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Are Biodegradable Biobased Plastics a Green Alternative?

Serão os Plásticos Biodegradáveis de Base Biológica uma Alternativa Verde?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia, realizada sob a orientação científica da Doutora Ana Luísa Patrício Silva, Investigadora Júnior do Departamento de Biologia e co-orientação científica da Doutora Teresa Rocha Santos, Investigadora Principal com Agregação do Departamento de Química, ambas do Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro.

À minha pedra de estimação pelo suporte firme.

o júri

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palavras-chave

Penicillium brevicompactum; Biodegradação; *Eisenia andrei*; Ecotoxicologia; Filmes de Adubo de Base Biológica; FTIR;

resumo

A sustentabilidade ambiental está a impulsionar uma intensa busca por "materiais verdes". Os plásticos de base biológica e biodegradáveis têm surgido como alternativas que visam reduzir a pressão ambiental causada pelos plásticos convencionais. Contudo, permanece duvidoso se estas alterativas são de facto amigas do ambiente, quando consideramos uma aplicação *in situ* sem requerer a sua remoção; como sejam os filmes aplicados em solos agrícolas.
Será que os plásticos de base biológica e biodegradáveis aplicados, por exemplo, na agricultura, sofrem biodegradação *in situ* como desejado? E durante esse processo, será que afetam negativamente a fauna local?
Para responder a estas questões, selecionou-se um biofilme biodegradável, certificado e comercialmente disponível, como objeto de estudo. Usando microplásticos deste biofilme, avaliou-se a sua biodegradação e ecotoxicidade em solos agrícolas. Para este efeito, os testes de biodegradação decorreram na presença do fungo *Penicillium brevicompactum* e os testes de ecotoxicidade com a minhoca *Eisenia andrei*, sendo ambos organismos-chave (relevância ecológica e elevada biomassa) nestes solos.
Nos ensaios de biodegradação em solo, o fungo *P. brevicompactum* interagiu com o biofilme, embora não tenha resultado numa evidente perda de massa. Contudo, a análise FTIR-ATR sugere afinidade entre o fungo e o plástico, com variações nas reservas de carbohidratos no fungo e pelo aumento de esteres de baixo peso molecular no biofilme que sugere degradação.
Nos testes de ecotoxicidade, que envolveram com microplásticos de biofilme na sua forma pristina ou envelhecida à luz ultravioleta, os resultados foram díspares. A presença de microplásticos pristinos não afetou sobrevivência de *E. andrei*, mas induziu um decréscimo significativo (de 28% a 44%) no número de juvenis. Já os microplásticos envelhecidos sob luz ultravioleta não afetaram a sobrevivência nem a reprodução. A análise dos espectros de FTIR-ATR nos microplásticos envelhecidos indica ligeiras alterações químicas que poderão ser responsáveis pela perda de toxicidade, enquanto a análise dos espectros de FTIR nos organismos adultos expostos a microplásticos pristinos sugerem alterações fisiológicas a nível das reservas energéticas, com défices aparentes em carbohidratos, proteínas e lípidos.
Os testes de biodegradação e ecotoxicidade utilizados na certificação de plásticos de base biológica e/ou biodegradáveis ainda carecem de relevância ecológica, uma vez que decorrem em condições muito distintas aos encontrados no ambiente natural onde se vão aplicar (ex: elevada temperatura, tipos de microorganismos). De igual forma, os testes de ecotoxicidade aplicados visam avaliar preferencialmente os efeitos agudos (ex: sobrevivência), negligenciando os seus efeitos crónicos (reprodução). Este trabalho traz, assim, novas evidências quanto à sua biodegradação e ecotoxicidade em solos agrícolas, considerando organismos ecologicamente relevantes. Ainda assim, embora os testes de biodegradação com o fungo requeiram estudos adicionais, os testes de ecotoxicidade indicam que, em cenários ambientalmente relevantes, os microplásticos de biofilme apresentam ausência de toxicidade aguda e crónica para *E. andrei*.

keywords

Penicillium brevicompactum; Biodegradation; *Eisenia andrei*; Ecotoxicology; Biobased Mulch Film; FTIR;

abstract

Environmental sustainability is driving an intense search for “green materials”. Bio-based and biodegradable plastics have emerged as alternatives that aim to reduce the environmental pressure caused by conventional plastics. However, it remains doubtful whether these alternatives are environmentally friendly when we consider an *in situ* application without requiring their removal, such as mulch films applied in crops. Do these materials safely biodegrade in situ as expected? In the meantime, do they negatively impact the local fauna?

A certified and commercially available biodegradable biofilm was selected as the object of study to answer these questions. Using microplastics from this biofilm, its potential biodegradation and ecotoxicity in agricultural soils were evaluated. For this purpose, the biodegradation tests occurred in the presence of the fungus *Penicillium brevicompactum*, and the ecotoxicity tests were performed with the earthworm *Eisenia andrei*, both key organisms (i.e., high ecological relevance and biomass) in these environments.

In the biodegradation experiments in soil, the fungus *P. brevicompactum* interacted with the biofilm, although not so evident on its mass loss. Notwithstanding, the FTIR-ATR analysis suggests affinity between the fungi and the plastic material, with changes in fungi carbohydrate contents, and an apparent increase in low molecular weight esters in microplastics exposed to fungal material suggesting biodegradation.

In the ecotoxicity tests, which involved pristine or UV aged microplastics, the results were distinct. The presence of pristine microplastics did not affect *E. andrei* survival but induced a significant decrease (from 28% to 44%) in the number of juveniles. Aged microplastics did not affect survival or reproduction. The analysis of FTIR-ATR spectra (prior bioassays) from weathered microplastics revealed some chemical changes that might be responsible for the lack of toxicity; whereas the FTIR-ATR spectra from earthworms exposed to pristine microplastics suggests a decline of energy reserves, reflected by a decrease in carbohydrates, lipids and proteins.

The biodegradation and ecotoxicity tests required for the certification of bio-based and/or biodegradable plastics still lack ecological relevance since they occur under very different conditions than those found in the natural environment where they will be applied (e.g., high temperature, types of microorganisms). Similarly, the ecotoxicity tests applied aim at assessing preferentially acute effects (e.g., survival), neglecting their chronic effects (reproduction). This project brings new evidence regarding their biodegradation and ecotoxicity in agricultural soils considering ecologically relevant species. Although biodegradation tests with the fungus require further studies, ecotoxicity tests indicate that biofilm microplastics in environmentally relevant scenarios show an absence of acute and chronic toxicity for *E. andrei*.

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List of Abbreviations

In Alphabetical Order

ANOVA – Analysis of variance
ATBC – Acetyl tributyl citrate
Bio-PA11 – Bio-Polyamide
Bio-PEF – Bio-Polyethylene Furanoate
Bio-PET – Bio-Polyethylene Terephthalate
Bio-PE – Bio-Polyethylene
Bio-PP – Bio-Propylene
Ct – Control
EVOH – Ethylene Vinyl Alcohol
FTIR-ATR – Fourier-Transform Infrared Spectroscopy Attenuated Total Reflection
GHG – Greenhouse Gases
L,M,H – Low, Medium, High Plastic Concentrations, respectively
MPs – Microplastics
Mt – Million Tons
PBAT – Polybutylene Adipate Terephthalate
PBS – Polybutylene Succinate
PEF – Polyethylene furanoate
PEG – Polyethylene glycol
PET – Polyethylene Terephthalate
PET – Polyethylene Terephthalate
PHA – Polyhydroxyalkanoate
PHB – Polyhydroxybutyrate
PLA – Polylactic Acid
PTT – Polytrimethylene Therephthalate
TPS – Thermoplastic Starch
UV (C) – Ultra-Violet [radiation] (Type C)
WWTP – Wastewater Treatment Plants

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Context, Aims and Outline for this Thesis

Bioplastics emerged as a Circular Economy-compatible solution for humanity's plastic woes. Bioplastic manufacturers race to gain an edge over traditional producers by touting their increased sustainability in an increasingly environmentally aware world, and these green plastics are indeed, steadily gaining recognition [1]. The European Union is perhaps the major economic power openly showing more interest in these new technologies – documents such as the Roadmap for a Strategy on a Circular Economy leave that fact clear [2]. Notwithstanding, bioplastics, which in their variety include petrochemical and biobased plastics, biodegradable or not, as per **Figure 0.1**, can still be grossly misused in a way that threatens to undo any significant progress their implementation might bring.

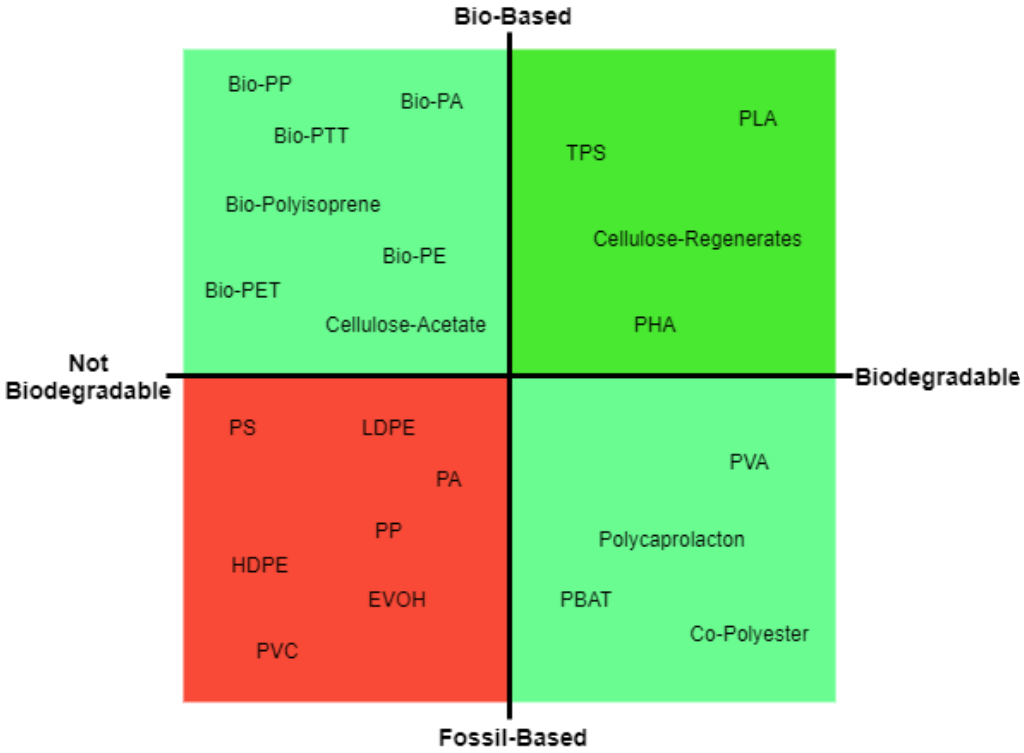

Figure 0.1. Material coordinate system of plastics, with the green quadrants denoting what are considered bioplastics. EVOH: Ethylene Vinyl Alcohol; PBAT: Polybuthylene Adipate Terephthalate; Polyhydroxyalkanoate; PHB: Polyhydroxybutyrate; PLA: Polylactic Acid; PTT – Polytrimethylene Therephthalate; TPS: Thermoplastic Starch; modified from Rujnić-Sokele, M., & Pilipović, A., 2017 [3].

Biobased plastics, which, as the name indicates, are plastics derived from renewable sources. Biodegradable or not, they have the key advantage of being able to be produced without the utilization of polluting and unrenewable fossil resources. At a time where the necessity to achieve carbon neutrality is more and more present in the social consciousness [1], the utilization of renewable resources, and later their reuse and repurposing and in a circular economy framework provides a powerful solution to the carbon economy's current unsustainability, with the ability of dramatically reducing carbon footprints of previously heavy polluting industries, precisely such as the plastics economy [4]. In order to maximize

these aspects, however, an effort must be made to install a competent waste management infrastructure capable, in order to maximize the reuse and repurposing of the biobased materials, minimizing production demands, which are both a technical and social challenge, after the relative recency and the renewable feedstock demands they impose on already strained markets such as the food sector, thus minimizing material waste and possible avenues of pollution [5,6].

Biodegradable plastics, on the other hand, have also been touted as a possible mitigator of plastic pollution effects. However, their biodegradability and environmental friendliness has come under the spotlight [7]. Plastic is considered biodegradable if it meets the requirements of international standards (e.g. EN ISO 14851:2019, ISO 18830:2016, ASTM D5988-12 [8,9,10]), but the reliability of the results when considering real environmental scenarios is very limited, as the evaluation is mostly based on respirometry measurements (i.e. CO_2 production), and tends to occur under unrealistic testing conditions (e.g. liquid/solid culture media, under controlled conditions or in anaerobic digesting sludges) [11]. Meanwhile, plastic biodegradation is deeply tied to environmental conditions that, if not met, may result in high environmental persistence, the same seen with other traditional polymers [7].

On the other hand, and even though in this sense they still appear to be superior to traditional petrochemical plastics, bioplastics', and especially biodegradable plastics' environmentally-friendliness remains somewhat of a murky subject. The biodegradation process of a material is not a static quality, instead depending on a plethora of environmental factors, and it is a difficult process to accurately characterize, given the limitations and biases that are tied with laboratorial and test trial conditions [12]; same applies when it comes to their toxicological properties as they might be designed for *in situ* degradation. These limitations are further amplified by the relative recency of the technology, which inevitably results in a variety of blind spots in the state of the art and knowledge around this subject.

As such, it is of special concern that biodegradable plastics, a group whose classification that has the possibility of being based on flimsy criteria, are predicted to be directly applied, for the most part (70% of production volume) in "short-life" applications, such as packaging and agricultural mulch films [5]. While specialists themselves may have limited knowledge about its ramifications, most of these materials will be placed in the hands of general consumers, who cannot be expected to share the same knowledge on the state of the art, and rather will be misinformed by the "green" packaging and a biodegradability certification [13]. As such, it becomes vital to study these dynamics more deeply, and in increasingly environmentally relevant conditions.

It is in this context that this project is integrated, aiming at studying the biodegradability and potential toxicity of biodegradable biobased plastics. For this purpose, it was selected an agricultural mulch biofilm as a case study (i.e., a certified and commercially available biodegradable biobased plastic) along with two key-organisms present in agroecosystems.

This thesis is thus organized in four main chapters:

Chapter I – Are Biobased Plastics Green Alternatives? – a critical review.

This chapter is composed of a critical overview on the recent advances in biobased polymers chemistry, emerging (bio)technologies that underpin their production, and discusses the potential for biodegradation, recycling, environmental safety and toxicity of these biobased solutions. This critical overview is published in the *International Journal of Environmental Research and Public Health* (https://doi.org/10.3390/ijerph18157729)

Chapter II – Biodegradation of an agricultural mulch biofilm by the fungi, *Penicillium brevicompactum*

This chapter addressed the biodegradation of the agricultural biofilm by a naturally occurring fungus in agricultural soils – *P. brevicompactum*, both in solid culture medium and in soil substrate. Data obtained will bring new insights on the biodegradation of biobased mulch plastics by this ecologically relevant species, while considering more realistic scenarios (in this case, soil itself).

Chapter III – Ecotoxicological of an agricultural mulch biofilm on the earthworm *Eisenia andrei*

This chapter assessed the ecotoxicity of the mulch biofilm, weathered under UV-C radiation or not, in a key-species in agricultural soils - the earthworm *E. andrei*. Survival (the most evaluated endpoint as per the international standards), microplastic ingestion/egestion, effects on homeostasis of adults, and reproduction (number of juveniles) were evaluated. Knowledge on this topic brought new insights on the chronic and molecular effects of this plastic in a key species in agroecosystems.

Chapter IV – Final Remarks and Future Research Perspectives

This final chapter provides a general discussion on the main findings and obstacles of this research, while addressing research needed to overcome them, as well as commenting on future research possibilities that arise from the implications of this work's results, conclusions, and uncertainties.

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Chapter I

1. Are Biobased Plastics Green Alternatives? A Critical Review

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1.1. Abstract

Environmental sustainability is driving an intense search for “green materials”. Biobased plastics have emerged as a promising alternative. Their building blocks can now be obtained from diverse biomass, by-products, and organic residues due to the advances in biorefineries and bioprocessing technologies, decreasing the demand for fossil fuel resources and carbon footprint. Novel biobased polymers with high added value and improved properties and functionalities have been developed to apply diverse economic sectors. However, the real opportunities and risks of such novel biobased plastic solutions have raised scientific and public awareness. This paper provides a critical review on the recent advances in biobased polymers chemistry and emerging (bio)technologies that underpin their production and discusses the potential for biodegradation, recycling, environmental safety, and toxicity of these biobased solutions.

1.2. Keywords

plastic pollution; bioplastics; circular economy; biodegradation; sustainability

1.3. Introduction

Since the introduction of plastics into the markets, their role in the world economy has grown immensely, now being omnipresent in several sectors, including construction, agriculture, medicine, and many others [1]. Diversity, malleability, durability, and a high degree of personalization are among plastics’ best qualities, leading the dependence upon these materials to naturally increase throughout the last century. This preference, together with the growth in population during this period, has led to massive production of these materials, resulting in equally huge waste generation and greenhouse gas emissions (GHG) [2,3]. In 2019, plastics production accounted for 10% of the global fossil feedstocks and reached a global production of approximately 370 million tons (Mt) [4,5]. A global generation of 150 Mt of post-consumer plastic waste and an emission of 390 Mt of CO_2 were estimated in a World Economic Forum report for the year 2012 alone, and it should be noted that since then, plastic production has steadily increased [6]. If plastic usage continues at such a rate, plastics are expected to account for 20% of total fossil oil consumption and 15% of the total

carbon budget, compared to approximately 1% at the time of writing that report. These numbers can be aggravated if we consider pandemic scenarios without implementing sustainable solutions [7].

Waste management infrastructures are still failing to cope with the waste generated from the continuous production and consumption of plastics, contributing to intensive loads of plastic waste ending up improperly managed [4]. Ideally, the plastics economy should be circularized to reduce plastic pollution worldwide; however, a significant share of plastic waste (around 79%) end up in landfills or improperly discarded in natural environments [1,8]. There, they can persist for hundreds to thousands of years, threatening animal and human health and affecting the balance of ecosystems [9,10].

To solve these shortcomings and reduce the plastic economy's strain in the areas of environmental pollution and climate change, the modern plastics economy must be converted into a sustainable, circular framework [11]. Such a transition was prioritized by the United Nations in their 2030 Agenda for Sustainable Development, with goals such as 11 to 14 highlighting the need for the widespread implementation of measures to increase balance and sustainability in resource exploration and waste generation, and the importance of said measures for both environmental issues, such as ecosystem pollution and climate change, and societal issues, such as social cohesion and precarity, which can draw heavily from the former [12]. Several advances have been made, for example, in plastics recycling, with new technologies increasing the amount of plastic types that can be reconverted. Still, perhaps the most promising of these advances are biobased plastics [13,14]. However, focusing only on the fact that this next generation of “green” plastics can be produced free from fossil fuel intervention might be mistaking the forest for the trees, perhaps conveniently ignoring (in a purely market-oriented perspective) the issues of plastic recycling and reconversion, which are vital for the circularization of the plastics market, as well as those of environmental friendliness, to promote the marketable idea that these “green” polymers are the solution to humanity’s plastics woes [15].

This critical review is focused on the recent advances in biobased polymers chemistry and emerging (bio)technologies that underpin their production, addressing their opportunities and challenges when envisioning a sustainable and circular economy. It also discusses the potential biodegradation, environmental safety, and toxicity of these biobased solutions.

1.4. Plastic pollution: a social, economic, and environmental problem

Since the 1950s, the volume of produced plastics has increased dramatically, from 2 Mt per year to 370 Mt in 2019, an over 190-fold increase that dwarfs the roughly tripling of the human population in the same timeframe [1,16]. Meanwhile, only 600 Mt of all the estimated virgin plastics produced ended up being recycled, with the vast majority being landfilled instead [1]. Plastic waste processing infrastructures worldwide have, therefore, proven incapable of adequately dealing with the sheer amount of incoming residual plastics, courtesy of today's largely linear plastics economy, which emphasizes continuous production of new plastic over reconversion of used materials. On the other hand, even if the infrastructure in place could deal with the entirety of the incoming waste volume, the ability to recycle the plastics would be limited by the available methods of sorting and recyclability, which limit yields and, consequently, economic attractiveness. For instance,

the different melting and glass transition temperatures of biobased PLA (Polylactic Acid) and fuel-based PET (Polyethylene Terephthalate) can interfere with drying and processing steps, resulting in lower-quality recycled PET [17,18]. Considering the economic point of view, it is also essential to keep in mind that failure to recycle plastics costs EUR 105 billion in the EU alone [19]. As such, this is a problem with multiple fronts beyond just the scientific, with economic, political, and social factors that must be dealt with to curb plastic pollution and the contamination of the ecosystems and food stocks, helping to minimize financial losses while at it.

In addition, it is vital to encompass other regions' socioeconomic contexts to minimize plastic pollution and leakage. The EU is one of the richest areas of the world, but pales, population-wise, compared to the current developing regions, such as Brazil (over 212 million) and India (over 1380 million), put together [20]. In addition, whereas European citizens might be more economically comfortable and aware of plastics' environmental footprint, developing regions are busy playing catch-up socioeconomically and thus less capable of implementing the sweeping reforms and infrastructure needed to deal with a tremendous waste output, especially when considering the lack of immediate economic benefits [21]. The Brazilian government's position on Amazon development is a prime example of promoting economic opportunity near a vital ecosystem, with possible disastrous ecological consequences [22]. Rapid populational growth and a focus on exploration and economic development, combined with severe waste processing shortcomings, turn communities such as Manaus, population 2.2 million, in the Middle Amazon Basin, into waste generation behemoths; the result is a (conservatively) estimated 180,000 Mt of plastic wastes discarded into Amazonian environments yearly. Effects of this waste mismanagement might already be popping up downstream, with reports of fish, sea anemones, and stingrays being affected by plastic debris, the former in the Amazon River Estuary and the latter two from the Amazon Coast [23–25]. India also has quite the predicament, with estimates ranging between 4.8 and 12.7 Mt of discarded plastic entering the ocean yearly; this environmental situation is not helped by the fact India is crossed by heavily polluted rivers from other Asian countries, and that the Indian Ocean is also bordered by 10 of the 20 biggest plastic polluting nations worldwide [26]. Additionally, despite a growing interest in the long-lasting effects of environmental plastic pollution, the country's waste management and regulation situation is expected to remain dire, thanks to high levels of single-use plastic consumption, ineffective legislation, insufficient infrastructure, and the low prioritization of this problem. Slowly, legislation is being enacted to reduce this problem, but great challenges remain for India in this regard.

Plastic pollution of the environment entails a wide range of negative consequences to animal and human health (Figure 1) [10]. For instance, due to their hydrophobic surface and longer half-life than most natural substrates, plastics in the environment slowly start being colonized by a diverse microbial community of heterotrophs, autotrophs, predators, pathogens, and symbionts, constituting the "Plastisphere" [27]. Such plastics and plastisphere can, therefore, promote the distribution of potentially non-native/allochthonous organisms/pathogens to other environments. In addition, plastic waste accumulation in soil systems can create a conducive environment for biological disease vectors [28] and affect water percolation and normal soils aeration, with repercussions on land productivity, as reviewed by Alabi et al. [29]. In addition, organisms can interact with plastic wastes: more than 260 different species of vertebrate and invertebrate animals were reported to have

ingested plastics or have gotten entangled by plastic or plastic products, resulting in more than 400,000 deaths. Additionally, ingestion of plastic wastes/debris by animals often induces physiological effects such as perforation of digestive tracts, false satiation, and obstipation [30,31].

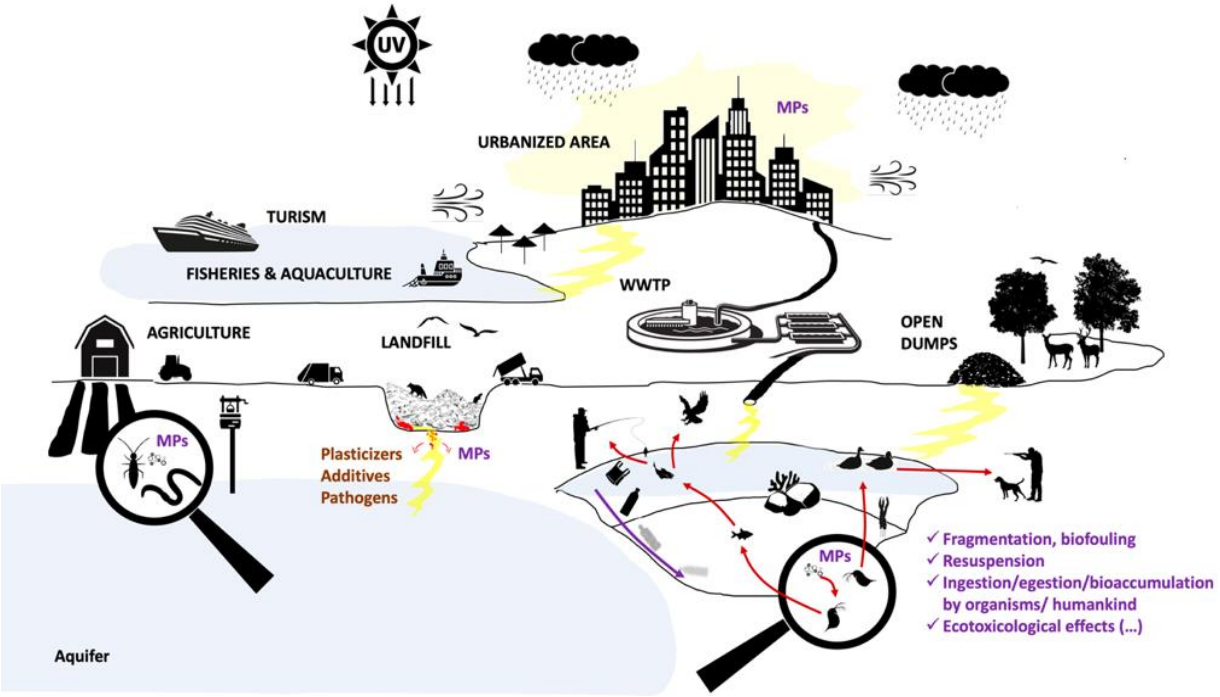

Figure 1.1. Schematic representation of sources, fate, and effects of plastic pollution on environmental and human health. MPs—Microplastics; UV- Ultraviolet (radiation); WWTP—Wastewater Treatment Plants

Regardless of initial dimensions, plastic debris can suffer degradation to various degrees in natural environments, slowly becoming smaller (from micro- to nanosized) and bioavailable to small-sized organisms [32]. This problem is amplified by the fact that plastics debris does not resist natural transport when in the environment, in other words meeting no borders. Plastic debris has been found in remote or guarded environments such as human-protected sanctuaries, such as the Pelagos Sanctuary in the Mediterranean Sea surrounding Corsica (France), or Gray's Reef, off the coast of Georgia, USA, and Trindade, an island part of a remote Brazilian archipelago in the Atlantic, courtesy of economic and touristic activities for the former two, and the South Atlantic Gyre for the latter [33–35]. The ease of migration of this debris can pose an urgent threat to the health of watched and endangered species and, consequently, to the health of their ecosystems as a whole.

The effect of microplastics and nanoplastics (microplastics: 1 µm–5 mm in size; nanoplastics: <1 µm in size, with colloidal behavior [36]) on organisms and human health remain largely unknown; notwithstanding, studies conducted in controlled conditions on various organisms, including human and other animal cells, point to harmful effects when these are exposed to concentrations higher than reported in the field, thus exposing the potentially detrimental effect of these materials [37]. For example, both in vertebrates and invertebrates (with different feeding guilds), microplastics were found to affect feeding patterns, and therefore energy availability at best, or to trigger more severe symptoms in

worse case scenarios—these can include severe inflammations and the triggering of stress pathways, endocrine disruptions, reduction in reproductive performance or even death events [22,38–40].

Given the tendency of persistence of these particles in organisms' guts or other organs, bioaccumulation can also result in the effective poisoning of entire food webs, on which many human populations also rely. Humans are exposed to microplastics through various media, but their potential toxic effects still remain largely uncovered [41], although these materials seem to be able to trigger a range of inflammatory and cytotoxic events in human cells [42].

The risks plastic and microplastic ingestion pose for the ecosystem, and public health is even broader, however. Although plastic debris is considered biochemically inert, plastic additives are incorporated during manufacturing processes to improve plastics properties [43]. Furthermore, plastic debris can also act as a vector for other harmful chemical compounds such as heavy metals and biological pathogens, such as *Vibrio cholerae* and harmful algal bloom-generating organisms [44]. Plastic additives and/or absorbed contaminants can then leach out and eventually percolate into various environmental compartments, decreasing soil and water quality and inducing adverse chemical effects (summing up to the physical effects) on terrestrial and aquatic biota at different levels of biological organization [45].

Thus, the increase of plastic matter in ecosystems, the resulting incomplete and unsafe degradation into small-sized particles such as microplastics, their spread in the environments, and the resulting increased bioavailability to wide food webs become a severe health risk for chronically neglected ecosystems and public health.

1.5. Biobased plastics and circular bioeconomy - the road ahead

Despite the various benefits plastics have in society, problems with plastic pollution (originating in waste or not) are some of the biggest challenges of our time. Once in the environment, plastic debris is somewhat difficult to recover. Research indicates that the best strategies for recovery consist in focusing on coastal areas, but in the EU alone, one of the regions in the world with the highest share of recycled plastic, those efforts can cost an estimated yearly EUR 630 million—a sum that will not turn a profit or reduce future economic damage, making it more challenging to approve and raise funding for these initiatives [19,46]. Throughout the last decades, plastics have become not only commonplace but entirely essential to a wide diversity of economic sectors, to the point that a carpet ban on these materials for the sake of the environment just is not feasible. Thus, one of the most valuable solutions to mitigate plastic litter inputs while restoring natural environments is by source-reduction and effective waste management to engage a more circular plastics economy. Beyond new waste processing methods, which are arguably not enough to sustain the ever-growing demand for these materials and the resulting influx of waste, the production of fossil fuel-independent plastics is also being touted as one of the key solutions in the plastics market reconversion that needs to occur in the coming years or decades [47].

Biobased plastics, as they have been dubbed, can be obtained from different renewable resources (e.g., plant-, algae-, residues-based) and, according to cradle-to-grave life cycle assessments, they seem to be generally advantageous in terms of saving fossil resources and reducing GHG emissions, as reviewed by Hatti-Kaul et al. [48]. As an example,

significant savings of fossil fuel (40–50%) and GHG emissions (45–55%) have been reported for PEF (polyethylene furanoate) production when compared to PET (polyethylene terephthalate) [49]. Despite their apparent environmental attractiveness, biobased plastics currently account for merely 1% of the overall plastics market, or 3.8 Mt, although significant gains are expected in coming years [50]. These new materials must play catch-up against a well-established industry with over half a century of research, development and dominating market presence to its name—conventional petrochemical plastics have been continuously refined over the years to achieve the ideal properties for a range of different uses. Meanwhile, biobased plastics sometimes fall short when it comes to physical and chemical properties, highlighting the need for further research and funding and again hurting their short-term viability.

Notwithstanding, these new materials boast more attractive properties than the traditional alternatives. Still, considering the example of PEF, this polymer offers better performances reported for qualities such as permeability to oxygen and carbon dioxide than its fossil-based counterpart/competitor, PET [51]. Still, if a wider substitution of petrochemical plastics by biobased alternatives is to be achieved, biobased polymers with properties on par with other types of plastics must be developed. To that end, legislative and regulatory action is needed to boost the attractiveness of these emerging markets, thus incentivizing research and investment, which are often bottlenecks in biotechnological industries, especially in biobased instances such as this one [52].

Doing so will allow for better characterization and streamlining of production and end-of-life processes for these emerging biobased alternatives, such as those presented in **Table 1.1**, thus easing their entry into the broader markets.

Table 1.1. Production, usage, and end-of-life options for commercially available (or soon to be available) biobased polymers. [101]

Polymer	Synthesis	Market Application	End-of-Life/ Biodegradability	References
Agrobiofilm®	Formulated using a starch base complemented with renewable raw materials from vegetable oils.	Used as additives in horticultural and perennial soils	Biodegradable and intended for in situ degradation	[53]
Bio-PA11	Synthesized using 11-aminoundecanoic acid from castor oil	Automotive and fuel tubings, electrical components, coatings	Non-Biodegradable, Chemical Recycling and Mechanical Recycling	[54–56]
Bio-PE	Dehydration of bioethanol from glucose	Food Packaging, Automotive applications, toy production, cosmetics and other industrial and agricultural applications	Non-biodegradable, mechanical recycling	[56,57]
Bio-PEF	Derived from 2,5-furandicarboxylic acid, which can be generated entirely from sugars such as cellulose	Being developed as a competitor to PET, mostly for packaging applications	Non-biodegradable, enzymatic depolymerization	[51,56,58]
Bio-PET	Synthesized using bio-ethylene glycol or bio-	Fibres and a variety of packaging applications	Non-biodegradable,	[56,57,59]

	terephthalic acid from Glucose and Fructose		chemical recycling, mechanical recycling and enzymatic depolymerization	
Bio-PP	Butylene dehydration of bio-isobutanol from glucose	Not yet industrially produced, confidential pilot plant phase	Non-biodegradable, mechanical recycling	[56,57]
PBS	Produced with succinic acid derived from biomass	A variety of packaging applications, including food packaging, as well as agricultural mulch films	Biodegradable, chemical recycling and enzymatic depolymerization	[56,60,61]
PHA and PHB	Bioproduction within micro-algae, bacteria and archaea	Various packaging, agricultural and medical applications	Biodegradable, home and industrial composting, anaerobic digestion and chemical recycling	[56,62,63]
PLA	Derived from microbial-produced lactic acid	Food packaging, electronic components, and 3D printing materials	Biodegradable, mechanical recycling, chemical recycling, and industrial composting	[56,64,65]
Chitosan	Derived from exoskeletons of crustaceans, insects, cell walls of fungi and yeast.	Various packaging, agricultural and medical applications	Biodegradable, anaerobic digestion and chemical recycling	[66]

Bio-PA11—Bio-Polyamide; 1Bio-PE—Bio-Polyethylene; Bio-PEF—Bio-Polyethylene Furanoate; PET—Polyethylene Terephthalate; Bio-PET—Bio-Polyethylene Terephthalate; Bio-PP—Bio-Propylene; PBS—Polybutylene Succinate; PHA/B—Polyhydroxyalkanoate/Polyhydroxybutirate; PLA—Polylactic Acid.

Slowly but surely, governments are realizing the vital importance of the reconversion of the plastics economy away from fossil fuel exploration. In 2016, the French government published a decree on energy transition and green growth mandating the use of bioplastics in certain packaging applications, specifically biobased and home composting polymers [67]; the European Union, despite as of yet lacking specific legislation comprehensively regulating biobased, biodegradable, and compostable plastics, introduced in its European Green Deal and Circular Economy Action Plan (2019 and 2020, respectively) a policy framework regarding the main issues of sourcing, labelling and uses of these materials [68]; the United Kingdom, on the other hand, claims commitment to the tackling of plastic pollution, but still raises pertinent concerns with production and waste management, and highlights the need for more research to explore this issue further [69]. However, the legislation seems limited, and these deliberations seem to be only the exception to the rule [70]. Should these initiatives succeed and be adopted by more and more authorities, however, the biobased plastic sector can expect an increasingly favorable regulatory

situation compared with traditional plastics' going forward, adding the factor of economic attractiveness to the ecological perspective.

1.6. Biobased plastics: environmentally friendly or possible foe?

Biobased plastics are touted as solutions to the environmental problems caused by conventional plastics production and waste (mis)management. However, they might come with a handful of environmental downsides of their own, and ignoring them can hinder the potential they have to curb the plastics economy's large environmental footprint (**Figure 1.2**).

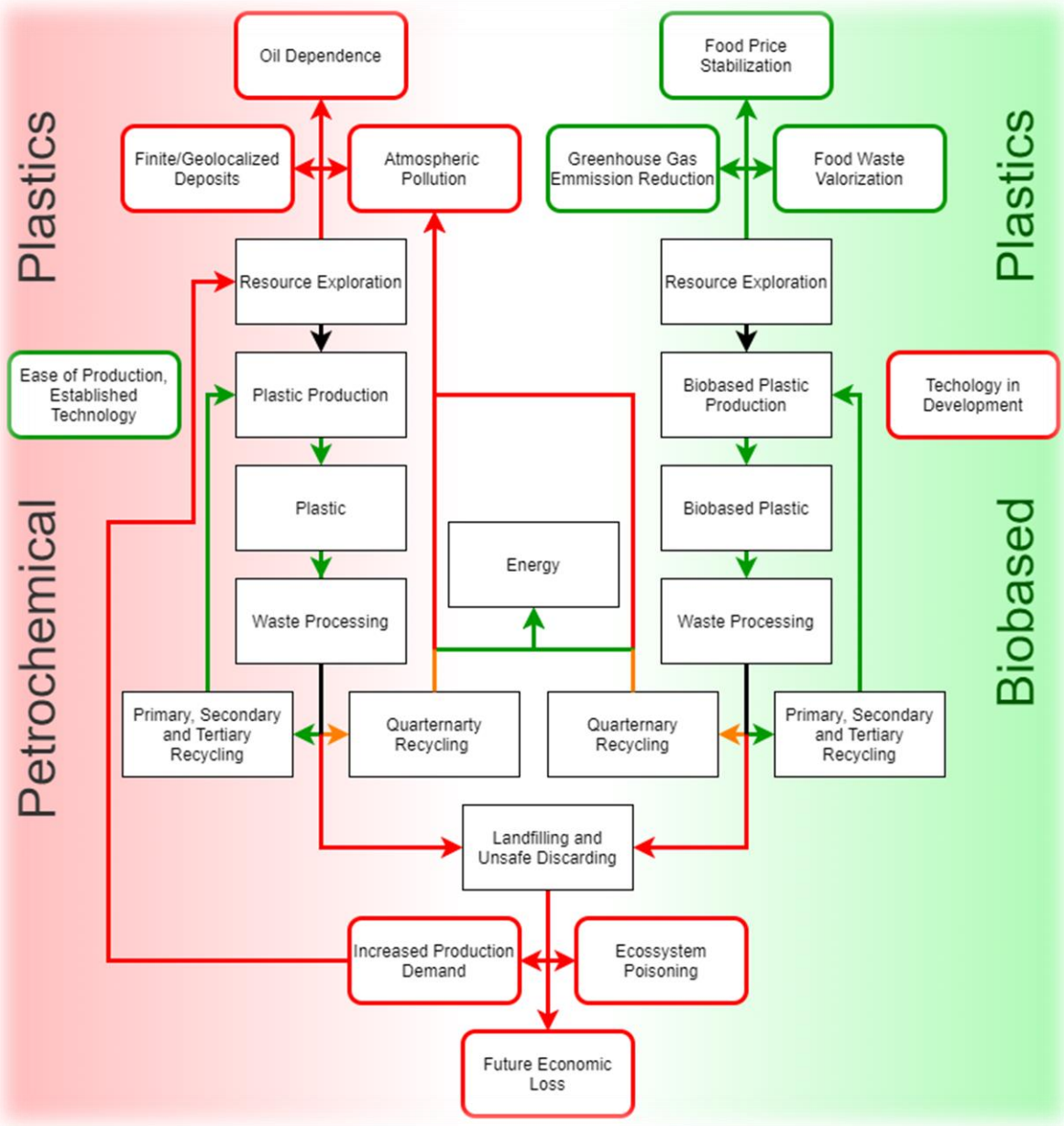

Figure 1.2. Schematic representation of petrochemical and biobased plastic life cycles, denoting some positive (green arrows) and negative (red arrows) effects of their use and disposal options.

The environmental attractiveness of current biobased plastics remains controversial among academics and different stakeholders. Current production patterns for biobased plastics still presents considerable limitations that underline their weaknesses in the markets (**Table 1.2**). The production of biobased plastics remains associated with energy requirements (with most being dependent on fossil fuel resources), leading to controversies regarding their carbon emissions. For example, and as reviewed by Gerassimidou et al. [71], ethanol production from corn can be more energy-intensive than petrochemical plastic resin production, but the production of bio-PE leads to approximately 140% savings in CO_2eq compared to high-density PE derived from fossil resources. In addition, woody feedstocks are highly lignocellulosic and resistant to degradation, so their conversion to a bio-based polymer resin requires an integrated biorefining process that involves the pre-treatment, enzymatic hydrolysis, fermentation, and further processing to iso-butanol (i.e., the starting monomer of bio-based plastic), which produces more GHG emissions and higher ecotoxicological impacts when compared with fossil-based plastics [72]. The replacement of fuel-based energy by renewable energy sources (e.g., solar, hydro, wind) and the development of microorganisms/enzymes to improve bioprocessing can reduce such limitations.

Table 1.2. Summary of pros, cons, and emerging solutions regarding biobased plastics (from cradle to crate). [101]

Pros	Cons	Emerging Solutions
	Costly manufacturing	Biorefinery technology
	(Partly) use of genetically modified organisms	New strains of microorganisms/enzymes required to improve bioprocessing
	Use of arable land, fertilizers, and pesticides for crops (which results in soil erosion and degradation)	Algae, waste residues, by-products as sources to retrieve building blocks
(Partly) based on natural feedstock		Implementation of renewable energy sources (e.g., solar, geothermic) for plastic production
Generally, lower GHG emissions	Potential food competition	
Lower dependence on crude oil	Narrow processing window (e.g., lower melting temperature)	Advances in nanotechnology (e.g., application of nanocomposites such as clays) to improve physicochemical and mechanical properties
Favorable policy landscape (e.g., EU plastic strategy)	Brittleness	
	Thermal degradation	
	Bioconversion requires a high amount of energy	Production of biobased plastics of pure polymers, or blended with compounds free of (eco)toxic effects
Biodegradable options can simplify waste management and returns carbon to the soil, potentially mitigating plastic pollution	Potential for harmful effects on biota (similar to petrochemical counterparts)	Dedicated recycling streams and adequate labelling
	Potential to contaminate recycling streams	Appropriate and coordinated international guidelines for product certification
	Uncertainty regarding biodegradability in open environments (due to current and limited international guidelines for product certification)	Increased public awareness and education efforts
		Increase in financial programs for sustainable plastics production and management of wastes

GHG—Greenhouse Gas.

The use of chemical compounds (additives/fillers) at the polymerization stage of biobased polymer resins can impact environmental and human health. For example, acetyl tributyl citrate (ATBC) or polyethylene glycol (PEG) may be intentionally added to deal with PLA's brittleness, high oxygen permeation, and poor thermal properties [73], which can aggravate both their biodegradability and ecotoxicity if discarded in open environments. Applying less toxic compounds, such as nanoclays and environmentally friendly nanocomposites (due to advances in nanotechnology as further discussed in Section 4), can improve biobased plastics properties [74].

End-of-life processing options for biobased plastics also raises environmental and economic concerns, as they are often misunderstood [15]. Although biobased plastics are, as the name indicates, plastics derived from renewable biological resources, that does not mean that their biodegradability is guaranteed. Some biobased plastics present resistance to degradation, such as PEF, some PLA options, Bio-PE, and Bio-PET, among others (Table 1). Hence, carelessly branding biobased plastics as green plastics might instil the wrong ideas in the minds of the consumers—the consequences of discarding these plastics, biodegradable or not, might be unintentionally ignored by the consumer lulled by the false sense of security given off by that green branding [75]; even certified biodegradable plastics are so only under specific conditions (e.g., in industrial composting facilities/bioreactors). For instance, PHA is biodegradable, but the extent of such biodegradability in aquatic environments was shown to depend on the inorganic water composition, water temperature, and polymeric chemical structure [76]. Some PLA options can also be biodegradable, but if discarded in marine environments, such polymers can take centuries to break down (weight loss of 2.5% was observed in a simulated marine environment over 600 days) [77]. Thus, careless discarding of these polymeric materials into the environment could have virtually the same effect as the "environmentally harmful" traditional petrochemical plastics, with toxicity assays demonstrating in vivo and in vitro toxicity [78,79]. For example, for PLA, Souza et al. found cytotoxic and genotoxic effects on the common onion (*Allium cepa*) [80], whereas Adhikari et al. detected inhibition of microbial activity caused by PLA films after 84 days of incubation in soil [81]. Huerta-Lwanga et al. [82] found that 1% PLA in composts resulted in significant mortality in earthworms (*Lumbricus terrestris*). The toxicity of PLA can be attributed to additives that are included in polymerization to improve mechanical properties. For example, substances such as tributyl citrate or PEG are commonly added to PLA for plasticization; additionally, to improve impact resistance, isocyanates can also be added as chain extension agents by forming a polyurethane bond with the terminal hydroxyl group of PLA [83,84].

On large scales, these attitudes could end up offsetting any positive impact biobased plastics might achieve. To solve this problem, the consumer base must be thoroughly educated on these materials and their waste management practices. This might seem at first like an obvious point. However, its importance is backed up by data that suggests that consumers are somewhat unfamiliar with the concept of biobased plastics, which is in their minds is more associated with environmental issues rather than technical ones—keywords such as "biodegradable" and "environmentally friendly" being more linked to these plastics than "independent from oil", one of their actual defining features, highlighting how easy it is to misrepresent biobased plastics [85].

Regulating authorities also have the responsibility to demand clear labelling to easily relay the proper disposal methods to the consumers to convert them into active participating members of the plastic waste processing infrastructure. For such correct labelling, international guidelines must also be updated. International standards specify the requirements for biodegradable plastics in composting, home composting, and soil or water compartments (e.g., EN 13432, ASTM D6400, Vinçotte OK Biodegradable Soil/Water). Typically, full biodegradation is assessed as the first tier of testing, and ecotoxicity is addressed as the second tier of testing [86]. However, as reviewed by Kjeldsen et al. [86], such international guidelines have several issues that can limit their reliability when attempting to predict biodegradation in environmental scenarios, such as limited methodology (primarily based on respirometry measurements), unrealistic testing conditions (e.g., aqueous/soil medium, controlled conditions or anaerobic digesting sludge), lack of guidance for employing different test materials (e.g., powder, film), insufficient statistical power from limited replicates (often < 3), unsuitable procedures for aquatic environments, many related to wastewater treatment plants (WWTP) situation, and flaws in toxicity testing that are often based on single-(model) species assays, without considering the impact of plastic litter and potential persistent compounds from the biodegradation process on multispecies communities, biochemical processes and ecosystem functioning.

For example, Mater-Bi® (a starch-based plastic) can achieve up to 80% biodegradation in 90 days in aerobic compost conditions (according to EN14045, ISO14851) [87,88]; however, in soil and aquatic conditions, this bio-based plastic only achieves 3.4% and 1.5% biodegradability, respectively, in the same timeframe [89]. Based on single-species tests, such bio-based plastics seem to present no ecotoxicity [90], though the effects at lower (cellular and biochemical level) and higher (community and ecosystem level) biological organization remain poorly covered.

In addition to the implementation of adequate guidelines and correct labelling, an adaptation of the existing recycling infrastructure is needed to accommodate these new materials, including new recycling procedures, and sorting mechanisms, which can prove to be somewhat of a challenge given the (intentionally) similar characteristics between specific plastics and some candidates for substitutes [18]. For example, PLA can be applied in transparent bottles (visually like PET bottles) and can end up on PET recycling streams, and even a 2% contamination would interfere with drying and processing steps, resulting in poor-quality recycled PET (rPET).

This fact, in turn, puts pressure on the waste management facilities, and without support, they may be less than willing to accept these wastes. The United Kingdom's government has recognized that facilities related to composting and anaerobic digestion sometimes show reluctance in even accepting the waste materials in the first place [69]. As such, adequate incentives are needed to update and expand the underlying recycling infrastructure to accommodate biobased plastics without risking causing possibly severe plastic pollution increases due to these new, conditionally, eco-friendly materials.

1.7. New sources and (bio)technological approaches for improving biobased polymers engineering and properties.

Recent advances in biorefinery and polymer chemistry have been applied to produce biobased solutions from alternative biomass (e.g., algae) and residues, with improved design, properties, and functionalities for their successful introduction to the markets and ensure their recyclability (chemical or mechanical).

The use of alternative biomass, by-products and wastes for green valorization provides a substantial ecological advantage when comparing with plant-based biomass sources, as they reduce arable land pressure and help lessen the issue of competition for food production, as well as the intensive use of fertilizers, pesticides and water, while reducing carbon footprints related to waste generation through their reuse as new raw materials [91]. Several compounds, such as lipids, flavonoids, lignocellulose, and phenolic compounds, can be extracted from agro-industrial, forest and even food wastes to produce high value-added biobased products via bioprocessing within a biorefinery framework (as reviewed by Patrício Silva, 2021 [17]). Concomitantly, chitin and chitosan have come up as value-added products that can be retrieved from industrial seafood waste, presenting several appreciated properties such as antimicrobial activity, chelation properties, film formation, and decent mechanical strength as a potential competitor for food packaging material [66,92–94]. Furthermore, it has also been used in edible coatings to enhance the shelf life of fresh produce or processed fruit, vegetables, poultry, and dairy products on a lab-scale without interfering with their sensory attributes.

In addition, algae-based biomass has been highlighted as an alternative approach to achieving sustainable plastic production while contributing to reduce the environmental footprint of production [95]. Algae (micro to macro) possess rapid growth, plasticity, reduced cultivation costs, and autotrophy that contribute to reducing the GHG emission by sequestering CO_2 (up to 1.8 lb) and releasing oxygen (>75%) [96]). Polyhydroxyalkanoates (PHAs) and homopolymers, such as polyhydroxybutyrates (PHBs), can be algal-based, and both can present similar physicochemical and mechanical properties as their closest petrochemical counterparts (e.g., polypropylene, polyethylene terephthalate, and polyethylene) with potential applications in industry, agriculture, and packaging [97]. Microalgae can be used as biofillers to improve mechanical properties in novel thermoplastic biocompounds from gluten [98].

