylcholinesterase (AChE), Electron Transport System (ETS) activity, Catalase (CAT), Glutathione-S-transferase (GST), and energy reserves (protein, carbohydrate, and lipid levels). Briefly, samples (i.e., each replicate of each treatment, containing 15 larvae) were sonicated with 1600 µL of ultra-pure water for 3 min, with a 2-s pulse (Model CL-18, Fisherbrand). From each sample, aliquots of 300 µL were collected to evaluate the energy reserves and the aerobic energy production measured through ETS activity. An extra aliquot of 200 µL was used to measure LPO, to which 4 µL of 4 % BHT (2,6-Di-tert-butyl-4-methyl-phenol) in methanol was added before processing the samples. From the remaining homogenate, 500 µL was

diluted with the same volume of K-phosphate buffer (0.2 M, pH 7.4) and centrifuged at 10,000 ×*g* for 20 min at 4 °C to isolate the Post Mitochondrial Supernatant (PMS), which was immediately divided into micro tubes for the protein and enzymatic determination (CAT, GST, and AChE), and kept at −80 °C.

Lipid peroxidation (LPO) was assessed via the production of thiobarbituric acid-reactive substances (TBARS) at 535 nm (according to Bird and Draper[30]). Glutathione-s-transferase (GST) was determined by measuring the absorbance (340 nm) of the colourimetric reaction of conjugation of GSH with 1-chloro-2,4-dinitrobenzene[31]. Catalase (CAT) activity was evaluated at 240 nm through the reaction of decomposition of the substrate H_2O_2 as described by Claiborne[32]. Acetylcholinesterase (AChE) activity was assessed following the Ellman method[33], using acetylcholine as substrate and reading the reaction at 412 nm. To estimate the protein concentration, the Bradford method followed, using bovine γ-globulin as standard (adapted from BioRad's Bradford colourimetric microassay set up in a microplate)[34]. Energy reserves (proteins, carbohydrates, and lipids) and aerobic metabolism (electron transfer system, ETS) were assessed following De Coen and Janssen[35].

Briefly, absorbance readings were made for standards solutions, blanks, and samples at 492 nm and 375 nm, for carbohydrates and lipid levels, respectively. A calibration curve was made for each case, and their levels (concentration) were estimated. ETS activity was evaluated through absorbance kinetics of formation of INT-formazan, at 490 nm over 3 min.

2.4.2. Extraction and quantification of bio-MPs in larvae

Two replicates per treatment obtained in the short-term exposure (samples consisting of 15 larvae) were subjected to tissue digestion to prove bio-MP ingestion by C. riparius larvae. For this purpose, samples were thawed, mixed with ultra-pure water (1600 µL), and homogenised using a sonicator (model CL-18, Fisherbrand). The samples were transferred to glass tubes to start digestion, and 2 mL of potassium hydroxide (KOH; 20 %) was added to help digest the larval exoskeleton's remains. After a reaction period of 48 h, 2 mL of hydrogen peroxide (H2O2; 10 %) was added to terminate this process. After 3 h, the final solution was vacuum-filtered onto white glass microfiber filters (Whatman, No.1825–047, diameter 47 mm, 1.2 µm porosity). The filters were then

stored in glass Petri dishes, where they were allowed to dry at room temperature (~20 °C) for 2–3 days. Bio-MPs were photographed and quantified under a compound optical microscope (Olympus BX4; 100× magnification).

2.4.3. Statistical analyses

For biochemical biomarkers data, the differences between treatments using each type of bio-MP (UV-aged, pristine) were analysed through a one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test. The data's normality and variances homogeneity were assessed by performing Shapiro-Wilk and Levene's tests, respectively. In case of non-normal distribution and the heterogeneous variances of the data set, non-parametric analysis was performed, i.e., Mann-Whitney tests and Kruskal-Wallis tests with Dunn's multiple comparison tests to assess significant differences. Statistical differences were considered at $p < 0.05$. All statistical analyses were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

2.5. Quality assessment, quality control

Glass material and stainless-steel utensils were prioritised and previously sterilised throughout testing, sampling, organism manipulation, and sample processing. Cotton lab coats were used, all the glass material was previously acid-washed before use, and samples were covered with aluminium foil when not used or processed. All solutions were previously filtered before being used. All working spaces were thoroughly disinfected (alcohol). Fungus manipulation was performed with appropriate precautions to minimise alterations in growth characteristics and contamination. For this purpose, all materials were previously sterillised, and the culture or test medium were autoclaved (Hanshin Medical HS-2522SD) at 121 °C for 30 min.

Air temperature, humidity, rotation (in case of biodegradation assays) and water quality parameters were monitored throughout the experiments using a data logger (EL-USB-2).

3. Results and discussion

3.1. Biodegradation of bio-MPs by *Penicillium brevicompactum*

3.1.1. *P. brevicompactum* growth

P. brevicompactum was able to thrive in the absence and presence of bio-MPs, with total biomass reaching a plateau after 7 days and remaining stable throughout the experiment and independently of the treatment (Fig. S2, section II, supplementary information). This is indicative that the presence of bio-MPs and potential degradation products did not affect the *P. brevicompactum*'s homeostasis. The FTIR spectra of *P. brevicompactum*, grown both in the presence and absence of bio-MPs, can be observed in Fig. 2. In the region between 3600 and 3000 cm^{-1}, bands attributable to OH bonds (present in hydroxyl, carboxyl or phenol groups) and NH bonds can be observed. A band found at 2925 cm^{-1} can be attributed to an asymmetric stretching of CH_2 and a less defined peak at 2852 cm^{-1}, possibly indicative of the symmetric stretching of the same group. These peaks have been associated with the presence of lipids[23,36]. In the 1800-1460 cm^{-1} range, the peaks at 1637 cm^{-1} and 1542 cm^{-1} may be attributed to the amide I and amide II groups, respectively, which may be associated with the presence of proteins[23,36]. Still in this range, a peak can be observed at 1742 cm^{-1}, consistent with CO bond stretching of carboxyl groups with OH bond deformations, which can also explain the presence of the peak at 1240 cm^{-1}. A group of peaks at approximately 1730 cm^{-1} and 1600 cm^{-1}, and between 1400 cm^{-1} and 137 cm^{-1}, are consistent with the presence of COO^- ions. The 1654 cm^{-1} and 1648 cm^{-1} peaks may indicate $C-C, C=C$, and $C\equiv C$ bonds, quinones and ketones. In the region between 1260 and 1180 cm^{-1}, bands attributable to polysaccharides with COC and COP groups are present (IR Spectrum Table & Chart - Merck). Small bands can also be observed between 1450 and 1250 cm^{-1}, attributable to CH_2, CH_3 and PO groups, which may be especially found in proteins and lipids. High peaks can also be found between 1100 and 1070 cm^{-1} can also be found, which may indicate a C-O stretching and a CH_2 bond. Finally, a band appears at 773 cm^{-1}, attributable to a C=C bond (IR Spectrum Table & Chart - Merck).

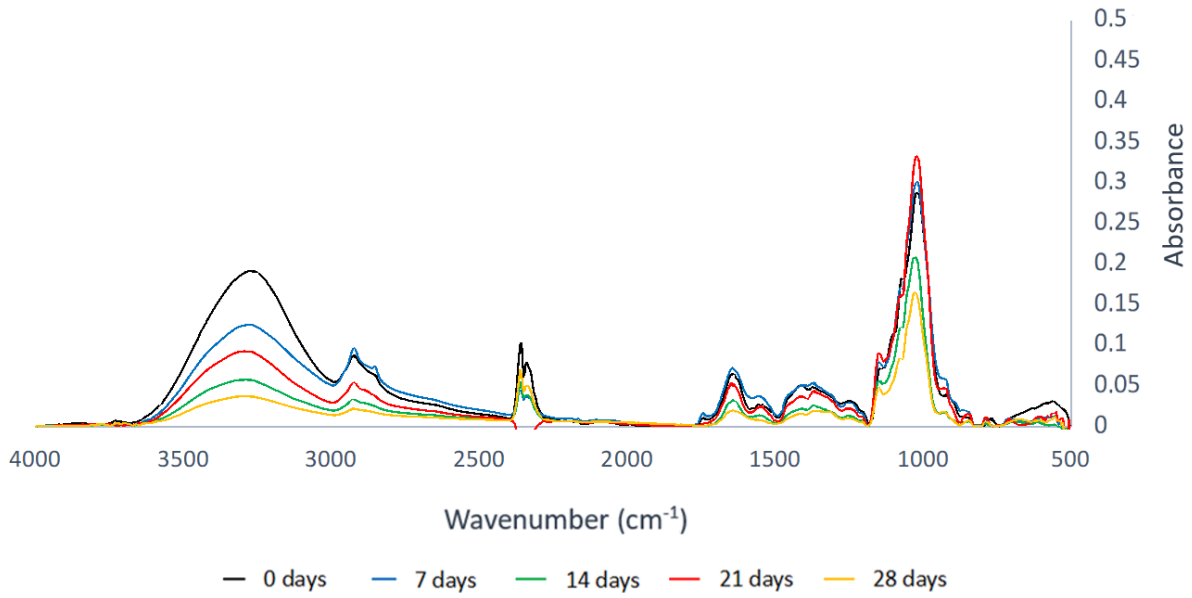

Figure 2 - FTIR-ATR spectra in the region 400–4000 cm^{-1} from *Penicillium brevicompactum* after 7, 14, 21 and 28 days of exposure to bio-based mulch films (bio-MPs) microplastics in Artificial Pond Water (Perkin Elmer (USA) Spectrum BX).

Considering *P. brevicompactum* grown in the presence of bio-MPs, a general decrease in peak height in the region between 3600-3000 cm^{-1} can be observed (except for the 21st-day peaks, higher in intensity than the 14th-day peaks), which could be indicative of carbohydrates' consumption over time. No apparent changes in lipids and protein content were observed. These results contrast with previous investigations, where carbohydrate contents in fungi remained similar throughout the incubation period with microplastics. Specifying, only a decrease in lipids and proteins was reported by Paço et al.[23], on the fungus *Zalerion maritimum* when in contact with polyethylene (PE) microplastics and by Ferreira-Filipe et al.[13] on *P. brevicompactum* when in contact with microplastics from the agriculture mulch biofilm. According to the authors, the decrease in lipids and proteins was attributed to the changes caused in the fungal metabolism when exposed to a medium with a reduced carbon or nitrogen source. In the present study, the APW medium was deficient in nutrients, particularly carbohydrates, compared to Paço et al.[23] and Ferreira-Filipe et al.[13]. A potential explanation for the decrease in the carbohydrate content in *P. brevicompactum* in the presence of bio-MPs could be indicative of a stress response in the fungus (e.g., oxidative stress), which could lead to changes in the carbohydrate metabolism (using it as a primary energy source).

The mechanism by which microplastics alter energy reserves and their allocation in fungi remained uncovered in most of the microplastics' biodegradation studies conducted so far, highlighting the need for further research to determine the precise effects of microplastics on fungal metabolism.

3.1.2. Bio-MPs removal and/or changes in their functional groups using FTIR

P. brevicompactum easily adhered to the bio-MPs' surface, being difficult to remove even after a chemical digestion procedure, as shown in Fig. 3. Such adherence compromised the weighting of the recovered bio-MPs. Even after a digestion procedure with 30 % H_2O_2 (for 24 h at 60 °C), some fungal mass still perdured, resulting in a bio-MP mass increase (rather than a decrease as expected, which would indicate bio-MP biodegradation/removal). Therefore, due to its chemical nature (prone to degradation by digestion procedures), no data could be presented for the bio-MP recovery.

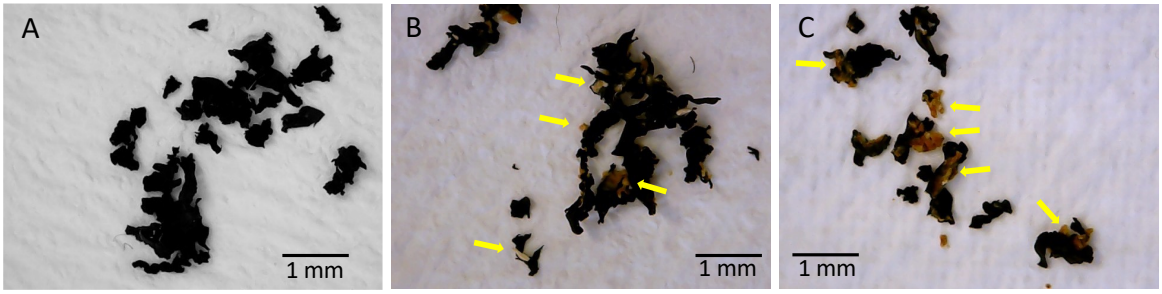

Figure 3 - *V*isual appearance and observed changes of bio-MPs after the digestion procedure with 30 % H_2O_2 for 24 h at 60 °C, in the absence (A) and presence (B – 14 days of contact; C – 28 days of contact) of *Penicillium brevicompactum*. The yellow arrows indicate the spots where *P. brevicompactum* remained attached to bio-MPs even after the digestion procedure. Images obtained *using a* USB microscope (*NORTHIX* 1600x).

A preliminary study was performed where several digestion procedures were tested, aiming to select a digestion procedure enabling the preservation of the chemical integrity of the microplastics (section III, supplementary information). The use of 30% H_2O_2 for 24 h was selected as the best option, thus, being used for the digestions. Some of the digestion procedures (e.g., 10% KOH, 7% bleach) induced chemical changes in bio-MPs, resulting in higher fragmentation and/or higher leaching of its additives (black

dye), among others, altering their chemical composition (Figure S3, section III, supplementary information). As previously mentioned, FTIR analysis of bio-MPs was performed only in bio-MPs successfully separated from the fungal biomass. Indeed, if another digestion procedure had been used, affecting bio-MPs chemical composition, it would not have been possible to make any inferences about its biodegradation – raising the question of whether the chemical changes observed in the spectra resulted from the digestion procedure or the fungal activity.

Although the bio-MPs removal by *P. brevicompactum* could not be proved in this study (i.e. in a simulated freshwater environment), due to the persistent attachment of fungal material to the bio-MPs, changes in functional groups of bio-MPs could be observed in the FTIR spectra as observed in Fig. 4 and further discussed in this section. In another study, the same agricultural mulch biofilm (starch-based/Mater-bi® based) presented 34 % removal by *P. brevicompactum* in the soil matrix, after 28 days of exposure at 20 °C[13]. Other such as Urbanek et al.[37], reported the removal of 12.1 % by *Trichoderma* sp. for starch-based polymer (film squares, 2 cm × 2 cm) after 30 days at 28 °C in liquid medium. In the same study, *Clonostachys rosea* had the capacity to remove 100 % of starch-based plastics in just 16 days under the same conditions (28 °C in liquid medium). It is important to mention that, to our knowledge, the existing studies were performed under distinct experimental conditions, thus not allowing an adequate comparison of the results. Aside from the experimental conditions, microplastics removal also depends on the fungi's enzymatic capacity and plasticity.

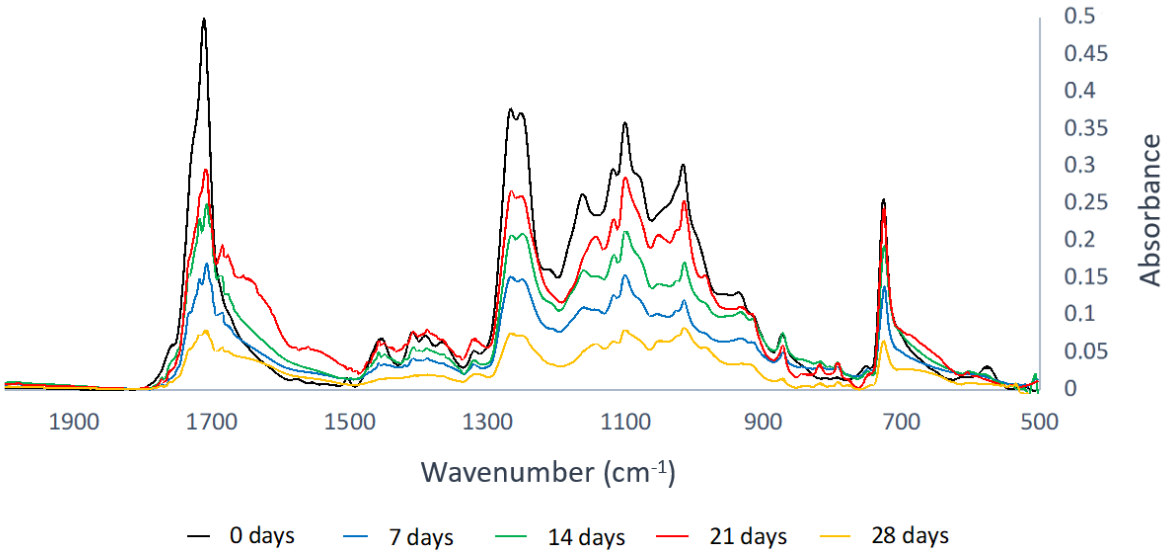

Figure 4 - FTIR-ATR spectra of bio-based mulch films microplastics before (0 days) and after 7, 14, 21 and 28 days of exposure to *Penicillium brevicompactum* in Artificial Pond Water, in the 500-2000 cm^{-1} region (Perkin Elmer (USA) Spectrum BX).

Analysis of the FTIR spectra of bio-MPs exposed to the fungi revealed potential changes to the chemical structure of the polymer that may be indicative of its biodegradation during the experiment. The FTIR spectra of Bio-MPs, both exposed and not exposed to *P. brevicompactum,* are depicted in Fig. 4. The following major peaks can be observed[38–40]: one high intensity peak at 1721–1717 cm^{-1}, attributable to CO; at 1850–1550 cm^{-1} attributed to a broad carbonyl; a peak at approximately 1456 cm^{-1} possibly indicative of phenylene, followed by another peak around 1274 cm^{-1}, attributable to ester linkages; at approximately 732 cm^{-1}, which can be attributed to CH planes from benzene groups; at 1118–1081 cm^{-1}, that can be attributed to CO groups; and at approximately 1063 cm^{-1}, which can denote CH_2OH. From the general FTIR spectrum, the chemical composition of the tested polymer, which involves poly(butylene adipate-co-terephthalate) (PBAT) and starch, can be inferred[39,41].

The spectra of bio-MPs that were in contact with *P. brevicompactum* (for 7, 14, 21, and 28 days), demonstrate increased intensities on the right (low wavenumber) side of the peak around 1649 cm^{-1}, relative to the main peak's intensity. These increased intensities could be an indication of the formation of lower molecular weight compounds from the degradation of PBAT, namely PBA (poly(butylene adipate) and PBT (poly(butylene

terephthalate))[39,41]; thus, such changes in the FTIR spectra could be indicative of bio-MPs biodegradation by the fungi. This polymer also presented similar alterations in their FTIR spectra after contact with *P. brevicompactum* in agricultural soils[13]. Aside from changes in peak intensities, no novel peaks were found to appear in the bio-MPs' spectra after exposure to *P. brevicompactum* in this study, compared to soil media. Tseng et al.[42] also found changes in peak intensities in the FTIR spectra of PBAT films after exposure to *Purpureocillium lilacinum* BA1S in soil, compared with those before degradation.

3.2. Ecotoxicity to *Chironomus riparius*

3.2.1. Acute effects on chironomids

After 48 h exposure, 4th instar larvae presented mulch bio-MPs in their gut after exposure to their pristine or aged form, as shown in Table 1. The number of bio-MPs was up to 1 in larvae exposed to concentrations up to 20 mg/kg sediment, and 1–2 bio-MPs in larvae exposed to the highest tested concentration (200 mg/kg sediment), independently of the polymer form (pristine or UV-aged). Most ingested particles presented long fibrous shapes, with only a few smaller irregularly shaped particles.

Table 1 - Number of bio-MPs present in *Chironomus riparius* fourth instar larvae, after 48h exposure to 0, 2, 20, and 200 mg of pristine or aged bio-MPs /kg of sediment.

Bio-MPs ageing	Bio-MPs concentration (mg/kg)	Nr of Bio-MPs/org (min-max)	Nr of Bio-MPs/org (average ± StDev)
Pristine	0	0	0 ± 0
	2	0 to 1	0.279 ± 0.009
	20	0 to 1	0.4 ± 0.3
	200	1 to 2	1.1 ± 0.6
UV-aged	0	0	0 ± 0
	2	0 to 1	0.38 ± 0.09
	20	0 to 1	0.45 ± 0.03
	200	1 to 2	1.0 ± 0.4

C. riparius larvae are freshwater invertebrate detritivores which ingest sediment in a non-selective manner and are vulnerable to potential exposure and uptake (in the same range as the natural particles ingested by them) of MPs that accumulate in freshwater sediment[43,44]. The number of ingested particles by *C. riparius* in the present study was, however, considerably lower when compared to a previous study with *C. riparius* 4th

instar larvae exposed to pristine MOs of PE ((the petrochemical counterpart used in agriculture mulch plastics) of similar size (e.g., 125 µm in size, ~90 polyethylene particles/org)[17]. However, the tested concentration in our study was considerably lower than the ones previously tested (*ca* 6 times lower), which partially explains the lower number of ingested particles as the encounter rate with the target particle is also inferior. In addition, microplastics may change sediment's physicochemical properties (through their concentrations or desorbed chemicals), leading to changes in chironomid foraging and feeding activity and tube-building effort[44]. As reviewed by Prata et al.[44] , the ingestion and ecotoxicity of microplastics in freshwater chironomids are tightly related to particle size, organisms feeding behaviour, tested concentrations, sediment and water physicochemical properties, among other parameters.

The effect of a short-term exposure to bio-MPs, in their pristine and UV-aged form, on aerobic energy production (ETS), catalase (CAT), glutathione-S-transferase (GST), lipid peroxidation (LPO), and acetylcholinesterase activity (AChE) of *Chironomus riparius* 4th instar larvae is presented in Fig. 5. Generalising, chironomid larvae exposed to pristine bio-MPs revealed a slight decrease in ETS ($F_{3,16}$ = 4.611; p = 0.0165), accompanied by a significant increase (p < 0.001) in AChE activity when exposed to 2 mg of pristine bio-MPs/kg sediment ($F_{3,16}$ = 7.852; p = 0.0019), remaining modestly higher at 20 and 200 mg pristine bio-MPs/kg compared to control conditions. Such larvae also revealed no significant alteration in CAT and GST along with decreased LPO levels ($F_{3,16}$ = 3.164; p = 0.0555), which was statistically significant for the 20 mg bio-MPs/kg sediment concentration. Chironomid larvae exposed to UV-aged bio-MP revealed a significant increase in ETS ($F_{3,16}$ = 6.448; p = 0.0045), and an activation of CAT activity ($F_{3,16}$ = 9.382, P = 0.0008), particularly at 20 and 200 mg bio-MPs/kg sediment. In these larvae, no significant alterations were recorded for GST and AChE activities and in LPO.

