ASSESSMENT OF THE SUITABILITY OF STANDARD SOIL ASSAYS TO STUDY THE BIODEGRADATION OF POLYMER MICROPARTICLES.

By

GRACE DAVIES

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Abstract

Biodegradable polymers are increasingly suggested as alternatives for conventional polymers, particularly in agricultural applications where soil is the end-of-life environment. Complete environmental biodegradation is required to ensure that persistent micro and nano plastics do not form, hence reproducible and accurate standard tests for measuring biodegradation are critical. Polymer biodegradation in the environment is measured using standard test methods, with the International Organisation for Standardisation (ISO) test guideline ISO 17556:2019 being applicable for the soil environment. The respirometric endpoints of biological oxygen demand and carbon dioxide (CO_2) evolution are used to indirectly measure polymer biodegradation. However, the suitability of respirometric endpoints for measuring polymer biodegradation is uncertain because they were developed using the principles of small molecule biodegradation and lack extensive testing on high molecular weight, insoluble polymers. Further, respirometric endpoints only measure the fraction of a polymer that is completely mineralised, and do not provide information on the environmental fate of the non-mineralised polymer fraction which includes the bioassimilation of the polymer by soil microbes or residual polymer that persists in the soil. The non-mineralised polymer fraction is important to determine, as there are concerns that incomplete biodegradation could lead to persistent micro and nano plastic pollution, with potentially negative impacts for soils. This research investigated the suitability of respirometric tests to measure the biodegradation of the polymer microcrystalline cellulose in both natural and standard (i.e. artificial) soils and identified the potential causes of poor reproducibility within the ISO 17556:2019 test method. Additionally, the suitability of soil microplastic extraction methods to identify non-mineralised residual polymers, were investigated, to be used alongside the respirometric methods to develop a greater understanding of the environmental fate of polymers. The reproducibility of the standard test was found to be poor, both between soils and between respirometric measurement endpoints. Significant variation within key soil parameters such as

soil water content and nutrient availability are permitted, which contributes to widely varying test conditions. Thus, a key recommendation is that reducing the acceptable range of variability for test parameters would likely improve test reproducibility. Additionally, the use of respirometric endpoints means that the measurement of oxygen demand or evolved CO_2 cannot be directly attributed to the polymer. Soil microplastic extraction methods were considered as a potential method to identify residual polymers but were found to induce degradation in the biodegradable polymers polylactic acid (PLA) and polyhydroxybutyrate (PHB), therefore this was not considered an appropriate method for further identification of the residual polymer. Pyrolysis gas chromatography-mass spectrometry (Py-GC-MS) was also investigated and shown to be a useful analytical method for directly identifying polymers in soil, that could provide insights into the degradation mechanisms of polymers. This work also highlighted how additional analysis alongside respirometric techniques can provide insights regarding the assumptions of respirometric methods. For example, Py-GC-MS analysis showed no evidence of PLA degradation, despite an increase in measured CO_2, which provides evidence to suggest that part of the measured CO_2 can be soil derived whereas respirometric techniques assume all measured CO_2 is due to polymer biodegradation. Overall, it is important to continually develop methodologies that can accurately determine the environmental fate of biodegradable polymers to ensure better regulation and minimise environmental risk.

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List of Abbreviations

°C	Degree Celsius
µg	Microgram
µL	Microlitre
18A	18 Acres
ASE	Accelerated Solvent Extraction
ASTM	American Society for Testing and Materials
ATR-FTIR	Attenuated total reflectance Fourier transform infrared spectroscopy
$Ba(OH)_2$	Barium hydroxide
BOD	Biological oxygen demand
C	Carbon
CEN	European Committee for Standardization
CH_4	Methane
CO_2	Carbon dioxide
CUE	Carbon use efficiency
D	Dalton
Đ	Dispersity
DCM	Dichloromethane
DSC	Differential scanning calorimetry
ECHA	European Chemicals Agency
EU	European Union
FAO	Food and Agriculture Organisation of the United Nations
FTIR	Fourier transform infrared spectroscopy
g	Gram
GAR	Gartenacker
GC	Gas Chromatography
GPC	Gel permeation chromatography
H_2O	Water
HCl	Hydrochloric acid
HDPE	High-density polyethylene
hPa	hectopascal
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
Kg	Kilogram
KOH	Potassium hydroxide
kPa	kilopascal
L	Litre
LDPE	Low-density polyethylene
m/z	Mass-to-charge ratio
MeOH	Methanol
Mg	Milligram
min	Minute
mL	Millilitre