The production of polymer building blocks from biomass or residue components relies on enzymatic tools (as reviewed by Hatti-Kaul [48]), and enormous efforts have gone into the screening and development of enzymes that hydrolyze different components of the biomass/residues. This process is costly, however, mainly due to the high energy demand and biological activity. Consolidated bioprocessing that involves the development of microbial strains with engineered degrading activities or the development of co-cultures that would allow the direct conversion of the biomass/residue to the target molecule have been gaining momentum to overcome such limitations. Metabolic engineering strategies could also include pathway prioritizations by changing substrate preferences, managing redox balances, easing the transport of metabolites, and improving resistance to inhibitory factors such as reaction product concentration or pH, among others to achieve high product yields and selectivity [48].

Meanwhile, advances in nanotechnology have revealed its potential to play an essential role in the polymerization process to improve biobased plastics' functionalities and properties. The inclusion of nanocomposites (e.g., nanoclays) in the polymerization process can result in materials with an improved balance between permeabilities for oxygen, carbon dioxide, nitrogen, and water vapor, with lower costs compared to other nanomaterials and chemical additives [74]. A significant contribution of such an application was observed with PLA microlayer films, which solved problems associated with loss of transparency and heat resistance by obtaining the flexibility required for packaging applications. Still, their environmental friendliness remains to be seen.

High-performance biobased polymers with desirable material features for recycling are also in demand. The glass transition temperature (Tg) is one of the most important thermal properties of amorphous plastic materials, determining their physical, mechanical, and rheological properties and, hence, their range of applications (as reviewed by [48]). Commercial biodegradable polymers generally have a low Tg—notably, for PHA, this parameter can reach a relatively low value when comparing to other plastics of −28 to −55 °C. [99,100]. However, the introduction of aromatic units (e.g., phenyl, phenoxy, and benzoyl) in the PHA chain can significantly increase the Tg of the polymer, which in some cases can then reach beyond the 20 °C threshold, depending on the aromatic group. Thus, improvements in Tg of aliphatic polyesters can be an effective strategy for increasing their performance and recycling possibilities and even their optical transparency. PEF presents yet another example—resorting to the use of the FDCA dimer 2,2′-bifuran-5,5′-dicarboxylate as the monomer can significantly improve the Tg of fully biobased PEF (from 86 to 107 °C), even though this parameter was already higher in this plastic than its competitor, PET (74 °C) [8].

Biobased polymers are getting closer to the reality of replacing their petrochemical counterparts than ever before, paving the way towards a more sustainable and circular economy. It is expected that soon, these materials will be used in several areas, from commodities to advanced applications, thanks to developments in biotechnology and bioprocessing.

1.8. Final considerations

Despite as of this point facing a somewhat uphill battle to secure a significant foothold in the plastics market, in coming times, the biobased plastics sector is expected to grow, lifted by an increasingly environmentally aware consumer base and more forgiving regulatory circumstances, potentially helping reduce the carbon footprint associated with the whole plastics industry.

However, simply taking these new "green" plastics at face value is risking taking a step backwards in the fight against plastic-related pollution. Data indicate that consumers, even in regions thought to be highly developed and educated, seem to easily mischaracterize what terms relate to these new generation plastics. Before biobased plastics become truly commonplace, though, rules and regulations should be put in place that incentivize manufacturers to integrate environmental performance in the development of new polymers and demand rigorous toxicity and life-cycle safety assessments for the new products. Furthermore, when it comes to waste management, the regulatory frameworks must strongly enforce the reutilization and recycling routes for these new materials, or, as a last

resource, the quaternary recycling to produce energy. This, in turn, means more investment will be necessary to properly integrate biobased plastic recycling methodology with the current capacities, given that sorting different plastics that were designed to behave similarly to existing ones is a hurdle that must be overcome to maximize the efficiency of the incoming plastics' reconversion.

Landfilling is a waste management solution that must be avoided at all costs, and aggressive action against littering is a must, given that even the subset of plastics deemed biodegradable by the admittedly lacking regulations on this matter require somewhat specific environmental conditions to degrade in the environment safely; as such, biodegradable options might further risk lulling the consumers into a false sense of security concerning their ecological safety. Once again, the importance of education efforts is highlighted but shows that prioritizing biodegradability rather than biological production might be misguided. Some certified biodegradable materials are chemically harmful after said degradation. As such, since biodegradability only ever so slightly reduces the environmental harm of littered plastics, all the while limiting the circularization of the plastics economy by diminishing consumers' worries about plastic discarding and landfilling rather than recycling, biobased plastic production might just be the better bet of the two to realize the ideal of a sustainable, circular plastics economy.

In sum, to truly begin to fix the problem of plastic pollution and its ramifications on the climate, ecosystems, and public health, the plastics economy must be rethought from a sustainable, circular, and low carbon perspective. Biobased plastics can emerge as tools with high potential for this conversion, although not without issues, both inherent and related to their relative novelty. As such, this subset of the plastics industry must be scaled up responsibly, always considering the economic, legislative, and social sides of this equation so that it may have the opportunity of truly fulfilling its perceived potential.

1.9. References

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Chapter II

Biodegradation of an agricultural mulch biofilm by *Penicillium brevicompactum*

2.1. Introduction

Intensive agriculture, combined with the effects of climate change and associated atypical weather events, results in imbalances in agricultural systems, which need to be addressed to secure production capacity. It was in this context that mulching emerged as a relatively practical solution to these problems – with the reported ability to manage soil temperatures and moistures, as well as being a microbial activity enhancer and as a weed growth manager, presenting themselves as a versatile tool for agricultural businesses [1].

Mulches can be both organic or synthetic, depending on if they consist of biological residues or use synthetic compounds such as plastics; in the latter case, polyethylene mulches have grown much in popularity, thanks to its high affordability and versatility. Biobased mulch films have been commercialized to the agricultural sectors, aiming to compete with the significantly more environmentally harmful non-biodegradable polyethylene mulches, by trumping them when it comes to environmental friendliness and, in the case of biobased and biodegradable mulch plastics, also on clean-up cost effectiveness (meaning the absence of said cost) [2]. However, current standards for the certification of biodegradable plastics for market use (e.g., EN 13432, ASTM D6400, [3]) are unable to predict polymer biodegradability in natural environments due to shortcomings in experimental procedures (e.g., prioritizing anaerobic digesting sludge conditions, biodegradation inferred through respirometric assays, i.e., CO_2 release) [3,4]. Therefore, it becomes important to continuously try to achieve experimental conditions as close to the appropriate environments as possible, to accurately assess biodegradable polymers' true environmental friendliness.

Biodegradation processes of mulch films by naturally occurring soil organisms (e.g., fungi), and the general area of soil microplastic pollution arguably remains poorly covered, in comparison with research focusing on aquatic environments [5]. As such, this study aimed to further understand to which extent a commercially available mulch biofilm biodegrades when in contact with common earth-dwelling fungal organisms.

Penicillium brevicompactum (phylum, class, subclass, order and family of Ascomycota, Eurotiomycetes Eurotiomycetidae, Eurotiales and Aspergillaceae, respectively [6]), is a common fungi species which can be found in agricultural environments, particularly in areas with decaying vegetation [7]. Here, in addition to their ecological role on decomposition of organic matter and nutrient cycling [7], they can bind and possibly damage plastic materials, such as PHB and PE [8], and even corrode metallic alloys in extreme environments [9]. Thus, using *P. brevicompactum* as test species, this study evaluated the biodegradation of a certified biodegradable and commercially available agricultural mulch film (starch-based), both in solid culture media and in agricultural soil. The material form used for testing were microplastics, as it is the common form, i.e., powder or granulates, applied in the current international standards (as in OK biodegradable soil standard), and due to increased surface/volume ratio.

2.2. Materials and Methods

2.2.1. Microplastics

The mulch film used for testing was gently provided by a local company. To obtain microplastics, the mulch film was cut in 1 m^2 sheets, folded repeatedly, and ground using a conventional stainless-steel shredder. The resulting fragments were sieved with stainless sieves, by hand, to obtain de desired size range between 0,5 mm and 1 mm.

2.2.2. Soil

The soil used for the biodegradation trials was kindly provided by the Agrarian School of Coimbra (Coimbra, Central Portugal), already fully characterized (properties can be found in **Table 2.1.**). Prior tests, the soil was sieved with a 5 mm mesh, defaunated from macrobiota (by hand) and then kept at -20ºC for 2 weeks minimum.

Table 2.1. Main properties of the tested soil according to Chelinho et al. [10].

pH (KCl) 1M)	OM(%)	Sand (%)	Silt (%)	Clay (%)	Total N (mg/g)	CEC (cmol/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.

2.2.3. Biological material and culture conditions

Penicillium brevicompactum was grown at 25 ºC in agitated batch reactors (250 mL Erlenmeyer flasks), with a liquid growth medium consisting in 1 g/L of Peptone (Sigma Aldrich), 20 g/L of glucose (LabKem) and 20 g/L of malt extract (Oxoid).

P. brevicompactum was also cultured at 25 ºC in solid culture medium in petri dishes. Solid culture medium consisted in 1 g/L of peptone, 10 g/L of agar (Sigma Aldrich), 20 g/L of glucose and 20 g/L of malt extract.

Both liquid or solid medium was autoclaved at 121 ºC for 30 minutes; and incubation occurred in a laminar flow cabinet using sterilized stainless materials.

2.2.4. Biodegradation assays

Biodegradation assays ran in solid culture medium and in soil.

For the biodegradation assay that ran in solid culture medium, 10 times diluted medium was used (see section 2.2.3. for specifications). Two controls for fungi (each containing 1 cm^2 section of the fungi grown solid medium), two controls for microplastics (each containing approximately 0.0045 g of microplastics dispersed randomly), and four replicates containing both microplastics and fungi as described were prepared for each sampling day (5, 10, and 15 days of incubation).

The biodegradation assay setup that ran on soil was based on a preliminary experiment (see supplementary information, **Annex I**). For this purpose, soil with nutritional supplement

was used to favour fungi growth. Several replicates of 40 g of soil were therefore 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 a quantity to fulfil 50% of the soil WHC. After thoroughly mixed, the soil portions were autoclaved, and distributed neatly in petri dishes under the laminar flow cabinet. Biodegradation soil assays consisted in two controls for fungi (each containing 5 small fragments of fungi retrieved from the batch reactors, roughly 0.25-0.5 cm each), two controls for microplastics (between 0.0030-0.0050 g), and four replicates containing microplastics and fungi (distributed somewhat randomly on the soil surface as per **Figure 2.1**) 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 (accuracy 0.0 mg).

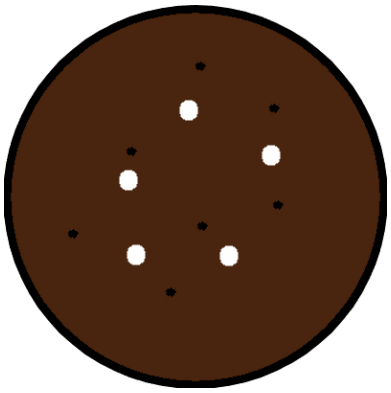

Figure 2.1. Diagram of a possible placement of components in a Soil Trial Petri dish, with microplastics (in black) spread randomly and fungal samples (in white) placed in somewhat circular fashion around halfway through the radius of the dish, not too close to the previously placed plastic.

2.2.5. Sample Separation and Analysis

Prior separation and extraction of fungi or microplastics from both solid medium and soil, all samples were photographed (Huawei P9 Lite, 13 Megapixels) to calculate the fungi surface area. The separation of the fungi and microplastics was done in two ways, depending on the test medium.

In solid culture medium, the whole contents of each petri dish were transferred into beakers, where boiling water dissolved the solid matrix, allowing for the retrieval of the floating fungal matter with a spoon, after which it was left to dry for a few hours at room temperature. In soil, fungi was retrieved directly by eye with sterilised pincers (although its complete retrieval was limited by the fungal spreading), and checked for the presence of microplastics. Collected biological material (in both biodegradation assays) was stored in small glass vials, frozen at -20ºC, freeze-dried, and further analysed by FTIR-ATR spectroscopy (PerkinElmer Spectrum BX spectrometer and the accompanying Spectrum v 5.3.1. program, by scanning their absorbance spectra in the 4000 cm^{-1} to 500 cm^{-1} range at a 4.0 cm^{-1} resolution).

The separation of the microplastics in both types of trials started with the retrieval, with pincers, of those with no medium or fungal matter attached. In the solid culture media trials, the rest were submitted to the same boiling water procedure as the fungal matter, and then retrieved with a pincer from the water surface. In the soil trials, microplastics were retrieved with a density separation procedure using a near-saturated saline solution (300 g NaCl per litre of distilled water). For this purpose, soil contents of each petri dish were thoroughly mixed with the saline solution for several minutes, allowing to settle for 20-30 minutes, filtered through cellulose filters, and observed (and photographed) under with a 1600x

digital microscope. Retrieved microplastics (from both solid medium and soil) were then transferred into glass bottles, placed within a close container to avoid contaminations or spills, and left to dry at least overnight. Afterwards, the mass of microplastics per treatment (and sampling day) were measured (Radwag MYA 2.3Y microbalance) to determine the mass loss due to biodegradation during the incubation periods. After completely dried, all microplastics samples were also analysed through Fourier-Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR) as previously described.

2.2.6. Fungal areas calculation

The ImageJ software was used to calculate fungi spreading areas. Briefly, the petri dish diameter in pixels (photo) was converted to cm based on a scale. Then, using the command “analyse particles”, a list of particles areas is given per petri dish. The sum of all particles areas on each petri dish corresponded to the total spreading area of the fungi. See **Figure 2.2.** for detailed procedure executed for replica 4, after 5 days of incubation in solid culture media trials.

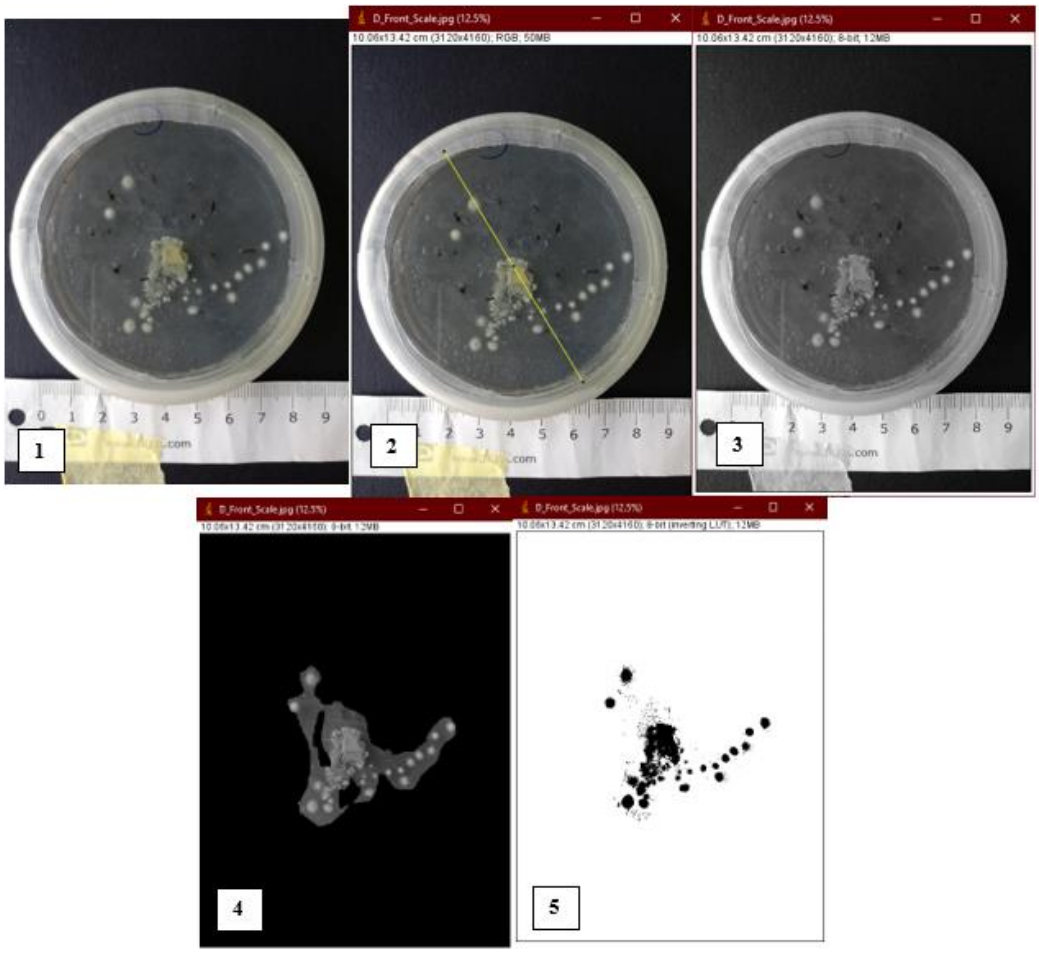

Figure 2.2. Graphical transition history of the photograph Sample D (solid culture media). 1. Sample D original photograph; 2. Diameter measurement (confirmable using the scale); 3. Conversion to 8-bit; 4. Growth area selected and outside removed to reduce the presence of visual artifacts; 5. Threshold adjusted, ready for the “Analyze Particles” command.

2.3. Results and Discussion

2.3.1. Mulch Film Degradation

The percentage of microplastics removal on solid media experiment can be found in **Table 2.2.** Results showed a clear outlier in the microplastic-controls sampled on the 5th-day, with a recorded average mass variation of 33 ± 10%, considerably higher than in other replicas or controls, even after longer periods of time.

Table 2.2. Removal (%) of biofilm microplastics after incubation period, in the absence (containing only microplastics, Controls) or presence of *Penicillium brevicompactum* (microplastics plus fungi, Replicates), in solid culture medium. Data is presented as average mass (g) or in percentage (%) ± standard deviation.

Retrieval Timepoint (Days)	*Replica*	*MPs (g)*	*Recovered MPs (g)*	*MPs removal (average ± std) (g)*	*MPs removal (%)*	*MPs removal (average ± std) (%)*
5	Replica 1	0.0045	0.0042	0.0003 ± 0.0001	6.7	6 ± 2
	Replica 2	0.0043	0.0041		4.7	
	Replica 3	0.0043	0.0041		4.7	
	Replica 4	0.0047	0.0043		8.5	
	Control 1	0.0045	0.0027	0.0015 ± 0.0004	40	33 ± 10
	Control 2	0.0047	0.0035		26	
10	Replica 1	0.0048	0.0036	0.0006 ± 0.0005	25	12 ± 10
	Replica 2	0.0046	0.0045		2.2	
	Replica 3	0.0044	0.0042		4.5	
	Replica 4	0.0044	0.0037		16	
	Control 1	0.0045	0.0044	0.0004 ± 0.0004	2.2	9 ± 10
	Control 2	0.0045	0.0038		16	
15	Replica 1	0.0046	0.0035	0.0010 ± 0.0004	24	21 ± 9
	Replica 2	0.0045	0.0032		28	
	Replica 3	0.0047	0.0042		11	
	Replica 4	0.0047	0.0051		--	
	Control 1	0.0046	0.0039	0.0004 ± 0.0004	15	9 ± 9
	Control 2	0.0045	0.0044		2.2	

Microplastics mass variation was observed in the absence of *P. brevicompactum* after 10 days of exposure, remaining similar after 15 days of exposure. The microplastics removal was observed in the presence of *P. brevicompactum*, here to a higher extent compared to control groups, increasing with the time of exposure (6 ± 2 to 21 ± 9, from 5th to 15th day). The microplastics removal observed in control conditions 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. The higher microplastics removal observed in the presence of *P. brevicompactum* when compared to controls with microplastics, highlights their role on boosting biofilm's degradation, which presented an exponential curve with time as represented in **Figure 2.3**.

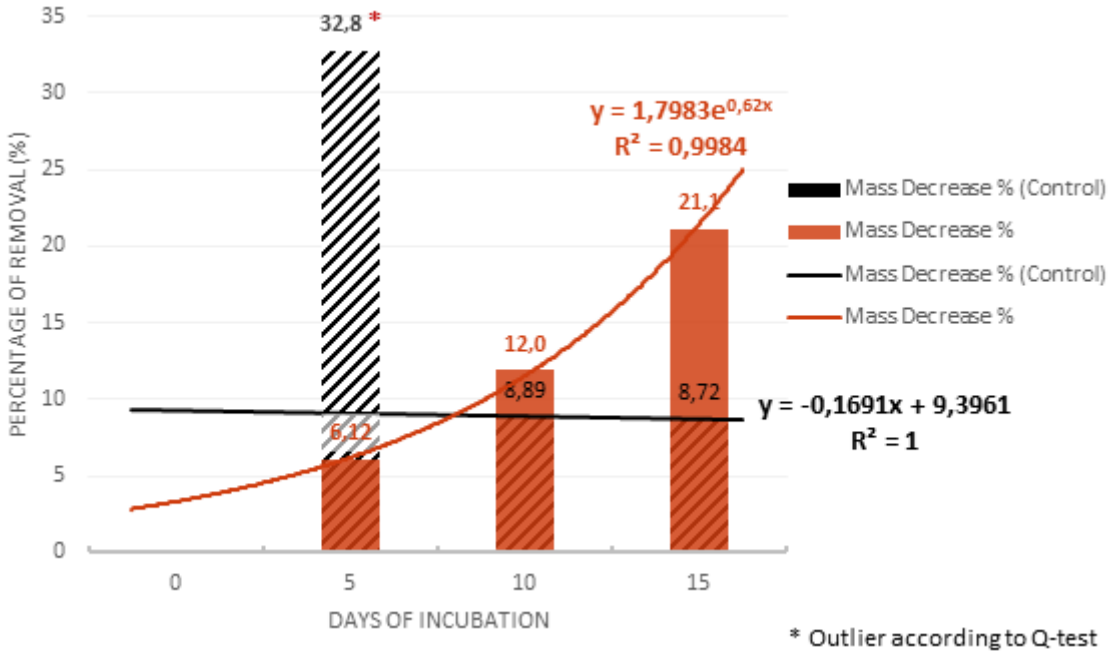

Figure 2.3. Microplastics removal throughout the Solid Culture Media Trials. In this representation, the outlier that was the 5-day control average was ignored, as was the previously referenced sample T, when constructing the respective trendlines, as well as the 15-day Mass Decrease % bar.

When it comes to percentage of microplastics removal, a clear, apparently exponential trend can be observed for microplastics exposed to *P. brevicompactum* when compared to control conditions. Noticeably, on the other hand, the high degree of microplastics removal observed in the first timepoint control was deemed an outlier by a Q-test at 95% confidence, and thus discarded when calculating a trend for mass loss in this experiment's controls, which then became an approximately constant rate. This could mean that the amounts of mass loss under control conditions vary slightly throughout the experiment, whereas in the presence of *P. brevicompactum* that rate is much more clear and defined, suggesting some degree of interaction between the two. The possibility of there being different rates of microplastics removal in control conditions cannot be discarded either, given the uneven morphologies of the particles used (**Figure 2.4**), meaning possibly high differences in surface area compared to volume in each particle, a factor that could contribute to less clear results throughout this section of the investigation, especially when it comes to controls such as these.

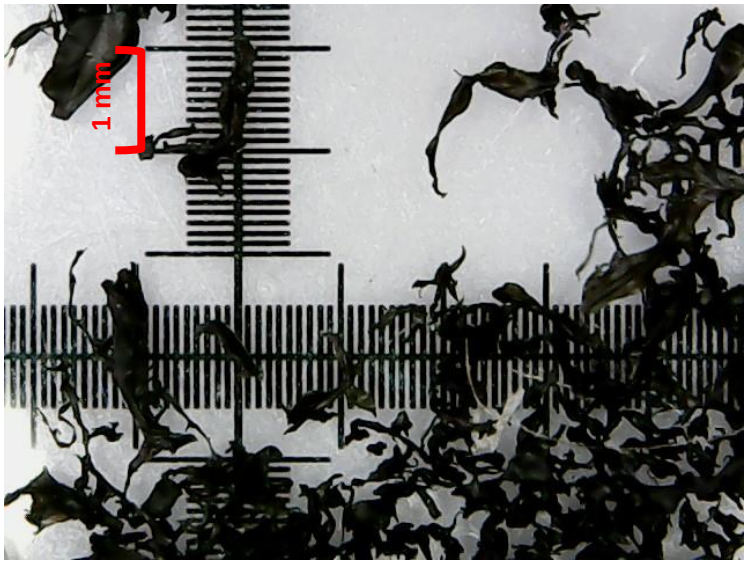

Figure 2.4. Variety of microplastic morphologies, which could lead to different rates of degradation, on a millimetric scale.

Considering the above information, several conclusions and possibilities can be raised. *P. brevicompactum* seems to influence this mulch film's biodegradation, with an increase in the removal percentage as the trials proceeded, despite the stable control mass values, if the 5-day value, rejected by Q-test, is not considered. Furthermore, this data appears to be backed by the high correlation of the mass loss average data in an exponential curve, despite all the arguably possible sources of variability within the trials. On the other hand, to say that biodegradation did not occur at all during these 15 days, including under control conditions, would be premature. Therefore, FTIR-ATR analysis was performed on both control and replica microplastics, and the results are discussed later in this chapter. When it comes to both Soil Media experiments, however, the results of percentage of removal were inconclusive, as it can be depicted in **Table 2.3.**, referring to the 28-day experiment (data relative to the 15-day soil trial can be found in **Annex I**). Unlike the transparent, easily removable agar media used previously, soil's opaqueness and grainy nature presented an obstacle to the successfully retrieve of both plastic particles and fungal matter, thus skewing the results in unpredictable fashion, rendering them, in general, unreliable.

Table 2.3. Removal (%) of biofilm microplastics after incubation period, in the absence (containing only microplastics, Controls) or presence of *Penicillium brevicompactum* (microplastics plus fungi, Replicates), in soil matrix. Data is presented as average mass (g) or in percentage (%) ± standard deviation.

Retrieval Timepoint (Days)	***Replica***	***MPs (g)***	***Recovered MPs (g)***	***MPs removal (average ± std) (g)***	***MPs removal (%)***	***MPs removal (average ± std) (%)***
7	Replica 1	0.0032	0.0015	0.0008 ± 0.0007	54	30 ± 19
	Replica 2	0.0021	0.0018		15	
	Replica 3	0.0020	0.0017		15	
	Replica 4	0.0020	0.0013		35	
	Control 1	0.0021	0.0013	0.0009 ± 0.0002	37	46 ± 13
	Control 2	0.0019	0.00085		55	
14	Replica 1	0.0032	0.0015	0.0010 ± 0.0009	53	36 ± 24
	Replica 2	0.0018	0.0025		-	
	Replica 3	0.0032	0.0035		-	
	Replica 4	0.0021	0.0017		19	
	Control 1	0.0023	0.0022	0.0005 ± 0.0005	4.3	21 ± 23
	Control 2	0.0022	0.0014		37	
21	Replica 1	0.0020	0.0017	0.0005 ± 0.0002	15	24 ± 13
	Replica 2*	-			-	
	Replica 3	0.0019	0.0013		33	
	Replica 4	0.0024	0.0025		-	
	Control 1	0.0018	0.0023	0.0005	-	25
	Control 2	0.0021	0.0016		25	
28	Replica 1	0.0021	0.0020	0.0007 ± 0.0008	6.8	34 ± 39
	Replica 2	0.0022	0.0023		-	
	Replica 3	0.0024	0.0026		-	
	Replica 4	0.0021	0.0008		62	
	Control 1*	-		0.0005	-	26
	Control 2	0.0018	0.0013		26	

** Sample Lost*

After the inconclusive data obtained from first experiment (15 days) using soil, several adaptations on the procedures for the second trial (28 days) were made to reduce shortcomings related to the extraction of both microplastics and fungi, and quantification of microplastics. The major change was the selection of a set number of microplastics, rather than trying to use mass alone as a criterion. Theoretically, this would have made the retrieval process much easier, simply requiring the same number of introduced particles to be retrieved after a trial's end, rather than all resulting filters needing to be thoroughly probed for the presence of all the introduced plastic material, without any sort of indication of how the process was going, making the process more time consuming. Other great challenge related with the microplastics extraction from the soil matrix was the presence of unidentified egg-like particles that closely resemble our microplastics (black colour, similar size). The main distinctive factor of those egg-shape particles was their "balloon" shape, although not a reliable indicator of their identity, since they often broke and started displaying a fragment shape that was all too easy to confound with a microplastic particle. Moreover, *P. brevicompactum* also seems able to bind to these egg-shape particles. Examples of these unidentified particles can be found in **Figures 2.5**. Furthermore, these materials had their FTIR spectra scanned to compare with the mulch biofilm's. The in-depth discussion of the FTIR results will follow later, but a comparison of the spectra of these two materials follows below in **Figure 2.6.**, which demonstrates that these particles, despite high visual similarities to the microplastic particles, were not such, raising concerns over possible contaminations during the FTIR scanning of plastic samples.

Figure 2.5. Unidentified egg-like particles found post-soil filtrations.

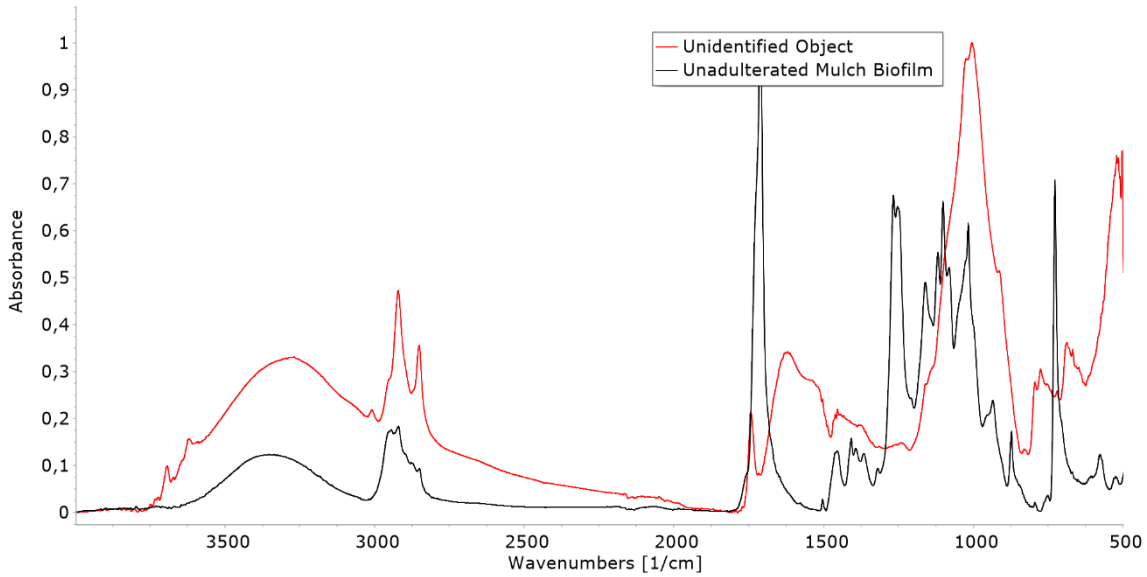

Figure 2.6. FTIR spectra comparison between that of the studied mulch film and the one pertaining to the unidentified objects found during the soil biodegradation experiments.

In addition, hetero-aggregation of microplastics to soil organominerals also difficulted the retrieval of microplastics from soil matrixes through density separation; by promoting their sinkage even under saturated saline solutions (where floatation was expected). Even after a digestion procedure with Fenton reagent (Fe(III) + H_2O_2), such hetero-aggregation remained mostly unaffected. Future soil biodegradation experiments will have to take these limitations into consideration in their design.

Moreover, the size and morphology of the microplastic particles must be even more carefully selected, to allow an even more reliable retrieval using the hypersaline solution; the amount of microplastics used could also be reconsidered, in order to make the mass variations less dependent on equipment uncertainties and punctual material losses during retrieval. Finally, the soil composition must also be looked into more closely, in order to avoid confusing factors such as those egg-like particles from affecting future trial results.

2.3.2. Fungal Growth

Throughout the biodegradation trials, fungal growth was also monitored. Although originally, the mass of the totality of the fungal samples was expected to be used, said measure later proved unreliable on the soil growth trials, due to both its spread across an uneven, disaggregated environment, and its adhesion to individual sediments, making their full retrieval virtually impossible, and even in such cases, their weighting misleading on the account of the presence of impurities, much more so than was the case with plastics, with which similar difficulties were found. Still, the mass values of all retrieved fungal samples were registered, including those of the soil trials. Firstly, the data relative to the solid culture media experiment can be found in **Table 2.4.**

Table 2.4. Biomass variation of *P. brevicompactum* from the solid culture media experiment, in the presence (replica) or absence (control) of biofilm microplastics.

Retrieval timepoint (days)	***Sample Type and Number***	***Initial Biomass (mg)***	***Final Biomass (mg)***	***Biomass Variation (mean ± std) (mg)***
5	Replica 1	34	56	42 ± 22
	Replica 2		62	
	Replica 3		59	
	Replica 4*		24	
	Control 1		44	50 ± 8
	Control 2		55	
10	Replica 1		159	151 ± 35
	Replica 2		152	
	Replica 3		103	
	Replica 4		188	
	Control 1		141	146 ± 6
	Control 2		150	
15	Replica 1		217	207 ± 27
	Replica 2		201	
	Replica 3		237	
	Replica 4		174	
	Control 1		181	156 ± 36
	Control 2		131	

**Rejected by Q-test*

In the solid culture media experiments, all soil grafts had a standardized size, given the impossibility of calculating initial values for each sample. Considering this, initial masses were determined resorting to a 1 cm^2 section of fungi, after melting the culture medium and drying. Thus, the mass value representative of the very beginning of the experiment in these solid culture media trials for all samples was 34 mg, as per the above table. For further visualization of the data, below in **Figure 2.7** follows a plot of both replica and controls' biomass progression throughout the trials.

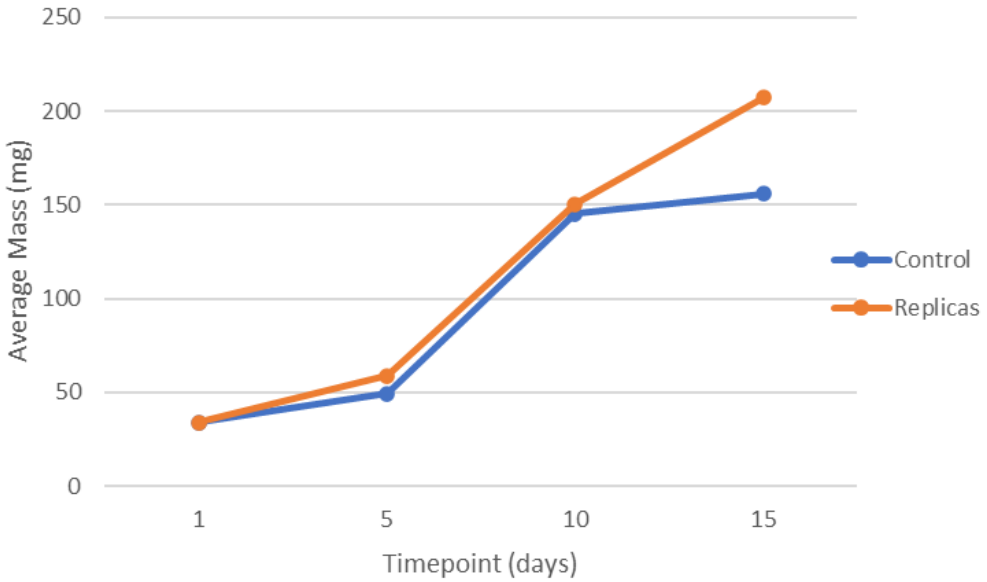

Figure 2.7. Average Biomass progression throughout the solid culture media biodegradation experiment.

According to these results, it can be observed that the fungi followed a growth pattern when in the minimal solid culture media. After an initial acclimation, its growth accelerates from a lagging-like phase to an equivalent of a logarithmic-like phase in the 5 to 10-day period, only to start deaccelerating shortly thereafter, with results from replicas notably outpacing those from control conditions in the later timepoints. In itself, this could be a sign that somewhere between the 5 and 10-day timepoint, the fungi consumes a significant part of the minimal media, making its growth rate unsustainable in the near future. Moreover, the higher growth rates in the environments containing the mulch biofilm suggest that this growth might have been fuelled by the presence of this alternative source of carbon. On the other hand, fungal growth rate might have been also affected by the introduction of a higher quantity of fungal material, the agitation of the petri dishes and such random uncontrollable factors. In order to truly confirm the hypothesis that the mulch biofilm might have been bioavailable as a carbon source for the fungi, FTIR-ATR spectra scanning was performed on the resulting fungi and microplastics from all experiments, and its analysis follows later on. The seemingly clear results were not confirmed by those of the soil experiments, however. The results relative to the 28-day soil experiment can be found displayed in **Table 2.5**, while results from the first soil experiment can once again be found in **Annex I**.

Unlike with the previous experiments, unfortunately, obtaining an accurate measurement of the initial fungus mass was impossible, due precisely to the way it was used in the inoculation of the petri dish – fungal fragments were taken from liquid media and immediately torn and placed in the soil matrix. In this first soil experiment, some masses were registered, but these did not represent their dry weight and, as such, their utility did not go beyond proving the uniformity of the initial masses – as per the table, it is possible to see the apparent uniformity of these starting biomasses, despite the final results. This measurement of the initial mass values was not repeated for the 28-day soil experiment due to time constraints; however, the high uniformity and the fact the methodology remained unchanged means that comparable initial biomass values were used in both instances**.**

On the other hand, the final mass measurements cannot be deemed reflective of the fungal samples. Given the way the fungal material expanded in the soil matrix, expanding downwards, and entrenching itself in the opaque matter through the gaps and crevices, as well as surrounding and adhering to a variety of sediments, the separation of the fungal matter from the environmental contents was, much unlike with the solid culture media, an impossibility. Neither trying to pick up the samples manually with a pincer, impossible due to the opaqueness of the medium and the attachment of the fungi nor the utilization of the saturated saline solution worked, with the latter suffering from the fact that the density of the fungi was increased by the soil sediments, thus rendering it unable to float (This effect can be verified in **Figure 2.8**); furthermore, its location was also often impossible, due to the colonies being surrounded by soil and thus not visible. All these factors contributed to the impossibility of determining if all fungal matter had been retrieved, making this analysis unreliable.

Table 2.5. Biomass variation of *P. brevicompactum* from the soil experiment, in the presence (replica) or absence (control) of biofilm microplastics.

Retrieval timepoint (days)	***Sample type/number***	***Final Biomass (mg)***
7	Replica 1	67.563
	Replica 2	28.730
	Replica 3	19.965
	Replica 4*	11.341
	Control 1	63.663
	Control 2	*lost
14	Replica 1	29.849
	Replica 2	20.437
	Replica 3	19.797
	Replica 4	14.471
	Control 1	35.584
	Control 2	27.570
21	Replica 1	54.885
	Replica 2	21.161
	Replica 3	40.890
	Replica 4	81.524
	Control 1	36.819
	Control 2	18.044
28	Replica 1	14.079
	Replica 2	27.935
	Replica 3	15.662
	Replica 4	17.836
	Control 1	31.849
	Control 2	*lost

Figure 2.8.
Fungal biomass entrenched in the soil during saline solution immersion.

In order to complement the shortcomings associated with the fungal material retrieval and the measuring of its mass as an indicator of fungal development throughout the experiments, the fungal spread in all Petri dishes was also observed, and the superficial areas of each calculated. The resulting areas for the solid culture media experiment can be found in **Table 2.6**. Furthermore, the pictures used in order to determine these areas can be found in **Annex II**.

Table 2.6. Fungal expansion areas from the solid culture media experiment.

Retrieval timepoint (days)	*Sample type/number*	*Area (cm^2)*	*Area (Average± std) (cm^2)*
5	Replica 1	16.26813	11 ± 7
	Replica 2	4.91008	
	Replica 3	17.10048	
	Replica 4	4.24020	
	Control 1	8.53632	7 ± 3
	Control 2	4.88054	
10	Replica 1	6.06390	10 ± 4
	Replica 2	6.71445	
	Replica 3	13.63451	
	Replica 4	11.96069	
	Control 1	10.92420	10 ± 1
	Control 2	10.00974	
15	Replica 1	16.85337	17 ± 6
	Replica 2	10.61522	
	Replica 3	14.26180	
	Replica 4	24.39444	
	Control 1	14.86265	13 ± 3
	Control 2	10.27309	

Areas calculated for replicas and controls throughout this trial seem to show a generally upwards trend, despite a small slump in the averaged areas of the 10-day replicas, which emerges as an apparent outlier. Thus, these results seem to act as confirmation of the solid media biomass results, indicating that throughout the relatively short trials this minimal media remains nutritional enough to support a continuous growth of *P. brevicompactum*, regardless of the presence of biofilm microplastics, although growth rates seem to slow down towards the end, regardless of the presence of the mulch biofilm. However, the process seems to show a high degree of variability, with highly variable results even for same experimental conditions and retrieval timepoints, often displaying high standard deviation to mean ratios.

The problem verified on Solid Culture Media was amplified on the soil experiments (whose results can be consulted in **Table 2.7** as well as **Annex I**), due to all the visual artifacts, which further contribute to the variability of this methodology's results. Still, and especially given the similarity in results obtained for the solid culture media experiments, this method can be a more representative alternative when comparing to the to the measurement of biomasses, given the impossibility of properly recovering all the fungal bits, either from the soil directly or after submersion in the saturated saline solution, as discussed before.

Just like with biomass measurements, superficial area is not as useful an indicator in these soil trials as it was in the experiments using solid culture media when evaluating the total growth of the fungi – in those, the space in which the fungi could spread was much more limited, only a flat surface of solid media was available for their spread, so their vertical expansion could be considered for all intents and purposes null. On soil environments, on the other hand, *P. brevicompactum* also spread inside the soil media itself, whereas superficial expansion only accounts for two out of three possible dimensions of fungal expansion. Still, the expansion area became the more reliable indicator for fungal development towards the microplastics, which were placed exclusively on the soil's surface, despite its inherent variability.

Table 2.7. Fungal expansion areas from the 28-day soil experiment.

	28 Day Trial		
Sample Type and Number	***Retrieval timepoint (Days)***	***Area (cm^2)***	***Area (average ± std) (cm^2)***
Replica 1	***7***	*	
Replica 2*			
Replica 3			
Replica 4			
Control 1			
Control 2			
Replica 1	***14***	1.37641	2 ± 1
Replica 2		1.58860	
Replica 3		2.18160	
Replica 4		4.09640	
Control 1		2.86572	2.7 ± 0.3
Control 2		2.49185	
Replica 1	***21***	**	
Replica 2		1.01805	0.9 ± 0.1
Replica 3		0.82712	
Replica 4		0.87420	
Control 1		1.00845	0.9 ± 0.09
Control 2		0.87609	
Replica 1	***28***	2.00300	1.7 ± 0.5
Replica 2		1.03518	
Replica 3		2.15271	
Replica 4		1.66344	
Control 1		0.35532	0.6 ± 0.4
Control 2		0.85170	

** Photographs lost*

*** Soil Shaken, Surface Area Lost*

The data provided in **Table 2.7** seems to demonstrate a general downwards trend of surface area throughout the trials, although said trend cannot be classified as strong, with instances such as the upswing in the 28-day after the 21-day low in the Second Soil experiment. In the general sense, though, the data allows to infer that the surface area expansion is not necessarily correlated with incubation time, with high swings in experiments retrieved in the same timepoints, and higher areas reported in shorter timeframes. These results naturally lend themselves to the next premise, despite the high degrees of variability – just like masses were not completely demonstrative of fungal development, especially in samples from the soil experiment, area calculation is very user-dependent, and thus these deliberations can still be somewhat flawed, although the fact these calculations were made by the same person and in sequence should indicate that this

associated deviance's effect on the results as a whole should be minimized. In this experiment, where microplastics are laid on the surface of the medium, area is most likely the most interesting criteria to analyse, and to that end, solutions must be found to facilitate the area calculations, mainly the reduction of visual artifacts by tightly pressing the soil in each petri dish, for example. Soil nutrition easily sustains surface fungal growth for the first week, with growth seeming to stall or recede starting at 10 days, when comparing to surface areas corresponding to earlier timepoints, even when accounting for the high variability. This assessment's variability also stems from the fact that each sample petri dish was its own independent system, receiving fungal matter independently and being handled differently as well, which could have a significant effect on area spread, as explained in the solid culture media area discussion. To better understand the fungal expansion pattern, as well as the evolution of its area, which could prove insightful on how it behaves in the presence of alternative feedstocks such as this mulch biofilm, longer trials with a higher number of samples, based on the continuous monitoring of the same systems (petri dishes) could be a sensible choice. This tendency for decline, which at first appears to run against what happened in the solid culture media trials, might be a sign not of nutritional exhaustion, but of the fungi migrating downwards into the soil matrix, hiding its real growth – while in the previous experiment, fungal growth happened only superficially, in soil it can entrench itself downwards. The nutritional media is also expected to have migrated downwards over time, being a liquid solution, thus explaining this growth by the fungus. What fungal material remained on the surface, however, sometimes contacted with the placed microplastics (as well as the previously described contaminants), hinting at the possibility *P. brevicompactum* might indeed be able to use it as a source of nutrition. Additional steps could also be taken to limit the vertical expansion, with the placement of lower amounts of soil, making the growth matrix a thinner layer upon which the fungal growth would be more easily trackable, and better represented by the superficial areas.

2.3.3. FTIR-ATR Analysis

2.3.3.1. *P. brevicompactum*

Fourier Transform Infrared Spectroscopy with Attenuated Total Reflection can provide information about the presence of functional groups, along with an insight on chemical structures present on the analyzed sample [11,12,13]. Thus, FTIR-ATR was used to both qualitatively assess chemical changes over time, comparing to those borne of different experimental conditions, as well as later to comment on the trends observed in certain functional groups. **Figure 2.9** shows the FTIR spectra obtained for *P. brevicompactum* samples collected after 0, 5, 10 and 15 days of contact with mulch film microplastics in solid culture media. In **Figure 2.10** can be observed the FTIR spectra for *P. brevicompactum* samples collected after 0, 7, 14, 21 and 28 days of exposure to microplastics from mulch film in soil. The FTIR spectra obtained for the experiment of 15 days of fungi exposition to mulch film microplastics can be found in **Annex I**. The FTIR spectra for control samples for all timepoints in both experiments (solid culture media and soil) can be found in **Annex II**.

In the *P. brevicompactum* spectra (0 days sample in **Figures 2.9 and 2.10**) a broad peak can be observed in the 3650-3000 cm^{-1} region, corresponding to OH bond vibrations from various possible sources, such as carboxyl, hydroxyl and phenol groups, as well as

amides' NH vibrations. The band approximately 2918 and 2851 cm^{-1} were assigned to the asymmetrical stretching of methylene groups and CH_2 symmetrical stretching, respectively, which can used to monitor lipids, as well as proteins. The 1800-1460 cm^{-1} region has been assigned for proteins, the main molecules carrying the amide I and II functional groups (~1630 and ~1540 cm^{-1}, respectively), and also includes a peak at 1745-1720 cm^{-1}, consistent with carboxyl groups' CO elongation and OH deformations, which can also be related to a peak observable at approximately 1230 cm^{-1}, while the bands between 1450 and 1260 cm^{-1} have been attributed to proteins and lipids with CH_2, CH_3 and phosphate compounds (with the group PO). A peak between 1462 and 1454 cm^{-1} can be attributed to symmetric CH deformation from CH_2 groups, OH deformation and CO elongation from phenolic groups. The small indentation band in these regions, between 1745 and 1720 cm^{-1}, as well as those at 1600 cm^{-1} and in the vicinity of 1575-1540 cm^{-1} and 1390-1375 cm^{-1} are characteristic of COO- ions, while absorption at 1660-1620 cm^{-1} can typically be attributed to CC vibrations, in addition to quinines, conjugated carboxyl groups and ketones. The 1260-1180 cm^{-1} region corresponds to polysaccharides with the COC and COP functional groups. Peaks can be found at 1150 and 1070 cm^{-1} corresponding to -C-O stretching (as well as CH_2 bending, in the case of the former peak). Finally, a peak at approximately 810 cm^{-1} can denote CH bending, whereas the more pronounced band at 750-600 cm^{-1} pertains to alkene (C=C) bending.

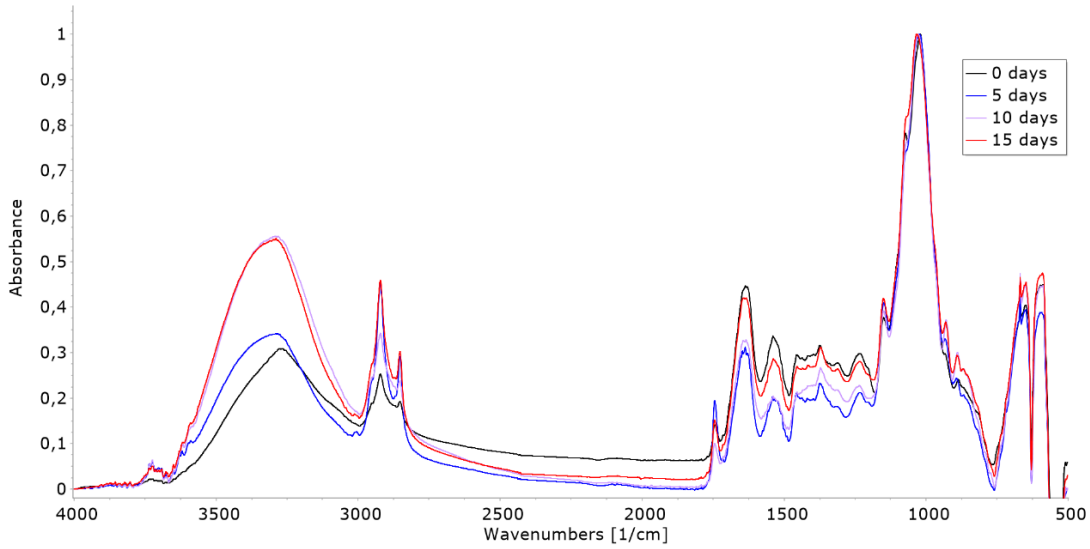

Figure 2.9. Spectra from FTIR analysis of *Penicillium brevicompactum* samples after 0, 5, 10 and 15 days of exposure to mulch film microplastics in the solid culture media experiment.