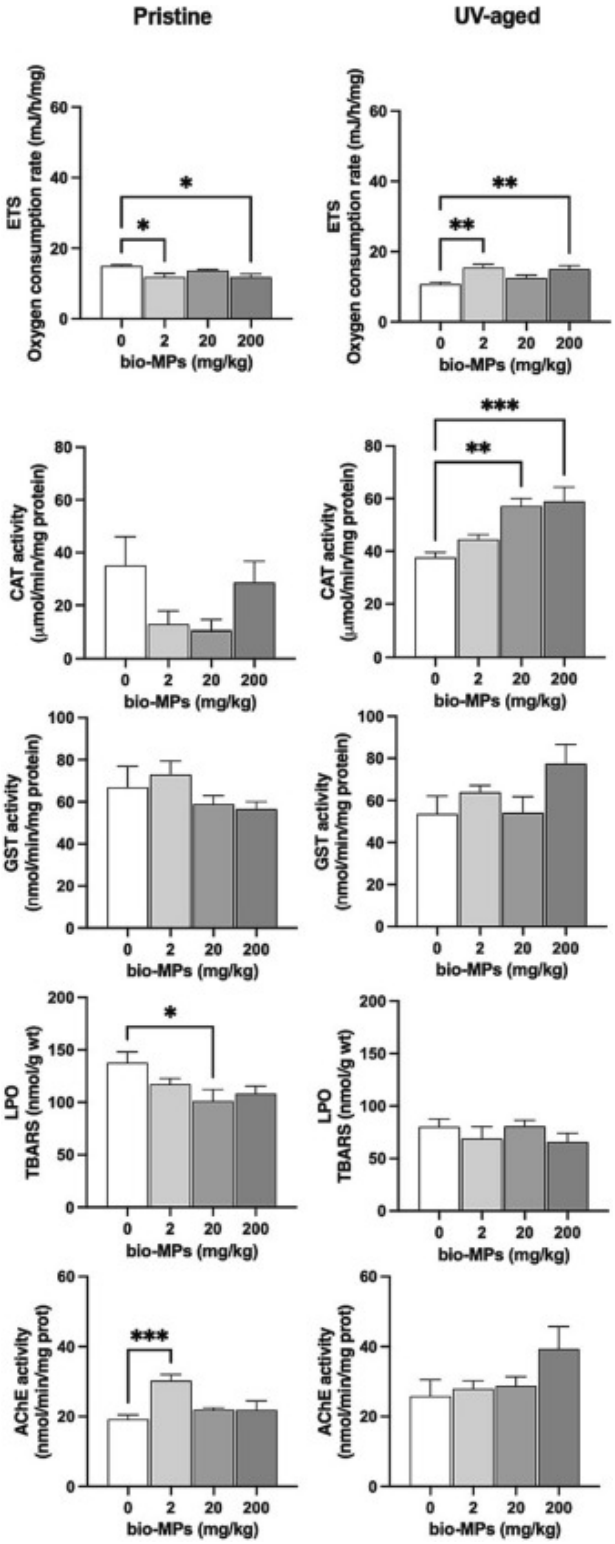

Figure 5 - Effect of short-term exposure (48 h) to bio-based mulch films microplastics (bio-MPs, 0, 2, 20 and 200 mg/kg, in their pristine and UV-aged form) on aerobic energy production (ETS), catalase (CAT), glutathione-S-transferase (GST), lipid peroxidation (LPO), and acetylcholinesterase activity (AChE) of *Chironomus riparius* 4th instar larvae. All values are presented as mean ± standard error of the mean (n = 5). *, **, and *** denote significant differences to the control group (ANOVA and post hoc Dunnett's test) with $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

The ingestion of microplastics seemed to trigger inflammatory processes in *C. riparius* larvae's body due to the blockages caused and possible wounds in the gut due to their irregular shape, as observed for PE microplastics[18]. Concomitantly, biochemical and metabolic responses can also be triggered by potential adsorbed chemical compounds that might have leached from bio-MPs during their residence time in the larval gut[45], and/or the potential alteration of gut microbiome[46]. Any of these processes can cause an imbalance in the organism, having repercussions in terms of oxidative stress due to the increase in reactive oxygen species (ROS) and consequently increasing organisms' energy expenditure to maintain cellular homeostasis[19,47]. The electron transport system (ETS) activity provides insights on the aerobic metabolism, as it is related to energy production in the presence of oxygen. In our study, the increase in aerobic metabolism (inferred by an increment of the ETS activity) observed in larvae exposed to UV-aged bio-MPs can be related to the activation of immune responses and consequent antioxidant defences to fight reactive oxygen species (ROS)[28]. This can be a result of the activation of the antioxidant and detoxification capacities. A significant increase in CAT activity (~30%) was, in fact, observed in larvae exposed to UV-aged bio-MPs exposed to 20 and 200 mg/kg concentrations (Fig. 5). As CAT is an antioxidant enzyme involved in decomposing hydrogen peroxide ($H2O2$) (a precursor of oxidative stress-inducing free radicals), these results support the idea that organisms could mobilise their antioxidant defences when exposed to these weathered MPs. Concomitantly, despite lacking statistical significance, GST increased > 20% at the highest tested concentration, indicating activation of antioxidant and detoxification systems. The CAT and GST activation implies higher energy expenditure, which supports the results obtained in the evaluation of ETS for larvae exposed to UV-aged bio.MPs. The activation of antioxidants (such as CAT and GST) was found in several animal models on different trophic levels, including *C. riparius*, following the exposure of microplastics (as reviewed by Hu and Palic[48] and Prata et al.[44]), as a direct response to the increase on ROS potentially triggered by the activation of immune response, or other response mechanisms to fight the physical and/or chemical toxicity of the synthetic particles.

Regarding larvae exposed to pristine bio-MPs, inhibition of CAT activity (> 50%) was observed in larvae exposed to 2 and 20 mg/kg and in GST activity (5-8%) in larvae

exposed to 20 and 200 mg/kg, despite the lack of statistical significance. A decrease in the activity of enzymes like CAT and GST can indirectly affect the electron transport system (ETS) and likely energy expenditure (aerobic metabolism), which, here, resulted in a slight decrease in ETS.

The alterations in the aerobic metabolism and the antioxidant capacities of *C. riparius* larvae did not result in lipid peroxidation (LPO), which indicates no lipid damage (Fig. 5). In fact, a trend for decreased LPO levels in larvae exposed to pristine bio-MPs/kg sediment was even observed, which was statistically significant for the concentration of 20 mg bio-MPs/kg sediment. This decrease in LPO is relatively uncharacteristic in *Chironomus riparius* exposed to conventional microplastics where most studies report an increase or no changes (e.g., polyurethane and PE microplastics)[19,49,50]. Notwithstanding, a follow-up study focusing on *Girardia tigrina* planarians feeding on contaminated *C. riparius*with polyurethane (PU) indicated a decrease in LPO compared to those that fed on non-contaminated midges[51]. A decrease in LPO might be related to a potential decrease in lipid content (which was not assessed in the present study). The ingestion of MPs has already been shown to cause a false sensation of satiation, which can induce changes in feeding behaviour and lead to weight loss[47,52–54]. The inclusion of antioxidants in a variety of polymeric materials and formulations used in a variety of fields, such as polyethylene (PE) and polypropylene (PP), is not infrequent[55]. Their inclusion in the formulation would allow microplastics to scavenge free radicals and reduce oxidative stress, decreasing lipid peroxidation. However, it is essential to note that the exact mechanisms of how microplastics might act as antioxidants are not yet fully understood, and further research is required to explore this possibility in detail. Lastly, another possible explanation for the decrease in LPO could be related to the regulation of the reactive oxygen species (ROS) and reactive nitrogen species (RNS), potentially triggered by an immune response. However, the immune system often regulates ROS/RNS production effectively, principally under stress, leading to a higher decrease in lipid peroxidation[56]. As such, the absence of oxidative damage in *C. riparius* exposed to pristine or UV-aged bio-MPs could also indicate that larvae antioxidant defences can successfully handle ROS.

Chironomus riparius larvae showed a significant increase in AChE activity when exposed to 2 mg of pristine bio-MPs/kg sediment, remaining modestly higher at 20 and 200 mg pristine bio-MPs/kg compared to control conditions (Fig. 5). An increase in AChE was also observed in larvae exposed to UV-aged bio-MPs, particularly at the highest concentration (with >30 % increment, despite the lack of statistical significance), which could contribute to explaining the increase in energy expenditure in such larvae. The increase in AChE activity can be explained by the increase in peristaltic movements to egest larger-diameter or aggregated particles or by the inflammatory processes triggered in cells and tissues since acetylcholine (AChE substrate) is a neurotransmitter with a vasodilating effect that accumulates in these cells to initiate an anti-inflammatory response[18]. The response of AChE activity to microplastic exposure can vary among different species and tissues within the same organism (as reviewed by Hu and Palić[48]). The mechanisms underlying these changes in AChE activity are not yet fully understood and require further investigation.

3.2.2. Chronic effects on chironomids

Given the more pronounced biochemical responses in *C. riparius* larvae exposed to UV-aged bio-MPs, allied to the higher environmental relevance of such microplastics compared to their pristine form, the potential ecotoxicity of such bio-MPs was further investigated at the organism (apical) level (larval growth and imagoes emergence) (Fig. 6).

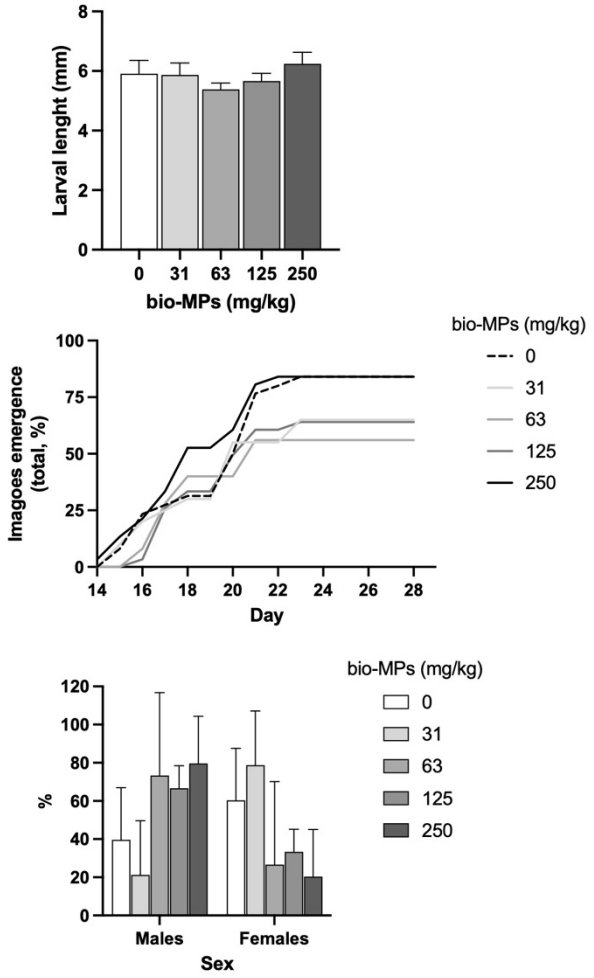

Figure 6 - Effect of UV-aged bio-based biodegradable microplastics (bio-MPs, 0, 31, 63, 125, 250 mg/kg) on larval length (top chart), imagoes emergence (middle chart), and imagoes sex composition (bottom chart) of *Chironomus riparius*, after 28 days of exposure. All values are presented as mean ± standard error of the mean (n = 5).

Exposure to UV-aged mulch biofilm microplastics did not affect survival, larval growth, and emergence. Considering the tested concentrations, the absence of effects on these apical parameters was also observed in chironomids exposed to other polymers (polyethylene) of similar size[17]. However, despite not being statistically significant, a decrease in the proportion of females to males was observed with an increase in the concentration of UV-aged bio-MPs, suggesting a male-biased emergence. A predominance of males in *C. riparius* was also observed after exposure of 3rd and 4thinstars to <180 µm polyamide (100 and 1000 mg/ kg)[57]. This sex shift/dominance in chironomids, driven by the presence of microplastics, might underline the potential

endocrine disruption caused by the chemicals/additives present in the tested bio-based biodegradable mulch film. Plastic debris may be able to sorb or leach harmful substances that can cause endocrine disruption in organisms[58]. Despite the absence of effects on larval survival, growth, and global emergence, a male-biased emergence (and considering that the tested concentrations were environmentally relevant for small-sized particles[59]) could have implications for the population dynamics of chironomids in a longer run. If the skewed sex ratio persists in subsequent generations, it might lead to reduced reproductive success and population decline. As such, these results warrant further investigations to evaluate longer-term/multi-generational effects of (bio)-MP pollution on *C. riparius*.

4. Conclusions and final remarks

Bio-based mulch films are increasingly used in agriculture as a more sustainable alternative to conventional plastic mulch films. However, such bio-based mulch films can enter freshwater ecosystems through runoff processes from agricultural fields or direct disposal into waterways, potentially breaking down into smaller pieces (microplastics) and affecting freshwater biota at different levels of biological organisation. This study provides the first integrative knowledge on the potential effects of a selected bio-based biodegradable mulch film plastic in freshwaters.

This study suggests that microplastics from a commercially available bio-based mulch film – tested bio-MPs – were prone to biodegradation by naturally occurring fungi such as *P. brevicompactum*. However, some adverse effects were observed upon exposure of *C. riparius* larvae to bio-based MPs, particularly in their UV-aged form. Exposure to environmentally relevant UV-aged bio-MP concentrations resulted in enzymatic and metabolic changes without oxidative damage in a short-term exposure, and sex composition and emergence impairment in longer-term exposure. Although the changes to sex composition were not found to be statistically significant, the overall, consistent increase in the number of males and an equivalent decrease in the number of females as microplastic concentrations increased could be an indication of the bio-MPs' potential to interfere with the normal development of *C. riparius*, which could eventually have populational implications over several generations. Thus, the present

results are indicative of potential, though low, sub-lethal ecotoxicological effects of agricultural bio-based mulch biofilms on adjacent environments if such films have a relatively short application in time, but potential for some endocrine disruption for longer exposures to UV-aged bio-MPs in this species. These findings, however, warrant further clarification studies to arrive at more robust results and clearer conclusions.

The studies conducted on both biodegradation and ecotoxicity of bio-based plastics remain scarce and scattered, compromising predictions of their long-term impacts, life cycle assessment, informed decision-making, and regulatory frameworks. Given that this agricultural mulch biofilm is exposed to UV radiation for months and may leak into nearby freshwater environments, further investigations are warranted to assess its long-term effects on freshwater ecosystems, ensuring the safety and sustainability of these bio-based biodegradable products. Complementary studies (e.g., more prolonged exposures, analysis of by-products resultant from the biodegradation process, and the use of other model organisms) are, therefore, welcome for a better and more in-depth understanding of the biodegradation and ecotoxicity (with particular emphasis towards its potential for endocrine disruption) of such and similar polymeric materials. Such knowledge would contribute to developing suitable management practices and new regulations for using bio-based mulch films.

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d. Facemasks: An insight into their abundance in wetlands, degradation, and potential ecotoxicity

Abstract

Disposable facemasks represent a new form of environmental contamination worldwide. This study aimed at addressing the abundance of facemasks in an overlooked natural environment with high ecological and economic value – the wetlands (Ria de Aveiro, Portugal, as study case), evaluating their potential biodegradation using naturally occurring fungi and assessing the potential ecotoxicity of released microfibres on local bivalves.

All masks collected within 6500 m^2 area of Aveiro wetland were 100% disposable ones (PP-based, confirmed by Fourier transform infrared spectroscopy - FTIR) with an initial abundance of 0.0023 items/m2 in Sept. 2021, which was reduced by ~40% in Apr. 2022 and ~87% in Sept. 2022, as a reflection of the government policies. Analysis of the carbonyl index (0.03 to 1.79) underlined their state of degradation, primarily due to sun exposure during low tides. In laboratory conditions, 1 mm^2 microplastics obtained from new disposable facemasks were prone to biodegradation by *Penicillium brevicompactum* and *Zalerion maritimum* inferred from microplastics mass loss (~22 to − 26% and ~40 to 50%, respectively) and FTIR spectra (particularly in the hydroxyl and carbonyl groups). In addition, microfibres released from facemasks induced sublethal effects on the clam, *Venerupis corrugata*, mostly in their UV-aged form when compared to pristine ones, characterised by a decrease in cellular energy allocation (CEA) and an increase in aerobic energy metabolism (ETS). Concomitantly, clams exposed to 1250 items/L of UV-aged microplastics (similar to field-reported concentrations) expressed greater clearance capacity, indicating a need to compensate for the potential energy unbalance.

This study provides the first baseline monitoring of facemasks in wetlands while bringing new evidence on their biodegradation and ecotoxicity, considering environmentally relevant conditions and keystone organisms in such environments. Such studies require scientific attention for rapid regulatory action against this emerging and persistent

pollutant, also targeting remediation and mitigation strategies considering these items under pandemic scenarios.

Keywords: Plastic pollution, microplastics, protective equipment, environmental implication

1. Introduction

The use of facemasks during the COVID-19 pandemic was essential to control the spread of the SARS-CoV-2 virus[1]. The demand, production, and usage of these items by the general public skyrocketed, particularly in the first year of the pandemic[2]. Among the available facemask options (reusable, surgical, and respirators), surgical ones have been preferred by ordinary citizens as they are more practical than reusable masks (no need to wash after each usage) and more breathable than respirators[2]. The production levels of this type of mask reached unprecedented levels, and since it is a single-use item, most of them ended up discarded along with municipal solid waste[3]. Due to the waste management system's overload or incorrect disposal by ordinary citizens[4,5], it has become quite common to see these items discarded in all types of environments, whether urban or not[6,7].

In open environments, facemasks, like any other plastic material, are often exposed to weather conditions and, consequently, prone to fragmentation and, eventually, to (bio)degradation[7]. Previous studies have already proven the deterioration of facemasks by thermal-, photo- and oxidative degradation [8–10], and even derived from contact with living organisms in terrestrial[11,12] and in aquatic environments[13]. When in contact with microorganisms, facemasks present clear signs of ageing (increased roughness and decreased molecular weight), and bacteria families such as *Rhodobacteraceae*, *Flavobacteriaceae* and *Vibrionaceae* seemed to heavily colonise the material forming a thick biofilm[14] which may lead to degradation by enzymatic activity[15].

Despite the colonisation of facemasks by bacteria in controlled terrestrial and aquatic environments, biodegradation itself remains undressed. Aside from bacteria, fungi species can also colonise plastic debris, and laboratory studies proved their higher

efficiency in using plastics backbone as a carbon source, biodegrading them[16]. Among the ones commonly found in wetlands environments, *Penicillium* sp. (including *P. brevicompactum*) and *Zalerion maritimum* are highlighted due to their ecological relevance but also their ability to degrade plastics (e.g., polyethylene teraphtalate- PET, polyethylene-PE, polypropylene-PP) and bioplastics (starch-based)[17–20]. Notwithstanding, isotactic polypropylene (i-PP), the principal component of disposable facemasks[21] has between 40 % and 70 % crystallinity and, in consequence, higher melting point (160 °C to 180 °C), higher density, and higher strength[22], that might hinder biodegradation. Instead, disposable facemasks might fragment into smaller-size microplastics and microfibres. In aqueous solutions, facemasks can release a considerable amount of microplastics and microfibres (in the magnitude of 10^5 particles/mask in aqueous environments (e.g., Wang et al.[23]), which can be aggravated with air exposure periods[24,25] as commonly experienced in wetlands. Thus, facemasks deterioration in wetlands seriously threatens their inhabitants.

Several studies have already confirmed the ecotoxicological effects of facemasks' microplastics and microfibres (degraded or not) on aquatic organisms, such as water fleas, fish larvae, zooplankton, and diatoms, mainly related to the impairment of reproduction/maturation/fecundity[7,26–28]. Nevertheless, the potential ecotoxicological effects of microplastics and/or microfibres released from disposable facemasks on organisms of economic and commercial value, such as bivalves, remained so far uncovered. Bivalves present a wide distribution, are sessile, and, as filter feeders, are prone to be easily contaminated[29,30]. Sediment-dwelling clams, such as *Venerupis corrugata*, are a regular presence in marine and transitional environments such as wetlands or wave-protected areas, and their economic value approaches 20.6 billion US$ per year[31]. Their natural populations have declined in the last decade due to climate change and persistent contaminants[32]. Exposure to microplastics and/or microfibres may also contribute to such decline by threatening clams' physiological status and consequently compromising their nutritional quality.

In order to comprehensively understand the potential environmental implications of disposable facemasks in wetlands, this study pursued three main objectives: i) to provide a baseline monitoring survey on facemasks in a 6500 m^2 area in Aveiro wetland

(protected area under Natura 2000 network); 2) to address the potential facemasks biodegradation in the presence of the fungus *P. brevicompactum* and *Z. maritimum* in saltwater medium; and 3) to evaluate the potential effects of microplastics released from disposable masks (in their pristine form or UV-aged to increase environmental relevance) in *V. corrugata*, addressing their clearance capacity, antioxidant and detoxification capacity, oxidative damage, aerobic energy production, and energy reserves after short-term exposure (96 h).

These objectives were formulated based on the following alternative hypotheses: a) the abundance of disposable facemasks in wetlands varies between seasons, reflecting potential influences of human activities, governmental laws, and weather patterns; b) microplastics from disposable facemasks are susceptible to biodegradation by the fungi *P. brevicompactum* and *Z. maritimum*, suggesting a promising approach for mitigating facemask pollution; c) short-term exposure to microplastics/microfibres released from disposable facemasks, whether in pristine form or UV-aged to simulate environmental conditions, leads to significant changes in *V. corrugata*'s physiological and biochemical parameters. This indicates potential adverse effects on the organism, raising concerns about the impact of facemask-derived microplastics on wetland biota.

By addressing these objectives and hypotheses, we aim to contribute to the broader understanding of the ecological consequences of facemask pollution in sensitive wetland ecosystems, supporting informed conservation and management efforts.

2. Material and methods

2.1. Baseline monitoring of facemasks in Aveiro wetland

Three monitoring actions in the space of a year were carried out in Ria de Aveiro Lagoon, Portugal: one on the 27th of September 2021 (mandatory use of facemasks), one on the 28th of April 2022 (no mandatory use of facemasks, restriction withdraw on 22nd April), and the last on the 6th of September 2022 (no mandatory use of facemasks).

The transect extended for 1180 m starting at the quay of Ribeira de Esgueira (N 40 39.439' W 008 38.198') (Fig. 1). The sampling area was the walkway itself (2,5 m) and covered 1 m on the left and 2 m on the right. Thus, an area of approx. 6 500 m^2 was sampled. Sampling was done during the low tide, and facemasks were identified by eye

while walking along the transect, picked up with the aid of object-collecting tweezers and stored in aluminium foil until further analysis in the lab.

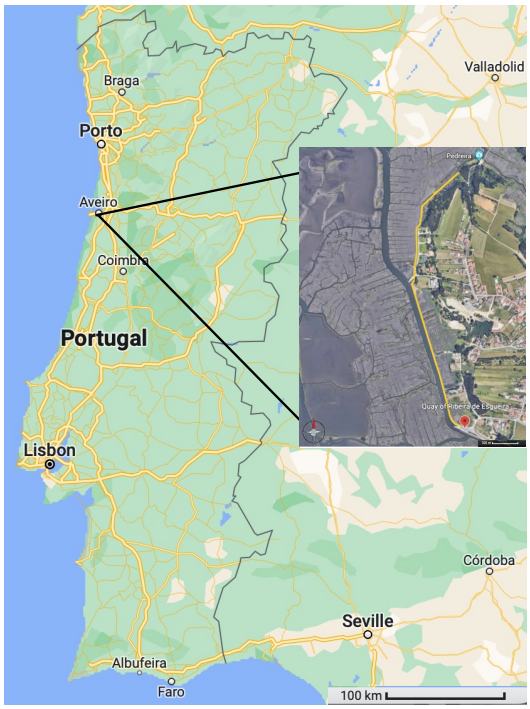

Figure 1 - Sampling transect in facemasks sampling actions.