mm	Millimetre
M_n	Number average molecular weight
MNP	Micro and nano plastic
MP	Microplastic
Mr	Relative molecular mass
MS	Mass Spectrometry
Mt	Million tonnes
M_w	Weight average molecular weight
NMR	Nuclear Magnetic Resonance
O_2	Oxygen
OECD	Organisation for Economic Co-operation and Development
PAE	Phthalate acid esters
PBAT	Polybutylene adipate terephthalate
PBS	Polybutylene succinate
PBSA	Poly(butylene succinate-co-adipate)
PC	Polycarbonate
PCL	Polycaprolactone
PE	Polyethylene
PET	Polyethylene terephthalate
pF	Field capacity
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PLA	Polylactic acid
PP	Polypropylene
PS	Polystyrene
PVA	Polyvinyl alcohol
Py-GC-MS	Pyrolysis Gas Chromatography Mass Spectrometry
q^{-1}H NMR	Quantitative Proton Nuclear Magnetic Resonance
REACH	Restriction, Evaluation, Authorisation and Restriction of Chemicals
SDS	Sodium dodecyl sulphate
sec	Second
STS	Standardised soil
SWC	Soil water content
TED GC MS	Thermal Extraction Desorption Gas Chromatography Mass Spectrometry
TG	Test guideline
T_g	Glass transition temperature
$ThCO_2$	Theoretical carbon dioxide evolved
ThOD	Theoretical oxygen demand
T_m	Melting temperature
TMAH	Tetramethylammonium hydroxide
TPU	Thermoplastic Polyurethanes
UN	United Nations

USDA	United States Department of Agriculture
UV	Ultraviolet
WHC	Water holding capacity
$ZnCl_2$	Zinc chloride
ΔH_f	Enthalpy of Fusion

Chapter 1. Introduction and Literature Review

Biodegradable polymers are considered as alternatives for conventional plastics, due to increasing concerns regarding the impacts of plastic pollution on the environment and human health (Borrelle et al., 2020; Geyer et al., 2017; Hurley and Nizzetto, 2018; Jambeck et al., 2015; Krause et al., 2024; Li et al., 2023; Rahman et al., 2021; Rochman, 2018; Rochman et al., 2013; Thompson et al., 2004, 2009; Wright and Kelly, 2017). Agriculture is a key user of plastic, with plastic use contributing to increased agricultural productivity (FAO, 2021a). While beneficial, this can result in micro and nano plastics (MNPs) entering agricultural soils, which can have negative impacts on soil physicochemical characteristics and biota (Hurley and Nizzetto, 2018; Okoffo et al., 2021; Pinto da Costa et al., 2019; Rillig, 2012; de Souza Machado et al., 2018a, 2018b, 2019; Zhang et al., 2020; Zhou et al., 2020). Due to the potential for negative impacts, there is an increasing need for plastic usage in agriculture to be more sustainable, with biodegradability in the environment considered a key part of the solution to increase their sustainability (Hofmann et al., 2023). However, the suitability of currently used methodologies for measuring the biodegradation of polymers in soil is uncertain, resulting in increased concern regarding the potential for incomplete biodegradation and the formation of persistent MNPs (Afshar et al., 2024; Agarwal, 2020; Briassoulis and Mistriotis, 2018; Briassoulis et al., 2014; Briassoulis and Degli Innocenti, 2017; Briassoulis and Dejean, 2010; Haider et al., 2019; Kaur and Chauhan, 2024; Liao and Chen, 2021; Sintim et al., 2019). Therefore, it is important to consider the suitability of current methods used to measure polymer biodegradation in soils.

1.1. Plastic Pollution and Impacts

The demand for plastic, and therefore production, has increased due to its versatility and durability. However, such characteristics also result in its environmental persistence, especially when combined with poor waste management (Borrelle et al., 2020; Geyer et al., 2017; Jambeck

et al., 2015; Thompson et al., 2004, 2009). Global plastic production was 400.3 million tonnes (Mt) in 2022, and this is expected to increase to approximately 764 Mt by 2040 (Nordic Council, 2023; Plastics Europe, 2023). The unsustainable production of plastic is paralleled by increasing plastic pollution, making this a critical global challenge to address (Walker and Fequet, 2023).

Plastic pollution is typically categorised by size, with macro (>5 mm), micro (1 μm – 5 mm) and nano (<1 μm) scale plastic debris (Hartmann et al., 2019). MNPs have been extensively documented in all environments, with recent initial evidence of MNPs in human tissues (Allen et al., 2019, 2022; Amato-Lourenço et al., 2021; Braun et al., 2021; Materić et al., 2021; Napper et al., 2020). Whilst there is evidence suggesting that MNPs have negative impacts for ecosystems and organisms, resulting from both particle characteristics and plastic associated chemicals, the extent of risk posed to human health is currently unknown (Catarino et al., 2021; Dissanayake et al., 2022; Guzzetti et al., 2018; Krause et al., 2024; Rahman et al., 2021; de Sá et al., 2018). Therefore, there is increasing concern regarding the impact of MNPs, which has prompted increased legislative efforts globally to mitigate plastic pollution. Global action is being developed, with the United Nations currently establishing an international plastics treaty, numerous countries imposing bans on single use plastics (e.g. plastic bags and straws), and the European Union (EU) imposing a wide-ranging restriction on the use of intentionally added microplastics (DEFRA, 2024; European Union, 2023; United Nations, 2024).