Through the FTIR spectra relative to samples of *P. brevicompactum* collected at diferent time (0, 5, 10 and 15 days) of exposure to mulch film microplastics in solid culture media, it is possible to observe that no new peaks seem to have appeared throughout the experiment's runtime. There is a noticeable difference in peak heights (absorvance) in the 3650-3000 cm^{-1} region, which seems to show an upwards trend stabilizing after the 10^{th} day, which could be indicative of an increase in carbohydrate contents throughout. That said, the rest of the several spectra generally show a clearer resemblance, with hader to define trends throughout the experiment, which can signal limited changes in other components.

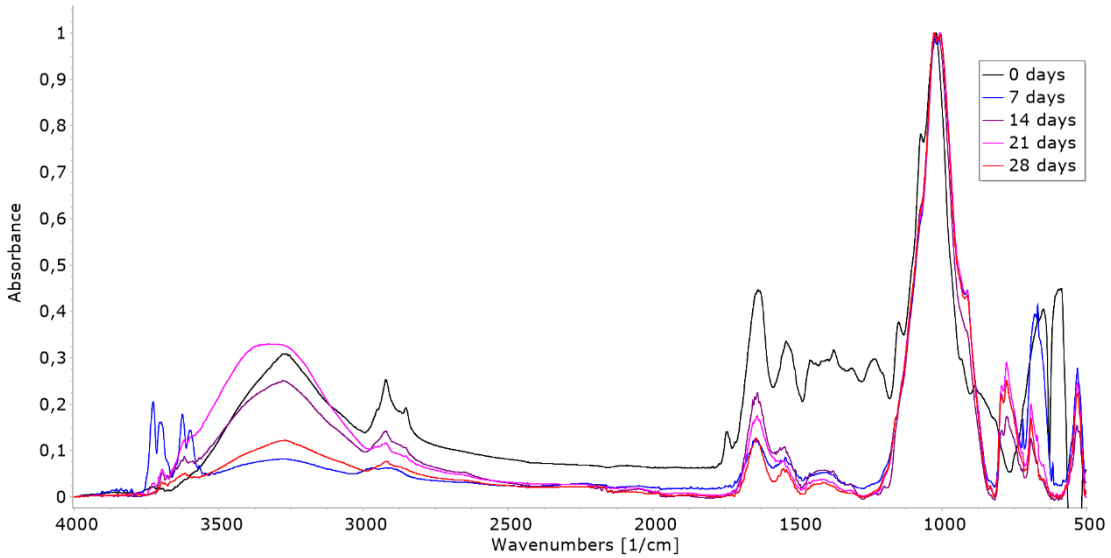

Figure 2.10. Spectra from FTIR analysis of *Penicillium brevicompactum* samples after 0, 7, 14, 21 and 28 days of exposure to mulch film microplastics in the 28-day soil experiment.

It can be observed in **Figure 2.10** for the fungi samples after 0, 7, 14, 21 and 28 days of exposure to mulch film microplastics in soil a very similar pattern for all the peaks, except for the ones located at approximately 1745 cm^{-1} and 1245 cm^{-1}. These changes could be indicative of a reduction of the protein contents of *P. brevicompactum*. The disappearance of that peak was verified for both samples and control and not verified for the experiment of exposition to mulch film microplastics under solid culture media. This could be attributed to a physiological adaptation to soil matrix, since fungi was transferred directly from the liquid media to the soil, which could have caused initial stress, and thus driven the fungi to increase its metabolic expenditure to overcome that situation. Furthermore, despite the thorough defaunation and sterilization process, which makes use of both low and high temperatures, the possible persistence of bacteria might contribute to fungal suppression due to competitive interactions in the growth environment, adding yet another possible source of stress for the fungi. These results are in agreement with Paço *et al.* [14] that tested the ability of *Zalerium maritimum* to biodegrade polyethylene and reported through the observation of FTIR and NMR spectra initial losses in lipidic contents, as well as proteins attributed to the fungi's acclimation into a more nutritionally restricted environment – this metabolic behaviour has been characterized in fungi in media with reduced carbon and nitrogen sources, inducing a large-scale endogenous biomolecule and nutrient recycling, precisely including the reassignment of proteins through the engagement of proteolytic enzymes, as well as other strategies such as the self-cannibalism of its own cell walls by a variety of other enzymes in order to further accumulate carbohydrates in an endogenous search for energy, as reported for several fungi, such as *Z. maritimum* and *Aspergillus niger* [14,15]. Other than these situations, peak absorbances seem to vary somewhat randomly throughout the experiment, with no discernible, clear progressions for the previously referenced regions and peaks of interest, which indicates these variations could be attributed to how the fungi developed differently in each independent system. The interference by soil particles entrenched in the fungal samples might have contributed to

these unclear results, with possible effects on the peaks themselves, as well as in the definition of the spectra, especially given these minerals could have impeded the full closing of the FTIR system.

Several improvements can be considered to try to obtain clearer results for the FTIR-ATR analysis of *P. brevicompactum*, and the overall assessment of its interactions with the mulch biofilm. The seemingly disparate results obtained in the solid culture media and soil experiments could be clarified by using an acclimation step to the soil matrix, by growing the fungi in soil enriched with concentrated growth media – should the trends stay the same in the soil FTIR analysis. In addition, the separation process in each sampling moment must also be reassessed, with a special focus on the separation of fungal material from soil. The utilization of more samples per timepoint, as well as the planning of a longer experiment that does not sacrifice resolution by increasing the time between each retrieval could also contribute to obtaining clearer results and clearer trends overall. Finally, proving interaction and metabolization of the microplastic by this fungal species could also be demonstrated by the measuring of expression levels of selected genes associated with enzyme production and the internal transport of nutrients, such as those coding for Pectinase, Xylanase and β-Glucosidase (the last of which was easily detected in previous studies with *P. brevicompactum*), through real time PCR, for example [16].

2.3.3.2. Mulch Biofilm

For the characterization of the FTIR spectra obtained for mulch biofilm's (**Figures 2.11 and 2.12**), a new set of regions of interest was considered, based on known regions of interest of compounds used in its formulation, such as (Polybutylene Adipate Terephthalate) PBAT and starch, [15,17,18,19,20,21,22]. PBAT displays peaks at approximately 1721-1717 cm^{-1} that can be attributed to C=O groups, followed by a broad carbonyl peak from 1850 to 1550 cm^{-1}, which tends to widen as more low molecular weight O-C=O groups form. A peak at approximately 1456 cm^{-1} has been previously attributed to phenylene, whereas the peak at approximately 1274 cm^{-1}, can be indicative of ester linkages. The sharp peak surrounding the approximately 732 cm^{-1} region can be attributed to Benzene's CH plane. For the second main component, Starch, two main regions were considered. Firstly, the peaks found close to the 1118-1081 cm^{-1} wavenumbers, which can be attributed to C-O groups, as well as the region at approximately 1063 cm^{-1} region, which can be indicative of CH_2OH groups.

The FTIR spectra obtained from the samples used in the solid culture media experiment and the 28-day soil experiment can be found below, in **Figures 2.11 and 2.12**, respectively, whereas the spectra relative to the 15-day soil experiment is present in **Annex III**.

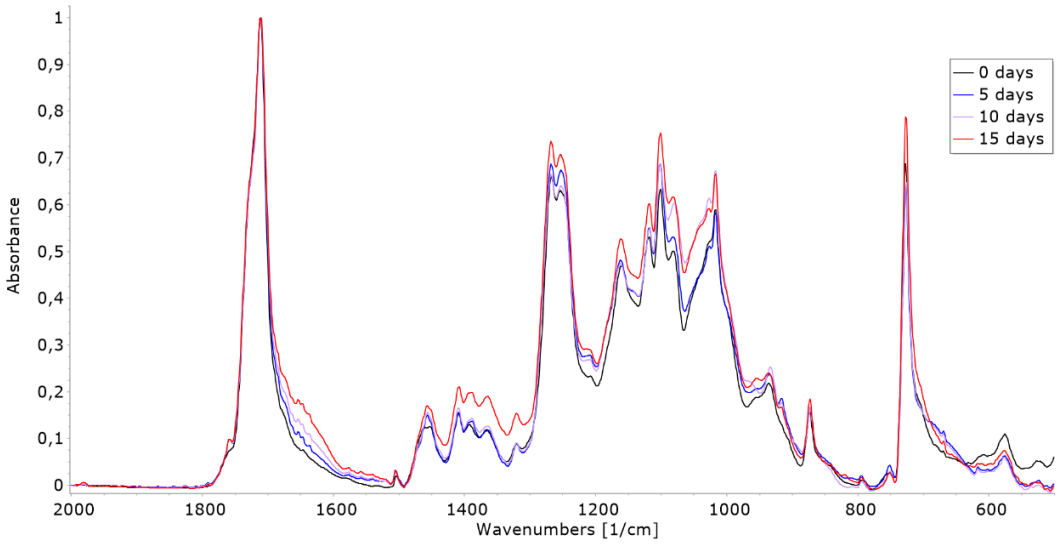

Figure 2.11. Spectra from FTIR analysis of the mulch biofilm samples after 0, 5, 10 and 15 days of exposure to *Penicillium brevicompactum* in the solid culture media.

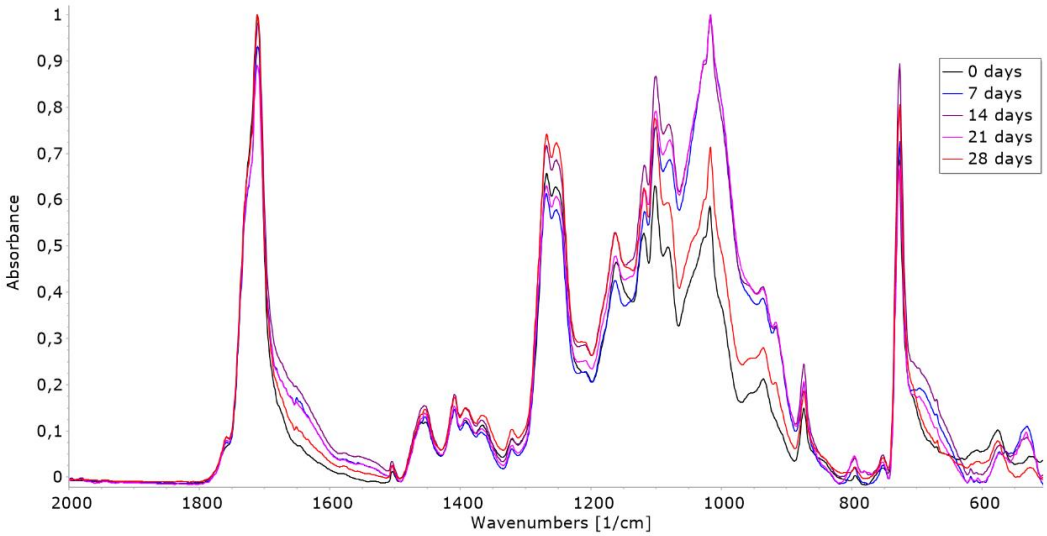

Figure 2.12. Spectra from FTIR analysis of the mulch biofilm samples after 0, 7, 14, 21 and 28 days of exposure to *Penicillium brevicompactum* in soil.

The peaks and bands in the spectra seem to remain overall unchanged, with no peaks appearing or disappearing, although some relatively time-proportional trends can be observed in some regions. Although evidence of degradation is somehwat limited, the situation observable in most FTIR spectra, and much more noticeable in samples that were exposed to *P. brevicompactum*, is the increase in intensity, proportionally with incubation time, of the right side of the approximately 1720 cm^{-1} peak, attributed to ester groups – this increase can be significant, since it signals an increase in low molecular weight esters, a group that also increases when one of the main components of the mulch film, PBAT, degrades, forming two molecules with lower molecular weight, PBA and PBT, both containing ester groups [17]. Furthermore, evidence was found throughout the experiments that the fungi attaches to the plastics (**Figure 2.13**), and mass losses were observed throughout all trials. Moreover, microplastic particles with gaps were found on the 21st day

of the solid culture media experiments (**Figure 2.14**), after the removal of fungal material and culture media. Thus, further study is required in order to accurately determine to which extent the mulch biofilm is biodegrades when in the presence of this fungi. The design of longer experiments, with more retrieval timepoints for higher accuracy, and, ideally, more samples for each timepoint could result in a more representative sample pool, which could contribute to clearer results in this FTIR analysis, in addition to the mass loss study.

Figure 2.13. *Penicillium brevicompactum* attachment to mulch biofilm microplastis, after 28 days exposure in soil.

Figure 2.14. Pierced microplastic particles after 21 days of exposure to fungi in soil.

As a final note on the subject of the sometimes contradictory and erratic results, fluctuations in results from all experiments, in both fungal and plastic samples can also be attributed to uncontrolled variables in the experiment. Unlike an agitated batch reactor, where carbon sources and fungal colonies are in constant motion, and thus more bioavailable to each other, this experiment relied on the random release of spores by several static fungal material. Yet another factor was petri dish agitation – during incubation they remained mostly static, when executing weekly checks, in order to probe for the appearance of contaminations, or merely to check the sealing of the petri dish, the individual environments would invariably suffer different, random degrees of vibration, helping the fungus spread at different rates. The effect caused by these random, uncontrollable

variables is clearly reflected in the spread area analysis executed previously, with similar timepoints displaying wildly varying levels of fungal spread (although possibly amplified by the area calculation method). As such, once again, the increase of the numbers of replicas and controls in each timepoint would certainly increase the chances the batch of results from each timepoint more accurately represent the real situation.

In sum, the FTIR spectra analysis seems to confirm that *P. brevicompactum* is indeed able to interact with this mulch biofilm, given the higher degree of changes in the spectra scanned from samples exposed to fungal material, namely the higher rates of what seems like the appearance of low molecular weight esters.

Still, the mixed results seen in the three experiments, overall, could benefit from a redesign of the experimental method, in order to try and reduce the shock experienced by *P. brevicompactum* upon its introduction to the soil, as well as to obtain an overall clearer image of what happens to the fungal homeostasis and the polymer's chemical structure during longer incubation periods, countering the high volatility of the results by increasing the number of replicas and controls retrieved at each timepoint.

2.4. Chapter Conclusions

Considering the results obtained in this chapter, several conclusions may be reached in regard to this mulch biofilm's biodegradation performance when in contact with prolific soil fungal species *P. brevicompactum*:

1. *P. brevicompactum* seems to positively influence the tested agricultural mulch biofilm's biodegradation process, as per the solid culture media trial results, a result observable in all experiments. The removal of microplastics under control conditions are also apparently confirmed by the elongation of the ester peak in the mulch biofilm's spectra, although on a lower scale than what happens with microplastics exposed to the fungi.

2. The soil biodegradation experiments, having been largely unsuccessful when it comes to retrieval of the fungal material and microplastics, should be redone considering the experienced shortcomings. Several changes can be made to reduce the chances microplastics would be unknowingly discarded, such as using lower quantities of soil, only enough to fully cover the surface of the petri dishes, to the usage of plastic particles with easily recognizable shapes, to avoid confusing them with the unidentified objects found in the soil, as well as the recording the exact positions of the placed microplastics, in order to ensure the retrieval of all fragments.

3. Fungal development seemingly stalled before the conclusion in the soil experiments. While this is in line with the apparent exhaustion of the nutrition media, this stall in growth might have been disguised by fungal growth within the soil matrix, following the sinking nutrients, acting as a confounder when it comes to the assessment of the fungal development throughout the incubation periods. Once again, the reduction of soil used in each petri dish could prove useful to mitigate this possibility and help to assess how biocompatible the mulch biofilm and *P. brevicompactum* really are.

4. Although short term mass decrease trends seem relatively robust, it is precisely the short time frame that can affect the credibility of this data. Furthermore, the study of more long-term mass variation trends, with and without the influence of *P. brevicompactum* is of interest in itself, since, admittedly, degradation does not occur at the same rate throughout the entirety of the process. As such, the preliminary results obtained should be regarded as a prelude for a longer term, more in-depth observation of this mulch biofilm's biodegradation, assisted and not by this fungal species.

5. None of the FTIR spectra obtained in any trial seemed to indicate the appearance of brand-new peaks when comparing to respective or initial controls. When doing a peak intensity analysis, however, certain trends were observed that suggest that *P. brevicompactum* placed in soil firstly engages in an internal search for energy when put in a nutritionally poor environment, but after some time, further changes in its FTIR spectra suggest it finds an alternate source of energy. This could be due to stress upon entering a new environment, or the effect of biotic factors that resisted the several steps of sterilization and then interfere with its growth. Either way, the experimental methods must be revised in future studies in order to account for these possibilities. As for the plastics, the possible inferences add yet another confounding factor into the mix – as a biodegradable polymer, it is uncertain at which rate it degrades in storage, and thus is unclear if the biodegradation process had already started before the factual start of the experiments. As such, steps should be taken in order to mitigate this possible problem, such as obtaining the biofilm directly from the manufacturer, and minimizing the wait before the start of the biodegradation trials in future experiments.

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Chapter III

Ecotoxicological Effects of a Mulch Biofilm on the earthworm *Eisenia andrei*

3.1. Introduction

The application of plastic films for agricultural mulching continues to increase significantly, worldwide; this application brings several benefits, as it improves crop yield, decreases pesticide' inputs to the field, maintains stable the soil moisture, and contributes to tackle the food demand for the growing world population [1]. However, most plastic mulching is polyethylene based, resulting in intense loads of polyethylene residues on agricultural soils, contaminating these ecosystems and perpetuating the massive worldwide plastic pollution. Biodegradable biobased plastic mulches have, therefore, emerged as a promising alternative to alleviate plastic pollution and environmental degradation [2].

Several international standards specify the requirements for biodegradable plastics in composting, home composting, and soil or water compartments (e.g., EN 13432, ASTM D6400) [3,4]. Typically, full biodegradation is assessed as the 1st tier of testing, and ecotoxicity is addressed as the 2nd tier of testing. However, most of these standards have several limitations (e.g., non-realistic testing conditions, such as using sludge in the biodegradation assays, and the use of acute toxicity tests) that can limit their reliability when attempting to predict their environmental friendliness in environmental scenarios. In fact, 'biodegradable plastics' that perform well in biodegradability tests might not necessarily degrade appropriately when in the natural environment and be free of (eco)toxicological effects [5]. The scientific community is raising attention to this topic, as well as to the fact that current ecotoxicological trials somehow irresponsibly ignore chronic endpoints such as growth and reproduction in favour of quicker, more easily tested, acute endpoints (e.g., survival). Current ecotoxicological acute tests used for products certification are, therefore, insufficient to protect ecosystem health, and possibly, down the line, human health.

Few investigations addressed the effects of biodegradable mulches on soil organisms despite their great importance in agroecosystems [6] – in fact, microplastic studies in soil, despite a recent upswing, still noticeably lag those performed in aquatic and mixed environments [7]. Among key-species in agroecosystems are the earthworms, one of the most important ecosystem engineers due to their ability to improve soil properties, such as aeration, nutrients cycling, among others [8]. Their great biomass is behind the provisioning of these ecosystem services, and they are also crucial for terrestrial food-webs [9]. Earthworms are detritivores, i.e., ingest large amounts of soil or specific fractions of soil (i.e., organic matter); thus, being able to involuntarily ingest microplastics through feeding activity [10]. The ingestion of both petrochemical and biobased originated microplastics has the potential to compromise their development and reproduction [11], as well as biochemical homeostasis and to induce a variety of chemical and metabolic changes [10]. However, such studies only tested pristine microplastics, neglecting the fact that these materials undergo ageing processes through, for example, UV radiation while in the environment, which can alter their ecotoxicity. In this sense, this research aimed to investigate the chronic effects of a novel biodegradable agricultural mulch film's microplastics, pristine and aged by UV radiation, on the earthworm *Eisenia andrei* survival, reproduction, and main molecular responses.

3.2. Materials and Methods

3.2.1. Microplastic Preparation

The preparation of microplastics for this investigation followed the same procedure applied in the biodegradation trials. Briefly, sheets of this biofilm were shredded using a stainless-steel grater, on top of 4 sieves, with a mesh-size of 2 mm, 1 mm, 500 µm and 65 µm. of which those between 65 and 500 µm were used. Microplastics were separated into two groups: pristine (without any ageing process) and weathered (aged with UV radiation, as described in the next section). Supplemental information can also be found in **Annex V**.

3.2.2. Microplastics ageing with UV radiation

Biofilm microplastics were subjected to UV degradation (UVC: 240 nm) for 21 days, to increase their environmental relevance on tests, as most plastic particles resulting from agricultural films fragmentation are commonly exposed to UV radiation for months. This was performed by placing the microplastics in open Petri dishes under a UV-C lamp (here only just for few days to mimic a long-term exposure under UV-A/B radiation in open fields), inside of a totally opaque black box, to block interfering radiation. Petri dishes regularly switched places under UV-C radiation, and the microplastics were regularly revolved and mixed inside their respective Petri dish, to guarantee a uniform rate of UV degradation within each microplastic sample. Radiation intensity was also registered every 3-4 days using a VLX-3W Radiometer, and weathered plastics were analysed through FTIR-ATR, using the same procedure as previously described (**Section 2.2.5**).

3.2.3. Test Species and culture conditions

The culture of the earthworm species *Eisenia andrei,* followed both international guidelines (OECD nr. 207; OECD nr. 222) [12,13] and Good Laboratory Practices (GPL), maintained in a medium constituted by: 1kg of sphagnum peat, 1kg of cow manure (defaunated with 2 cycles freeze/unfreeze) and 1L water. Synchronized and mature organisms (over 300 earthworms, < 12 months old, with 1 month difference and with well-developed clitellum), with a mean length between 60 and 120 mm and a diameter of 3 to 6 mm (as described in Jänsch et al., 2005 [14]) were allowed to acclimate to test soil (properties found in **Table 2.1**, in **Chapter II**) for 48 h prior tests. Organisms with a fresh weight between 250 g and 600 mg were selected and used for testing.

3.2.4. Test soil contamination

The soil used for the ecotoxicity trials was the same used in the biodegradation trials. As in the previous investigation and prior tests, the soil was defaunated from macrobiota (by hand), and frozen at -20ºC for a minimum of two weeks. After that period, the soil was thawed, sieved with a 5 mm mesh, and spiked with pristine or weathered microplastics in a

stainless-steel bowl to obtain the desired concentrations, at which point the contents were mixed with a small stainless-steel rototiller.

For each microplastic type (pristine or aged), the tested concentrations were 0,125 g, 0,250 g and 0,500 g of plastic per kilogram of soil, dubbed “low”, ”medium” and ”high”. A total of six treatments of four replicates, plus eight controls were prepared, as resumed in **Table 3.1**.

Table 3.1. Composition of the Ecotoxicological Trials, as well as their denominations (in parenthesis).

TRIAL	VIRGIN PLASTIC TRIAL (NM)				UV-C DEGRADED PLASTIC TRIAL			
SAMPLE TYPE	0 g (Control)	0.125 g (Low)	0.250 g (Medium)	0.500 g (High)	0 g (Control)	0.125 g (Low)	0.250 g (Medium)	0.500 g (High)
REPLICAS	1	1	1	1	1	1	1	1
	2	2	2	2	2	2	2	2
	3	3	3	3	3	3	3	3
	4	4	4	4	4	4	4	4

3.2.5. Bioassay Procedure

Ecotoxicity tests followed the OECD guideline Nº222: Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*) [13]. Briefly, each treatment, both control and spiked soils, consisted in four replicates (glass vials), each containing 500g (DW) of control or contaminated soil, as per **Table 3.1,** along with 10 adult earthworms, synchronized and with a well-developed clitellum. Tests ran in a $16h^{L}$:$8h^{D}$ light cycle at 19ºC. Defaunated (3 frost cycles at -20ºC) cow dung was humidified and used to feed each vial’s population every week (15 g after humidified in each vial). If needed, some additional water was sprinkled in to further replenish water content. In cases where little flora sprouted within the vials, it would be taken out before feeding, in order not to interfere with weight control.

After 28 days of exposure, living adults were removed and gently rinsed with distilled water. Here, 3 adult earthworms per replicate were allowed to purge, individually, in glass petri dishes containing a humidified cellulose filter paper for 24h, in the dark, at room temperature. The remaining survivors were stored for further biochemical analysis (out of the scope of this thesis).

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 54ºC to force the juveniles to migrate to the soil surface, without being roasted to death. Juveniles were collected and stored in 70% ethanol for further quantification. Each vial was then double checked for the presence of any remaining juveniles.

3.2.6. Examination of the Earthworm Purging

As previously mentioned, 3 earthworms were placed, individually, in a petri dish containing moistened filter paper and allowed to purge their gut for 24 h. After the purging period, the earthworms were sorted into individual 2 ml Falcon ® tubes, and then frozen in

a -20ºC for further analysis (**Section 3.2.8**). Each Petri dish and filter paper containing egestion residues were carefully observed under a dissection USB microscope (1600X 8 LED Zoom USB Microscope Digital Magnifier). Faeces suspected to contain microplastics from each earthworm were scrapped from the surfaces and collected with stainless steel tweezers and transferred into glass tubes. Organic matter was degraded using a Fenton reaction (Fe(II)+H_2O_2 1:1 solution), prepared by transferring 1 ml of both Fe(II) 0.01M (pH =6) and H_2O_2 30%, in that order, into the vials where the organic matter was collected, and which was left reacting overnight in the oven at 50ºC, after which the reaction was stopped by adding a volume of either NaCl 300 g/L solution, previously filtered, matching the total volume of the former reagents (2 ml). Samples were then mixed by shaking the tubes, and then immediately filtered into previously burned glass glass microfibre filter (Whatman® glass microfiber filters GF/C, 47 mm). Filters were then left drying at room temperature, overnight, and then checked for the presence of microplastics using the USB microscope.

3.2.7. Soil pH and moisture

Soil samples were retrieved in the beginning and end of the trials for each of the control and microplastic-contaminated flasks to assess its moisture and pH. For moisture, soil samples were dried in the oven, at 105ºC for 24h; the weight of the dry soil was then subtracted from the weight of the moist soil, and then dividing by the weight of the dry soil.

For soil pH assessment, the soil samples were dried at room temperature, macerated, weighed into glass vials, 5 g for each sample. Then, 25 ml of 0.01 M $CaCl_2$ Solution was transferred into each vial, and the contents were vigorously shaken for 1 minute, being left lying undisturbed for the next 2 hours. Immediately after these 2 hours, the supernatant suspensions were separately transferred into a beaker, agitated, and had their pH measured using a properly calibrated Hanna HI98194 pH Probe.

3.2.8. FTIR-ATR Analysis of Adult Earthworms

After being freeze-dried for 2 days, the adult earthworms were 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 (PerkinElmer Spectrum BX spectrometer accompanied by Spectrum v 5.3.1. program) at a 4 cm^{-1} resolution within the 4000–500 cm^{-1} range.

3.2.9. Statistical Analysis

Reproduction data was analysed for normality (Kolmogorov-Smirnov test) and for variance homogeneity (Levene's test). The effect of microplastic concentrations on earthworms' reproduction was assessed with one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test for multiples comparison against a control (absence of microplastics) using a 95% confidence level. All the statistical analysis was performed using the program SigmaPlot 14.5, 2021.

3.3. Results and Discussion

3.3.1. UV-C Degradation of the Mulch Biofilm

Throughout and after the end of the weathering process of the microplastics, visual indicators of UV degradation such as colour, texture, and integrity changes, were found lacking when comparing to pristine particles (although a loss of integrity would be somewhat difficult to determine in fragments under 0.5 mm of diameter). A chemical analysis through FTIR was conducted, and the resulting spectra were then compared with ones generated from pristine samples. Plots detailing the progression of the FTIR spectra of both pristine and UV weathered plastics, as well as a comparison between both types can be seen below, in **Figure 3.1.**

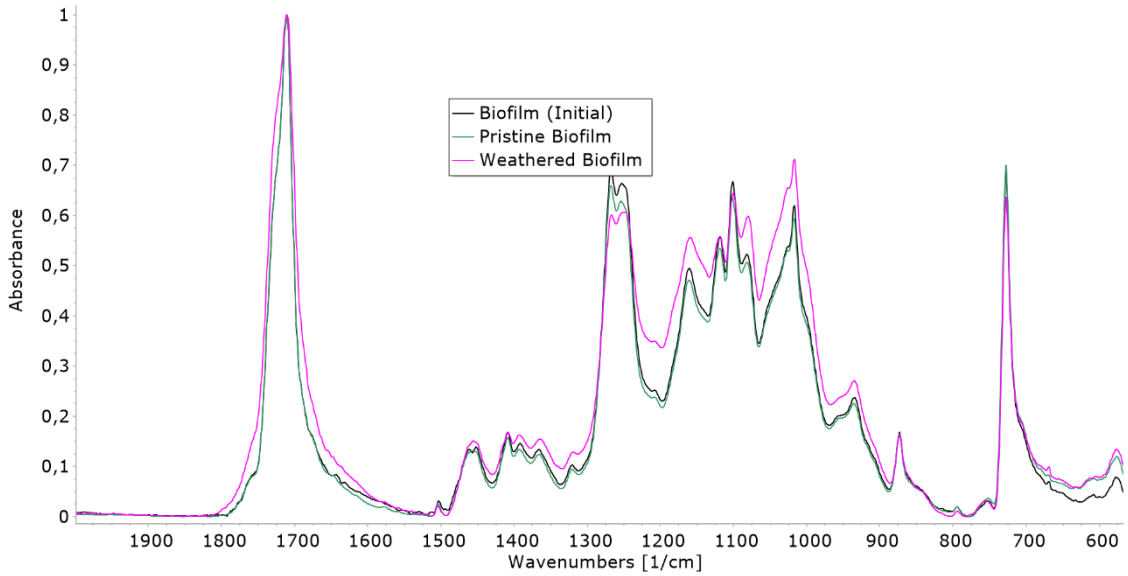


Figure 3.1. FTIR spectra of the mulch biofilm samples prior and after UV type C weathering process.

Through simple observation, it seems clear that the UV weathering process provokes changes in the FTIR spectra of the mulch film microplastics, leading to an apparent cave-in and widening of some peaks throughout the spectra in favour of higher relative intensities in between, whereas the differences between the initial and the pristine plastic are minimal. To exemplify this a ratio was calculated, for all spectra, between the height of the 1101 cm^{-1} peak, one of the most stable throughout the experiment, and the height of the lowest point in the depression observed at approximately 1197 cm^{-1}, using ImageJ's "set scale" command. The ratios are displayed below in **Table 3.2**.

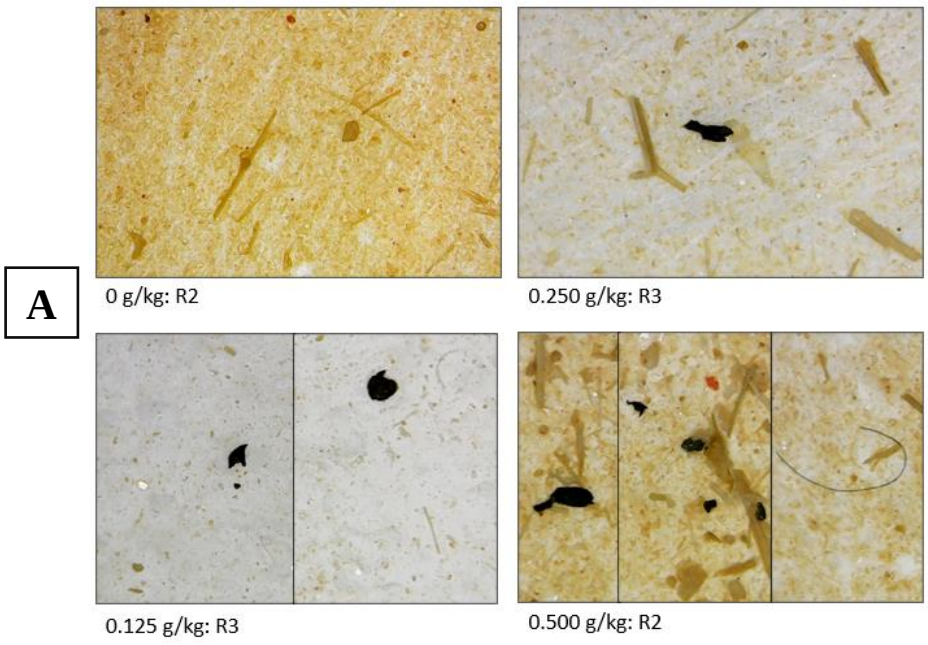
Table 3.2. Height ratio between the height of the depression at 1197 cm^{-1} and the height of the 1101 cm^{-1} peak.

Microplastic Type	*Ratio*
Initial	0.35
Pristine	0.35
Weathered	0.55

As per the above table, it does seem confirmed that plastics subjected to continuous UV-C radiation had their FTIR spectra suffer a collapse of their peaks. This might be a signal of the changes occurring at a molecular level in the mulch biofilm, whose composition includes PBAT, a material with a high density of UV-absorbing ester linkages [15]. Upon the absorbance of these radiations, PBAT-based materials sometimes undergo Norrish reactions, leading to scission and crosslinking events. Were the tested material not composed of microplastics, it is likely it would be visibly brittle and structurally weakened. Although it is impossible to judge those qualities in microplastics under 0.5 mm of diameter, the FTIR-ATR analysis seems to confirm this photochemical degradation.

3.3.2. Microplastic Egestion

The number of egested microplastics can be depicted in **Table 3.3**. Earthworms were able to ingest microplastics (independently of their weathering condition), and ingestion increased linearly with the microplastics concentration in the soil (**Figure 3.3.**). Below, in **Figure 3.2**, are displayed microscope images of the isolated plastic particles on filter paper after the removal of organic matter, determined to be so through a hot needle test, as well as a visually similar particle that could have originated from the unidentified objects discussed in **Chapter II**, denoted by an asterisk.



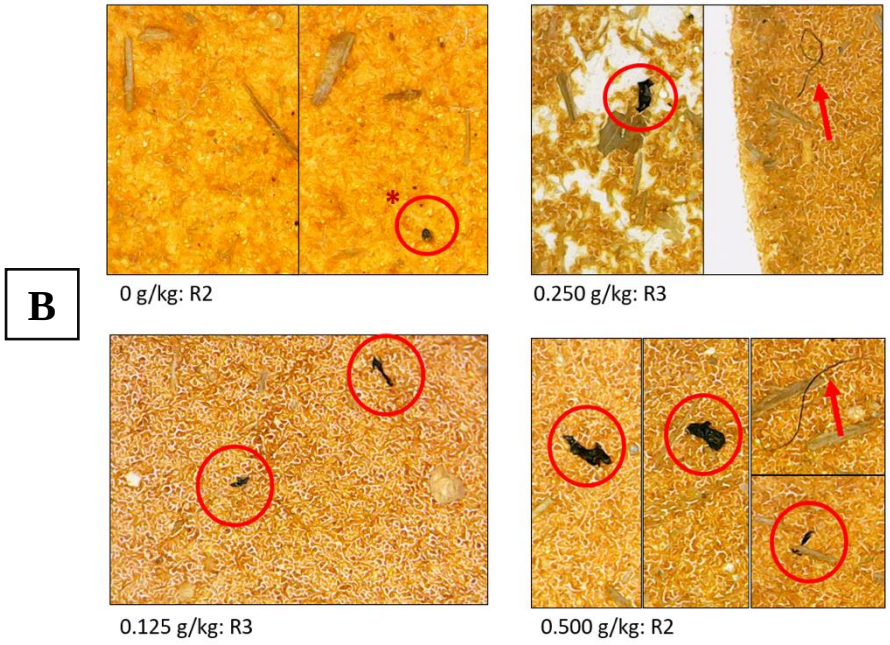


Figure 3.2. Egested microplastics by *Eisenia andrei* from each experimental conditions (the code referring to the organism that egested said particles). (A – Pristine Experiments; B – Weathered Experiments). Particles with suspected shape (*) were tested with hot needle test to confirm its polymeric nature.

Table 3.3. Instances of egested plastic particles found after the Purging step in both Pristine and UV-C Exposed P1 Ecotoxicological Trials.

Sample		***PRISTINE PLASTIC TRIALS***					***UV-C EXPOSED PLASTIC TRIALS***				
		A	***B***	***C***	***Average***	***Group Average ± std***	***A***	***B***	***C***	***Average***	***Group Average ± std***
CT	1	0	0	0	0	0	0	0	0	0	0
	2	0	0	0	0		0	0	0	0	
	3	0	0	0	0		0	0	0	0	
	4	0	0	0	0		0	0	0	0	
L	1	0	0	0	0	0.5 ± 0.6	3	2	1	2.0	0.8 ± 0.8
	2	0	0	0	0		2	0	0	0.67	
	3	1	2	1	1.3		0	2	0	0.67	
	4	1	0	1	0.67		0	0	0	0	
M	1	3	3	0	2.0	2.1 ± 0.9	5	1	2	2.7	1.7 ± 0.7
	2	8	1	1	3.3		3	0	1	1.3	
	3	2	1	2	1.7		3	0	1	1.3	
	4	0	4	0	1.3		1	1	2	1.3	
H	1	2	4	1	2.3	2.7 ± 0.9	1	3	0	1.3	2.5 ± 0.2
	2	4	2	6	4.0		5	0	4	3.0	
	3	2	1	1	1.3		4	0	5	3.0	
	4	5	2	2	3.0		1	4	3	2.7	

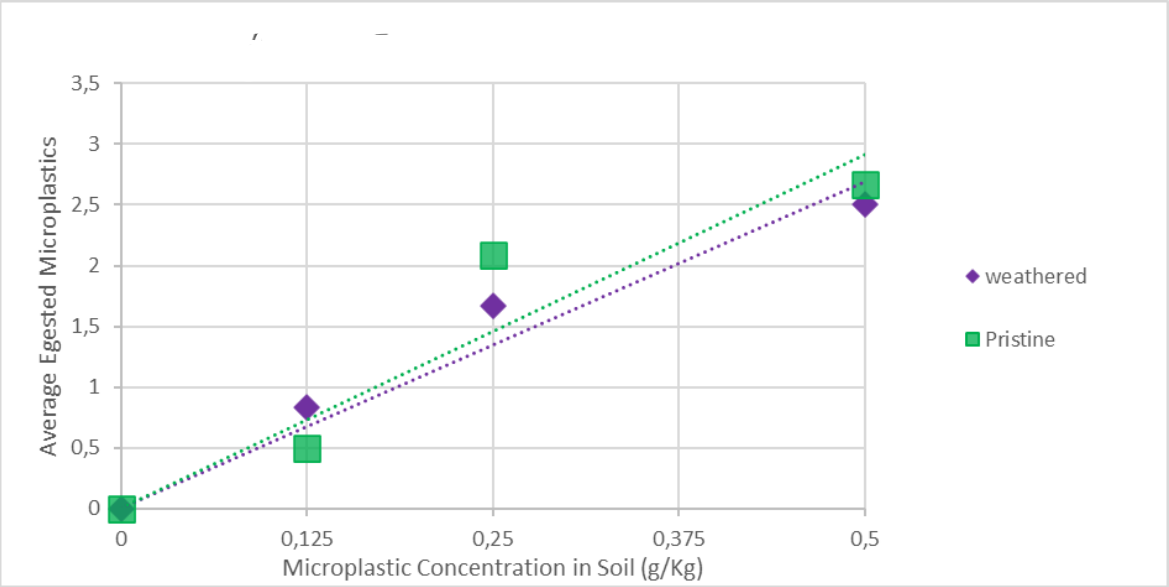


Figure 3.3. Average Egested Biofilm Particles during a 24h purging activity in the several Conditions in each Trial.

The variability found within each group of samples can be explained by the heterogeneous distribution of microplastics in soil and how close those particles are to the organisms. With the decrease of the microplastics concentration, the probability that earthworms encounter and ingest the particles also decreases; thus, the number of plastics ingested/egested at lower concentrations is more variable than in higher concentrations, as signalled by the standard deviation to mean ratios (with standard deviations equal or even higher than means in the lower concentration experimental setting).

Microplastic ingestion is a common occurrence in earthworms. The average earthworm species from temperate regions has been reported to have a mouth aperture of around 3 mm; theoretically they could ingest materials up to those dimensions, but previous studies have shown a degree of situational avoidance of microplastics. Microplastics of LDPE (100-200 µm in size) was found on the casts (faeces) of earthworm *Eisenia fetida*, also in a dose dependent manner (e.g., < 1 items at 0.1 g/kg to 0.8-1.2 items at 0.5 g/kg) [16].

Previous studies reported that ingested particles had relatively lower sizes than the particles used to spike the soil, suggesting a selective ingestion of these contaminants by earthworms (*E. Fetida*, *Lumbricus terrestris* [16,17,18]). Said lower particle sizes can be attributed to the breakdown of these particles inside of the organisms' gastrointestinal tracts and/or potential degradation due to the presence of actino-bacteria and firmicutes isolated from the earthworm's gut [18]. However, in this study, microplastics as large as 0.5 mm in diameter (as explored in **Annex V**) were found among the egested particles, thus it remains unclear if any breakdown of the biofilm microplastics happened. In order to deepen knowledge on this topic, electronic microscopy and histopathological evaluations could prove useful.

3.3.3. Ecotoxicological Analysis – Stress, Mortality and Reproduction

The tests fulfilled the validity criteria as described by the previously mentioned standard guidelines. Soil pH remained similar throughout the text, and, as such, there was no influence of the microplastic contamination on this factor (6.11 < pH < 6.53, the highest pH variation between beginning and end being an increase of 0.42 pH units, as per **Table 3.4.**).

Table 3.4. Initial (I) and Final (F) soil pH values per sample type (Ct – Control; L – Low, 0.125g/Kg; M – Medium, 0.250 g/Kg; H – High, 0.500 g/Kg).

SAMPLE		pH (I)	pH (F)
PRISTINE EXPERIMENT SOIL	Ct	6.30	6.47
	L	6.17	6.43
	M	6.11	6.36
	H	6.12	6.53
WEATHERED EXPERIMENT SOIL	Ct	6.19	6.31
	L	6.60	6.45
	M	6.21	6.53
	H	6.19	6.34

Generally, the earthworms' survival was not significantly affected by either pristine or weathered microplastics. Nonetheless, high sensibility to physical contact was observed, particularly in treatments containing high concentration of pristine microplastics (although some cases were also observed in the weathered experiment, as can be seen in the supplemental photographs in **Annex VI**). Contaminated earthworms from both experiments presented greater sensitivity to touch (vigorous contouring movements, and not just enrolling as commonly observed in healthy worms) when comparing to both control and culture organisms.

Beyond sensitivity, and as per **Figure 3.4**, many cases of contaminated earthworms displayed yellowing in their derma and extremities. This yellowing of its surface is often interpreted as a sign of the excretion and accumulation of coelomic fluids, which are home to important immune cells in these organisms, coelomocytes, deployed in high stress situations, such as exposure to irritant factors or predation [10,19,20]. Such yellowish colour (accumulation of coelomic fluid) in their rear extremity is often related with possible fragmentation during physical or chemical stress.

Figures 3.4. Yellow patches observed in microplastic-contaminated trial parental earthworms from the pristine experiments, suspected to be coelomic fluid accumulations.

Other signs of stress were also found, with many contaminated earthworms displaying ring deformations reminiscent of premature cleavage furrows, and something that was also especially noticeable while they moved. Examples of these deformations can be seen displayed in **Figure 3.5.** These cleavage furrows constitute a process through which the earthworm self-amputates, something that generally happens to isolate the more critical parts of its organism after accumulating toxic matter in its rear end [22].

Figure 3.5. Retrieved P1 earthworms from the pristine experiments, sporting ring deformations with varying degrees of severity.

Finally, cases of severe morbidity were also observed, particularly at the highest concentration of pristine microplastics, with examples of earthworms with ring deformations so severe that they were barely attached at all. Moreover, some others were also very fragile, with several instances of earthworms' bodies getting detached during their retrieval, as can be found displayed in **Figure 3.6.** The number of instances, dubbed "morbidity" can be found in **Figure 3.7.**, along with recorded cases of mortality before retrieval. These tail detachments are a natural consequence of the previously described cleavage furrows, themselves caused by exposure to intense stress [22]. The appearance of these depressed areas helps explain how easy it was to accidentally provoke a detachment upon their retrieval. Still, it must be stated that, given the lack of fluids and haemolymph released upon their accidental detachment, that the cleavage process was nearing its completion to begin with.

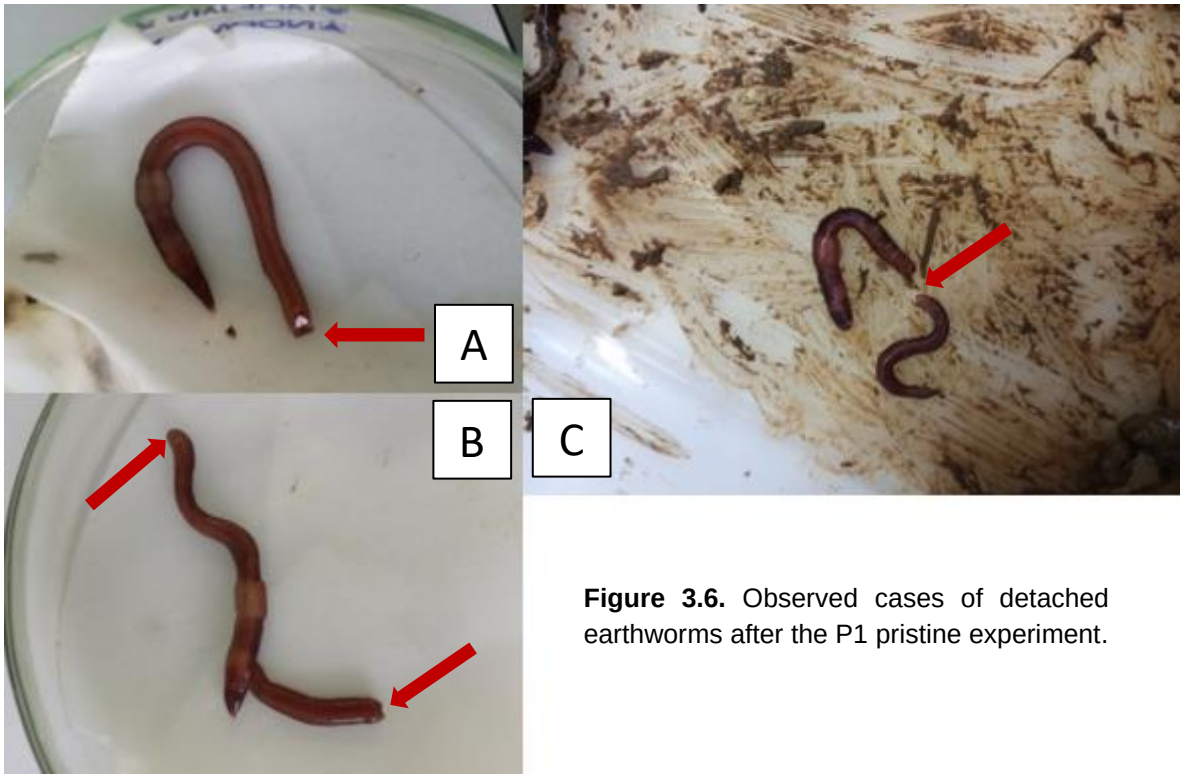


Figure 3.6. Observed cases of detached earthworms after the P1 pristine experiment.

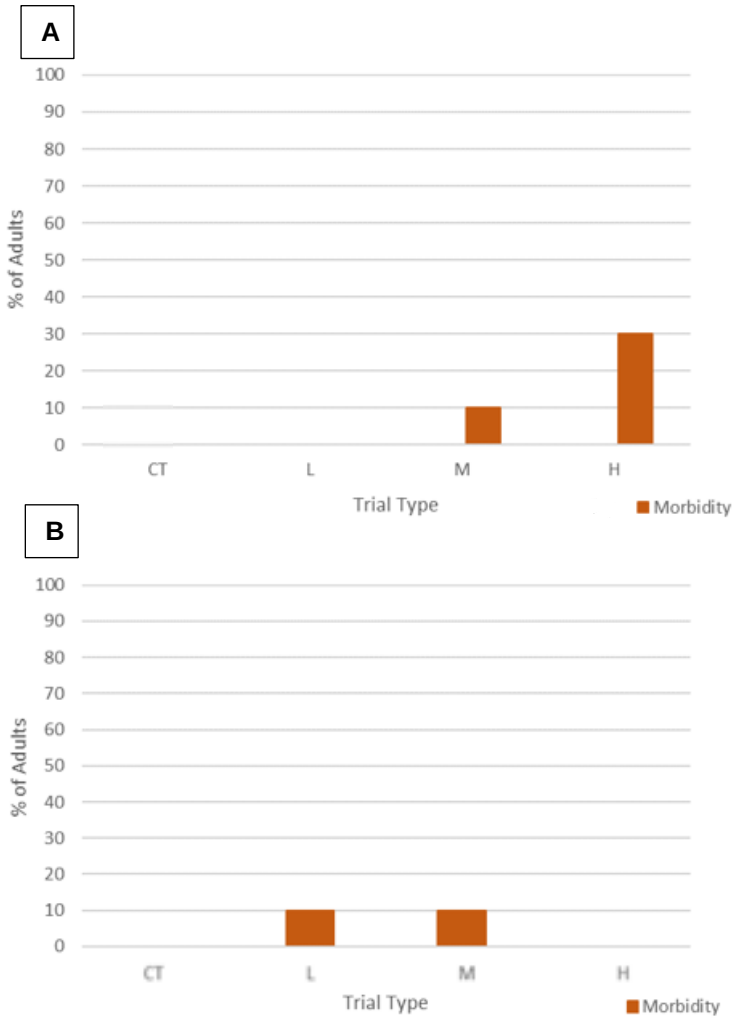


Figure 3.7.
Parental Morbidity Events at the end of the Pristine (A) and Weathered (B) Trials.