Facemasks' density was calculated using the following formula: $C = n/A$ [33], where C is the density of facemasks per m2, n is the number of facemasks counted, and A the surveyed area.

2.1.1. Chemical characterisation with Fourier transform infrared spectroscopy (FTIR-ATR)

Once at the laboratory, collected facemasks were carefully washed with distilled water to remove as much litter as possible and then airdried (although covered with aluminium foil). Afterwards, all facemasks were analysed by Fourier transform infrared spectroscopy (FTIR-ATR) with a Perkin Elmer (USA) Spectrum BX FTIR instrument to determine their state of degradation. Thus, two fragments were cut from the outer layer fabric, and the readings were carried out in absorbance mode in wavelengths between 400 and 4000 cm^{-1}, being performed 32 scans, with a resolution of 4 cm^{-1} and an interval of 2 cm^{-1}. In addition, the FTIR spectra were used to calculate the individual

Carbonyl Index (CI) for degraded samples using the equation $\boldsymbol{CI}=\boldsymbol{Abs}(\mathbf{1})/\boldsymbol{Abs}(\mathbf{2})$, where Abs(1) is the absorbance at 1600-1684 cm^{-1} for carbonyl group and Abs(2) is the absorbance at 1453 cm^{-1} for PP reference peak (adapted from Rodrigues et al.[34]). The obtained spectra and CI from each sample were compared with those from virgin polypropylene to identify the occurrence of degradation.

2.2. Biodegradation assay

2.2.1. Microorganisms culture conditions

Penicillium brevicompactum (CMG 72) and *Zalerion maritimum* (ATCC 34329) were grown at about 20 ^{o}C in agitated batch reactors (250 mL Erlenmeyer flasks; 120 rpm), with a liquid growth medium consisting of 35 g/L of salt (LabKem), 1 g/L of peptone (Sigma Aldrich), 20 g/L of glucose (LabKem), and 20 g/L of malt extract (Oxoid)[18].

2.2.2. Microplastics preparation and characterization

Microplastics used for testing (PP based, according to the manufacturer) were obtained by cutting squares of approximately 1 mm^2 from a disposable surgical facemask outer layer (made in China and distributed by ATSFARMA II). To confirm their chemical composition, such microplastics were then characterised by FTIR-ATR as previously described (section 2.1.1.).

2.2.3. Experimental conditions

The culture medium used for testing consisted of 35 g/L of salt, 2 g/L of glucose, 2 g/L of malt extract and 0.1 g/L of peptone, as seen in Paço[35] (sec. 3.2.2). The experiment was prepared to run for 28 days, with sampling day every 7 days (i.e., sampling day 7, 14, 21, and 28). For this purpose, two incubators (one for experiments with *P. brevicompactum* and one for *Z. maritimum*) were fulfilled with thirty-six batch reactors of 100 mL containing 50 mL of culture such medium were prepared, per test species, to accomplish the following testing conditions: 8 control conditions containing only fungus (0.5 g of fungus; *N*=2 per sampling day), 12 control conditions containing only microplastics (0.015 g; *N*=3 per sampling day) and 16 for batch reactors containing microplastics and fungus (0.015 g of microplastics + 0.5 g of *P. brevicompactum* or *Z.*

maritimum; *N*=4 per sampling day). The specific initial weight of fungi and microplastics can be depicted in Table S2 in the supplementary material. The number of replicates (N) was based on previous investigations[35] and conclusions obtained through preliminary tests.

All batch reactors were autoclaved at 121 °C for 30 min previously the inoculation with *P. brevicompactum* or *Z. maritimum* (for those that should receive the fungus). Subsequently, and after inoculation, the 36 batch reactors per incubator (i.e., per species) were placed in an incubator at 120 rpm at approximately 20 °C. Fig. 2 shows a schematic representation of the experiment to assess face mask microplastics' biodegradation by fungi.

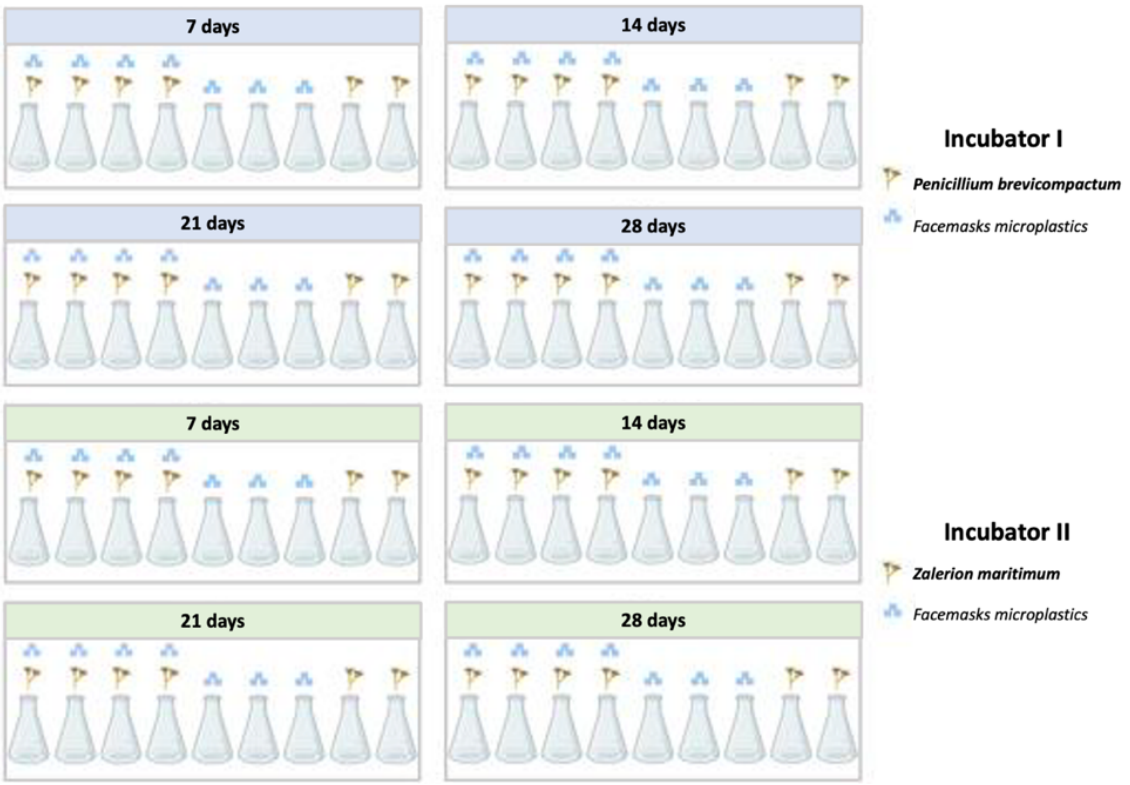

Figure 2 - Schematic representation of the experiment to assess facemask microplastics' biodegradation by *Penicillium brevicompactum* and *Zalerion maritimum*.

2.2.4. Separation of microplastics from fungi

On each sampling day (7, 14, 21, and 28), nine batch reactors (2 batch reactors containing only fungus, 3 batch reactors containing only microplastics, and 4 batch reactors containing both microplastics and fungus) were removed from each incubator

and subjected to a gravity filtration process using a filter of 90 mm diameter and 200 µm pore (Prat dumas, A009210). During filtration, and when possible, microplastics were separated from fungi and placed in one previously weighted 2 mL glass flask; mycelium was also placed in a previously weighted glass flask, frozen and later lyophilised. The lyophilised mycelium was analysed and the remaining microplastics were retrieved. The particles that were not successfully detached from fungi were submitted to a 48h digestion with 10% (v/v) sodium hypochlorite (NaClO; Fisher Chemical) and vacuum filtered onto black polycarbonate filters (47 mm, 0.2 µm pore size, Watman®). Microplastics collected and/or separated from fungi were then oven-dried at 50 ^{o}C until the weight was kept constant. Afterwards, microplastics were carefully weighted.

2.2.5. Analysis of microplastics after contact with fungi

The potential degradation of facemasks microplastics was analysed via FTIR-ATR as previously described (Section 2.1.1.) The obtained spectra and Carbonyl Index from each sample were also compared with those from virgin polypropylene to identify the degradation occurrence.

2.3. Ecotoxicity assay

2.3.1. Microplastics (including microfibers) preparation and characterisation

Three disposable facemasks (Mobiclinic; 50 items pack), except for the nose metal and earloops, were shredded with a stainless-steel grater into a system of two stainless-steel sieves, with 1 and 0.5 mm meshes, respectively, being the microplastics and microfibres selected between these two sizes. Then, such small particles were divided into two groups of ~0,6011 g each. One group underwent the ageing process by UV-C light (denominated by UV treatment), whereas the other remained as controls without any conditioned ageing (denominated pristine treatment). Although recognising that UV-C radiation does not reach the Earth's surface, this procedure was carried out to accelerate the ageing of particles by solar radiation. This exposure was based, with the necessary adaptations, on previous studies on petrochemical microplastics[36].

Briefly, facemasks' microplastics to be aged were placed in a rounded glass container of 2 L with distilled water on a magnetic stirrer to be kept under constant movement to ensure uniform ageing between the particles. Then, such a glass container was enlightened with a UV-C light lamp (245 nm; Uvitec LF-206.LS lamp) inside a completely opaque black box. For pristine microplastics, the same procedure was performed without UV-C light. The exposure period endured 12 days. After this period, the two groups of microplastics were vacuum filtered separately with a Buchner funnel and a 125 µm stainless-steel filter to retain particles larger than the filter mesh size and allow to pass small-sized microplastics – essentially microfibres. Afterwards, distilled water containing microfibres was vacuum filtrated onto black polycarbonate filters (Whatman® Nuclepore™ Track-Etched Membranes, diam. 47 mm, pore size 0.2 µm) and chemically characterised by FTIR-ATR as described in Section 2.1.1.

2.3.2. Microfibers quantification and preparation of stock solutions

After chemical characterisation via FTIR, microfibres with and without UV treatment retained on the black filters were weighted, resuspended in 100 mL of distilled water, and exposed to an ultrasonic bath at 25 °C for 5min to stimulate their disaggregation for further use. Then, five replicates of 500 µL of each sample were pipetted, filtered (with black polycarbonate filters), and photographed under a stereomicroscope (Olympus BX41; objective 10X) coupled with a Canon EOS 1200D camera to determine the number of fibres in that volume. From this knowledge, three stock solutions of each microplastics' treatment were prepared (3330 items/L- 33330 items/L- 333330 items/L).

2.3.3. Test species and culture conditions

Venerupis corrugata adult individuals were obtained from Ria de Aveiro coastal lagoon. Upon arrival at the laboratory, clams were placed in a depuration system as described in Silvestre et al.[37] (Section 2.2), where they were kept for seven days at 15 ^{o}C and 35 PSU. Afterwards, organisms with relatively uniform sizes were selected and used for testing. Additionally, five clams of similar size were weighted and stored at -80 ^{o}C further to determine the clams' initial physiological and biochemical status.

2.3.4. Exposure conditions

The artificial saltwater (ASW) was prepared with Ocean Fish salt (PRODAC: item code OC30KG) and then filtered with 90 mm diameter and 200 µm pore (Prat dumas, REF A009210) filters to remove possible impurities that could interfere with the experience. For each microplastic type (pristine or aged), the tested concentrations were 0 (control), 50, 250 and 1250 fibres/L of ASW. Five replicates of 1 L per treatment were prepared. For this purpose, 1 L flask contained 997 mL of ASW and 3 mL from the respective contamination stock (e.g., 3 mL from the stock solution of 3330 items/L to achieve the final concentration of 50 items/L). To confirm nominal set concentrations, water subsamples were collected (3-5 mL; *N*=3), vacuum filtrated onto black polycarbonate filters, and microfibres from each treatment and replicate were counted under a stereomicroscope (Olympus BX41; objective 10X) coupled with a Canon EOS 1200D camera.

For the exposure, three adult clams of similar size were gently added to each replicate of each treatment. The tests ran for 96 h, in the absence of food, in an ambient photoperiod (14 h light: 10 h dark) at 19 ^{o}C. After exposure, one clam per replicate and treatment was individually transferred to glass flasks containing 300 mL of uncontaminated ASW to assess the clearance capacity. The remaining survivors were measured (biometric analysis), de-shelled, rinsed with ultrapure water to eliminate inorganic or organic particles adhered to their bodies, gently dried with filter paper, weighted (fresh weight, FW), snap frozen in liquid nitrogen, and stored at -80 ^{o}C for biochemical analysis.

2.3.5. Clearance capacity and biomarkers analysis

The clearance rate estimates the algae concentration cleared by the clams during a period of time[38]. For this purpose, encapsulated *Nannochloropsis* microalga, ϕ 40 µm, was used as a food source and prepared according to manufacturer instructions. A standard curve was prepared by a series of dilutions with a factor of two, downwards from a stock solution of 1 mg/mL. Cell concentration was determined by spectrophotometry (650 nm) in a microplate reader (Biotek® Synergy HT, VT, USA)[39]. The algae concentration per mL in each treatment was then quantified through

spectrophotometry, considering 200 µL subsamples of overlying seawater collected at 0-, 15-, 45-, and 120-min. Absorbance was then converted into cell concentration using the standard curve.

For biomarkers analysis, frozen clams were quickly shredded with a sterilised stainless-steel scalpel to ease their homogenisation in 10 mL of ultra-pure water with a sonicator (used mode of 10% for 30s, 250 Sonifier, Branson Ultrasonics). From each homogenate, three aliquots of 300 µL were stored in 2 mL microtube to assess: i) lipids, ii) proteins and carbohydrates, and iii) aerobic energy production (via Electron Transference System - ETS). An extra aliquot of 250 µL was stored with 4% butylated hydroxytoluene (BHT) in methanol for lipid peroxidation evaluation. The remaining homogenate was diluted with 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 15 min at 10 000 x g (4 °C). The post-mitochondrial supernatant (PMS) was then divided into several aliquots to evaluate catalase (CAT) and glutathione-S-transferase (GST) activities. All biomarkers determinations were performed spectrophotometrically, with a Microplate reader MultiSkan Spectrum (Thermo Fisher Scientific, Waltham, MA, USA), following previously described procedures (details in Silvestre et al.[37]).

2.3.6. Statistical analysis

For biochemical biomarkers data analysis, two parameters were considered (microfibres concentration and ageing), and, therefore, a two-way ANOVA was performed, followed by Šídák's multiple comparisons test. Shapiro-wilk test and Spearman's test were also performed to assess normality of residuals and heteroscedasticity, respectively. For the clearance capacity data analysis, and considering that aside from microfibres concentration and ageing, there was also the effect of time (0-, 15-, 45- and 120-min), a two-way ANOVA with mixed-effects model and multiple comparisons within each parameter (microfibre concentration and ageing) was done. The significance level was set at $p < 0.05$ for all statistical tests. All the statistical analysis was performed using the program Prism 8.2.1, 2019.

2.4. Quality assessment, quality control

Throughout testing, sampling, organism manipulation, and sample examination/process, the use of plastic material was avoided, using preferable glass or aluminium vials/equipment and stainless-steel utensils. Cotton lab coats were used, all the material was previously acid-washed before use, and samples were covered with aluminium foil when not used or processed. All solutions (ultrapure water, artificial seawater, among others) were previously filtrated before being used. All working spaces were thoroughly cleaned (alcohol). Procedural blanks were also applied.

3. Results and discussion

3.1. Facemasks occurrence and density in Aveiro wetland, Portugal

Overall, 26 facemasks were collected in total, 100% of disposable type with 2 or 3 layers (Fig. S1). From such total, 15 facemasks were collected in Sept. 2021, 9 facemasks were collected in Apr. 2022, and 2 facemasks were collected in Sept. 2022; indicating a ~ 87% reduction within one year of pandemic. The gradual reduction of the facemasks found in Aveiro wetland is possibly explained by two main factors: seasonality and government policies[40]. Weather conditions affect disposal since warmer seasons make it appealing to spend more time outdoors[41], that is, samplings carried out in the late summer season (as Sept. 2021) and in countries that have warmer climates as Portugal, a superior density of facemasks was antecipated. In addition, Government guidelines are a factor in determining the number of masks that can be found since, from country to country, measures to combat COVID-19 vary (e.g., mandatory use of facemasks) as well as the time at which they are a pplied[41,42]. In Portugal, the mandatory use of facemasks was withdrawn on 22nd April 2022, which supports the substantial decrease in their number in the second and third sampling moments (April and Sept. 2022).

The overall mean density was $1.4 \times 10^{-3} \pm 1 \times 10^{-3}$ facemasks.m^{-2}, which is comparable to previous reports, as reviewed in Table 1. For example, values reported along the Chilean coast[43] and on beaches in northern Morocco[44] are on the same scale of magnitude. However, other sampling campaigns have recorded lower mean values[40,41,45,46], meaning that, despite the reduced area in this study, the average density recorded is one of the highest.

Table 1 - Summary of the mean face mask densities in natural environments, until Sept. 2022.

Country	Sampling Site	Mean density (Face mask/m^2)	Reference
Chile	Beach	6.00×10^{-3}	Thiel et al.[43]
Morocco	Beach	1.09×10^{-5}	Haddad et al.[46]
Morocco	Beach	1.20×10^{-3}	Mghili et al.[44]
Argentina	Beach	3.35×10^{-4}	De-la-Torre et al.[45]
Peru	Beach	6.23×10^{-4}	De-la-Torre et al.[45]
Iran	Lake	9.75×10^{-5}	Hatami et al.[41]
Ethiopia	Lake	1.51×10^{-4}	Aragaw et al.[40]
Portugal	Wetland	1.40×10^{-3}	This study

The FTIR spectra of degraded disposable masks corroborated their PP-based nature due to the presence of many characteristic peaks of PP, such as the intense CH_3 and CH_2 symmetric and antisymmetric stretching peaks (2950, 2917, 2878, 2869 and 2838 cm^{-1}); CH_2 and CH_3 symmetric and antisymmetric bending peaks (1453 and 1376 cm^{-1}); CH bending at around 1166 cm^{-1}; CH_3 rocking at 970 cm^{-1}; C-C stretching at 998 cm^{-1}; CH_3, CH_2, C-C, and C-CH_3 vibrations peaks at 808 and 841 $cm^{-1;}$ (Fig. 3).

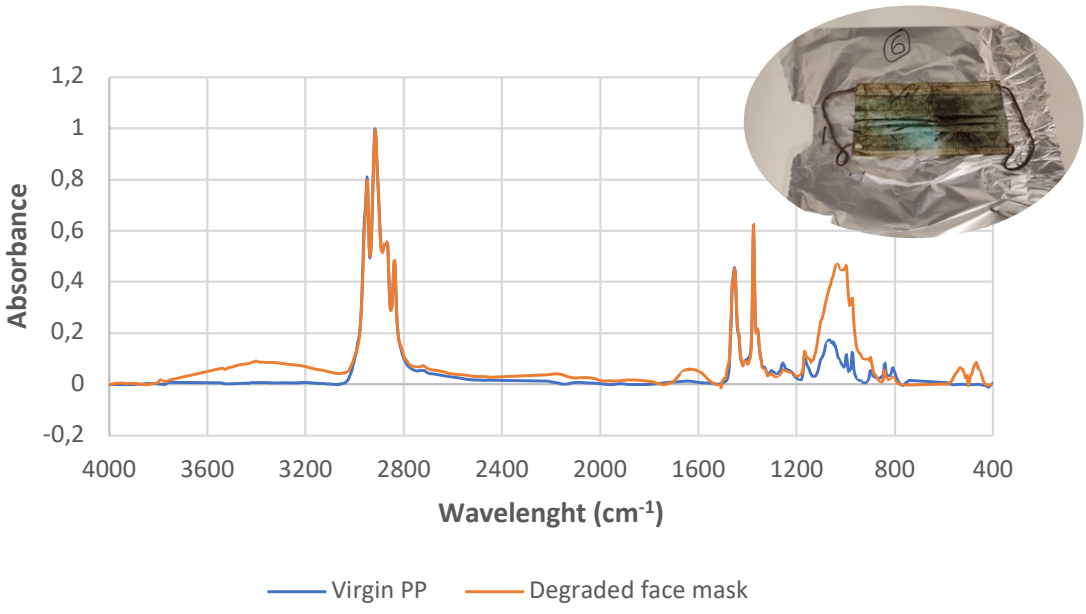

Figure 3 - FTIR spectra of both virgin PP and a subsample of a degraded facemask with its corresponding photograph.

Expectedly, collected facemasks also revealed different degrees of degradation, reflecting the time exposed to the environmental variables (such as sunlight, tidal cycles, and biological activities) (Fig. S1, Fig. 3). Signs of degradation were depicted by the presence of a broad band between 3065 and 3526 cm^{-1}, corresponding to hydroxyl groups, and a smaller and narrower band between 1540 and 1718 cm^{-1} attributed to carbonyl groups, both considered signs of oxidation due to UV light[8–10]. De-la-Torre et al.[8] further detected two peaks between 2300 and 2400 cm^{-1} and a broad peak between 650 and 700 cm^{-1}, however, in this case, they were most likely not recorded since they are in wavelengths where CO_2 peaks were identified and removed.

The carbonyl index (CI), a successful measure to evaluate the photooxidation of a polymer[47], was also calculated to assess masks' degradation state. Virgin facemasks CI ranged from 0.01 to 0.02, while those from weathered masks ranged from 0.03 to 1.79 with mean values per sampling (Table 2). Higher mean CI values were recorded in Sept. 2022, which, assuming that the masks were not in the sampling site longer than since the preceding sampling, can be explained by the greater exposure to UV radiation due to the longest photoperiods and higher UV rays' intensity, typical of spring and summer months. However, these values seem somewhat high when compared to other studies already carried out with facemasks in similar conditions (between 0.21 and 0.33 in cities in the southeast of Turkey[47], and between 0.1 and 0.4 on Italian beaches[10], possibly because these facemasks had suffered higher exposure to weathering agents.

Table 2 - Mean Carbonyl Index calculated for disposable masks collected in Aveiro wetland, per sampling action.

Date	Mean CI
Sept. 2021	0.3 ± 0.1
Apr. 2022	0.13 ± 0.02
Sept. 2022	0.92 ± 0.05

3.3. Disposable PP-based facemasks biodegradation by *Penicillium brevicompactum* and *Zalerion maritimum*

The percentage of microplastics removed from the batch reactors in the presence or absence of *P. brevicompactum* or *Z. maritimum*, throughout the 28 days of the experiment, can be observed in Fig. 4 (data also presented in Table S1). Here, the removal rate corresponds to the loss of microplastics mass, i.e., to what was possibly degraded by the fungi in the case of samples exposed to it, or, considering that we are dealing with microplastics made up of spunbonded polypropylene fibres, to their release to the liquid medium, which occurs very easily throughout all phases of the experience (also supported by previous investigations, e.g., Shen et al.[25].