Whilst MNPs have been detected in all environments, research has traditionally focused on the marine environment (Akdogan and Guven, 2019). However, awareness of MNPs in terrestrial soil ecosystems has increased in recent years because soil is expected to be a significant sink for MNPs (Hurley and Nizzetto, 2018; Nizzetto et al., 2016). MNPs can negatively affect soil properties, including the nutrient availability, aggregate stability and soil water content, which can lead to reduced crop yields (Chen et al., 2024; Graf et al., 2023; Lehmann et al., 2019, 2021; Qi et

al., 2020; de Souza Machado et al., 2018b, 2019). MNPs can also affect soil microbial community abundances and have negative ecotoxicological impacts on key soil organisms such as earthworms (Boots et al., 2019; Dong et al., 2024; Han et al., 2024; Huerta Lwanga et al., 2016; Huo et al., 2022; Zhou et al., 2021). Conversely, some studies have found no significant evidence of impacts from plastics on soil properties in field studies, suggesting that more research is needed to understand the reasons for the disparity in results (Brown et al., 2022; Chu et al., 2023; Greenfield et al., 2022). Further, as many plastics contain additives to improve their functionality, it is important to understand the physical versus chemical drivers of MNP impacts (Lambert et al., 2017). For example, a recent study found no short-term effects of polyethylene (PE) on soil biological functioning (Brown et al., 2022) but testing PE as a pure polymer is unlikely to represent the natural environment, as additives are expected to be present. Additives such as plasticisers, flame retardants, and stabilisers are widely used in conventional and biodegradable plastics (Hahladakis et al., 2018). As agricultural plastics degrade in the environment, such additives can leach into the soil where they can potentially have negative impacts on soil biota, such as reduced seed germination in plants, as well as oxidative stress and developmental toxicity in organisms (De Bruin et al., 2016; Cao et al., 2023; Lemos et al., 2010; Xue et al., 2009; Zhang et al., 2015).

Although biodegradability is considered a solution for plastic pollution, recent studies have shown negative ecotoxicological impacts of biodegradable polymers. For example, the addition of the biodegradable polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) to soil resulted in reduced soil microbial activity and alteration of the soil bacterial community, as well as a reduction in plant growth (Brown et al., 2023). Further, the impact of the biodegradable polymer polylactic acid (PLA) was found to have dose specific negative impacts on the growth and reproduction of the earthworm *Eisenia fetida* which are comparable to the negative impacts seen after exposure to conventional polymers (Courtene-Jones et al., 2024). It is important to determine the environmental fate and ecotoxicity of biodegradable polymers to enable a

comprehensive environmental risk assessment and avoid regrettable substitution (Drohmann and Hernández, 2020).

1.2. Agricultural Plastics

Productive and sustainable agricultural ecosystems are critical for global food security (Viana et al., 2022). Currently ~11 billion tonnes of food are produced by agricultural systems annually (FAO, 2021b). The expected increase of the global population to 9.8 billion in 2050, will place pressure on agriculture systems for increased food production (United Nations, 2019). Globally, an estimated 12.5Mt of plastic are utilised in agriculture annually, which increases productivity (FAO, 2021a), but results in high volumes of MNPs potentially entering agricultural soils, and therefore there is a drive to increase the sustainability of plastic usage in agriculture (Hofmann et al., 2023; Meizoso-Regueira et al., 2024).

All agricultural plastic usages have measurable benefits, including increased productivity and improved environmental and human safety. Plastics are heavily used for crop production, with examples including plastic mulching films, plastic pots, greenhouses, protective nets, irrigation, plastic containers for pesticides, and the use of polymer coated fertilisers and plant protection products (FAO, 2021a). Plastic mulching films represents ~50% of agricultural plastic use, with significant benefits including soil moisture and temperature maintenance, and enhanced nutrient uptake, which directly translates into increased crop yields and reduced demand for water and nutrients (Hofmann et al., 2023; Kasirajan and Ngouajio, 2012). Polymer encapsulation technology for fertilisers and pesticides contributes to improved environmental and human safety, by controlling release and efficiency, as well as minimising loss to the environment via agricultural runoff (Pinto et al., 2022; Shirley et al., 2001; Sikder et al., 2021; Sinha et al., 2019). However, all plastic usage can result in MNPs entering the environment.