In sum, although there was a casualty in a control setting (potentially a random death), stress and morbidity seem to be largely induced by the plastic particles – in the Pristine Plastic Trial, specifically, the number of severe morbidity cases seem to take off in the conditions with the highest added microplastic content.

Another important ecotoxicological effect was observed in the number of earthworms offspring. Here, pristine microplastics significantly decreased the number of juveniles, right from the lowest concentration (see **Table 3.5**, and **Figure 3.8,** whose one-way analysis of variance (ANOVA), as per **Equation 1** is detailed further down in **Table 3.6**). Conversely, weathered microplastics that did not induce significant changes in the number of juveniles earthworms. These findings are congruent with a previous study, which reported a negative impact of pristine microplastics of PE, PLA and PPC in biomass and reproduction on the closely related earthworm *Eisenia fetida*, [6]. Such take is still far from consensual, with other findings arguing in defence of some microplastics' innocuity even at high concentrations, including some using Mater-Bi® formulations, as is the case with the presently tested mulch biofilm [23]. Furthermore, said study also reported that biodegradable plastics such as PLA, didn't appear to show higher ecotoxicological effects than their non-biodegradable counterparts (in the case of that study, Polyethylene). Another finding was that microplastic concentrations over 40 g/kg start provoking these effects, at a time concentrations in the environments were shown to reach as high as 67 g/kg [6].

Biodegradable plastics (biobased or not) have, also been reported to cause chronic effects on a variety of soil organisms other than earthworms, such as nematodes, snails, isopods and others [11], proving that even these materials misuse can end up posing the same threats and the ones they were designed to suppress (i.e., the petrochemical ones). In addition, it has been recently reported ecotoxicity of pristine microplastics in soil invertebrates, mainly nematodes and earthworms, affecting gut system (75% of the cases), behavioural, sensory and neuromuscular functions (55% of the cases), species fitness, including reproduction, development and survival, (43% affected), Immune system responses (50% affected), and metabolic activity changes (46% affected), as reviewed by Ji et al, 2021 [11]. Earthworms mostly presented deviations in DNA and carbohydrate metabolism, as well as 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 used in this study [11] were non-biodegradable, biodegradable materials do not necessarily have less damaging effects, making the relative lack of studies designed around these materials much more concerning, despite their recent growth and future expectations.

In this study, considering that the ingestion/egestion of microplastics were similar on both pristine and aged microplastics treatment, the absence of chronic ecotoxicity of weathered microplastics when comparing to pristine microplastics could be related with a potential degradation of the polymer by UV radiation (chemical desorption of plasticizers/additives during ageing process). Indeed, it was previously established that the chemical composition of the material shifted under UV type C radiation exposure when comparing to pristine samples, with the occurrence of what are suspected to be Norrish reactions. As such, it is perhaps this breakdown of the mulch biofilm that allows it to decrease its impact on earthworm offspring. These results point out that microplastics from mulch biofilms under relevant scenarios (i.e., UV aged) might impose low- threat to earthworms' fitting. Notwithstanding, it also highlights that behavioural and chronic endpoints should be considered in ecotoxicity tests for plastic products certification. As observed here, survival was not affected but the behaviour and reproduction were significantly impacted, particularly in treatments of pristine microplastics, something that highlights the unreliability of the most commonly applied tests, which base verdicts of ecotoxicity and environmental friendliness of plastics based only on survival endpoint.

Table 3.5. Parental (P1) and Offspring (F1) numbers for each replica and trial type (microplastic concentration in soil) in both Virgin and UV-C Exposed Microplastics.

TEST TYPE [MPS] G/KG	VIRGIN PLASTIC TRIALS				WEATHERED PLASTIC TRIALS			
	Earthworm numbers		F1/P1	Average F1/P1 ± std	Earthworm Numbers		F1/P1	Average F1/P1 ± std
	P1	F1			P1	F1		
0 (CONTROL)	**10**	55	5.5	6.0 ± 2.0	10	52	5.2	7.0 ± 1.0
	9	45	5.0		10	67	6.7	
	10	53	5.3		10	65	6.5	
	10	82	8.2		10	75	7.5	
0.125 (LOW)	**10**	45	4.5	4.6 ± 0.6	10	57	5.7	6.0 ± 0.7
	10	51	5.1		10	55	5.5	
	10	38	3.8		10	71	7.1	
	10	48	4.8		10	57	5.7	
0.250 (MEDIUM)	**10**	39	3.9	3.6 ± 0.8	10	65	6.5	5.7 ± 0.6
	10	32	3.2		10	56	5.6	
	10	46	4.6		10	54	5.4	
	10	28	2.8		10	53	5.3	
0.500 (HIGH)	**10**	37	3.7	3.6 ± 0.2	10	82	8.2	7.0 ± 1.0
	10	35	3.5		10	71	7.1	
	10	37	3.7		10	54	5.4	
	10	33	3.3		10	58	5.8	

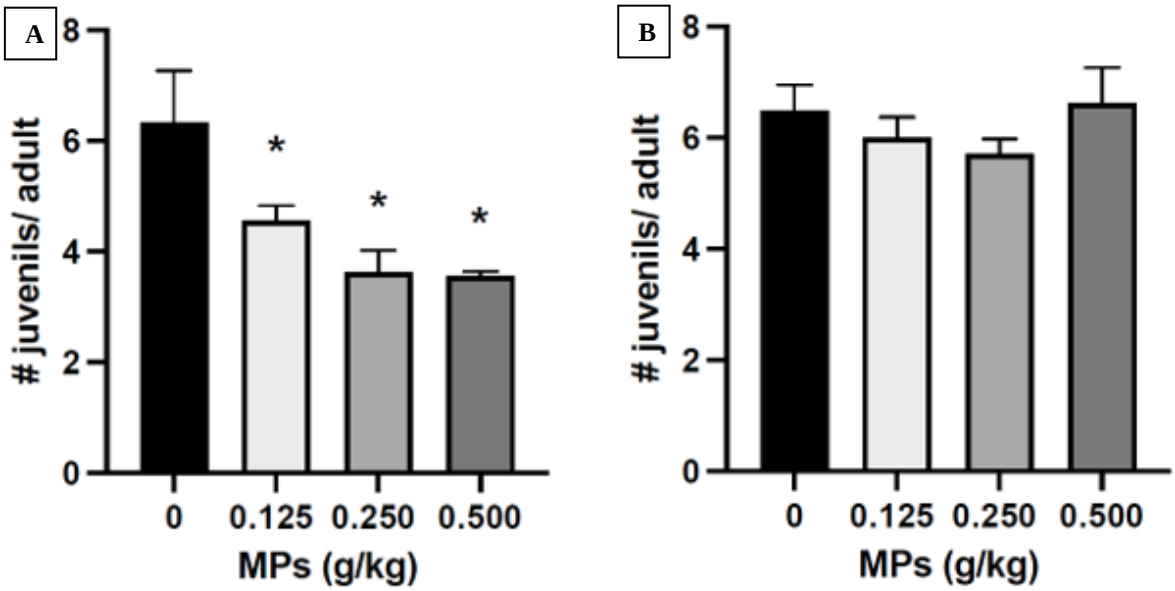


Figure 3.8. Offspring Numbers per Adult for each of the control and test conditions in both the Pristine Plastic **(A)** and UV-C Radiation-Exposed Trials **(B)**, with asterisks above columns denoting a statistically significant difference against control, as per Dunnett's method in the one-way ANOVA analysis.

Equation 3.1: ($F_{GDF,TDF} = F_{CV}$, P)

F_{CV} – F-Critical Value
P – P-Value
GDF – Degrees of Freedom between Groups
TDF – Total Degrees of Freedom

Table 3.6. Results from the one-way ANOVA analysis of both the pristine and weathered microplastics ecotoxicological experiments

Trial Type	*Analysis*	*GDF*	*TDF*	*F_{CV}*	*P*	*Significant (P < 0,05)*
Pristine	Between Groups	3	14	7.444	0.005	***Yes***
	Control vs Low				0.005	***Yes***
	Control vs Medium				0.004	***Yes***
	Control vs High				0.004	***Yes***
Weathered	Between Groups	3	15	0.861	0.488	***No***

3.3.4 FTIR-ATR Analysis of Parental Samples

The stress responses of organisms exposed to a variety of stressors has been studied through the observation of specific molecular changes (Muthukaruppan, 2015). As such, once again, a comparison of the FTIR spectra of adult earthworms, from both pristine and weathered experiments was performed. Said spectra, comparing samples from each experiment at different environmental microplastic concentrations, as well as comparing spectra relative to equal microplastic levels from both experiments, can be found in **Figure 3.9.**

In a typical spectra of *E. andrei***,** a broad peak was observed in the 3650-3000 cm^{-1} region, corresponding to OH bond vibrations from various possible sources, such as carboxyl, hydroxyl and phenol groups, as well as amides' NH vibrations. Next, the band with more defined peaks circa approximately 2918 and 2851 cm^{-1} were assigned to the asymmetrical stretching of methylene groups and CH_2 symmetrical stretching, respectively, which can used to monitor lipids, as well as proteins. The 1800-1460 cm^{-1} region has been assigned for proteins, the main molecules carrying the amide I and II functional groups (~1630 and ~1540 cm^{-1}, respectively), and also includes a peak at 1745-1720 cm^{-1}, consistent with carboxyl groups' CO elongation and OH deformations, which can also be related to a peak observable at approximately 1230 cm^{-1}, while the bands between 1450 and 1260 cm^{-1} have been attributed to proteins and lipids with CH_2, CH_3 and phosphate compounds (with the group PO). Near the lower extreme of the former region, a slim peak

between 1462 and 1454 cm^{-1}, which can be attributed to symmetric CH deformation from CH_2 groups, OH deformation and CO elongation from phenolic groups. The small indentation band in these regions, between 1745 and 1720 cm^{-1}, as well as those at 1600 cm^{-1} and in the vicinity of 1575-1540 cm^{-1} and 1390-1375 cm^{-1} are characteristic of COO- ions, while absorption at 1660-1620 cm^{-1} can typically be attributed to CC vibrations, in addition to quinines, conjugated carboxyl groups and ketones. The 1260-1180 cm^{-1} region corresponds to polysaccharides with the COC and COP functional groups. On lower wavenumbers, peaks can be found at 1150 and 1070 cm^{-1} corresponding to -C-O stretching (as well as CH_2 bending, in the case of the former peak). Finally, a peak at approximately 810 cm^{-1} can denote CH bending, whereas the more pronounced band at 750-600 cm^{-1} pertains to alkene (C=C) bending.

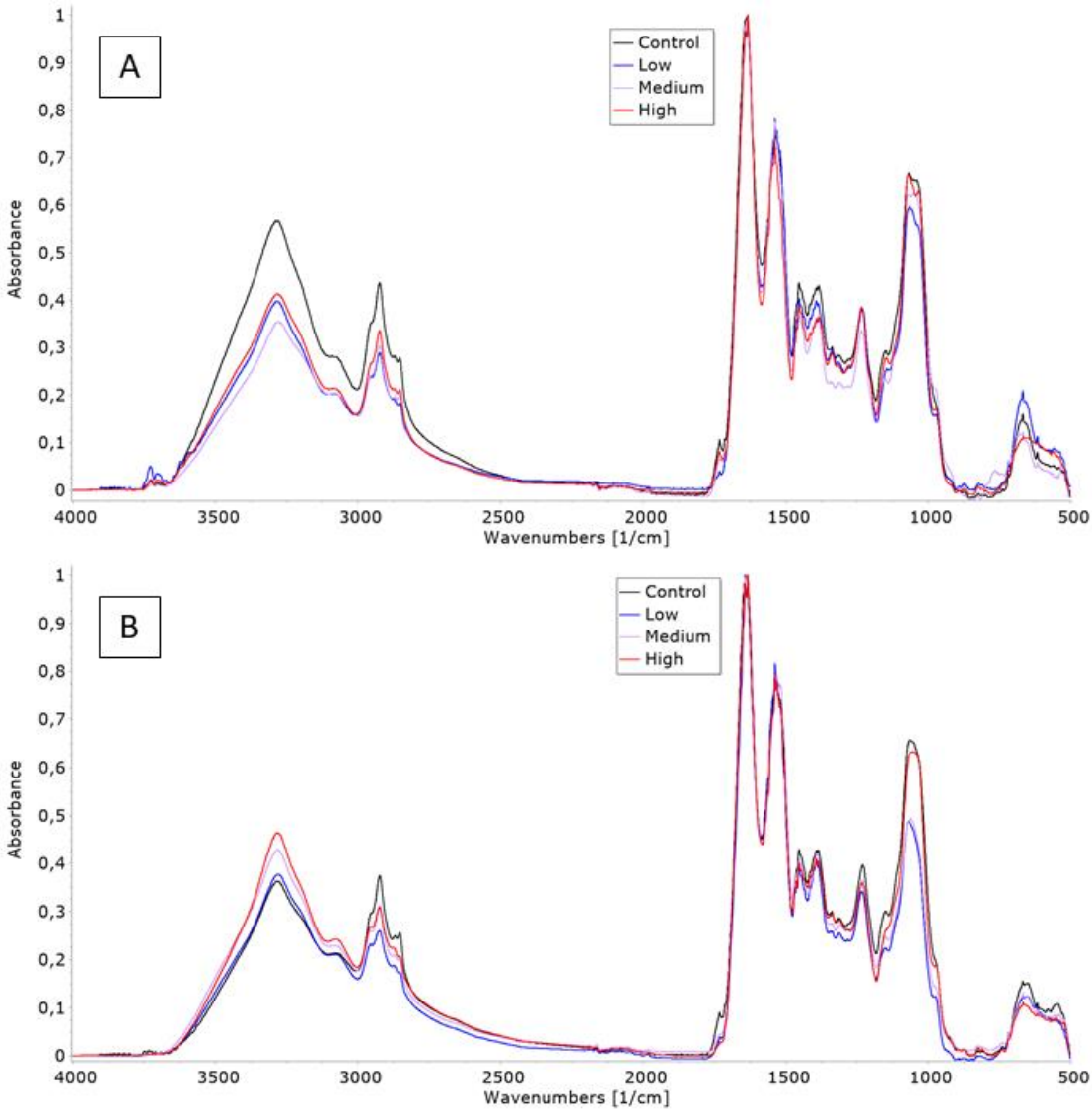


Figure 3.9. FTIR spectra of the *Eisenia andrei* exposed to 0.125, 0.250 and 0.500 g of pristine (A) or weathered (B) mulch biofilm microplastics per kg of dry soil.

FTIR spectra from earthworms exposed to pristine microplastics show no differences between themselves, regarding the present peaks in control and experimental samples. When it comes to intensities, however, some trends can be observed for several regions. The wide band centred at approximately 3275 cm^{-1} shows a decline with microplastics concentrations. The lowest recorded absorbances were relative to 0.250 mg of microplastics/kg samples, with samples from the low (0.125 mg/kg) and high (0.500 mg/kg) concentrations showing relatively close intensities, which suggests that this decrease stabilizes by the time concentrations reach the minimum level tested. The band immediately to the right, centred at 2925 cm^{-1} show similar declines, with control levels once again presenting higher absorbances whereas somewhat similar values, with an undiscernible concentration-dependent trend, were recorded for the different tested microplastic concentrations, results which lend themselves once again to the previous interpretation. As explained above, changes in these regions could be attributable to shifts in carbohydrate and lipidic contents, and thus the decrease in absorbances suggests a decrease in these energetic reserves that plateaus at or before the low concentration level of 0.125 grams of microplastics per kilogram of soil. The 1450-1260 cm^{-1} region also seems to show a decrease in absorbances as microplastic concentrations rise, with controls, low samples, and finally medium/high samples (relatively similar), in decreasing order of absorbance, which can be attributed to decreases in CH_2 and CH_3 groups commonly found in lipids and proteins. These results thus suggest a decrease in lipidic and protein reserves in tandem with the increase in microplastic concentrations up until around 0.250 g/Kg concentration, upon which these reserves seem to stabilize on these lower levels. Overall, the results obtained for the pristine plastic experiments suggest a negative impact on the energetic reserves from exposure to microplastic concentrations as low as 0.125 g/Kg, above which the observable further impacts on homeostasis seem to decline.

Such depletion on energy reserves on *E. andrei* (even in the lowest microplastic concentration) agree with the results observed in the reproduction, with a decrease on the number of juveniles in any of the microplastic treatment (i.e., low energy reserves low investment on the number of cocoons). Microplastics affecting the species' fitness have been reported in several studies, highlighting a decreased reproduction when organisms were exposed to increased doses of microplastics. For example, it has been reported that polyethylene microplastics can, upon ingestion, have direct adverse effects on the viability of earthworms' reproductive systems, namely in hindering spermatogenesis, as well as affecting important cells such as coelomocytes [24]. These result in impaired defense mechanisms, as well as on less successful reproduction, as observed in the pristine experiment. However, the impact on reproduction was not felt on the weathered experiment, in which the microplastics used were theoretically more brittle, and thus more likely to break down and internally affect the organisms, when comparing to the pristine plastics. As such, it is unlikely that the mulch biofilm replicates these results obtained using PE. It would, nevertheless, be interesting to analyze earthworms' reproductive systems after a pristine plastic experiment, in order to assess the impact on gametogenesis.

In the weathered microplastic experiment, conversely, in some regions absorbance values from samples retrieved from contaminated soil spiked over those corresponding to control conditions, suggesting energy reserves remained stable, or even increased in these organisms, as indicated by the increase of the ~3275 cm^{-1} band, proportionally to environmental microplastic concentrations (denoting an increase of carbohydrates) and the lack of discernible microplastic concentration-related trend (less so than the one observed for the pristine spectra) in the 1450-1260 cm^{-1} region, suggesting punctual variations of the lipidic and protein contents, although in lower levels than those found in organisms from

control conditions. This does not necessarily indicate that earthworms benefited nutritionally from the starch-based polymer, as this increase could be due to variations in the feeding of the earthworms, that is the unintentional placement of slightly higher amounts of feed in vials from the weathered experiments, coupled with the apparently negligible effects the mulch biofilm seems to have in its weathered form.

The passage of these particles along the organisms' gut might otherwise be a triggering factor in possible stress responses in earthworms, and more prolonged stay might be an amplifier, as microplastic ingestion has been reported to cause a several ailments in a diversity of species, with reports of a wide array of sub-lethal conditions, and metabolic changes in invertebrates, and varying impacts from gastrointensinal malfunctions due to blockage and perforation to blood chemistry alterations in vertebrates, as mentioned previously. In regard to earthworms specifically, the contact with microplastic particles and fibers such as nylon and polyethylene, with a size under 150 μm, has been correlated with increased stress events, oxidative stress levels, DNA damage, lower growth rates, higher mortality rates and even negative effects on reproduction on several earthworm species, such as *E. andrei, E. fetida*, *Enchytraeus crypticus* and *Lumbricus terrestris* [10,16,17,19].

As referenced before, in stress situations, earthworms such as *E. andrei* are known to employ coelomic fluid, which performs immunity-related roles, including the deployment of coelomocytes, and the transport of metabolites and proteins necessary for foreign body recognition and important enzyme cascades for the destruction of foreign material [10,20,21]. However, in neither situation was there a detection signalling the deployment of this fluid rich in proteins and other metabolites. Rather, whereas the absorbance levels of protein-associated group regions in *E. andrei'*s FTIR spectra in the weathered experiments fluctuated, in the apparently more stressful environments of the pristine experiments, the protein content of the analyzed organisms also seems to have decreased, contrary to previous reports studying the effects of PE microplastics on this species, which signalled an apparent increase.

To complement the analyses performed on the present study, other endpoints such as oxidative stress [23], energy reserves and metabolic reserves should be considered in future studies. In addition, for a more appropriate assessment of the metabolic effects of microplastic ingestion and persistence in earthworms' gastrointestinal tracts, which should be confirmed through histopathological analysis, by means of dissection and microscopy [10], the study of selective biomarkers could also provide key insights to the earthworm's apparently different response to the pristine and weathered mulch biofilm microplastic ingestion [25]. Finally, yet another possibility for studying stress response of *E. andrei* to contaminations by these particles could try to focus on gene expression of *in silico*-selected, stress response and regeneration-associated genes, through the real time PCR measuring of RNA expression when comparing to that of household genes [26]. More than just focusing on *E. andrei* itself, its own gut microbiota could also be the focus of further studies, given its possible role on the breakdown of ingested plastics [18].

3.4. Chapter Conclusions

Considering the results obtained in this chapter, several conclusions may be reached in regard to this specific biodegradable mulch biofilm's ecotoxicological effects on *E. andrei*, a species itself often used as indicator of soil health:

1. This mulch biofilm's weathering by UV radiation seems to induce a change in its chemical composition, as well as in its physical properties, although the latter are more difficult to assess when in the form of microplastics. Current literature points towards Norrish reactions, cleavage, and crosslinking events as a consequence of radiation absorption by photosensitive groups in the plastic.

2. The current ecotoxicological standards operate on too narrow a window to consider certified products as being completely environmentally friendly. Although the studied biofilm was certified, the presence of non-weathered plastic debris in soil seem to induce deleterious effects on worm physiology and reproductive output, with consequences comparable to those of non-biodegradable plastics. These behavioural and physiological changes observed in contaminated earthworms, under these treatments, cannot be neglected as they can impair future generations (and their reproductive output).

3. Results highlight that the inclusion of chronic endpoints should be prioritized on current standards for biodegradable products certification, together with particles size and shape of environmental relevance (e.g., test both pristine but also weathered microplastics, along with the biodegradation products from the biodegradation trials).

4. Despite the changes documented throughout this section, mostly induced by the presence of pristine particles in the testing environments, the practical relevance of said changes is diminished precisely by the nature of their application. Considering the practical application of this mulch film, its degradation sheltered from UV radiation and subsequent particle dispersion becomes exceedingly unlikely. Although it reinforced the importance of closely studying chronic endpoints due to possible ecotoxicological effects, under proper application, the ecotoxicological risk of this mulch biofilm is minimized.

3.5. References

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Chapter IV

Final Remarks and Future Research Perspectives

Throughout this thesis different sections, biobased plastics' potential for positive socioeconomical and environmental impacts have been highlighted, from reductions in fossil exploration and carbon footprints, to the propping up of local businesses. Considering the current environmental situation, a conversion of the plastics economy into a biobased, circular framework is imperative, if large scale health and environmental, and consequently socioeconomically disastrous scenarios are to be averted.

However, regarding biobased plastics as an absolute solution to today's plastic demand and environmental concerns can be counterproductive to the aim that is the establishment of a sustainable plastic economy. This stems from various factors, from humanitarian concerns over the use of feedstocks for plastic production rather than food, to the inadequacy of the currently installed plastic waste infrastructure. One of the bigger concerns is how the glamour of this new technology's green labelling could result in consumers neglecting the proper waste management practices. In the case of biodegradable plastics, where regulations were put in place specifying how this biodegradation process must play out, specific environmental conditions are key for their breakdown, so much so that in some scenarios, their rate of biodegradation is comparable to their non-biodegradable counterparts. And, with environmental persistence, so come potential ecotoxicological effects, made more dangerous for plastic's ability to propagate and accumulate throughout food webs.

To solve these issues, a firm and comprehensive regulatory framework must be put in place for plastics' certification and commercialization, and, to that end, the ecotoxicological testing standards must also be expanded significantly. Current standards have a heavy focus on acute endpoints, affecting individual survival, but weakly extend the same focus onto more endemic effects, such as populational health and reproduction. As such, to assess these concerns, this project was designed as two separate studies, each focusing either on the biodegradability or on ecotoxicological effects, using soil dwelling organisms, with the unifying factor being the tested polymer, a certified biodegradable, biobased agricultural mulch film.

in the first study, the biodegradation performance of this mulch film was evaluated when in contact with the fungal species *P. brevicompactum*, both in solid media and humidified soil, at 25ºC. While the solid culture media experiment seemed to indicate a positive influence of this fungal species on the biodegradation of the mulch film, the soil trial's results were inconclusive, when going by mass loss data. This was attributed to operator limitations, in the retrieval of the plastic material, given soil's more complex nature. The fact this was the first study in this laboratory using microplastics laid in soil made the experiment more challenging. This soil experiment was then redesigned, to increase its duration, and to try to better account for the number of microplastics laid out in each individual plot of soil. Despite the best efforts, this trial, too, returned inconclusive results going by the previously mentioned measurements. Thus, while the solid culture media trial (laboratory conditions) results seem to suggest that *P. brevicompactum* indeed assists this mulch biofilm's biodegradation process, the effort to study these processes and effects in conditions closer to the practical applications of the mulch film proved, at this point, fruitless. Extending the biodegradation trials over the tested 28 days could offer insight both on the long-term biodegradation trends, as well as on the accuracy of the recorded short term biodegradation results. The use of lower amounts of soil could help better understand fungal development and *P. brevicompactum*'s affinity towards the mulch film, especially after the complete consumption of the nutritional media, as well as lower the chances of accidental discarding of plastic material upon retrieval. Finally, a better monitorization of each individual microplastic particle in each soil sample surface could be crucial for the retrieval of all the originally placed plastic material, given the saline solution's proven unreliability. And to obtain the most accurate possible weight measurements, both initial and final weights should be obtained using a microbalance (0.000 mg).

Still considering this first research, FTIR spectra for both the mulch film and *P. brevicompactum* were also obtained, and cross compared, to determine whether or not one's presence and activity had a significant impact over the other's chemical composition. Qualitatively, no substantial differences were inferred using the obtained spectra for either the mulch film or the fungi other than the apparent collapse of peaks attributable to protein and lipid contents only in soil experiments, suggesting a physiological adaptation to a new growth medium, something that should be corrected in future experiments with an interim step of culture of the fungi in soil before the actual experiments. A closer analysis focus on the absorbances of regions and peaks of interest suggest an increase of carbohydrate contents in the fungi of the several experiments over time when in contact with the microplastics, with apparent decreases in protein and lipidic contents, whereas what appears to be an increase over time, and especially when in contact with fungi, in the amount of low molecular weight esters in the biofilm suggests higher levels of degradation in experimental conditions when comparing to samples from control conditions.

In sum, the results obtained in this section of the project could benefit from further studies expanding its scope, while also considering the shortcomings and acquired know-how from these initial trials, in order to safely determine *P. brevicompactum*'s role in the biodegradation of this agricultural mulch film, although these admittedly preliminary results are encouraging. Further studies might consider expanding the length and sample size of the experiments, as well as complementing the FTIR analysis with other approaches such as gene expression monitoring through real time PCR in order to better understand this fungal species' behaviour when in contact with this mulch biofilm.

In the second study, the ecotoxicological potential of this certified biodegradable agricultural mulch film, both in a pristine form and in a UV-C weathered condition, was evaluated, resorting to common organisms in agricultural environments, the earthworms *E. andrei*. Kept at 19ºC, under a $16h^{L}$:$8h^{D}$ light cycle after microplastic contamination, and fed recurringly every 7 days, the different earthworm parental populations were then analyzed for acute effects associated to the environmental contamination. Although mortality was non-existent in either study, thus safeguarding the mulch biofilm's certification, instances of mild to severe stress events were also observed, particularly as microplastic concentration in the environments went up. These ranged from the simple increase in sensitivity in some earthworms, to the more noticeable accumulations of coelomic fluids and instances of shape deformation, integral fragility or, in more extreme cases, bisections. On its own, the results from this first phase of the study would signal the potential need for an increase of the robustness of the regulatory framework for plastic biodegradability certifications, but the next phase returned more concerning results.

After the removal of the parental generations, the progenies were left incubating for an additional four weeks before retrieval and counting. While UV-C weathered microplastics did not seem to induce a significant decrease in offspring numbers when comparing to control populations, those exposed to pristine plastics suffered offspring losses of up to almost 44% in the worst tested scenario. Practically speaking, when utilized, this agricultural mulch biofilm can suffer significant UV radiation exposure, as it is standardly laid out in sheets over the soil. However, results from the pristine ecotoxicological trials are too concerning to be simply ignored, despite testing conditions using relatively high microplastic concentrations comparing to recorded occurrences – these testing conditions should not be considered environmentally unrealistic, so much as possibly a matter of time. And while this specific film is designed to fully biodegrade, it is not out of the question that other microplastics could have similar effects on progeny, and thus, the health of the species and the ecosystem.

A purging test was then performed to prove microplastic ingestion by these organisms. The results showed clear correlations between microplastic concentrations in the environment and egested microplastics, demonstrating *E. andrei* does not seem to avoid these particles during its feeding, at least in lower concentrations. With the egestion proven, the negative effects experienced through these trials were demonstrably tied to microplastic consumption. It would, nevertheless, be interesting to check whether microplastic particles remain within the organism after the purging process, and, if so, how often it occurs when comparing to egestion. Finding persistent plastics could give even more weight to the argument that these particles are indeed the cause of the toxicological effects reported on the observed *E. andrei* populations.

FTIR spectra were also recorded for these specimens, but no new peaks were found when comparing to control conditions. The analysis of peak intensities, on the other hand, reveals that earthworms raised in the pristine trials showed, in their FTIR spectra, stress-related symptoms, with what seems to be a decrease in energetic reserves across the board when comparing with samples from control conditions, with lower levels of carbohydrates, proteins and lipids. Results relative to organisms from the weathered experiments, however, seem to suggest an increase over time in carbohydrate contents, though lipids and proteins still suffer from a deficit when comparing to controls; although other studies speculate about earthworms' capabilities to metabolize biodegradable polymers, this increase could also

stem from user error in the feeding process. In any case, this is yet another possibility that could be explored further.

Considering these results, follow up tests should be conducted in order to confirm both of these experiments' results, especially in regard to offspring numbers: other trials previously conducted with Mater-Bi®, a formulation used for biodegradable mulch films, including the one tested in this work, returned dissimilar results, and, as such, this matter requires further investigation. Apart from the completion of the biochemical analysis of the remaining earthworms, with approaches such as histopathological analyses, monitoring of biomarkers and oxidative stress, as well as the study of gene expression, or even the study of the effects of the earthworm's gut microbiota on ingested microplastics, it would also be of interest to repeat these procedures with other plastic types, both biodegradable and not, in order to determine how different types of microplastics influence important biological factors not yet widely tested as part of certification processes, such as survival of progeny as done in this study. Should these findings, despite the practical ecotoxicological potential of this mulch biofilm being minimal, be replicated using other types of biodegradable plastics, and no significant differences found between the effects of biodegradable and non-biodegradable plastic particles, a strong, unignorable argument could be made for the strengthening of current standards, proven in this present thesis to be possibly fallible, in order to protect the fitness of soil dwelling species.

To sum it up, the presently used agricultural mulch biofilm's biodegradability was not called into question in this study, instead the obtained results point that its biodegradation performance could even be enhanced through the participation of key soil dwellers such as *P. brevicompactum*, despite the limited success on the soil biodegradation trials. More concerning, however, is the effect plastic particles can have on other soil dwelling species, such as *E. andrei*, somewhere between their fragmentation into sizes that allow ingestion by them, and the point full degradation is achieved. During said timeframe, species such as *E. andrei* are exposed to potentially toxic particles with effects on its reproduction, thus with high potential of ecosystem destabilization. As such, an insufficiency of currently applied certification standards was exposed, and pending confirmation studies with other types of plastics, the rectification of these oversights becomes extremely important in order to protect soil ecosystems' health.

Annex I

15-day soil biodegradation experiment results

Before the realization of the 28-day biodegradation experiment, another was conducted in the same experimental conditions, on a similar timeframe as the solid culture media experiment. Bellow follows the data and results obtained throughout this interim experiment, which help frame the results of the 28-day experiment. FTIR-ATR spectra relative to this experiment can be found later in **Annex III**.

Table I.1. Removal of microplastics during the 15-day soil biodegradation experiment.

Retrieval Timepoint (Days)	***Replica***	***MPs (g)***	***Recovered MPs (g)***	***MPs removal (average ± std) (g)***	***MPs removal (%)***	***MPs removal (average ± std) (%)***
5	Replica 1	0.0034	0.00034	0.002 ± 0.001	90	69 ± 30
	Replica 2	0.0032	0.0020		36	
	Replica 3	0.0030	0.0015		51	
	Replica 4	0.0030	0.000072		97	
	Control 1	0.0030	0.0018	0.002 ± 0.001	39	69 ± 42
	Control 2	0.0032	0.000042		99	
10	Replica 1	0.0033	0.0030	0.00034 ± 0.00005	9.2	10 ± 2
	Replica 2	0.0030	0.0026		13	
	Replica 3	0.0030	0.0027		10	
	Replica 4	0.0031	0.0016		50*	
	Control 1	0.0033	0.0039	-	-	-
	Control 2	0.0031	0.0038		-	
15	Replica 1	0.0031	0.0034	0.000088	-	3
	Replica 2	0.0031	0.0033		-	
	Replica 3	0.0032	0.0034		-	
	Replica 4	0.0033	0.0032		2.7	
	Control 1	0.0032	0.0030	0.00016	4.8	5
	Control 2	0.0032	0.0038		-	

**Rejected by Q-test*

Table I.2. Biomass of *P. brevicompactum* from the first soil experiment.

Retrieval timepoint (days)	***Sample Type and Number***	***Initial Biomass (mg)***	***Initial Biomass Average ± std (mg)***	***Final Biomass (mg)***	***Biomass Variation (mean ± std) (mg)***
5	Replica 1	120	118 ± 3	17.9	-91 ± 20
	Replica 2	120		18.2	
	Replica 3	116		17.7	
	Replica 4*	115		53.7	
	Control 1	120	133 ± 28	47.9	-103 ± 44
	Control 2	161		26.5	
10	Replica 1	126	132 ± 10	20.4	-98 ± 24
	Replica 2	125		39.1	
	Replica 3	129		55.7	
	Replica 4	146		17.8	
	Control 1	143	135 ± 6	13.2	-122 ± 11
	Control 2	135		21.0	
15	Replica 1	126	137 ± 13	8.38	-109 ± 21
	Replica 2	135		18.0	
	Replica 3	155		30.1	
	Replica 4	130		52.0	
	Control 1	109	122 ± 19	45.4	-76 ± 18
	Control 2	135		46.1	

Table I.3. Fungal areas from the soil biodegradation experiments.

	15 Day Trial		
Sample Type and Number	***Retrieval timepoint (days)***	***Area (cm^2)***	***Area (average ± std) (cm^2)***
Replica 1	***5***	3.29978	2.8 ± 0.4
Replica 2		7.93614*	
Replica 3		2.53748	
Replica 4		2.61040	
Control 1		12.9816	16 ± 4
Control 2		18.1646	
Replica 1	***10***	2.08004	1.5 ± 0.5
Replica 2		1.06860	
Replica 3		1.77760	
Replica 4		1.16892	
Control 1		14.3232	8 ± 9
Control 2		1.14780	
Replica 1	***15***	0.67588	1.1 ± 0.6
Replica 2		0.63685	
Replica 3		1.86208	
Replica 4		1.04858	
Control 1		1.39054	1.41 ± 0.02
Control 2		1.41975	

** Rejected by Q-test*

Annex II

Penicillium brevicompactum Growth Gallery

In this Annex, all photos taken in order to record the growth *P. brevicompactum,* as well as for the calculation of the superficial areas throughout all of the biodegradation experiments will be presented. All petri dishes had a diameter of 8 cm, used for setting the scale, and confirmed in some pictures by ruler.

As discussed in **Chapter II**, the pictures relative to the 7-day samples in the 28-day Soil Experiment were lost, due to hardware failure, and as such the section 28-day experiment section starts with the 14-day images.

Table II.1. Solid Culture Media Trial – Day 5

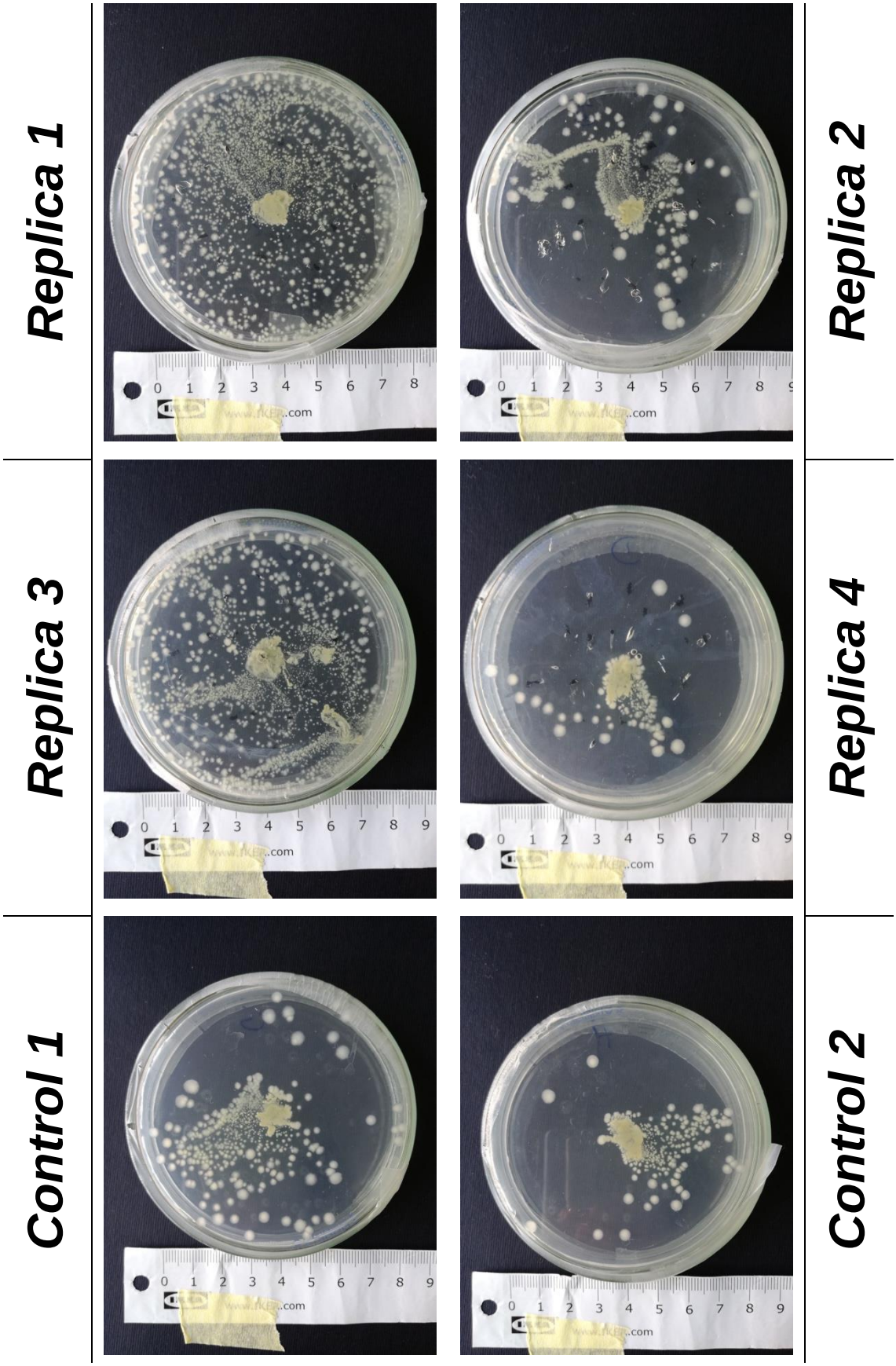


Table II.2. Solid Culture Media Trial – Day 10

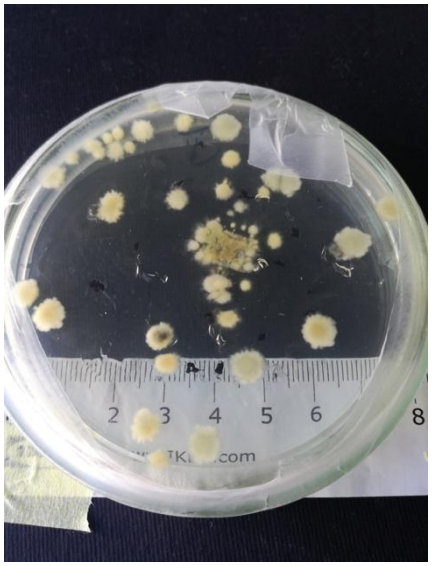
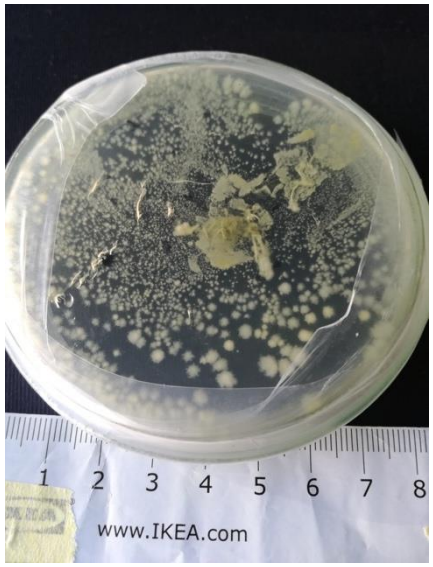
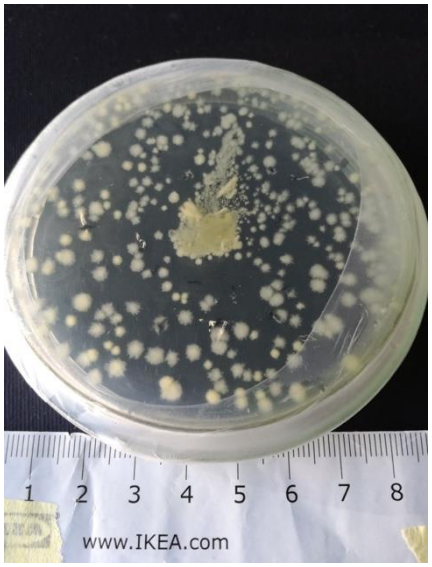
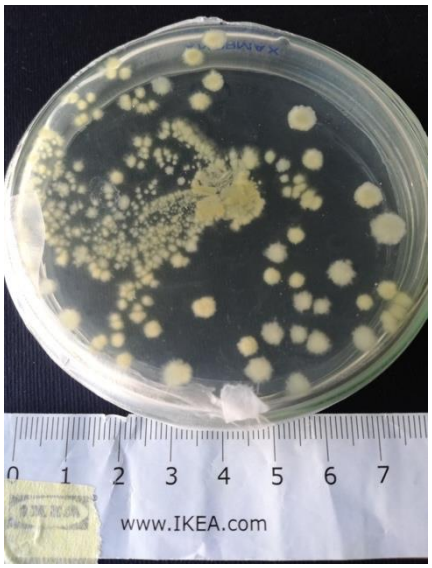
Replica 1			*Replica 2*
Replica 3			*Replica 4*
Control 1			*Control 2*

Table II.3. Solid Culture Media Trial – Day 15

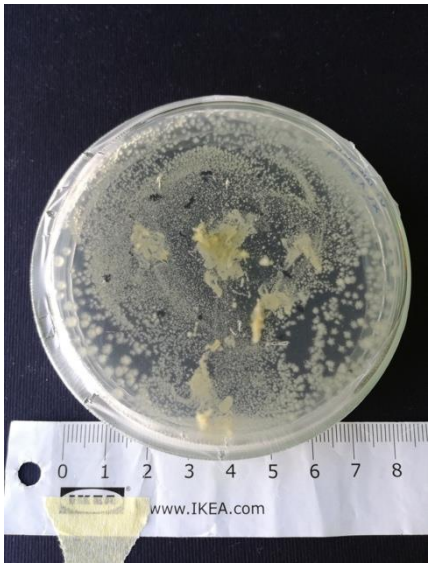
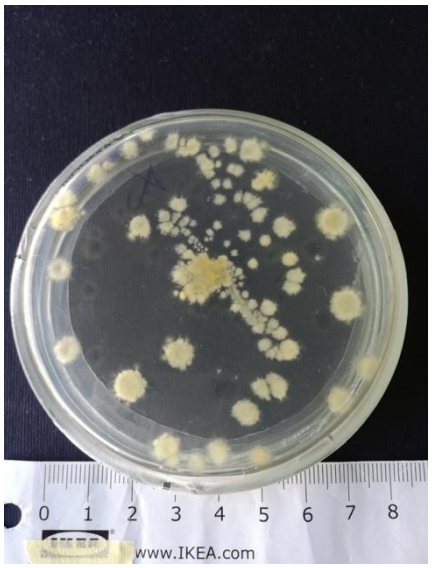
Replica 1		 *Replica 2*
Replica 3		 *Replica 4*
Control 1		 *Control 2*