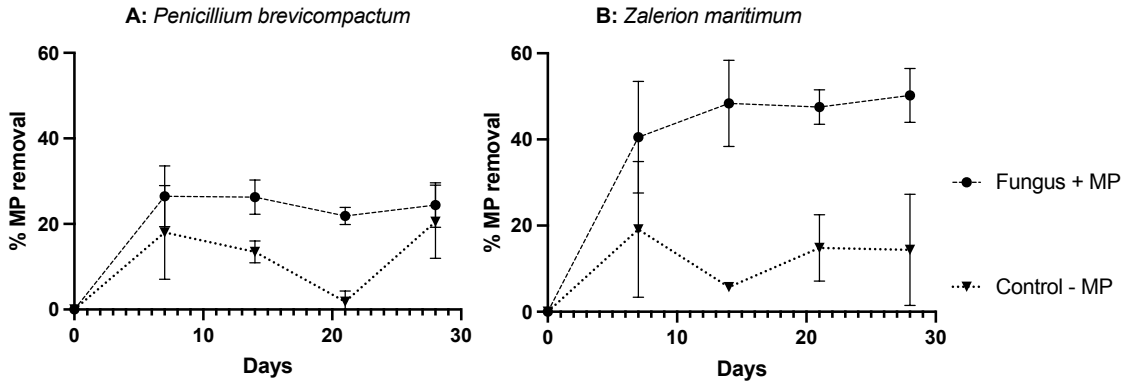

Figure 4 - Facemasks microplastics removal (in percentage, %) during the experiment with *Penicillium brevicompactum* (A) and *Zalerion maritimum* (B). Data are presented as mean ± standard deviation.

As observed in the batch reactors containing only microplastics (code; P_nC – Table S1), the removal percentage varied from 2 ± 3 to 21 ± 9 and from 3 ± 2 to 15 ± 8 in the experiments with *P. brevicompactum* and *Z. maritimum*, respectively, indicating loss of microfibres from the facemasks microplastics in the absence of fungi. Facemasks' microplastics are fragile, and microfibres released could be due to the agitation in the incubator and also somehow lost in the filtration procedure. Previous studies proved that disposable surgical facemasks exposed to water with or without agitation release microfibres[25], particularly the outer layer which seems more susceptible to fragmentation, releasing even more fibres[13].

Notwithstanding, in the presence of fungi (i.e., *P. brevicompactum* or *Z. maritimum*), microplastics removal is 9 to 70% higher than in the absence of them (i.e., compared with batch reactors containing only microplastics, Fig. 4, Table S1). *Zalerion maritimum* presented higher removal of facemasks microplastics (~2 times higher) compared to *Penicillium brevicompactum,* in all sampling days (Fig. 4, Table S1). Facemasks microplastics' removal by both fungi occurred, preferably, in the first week, remaining similar within the next three weeks, indicating that microplastics removal did not evolve beyond a certain point. It could be argued that the stagnation of microplastics removal could be due to a potential decrease in fungi biomass.

In the experiments with *Z. maritimum,* the growth was similar in the presence or absence of facemasks microplastics, with a decrease in weight in the first seven days followed by an increase in the following days (Table S2). However, in this experiment, the growth of *Z. maritimum* in contact with facemasks microplastics had slightly higher growth than *Z. maritimum* that had no contact with microplastics, indicating that such fungus might have used the carbon backbone of microplastics as carbon source. In the experiment with *P. brevicompactum*, the fungus that grew in batch reactors without microplastics, had continuous growth for twenty-one days, and decrease in the last seven days (Table S2), probably related to the lack of nutrients in the medium and the higher metabolism of this fungus compared to *Z. maritimum*. However, in batch reactors where *P. brevicompactum* contacted with microplastics, the fungus might also have used carbon backbone of microplastics as carbon source, justifying their less pronounced decrease in growth values when compared to *P. brevicompactum* growth value in the absence of microplastics, particularly after 28 days. An identical trend was observed in Ferreira-Filipe et al.[18], where *P. brevicompactum* was grown in a minimal solid culture media with mulch biofilm for 15 days and sampled every 5 days. In the first 10 days, *P. brevicompactum* grew with very similar biomass variations, in the presence or absence of microplastics, in soil and solid medium. Afterwards, even though *P. brevicompactum* growth slowed down in both treatments (with or without microplastics), *P. brevicompactum* that were in contact with microplastics presented greater growth values than *P. brevicompactum* controls[18].

Facemasks microplastics exposed to the fungi activity revealed changes in their functional groups, as seen in Fig. 5. The spectra show uncharacteristic peaks of polypropylene at 3525-3050 cm^{-1} and 1755-1490 cm^{-1}, that are probably due to the formation of hydroxyl and carbonyl groups, respectively, and that are normally associated to PP's degradation, as mentioned before. Unfortunately, the same changes can be found in the microplastics from control samples, proposing that there was also microplastics' ageing caused by the culture medium and the agitation. This was expected as, mentioned previously, the particles obtained form facemasks are fragile and have a tendence to fragment itself and, therefore, were very susceptible to mechanical degradation. The characteristic peaks of PP at 2800-3000 cm^{-1}, 1480-1350 cm^{-1}, 1180-1130 cm^{-1} and 1005-780 cm^{-1} do not show significant variations in any of the conditions, as seen with the relative areas presented in Table 3.

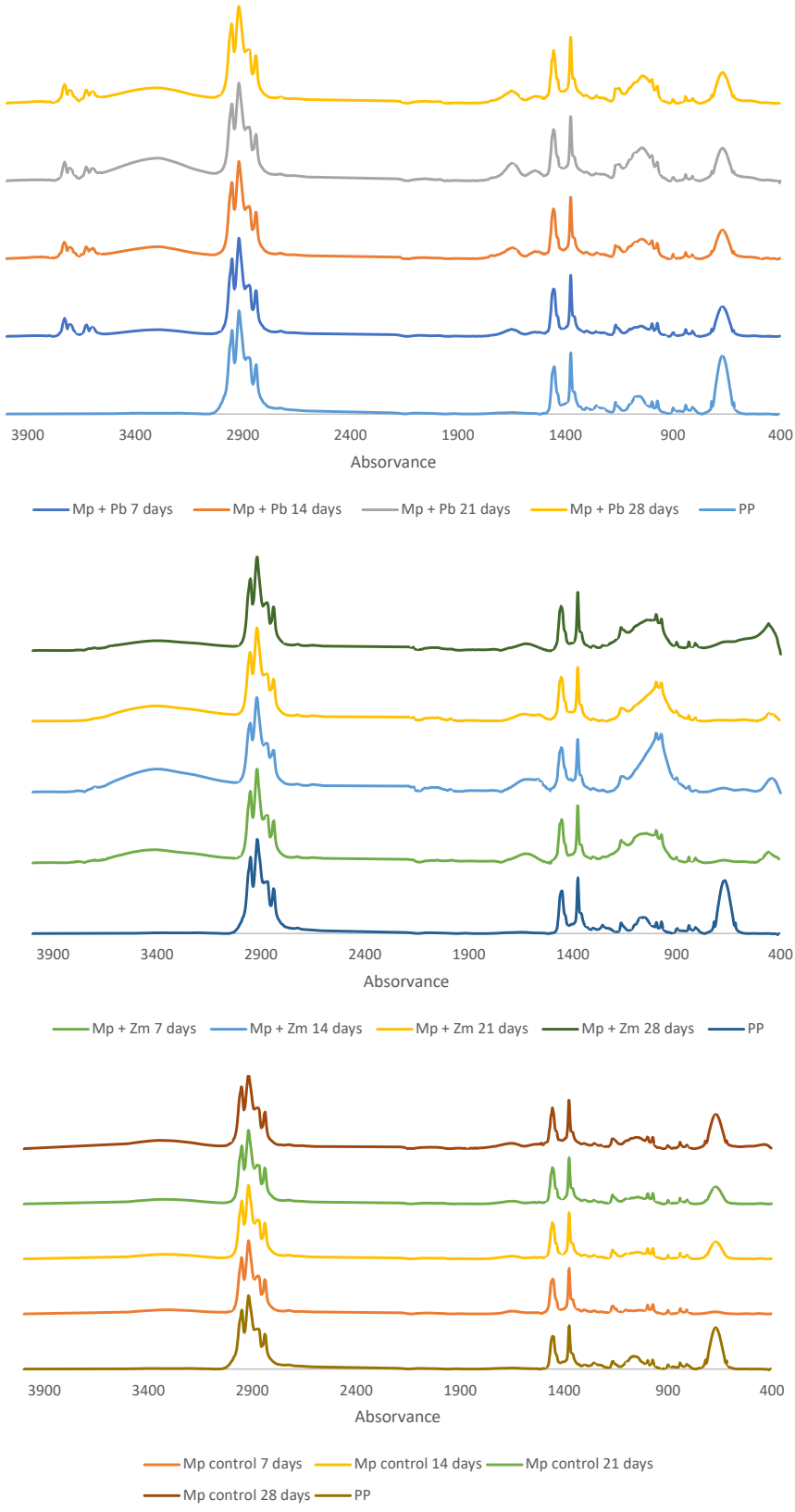

Figure 5 - FTIR-ATR spectra in the region 400–4000 cm^{-1} from microplastics collected from batch reactors containing *P. brevicompactum* (Top) and *Z. maritimum* (Middle, and control samples (Bottom) after 7, 14, 21 and 28 days of exposure, compared with virgin polypropylene.

Table 3 - Relative areas of the identified regions on the spectra obtained for microplastics collected from batch reactors containing *P. brevicompactum* (Pb), *Z. maritimum* (Zm), and control samples and for virgin polypropylene (MPP), for each sampling day (7, 14, 21 and 28 days).

	3650-3050	3000-2800	1755-1490	1480-1350	1180-780
MPP	2.868	93.49	2.166	27.62	25.96
MP Control 7 days	28.11	82.85	9.423	27.08	19.18
MP Control 14 days	46.9	84.97	15.01	28.59	30.55
MP Control 21 days	86.15	88.26	21.9	32.27	47.42
MP Control 28 days	61.59	88.9	15.92	32.32	39.51
MPs + Pb 7 days	59.19	85.65	12.25	27.93	64.63
MPs + Pb 14 days	106.4	93.96	21.23	31.13	93.66
MPs + Pb 21 days	71.93	89.01	11.91	28.47	62.91
MPs + Pb 28 days	46.78	87.81	9.21	28.22	70.33
MPs + Zm 7 days	21.92	85.84	5.145	27.69	15.44
MPs + Zm 14 days	13.08	85.39	8.716	28.07	14.35
MPs + Zm 21 days	23.75	87.45	5.758	28.22	19.87
MPs + Zm 28 days	46.58	94.94	13.99	33.4	28.35

Table 4 present the values of CI from a virgin PP, from the facemasks' microplastics exposed to both fungus for 14 and 28 days, and also the CI from the control samples. When considering the carbonyl index values, microplastics collected from batch reactors containing *P. brevicompactum* or *Z. maritimum* exhibited signs of degradation, as these microplastics present 10 times higher carbonyl index when compared to virgin PP. When compared to the microplastics that had no contact with fungi, exposed microplastics still have a higher CI, which, once again, suggest that there is effect of the fungi presence, even do there is also some mechanical degradation caused by the experimental conditions.

Table 4 - Carbonyl index (CI) for microplastics prior exposure (MPP), microplastics extracted from the batch reactors without fungi (Mp Control), microplastics extracted from the batch reactors containing fungi, *Penicillium brevicompactum* (Mps + Pb) or *Zalerion maritimum* (Mps + Zm), after 14 and 28 days of exposure.

	MPP	Mp Control 14 days	Mps + Pb 14 days	Mps + Zm 14 days	Mp Control 28 days	Mps + Pb 28 days	Mps + Zm 28 days
Abs (1)	0.01364	0.04569	0.1095	0.1342	0.07461	0.1211	0.07296
Abs (2)	0.4585	0.4719	0.4574	0.4671	0.5090	0.4703	0.4751
CI	0.02977	0.09682	0.2393	0.2874	0.1466	0.2574	0.1536

In the case of the microplastics exposed to *Z. maritimum*, the carbonyl index appears to decrease trough time, this has been reported before when biotic degradation occurs[48,49]. It is proposed that, with time and with the action of the enzymes, Norrish reactions take place and/or there is the formation of ester groups, which leads to a decrease in CI. Overall, both fungi appear to have potential to degrade the facemasks's microplastics, but *Z. maritimum* seems to be more promising, as it achieved higher removal rates and in general, based on the FTIR's spectra and CI, the fragments exposed to this species of fungus showed higher rates of degradation. This species of fungus has already showed promising results to the degradation of polyethylene[35], achieving 70% removal in 21 days, and showed affinity with polyurethane[50], growing on its surface and possibly using it as a substrate.

To the best of our knowledge, these were the first fungi to be tested for their potential to biodegrade facemasks, and also these were the first tests for biodegradation in a marine environment. In future studies, to improve facemasks' microplastics removal, would be interesting to increase exposure time and/or optimize the medium to avoid scarcity of nutrients.

3.4. Ecotoxicity of microfibers released from facemasks (pristine or UV-aged) on the clam, *Venerupis corrugata*

FTIR spectra from microplastics/microfibres released from facemasks that underwent UV-aging showed a peak at 1640 cm^{-1} and an increased carbonyl zone (approx. 1544-1684 cm^{-1}), both suggestive of higher degradation compared to microplastics from pristine treatment, as also observed in disposable facemasks collected in Ria de Aveiro, likely exposed to UV radiation and seawater (section 3.1.).

In ecotoxicity tests the testing concentrations of UV-aged or pristine microfibres were similar to nominal concentrations (Table S3), and within concentrations predicted in such environments[51]. Mortality was observed in 5 organisms (from 24 used for tests) but without relation with microfibres ageing nor concentration.

3.4.1. Effects on clams' energy reserves and aerobic energy production

In general, *Venerupis corrugata* adults did not reveal significant changes in energy reserves individually (i.e., lipids, carbohydrates, proteins) after 96 h exposure to pristine or UV-aged microfibres, even when considering concentrations > 1000 items/L (Table S4 and S5, SI). Notwithstanding, *V. corrugata* revealed a decrease in cellular energy allocation (> 20%) in all tested microfibres concentrations in the UV-aged treatment, significant for the lowest and highest tested concentrations, which reflects a potential decrease in energy acquisition or, in other terms, an increase in energy consumption (Fig. 6, Table S4 - SI). Considering that all organisms were deprived of food source during exposure (to ensure the same physiological status), the presence of a stressor such as microfibres would require mobilization of energy and, consequently, alteration of the aerobic energy metabolism. Such energy allocation was pronounced in organisms exposed to UV-aged microplastics, which underlines the significant interaction observed for ageing treatment and microfibres concentration in CEA parameter ($F_{(1, 24)}$ = 20,44 and $F_{(3, 24)}$ = 3,973, respectively; $p < 0.05$) (Fig. 6).

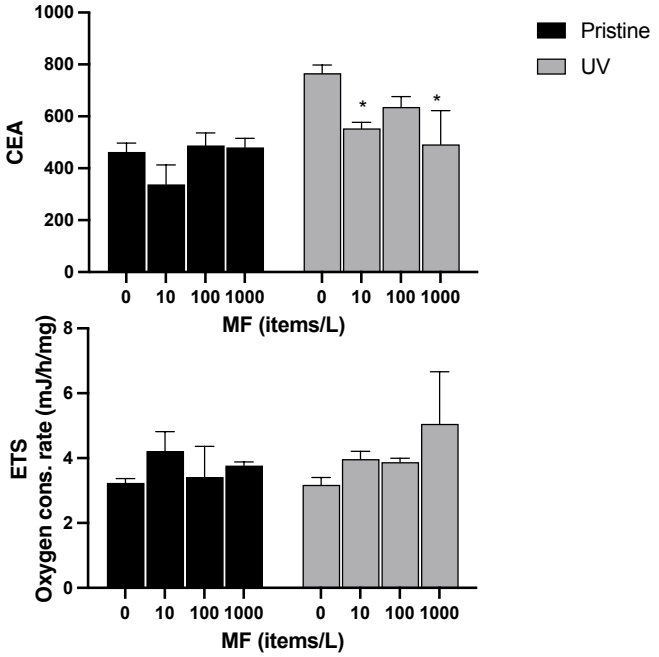

Figure 6 - Effects of face masks microfibres (MF) with (grey) or without (black) UV-C treatment on *Venerupis corrugata* adults after 96h exposure, considering Cellular Energy Allocation (CEA, top chart) and aerobic energy production (ETS, bottom chart). Data are presented as mean ± standard error of the mean. (*) denote significant statistical differences compared to the respective control, 0 MF/L (Šídák's multiple comparisons test, $p<0.05$).

An increase (by ~20-30%, despite the lack of statistical significance) in the mitochondrial ETS activity (a proxy of the aerobic energy metabolism, or, in other words, in energy consumption) was, indeed, in clams from the same treatment (UV-aged) where a decrease in energy allocation was observed (Fig. 6A). Thus, a decrease in *V. corrugata* CEA is more likely related to increased aerobic energy metabolism rather than a decreased energy assimilation. No significant interactions between microfibres treatments and microfibres concentration were denoted, for ETS (Table S4, SI).

Our results are congruent with previous works, particularly with sediment-dwelling bivalves. For example, no changes were observed in protein, carbohydrate, or lipid contents (individually) in *Ennucula tenuis* and *Abra nitida,* after exposure to polyethylene microplastics (here in the pristine form) in concentrations up to 10^6 in range, for four weeks[52]. Nonetheless, a general decrease in the total energy was also observed. Conversely, *Mytilus coruscus,* a bivalve commonly found in rocky crevices, revealed a decrease in energy reserves (particularly carbohydrates), also reflected in

terms of decreased cellular energy allocation after 7 days of exposure to polystyrene microplastics. However, such effects were only reported for concentrations also approximately 8 times higher than our highest tested concentration[53]. Distinct ecotoxicological effects on bivalves are often related to organism physiology, behaviour, and habitat, with the influence of the dose and shape of the tested particles[54].

In most marine species, exposure to (and ingestion of) microplastics is commonly related to a decrease in energy allocation (mainly energy acquisition), essentially by compromising food intake or clearance rate (in the case of bivalves, as also observed in our study). Another possible explanation is related to the high energetic cost related to impaired internal homeostasis and upregulation of energetically costly protective mechanisms, as for antioxidant and detoxification, against oxidative stress (as reviewed by Sá et al.[54]). The slight increase in the mitochondrial ETS activity observed in *V. corrugata* exposed to UV-aged microfibre indicates a potential activation of mitochondrial metabolism and an increase in ATP demand; thus, reflecting the cellular energy requirements of such clams to reinstall internal homeostasis through depletion of energy reserves in general (i.e., lipids+proteins+carbohydrates). Such increment in ETS activity (aerobic energy metabolism) is probably related to the clams' clearance activity and the capacity to counteract potential oxidative stress (as discussed in Section 3.3.2).

3.4.2. Oxidative stress and detoxication in clams

The clam *V. corrugata* presented similar antioxidant (CAT and GST) and detoxificant (also GST) capacities when exposed to different concentrations of pristine or UV-aged microfibres (Table S4, effect of microfibres concentrations, SI). Yet, a significant effect of the ageing process was denoted in CAT activity ($F_{(1, 25)} = 5{,}507$, $p<0.05$), with a higher fluctuation on such enzymatic biomarkers in clams exposed to UV-aged microfibres, which validates the higher energetic demand previously observed in clams of this treatment (previous section).

There was no lipid peroxidation (LPO) in exposed clams from all tested treatments. In fact, a significant decrease (~50%) was observed in the LPO of clams exposed to the highest concentration of UV-aged microfibres (1250 items/L) (Fig. 7). No interaction

between microfibres ageing and concentration was observed. The apparent no oxidative damage observed in *V. corrugata* exposed to pristine or UV-aged microfibres, might reflect (to some extent) the success in counteracting the potential oxidative stress triggered by the exposure to (and potential ingestion of) microfibres (UV-aged aged or not). However, the decrease itself in LPO, can also reflect stress. A decrease in LPO has been reported in several marine organisms (as reviewed by Hu and Palić[55], including bivalves such as *Scrobicularia plana* exposed to polystyrene microplastics (20 μm, 1 mg/L)[56]. However, the underlying mechanisms for such decrease, remains unclear; but several hypotheses have been raised such as the link with an i) increment on antioxidant defences, which consequently limit the attack of ROS to membrane lipids, ii) a decrease in lipid contents, and, in highly stressful situations, to iii) apoptosis.

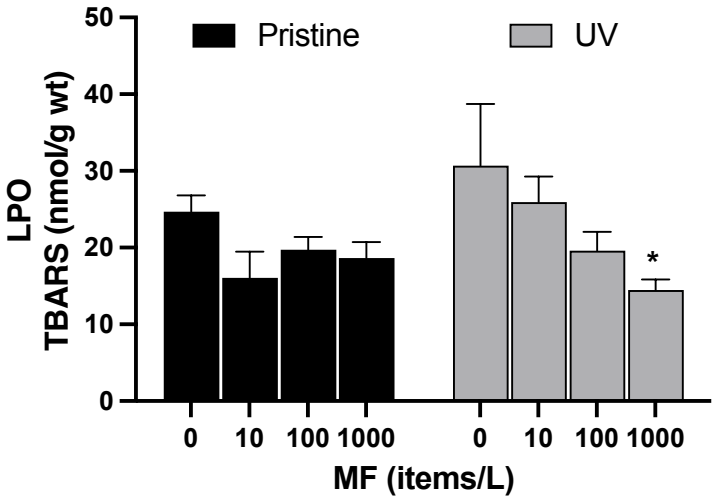

Figure 7 - Lipid peroxidation in adult *Venerupis corrugata*, after 96 h exposure to face masks microplastics (MF) with (grey) or without (black) UV-C treatment. Data are presented as mean ± standard error of the mean. (*) denote significant statistical differences compared to the respective control, 0 MF/L (Šídák's multiple comparisons test, $p<0.05$).

Microplastics have been shown to interfere with bivalves' digestive processes and bioenergetics, alter their microbiome, induce an immune response, and induce a cascade of cellular responses (as reviewed by Li et al.[57]. Oxidative stress has been proposed as mechanism to microplastic-induced stress and (eco)toxicity[55]. Elevated production of reactive oxygen species (ROS) can be triggered via microplastics uptake

and translocation, overwhelming the capacity of the cellular antioxidant system to detoxify ROS, which results in a misbalance between ROS generation and removal and consequently in oxidative damage (lipid and DNA damage)[57]. Several studies in marine bivalves (clams, mussels, oysters) support this hypothesised mechanism of microplastics (eco)toxicity, by revealing oxidative lesions to proteins and lipids and depletion of antioxidants such as catalase (CAT) and glutathione (GSH)[58,59]. However, other studies (such as this one) demonstrate no oxidative stress nor damage after microplastics exposure, also aligned with findings from Wang et al.[60] and Magni et al.[61], with other bivalve species. The assessment of oxidative stress mechanisms as a maker of microplastics-induced toxicity in marine bivalves seems, therefore, debatable. It can be arguable that the triggered oxidative stress responses are dependent (among others) on the number of particles retained in bivalves tissues, along with their size, shape, and potential leachates. Thus, particles quantification (potential distribution) and characterisation are crucial to better elucidate (and corroborate) physiological and/or biochemical target mechanisms triggered by such particles.

3.4.3. Clearance rate

The clearance capacity has been used as a practical indicator of feeding in bivalves[62]. Such capacity can be inferred from the algae concentration during a period of time. *V. corrugata* clearance capacity was addressed during 120 min, after 96 h exposure to 0, 50, 250, and 1250 items/L of pristine or UV-aged microplastics, and results are presented in Fig. 8.