MNPs are typically classed as primary or secondary, primary MNPs are when plastics are manufactured at the MNP scale, whereas secondary MNPs are produced from the degradation and fragmentation of plastic products in the environment. Both primary and secondary MNPs can reach the environment either directly or indirectly. Indirect sources of MNPs to agricultural soils can include plastic mulching films, the application of sewage sludge as fertiliser, or the use of wastewater for irrigation (Kumar et al., 2020; Lwanga et al., 2022; Moeck et al., 2023). Plastic mulch films will contribute to MNP formation through environmental degradation and fragmentation, making them an indirect source (Huang et al., 2020; Kasirajan and Ngouajio, 2012; Yang et al., 2022). Wastewater treatment plants use treatments which lead to the retention of MNPs in the sewage sludge, to minimise the outflow of MNPs into the environment. However, sewage sludge can then be applied as agricultural fertiliser, which then becomes an indirect source of MNPs to the soil (Hurley and Nizzetto, 2018; Zubris and Richards, 2005). Additionally, MNPs are not always completely removed from the wastewater, which can then be used for irrigation providing a further indirect source of MNPs to agricultural soils (Conley et al., 2019; Zhang et al., 2020). Direct sources of MNPs to agricultural soil include controlled release fertilisers and fertiliser additives, capsule suspension plant protection products, and coated seeds (European Chemicals Agency, 2019). Polymer encapsulation technology is widely used in these products which are directly produced at the microplastic size range; therefore, they will directly incorporate MNPs into agricultural soils (European Union, 2023; Machado et al., 2022; Woźniak-Budych et al., 2024; Zanino et al., 2024).

Whilst plastic usage in agriculture provides environmental and human safety benefits, there is a drive to make plastic usage in agriculture more sustainable and reduce MNP pollution (Hofmann et al., 2023). Therefore, biodegradable polymers have been suggested as an alternative option for agricultural plastics, as their use in theory would maintain key benefits of plastic and reduce soil contamination with MNPs (Agarwal, 2020; Hofmann et al., 2023).

1.3. Polymer Biodegradation: Definitions and Process

Polymer biodegradation is a desirable trait, however, there is inconsistency regarding the definition of key terms. It is important to explicitly define several commonly used terms to avoid confusion (Box 1.1). Polymers are large molecules composed of repeating units of monomers, whereas plastics are specific types of polymers that have been processed, typically under heat, to a final solid state (Rosenboom et al., 2022).

Box 1.1. Key Terms and Definitions. Adapted from FAO, 2021a; Lambert and Wagner, 2017; IUPAC, 2019.

Term	Definition
Bioassimilation	The metabolization of low molecular weight compounds (e.g. dimers, monomers) by transport through a cell membrane (Bher et al., 2022).
Bio based	Plastics made from polymers derived from non-petroleum, biological sources, including plant and microbial based polymers (FAO, 2021a). Being bio-based has no relation to whether the plastic is biodegradable.
Biodegradable	Polymer is susceptible to degradation by biological activity, with the degradation accompanied by a lowering of its molar mass (IUPAC, 2019). Biodegradation results from the actions of naturally occurring microorganisms such as bacteria and fungi, and ultimately results in the formation of biomass, water, and either carbon dioxide in aerobic conditions or methane in anaerobic conditions (FAO, 2021a).
Biodegradation	Breakdown of a substance catalysed by enzymes in vitro or in vivo. This may be characterized for purposes of hazard assessment as: 1. Primary. Alteration of the chemical structure of a substance resulting in loss of a specific property of that substance. 2. Environmentally acceptable. Biodegradation to such an extent as to remove undesirable properties of the compound. This often corresponds to primary biodegradation, but it depends on the circumstances under which the products are discharged into the environment. 3. Ultimate. Complete breakdown of a compound to either fully oxidized or reduced simple molecules (such as carbon dioxide/methane, nitrate/ammonium and water). It should be noted that the products of biodegradation can be more harmful than the substance degraded. (IUPAC, 2019)
Compostable	A subset of biodegradable polymers that undergo biological degradation under composting conditions. Industrial compositing conditions include temperatures greater than 55°C, high humidity and oxygen availability (FAO, 2021a).
Depolymerisation	The breakdown of polymer chains into small molecules such as oligomers, trimers, dimers, and monomers by the action of extracellular enzymes (Bher et al., 2022).
Mineralisation	The ultimate biodegradation of polymers to simple molecules (e.g. carbon dioxide, methane, water) and biomass (Bher et al., 2022).
Monomer	A substance composed of monomer molecules, which are molecules that can undergo polymerisation thereby contributing constitutional units to the essential structure of a macromolecule (IUPAC, 2019).

Oligomer	A substance composed of oligomer molecules, which are molecules of intermediate relative molecular mass, the structure of which essentially comprises a small plurality of units derived, actually or conceptually, from molecules of lower relative molecular mass (IUPAC, 2019).
Plastic	A material that contains as an essential ingredient one or more organic polymeric substances of large molecular weight, is solid in its finished state, and, at some stage in its manufacture or processing into finished articles, can be shaped by flow (Lambert and Wagner, 2017).
Polymer	A large molecule composed of repeating units of monomers (Lambert and Wagner, 2017).
Polymer Degradation	Chemical changes in a polymeric material that usually result in undesirable changes in the in-use properties of the material. In most cases degradation is accompanies by a decrease in molar mass. In some cases, degradation means changes in the chemical structure. Usually, degradation results in the loss of, or deterioration in, useful properties of the material (IUPAC, 2019).