Table II.4. 15-Day Soil Media Trial – Day 5

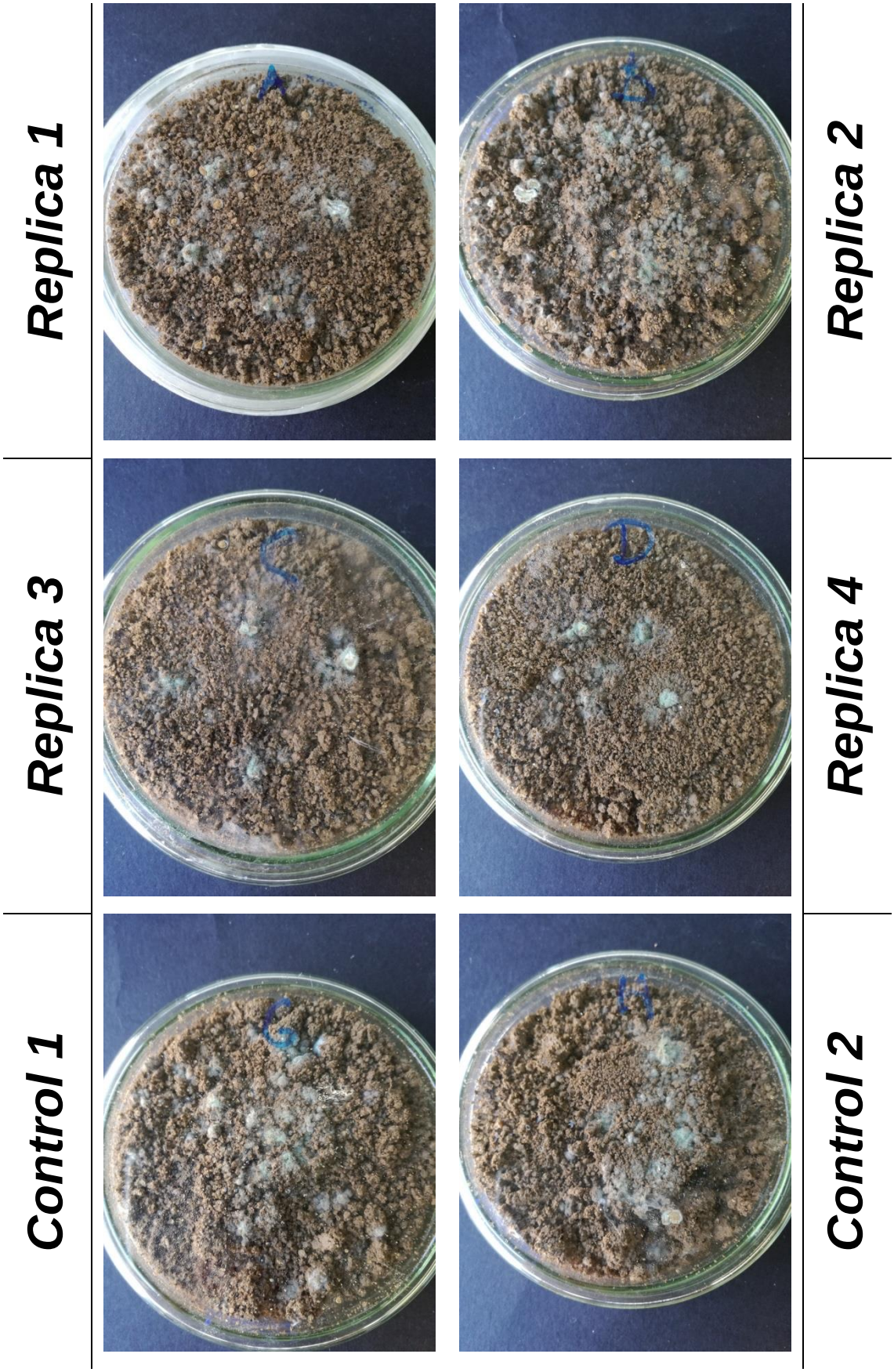


Table II.5. 15 Day Soil Media Trial – Day 10

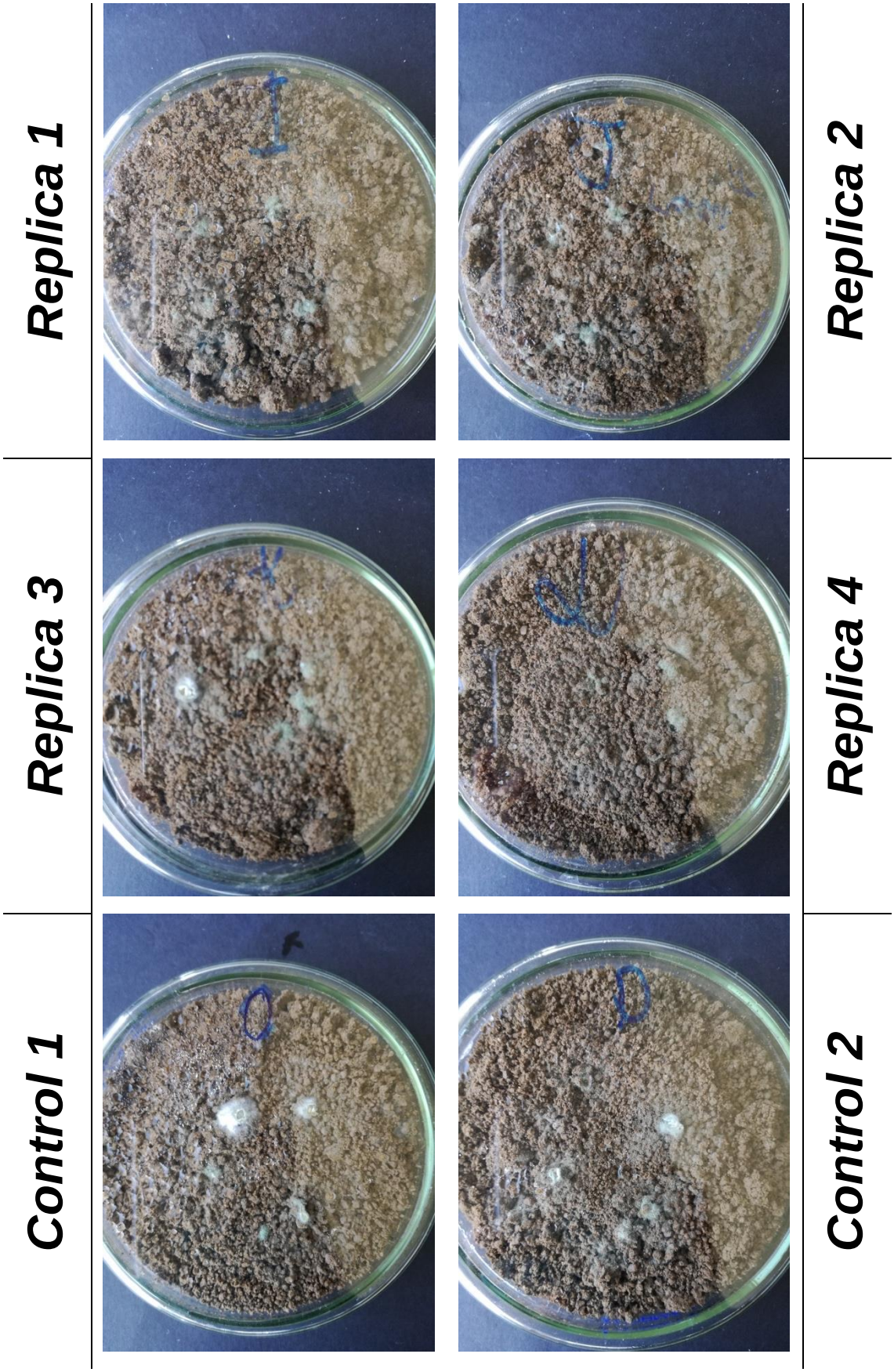


Table II.6. 15 Day Soil Media Trial – Day 15

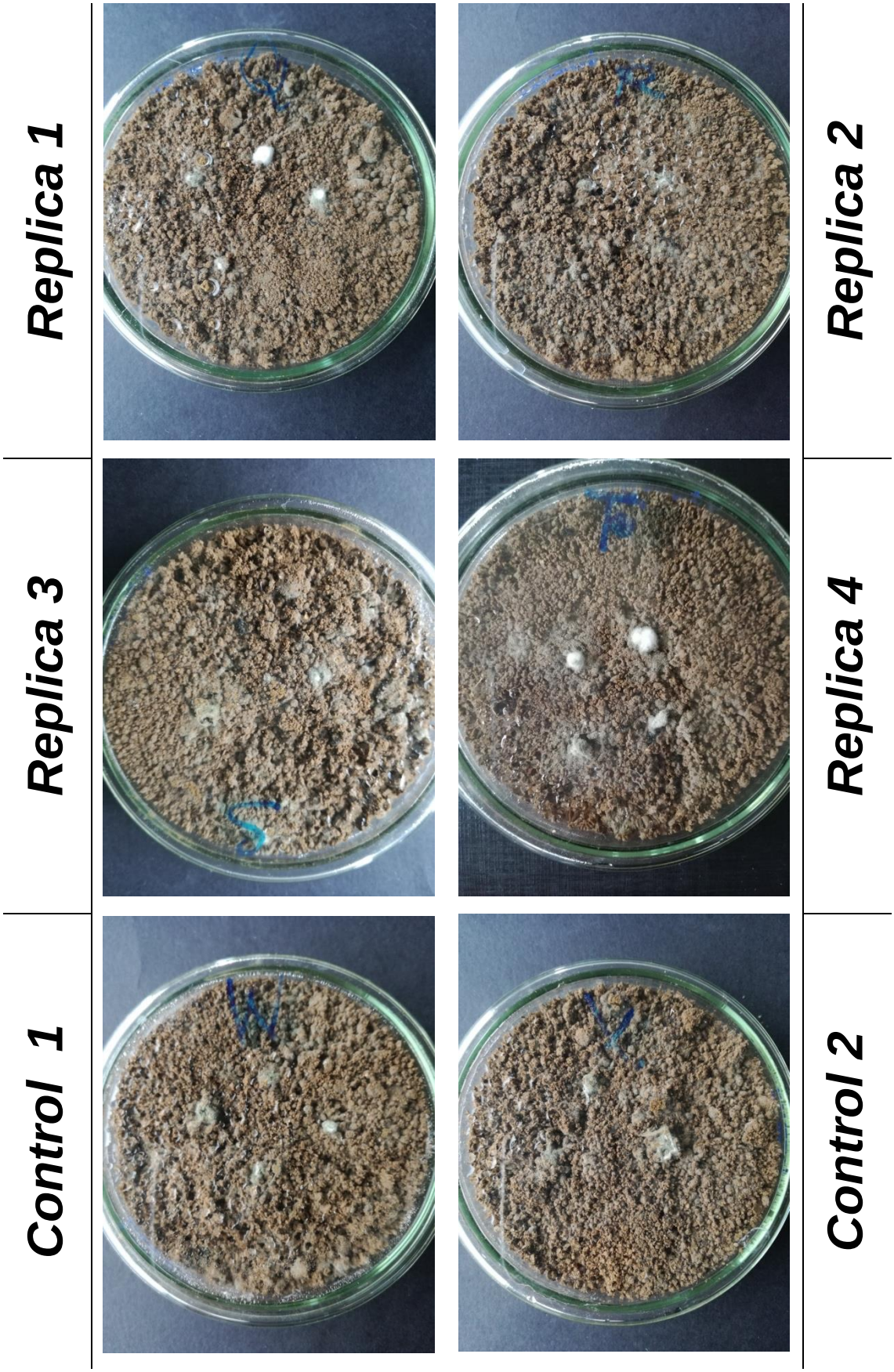


Table II.7. 28 Day Soil Media Trial – Day 14

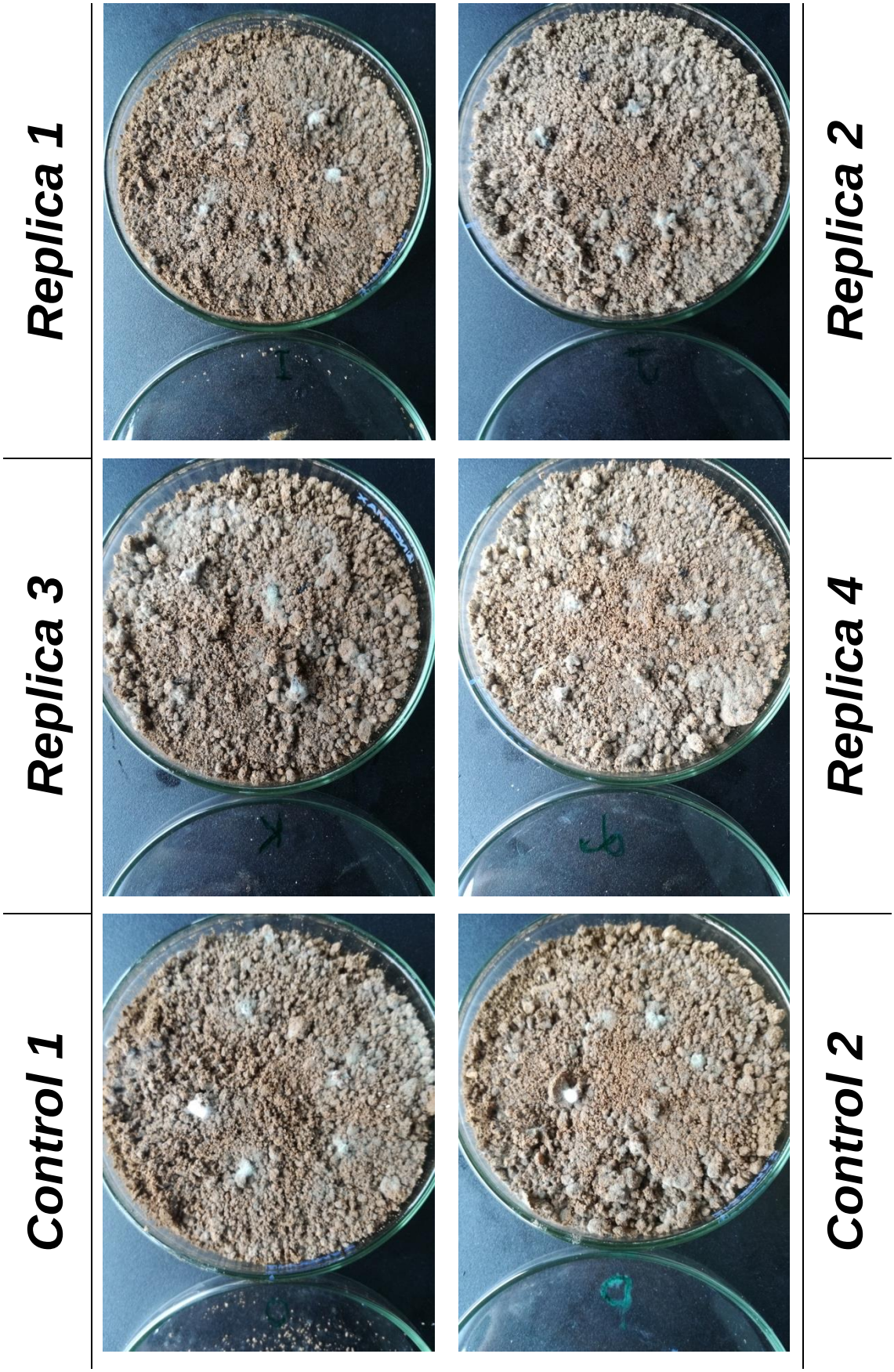
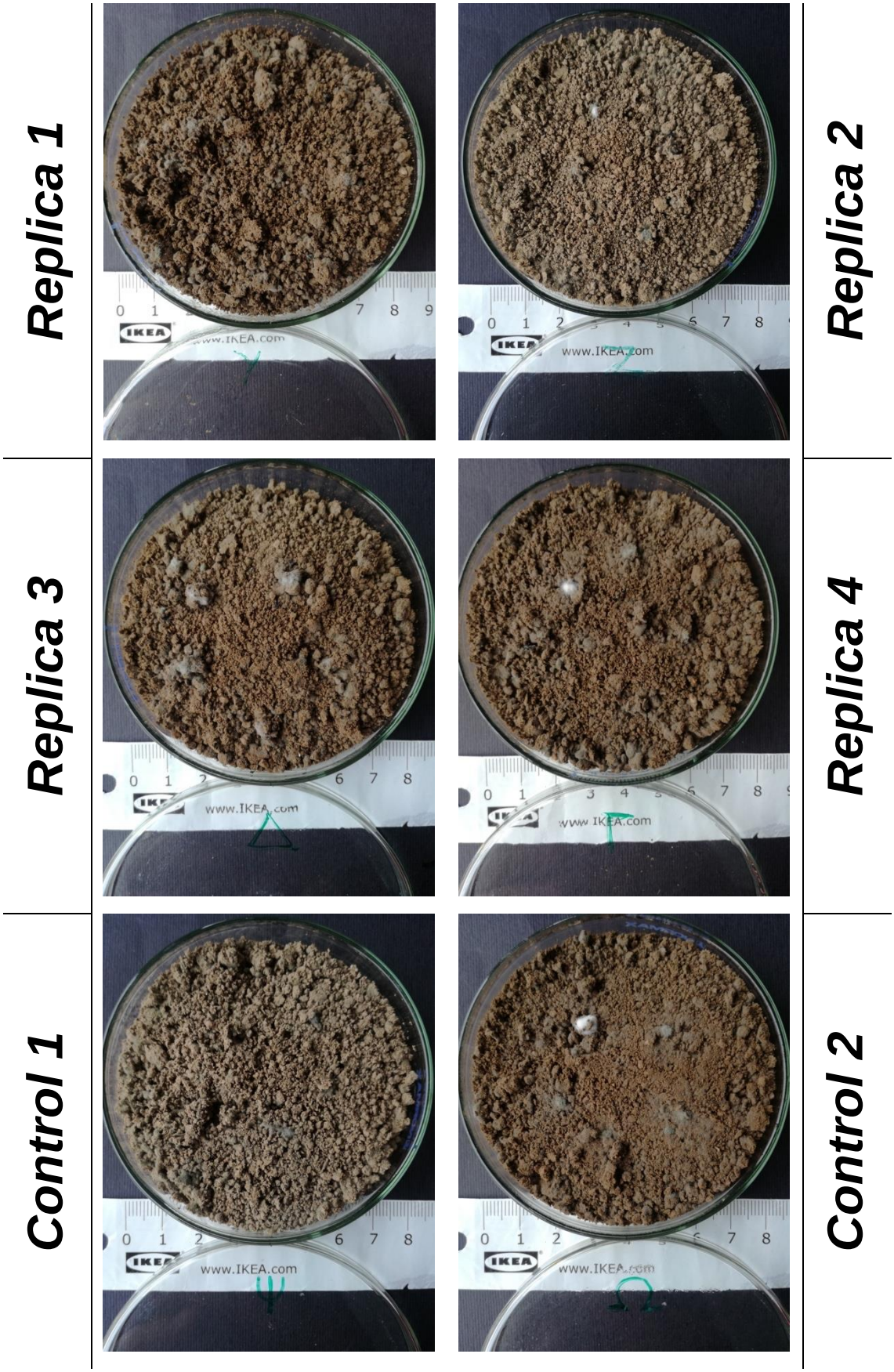


Table II.8. 28 Day Soil Media Trial – Day 21

Replica 1		 *Replica 2*
Replica 3		 *Replica 4*
Control 1		 *Control 2*

Table II.9. 28 Day Soil Media Trial – Day 28



Annex III

Replica and Control FTIR Spectra Comparison

In this Annex, the control sample plots used as term of comparison, as well as the previously displayed replica sample plots for both *P. brevicompactum* and the mulch biofilm can be found.

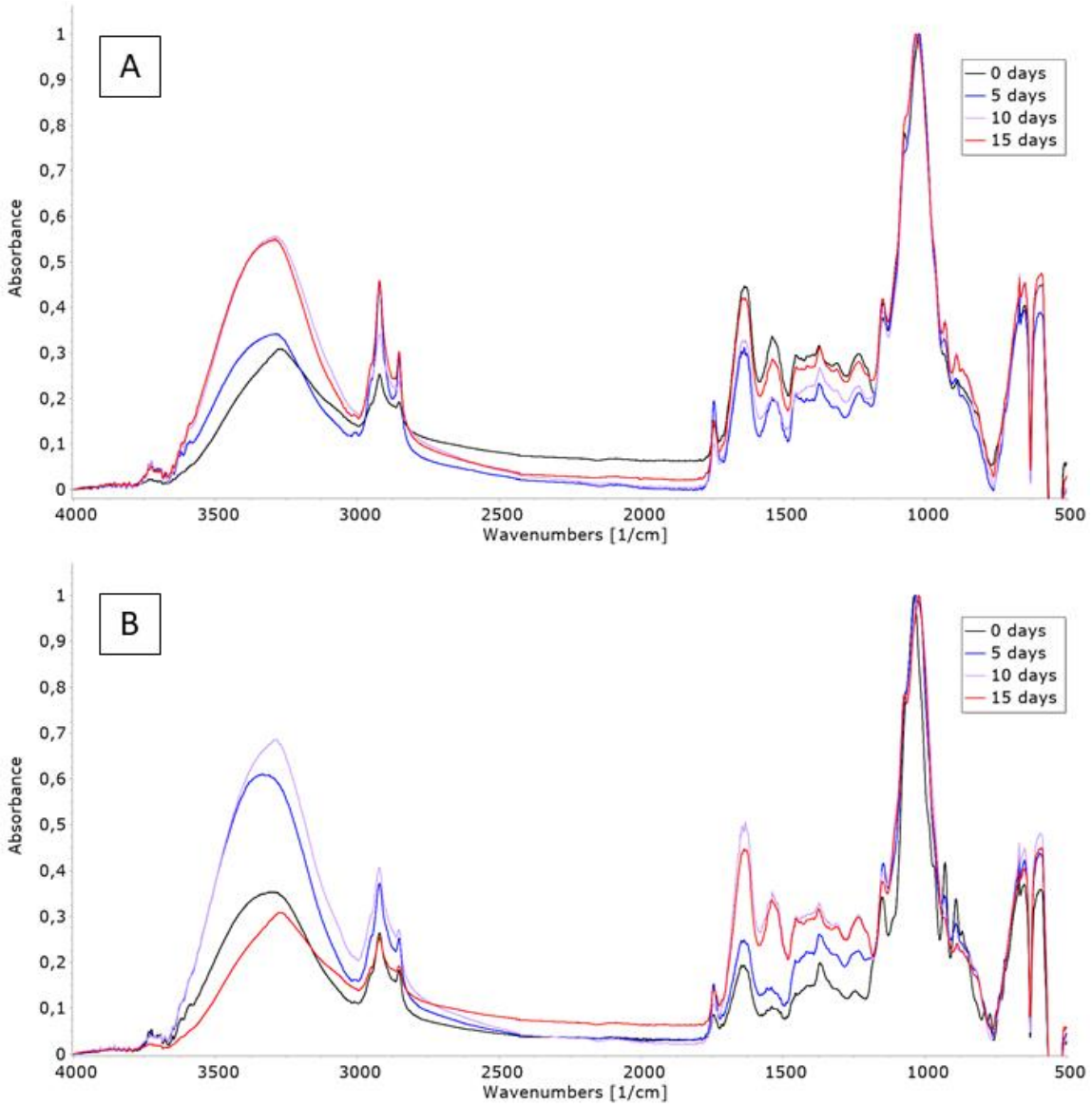


Figure III.1. Spectra from FTIR analysis of the fungal samples from control and replica conditions collected throughout the solid culture media experiment (A – Replicas, B – Controls)

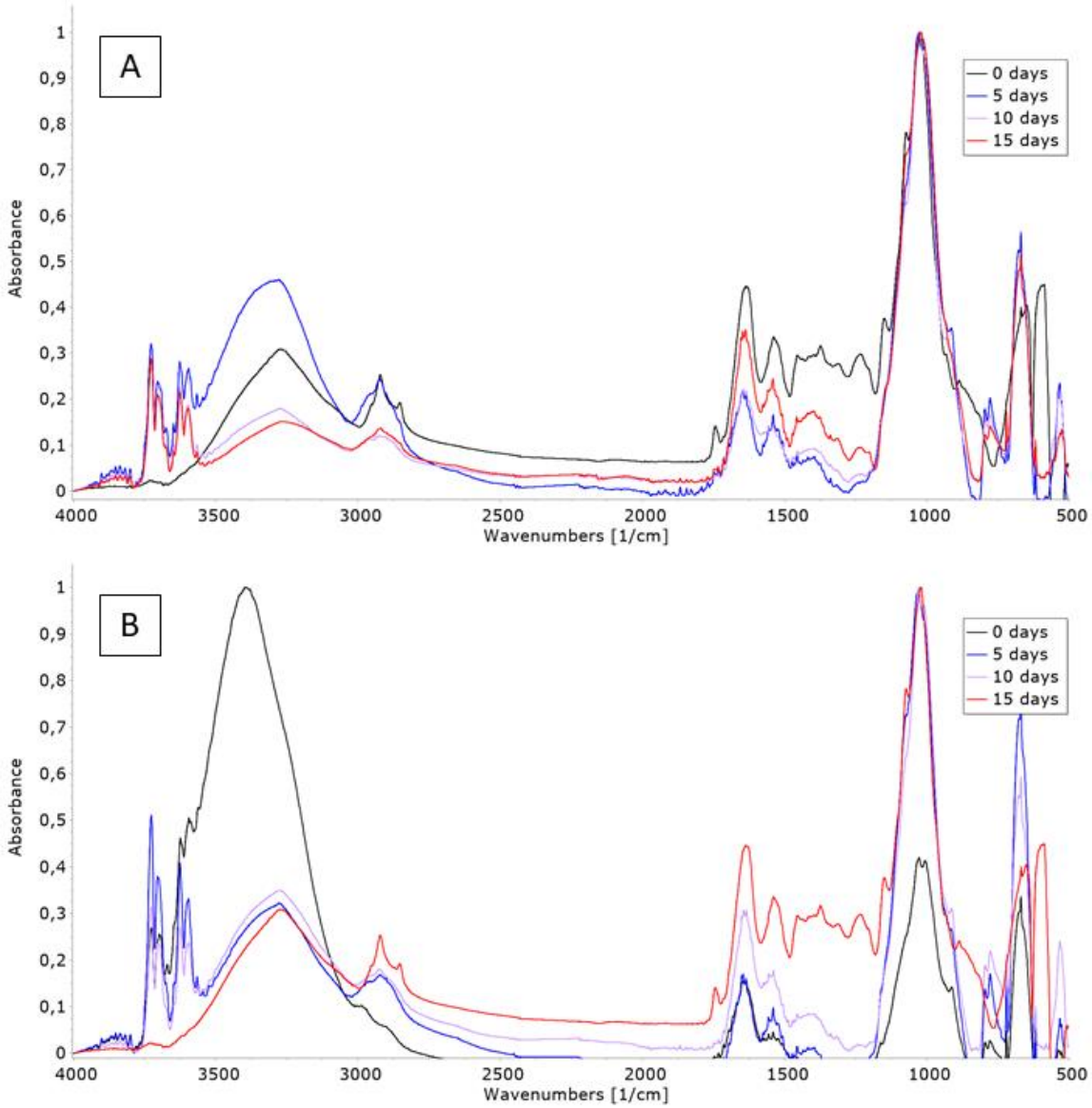


Figure III.2. Spectra from FTIR analysis of the fungal samples from control and replica conditions collected throughout the 15-day soil experiment (A – Replicas, B – Controls)

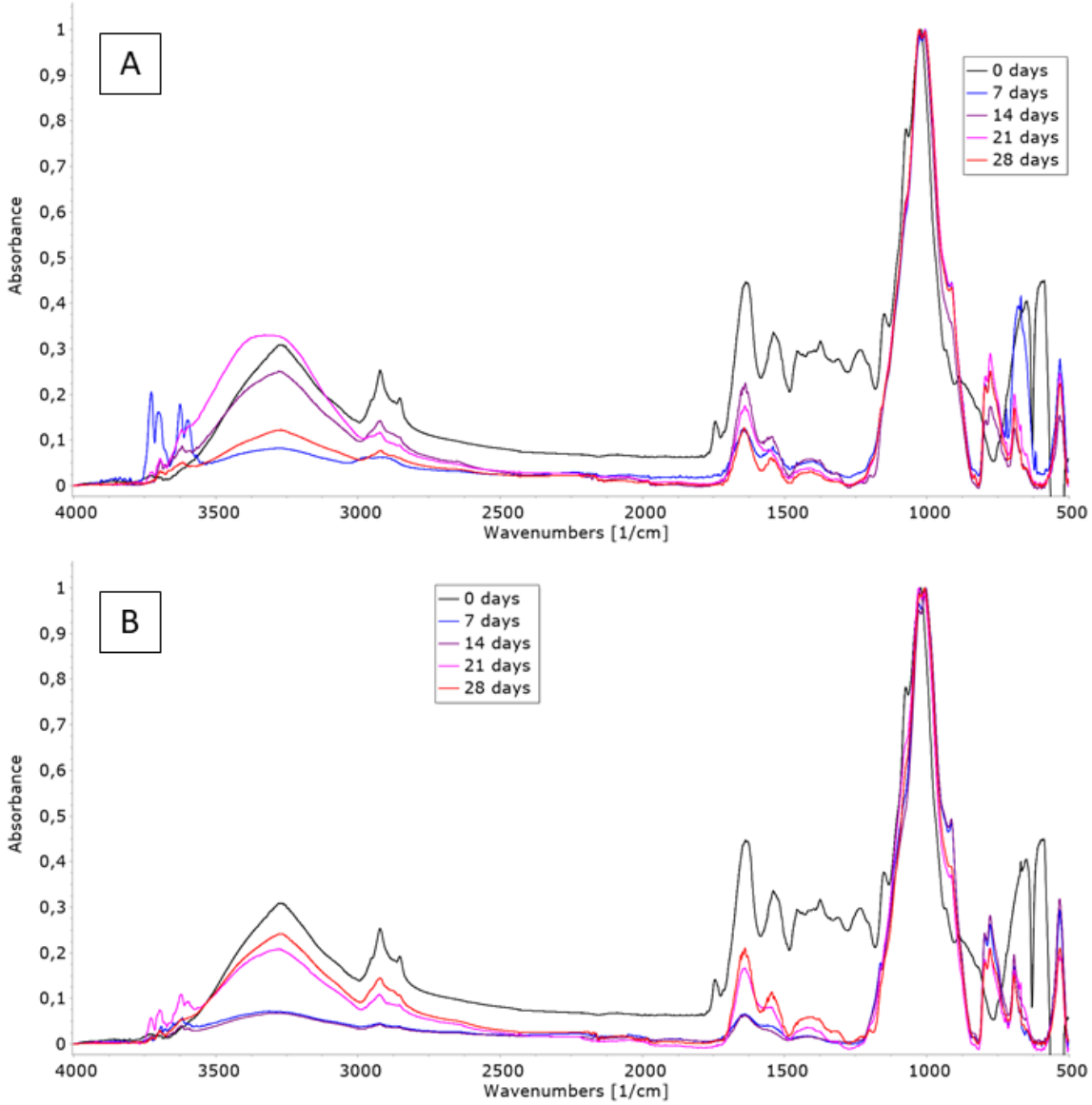


Figure III.3. Spectra from FTIR analysis of the fungal samples from control and replica conditions collected throughout the 28-day soil experiment (A – Replicas, B – Controls)

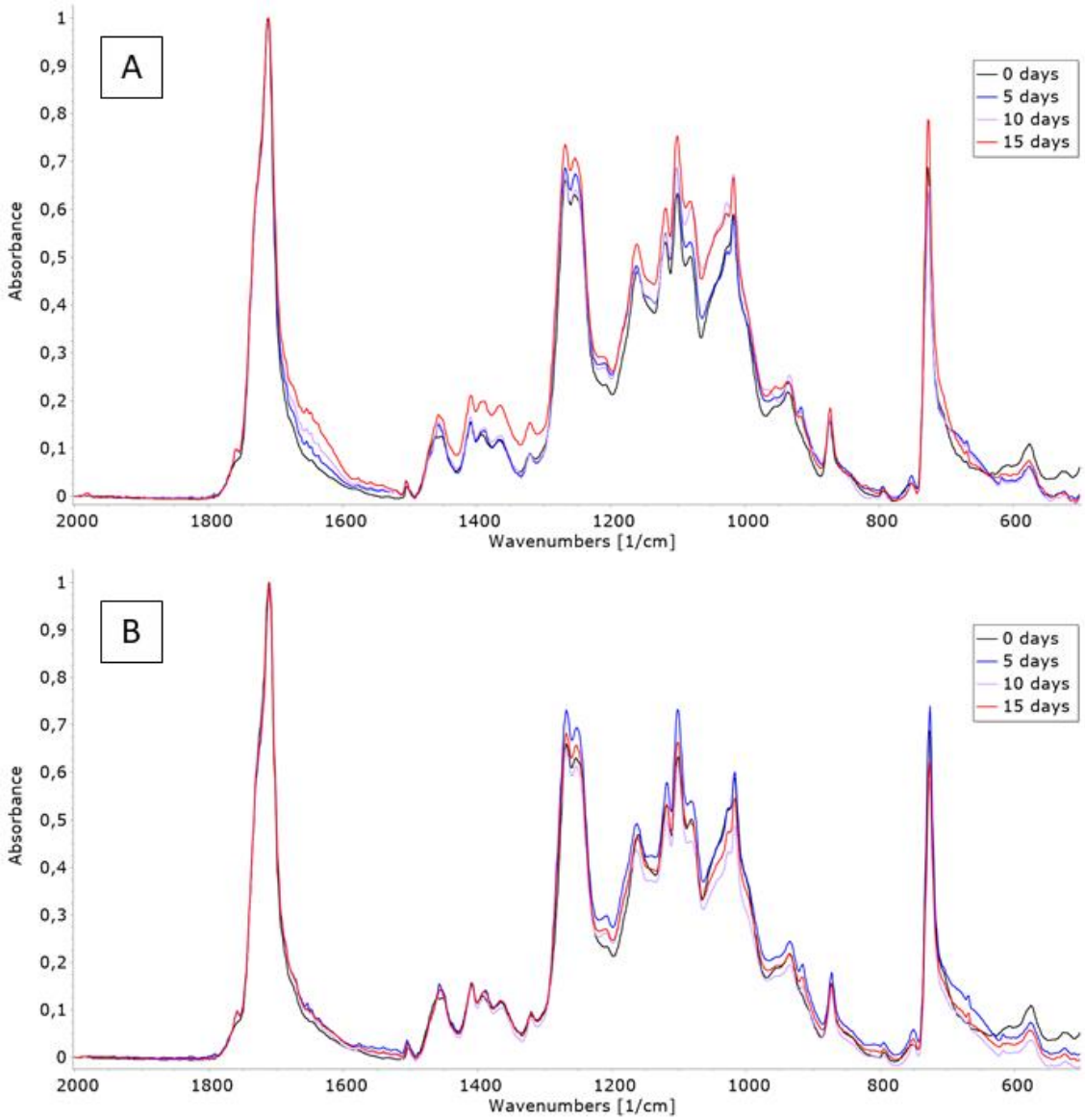


Figure III.4. Spectra from FTIR analysis of the mulch biofilm samples from control and replica conditions collected throughout the solid culture media experiment (A – Replicas, B – Controls)

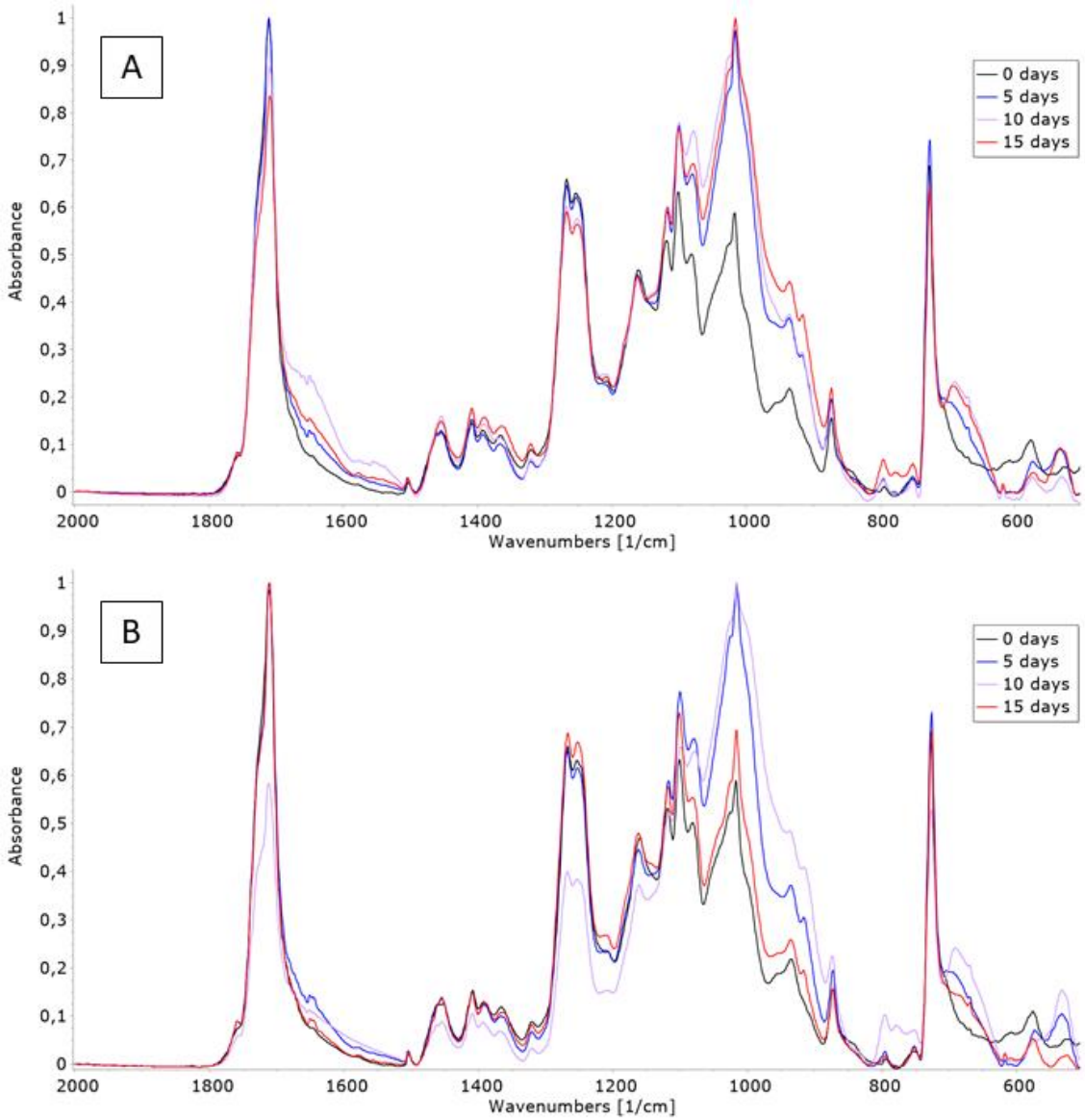


Figure III.5. Spectra from FTIR analysis of the mulch biofilm samples from control and replica conditions collected throughout the 15-day soil experiment (A – Replicas, B – Controls)

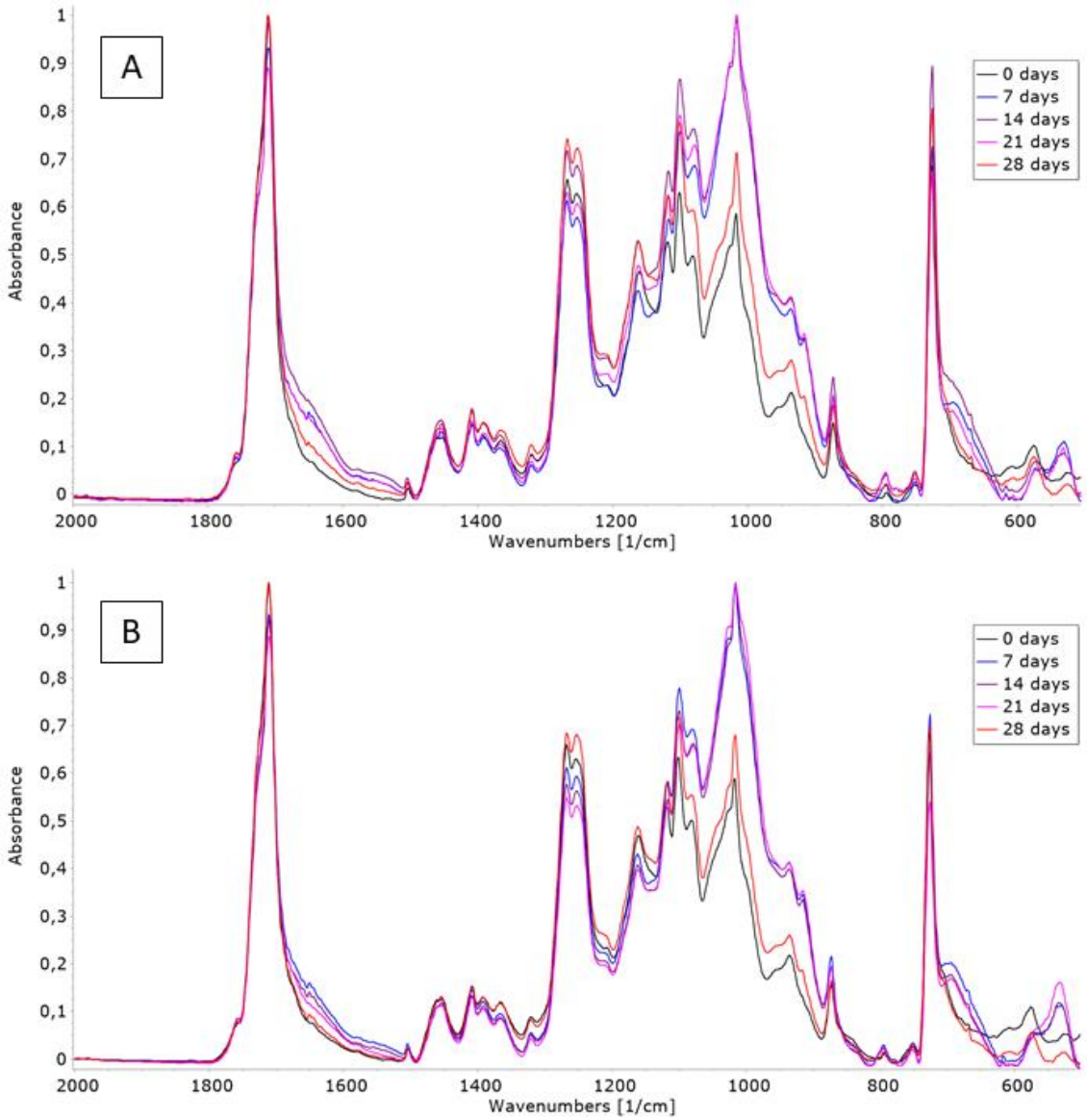


Figure III.6. Spectra from FTIR analysis of the mulch biofilm samples from control and replica conditions collected throughout the 28-day soil experiment (A – Replicas, B – Controls)

Annex IV

Penicillium brevicompactum FTIR Spectra

In this Annex, all *P. brevicompactum* spectra obtained throughout the several solid media and soil biodegradation trials can be found. Every spectrum group has been colour-coded so that within each image, replicas have a progressively darker shade of orange, and controls a progressively darker shade of blue, as per the letter codes used throughout the trials, which can be consulted below, in **Table IV**.

Table IV. List of denominations given to each sample, as presented in each of the following plots.

Sample Origin	Denomination			
	1st Timepoint	2nd Timepoint	3rd timepoint	4th Timepoint
Replica 1	A	I	Q	Y
Replica 2	B	J	R	Z
Replica 3	C	K	S	Γ
Replica 4	D	L	T	Δ
Plastic Control 1	E	M	U	Σ
Plastic Control 2	F	N	V	Φ
Fungi Control 1	G	O	W	Ψ
Fungi Control 2	H	P	X	Ω