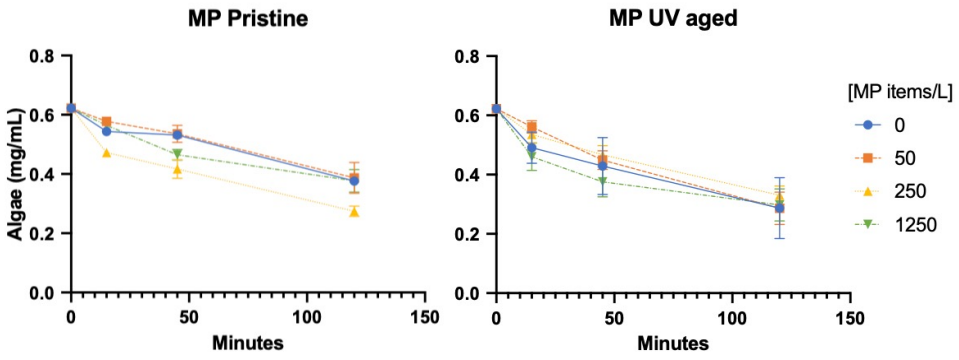

Figure 8 - Clearance capacity of *Venerupis corrugata* individuals during 120 min for pristine (on the left) and UV-aged (on the right) microfibres.

In general, there is a decrease in the concentration of algae in the medium, which indicates an increasing capacity for clearance by the clams. This clearance capacity appears to be specifically higher at higher concentrations (Tables S7, S8 SI), particularly in organisms exposed to UV-aged microfibres, which may be related to the greater stress felt by these clams in order to restore their energy levels. In fact, these organisms showed a decrease in CEA and an (although not significant) increase in energy consumption. Naturally, and after a period of 96 h of starvation, they were more prone to feeding and, therefore, increased their clearance rate. No interaction between microfibres concentration and time to which clams were exposed to algae was observed (Table S8, SI).

Our results are in agreement with other studies with bivalves exposed to microplastics. For example, *Dreissena polymorpha* mussels exposed to pristine polystyrene up to 100 000 p/mL for 42 days revealed an inversely proportional trend in the chlorophyll's concentration in the medium. From three days onwards, the longer the exposure time to polystyrene, the lower the concentration of chlorophyll in the medium (with significant results for the highest concentration)[62], therefore, the greater the clearance capacity. Also, algae consumption was significantly higher in adult oysters exposed to polystyrene microplastics that those not exposed[63], supporting the previous suggestion that high algae ingestion after interaction with microplastics results from an act of compensation.

4. Conclusion and final remarks

Disposable facemasks are a common plastic-based item commonly observed in urban but, most concerning, in natural environments such as Aveiro Lagoon. In such a transitional environment, facemasks can be exposed for long periods to different biotic and abiotic elements, which trigger their chemical degradation, altering the polypropylene backbone. Consequently, physicochemical degradation occurs, becoming visible through the yellowing of the mask fabrics, the appearance of cracks, the number of fibres they release (all visible to the naked eye), and the changes in their functional groups when analysed with spectroscopic methodologies (as FTIR).

When in contact with common fungi in aquatic environments *(Penicillium brevicompactum* or *Zalerion maritimum*) face masks' microplastics denote biodegradability (despite apparently low) within one or two weeks. Such low potential for biodegradability impulse the release of microfibres from such masks, which in their UV-aged form, might have the potential to induce ecotoxicity to native invertebrates that inhabit the wetlands - such as clams. A short-term exposure of *Venerupis corrugata* clam to microfibres from masks induced sub-lethal effects in clams, particularly to those exposed to UV-aged microplastics at the highest concentration that resulted in a decrease in cellular energy allocation (CEA) and lipid peroxidation (LPO), and an increase in clearance capacity. Considering that in a natural environment the exposure is continuous, studies with longer periods are one of the next steps to consider. In addition, quantification of ingested fibres should be done as well as to determine whether the clams can expel the fibres or if these are translocated to other tissues. Finally, it is also necessary to study face masks' chemical additives, their released and possible ecotoxicological effects on the organisms of the Ria de Aveiro.

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VI. General discussion

The main purpose of this thesis was to gather knowledge that could contribute to the discovery of a natural and sustainable approach for reducing (micro)plastics in the environment, particularly in the marine ecosystem. The search for new bioremediation processes to mitigate the impact of plastics on nature has been the focal point of intense research in the last few years. Fungi, recognized for their role as decomposers in nature, have emerged as promising candidates in this regard[1]. These organisms possess the capacity to produce a wide variety of enzymes involved in the degradation of recalcitrant substances and contaminants[2].

To achieve this goal, two fungi, *Z. maritimum* and *P. brevicompactum*, known for their potential to remove and biodegrade polyethylene (PE) (micro)plastics, underwent thorough investigation. This project made significant contributions, including the annotation of two fungal genomes, the optimization of a culture medium, a proteome study of filamentous fungi grown in the presence of PE, and insights into the biodegradability and ecotoxicity of two plastic materials widely used in the last few years.

In Chapter II, our primary focus was to gain a deeper understanding of the biotechnological potential of *Z. maritimum* and *P. brevicompactum* in the removal and biodegradation of plastics from the environment. To achieve this and unravel genetic information of both fungi, we sequenced, annotated, and analyzed their genomes. This work represents a valuable contribution to the growing field of fungal genomics. A noteworthy aspect is the sequencing and annotation of the genome of *Z. maritimum*, as marine fungal genomes remain relatively underrepresented in databases.

One of the main limitations of this work was the lack of available annotation for fungal genomes, especially those phylogenetically close to *Zalerion*. This limitation resulted in the annotation of many genes, in both genomes, as hypothetical proteins, indicating that for those sequences, the predictions of the encoded proteins have not been experimentally characterized yet, leaving our species not completely annotated[3].

The work described in this chapter involved a comprehensive examination of the genomes of both *Z. maritimum* and *P. brevicompactum*. Regarding the data obtained from annotation, it was possible to identify genes within the genomes of both species that encode enzymes previously associated with the degradation of plastics, like laccase and cutinases[2]. Notably, KEGG analysis unveiled pathways associated with the degradation of other chemical compounds, underscoring the potential utility of these fungi in bioremediation processes[4,5]. Nonetheless, the data derived from the annotation represent a first foundation, a "blueprint", for subsequent OMICs studies[6] in the bioremediation area using *Z. maritimum* and *P. brevicompactum*, some of which are also present in this thesis.

The information present in this chapter not only expand our understanding of the biotechnological potential of *Z. maritimum* and *P. brevicompactum* but also contributes to the broader scientific knowledge regarding fungal genomes and their applications in environmental bioremediation processes.

Chapter III presents the optimization of the chemical composition of a culture medium using uniform design to enhance the removal and degradation of PE microplastics by *Z. maritimum*. The choice of uniform design was motivated by it proven effectiveness in a prior study using *P. brevicompactum*[7]. In that study, a comparison between two experimental designs revealed uniform design as a more economical and effective option for this type of application, providing substantial information even when the regression model is unknown[8].

The outcome of the present study unveiled the biological limitations of *Z. maritimum* when exposed solely to PE microplastics. Notably, the fungus cannot thrive in such conditions, highlighting the importance of a supplementation in the medium. Malt extract proved to be the main regulatory factor influencing the removal of PE microplastics, with the highest optimal concentration of 11.5 g/L. While glucose and peptone did not significantly impact degradation, they proved essential for fungal growth, and their presence is necessary, even at lower concentrations. The optimal concentrations for glucose and for peptone, at 4.47 g/L and 0.458 g/L respectively, reflect a careful balance that supports growth without interfering with the removal and

degradation process. Since, (micro)plastics are proposed to serve as an alternative source of carbon, substituting glucose[9].

The key outcome of this study is an optimized medium applicable to scale-up approaches, crucial for the realistic and effective implementation of *Z. maritimum* in biodegradation applications. This optimized medium is valuable in various biodegradation studies, exemplified in Chapter IV where this medium was applied in the experiment used for obtaining the proteomic profile of fungi grown in the presence of PE microplastics.

It is essential to note that the medium optimization was only performed for *Z. maritimum*, as for *P. brevicompactum* had previously been made and it is detailed in "Biodegradation of microplastics: Optimization and Scale-up"[7]. Notably, malt extract emerged as the most significant medium component for both fungi, aligning with existing knowledge, describing it as essential for the growth and metabolism of fungi, serving as key nutrient source[10].

The study detailed in this chapter serves as guide for future research, offering insights needed for the development of strategies that consider the specific needs of fungi in practical biodegradation applications.

The primary objective of the work presented in Chapter IV was to understand potential changes in the cellular proteome of the two fungi, *Z. maritimum* and *P. brevicompactum* when grown in the presence of PE. This experiment used gathered information from the previous chapters, utilizing optimized mediums in the experiment and the genomic annotations to analyze the proteomic data obtained. A comparative analysis was conducted between the proteomic profile of the fungi grown in the presence of PE in the optimized medium and those grown solely in the optimized medium.

This work marks a significant contribution as the first proteomic characterization of filamentous fungi in contact with plastics and provides the first proteomics profiles of *Z. maritimum* and *P. brevicompactum*. Interestingly, the results indicate that, contrary to expectations, neither fungus exhibited a specific protein response when in contact with PE. However, variations in the abundance of certain proteins suggest that intracellular proteins involved in plastic biodegradation are constitutively expressed.

Similar to the genomic study of Chapter II, a notable limitation of this work was the identification of a large number of hypothetical proteins, as explained before, proteins with no functional annotation associated.

The fragments resulting from the PE degradation are likely utilized to produce energy through the cell's normal metabolism, such as the citrate cycle (TCA), as proposed in previous literature[4]. For instance, in *Z. maritimum*, it was observed that enzymes associated with the TCA cycle were more abundant in samples of fungi grown in the presence of PE. This suggests that these enzymes play a role in utilizing the fragments generated from PE biodegradation for energy production within the cell.

In summary, this study not only expands the frontiers of knowledge regarding fungal responses to plastic but also prompts further inquiries into the functional roles of identified proteins. It highlights the necessity to also study the extracellular proteome for a more comprehensive understanding of biodegradation of plastics by fungi.

Finally, in Chapter V, *P. brevicompactum* was studied for its ability to degrade real samples of polyethylene, mulch biofilm and facemasks. The ability of *Z. maritimum* to degrade facemasks was also tested. While the other three chapters of this thesis present studies that aimed to better understand the fungi themselves and unravel knowledge that would help to develop an integrated bioremediation process, this chapter presents how the fungi behave when in contact with (micro)plastics in environmental relevant conditions. The samples of plastics studied were already processed and shaped in a specific form used in our daily lives. The removal and degradability of PE from a yogurt bottle and from a bag were studied, along with the degradability and toxicity of a mulch biofilm, and the presence, degradability, and ecotoxicity of facemasks.

In the case of PE samples, the removal and degradation were tested using the optimized medium, unfortunately, neither the theoretical nor the percentages obtained with standard PE microplastics were achieved. Despite the percentages being lower than expected, *P. brevicompactum* showed great affinity to this type of sample, it was able to grow on its surface and the plastics showed signals of degradation. These results highlight the importance of understanding more about the mechanisms involved in the

biodegradation ability of the fungi since a simple change in the polymers itself affects the success of the process.

With the intent of reducing the use of conventional plastic mulch film in agriculture and, consequently, reducing soil contamination, biobased and biodegradable plastic mulch films (aka, mulch biofilm) have emerged as a sustainable alternative. As the environmental friendliness of these alternatives, mulch biofilm, has been questioned[11] with this work, we intended to understand the possible biodegradation and ecotoxicity of mulch biofilms in soil and freshwater systems. We choose these environments since mulch biofilms can remain on soil or enter freshwater ecosystems through runoff processes from agricultural fields or direct disposal into waterways, potentially breaking down into smaller pieces (microplastics) and affecting the soil and freshwater biota. We only tested the ability of *P. brevicompactum* in this case, as it is the only one that can be found in agricultural environments and that has been isolated from freshwater environments[12] and other authors have shown its ability to degrade PVA[13]. *Penicillium brevicompactum* showed great affinity to this polymer, and it was difficult to separate the fungi from the microplastics in both cases. Fourier Transform Infrared Spectroscopy (FTIR) spectra of the microplastics showed that the ones put in contact with *P. brevicompactum* showed evidence of degradation. The results obtained in this work suggest that *P. brevicompactum* is able to act as a bioremediation agent and accelerate the biodegradation process of the mulch biofilm. Unfortunately, it was difficult to understand in which environment this species would act as a better bioremediation agent. Since each had its challenges, it was impossible to evaluate the real ability. Furthermore, it is not possible to compare the growth or the removal percentages in each environment, as the values end up being calculated differently.

Regarding ecotoxicity, the earthworm *E. andrei* and the chironomid *C. riparius* were used as animal models, since both these model organisms, ingest large amounts of substrate while obtaining food, making them both susceptible to ingesting the plastic particles. *E. andrei* is commonly found in vermicomposting and agroecosystems and plays a pivotal role in aeration and nutrient cycling and are key organisms on terrestrial food webs. The chironomid *C. riparius*, particularly in its larval stage, is a benthic macroinvertebrate that plays an important role in nutrient cycling, and it is a key

organism in freshwater food webs. The pristine and UV-weathered microplastics had different effects in each model organism. Nevertheless, in agroecosystems, the mulch biofilm microplastics have no chronic effects and in freshwater environments, they have low deleterious effects, but potential for endocrine disruption in the case of UV-weathered microplastics.

During the process and work behind this thesis, the Covid-19 pandemic erupted and evolved, leading to the implementation of some safety protocols, including the use of facemasks, which were essential to control the spread of the virus. In several situations, the use of disposable facemasks was encouraged or preferred by people in their daily life[14]. Unfortunately, in some cases, the correct disposable of these facemasks was not made, and it was possible to find facemasks in several natural environments, including Ria de Aveiro. The principal component of disposable facemasks is isotactic polypropylene[15], making them a plastic material following in the scope of this thesis, and their potential for biodegradation was tested using both fungi, *P. brevicompactum* and *Z. maritimum*. The ecotoxicity potential of this item was also studied on local bivalves, *V. corrugata*. In the biodegradation tests of facemasks' particles performed with *P. brevicompactum* removal percentages around 20% were achieved. In the case of *Z. maritimum,* it was possible to achieve higher percentages, around 45%. In both cases, the FTIR spectra of the particles exposed to the fungi showed signs of degradation. Microfibers released from facemasks induced sublethal effects on the clam, especially the UV-weathered ones.

So, in Chapter V, we contributed information regarding the ability of *P. brevicompactum* to degrade different types of polymers, we tried to reach theoretical values for the degradation of PE, using microplastics from real samples, tested the degradability of a mulch biofilm and facemasks. In general, *P. brevicompactum* was able to remove and cause alterations in all the polymers, based on mass losses and FTIR spectra. In the case of *Z. maritimum*, which was only tested in contact with facemasks, we saw that this fungus was also able to achieve high percentages of removal when compared to *P. brevicompactum* and the previous results for this fungus in contact with PE microplastics[16]. The FTIR spectra of facemasks' particles in contact with *Z. maritimum* also had signs of degradation.

In terms of ecotoxicity, the three tests were in environments and with different organisms, the results showed that in general, all kinds of plastics have some kind of effect on the organisms, proving the importance of encountering a bioremediation process that will reduce these contaminants in the environment.

VII. Future perspectives

Plastics continue to play a significant role in our daily life, despite the European Union's efforts to reduce the use of this material. The production of plastics keeps increasing, and in developing countries, plastic remains an important material[17]. The Covid-19 pandemic further emphasized our dependence on plastic materials, which played a vital role in implementing measures to prevent virus' spread[18]. Given these challenges, finding innovative solutions and alternative materials to replace conventional plastics becomes imperative, along with effective methods for removing and recycling used plastics.

This thesis focuses on exploring a potential solution for plastic removal, the use of fungi and their ability to biodegrade (micro)plastics. While this research takes initial steps in unraveling the biological process behind this process, there is still much to explore. Specifically, study the extracellular proteome of both fungi when grown in the presence of PE and further investigate the cellular proteome. Continuous updates to genomic databases offer opportunities for annotating hypothetical proteins, and a metabolomics study can provide insights into the compounds involved in the biodegradation process. Additionally, characterizing the chemicals in the extracellular medium is vital to assessing potential stress or toxicity effects on fungi.

Our findings highlight *P. brevicompactum* as a promising candidate for various biodegradation processes due to its adaptability to diverse habitats, but *Z. maritimum* would be a better candidate in the marine environment. Yet, further investigations are necessary to understand how both fungi respond to polymer mixtures or polymers more challenging to degrade.

In conclusion, this thesis underscores the urgency for exploring alternative solutions to mitigate the environmental impact of plastics and represents a significant advance towards unraveling the biotechnological potential of *Z. maritimum* and *P. brevicompactum* in plastic waste remediation.

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Supplementary Information

Chapter II. Complete genome sequence of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329, two fungi with potential for biodegradation of microplastics

The supplementary tables of this chapter, Tables S1 to S8, are deposited in an excel file named "SupplementaryMaterial_ChapterII".

Chapter IV. Proteome analysis of *Penicillium brevicompactum* CMG 72 and *Zalerion maritimum* ATCC 34329, two fungi with potential for biodegradation of microplastics

Methods:

SignalP-6.0[1] was used to analyze the genome data, from Chapter II, regarding the enzymes normally associated with biodegradation (laccases, cutinases, monooxygenases, cytochrome P450, and alcohol dehydrogenase).

Results:

Table S1 – Identified proteins in samples of *Zalerion maritimum*, at 14 and 28 days of experiment, in both conditions, grown in the presence of PE and control. In blue are highlighted the proteins present in both time points.

Protein IDs - 14 days	Protein Ids - 28 days
KAJ2890462.1	KAJ2890462.1
KAJ2890611.1	KAJ2890611.1
KAJ2890968.1	KAJ2890968.1
KAJ2891120.1	KAJ2891120.1
KAJ2891223.1	KAJ2891223.1
KAJ2891229.1	KAJ2891229.1
KAJ2891430.1	KAJ2891430.1
KAJ2891553.1	KAJ2891553.1
KAJ2891611.1	KAJ2891611.1
KAJ2891625.1	KAJ2891625.1
KAJ2891641.1	KAJ2891641.1
KAJ2891911.1	KAJ2891911.1
KAJ2891952.1	KAJ2891952.1
KAJ2891954.1	KAJ2891954.1
KAJ2892090.1	KAJ2892090.1
KAJ2892153.1	KAJ2892153.1
KAJ2892188.1	KAJ2892188.1
KAJ2892200.1	KAJ2892200.1
KAJ2892233.1	KAJ2892233.1
KAJ2892266.1	KAJ2892266.1
KAJ2892474.1	KAJ2892474.1
KAJ2892550.1	KAJ2892550.1
KAJ2892555.1	KAJ2892555.1
KAJ2892571.1	KAJ2892571.1
KAJ2892576.1	KAJ2892576.1
KAJ2892592.1	KAJ2892592.1

KAJ2892602.1	KAJ2892602.1
KAJ2892603.1	KAJ2892603.1
KAJ2892695.1	KAJ2892695.1
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KAJ2906503.1
KAJ2906515.1
KAJ2906528.1

KAJ2906614.1
KAJ2906664.1
KAJ2906680.1
KAJ2906687.1
KAJ2906764.1
KAJ2906793.1
KAJ2906828.1
KAJ2906904.1
KAJ2906932.1
KAJ2906944.1
KAJ2906949.1
KAJ2907003.1
KAJ2907037.1
KAJ2907056.1
KAJ2907060.1
KAJ2907062.1
KAJ2907126.1
KAJ2907197.1
KAJ2907290.1

Table S2 – Identified proteins in samples of *Penicillium brevicompactum*, at 21 and 28 days of experiment, in both conditions, grown in the presence of PE and control. In blue are highlighted the proteins present in both time points.

Protein IDs 21 days	Protein IDs 28 days
KAJ5322412.1;XP_056805357.1;KAJ5318828.1;KAJ5354260.1	KAJ5322412.1;XP_056805357.1;KAJ5318828.1;KAJ5354260.1
KAJ5323160.1;XP_056806117.1;KAJ5319588.1;KAJ5353478.1	KAJ5323160.1;XP_056806117.1;KAJ5319588.1;KAJ5353478.1
KAJ5326586.1;XP_056808371.1;KAJ5341454.1	KAJ5326586.1;XP_056808371.1;KAJ5341454.1
KAJ5327918.1;XP_056815605.1;KAJ5347141.1;KAJ5346543.1	KAJ5327918.1;XP_056815605.1;KAJ5347141.1;KAJ5346543.1
KAJ5328585.1;KAJ5347807.1;XP_056816271.1;KAJ5367335.1	KAJ5328585.1;KAJ5347807.1;XP_056816271.1;KAJ5367335.1
KAJ5328676.1;XP_056816358.1;KAJ5347894.1;KAJ5367242.1	KAJ5328676.1;XP_056816358.1;KAJ5347894.1;KAJ5367242.1
KAJ5328837.1;XP_056816477.1;KAJ5367094.1;KAJ5348013.1	KAJ5328837.1;XP_056816477.1;KAJ5367094.1;KAJ5348013.1
KAJ5334996.1;XP_056812868.1;sp|F1DBB2.1|MPAF_PENBR;KAJ5357673.1;KAJ5336663.1;ADY00133.1;sp|A0A0B5L585.1|MPAF2_PENBR;AJG44383.1	KAJ5334996.1;XP_056812868.1;sp|F1DBB2.1|MPAF_PENBR;KAJ5357673.1;KAJ5336663.1;ADY00133.1;sp|A0A0B5L585.1|MPAF2_PENBR;AJG44383.1
KAJ5337981.1;XP_056808999.1;KAJ5327214.1;KAJ5340850.1	KAJ5337981.1;XP_056808999.1;KAJ5327214.1;KAJ5340850.1
KAJ5339430.1;XP_056807567.1;KAJ5325782.1;KAJ5342256.1	KAJ5339430.1;XP_056807567.1;KAJ5325782.1;KAJ5342256.1
KAJ5340465.1;KAJ5337600.1;XP_056809382.1;KAJ5327597.1	KAJ5340465.1;KAJ5337600.1;XP_056809382.1;KAJ5327597.1
KAJ5341097.1;KAJ5338236.1;XP_056808752.1;KAJ5326967.1	KAJ5341097.1;KAJ5338236.1;XP_056808752.1;KAJ5326967.1
KAJ5341869.1;KAJ5339018.1;XP_056807954.1;KAJ5326169.1	KAJ5341869.1;KAJ5339018.1;XP_056807954.1;KAJ5326169.1
KAJ5341910.1;KAJ5339056.1;XP_056807916.1;KAJ5326131.1	KAJ5341910.1;KAJ5339056.1;XP_056807916.1;KAJ5326131.1
KAJ5341916.1;XP_056807908.1;KAJ5326123.1;KAJ5339062.1	KAJ5341916.1;XP_056807908.1;KAJ5326123.1;KAJ5339062.1
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KAJ5345226.1;XP_056813849.1;KAJ5349991.1;KAJ5343038.1	KAJ5345226.1;XP_056813849.1;KAJ5349991.1;KAJ5343038.1
KAJ5345385.1	KAJ5345385.1
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KAJ5358016.1;XP_056813208.1;KAJ5337003.1;KAJ5335348.1	KAJ5358016.1;XP_056813208.1;KAJ5337003.1;KAJ5335348.1
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XP_056809128.1;KAJ5337852.1;KAJ5327343.1;KAJ5340721.1	XP_056809128.1;KAJ5337852.1;KAJ5327343.1;KAJ5340721.1
XP_056809133.1;KAJ5327348.1;KAJ5340716.1;KAJ5337847.1	XP_056809133.1;KAJ5327348.1;KAJ5340716.1;KAJ5337847.1
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Table S3 – Results obtained with SignalP-6.0 for the enzymes encoded by *Z. maritimum* and *P. brevicompactum*, associated with biodegradation of plastics.