Plastic products are often a blend of polymers and additive compounds, which are added to improve the functionality of the plastic product for its intended use. There are four general categories of additives: functional additives, colorants, filters, and reinforcements (Table 1.1, (Hahladakis et al., 2018; Lambert and Wagner, 2017)). In nearly all cases, the additives are not chemically bound to the polymer (Hahladakis et al., 2018).

Table 1.1. Non exhaustive list of exemplary commonly used additives, typical amounts of use, purposes and example compounds. Information presented in this table is adapted from Hahladakis et al., 2018 and Lambert and Wagner, 2017.

Type of Additive	Typical Amount (Range, % w/w)	Purpose	Example Compound (non-exhaustive)
Functional Additives			
Plasticisers	10-70	Improved flexibility, durability, and stretchability	Phthalic esters (PAEs), Bis (2-ethylhexyl) phthalate (DEHP), dipentyl phthalate (DPP), Benzyl butyl phthalate (BBP), di-(2-ethylhexyl) adipate (DEHA), di-octyladipate (DOA)
Flame retardants	3-25 (brominated) 0.7-3	Adjust the flammability	Organic non- reactive (e.g. phosphate esters, halogenated phosphate esters, halogenated hydrocarbons) Inorganic nonreactive (e.g. antimony oxide, aluminum oxide trihydrate, zinc borate, ammonium orthophosphate, ammonium sulfamate) Reactive (e.g. bromine and/or phosphorus containing polyols, halogenated phenols,

			tetrachlorophthalic anhydride, phosphonate esters, dibromoneopentyl alcohol)
Stabilisers, antioxidants and UV stabilisers	0.05-3	Increased durability by delaying overall oxidative degradation of plastics	Arylamines e.g. Butylated hydroxytoluene (BHT), 2- and 3-t-butyl-4 hydroxyanisole (BHA), Bisphenol A (BPA) Phenolics and organophosphites e.g. Tris-nonyl-phenyl phosphate (TNPP), tris (2, 4-di-tert-butylphenyl) phosphite, (Irgafos 168).
Heat stabilisers	0.5-3	Prevention of thermal degradation when exposed to elevated temperatures	Cadmium and lead compounds, Nonylphenol
Colorants			
Organic pigments	0.001-2.5	Gives a specific colour	Cobalt (II) diacetate
Inorganic pigments	0.01-10	Gives a specific colour	Cadmium compounds, chromium compounds, lead compounds
Fillers and Reinforcements			
Fillers	Up to 50		Calcium carbonate, clay, zinc oxide, metal powder
Reinforcements	15-30	Reinforced materials	Glass fibres, carbon fibres, aramide fibres

'Bioplastic' is a term often used interchangeably, to mean either a bio-based or biodegradable polymer, or both. However, this is a misleading term, and as such its use is discouraged (FAO, 2021a; Lambert and Wagner, 2017; Vert et al., 2012). Biodegradable polymers can be made from both fossil-based and bio-based feedstocks (the source of biomass). The biodegradability of a polymer is independent of the feedstock of polymer production. The chemical and physical structure of a polymer are key factors that control susceptibility to biodegradation (Figure 1.1).