Figure IV.1. Solid Culture Media Experiment – 5th day

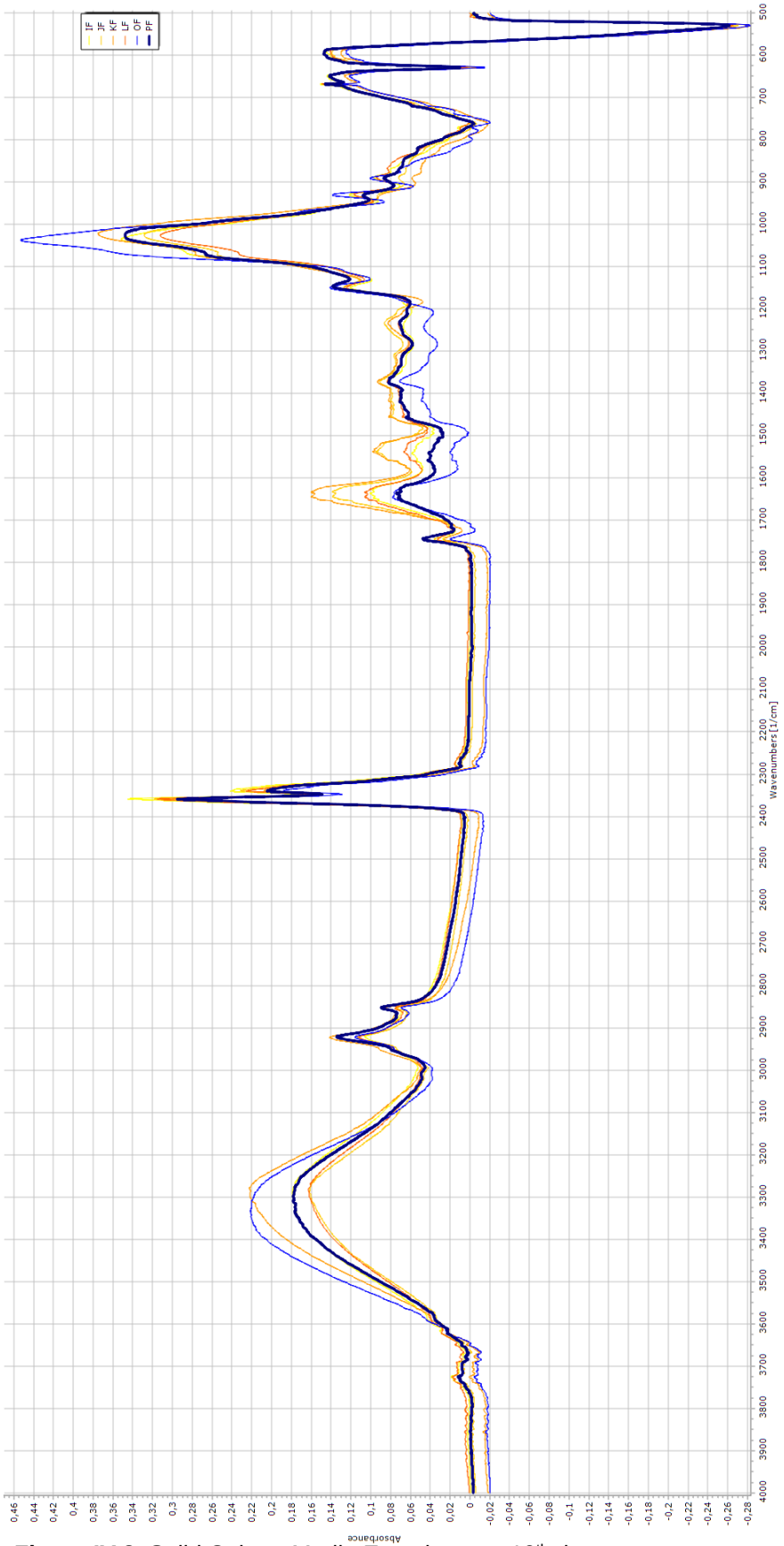


Figure IV.2. Solid Culture Media Experiment – 10th day

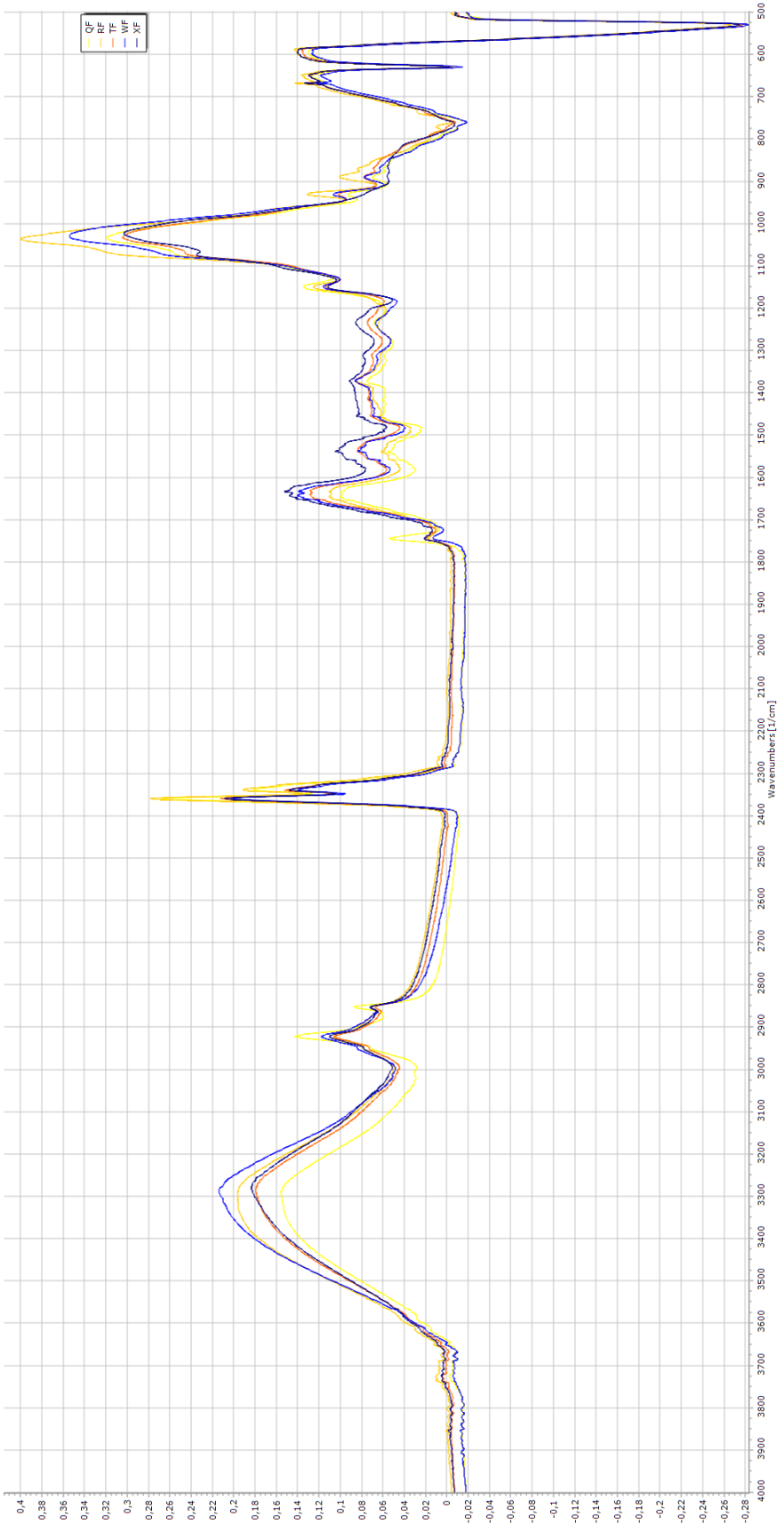


Figure IV.3. Solid Culture Media Experiment – 15th day

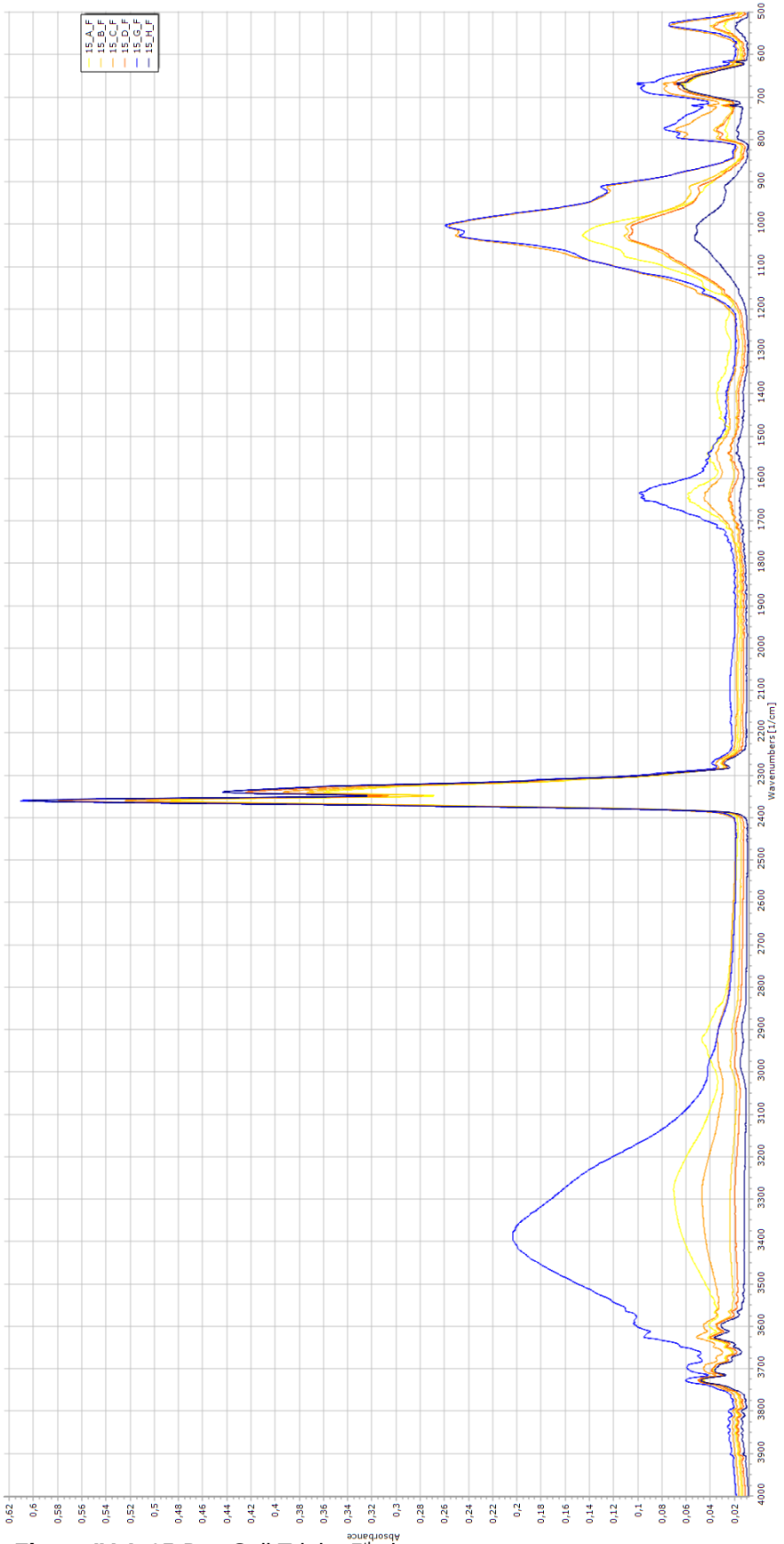


Figure IV.4. 15-Day Soil Trial – 5th day

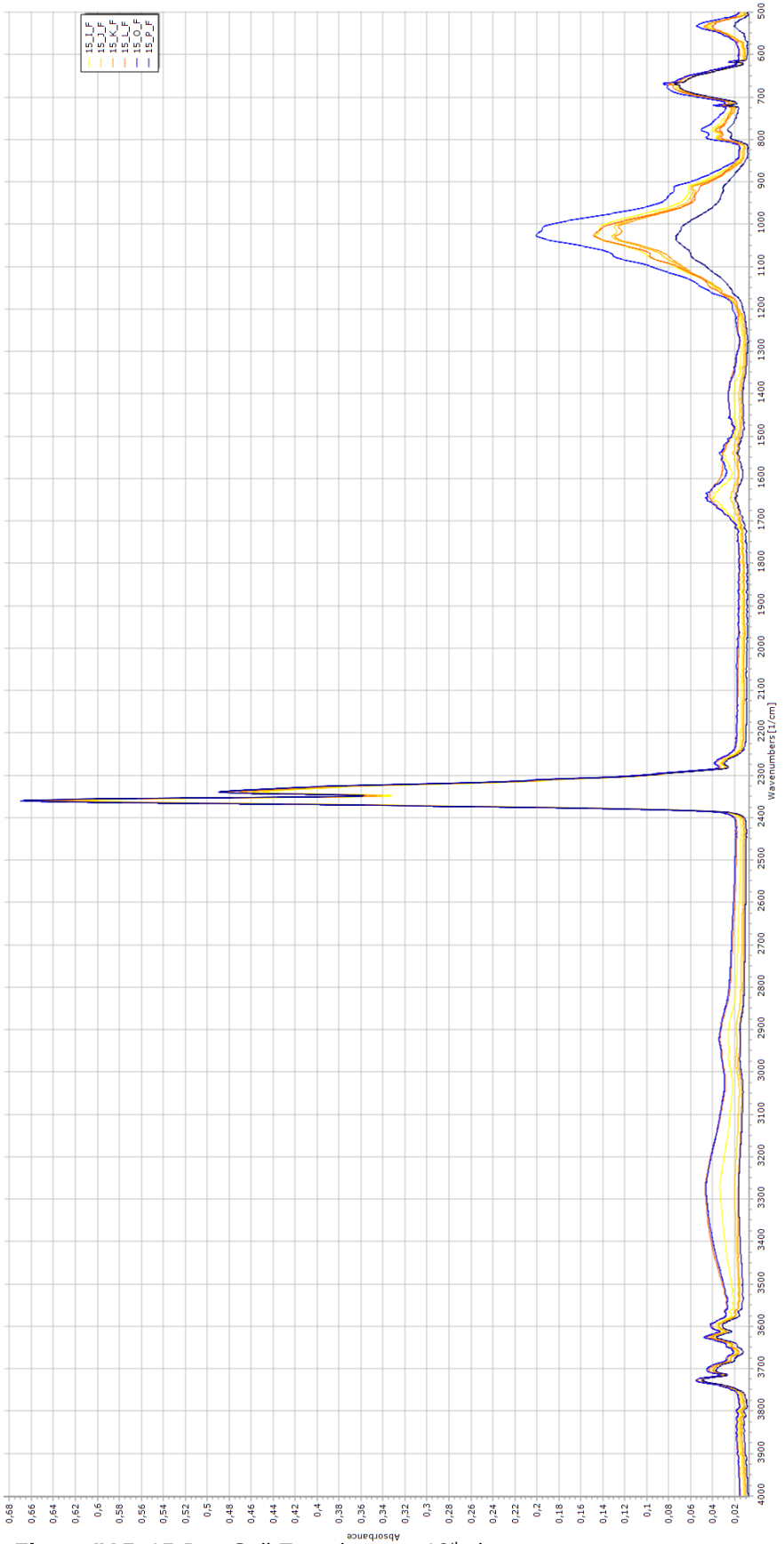


Figure IV.5. 15-Day Soil Experiment – 10th day

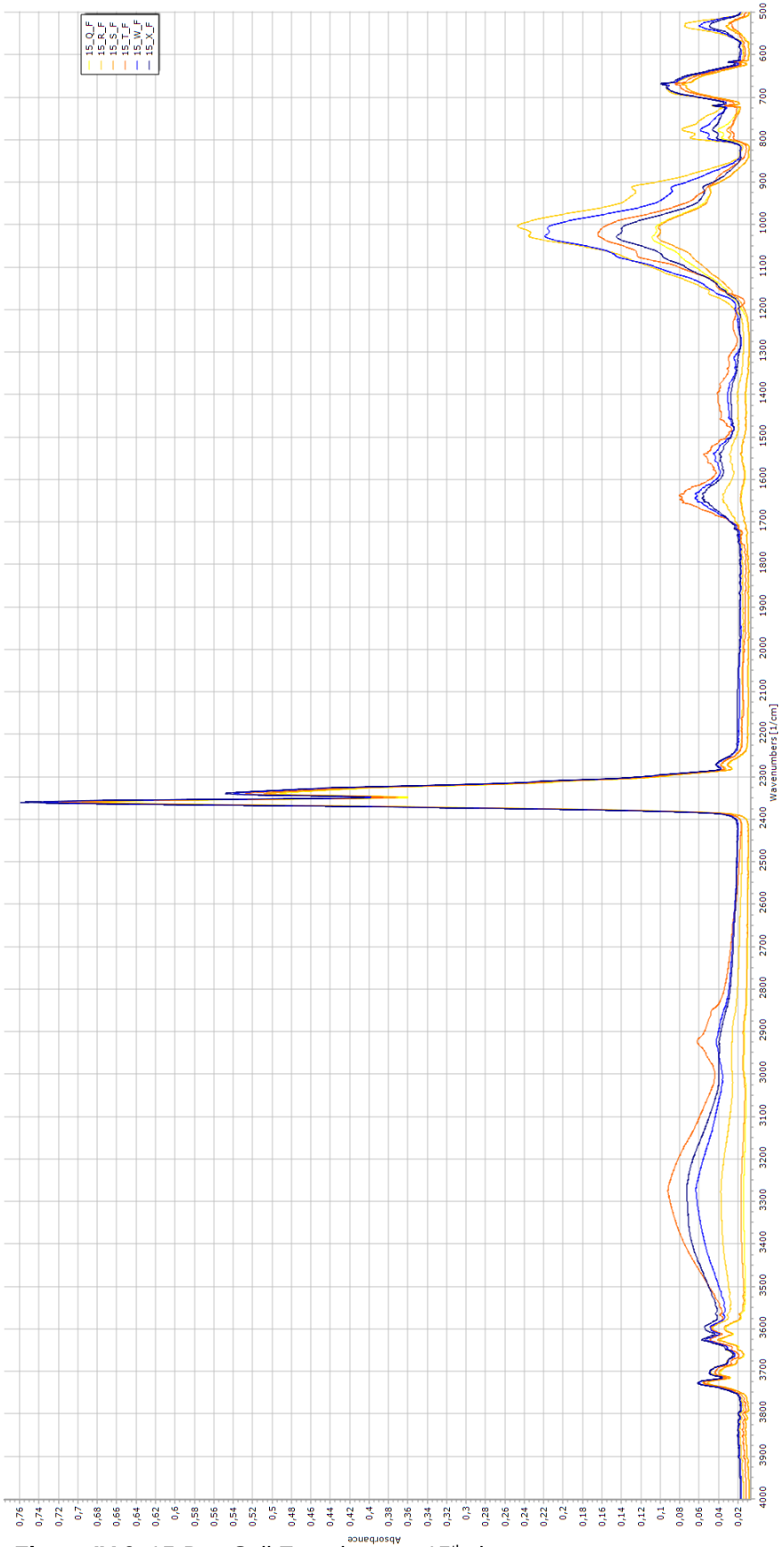


Figure IV.6. 15-Day Soil Experiment – 15th day

Figure IV.7. 28-Day Soil Experiment – 7th day

Figure IV.8. 28-Day Soil Experiment – 14th day

Figure IV.9. 28-Day Soil Experiment – 21st day

Figure IV.10. 28-Day Soil Experiment – 28th day

Annex V

Mulch Biofilm FTIR Spectra

In this Annex, all mulch biofilm spectra obtained throughout the several solid media and soil biodegradation trials can be found. Each spectrum has been colour-coded so that within each image, replicas have a progressively darker shade of orange, and controls a progressively darker shade of blue, as per the letter codes used throughout the trials, which can be consulted below, in **Table V**.

Table V. List of denominations given to each sample, as presented in each of the following plots.

Sample Origin	Denomination			
	1st Timepoint	2nd Timepoint	3rd timepoint	4th Timepoint
Replica 1	A	I	Q	Y
Replica 2	B	J	R	Z
Replica 3	C	K	S	Γ
Replica 4	D	L	T	Δ
Plastic Control 1	E	M	U	Σ
Plastic Control 2	F	N	V	Φ
Fungi Control 1	G	O	W	Ψ
Fungi Control 2	H	P	X	Ω

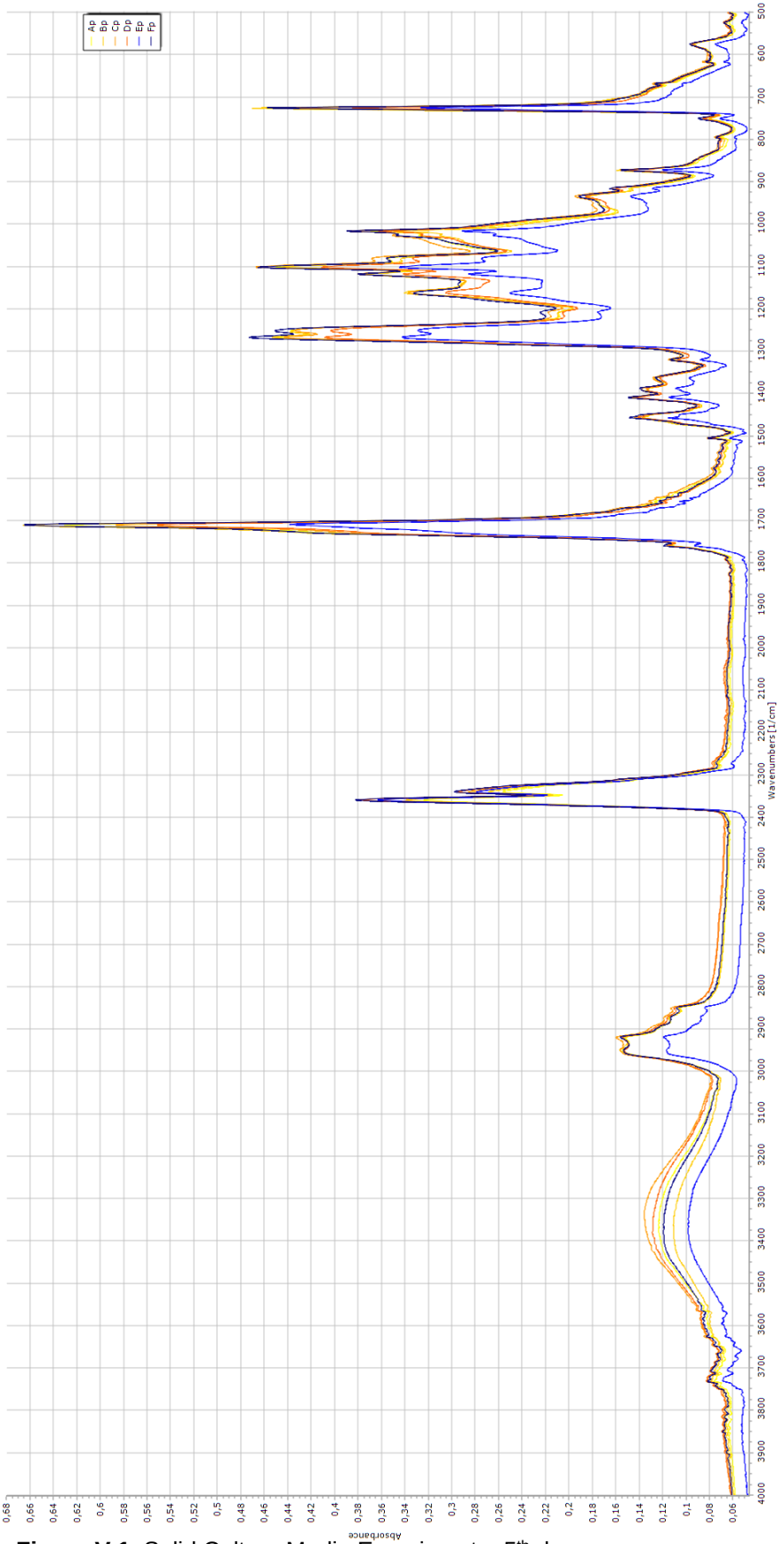


Figure V.1. Solid Culture Media Experiment – 5th day

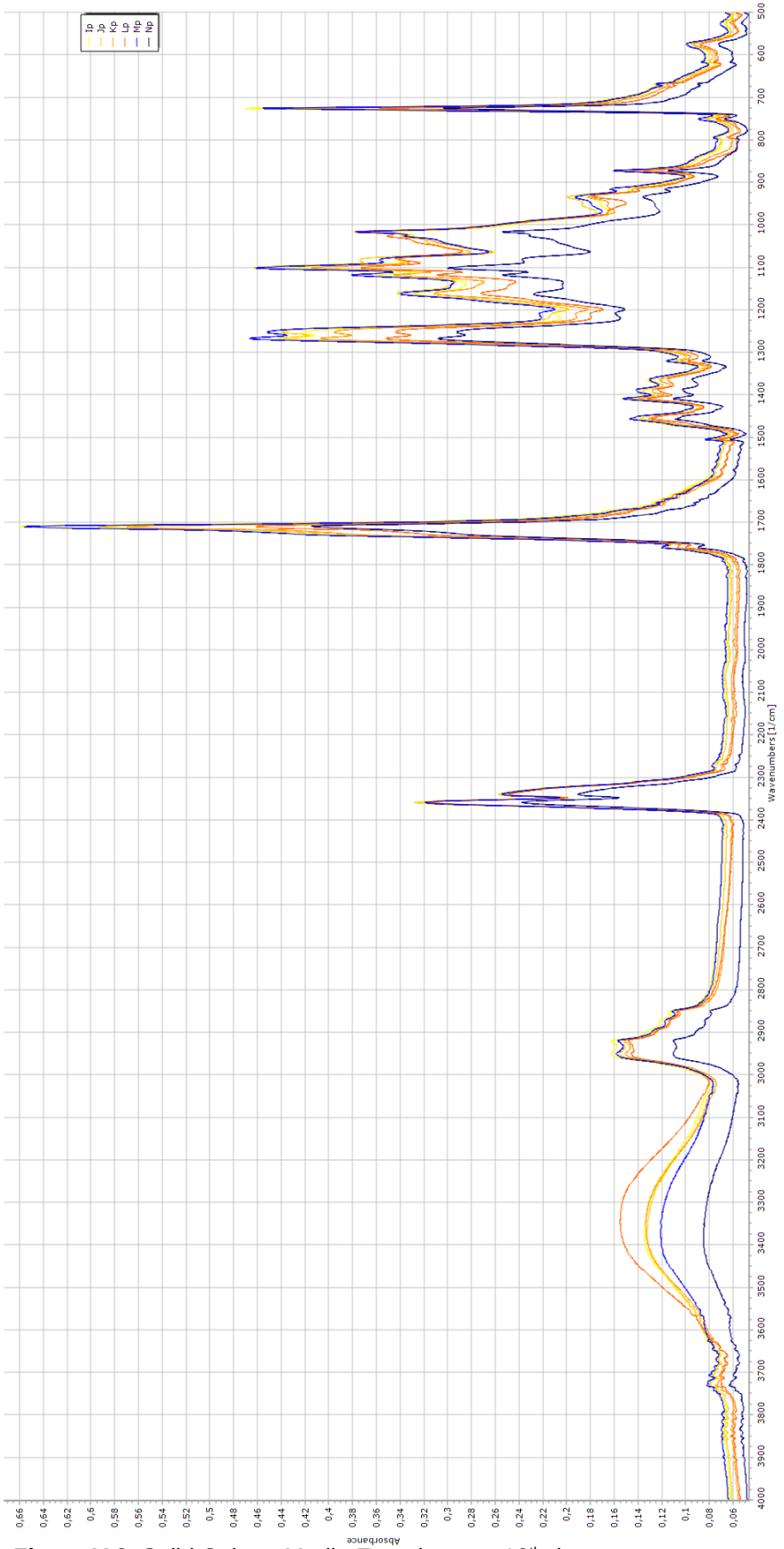


Figure V.2. Solid Culture Media Experiment – 10th day

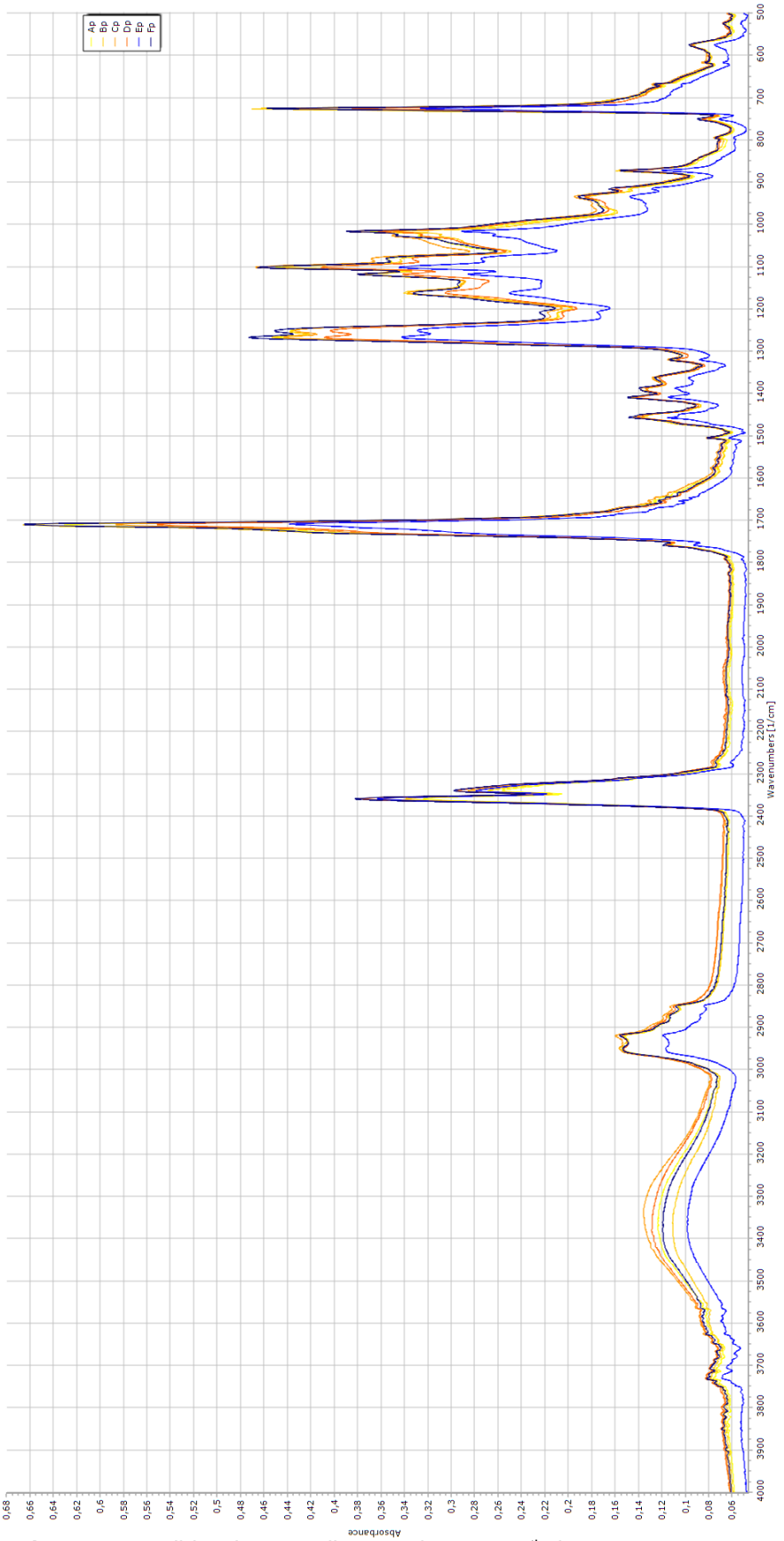


Figure V.3. Solid Culture Media Experiment – 15th day

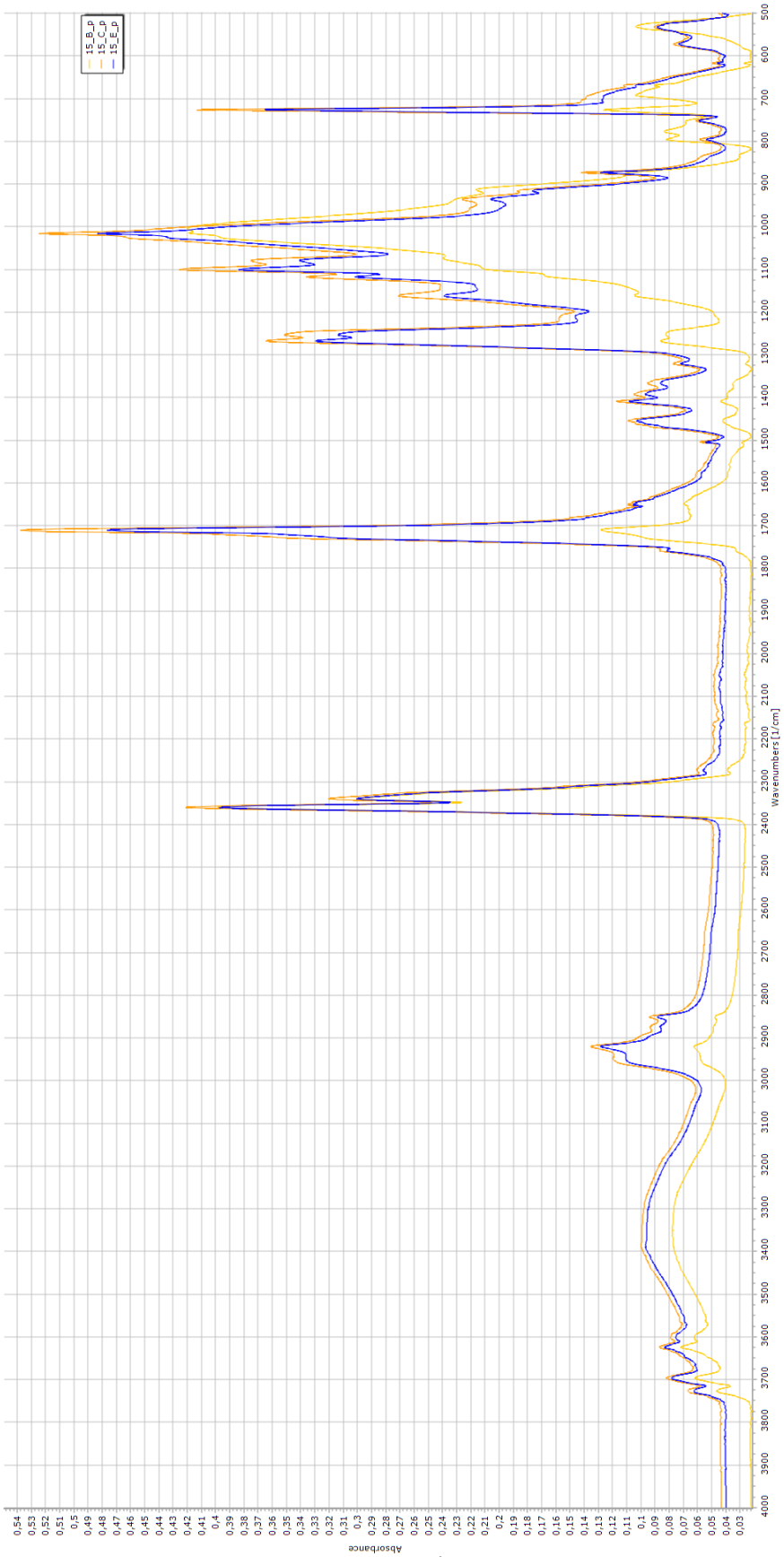


Figure V.4. 15-Day Soil Experiment – 5th day

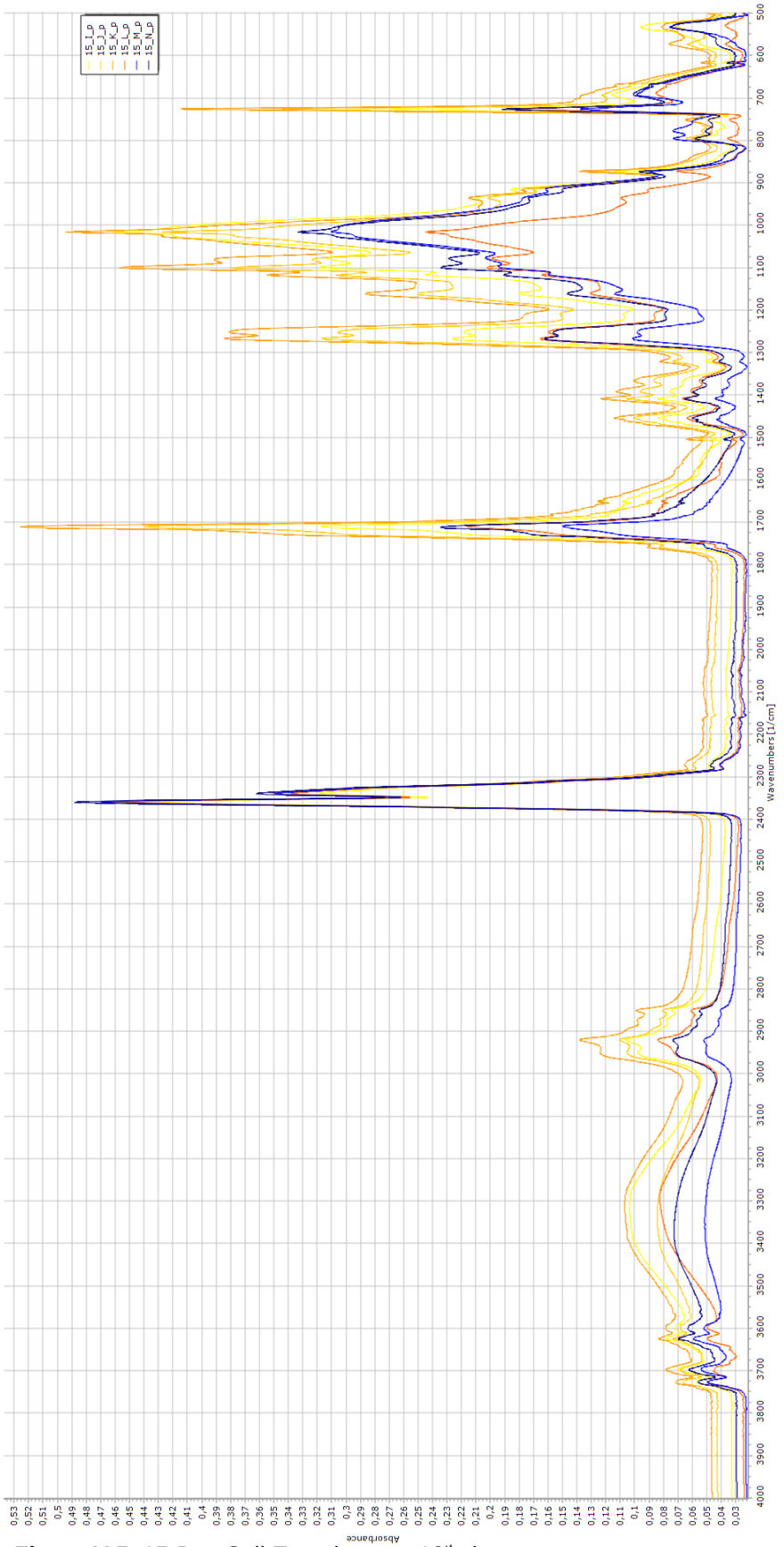


Figure V.5. 15-Day Soil Experiment – 10th day

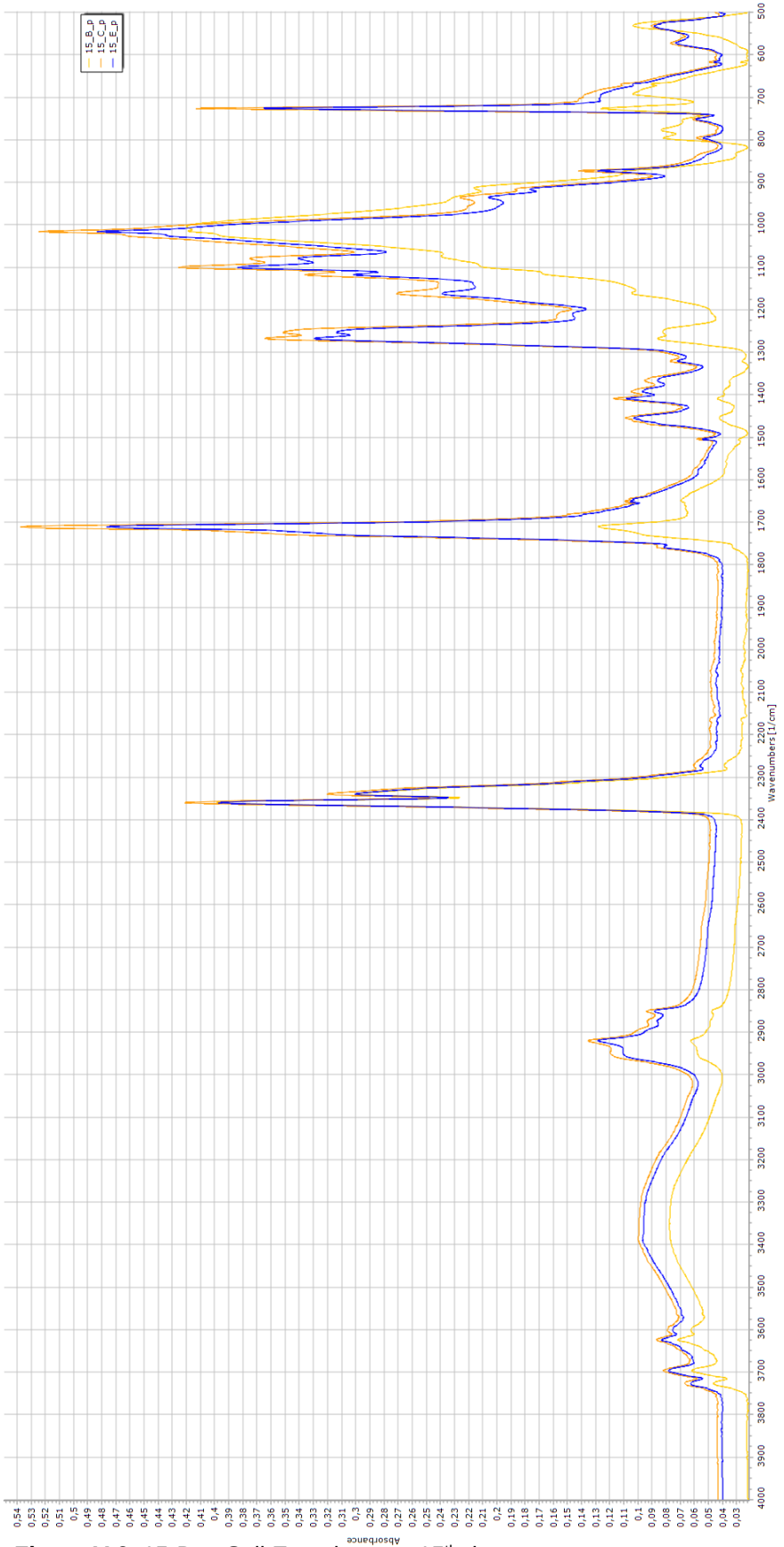


Figure V.6. 15-Day Soil Experiment – 15th day

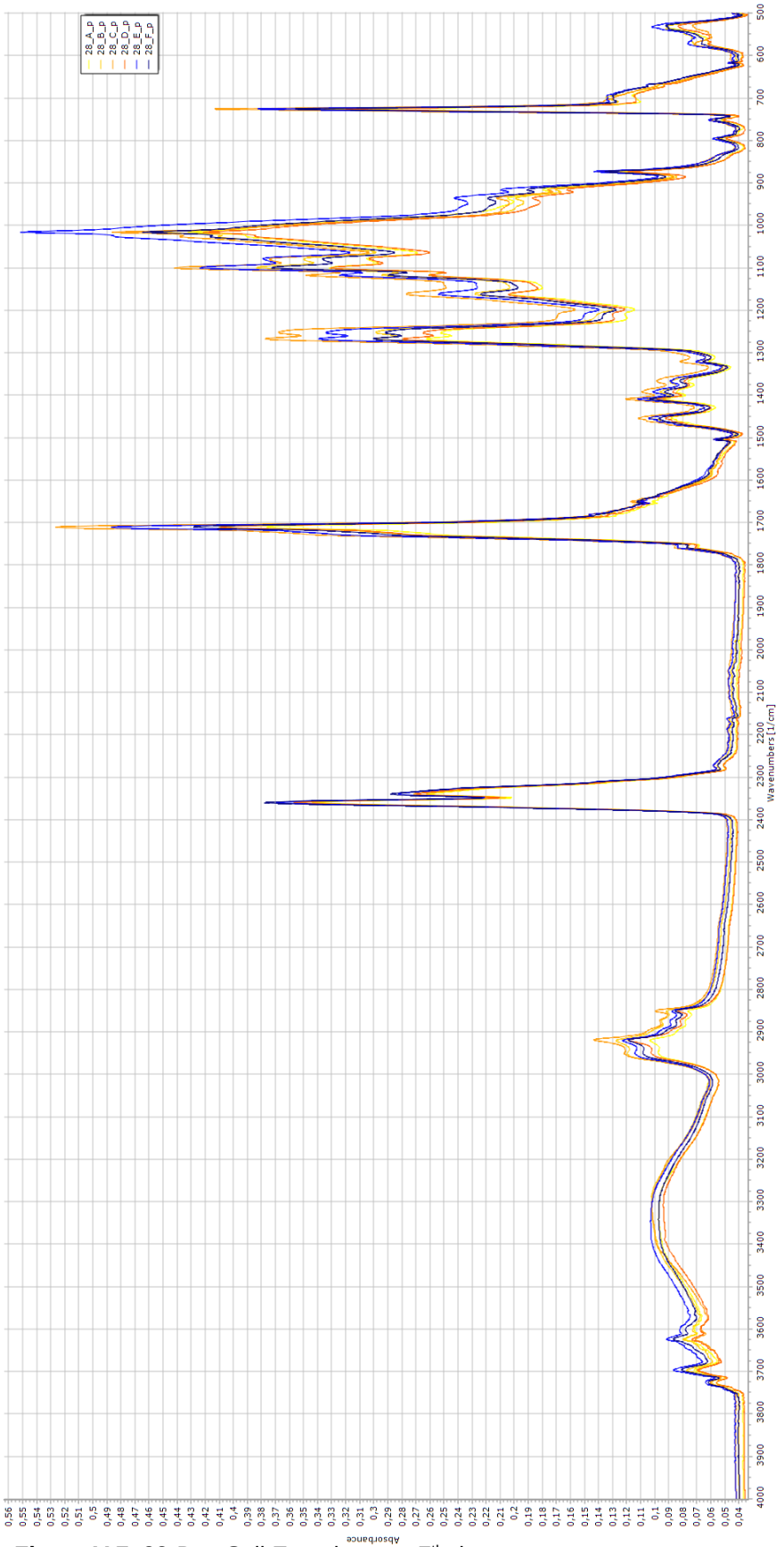


Figure V.7. 28-Day Soil Experiment – 7th day

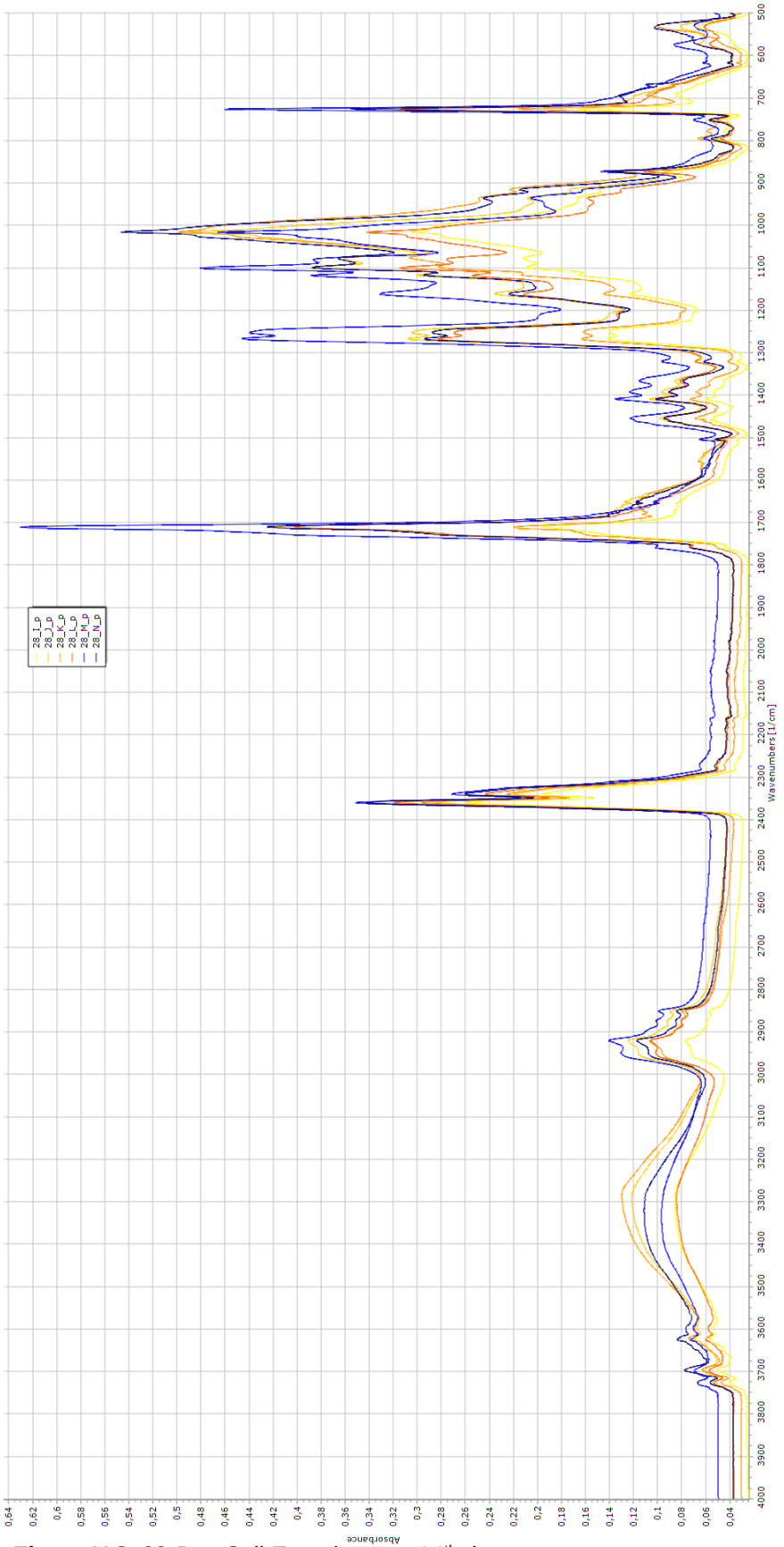


Figure V.8. 28-Day Soil Experiment – 14th day

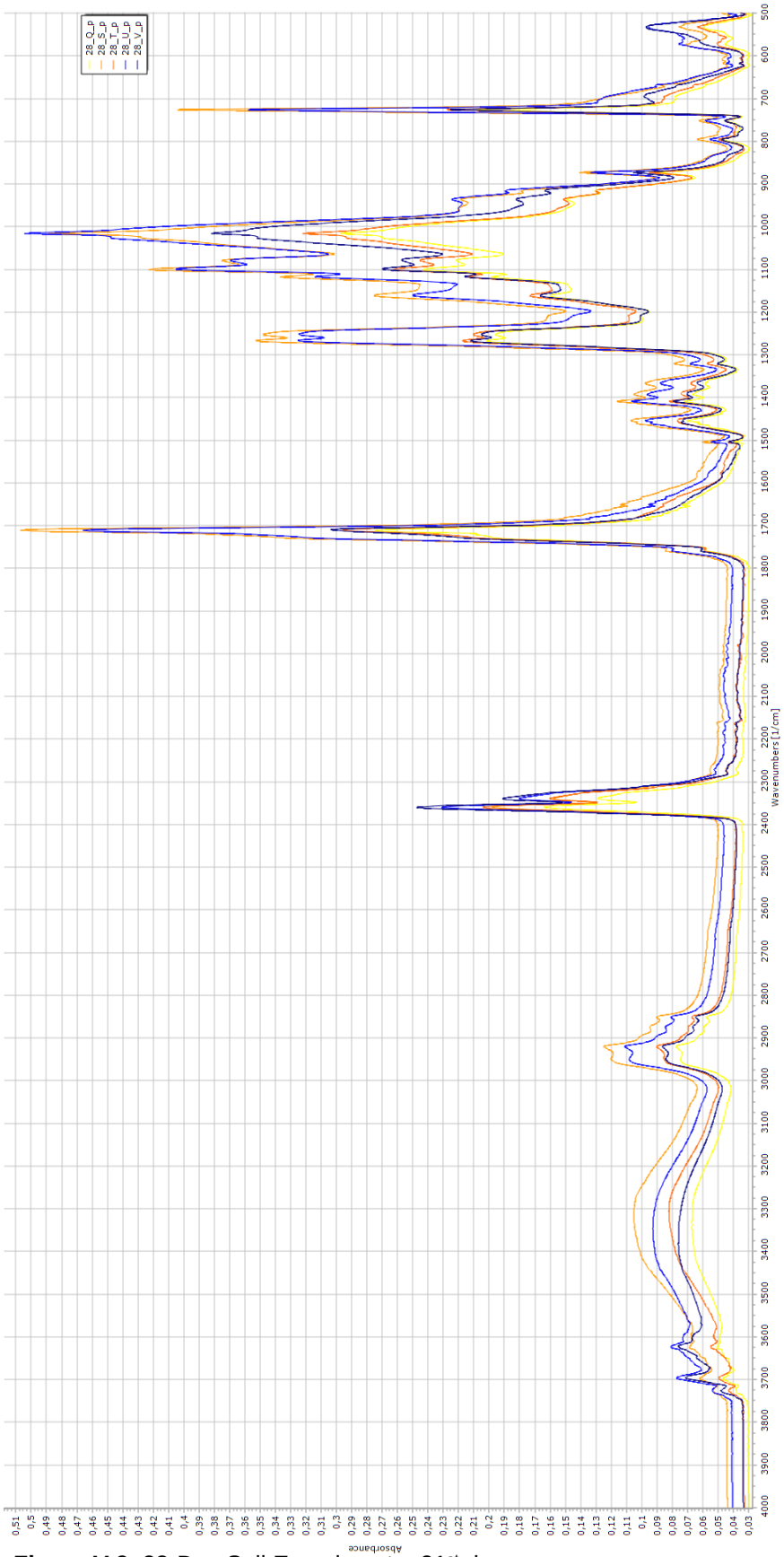


Figure V.9. 28-Day Soil Experiment – 21st day

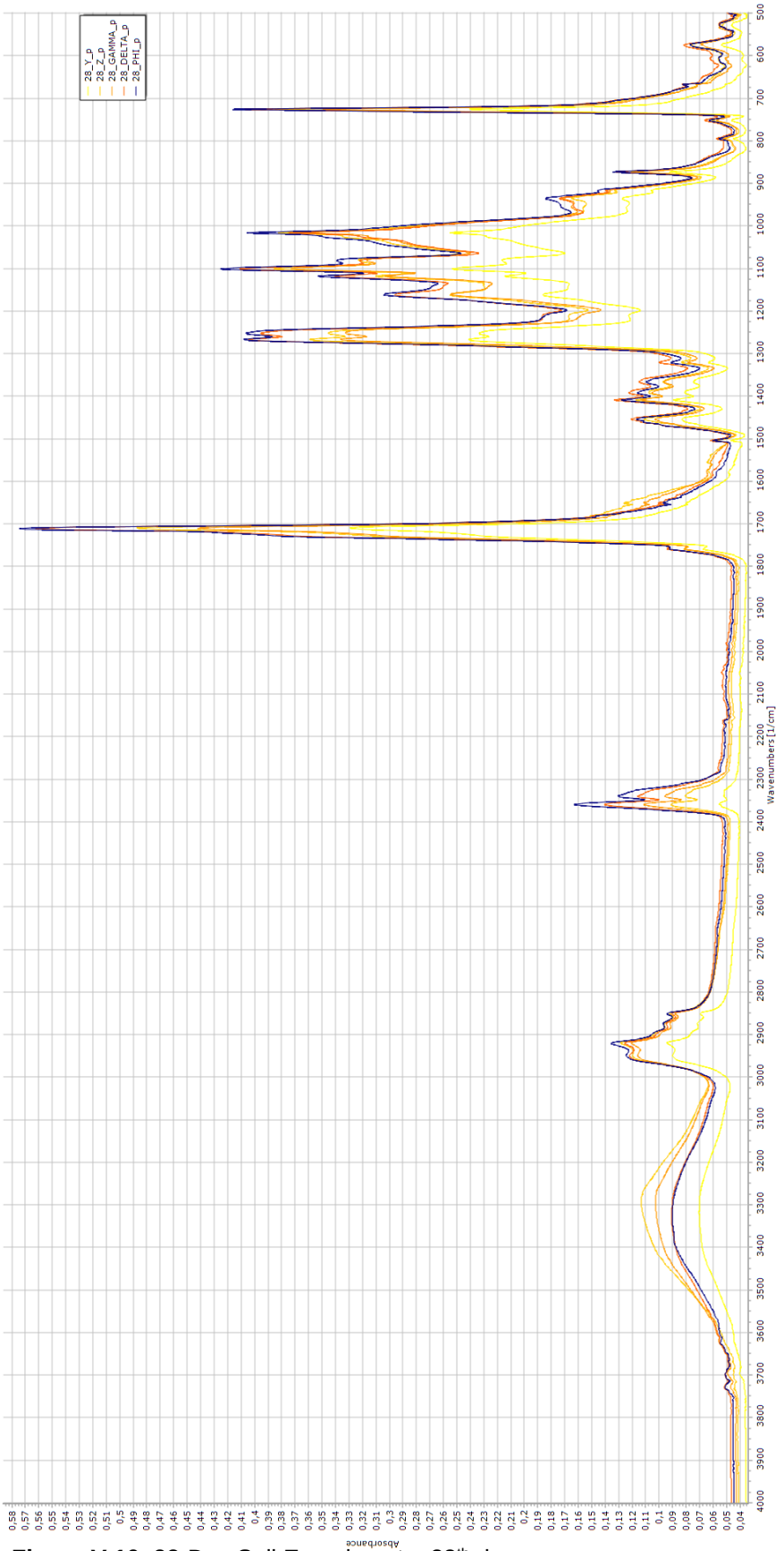


Figure V.10. 28-Day Soil Experiment – 28th day

Annex VI

Eisenia andrei Egested Microplastic Analysis

As explored in **Chapter III**, the egestion of microplastics was studied in order to demonstrate the ingestion and link to potential negative physiological effects. In this annex, an analysis of the size of microplastic particles egested by earthworms of several experimental conditions will be made using the same ImageJ scaling technique as used for the superficial area calculation of *P. brevicompactum*. Microplastic particles of different sizes found in filters corresponding to organisms submitted to different treatments, as well as a scale can be found in **Figure VI**.