Protein	Prediction	Likelihood
KAJ2901882.1_Laccase	Signal Peptide	0.999
KAJ2896927.1_Cutinase	other	0.5168
KAJ2897060.1_cutinase	Signal Peptide	0.9997
KAJ2902845.1_monooxygenase	other	0.9993
KAJ2907039.1_putative monooxygenase	other	1
KAJ2904869.1_FAD-dependent monooxygenase DEP4	other	0.9993
KAJ2898188.1_cytochrome P450 monooxygenase	other	0.9813
KAJ2898160.1_putative dimethylaniline monooxygenase	other	1
KAJ2898042.1_Dimethylaniline monooxygenase [N-oxide-forming] 2	other	1
KAJ2897448.1_putative FAD-dependent monooxygenase	other	0.9889
KAJ2897396.1_FAD-dependent monooxygenase OpS4	other	1
KAJ2894626.1_FAD-dependent monooxygenase	other	0.9991
KAJ2893039.1_Polysaccharide monooxygenase Cel61a	Signal Peptide	0.9997
KAJ2905396.1_cytochrome P450	other	1
KAJ2904616.1_cytochrome P450	other	1
KAJ2903944.1_cytochrome P45	other	0.9998
KAJ2903856.1_cytochrome P450	other	1
KAJ2903706.1_cytochrome p450	other	1.0001
KAJ2903702.1_Cytochrome p450	other	1.0001
KAJ2903469.1_cytochrome P450	other	1
KAJ2901741.1_cytochrome P450	other	1.0001
KAJ2901433.1_cytochrome p450	other	0.9999
KAJ2900598.1_cytochrome p450	other	1.0001
KAJ2900066.1_cytochrome P450	other	0.9912
KAJ2896435.1_cytochrome P450	other	0.9997
KAJ2895709.1_cytochrome P450	other	1
KAJ2902793.1_Cytochrome p450 family protein	other	1.0001
KAJ2902340.1_cytochrome p450 7a1	other	0.9998
KAJ2900281.1_cytochrome P450 4A12A	other	0.9999
KAJ2898739.1_Cytochrome P450 55A2	other	1
KAJ2897835.1_cytochrome P450 82A2	other	1.0001
KAJ2896395.1_cytochrome p450 6a1	other	1
KAJ2894835.1_Cytochrome P450 52A5	other	1
KAJ2892488.1_cytochrome P450 61	other	1
KAJ2906016.1_alcohol dehydrogenase	other	1
KAJ2894262.1_alcohol dehydrogenase	Signal Peptide	0.6002
KAJ2906950.1_alcohol dehydrogenase GroES-like domain-containing protein	other	1.0001
KAJ2904328.1_Alcohol dehydrogenase 1	other	1.0001
KAJ2903110.1 alcohol dehydrogenase -like domain-containing protein	other	1.0001
KAJ2902782.1_putative alcohol dehydrogenase	other	1.0001
KAJ2902251.1_Alcohol dehydrogenase GroES-like domain family protein	other	0.9848
KAJ2897707.1_alcohol dehydrogenase GroES-like domain-containing protein	other	1.0001
KAJ2891462.1_Alcohol dehydrogenase superfamily	other	1.0001
g9382.t1 Laccase putative	other	0.9994
g1791.t1 Cutinase	other	0.9978
g3426.t1 putative cutinase	other	0.9999
g2086.t1 Cytochrome P450 monooxygenase	other	1
g2644.t1 Baeyer-Villiger monooxygenase	other	0.9993
g3016.t1 Nitronate monooxygenase	other	0.9994
g3200.t1 putative monooxygenase	other	0.9995
g4034.t1 monooxygenase	other	0.9999
g6754.t1 Dimethylaniline monooxygenase	other	0.9998
g7318.t1 cytochrome P450 monooxygenase	other	1
g7633.t1 cytochrome P450 monooxygenase	other	0.9995
g8939.t1 Flavin monooxygenase	other	0.9999
g9155.t1 Dimethylaniline monooxygenase	other	0.9988
g10586.t1 Putative Cellulose monooxygenase	other	0.9996
g157.t1 Cytochrome P450 monooxygenase	other	0.9994
g292.t1 Cytochrome P450	other	0.9997
g5344.t1 Alcohol dehydrogenase superfamily	other	0.9997
g8176.t1 Alcohol dehydrogenase superfamily	other	0.9980

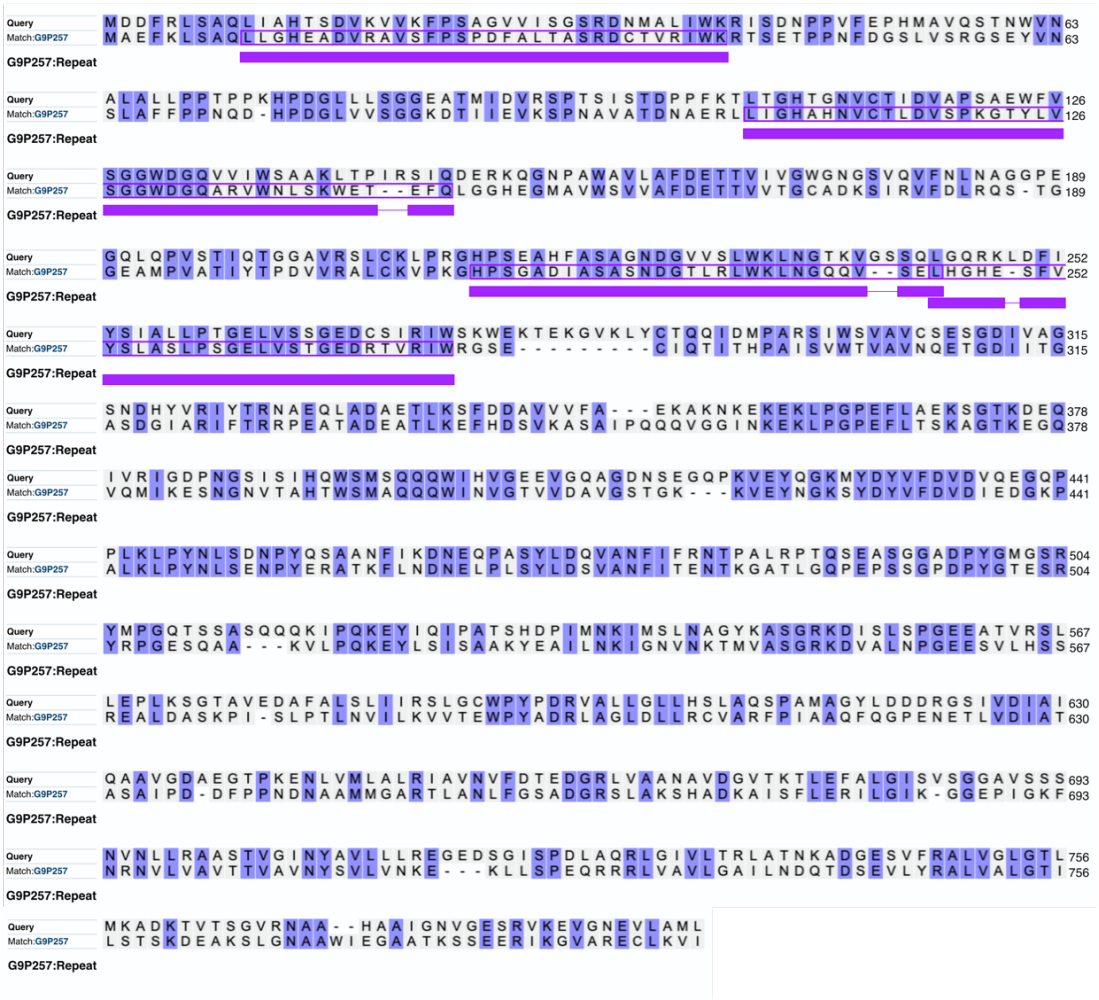

Figure S1 – Protein alignment of the identified protein KAJ2892233.1 and G9P257_HYPAI, from UNIPROT database. Alignment was generated by Blast tool of UNIPROT.

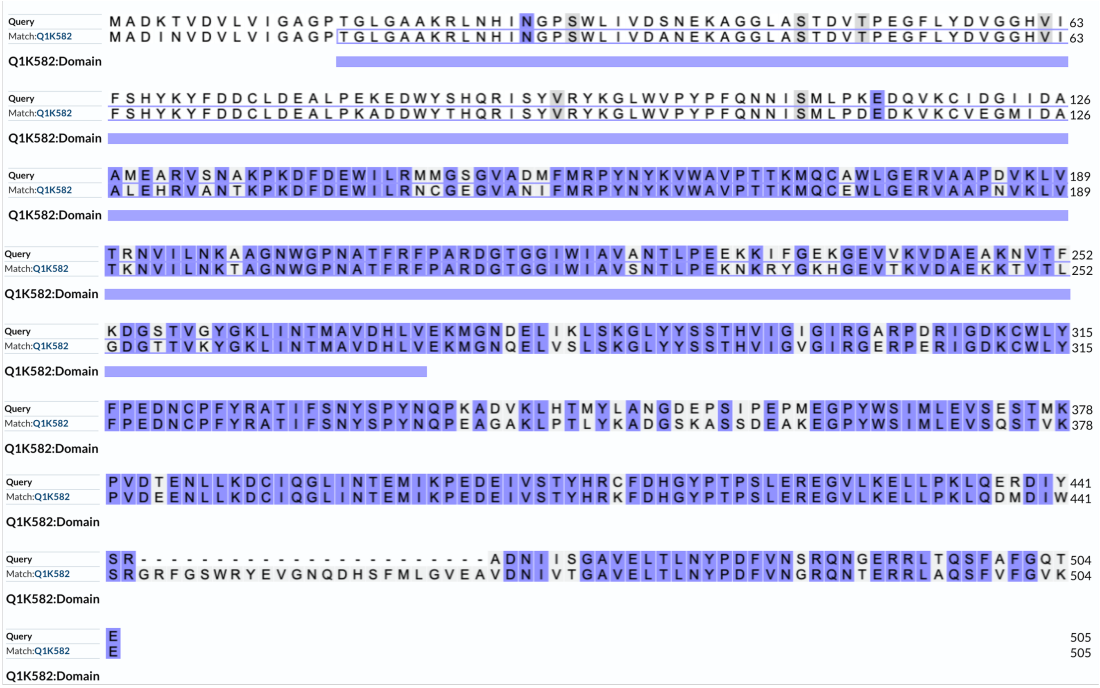

Figure S2 - Protein alignment of the identified protein KAJ2897813.1 and Q1K582_NEUCR, from UNIPROT database. Alignment was generated by Blast tool of UNIPROT.

Figure S3 - Protein alignment of the identified protein XP_056806292.1 and A0A1V6UY11_9EURO, from UNIPROT database. Alignment was generated by Blast tool of UNIPROT.

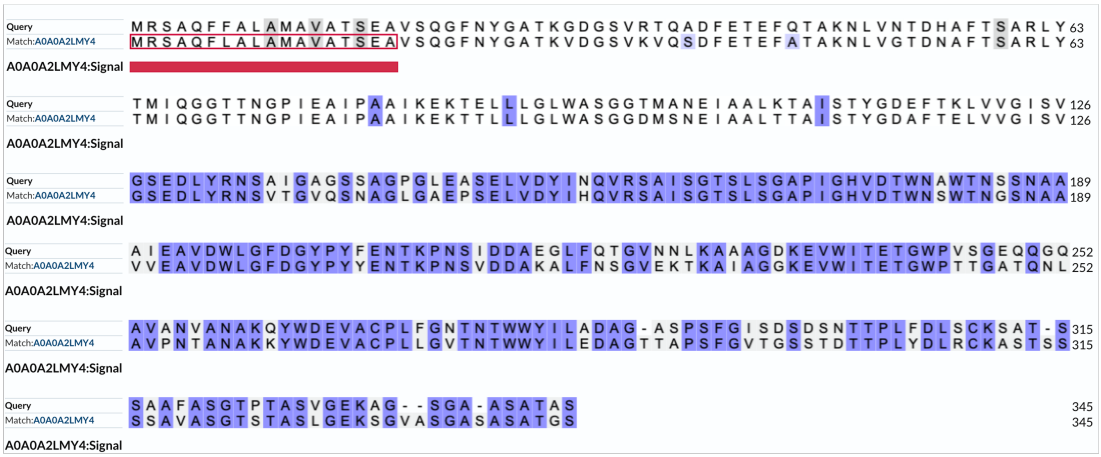

Figure S4 - Protein alignment of the identified protein KAJ5363048.1 and A0A0A2LMY4_PENIT, from UNIPROT database. Alignment was generated by Blast tool of UNIPROT.

Figure S5 - Protein alignment of the identified protein XP_056815070.1 and A0A1V6PWH8_9EURO, from UNIPROT database. Alignment was generated by Blast tool of UNIPROT.

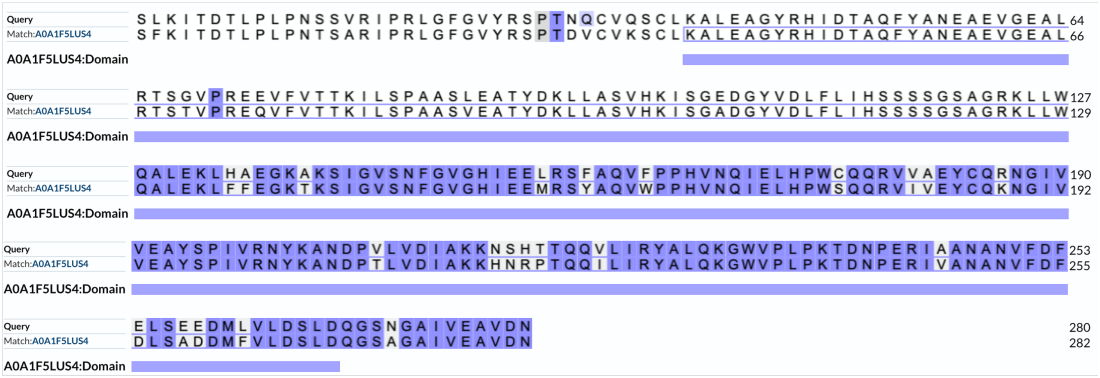

Figure S6 - Protein alignment of the identified protein XP_056805678.1 and A0A1F5LUS4_9EURO, from UNIPROT database. Alignment was generated by Blast tool of UNIPROT.

References:

1. Teufel, F.; Almagro Armenteros, J.J.; Johansen, A.R.; Gíslason, M.H.; Pihl, S.I.; Tsirigos, K.D.; Whinther, O.; Brunak, S.; Heijne, G.V. & Nielsen, H. SignalP 6.0 predicts all five types of signal peptides using protein language models. *Nature Biotechnology* **40**, 1023-1025 (2022). doi: 10.1038/s41587-021-01156-3

Chapter V. The use of *Penicillium brevicompactum* and *Zalerion maritimum* for the biodegradation of plastics in environmentally relevant conditions, and ecotoxicity studies of polymers in different habitats - Are mulch biofilms used in agriculture an environmentally friendly solution? - An insight into their biodegradability and ecotoxicity using key organisms in soil ecosystems

Table S1 - Main properties of the tested soil adapted from Chelinho et al.[1]. The pH was freshly measured before and after the performed tests.

pH (KCl, 1M)	**OM (%)**	**Sand (%)**	**Silt (%)**	**Clay (%)**	**Total N (mg/g)**	**CEC (mol/g)**	**WHC (5)**
6.4±0.2	3.10	62.4	21.2	16.4	0.83	0.0125	32.80±2.89

OM: organic matter, CEC cation exchange capacity, WHC water holding capacity. For pH and WHC, data are presented by mean ± SD (n=3).

Table S2 **-** Percentage of removed biofilm microplastics after the incubation period (here corresponding to a loss in mass), in the absence (containing only microplastics, MP) or presence of *Penicillium brevicompactum* (microplastics plus fungi, MP+F), in the preliminary soil matrix experiment that endured up to 15 days. Data are presented as average mass (g) or percentage (%) ± standard deviation. (*) denotes contamination of samples, with the retrieval of higher masses than those placed in the petri dish.

Sampling day	**Treatment**	**MP loss (g)**	**MP removal (%)**
5	MP+F	0.002±0.001	69±30
	MP	0.002±0.001	69±42
10	MP+F	0.00034±0.00005	10±2
	MP	*	*
15	MP+F	0.00088*	3*
	MP	0.00016*	5*

Table S3 - Biomass variation and spreading area of *Penicillium brevicompactum* after the incubation period, in the absence (containing only fungus, F) or presence of biofilm microplastics (microplastics plus fungi, MP+F), in the preliminary soil matrix experiment. Data are presented as average mass (g) or square centimetres (cm^2) ± standard deviation.

Sampling day	**Treatment**	**Final Biomass (g)**	**Area (cm^2)**
5	MP+F	17.9±0.3	2.8±0.4
	MP	37±15	16±4
10	MP+F	33±18	1.5±0.5
	MP	17±6	8±9
15	MP+F	27±19	1.1±0.6
	MP	45.8±0.5	1.40±0.02

Table S4 - Biomass variation and spreading area of *Penicillium brevicompactum* after the incubation period, in the absence (containing only fungus, F) or presence of biofilm microplastics (microplastics plus fungi, MP+F), in solid culture medium and soil matrix. Data are presented as average mass (g) or square centimetres (cm^2) ± standard deviation. (*) denotes invalid duplicate, and lack of mean and standard deviation calculation (**) denotes data loss due to equipment issues.

Experiment	**Sampling day**	**Treatment**	**Final Biomass (g)**	**Area (cm^2)**
Solid culture medium	5	MP+F	42±22	11±7
		MP	50±8	7±3
	10	MP+F	151±35	10±4
		MP	146±6	10±1
	15	MP+F	207±27	17±6
		MP	156±36	13±3
Soil	7	MP+F	32±25	**
		MP	64*	**
	14	MP+F	21±6	2±1
		MP	31±6	2.7±0.3
	21	MP+F	50±25	0.9±0.1
		MP	27±13	0.9±0.9
	28	MP+F	19±6	1.7±0.5
		MP	32*	0.6±0.4

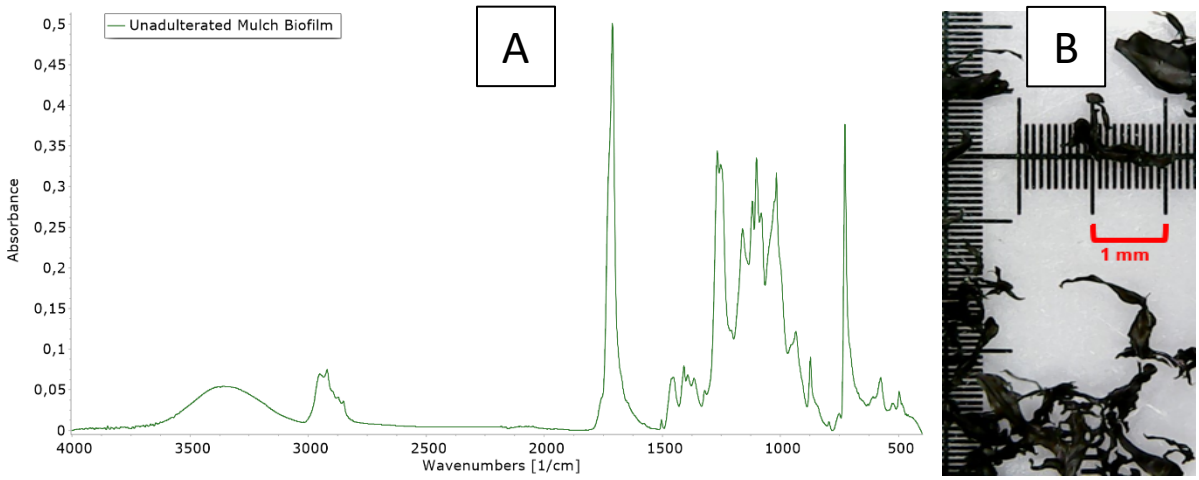

Figure S1 - Fourier-transform infrared spectroscopy spectra of the studied mulch biofilm (A), along with the variety of microplastic morphologies and sizes applied in both biodegradation and ecotoxicity tests (B).

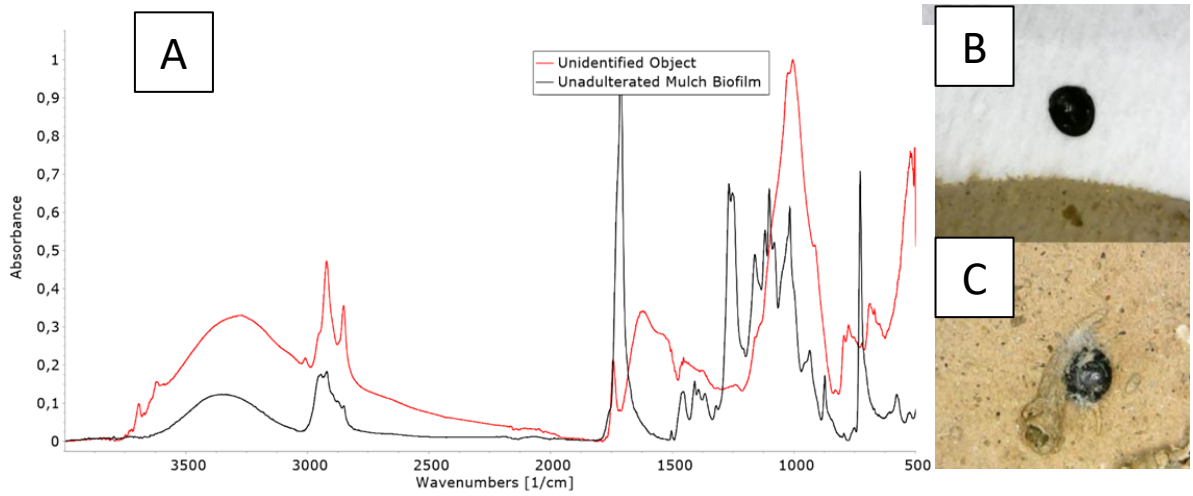

Figure S2 - Fourier-transform infrared spectroscopy spectra obtained from the tested biofilm (black) and from the undefined particles (red) that presented similar colour and size to microplastics used for testing (A), along with photographs of said particles, without (B) and with (C) fungal attachment.