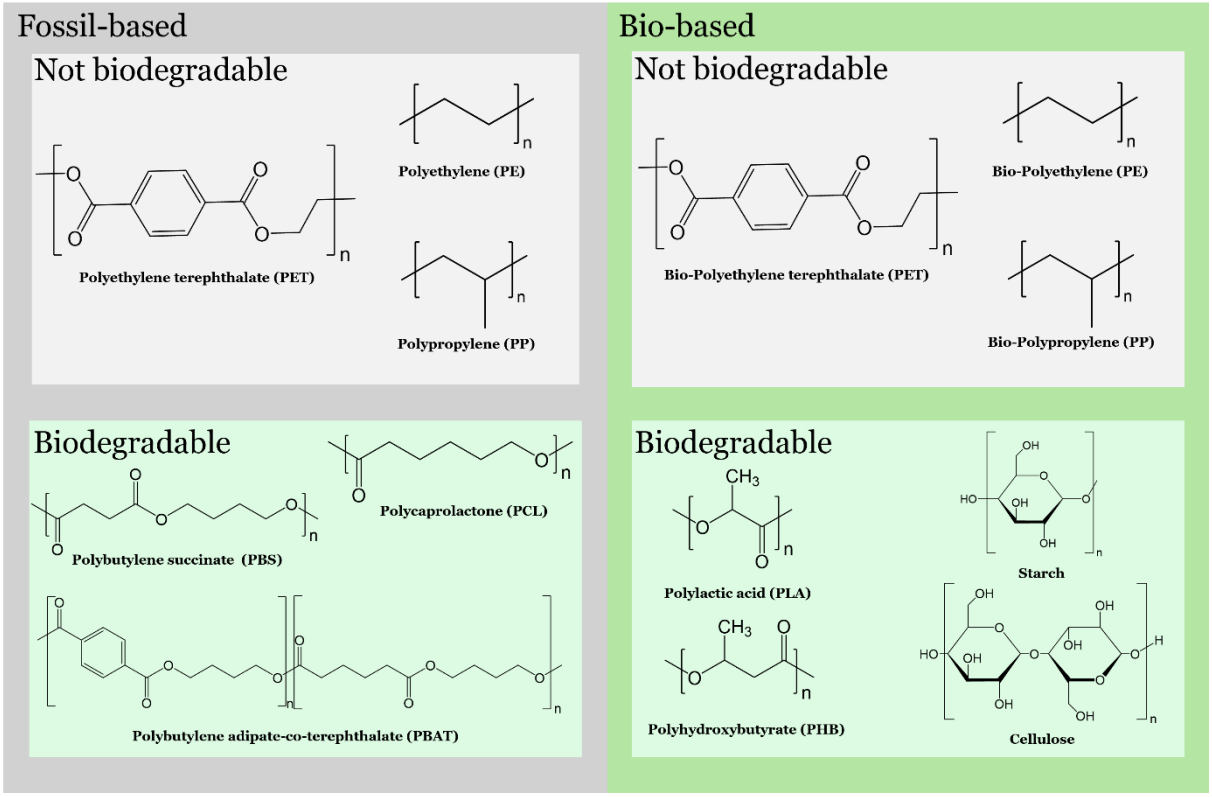


Figure 1.1. Classifications of biodegradability for common polymers and plastics derived from both fossil-based and bio-based feedstocks. PE, polyethylene; PET, polyethylene terephthalate; PP, polypropylene; PBAT, polybutylene adipate-co-terephthalate; PCL, polycaprolactone, PLA, polylactic acid; PBS, polybutylene succinate; PHA, polyhydroxyalkanoate. Figure based on works from Rosenboom et al. (2022) and FAO (2021a).

Biodegradation explicitly refers to a process, whereas biodegradability is specifically referring to the potential of a polymer to undergo the process of biodegradation under specific environmental conditions, within a given time frame (Chinaglia et al., 2024; Sander et al., 2023). It is critical to understand that biodegradability is dependent on both the intrinsic polymer properties and the environmental conditions where biodegradation occurs (Sander et al., 2023). Physicochemical polymer properties that influence a polymers susceptibility to biodegradation include the presence of hydrolysable bonds (e.g. an ester bond), crystallinity, form, surface area to volume ratio, the presence of additives and blends, polymer morphology, and molecular weight (Lucas et al., 2008; Samir et al., 2022; Sander et al., 2023). Environmental conditions that affect biodegradation are both abiotic and biotic, such as temperature, moisture, pH, oxygen availability,

nutrient availability, and the presence of appropriate microbial communities (Agarwal, 2020; Hahn and Hennecke, 2023; Lucas et al., 2008). With this knowledge, it is important that the environment in which a polymer is deemed biodegradable is specified (*see Section 1.4.2 and 1.5*).

In the environment, polymers will initially be degraded by abiotic (e.g. mechanical, thermal, photo, chemical) and/or biotic (e.g. extracellular enzymes) processes, which reduce the polymer size and enhance the availability of the polymer for bioassimilation by microbes prior to mineralisation (Agarwal, 2020; Bher et al., 2022; Emadian et al., 2017; Lucas et al., 2008). Abiotic degradation can increase the surface area and reduce the molecular weight of the polymer, which then increases the susceptibility of polymers to biotic degradation. Biotic enzymatic degradation has four main stages: biofilm formation, depolymerisation, bioassimilation, then mineralisation (Figure 1.2).