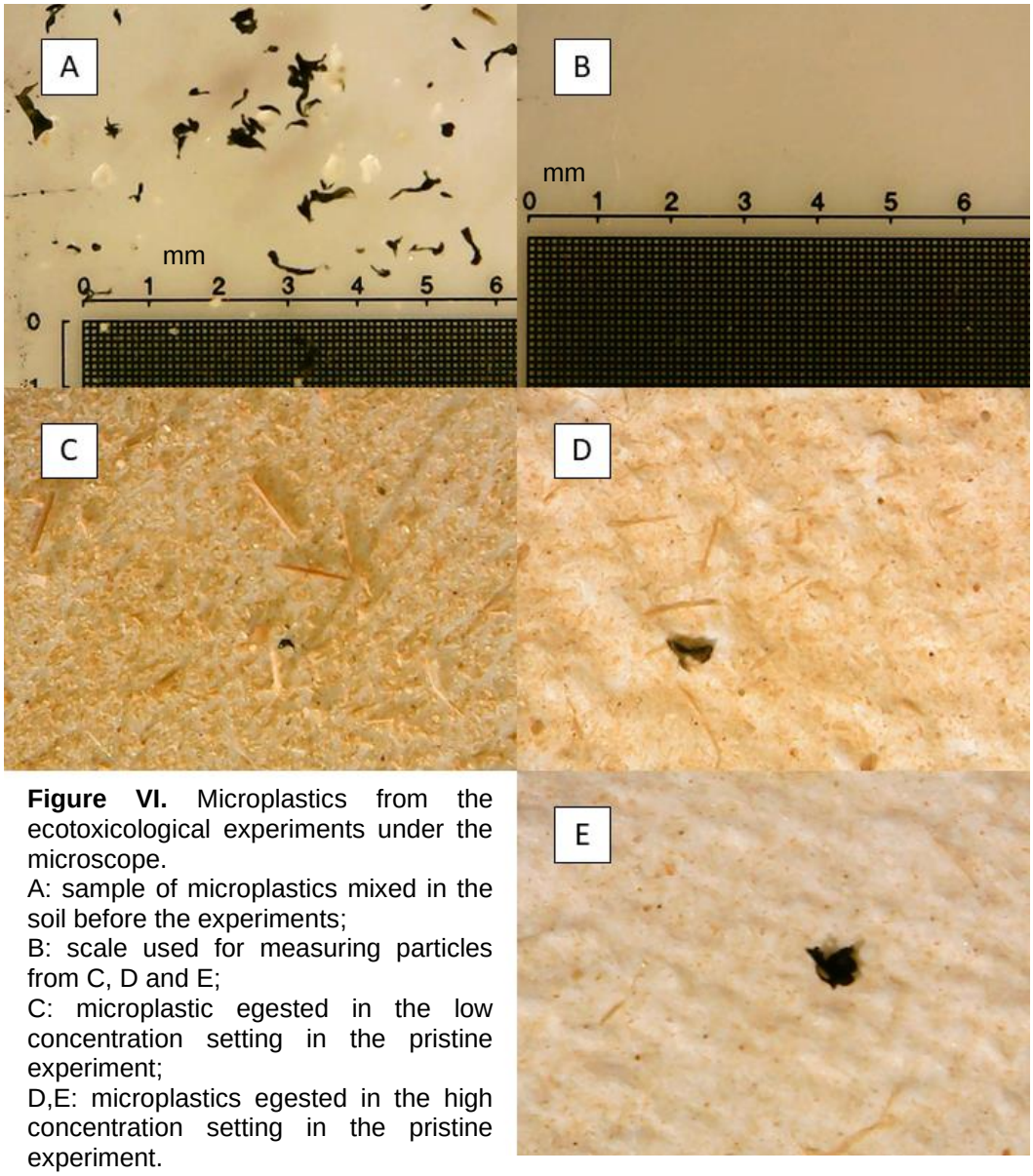


Figure VI. Microplastics from the ecotoxicological experiments under the microscope.
A: sample of microplastics mixed in the soil before the experiments;
B: scale used for measuring particles from C, D and E;
C: microplastic egested in the low concentration setting in the pristine experiment;
D,E: microplastics egested in the high concentration setting in the pristine experiment.

Using the above displayed scale, the microplastics' dimensions were measured resorting to ImageJ, using the same methodology as in the fungal area calculation. Microplastics used in the ecotoxicological experiments ranged in sizes from lengths of over 0.8 mm, usually in slimmer plastic particles that were thus able to pass through the restrictive sieve, to diameters under 0.1 mm on the lower ends.

As previously stated, it has been reported that most earthworms from temperate regions, as is the case of *E. andrei* have mouths of about 3 mm in diameter. As such, all particles displayed in **Figure VI-A** could theoretically be ingested by *E. andrei*. And indeed, as per **Figure VI-C to E**, whose scale can be found in **Figure VI-B**, the ingestion and subsequent egestion of plastic particles as small as 0,19 x 0,09 mm (C) and as large as 0.54 x 0.60 mm (E) was confirmed. Coincidentally, the smaller particle was found in the sample from lower microplastic concentration; however, such a link is unlikely, given the even, random distribution of microplastics through the several soil samples to be used in the different experimental settings.

The presence of larger microplastics such as the one found in **Figure VI-E** suggests that they did not fragment during their travel through *E. andrei*'s gastrointestinal tract, whereas the presence in the initial sample in **Figure VI-A** of microplastics with similar sizes to the one found after egestion in **Figure VI-C** demonstrates the possibility that egested particles of reduced dimensions could have been ingested that way, instead of being products of a physical breakdown during the experiment. Thus, as discussed previously, the realization of further evaluations is essential in order to confirm or deny that this biodegradable mulch film breaks down significantly inside *E. andrei*'s gut.

Annex VII

Eisenia Andrei Gallery

In this Annex, some examples photographs of *E. Andrei* collected during their retrieval and purging phases, documenting several apparent stress situations, are displayed, in order to complement the punctual examples given in the body of this thesis.

Coelomic Fluid Accumulations

Figure VII.1. Additional examples of coelomic fluid accumulations found in the pristine (A, B) and weathered (C) experiments.

Cleavage Furrows

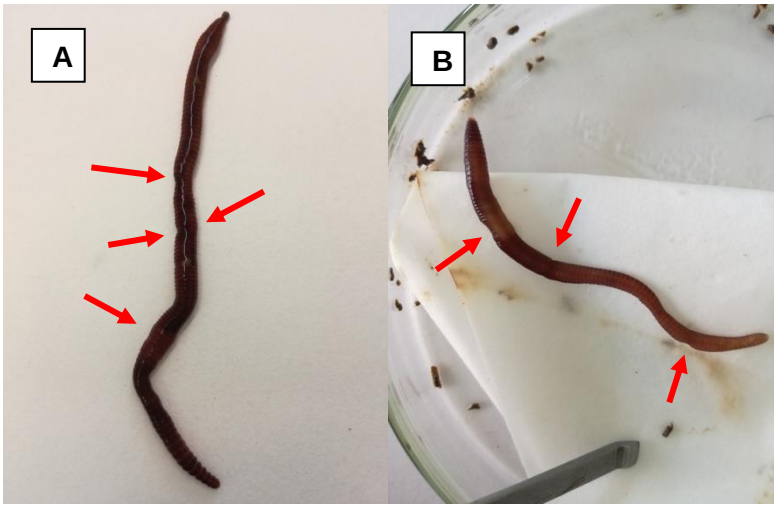


Figure VII.2. Additional examples of the appearance of cleavage furrows in specimens from the pristine (A) and weathered (B) experiments.

Annex VIII

Eisenia andrei FTIR Spectra

In this Annex, all *Eisenia andrei* spectra obtained throughout the several Ecotoxicological Trials, using both Pristine and UV-C Weathered Microplastics can be found. Each trial's (Pristine and UV-Weathered) spectra have been distributed throughout 18 plots (9 each), containing the spectra as indicated in the matrix represented in **Table VIII**.

Table VIII. Contents of each FTIR plot (e.g. Ct-3B, L-3B, M-3B and H-3B are all located in the 8th Plot of both the Pristine and the UV-Weathered Trials); Ct – Control, L – Low (0.125 g/Kg), M – Medium (0.250 g/Kg), H – High (0.500 g/Kg)

		A	**B**	**C**
CT + L + M + H	**1**	1st Plot	2nd Plot	3rd Plot
	2	4th Plot	5th Plot	6th Plot
	3	7th Plot	8th Plot	9th Plot
	4	10th Plot	11th Plot	12th Plot

In each plot, controls are represented in blue, and earthworms are displayed in progressively darker shades of orange according to the concentration of their test (higher microplastic concentrations represented by darker hues). In cases where earthworms migrated and Petri dishes had more than one inhabitant, both were included in the same plot, although the doubles were instead represented with progressively darker shades of pink/violet. The legend codes in each plot denote the type of environment (Ct, L, M, H), the number of the vial from which the organism was taken (1, 2, 3, 4), which organism (A, B, C), and the type of experiment (NM – pristine experiment, UV – weathered experiment).