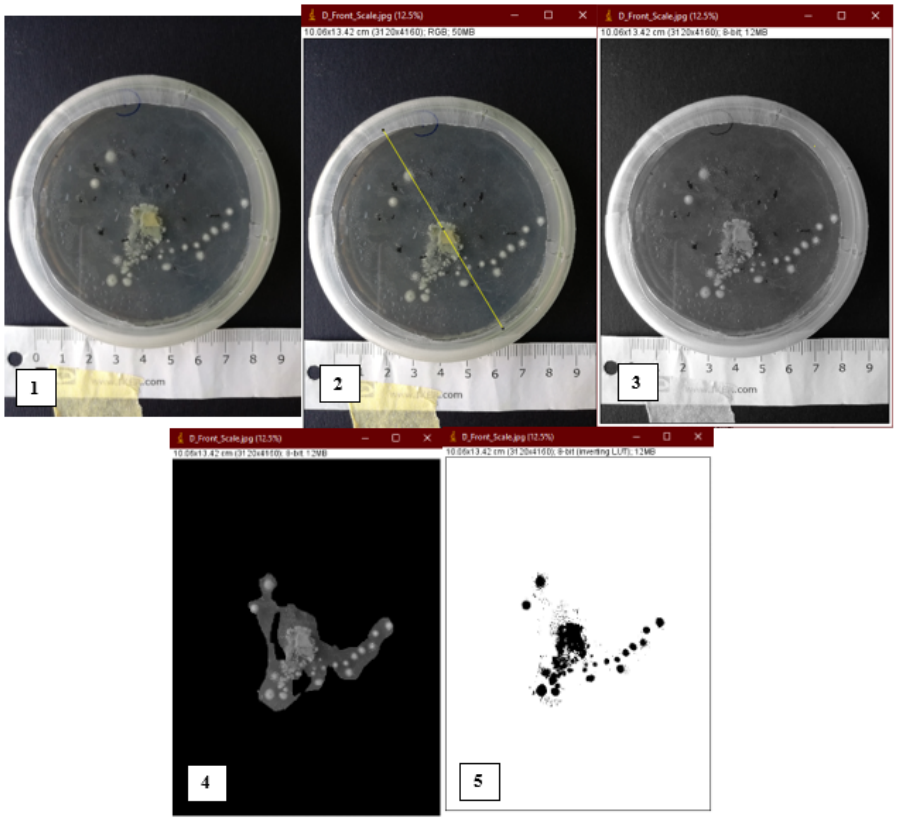

Figure S3 - Procedural steps to calculate the fungi area from sample photography, using Image J free software. 1. Photo of the entire petri dish, with a proper scale; 2. Diameter measurement (confirmable using the scale in the photo); 3. Conversion to 8-bit; 4. Growth area selected and outside removed to reduce the presence of visual artefacts; 5. The threshold was adjusted, ready for the "Analyze Particles" command. The sum of all particle areas on each petri dish corresponded to the total spreading area of the fungi.

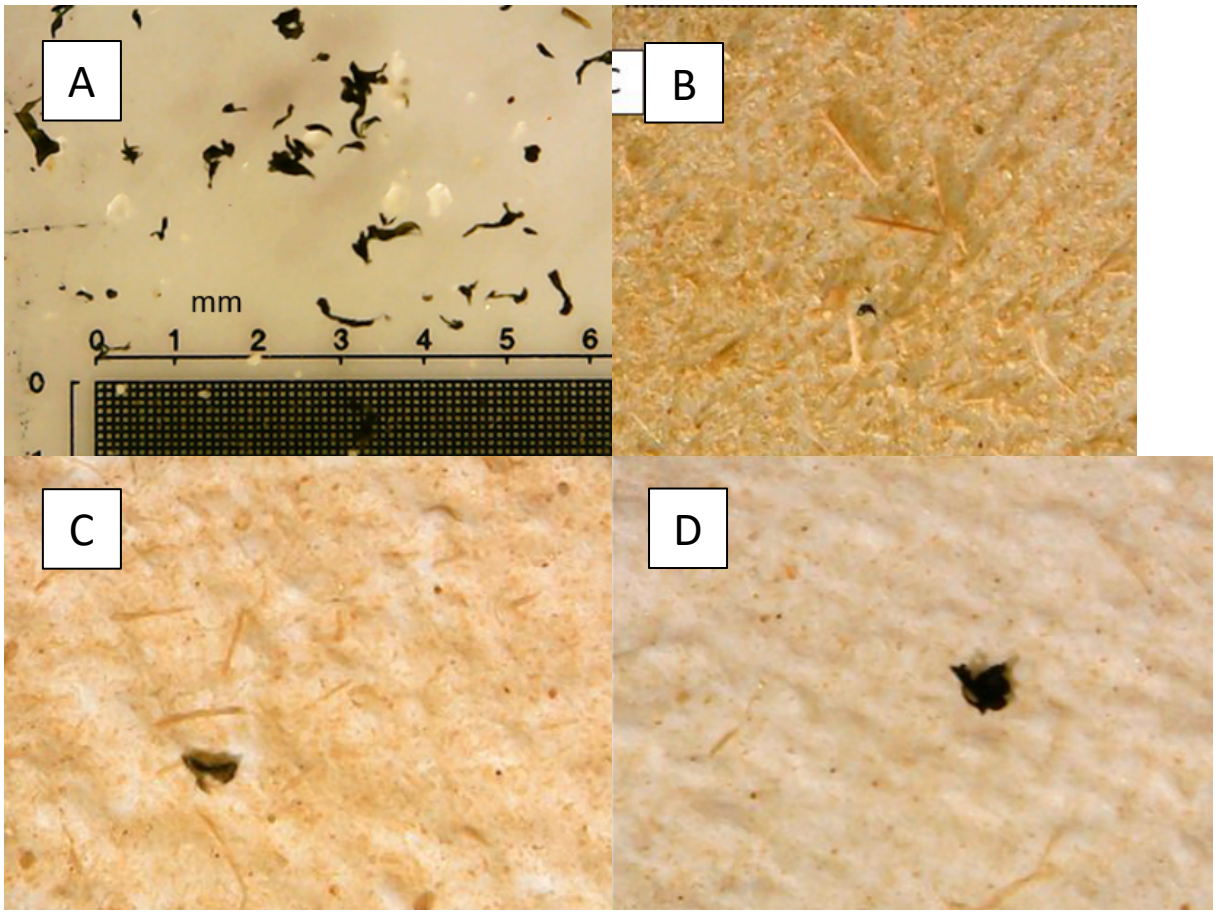

Figure S4 - Size analysis of egested microplastics from the pristine experiment. The scale used (A); microplastic egested in the lowest concentration setting (B); microplastics egested in the highest concentration setting (C, D).

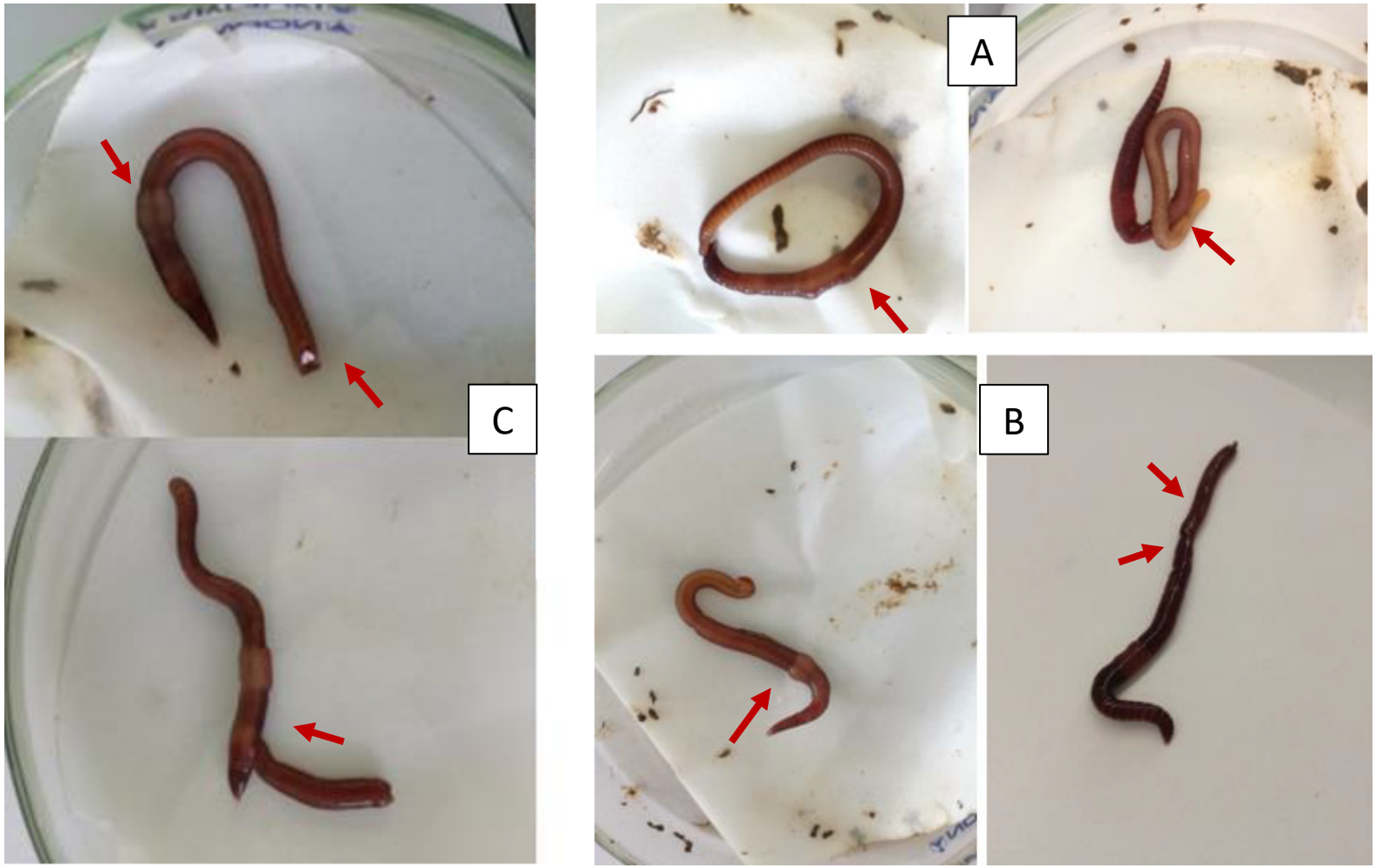

Figure S5 - Observed indicators of stress in adult earthworms after 21 days of exposure to pristine biofilm microplastics: coelomic fluid accumulations (A); cleavage furrows (B); tail detachments (C).

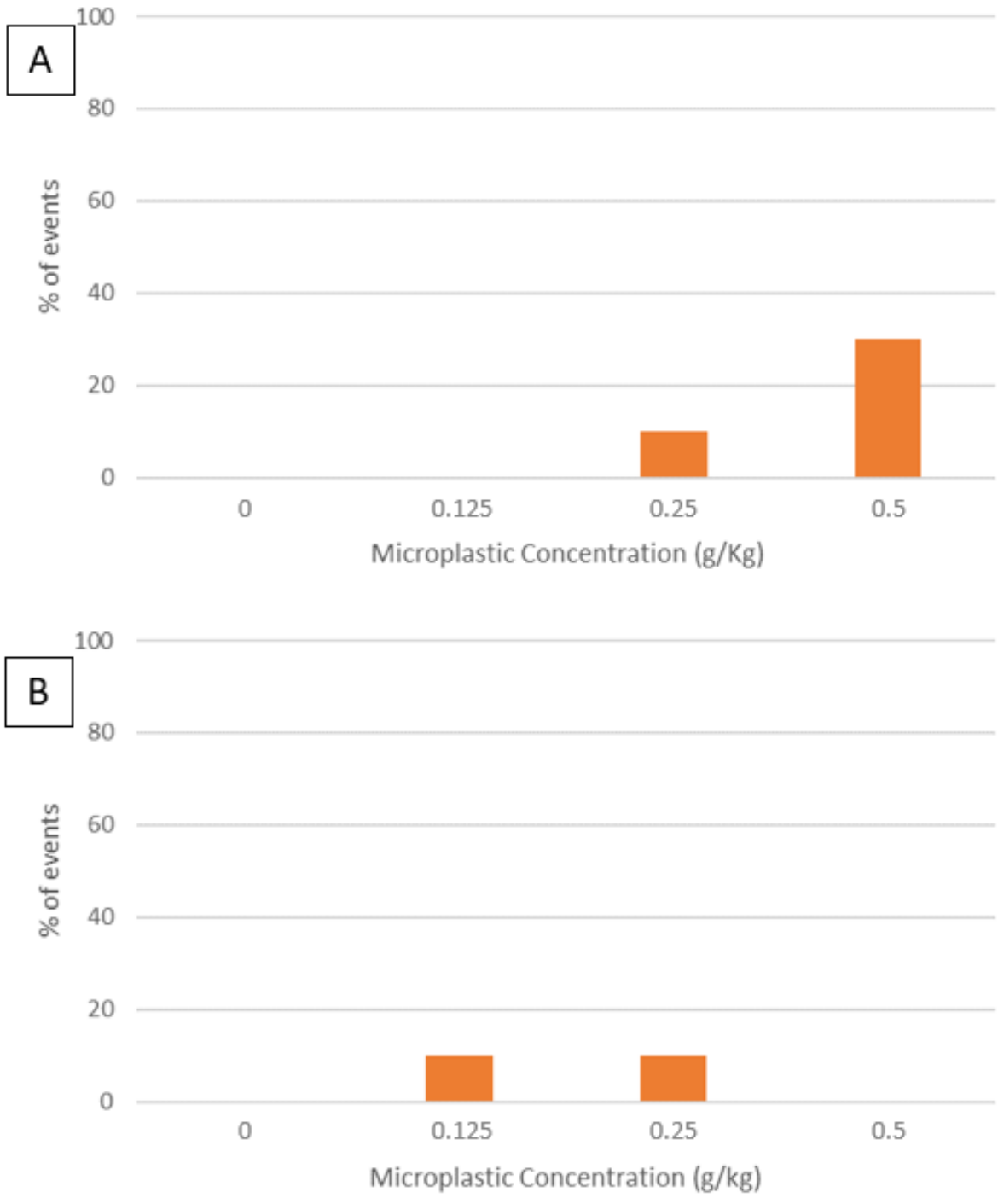

Figure S6 - Percentage of earthworms presenting tail detachment after 21 days of exposure to 0, 1.125, 0.250, and 0.500 g of pristine (A) or weathered (B) biofilm microplastics per kg of soil.

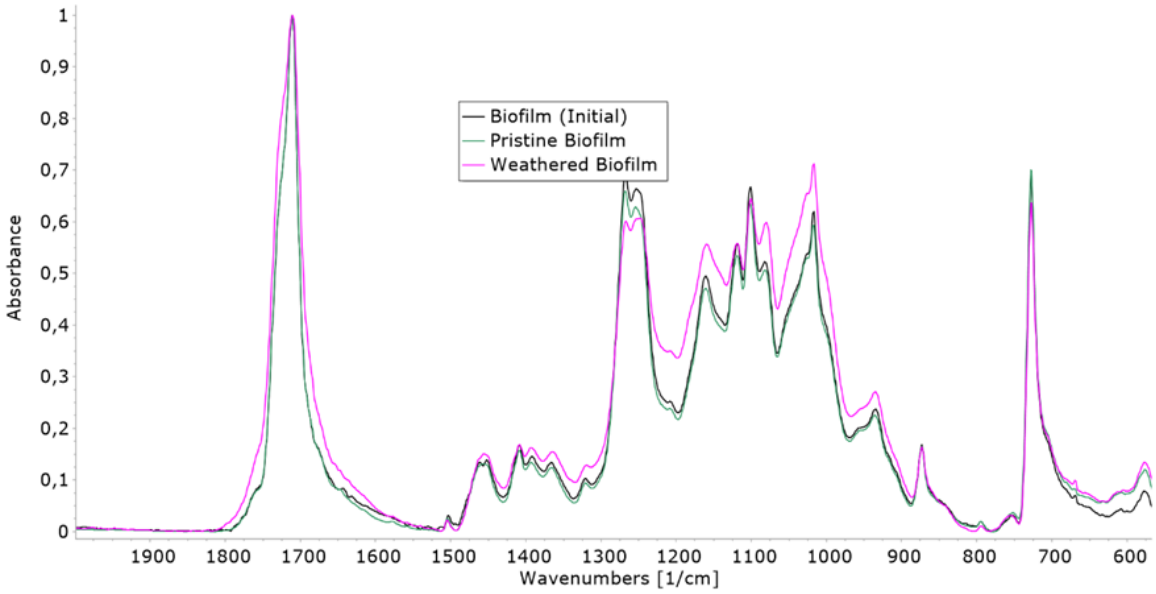

Figure S7 - FTIR spectra of the mulch biofilm samples prior to and after weathering process (UV-C radiation at 240 nm with a Uvitec LF-206.LS lamp for 21 days).

As per Figure S7, a noticeable reduction of peaks can be observed in samples from the weathered biofilm. Some of these losses of definition in the FTIR spectra, exemplified in this plot by the 1300-1000 cm^{-1} region, could be attributable to the occurrence of Norrish reactions. Norrish reactions result in scission and crosslinking events that fundamentally alter the chemical structure of the polymer, thus perhaps contributing to the higher biocompatibility of this mulch biofilm with *E. andrei*.

References:

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Chapter V. The use of *Penicillium brevicompactum* and *Zalerion maritimum* for the biodegradation of plastics in environmentally relevant conditions, and ecotoxicity studies of polymers in different habitats - Microplastics from agricultural mulch films: biodegradation and ecotoxicity in freshwater systems

Section I: UV-C weathering of Bio-MPs for ecotoxicity tests

Methods:

Before the experiments, the bio-MPs underwent a UV-C photodegradation process. Briefly, the bio-MPs were subjected to radiation of 240 nm (intensity daily assessed using a VLX-3W Radiometer) for 21 days at room temperature (20 ^{o}C), using a Uvitec LF-206.LS lamp inside an opaque box[1,2]. After the weathering process, chemical changes were assessed through Fourier-Transform Infrared Spectrometry with Attenuated Total Reflectance. The equipment was operated in the absorbance mode at wavenumbers between 400 and 4000 cm^{-1}, with 32 performed scans, a resolution of 4 cm^{-1}, and an interval of 2 cm^{-1}.

Results:

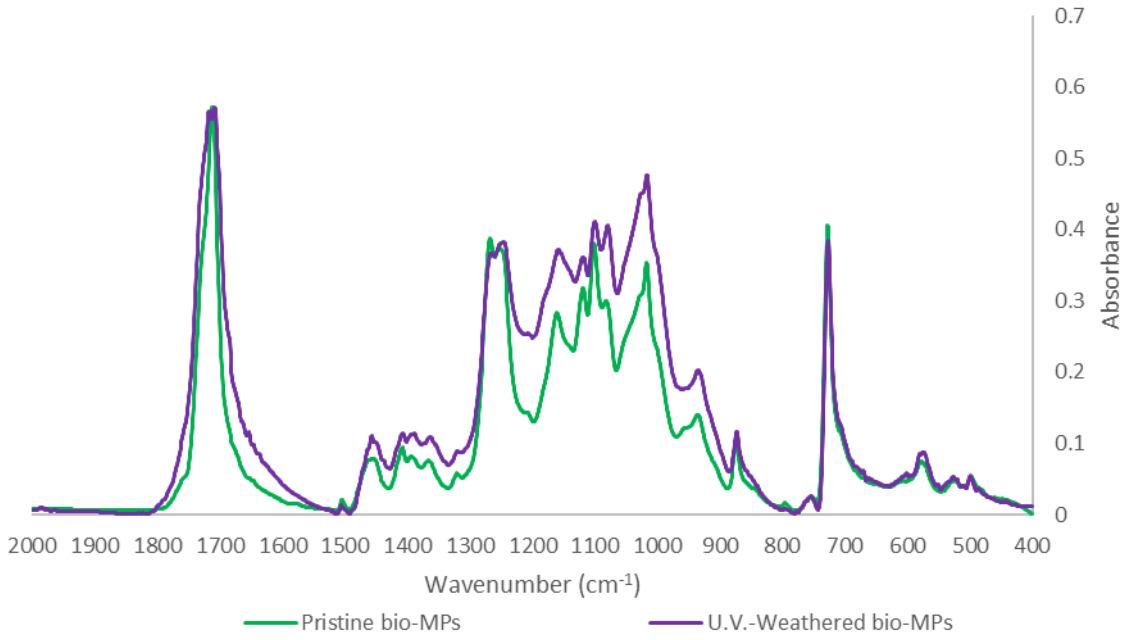

Figure S1 - Comparison between FTIR spectra of pristine and U.V.-weathered bio-MPs after 21 days of radiation exposure.

As evidenced in Figure S1, the U.V. degradation seems to induce changes in the FTIR spectra of the bio-MPs. These changes, including the widening and decrease in peak definition, could indicate the occurrence of changes to PBAT, one of the components of this polymer formulation. Scission and crosslinking events have been reported for PBAT-based materials after exposure to solar radiation, events which were attributed to high densities of ester linkages that make such materials prone to the absorption of high intensity (namely U.V.) radiation[3].

Section II: *Biomass variation throughout the experiment*

Fungal biomass variation (%) was calculated as follows:

Fungal growth = [(Biomass $_{FW}$ – Biomass $_{IW)}$ / Biomass $_{FW}$]

where " Biomass $_{FW}$ " refers to the amount of fungal biomass (g) at the end of an exposure period; " Biomass $_{IW}$ " is the amount of fungal biomass (g) at the beginning of the test.

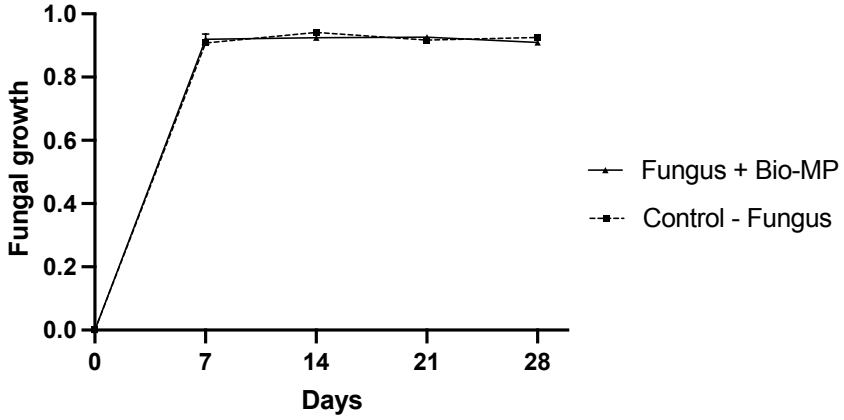

Figure S2 - Variation of *Penicillium brevicompactum* biomass throughout the experiment, in the presence and absence of bio-based mulch film microplastics (Bio-MPs). Data are presented as mean ± standard deviation.

Section II: *Methodology for bio-MP treatment for fungal mass removal*

Methods:

Before subjecting the bio-MPs present on each batch reactor to a digestion procedure to remove fungal mass attached to their surface, four methodologies were tested on pristine particles:

- 10% KOH, for 48 h at 50 ºC;
- Fenton reaction for 3 h at 50 ºC; followed by the addition of an equal volume of 30% H_2O_2 for more 24 h, at room temperature;
- 7% NaClO, for 48 h at 50 ºC;
- 30% H_2O_2 for 24h, at room temperature;

These methodologies successfully eliminated biological remains in previous studies[4-7]. To test each method, pre-weighted bio-MPs (approximately 0.0015 g, three replicates per treatment) were subjected to a total volume of 4 mL of each reagent for 24 to 48 h, depending on the procedure. Then, each sample was vacuum filtered onto pre-weighted glass microfibre filters (47 mm, 0.7 μm pore size, Watman®) and allowed to dry at room temperature for 1 week in glass Petri dishes (previously acid washed).

Bio-MP mass loss (%) was calculated as follows:

Bio-MP mass loss (%) = [(Bio-MP $_{IW}$ - Bio-MP $_{FW}$) / Bio-MP$_{IW}$] x 100

where " Bio-MP $_{IW}$" refers to the initial amount of bio-MP (mg); " Bio-MP $_{FW}$ " is the amount of bio-MP (mg) after the digestion period.

When dried, samples were photographed with a Canon EOS 200D DSLR Ds126671 camera, and the ones in which bio-MPs presented physical integrity (i.e., those exposed to H_2O_2 and Fenton, as per Figure S3) were further analysed by FTIR spectroscopy.