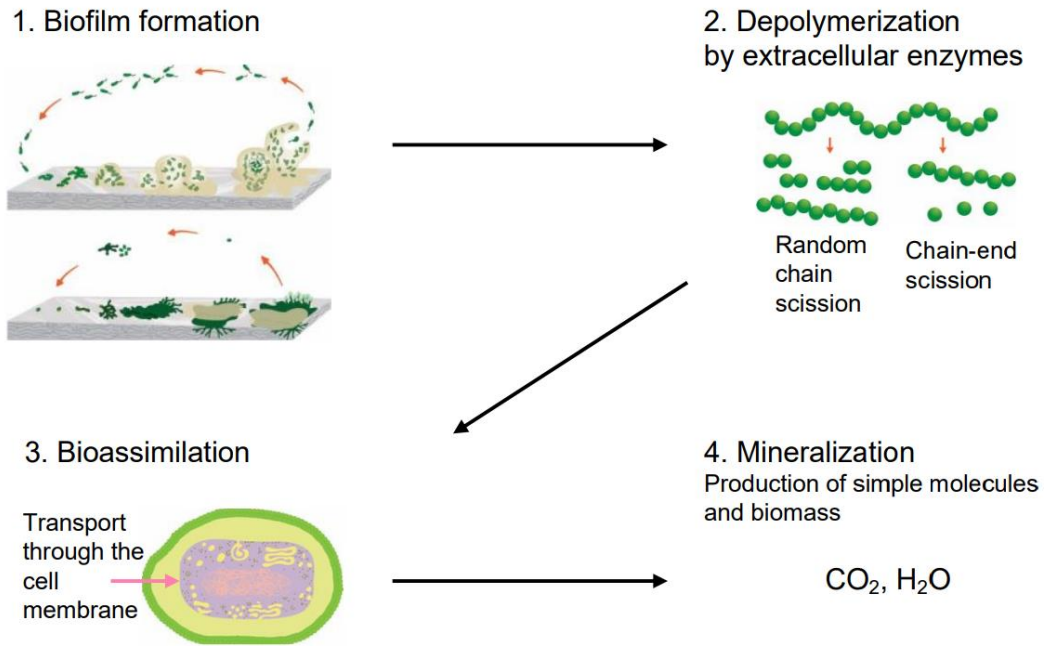


Figure 1.2. The four main stages involved in biotic degradation: (1) Biofilm formation, when microbial colonies form on the polymer surface. (2) Depolymerisation, the breakdown of polymer chains into small molecules by extracellular enzymes. (3) Bioassimilation, the metabolization of low molecular weight compounds by transport through cell membranes. (4) mineralisation, when carbon is biologically oxidised and simple molecules and microbial biomass are produced. This figure is originally from (Bher et al., 2022).

During the process of polymer degradation, there is the potential formation of interim micro and nano sized particles (Pfohl et al., 2022b, 2024; Wohlleben et al., 2023, 2024). If complete mineralisation occurs, then MNPs should be transient, but there is still a limited understanding of the process of polymer biodegradation in soil. There are concerns regarding the potential for persistent microplastic formation, with recent studies identifying biodegradable microplastics in the environment (Kaur and Chauhan, 2024; Lara-Topete et al., 2024; Qin et al., 2021; Sintim et al., 2019; Wei et al., 2022).

It is important to explicitly define polymer biodegradation, to avoid confusion. An accepted definition has been proposed by Sander et al. (2023):

"Polymer biodegradation is the process by which microorganisms completely metabolize the organic carbon in the polymer under formation of carbon dioxide and microbial biomass under oxic conditions, or carbon dioxide, methane, and microbial biomass, under anoxic conditions."

In aerobic (oxic) conditions, biodegradation can be simply expressed (Equation 1.1, (Chinaglia et al., 2018a).

$$C_{polymer} + O_2 \rightarrow C_{biomass} + CO_2 + H_2O$$

[Equation 1.1]

However, it is important to note that whilst Equation 1.1 is typically described as biodegradation, Equation 1.2 presents a more accurate depiction, where the conversion of carbon in the polymer ($C_{polymer}$) to carbon in microbial biomass ($C_{biomass}$) is biodegradation, whilst the conversion of $C_{biomass}$ to carbon dioxide (CO_2) is mineralisation (Chinaglia et al., 2018a).

$$C_{polymer} + O_2 \xrightarrow{a)\ biodegradation} C_{biomass} \xrightarrow{b)\ mineralisation} CO_2 + H_2O$$

[Equation 1.2]

The term mineralisation is commonly used interchangeably with biodegradation in the literature (Chinaglia et al., 2018b, 2024). Mineralisation is specifically the conversion of carbon from the

polymer (i.e. $C_{Polymer}$) into carbon in CO_2 (i.e. C_{CO2}) by microbial activity (Step 4 in Figure 1.2). Currently, standard test methods used to measure polymer biodegradation in soil focus on mineralisation (Section 1.4) and provide no information regarding the environmental fate of any transformation products (i.e. metabolites) or the original polymer. This is because bioassimilation (Step 3 in Figure 1.2) is more challenging to measure requiring the use of labelled polymers (e.g. ^{13}C, ^{14}C). In this thesis the term biodegradation will be used to refer to the mineralisation measured in respirometric tests (Chinaglia et al., 2018b, 2024).

1.4. European Legislation and Regulation

1.4.1. REACH Regulation

The use of chemicals in the EU is governed by the European Chemicals Agency (ECHA) via Regulation No 1907/2006 for the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (European Union, 2006). Historically, polymers and plastics were exempt from chemical legislation, as they were thought to be of lesser environmental concern (Abdolahpur Monikh et al., 2022). However, the regulatory landscape has changed, with polymers now being considered for regulation under REACH (European Commission, 2020). Additionally, since October 2023 there has been an amendment to REACH that restricts the intentional use of solid polymer microparticles (commonly referred to as microplastics) across multiple sectors, including agriculture (Box 1.2, REACH Annex XVII) (European Union, 2023). Amongst others, agricultural plastic applications impacted by REACH Annex XVII include the intentional use of microplastics in products such as controlled release fertilisers, and the polymer encapsulation of plant protection products, including treated seeds and biocides (European Union, 2023). Whilst the restriction entered into force in October 2023, it does not apply to certain agricultural products for a further 5 or 8 years to allow time for the development of alternative products that can maintain the benefits of plastic usage including improved environmental and human safety (Langlet et al., 2024). Plant protection products, treated seeds, and biocidal products will be

subject to this legislation from 17th October 2031. All other agricultural and horticultural products will be subject to this legislation from 17th October 2028. REACH Annex XVII has four derogations for exclusion from the restriction, including polymers that are proven to be degradable (Box 1.2) (European Union, 2023). Degradability is required to be proven in accordance with Appendix 15 of REACH, which lays out a series of standard test methods (European Union, 2023).