Pristine Trials

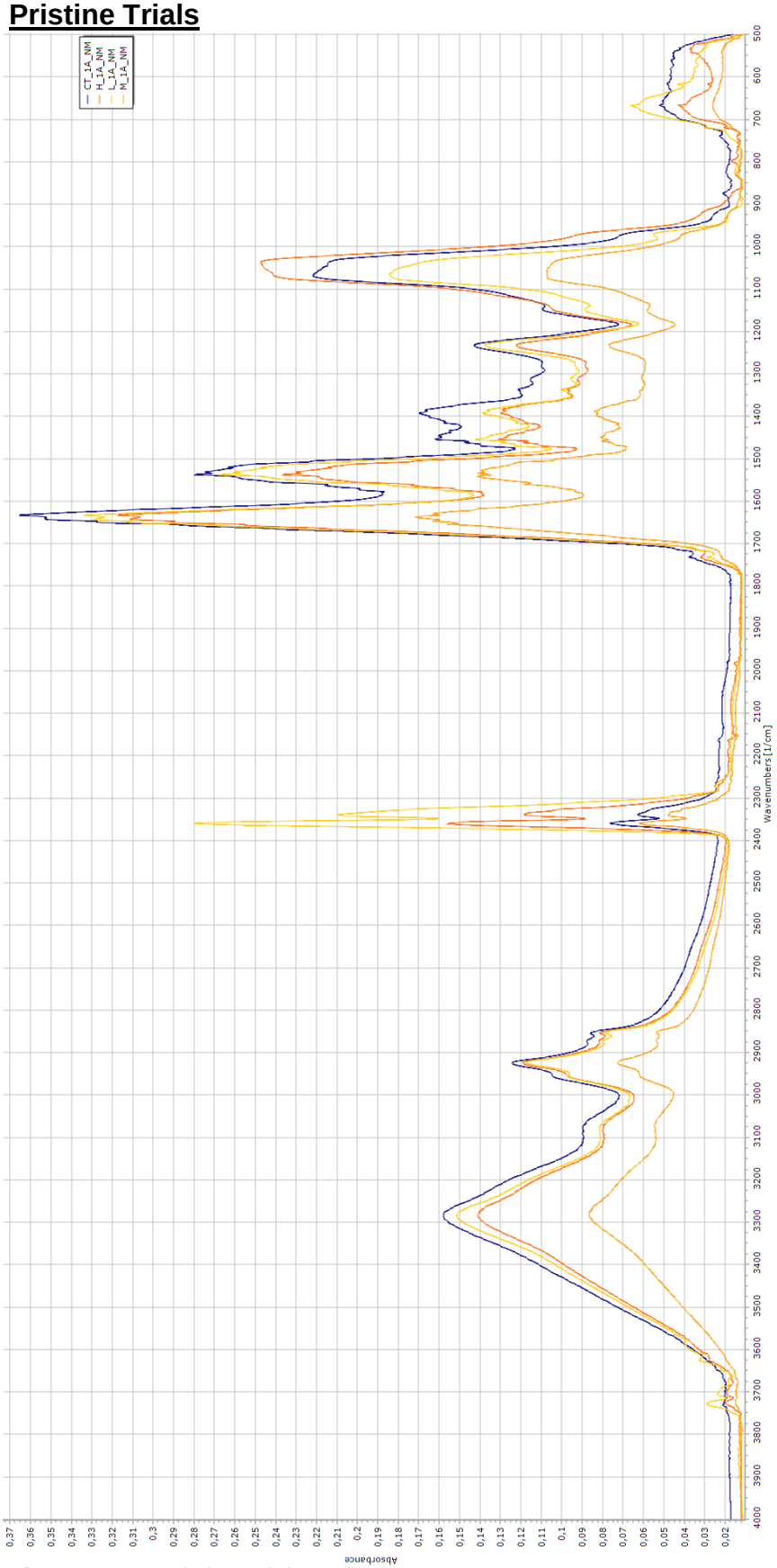


Figure VIII.1. Pristine Trials – Plot 1

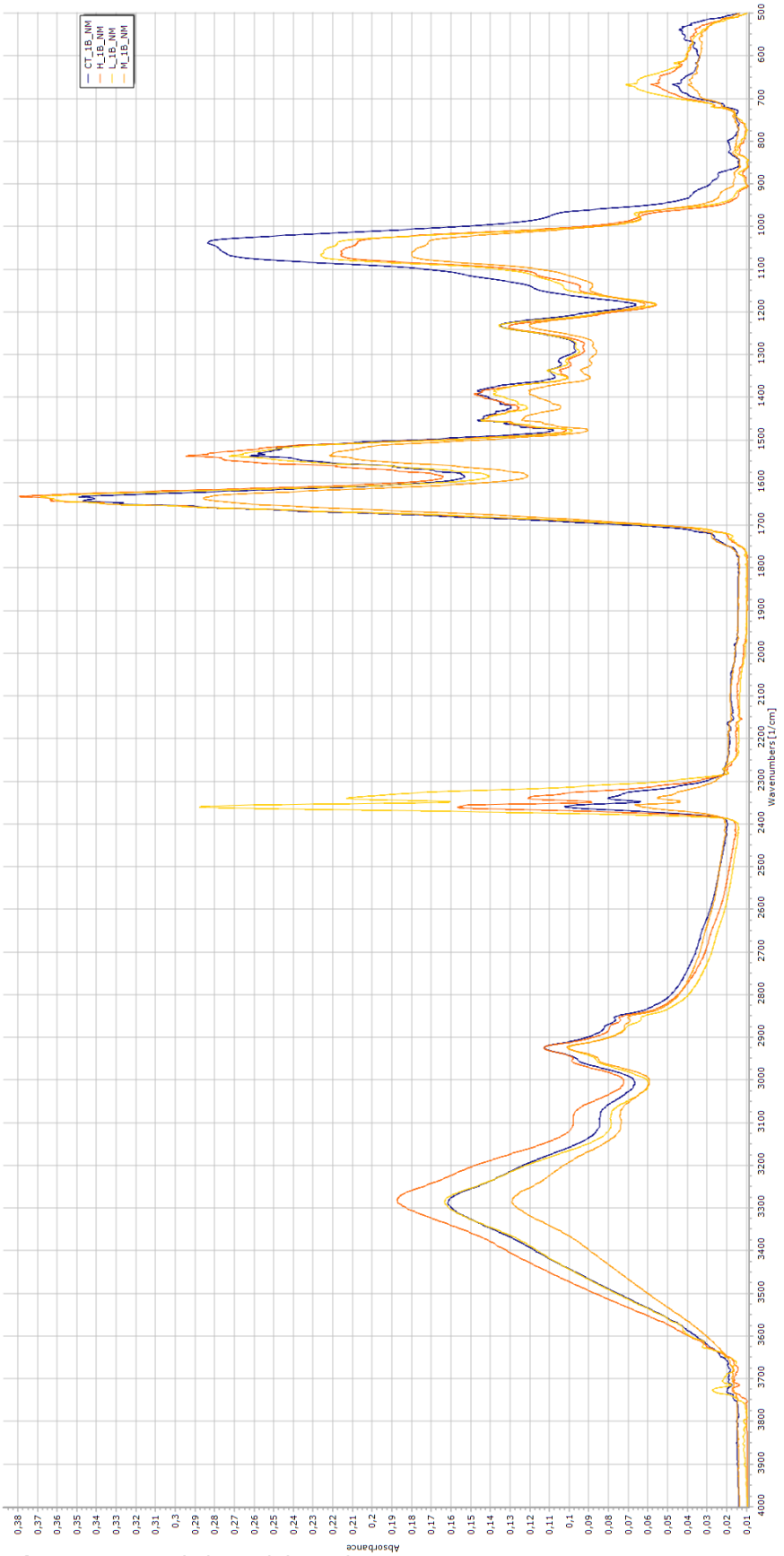


Figure VIII.2. Pristine Trials – Plot 2

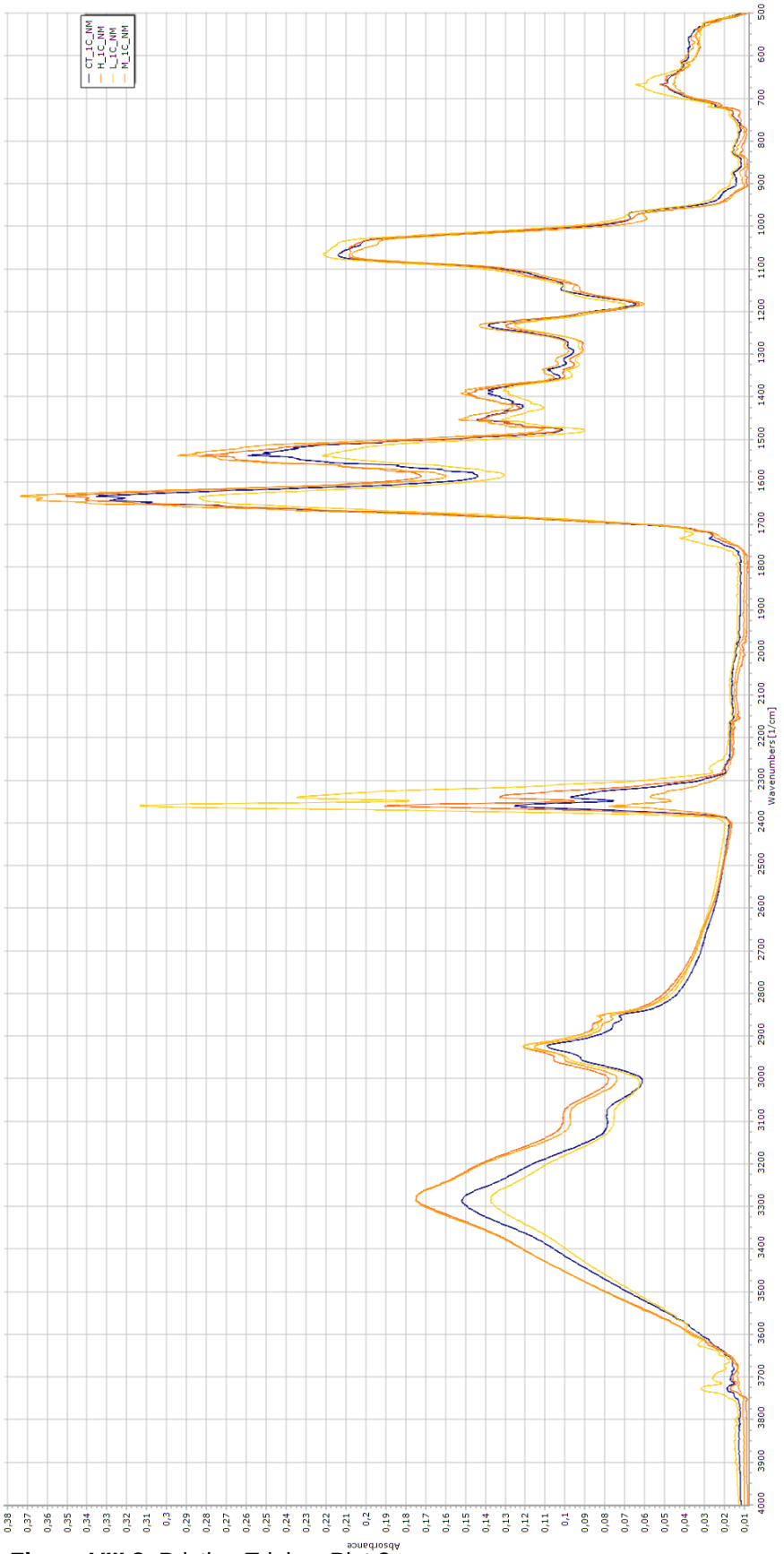


Figure VIII.3. Pristine Trials – Plot 3

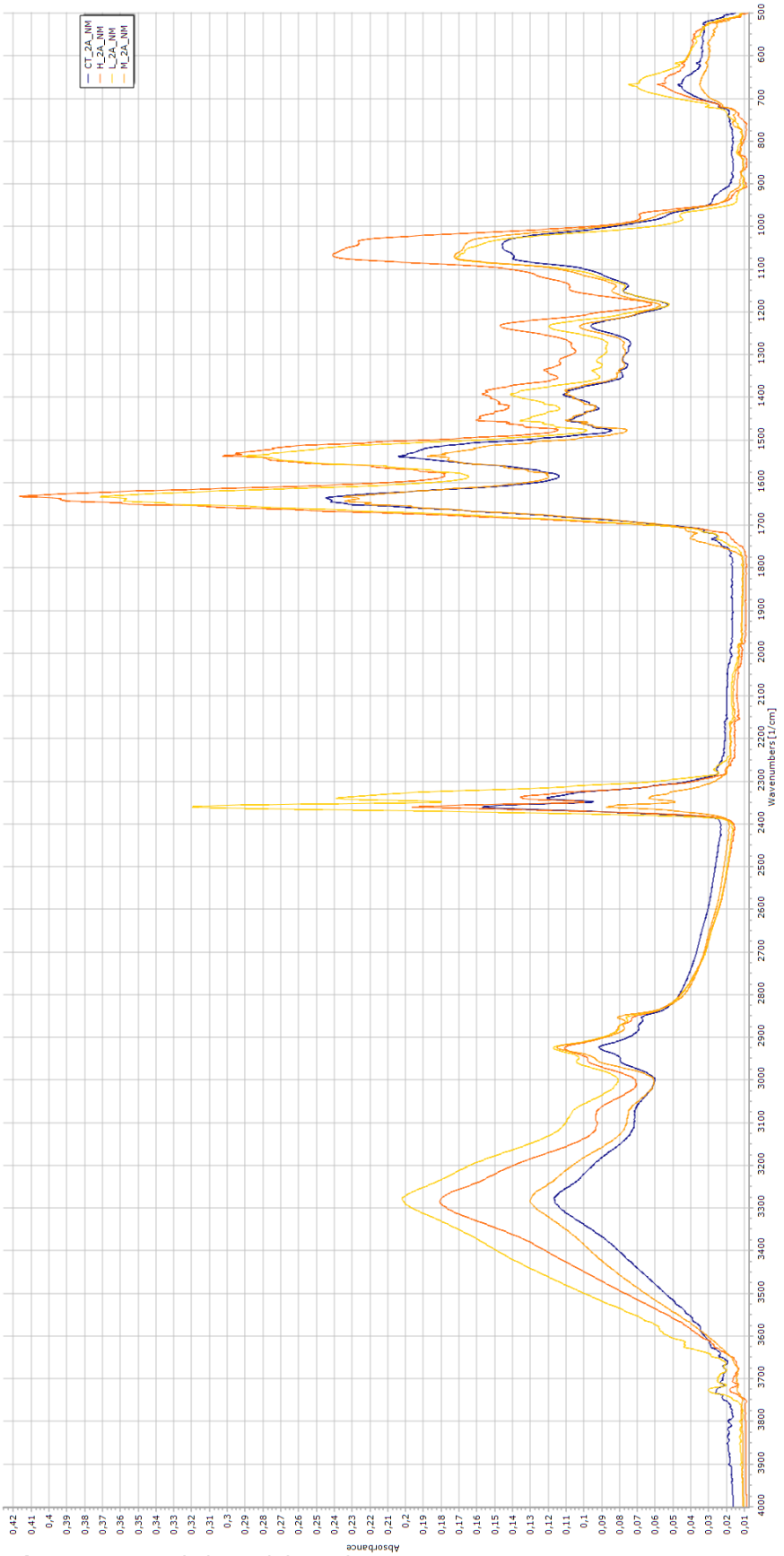


Figure VIII.4. Pristine Trials – Plot 4

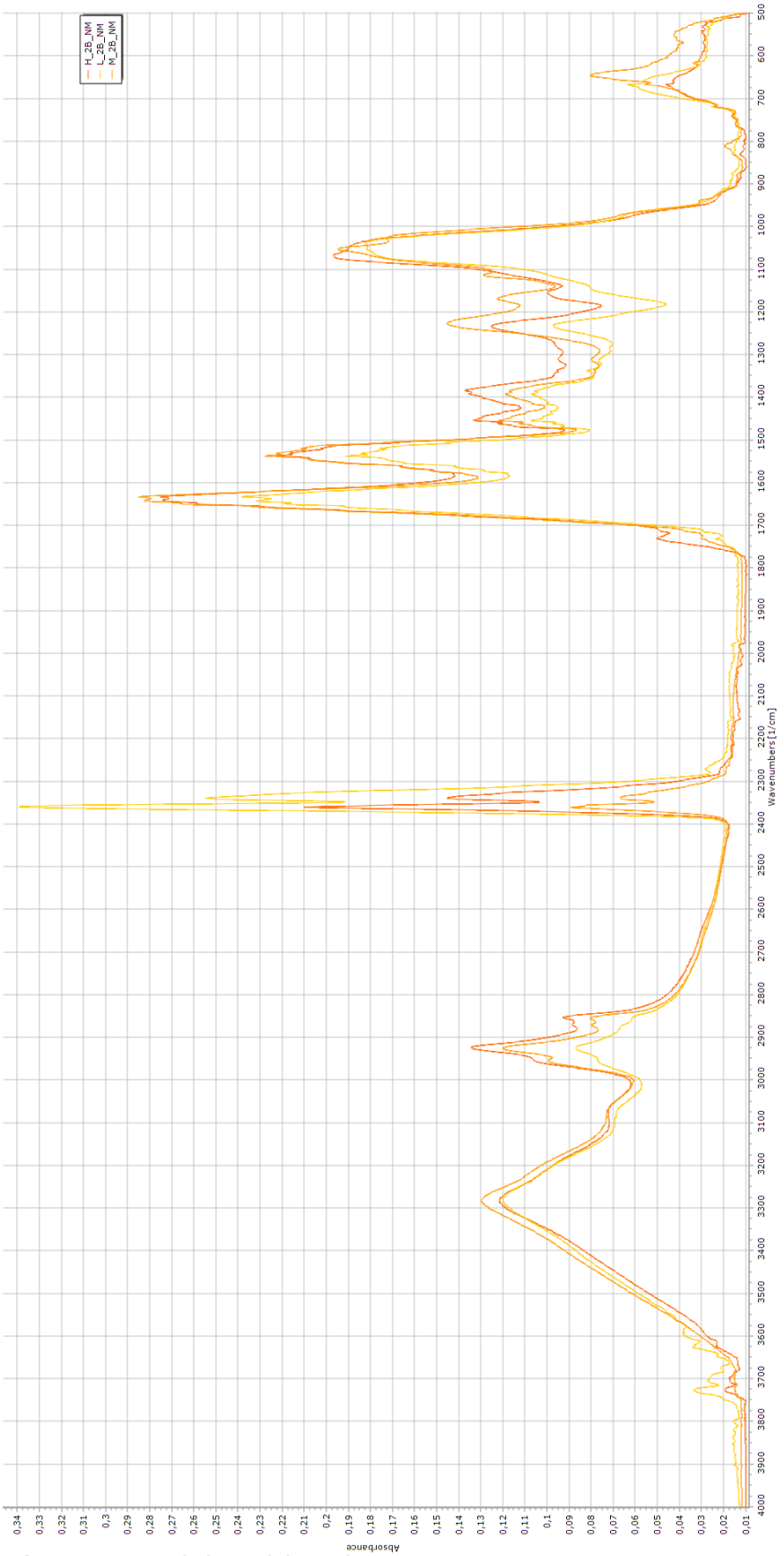


Figure VIII.5. Pristine Trials – Plot 5

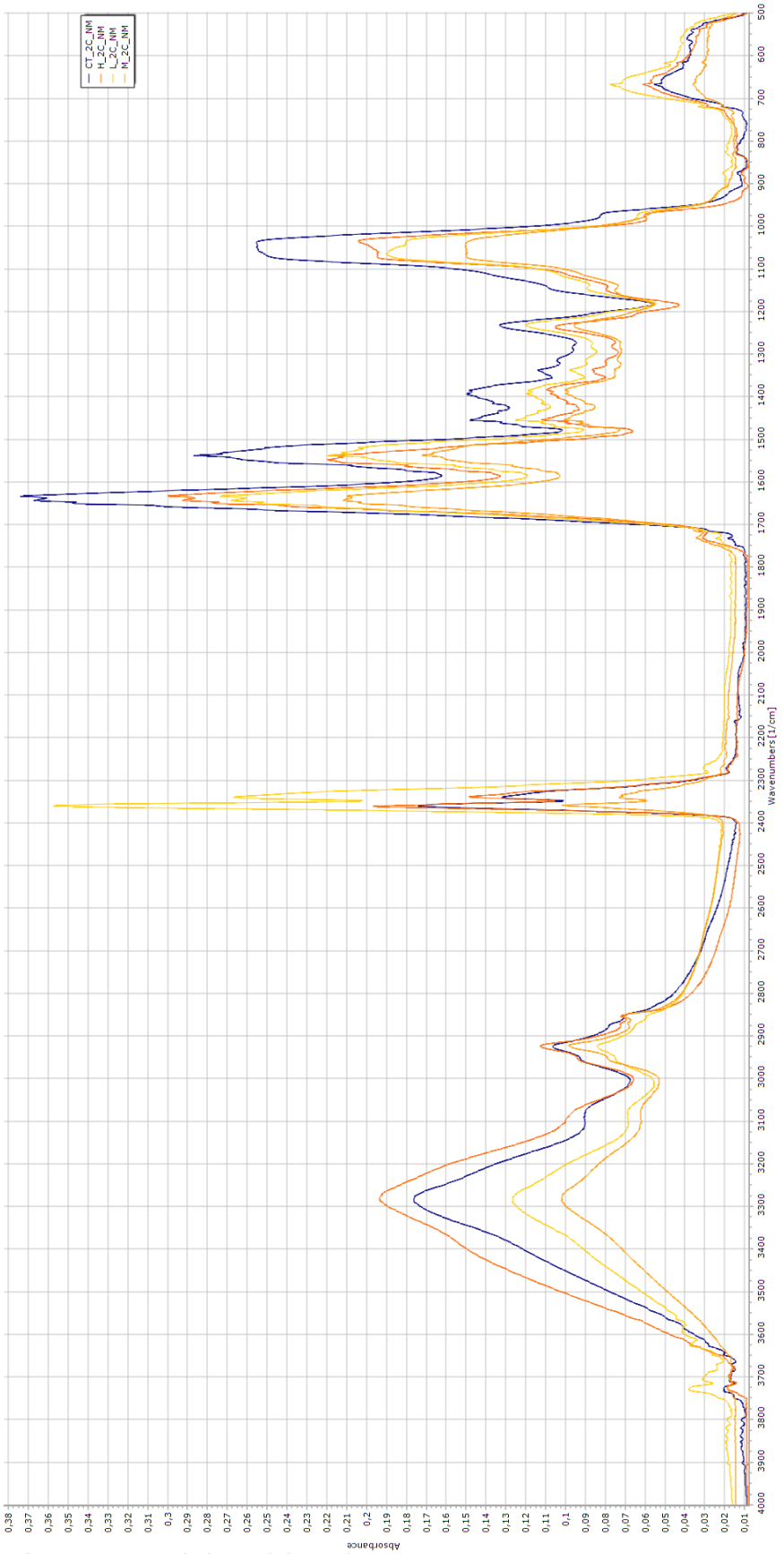


Figure VIII.6. Pristine Trials – Plot 6

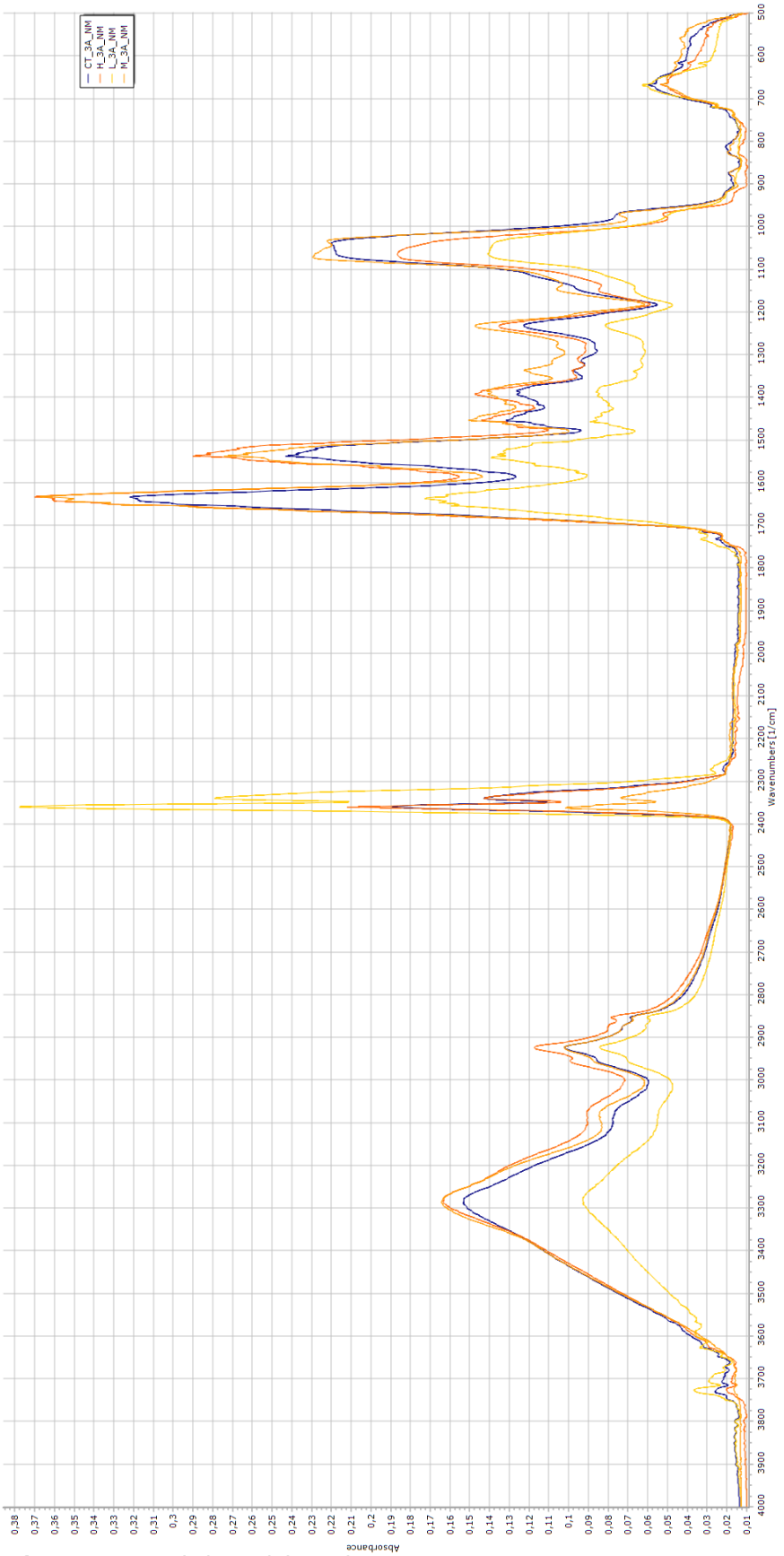


Figure VIII.7. Pristine Trials – Plot 7

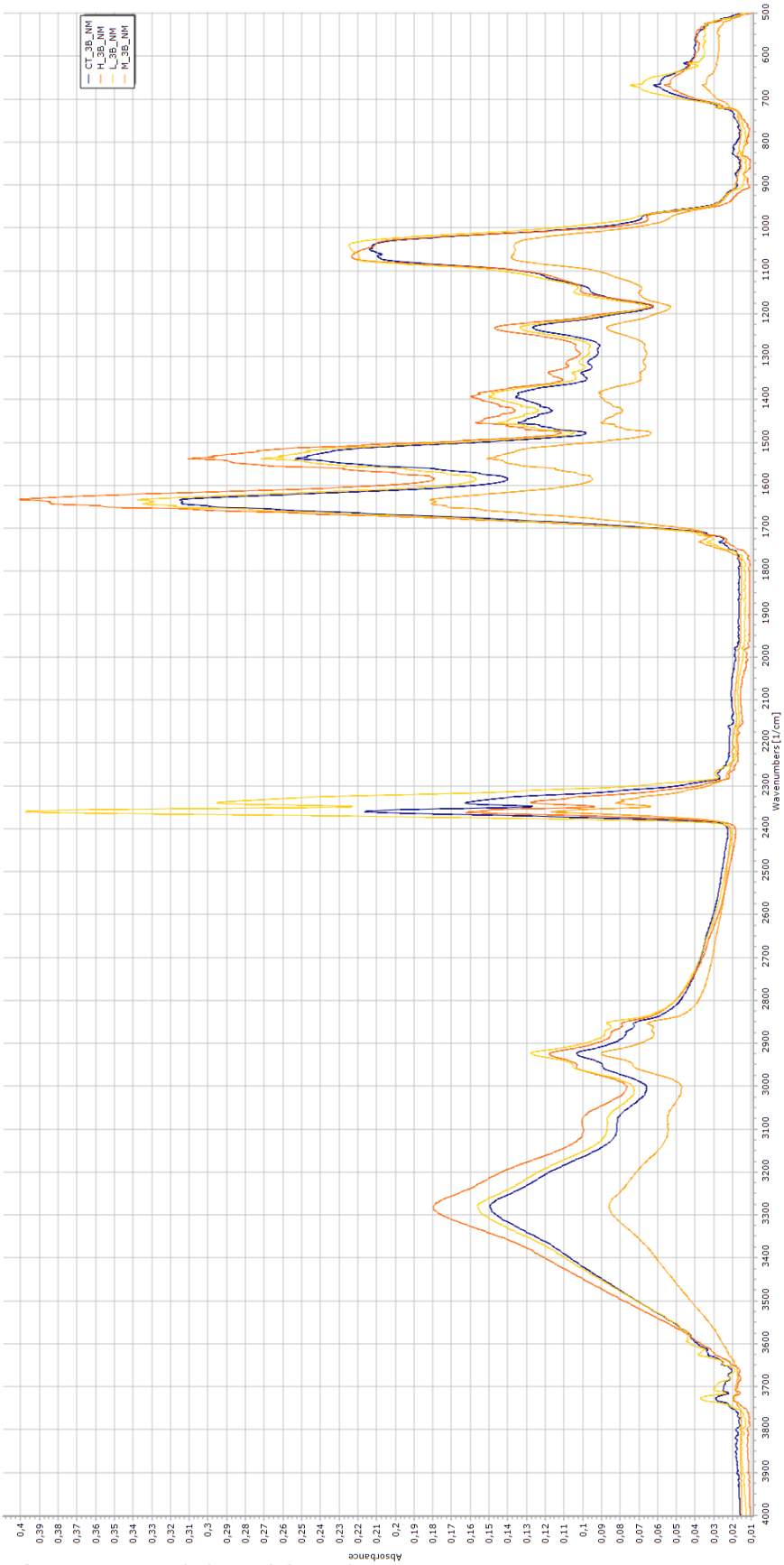


Figure VIII.8. Pristine Trials – Plot 8

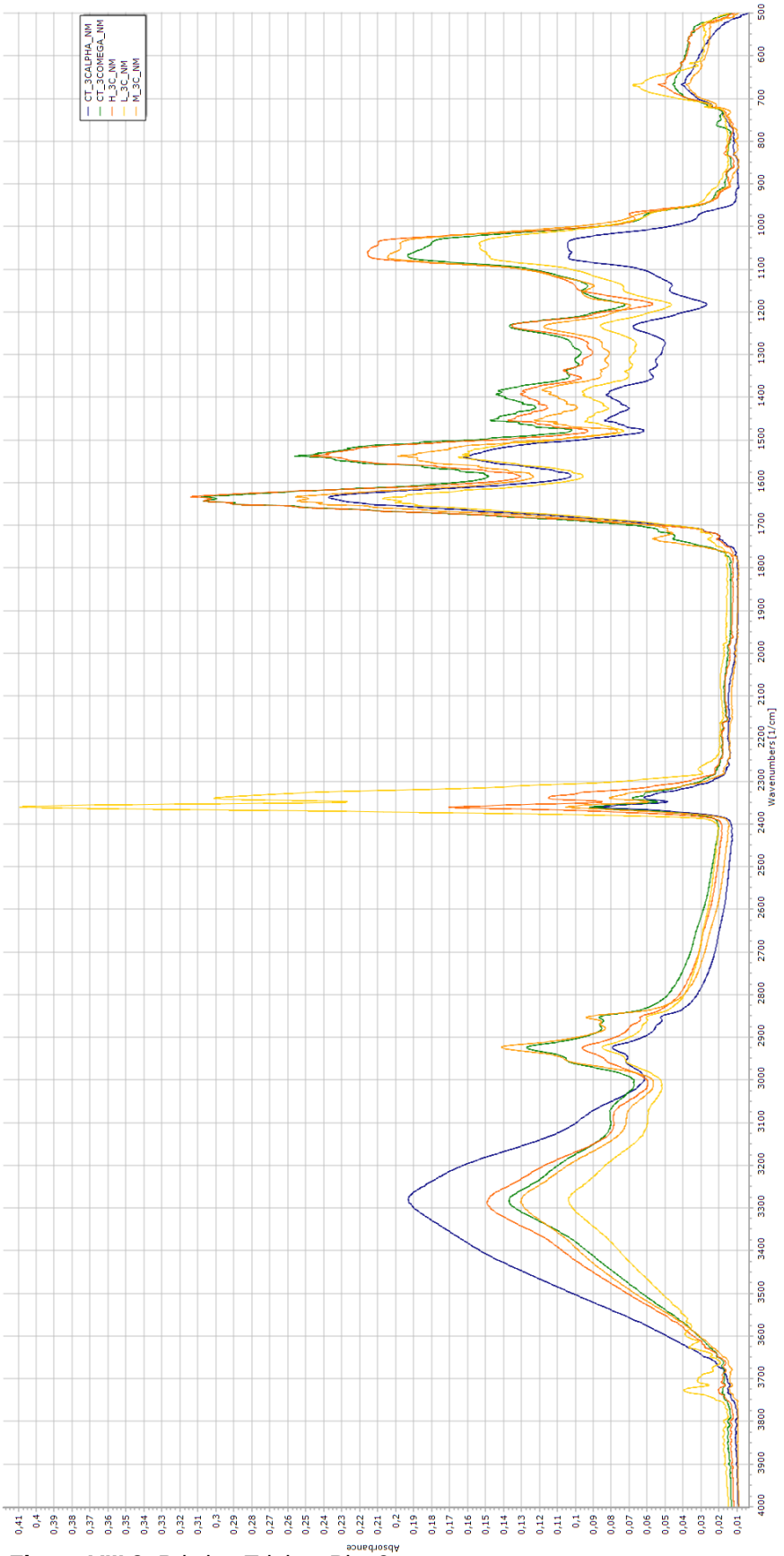


Figure VIII.9. Pristine Trials – Plot 9

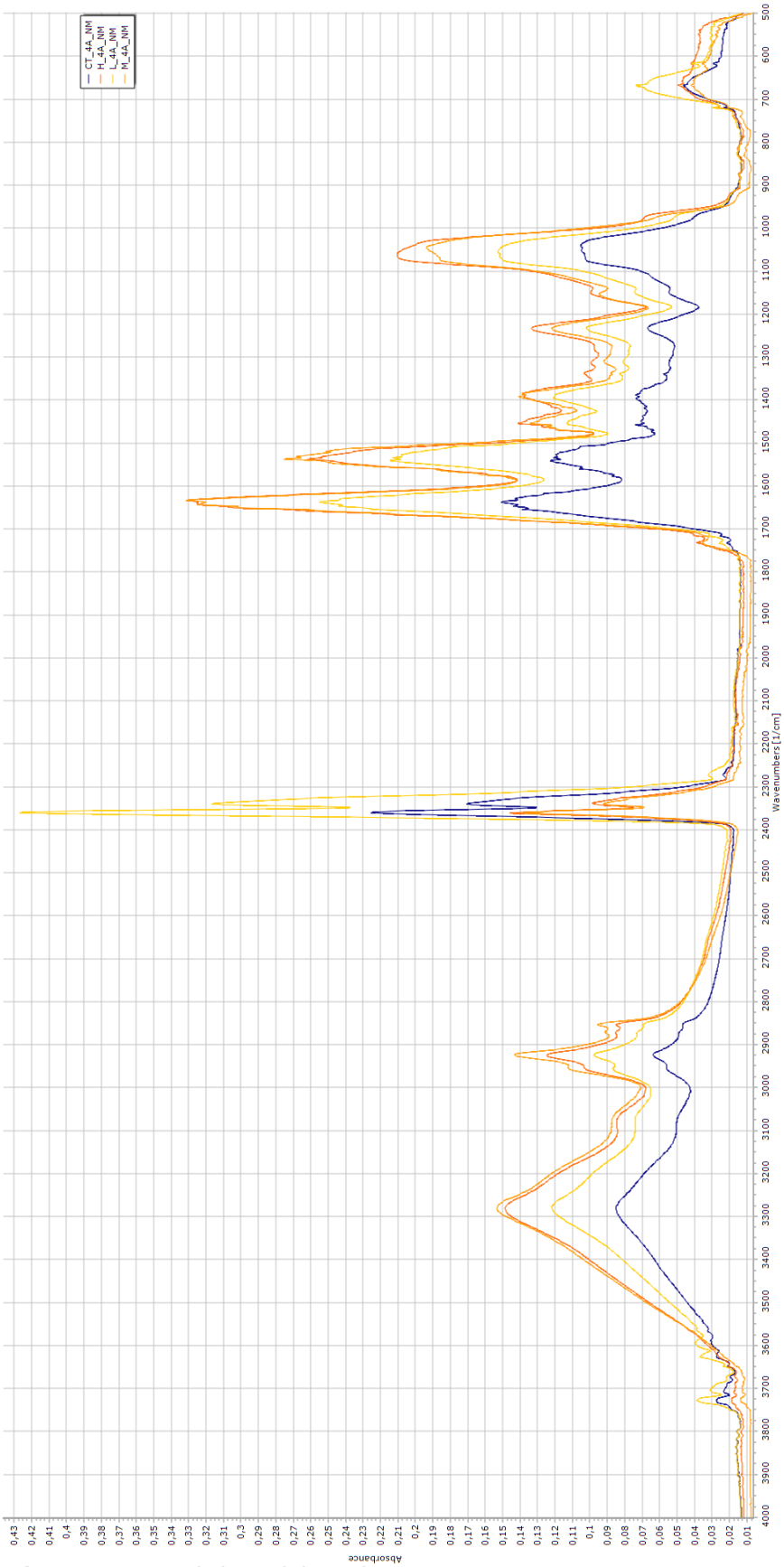


Figure VIII.10. Pristine Trials – Plot 10

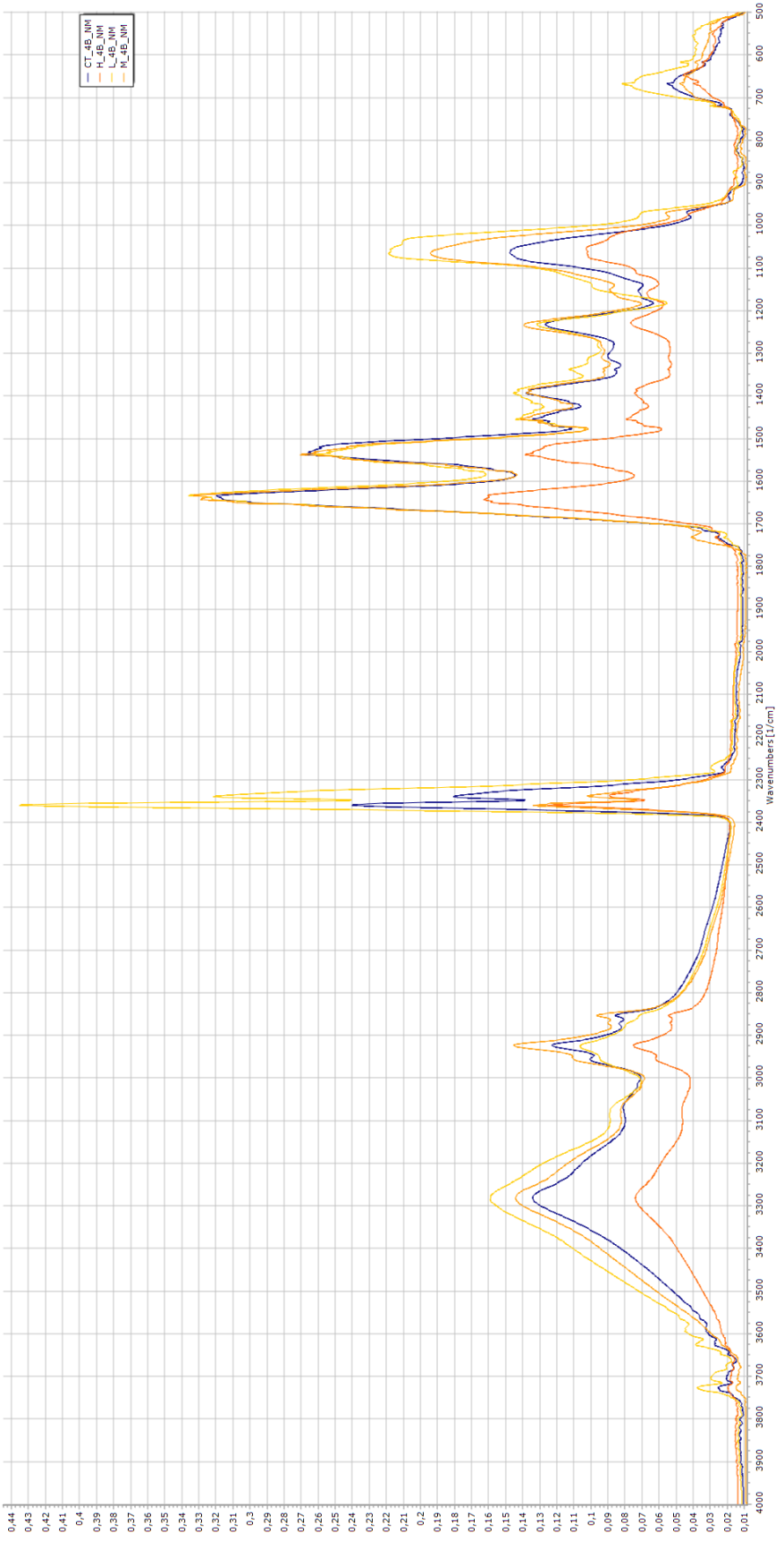


Figure VIII.11. Pristine Trials – Plot 11

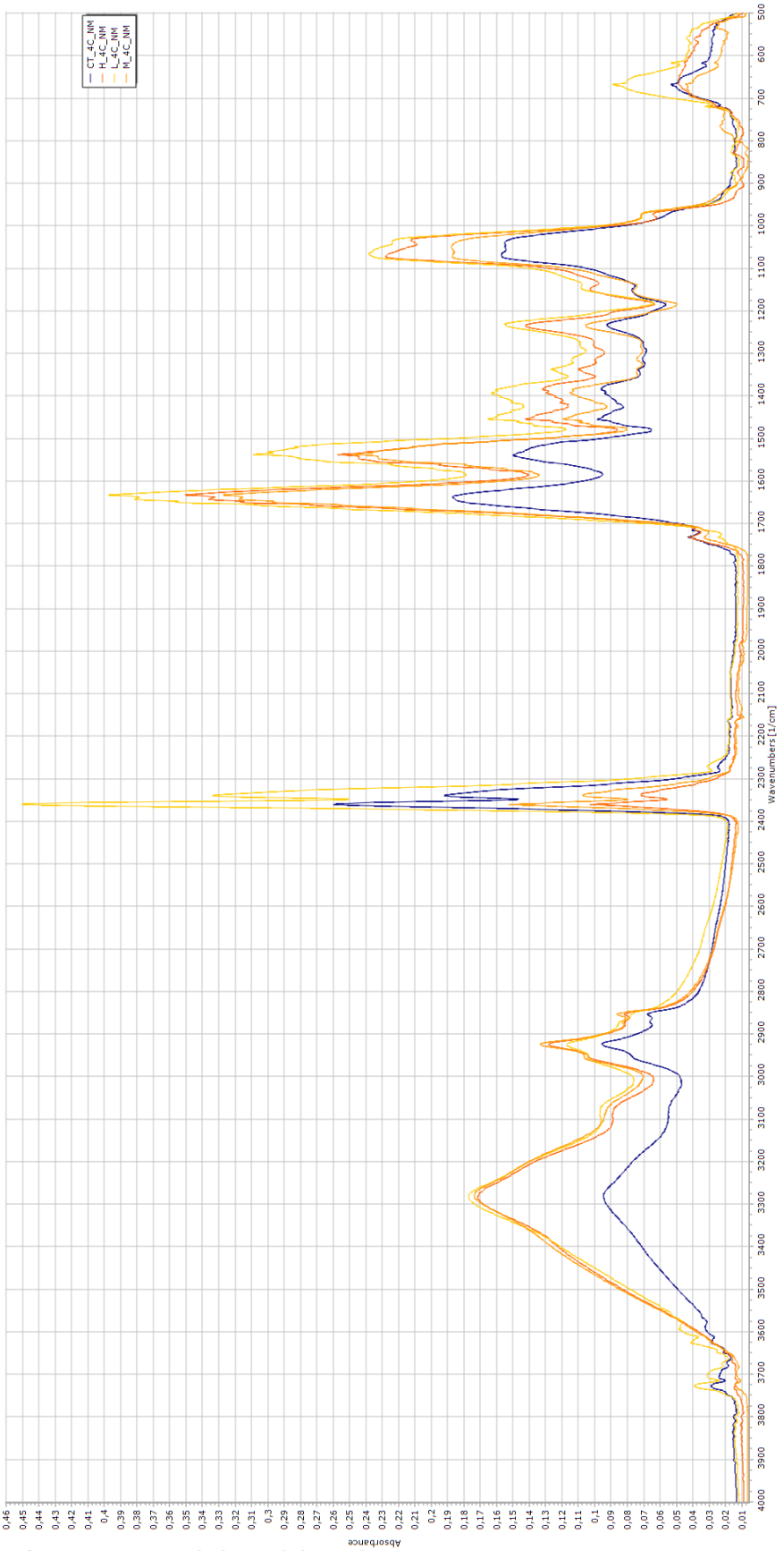


Figure VIII.12. Pristine Trials – Plot 12

UV-Weathered Trials

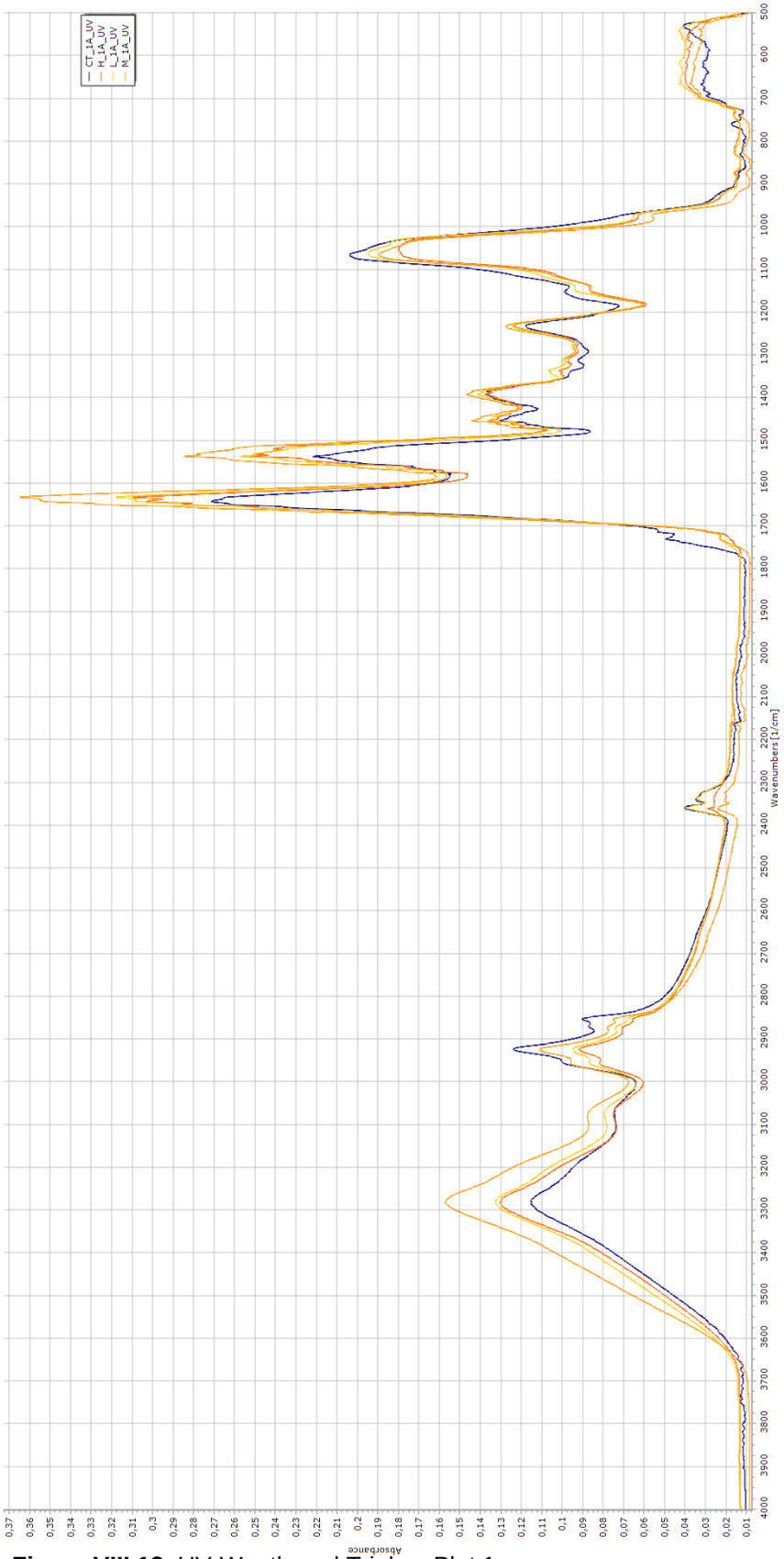


Figure VIII.13. UV-Weathered Trials – Plot 1

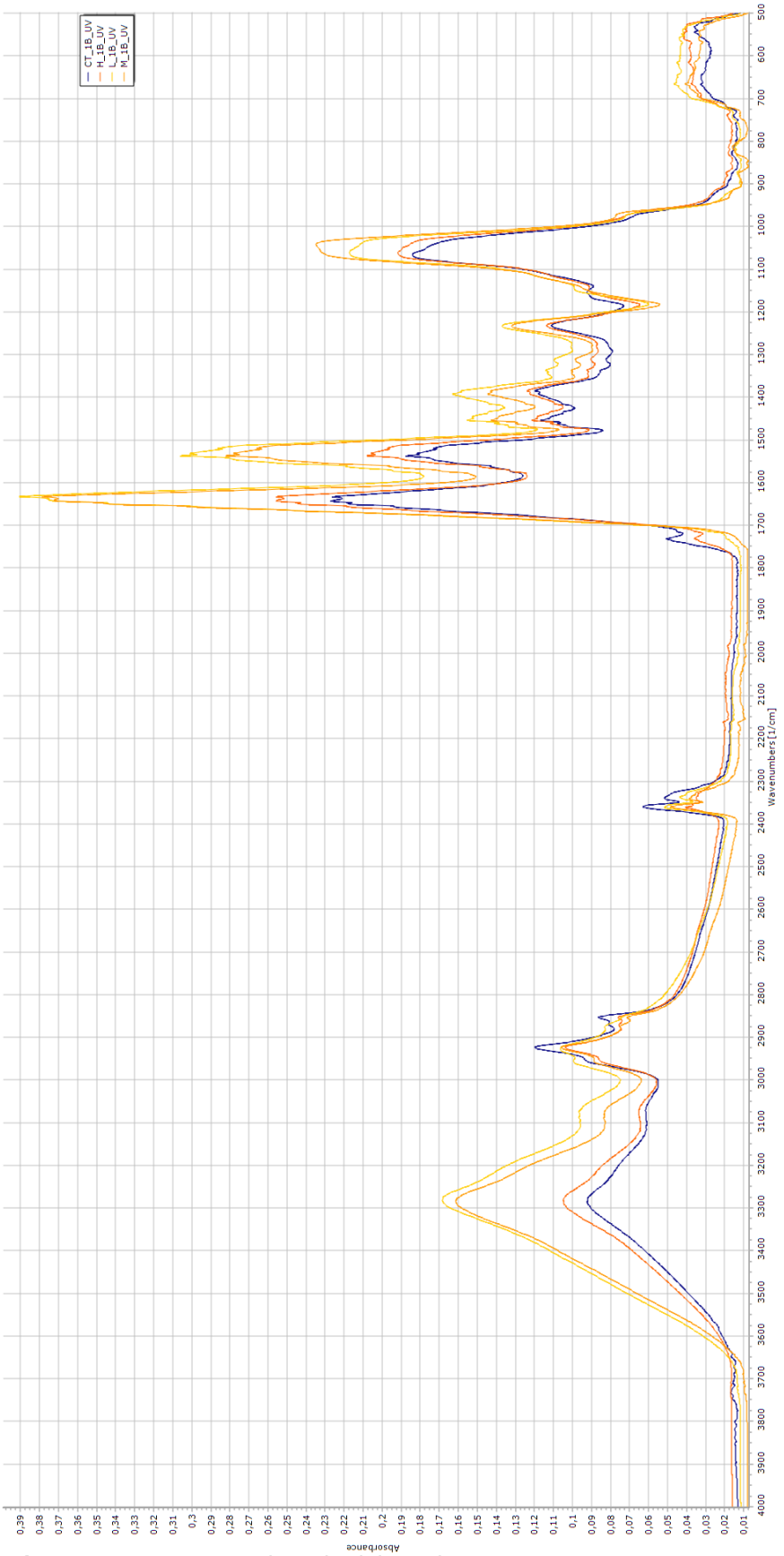


Figure VIII.14. UV-Weathered Trials – Plot 2

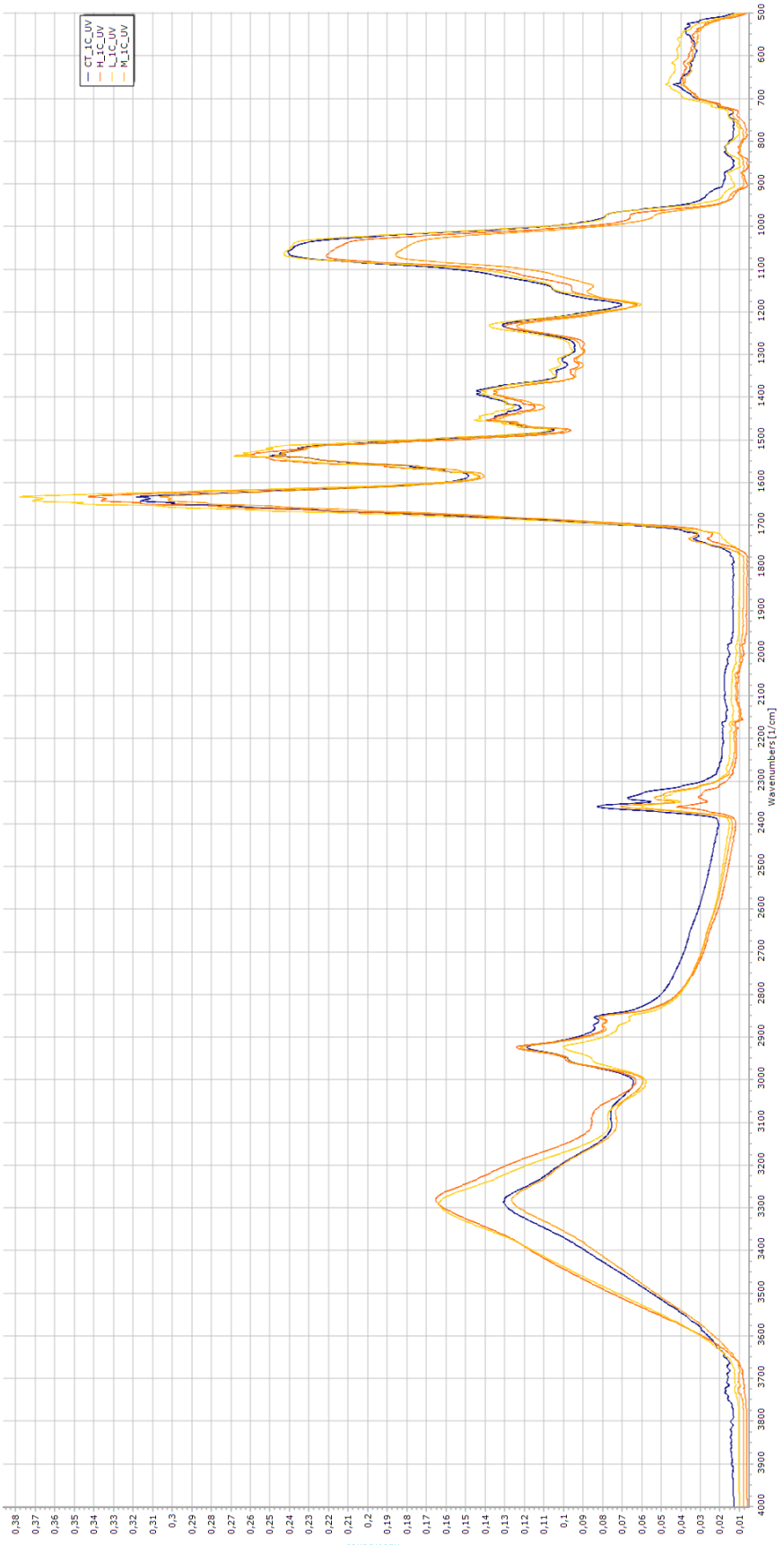


Figure VIII.15. UV-Weathered Trials – Plot 3

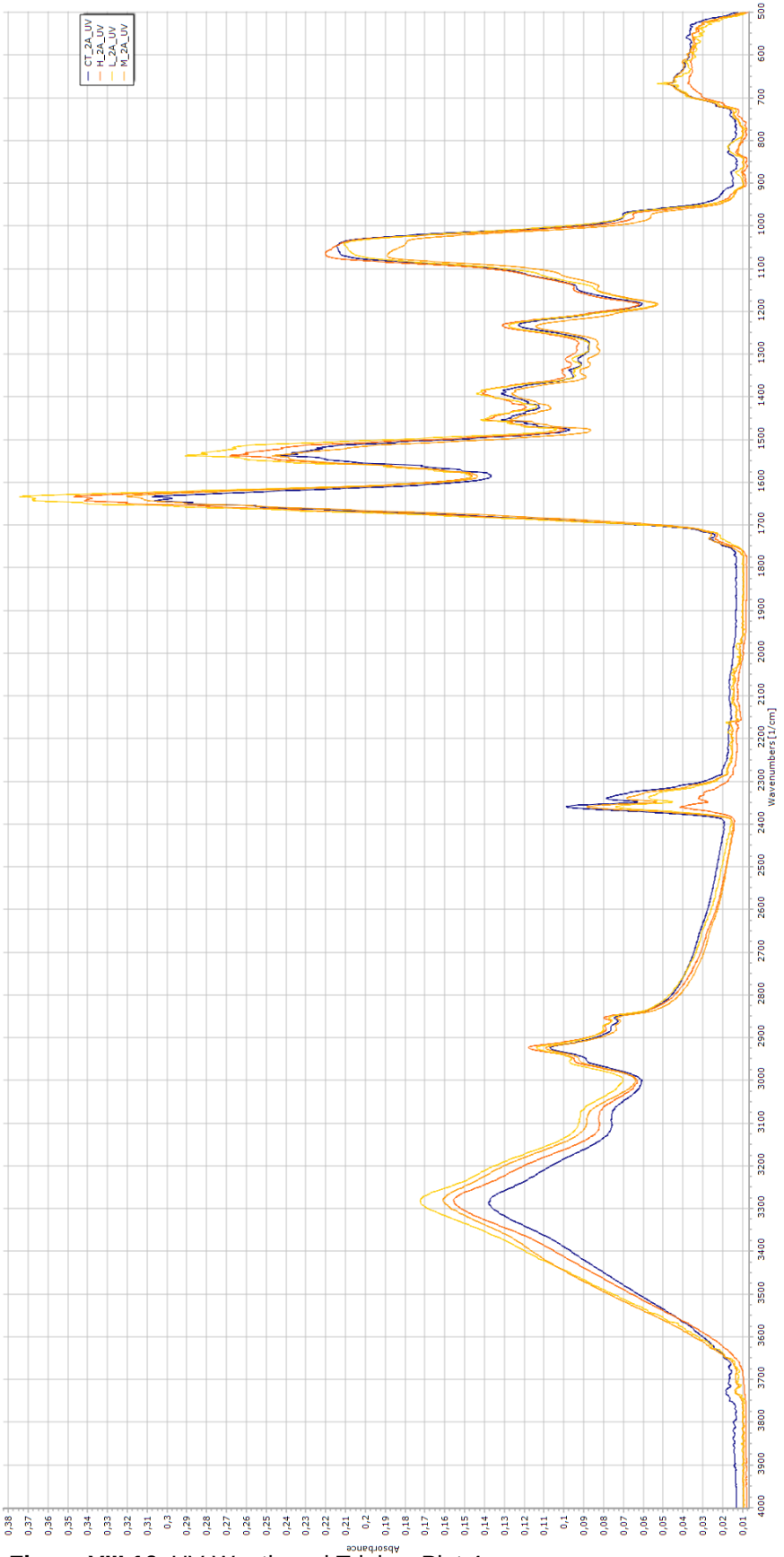


Figure VIII.16. UV-Weathered Trials – Plot 4

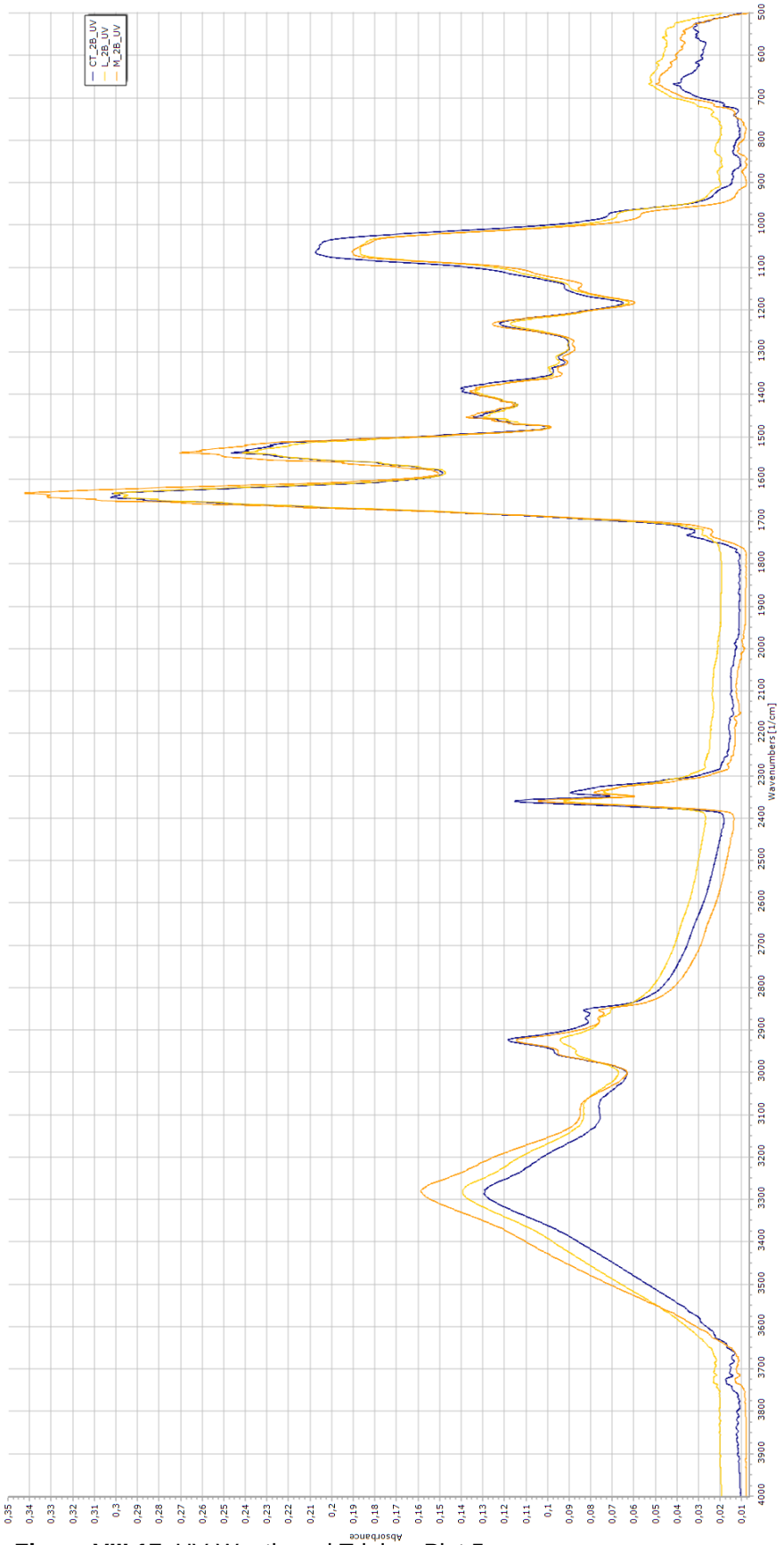


Figure VIII.17. UV-Weathered Trials – Plot 5

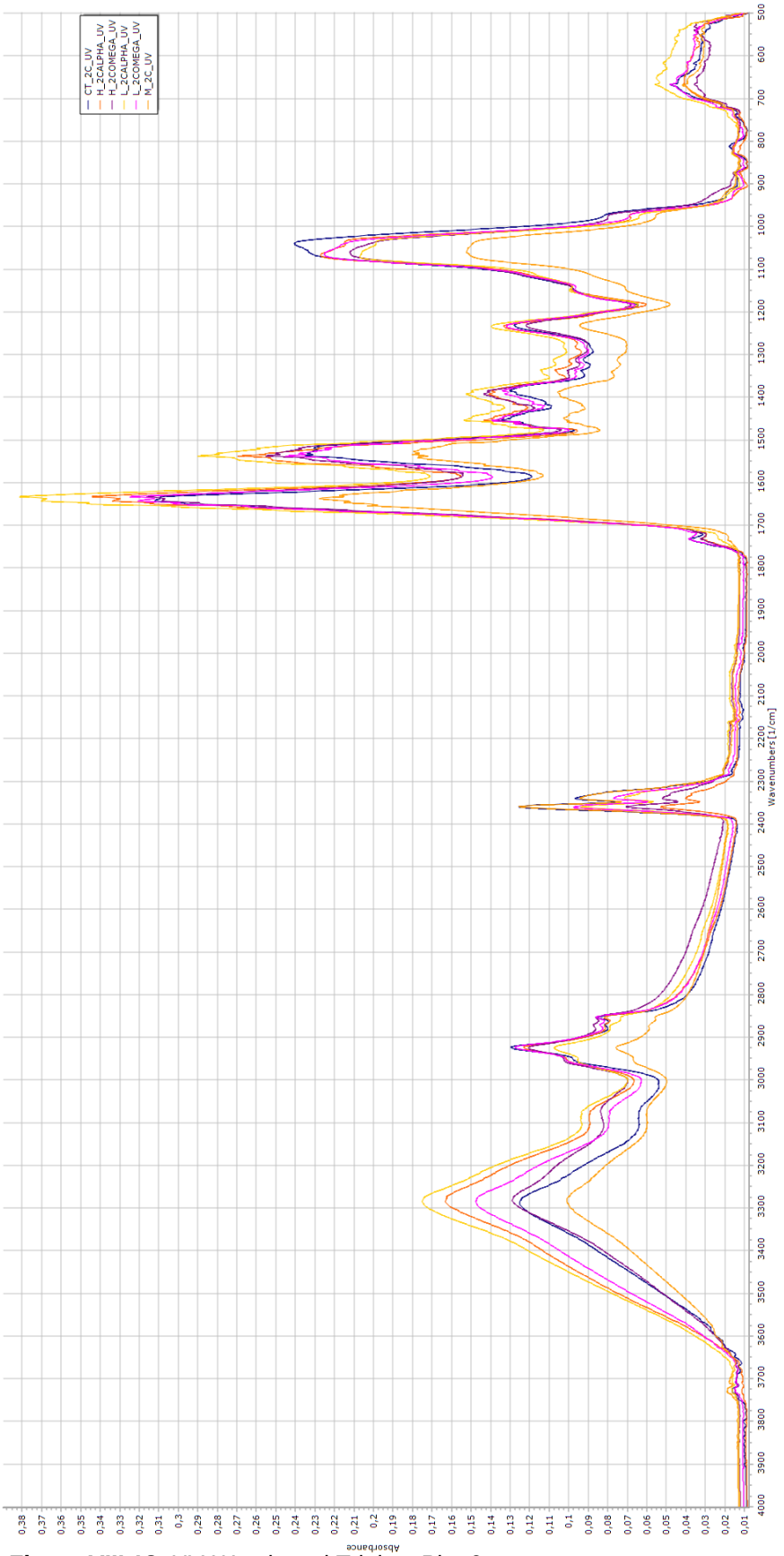


Figure VIII.18. UV-Weathered Trials – Plot 6

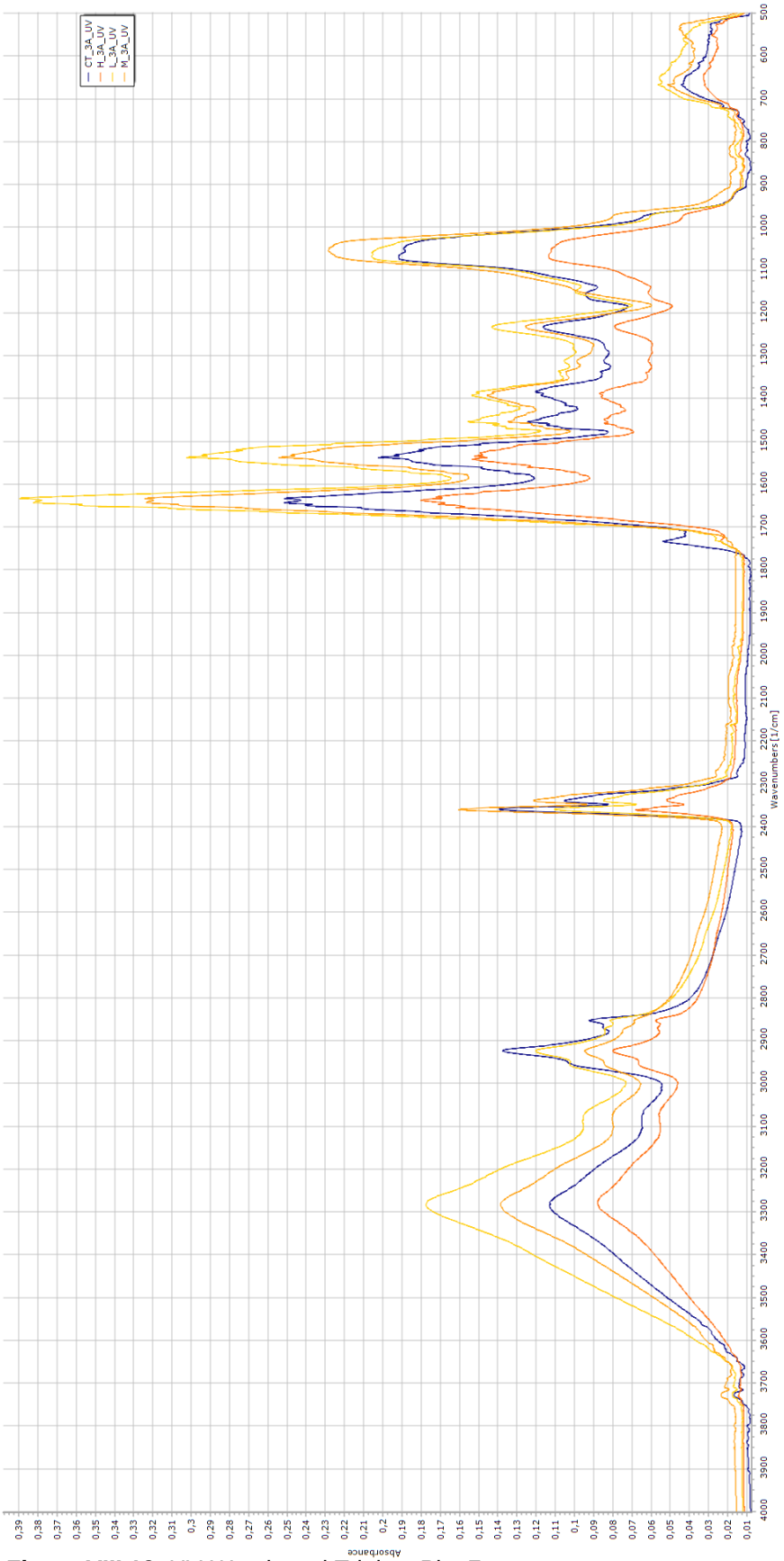


Figure VIII.19. UV-Weathered Trials – Plot 7

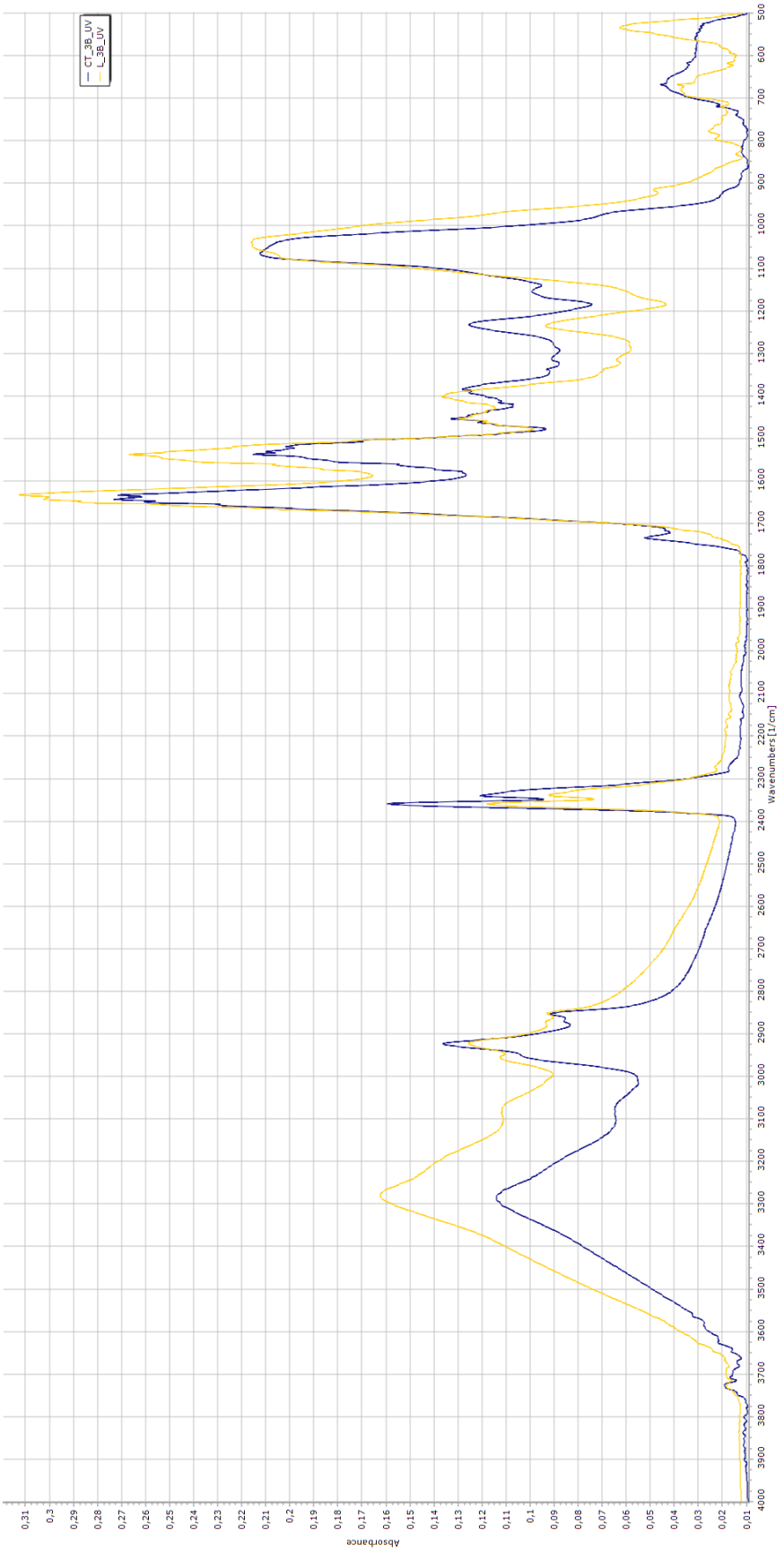


Figure VIII.20. UV-Weathered Trials – Plot 8

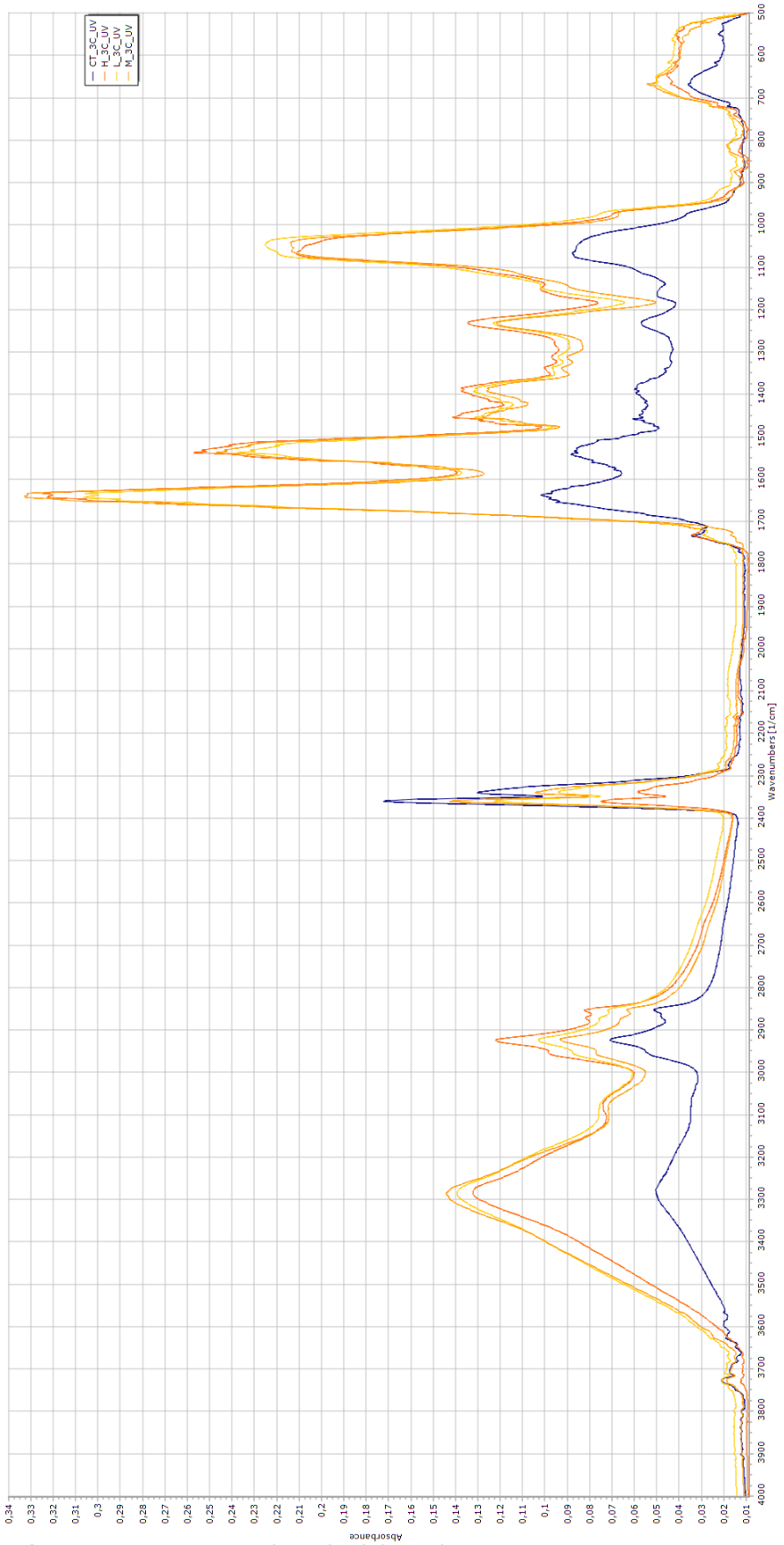


Figure VIII.21. UV-Weathered Trials – Plot 9

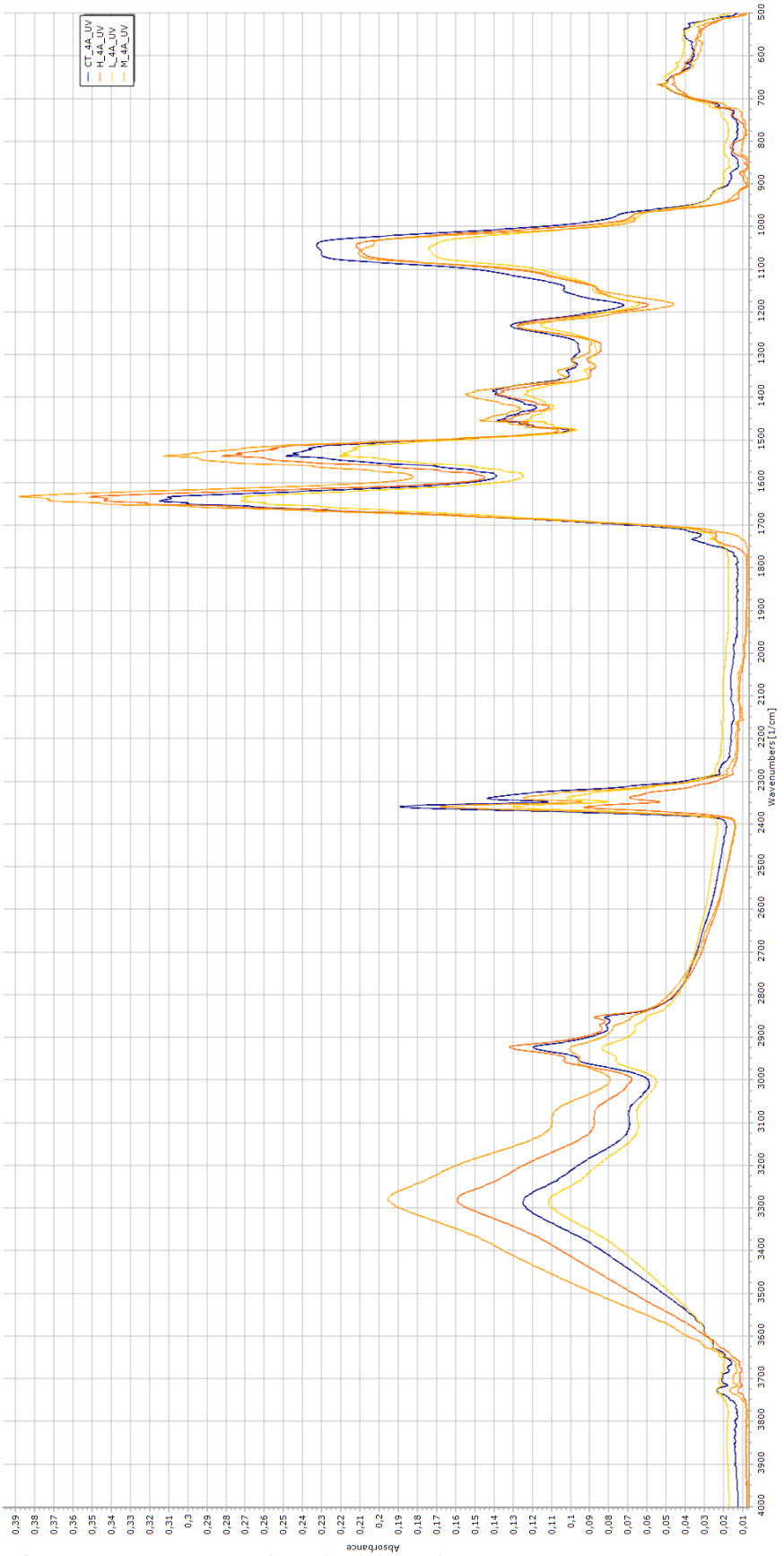


Figure VIII.22. UV-Weathered Trials – Plot 10

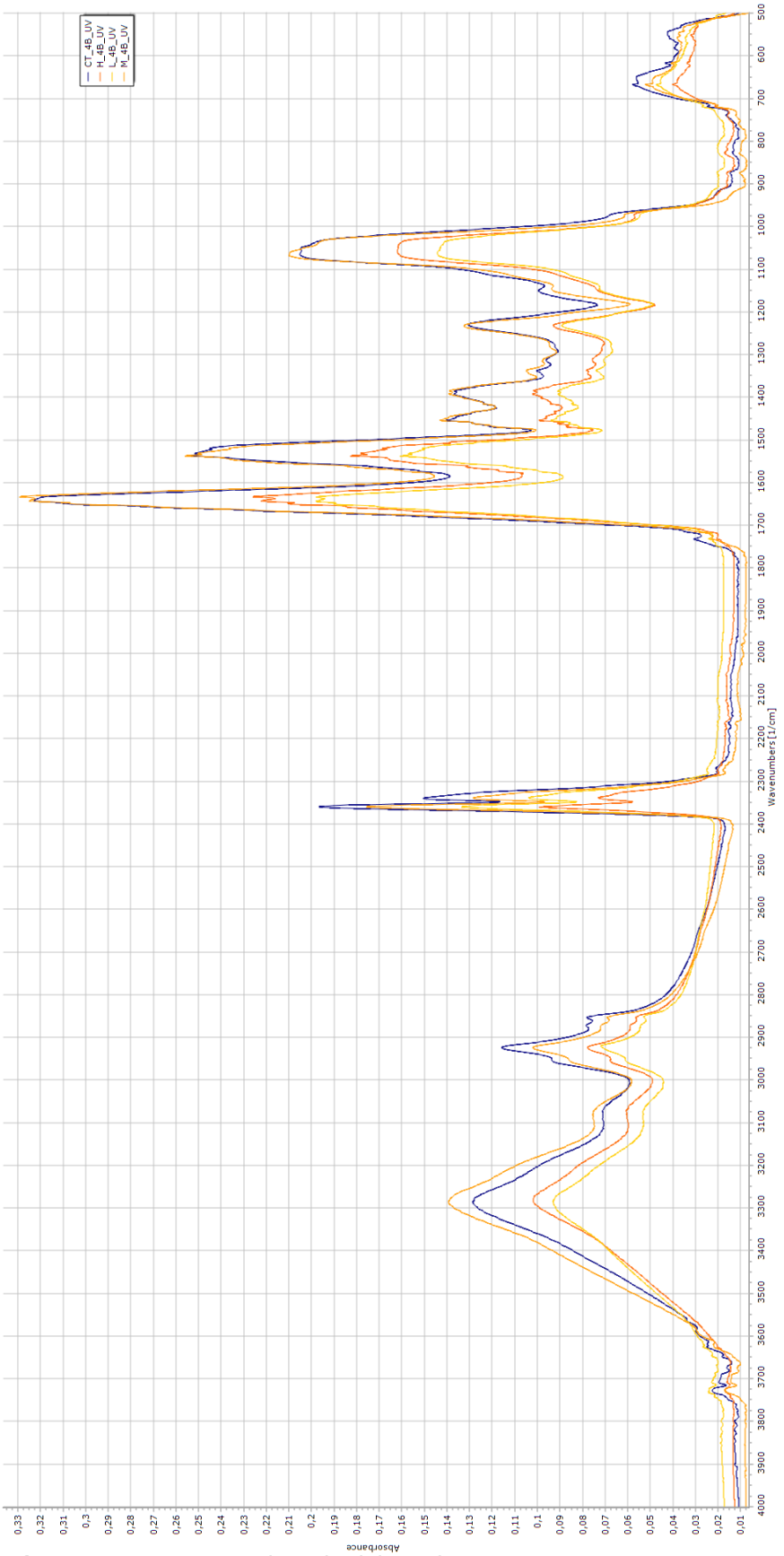


Figure VIII.23. UV-Weathered Trials – Plot 11

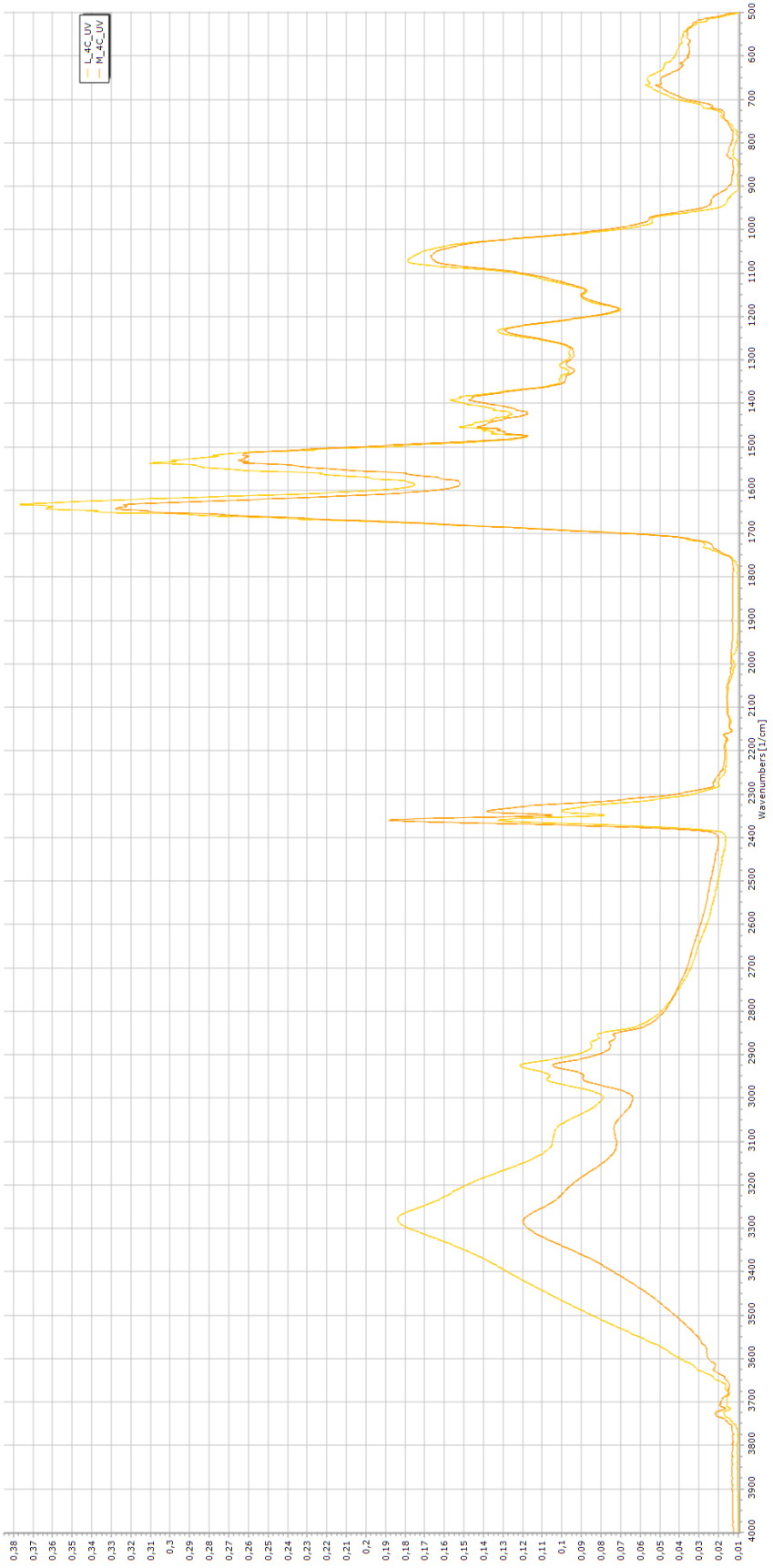


Figure VIII.24. UV-Weathered Trials – Plot 12