Results:

The appearance of the bio-MPs after each digestion methodology is presented in Figure S3; whereas the mass variation of bio-MPs is shown in Table S1.

Methodology

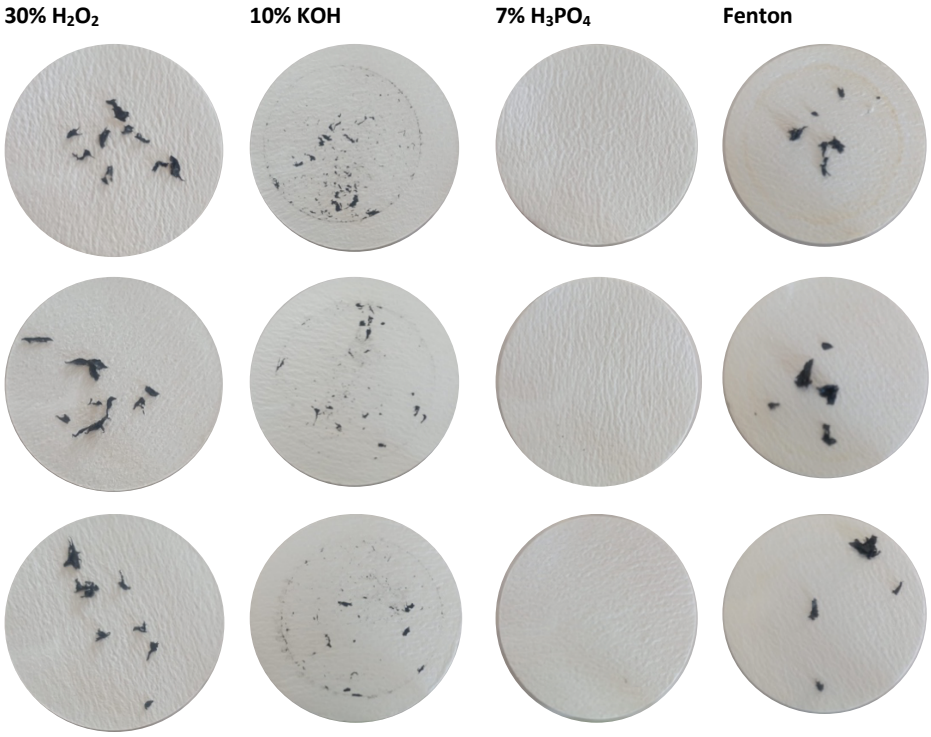

Figure S3 - Visual appearance of the bio-MPs after digestion with 30% H_2O_2, 10% KOH, 7% H_3PO_4, and Fenton.

Table S1 - Mass variation of pristine bio-MPs subjected to digestion procedures. *Replicate is not considered. Sample lost during weighing due to electrostatic energy.

Sample		**Mass (g)**		**Variation (g)**	
Procedure	**Replicate**	**Initial**	**Final**	**Initial-Final**	**Average**
30% H_2O_2	1	0.0015	0.0014	-0.0001	-0.0002 ± 0.0002
	2	0.0016	0.0012	-0.0004	
	3	0.0015	0.0015	0	
10% KOH	1	0.0016	0.0002	-0.0014	-0.0014 ± 0.0002
	2	0.0015	0.0003	-0.0012	
	3	0.0016	0	-0.0016	
7% H_3PO_4	1	0.0016	0.0001	-0.0015	-0.00160 ± 0.00007
	2	0.0017	0.0001	-0.0016	
	3	0.0017	*		
Fenton	1	0.0016	0.0014	-0.0002	-0.0001 ± 0.0001
	2	0.0015	0.0015	0	
	3	0.0017	0.0015	-0.0002	

As can be depicted in Figure S3 and Table S1, the digestion methodologies that applied 10% KOH and 7% H_3PO_4 resulted in a major bio-MP mass loss (> 85%), whereas the digestion with 30% H_2O_2 and Fenton resulted in a minor (~8-11%) bio-MP mass loss. For this reason, only bio-MPs that were subjected to 30% H_2O_2 and Fenton were further analysed by FTIR spectroscopy.

The FTIR spectra of bio-MPs in their pristine form and after the digestion procedure with 30% H_2O_2 and Fenton methodologies are presented in Figure S4.

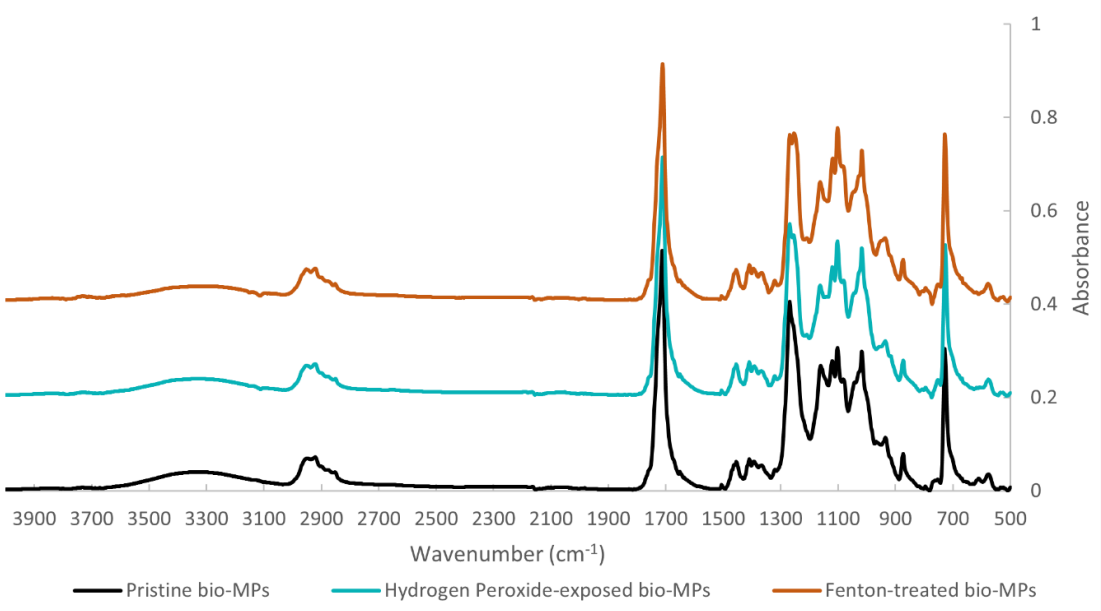

Figure S4 - Comparison between FTIR spectra of bio-MPs (both pristine and subjected to the 30% H_2O_2 and Fenton methodologies).

As per Figure S4, it was determined that safe for the disappearance of a peak at 608 cm^{-1}, which was common to both tested approaches, no further changes to the chemical structure of the bio-MPs were detected; that said, further assessments of chemical changes to the bio-MPs as part of the experiments will not consider this peak. Given the high efficacy of both in the removal of remaining organic matter and a similar effect on the bio-MP mass, it was determined that either approach would be equally adequate for application in the samples. Thus, 30%H_2O_2 was selected to remove the fungal biomass still attached to the bio-MPs.

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1. Doğan, M. Ultraviolet light accelerates the degradation of polyethylene plastics. *Microscroscopy Research and Techenique* **84**, 2774–2783 (2021). doi:10.1002/jemt.23838

2. Ferreira-Filipe, D. A.; Paço, A.; Natal-da-Luz, T.; Sousa, J. P.; Saraiva, J. A.; Duarte, A. C.; Rocha-Santos, T. & Patrício Silva, A. L. Are mulch biofilms used in agriculture an environmentally friendly solution? - An insight into their biodegradability and ecotoxicity using key organisms in soil ecosystems. *Science of The Total Environment* **828**, 154269 (2022). doi: 10.1016/j.scitotenv.2022.154269

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4. Monteiro, S.S., Rocha-Santos, T., Prata, J.C., Duarte, A.C., Girão, A.V., Lopes, P., Cristovão, T., Costa, J.P. A straightforward method for microplastic extraction from organic-rich freshwater samples. *Science of The Total Environment* **815**, 152941 (2022). doi: 10.1016/j.scitotenv.2022.152941

5. Prata, J. C., Costa, J.P. da, Duarte, A.C., Rocha-Santos, T. Methods for sampling and detection of microplastics in water and sediment: A critical review. *Trends in Analytical Chemistry* **110**, 150–159 (2019). doi: 10.1016/j.trac.2018.10.029

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7. Prata, J.C., Costa, J.P. da, Lopes, I., Duarte, A.C., Rocha-Santos, T. Effects of microplastics on microalgae populations: A critical review. *Science of The Total Environment* 665, 400–405 (2019). doi:10.1016/j.scitotenv.2019.02.132

Chapter V. The use of *Penicillium brevicompactum* and *Zalerion maritimum* for the biodegradation of plastics in environmentally relevant conditions, and ecotoxicity studies of polymers in different habitats - Facemasks: An insight into their abundance in wetlands, degradation, and potential ecotoxicity

Figure S1 - Facemasks collected throughout the three sampling actions.

Table S1 - Facemasks microplastics variation and removal during the experiment with *Penicillium brevicompactum* (MPs-microplastics; STD-Standard deviation from the Mean.).

Batch reactor treatment	Batch Reactor code	MPs Beginning (g)	MPs Recovered (g)	MPs Removed (Beginning-Recovered) (g)	% MPs Removed	% MPs Removed (Mean ± STD)
7 Days						
Microplastics + *P. brevicompactum*	R_1	0.0157	0.0099	0.0058	36.9	26 ± 7
	R_2	0.0149	0.0112	0.0037	24.8	
	R_3	0.0146	0.0113	0.0033	22.6	
	R_4	0.0153	0.0120	0.0033	21.6	
Microplastics	P_1C	0.0148	0.0140	0.0008	5.4	18 ± 11
	P_2C	0.0152	0.0116	0.0036	23.7	
	P_3C	0.0148	0.0111	0.0037	25.0	
14 Days						
Microplastics + *P. brevicompactum*	R_5	0.0149	0.0107	0.0042	28.2	26 ± 4
	R_6	0.0152	0.0105	0.0047	30.9	
	R_7	0.0150	0.0114	0.0036	24.0	
	R_8	0.0150	0.0117	0.0033	22.0	
Microplastics	P_4C	0.0148	0.0128	0.0020	13.5	13 ± 3
	P_5C	0.0147	0.0131	0.0016	10.9	
	P_6C	0.0150	0.0126	0.0024	16.0	
21 Days						
Microplastics + *P. brevicompactum*	R_9	0.0153	0.0124	0.0029	19.0	22 ± 2
	R_{10}	0.0160	0.0123	0.0037	23.1	
	R_{11}	0.0150	0.0115	0.0035	23.3	
	R_{12}	0.0149	0.0116	0.0033	22.1	
Microplastics	P_7C	0.0151	0.0150	0.0001	0.7	2 ± 3
	P_8C	0.0145	0.0145	0.0000	0.0	
	P_9C	0.0148	0.0141	0.0007	4.7	
28 Days						
Microplastics + *P. brevicompactum*	R_{13}	0.0146	0.0112	0.0034	23.3	24 ± 5
	R_{14}	0.0156	0.0106	0.0050	32.1	
	R_{15}	0.0153	0.0121	0.0032	20.9	
	R_{16}	0.0150	0.0118	0.0032	21.3	
Microplastics	$P_{10}C$	0.0151	0.0128	0.0023	15.2	21 ± 9
	$P_{11}C$	0.0156	0.0131	0.0025	16.0	
	$P_{12}C$	0.0148	0.0103	0.0045	30.4	

Batch reactor treatment	Batch Reactor code	MPs Beginning (g)	MPs Recovered (g)	MPs Removed (Beginning-Recovered) (g)	% MPs Removed	% MPs Removed (Mean ± STD)
7 Days						
Microplastics + *Z. maritimum*	R_1	0,0151	0,0087	0,0064	42,38	41 ± 13
	R_2	0,0152	0,0064	0,0088	57,89	
	R_3	0,0150	0,0100	0,0050	33,33	
	R_4	0,0151	0,0108	0,0043	28,48	
Microplastics	P_1C	0,0150	0,0103	0,0047	31,33	12 ± 16
	P_2C	0,0150	0,0148	0,0002	1,33	
	P_3C	0,0150	0,0145	0,0005	3,33	
14 Days						
Microplastics + *Z. maritimum*	R_5	0,0150	0,0081	0,0069	46,00	48 ± 10
	R_6	0,0150	0,0070	0,0080	53,33	
	R_7	0,0149	0,0096	0,0053	35,57	
	R_8	0,0150	0,0062	0,0088	58,67	
Microplastics	P_4C	0,0150	0,0143	0,0007	4,67	6 ± 1
	P_5C	0,0148	0,0140	0,0008	5,74	
	P_6C	0,0148	0,0138	0,0010	6,76	
21 Days						
Microplastics + *Z. maritimum*	R_9	0,0151	0,0088	0,0063	41,72	48 ± 4
	R_{10}	0,0150	0,0077	0,0073	48,67	
	R_{11}	0,0148	0,0076	0,0072	48,65	
	R_{12}	0,0151	0,0074	0,0077	50,99	
Microplastics	P_7C	0,0149	0,0128	0,0021	14,09	15 ± 8
	P_8C	0,0153	0,0118	0,0035	22,88	
	P_9C	0,0147	0,0136	0,0011	7,48	
28 Days						
Microplastics + *Z. maritimum*	R_{13}	0,0152	0,0079	0,0073	48,03	50 ± 6
	R_{14}	0,0149	0,0076	0,0073	48,99	
	R_{15}	0,0152	0,0062	0,0090	59,21	
	R_{16}	0,0152	0,0084	0,0068	44,74	
Microplastics	$P_{10}C$	0,0148	0,0146	0,0002	1,35	3 ± 2
	$P_{11}C$	0,0148	*	*	*	
	$P_{12}C$	0,0149	0,0143	0,0006	4,03	

Table S2 - Variation of fungi biomass throughout the experiment. Data are presented as mean ± standard deviation (* growth=(final weight- initial weight)/ final weight).

Test species	**Sampling day**	**Treatment**	**Inoculated biomass (FW, g)**	**Inoculated Biomass (DW, g)**	**Final Biomass (DW, g)**	**Variation of Biomass**	**Growth***
P. brevicompactum	7th	Fungus	0.53±0.03	0.053±0.003	0.09±0.01	0.03±0.02	0.38±0.09
		Fungus & MPs	0.52±0.02	0.052±0.002	0.064±0.006	0.012±0.006	0.18±0.04
	14th	Fungus	0.55±0.06	0.056±0.006	0.099±0.008	0.04±0.01	0.44±0.08
		Fungus & MPs	0.52±0.02	0.052±0.002	0.09±0.01	0.04±0.01	0.42±0.04
	21th	Fungus	0.51±0.05	0.052±0.005	0.09±0.02	0.04±0.03	0.4±0.1
		Fungus & MPs	0.6±0.2	0.06±0.02	0.087±0.008	0.03±0.01	0.309±0.008
	28th	Fungus	0.53±0.05	0.053±0.004	0.07±0.02	0.02±0.002	0.3±0.1
		Fungus & MPs	0.51±0.01	0.051±0.001	0.07±0.01	0.02±0.01	0.31±0.05
Z. maritimum	7th	Fungus	0.51 ± 0.02	0.062±0.002	0.04±0.02	-0.02±0.02	0
		Fungus & MPs	0.49 ± 0.02	0.058±0.003	0.06±0.02	0.00±0.02	0
	14th	Fungus	0.505±0.007	0.0604±0.0008	0.14±0.03	0.08±0.03	0.5 ± 0.1
		Fungus & MPs	0.55 ± 0.03	0.065±0.004	0.15±0.05	0.09±0.05	0.5 ± 0.1
	21th	Fungus	0.525±0.007	0.0628±0.0008	0.21±0.04	0.15±0.04	0.69 ± 0.06
		Fungus & MPs	0.52±0.08	0.06±0.01	0.19±0.03	0.12±0.04	0.6 ± 0.1
	28th	Fungus	0.52±0.00	0.06±0.00	0.17±0.04	0.11±0.04	0.62 ± 0.09
		Fungus & MPs	0.55±0.08	0.07±0.01	0.21±0.02	0.12±0.03	0.67 ± 0.09

Table S3 - Nominal vs testing concentrations of pristine and UV-aged microfibres in artificial salt water used for the ecotoxicity tests with *V. corrugata*.

	Nominal concentration (items/L)	**Testing concentration (items/L)**	**Mean testing concentration (items/L) ± SEM**
Pristine	50	56	54 ± 4
		59	
		46	
	250	258	273 ± 12
		264	
		297	
	1250	1767	1672 ± 154
		1371	
		1878	
UV-aged	50	59	53 ± 3
		50	
		52	
	250	360	378 ± 9
		390	
		384	
	1250	1788	1735 ± 33
		1674	
		1743	

Table S4 - Effects of face masks microfibres (MF) with or without UV-C treatment on *Venerupis corrugata* adults after 96h exposure, considering energy reserves (proteins, carbohydrates and lipids). Data are presented as mean ± standard error of the mean.

		Concentration (MF/L)	Mean ± SEM
Proteins (mg/mL)	Pristine	0	798 ± 88
		50	819 ± 208
		250	1178 ± 403
		1250	1083 ± 125
	UV	0	1239 ± 57
		50	1260 ± 52
		250	1110 ± 32
		1250	1120 ± 146
Lipids (mJ/mg organism)	Pristine	0	536 ± 37
		50	342 ± 59
		250	527 ± 49
		1250	405 ± 41
	UV	0	660 ± 104
		50	493 ± 16
		250	579 ± 52
		1250	650 ± 106
Carbohydrates (mJ/mg organism)	Pristine	0	159 ± 59
		50	191 ± 75
		250	338 ± 34
		1250	311 ± 34
	UV	0	533 ± 60
		50	438 ± 151
		250	763 ± 122
		1250	311 ± 36

Table S5 - Two-way ANOVA results testing for concentrations of face masks microplastics [MF/L], presence or absence of UV treatment (Ageing), and interaction between both on lipid peroxidation (LPO), catalase activity (CAT), glutathione-S-transferase activity (GST), proteins (Prot.), lipids (Lip.), carbohydrates (Carb.), aerobic energy production (ETS) and cellular energy allocation (CEA).

	ANOVA table	SS (Type III)	DF	MS	F (DFn, DFd)	P value
LPO	[MF/L]	555,1	3	185,0	$F_{(3, 25)} = 3,482$	0,0307
	Ageing	66,47	1	66,47	$F_{(1, 25)} = 1,251$	0,2740
	Interaction	217,8	3	72,61	$F_{(3, 25)} = 1,367$	0,2758
CAT	[MF/L]	45,70	3	15,23	$F_{(3, 25)} = 1,046$	0,3894
	Ageing	**80,19**	**1**	**80,19**	**$F_{(1, 25)} = 5,507$**	**0,0272**
	Interaction	37,58	3	12,53	$F_{(3, 25)} = 0,8604$	0,4745
GST	[MF/L]	3298	3	1099	$F_{(3, 26)} = 0,6493$	0,5905
	Ageing	312,7	1	312,7	$F_{(1, 26)} = 0,1847$	0,6709
	Interaction	5470	3	1823	$F_{(3, 26)} = 1,077$	0,3761
Prot.	[MF/L]	194199	3	64733	$F_{(3, 24)} = 0,4710$	0,7053
	Ageing	551672	1	551672	$F_{(1, 24)} = 5,833$	0,7053
	Interaction	658373	3	219458	$F_{(3, 24)} = 2,400$	0,0927
Lip.	[MF/L]	132405	3	44135	$F_{(3, 25)} = 2,884$	0,0557
	Ageing	162157	1	162157	$F_{(1, 25)} = 10,60$	0,0032
	Interaction	38580	3	12860	$F_{(3, 25)} = 0,8403$	0,4847
Carb.	[MF/L]	332475	3	110825	$F_{(3, 25)} = 4,212$	0,0153
	Ageing	541858	1	541858	$F_{(1, 25)} = 20,60$	0,0001
	Interaction	214661	3	71554	$F_{(3, 25)} = 2,720$	0,0659
ETS	[MF/L]	6,745	3	2,248	$F_{(3, 26)} = 1,480$	0,2432
	Ageing	1,049	1	1,049	$F_{(1, 26)} = 0,6907$	0,4135
	Interaction	2,650	3	0,8833	$F_{(3, 26)} = 0,5813$	0,6325
CEA	**[MF/L]**	**128263**	**3**	**42754**	**$F_{(3, 24)} = 3,973$**	**0,0197**
	Ageing	**219955**	**1**	**219955**	**$F_{(1, 24)} = 20,44$**	**0,0001**
	Interaction	90817	3	30272	$F_{(3, 24)} = 2,813$	0,0608

Table S6 - Tukey's multiple comparisons significant results for time effect on clearance capacity of *V. corrugata* for microplastics with (UV) or without (pristine) ageing treatment ($p < 0.05$).

	[MF/L]	Tukey's multiple comparisons tests	Mean diff	95.00% CI of diff	Adjusted P value
UV	**250**	15.0 vs. 45.0	0.06872	0.02060 to 0.1168	0.0149
		15.0 vs. 120.0	0.2052	0.1252 to 0.2853	0.0017
		45.0 vs. 120.0	0.1365	0.07429 to 0.1987	0.0030
Pristine	**0**	0.0 vs. 120.0	0.2462	0.06965 to 0.4227	0.0300
		15.0 vs. 120.0	0.1670	0.1143 to 0.2198	0.0007
		45.0 vs. 120.0	0.1548	0.1047 to 0.2049	0.0008
	50	45.0 vs. 120.0	0.1488	0.03004 to 0.2676	0.0271
	250	15.0 vs. 120.0	0.1978	0.1637 to 0.2319	0.0003
		45.0 vs. 120.0	0.1427	0.02791 to 0.2575	0.0277
	1250	15.0 vs. 120.0	0.1875	0.02106 to 0.3539	0.0338

Table S7 - Tukey's multiple comparisons significant results for concentration effect on clearance capacity of *V. corrugata* for microplastics with (UV) or without (pristine) ageing treatment ($p < 0.05$).

	Time	Turkey's multiple comparisons test	Mean diff	95.00% CI of diff	Adjusted P value
Pristine	**15.0**	0 vs. 250	0,07126	0,01114 to 0,1314	0,0231
		50 vs. 250	0,1051	0,05165 to 0,1586	0,0037
		250 vs. 1250	-0,09231	-0,1548 to -0,02980	0,0074
	120.0	0 vs. 250	0,1021	0,02198 to 0,1821	0,0242

Table S8 - Two-way ANOVA results testing for concentrations of face masks microplastics [MF/L], exposure time to algae (Time), and interaction between both on clearance capacity of *V. corrugata* exposed to microplastics with (UV) and without (pristine) ageing treatment.

	Fixed effects (type III)	P value	P value summary	Statistically significant (P < 0,05)?	F (DFn, DFd)	Geisser-Greenhouse's epsilon
UV	Time	<0,0001	****	Yes	$F_{(1.697,\ 15.28)}$ = 67,63	0,5658
	[MF/L]	0,8763	ns	No	$F_{(3, 16)}$ = 0,2269	
	Interaction	0,1391	ns	No	$F_{(9, 27)}$ = 1,695	
Pristine	Time	<0,0001	****	Yes	$F_{(1.550,\ 15.50)}$ = 102,7	0,5168
	[MF/L]	0,0152	*	Yes	$F_{(3, 16)}$ = 4,716	
	Interaction	0,1401	ns	No	$F_{(9, 30)}$ = 1,672	