Box 1.2. REACH Annex XVII regarding the restriction of synthetic polymer microparticles, with key information reproduced regarding the definition of synthetic polymer microparticles, and the main derogations. Text is reproduced from the Commission Regulation (EU) 2023/2055 of 25 September 2023 amending Annex XVII to Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards synthetic polymer microparticles (European Union, 2023). Appendix 15 and Appendix 16 refer to appendices within REACH.

Annex XVII to REACH. **Restrictions on the manufacture, placing on the market and use of certain dangerous substances, mixtures, and articles.**
Entry 78
Synthetic polymer microparticles: polymers that are solid and which fulfil both of the following conditions: (a) are contained in particles and constitute at least 1 % by weight of those particles; or build a continuous surface coating on particles; (b) at least 1 % by weight of the particles referred to in point (a) fulfil either of the following conditions: (i) all dimensions of the particles are equal to or less than 5 mm; (ii) the length of the particles is equal to or less than 15 mm and their length to diameter ratio is greater than 3. The following polymers are excluded from this designation: (a) polymers that are the result of a polymerisation process that has taken place in nature, independently of the process through which they have been extracted, which are not chemically modified substances; (b) polymers that are degradable as proved in accordance with Appendix 15; (c) polymers that have a solubility greater than 2 g/L as proved in accordance with Appendix 16; (d) polymers that do not contain carbon atoms in their chemical structure.

1.4.2. Proposed Standard Test Methods for Polymer Biodegradation

Multiple groups are responsible for the implementation of standard tests and certification of polymer biodegradability. Key groups are the Organisation for Economic Cooperation and Development (OECD), the International Organisation for Standardisation (ISO), the American Society for Testing and Materials International (ASTM), and the European Committee for Standardisation (CEN). OECD have several accepted guidelines for the testing of chemical persistence in the environment. However, these are not directly applicable to the biodegradation of polymers and plastics, which resulted in the development of specific biodegradation tests by ASTM, ISO, and CEN (Folino et al., 2023; Kjeldsen et al., 2019).

To demonstrate degradability in accordance with Appendix 15 of REACH, and therefore exemption from REACH Annex XVII, a hierarchy of standardised tests are proposed within five groups, with tests developed by OECD, ISO, and CEN (Table 1.2). The standardised tests are split into screening (Groups 1-4) and simulation (Group 5) studies, with increasing levels of complexity and environmental relevance. The standardised tests in groups 1, 2, 3, and 5 were designed for chemical testing of small molecules, meaning their suitability for testing polymers is uncertain. Group 4 tests were designed by ISO using the principles of small molecule biodegradation tests, to test the biodegradation of plastics in the environment. The focus of the tests is to measure biotic degradation, but it is noted that abiotic degradation cannot always be excluded.

Passing a test from groups 1, 2, or 3 is deemed sufficient to prove degradability in all environments. Whereas the use of tests in groups 4 and 5 requires biodegradability to be proven in more than one environment because each test specifies exact environmental conditions. Therefore, when a test is passed, the polymer is only proven biodegradable under the specific test conditions. Polymers that are used in agricultural products are required to have proven degradability in at least two environmental compartments: soil, and either fresh, estuarine, or marine water. This differs to other polymer applications, which also require testing in a third

compartment that is fresh, estuarine, or marine sediment, or the water/sediment interface. Additionally, for polymers used in agriculture, there is an adjustment in the minimum pass level based on the functionality period of the polymer, which is the time after application when the product performs its primary function. In soil, ≥90% degradation relative to the degradation of a reference material should occur within 48-months after the functionality period. In water, ≥90% degradation relative to the degradation of a reference material is required 12 months after the functionality period for group 4 tests, or 16 months after the functionality period for group 5 tests.

Table 1.2. Permitted test methods, pass criteria, and further detailed information regarding the rules for proving degradability of polymers given in Appendix 15 of REACH to prove the degradability of polymers in relation to Annex XVII. T: Test; TG: Test Guideline.

Group	Permitted test methods	Pass criteria	Additional Information from Appendix 15
1) Screening test methods and pass criteria to demonstrate ready biodegradation	T1) "Ready biodegradability" (OECD TG 301 B, C, D, F) (OECD, 1992a). T2) "Ready biodegradability – CO_2 in sealed vessels (headspace Test)" (OECD TG 310) (OECD, 2014).	60% mineralisation measured over 28 days as evolved CO_2 or consumed O_2	The 10-day window requirement mentioned in T1 and T2 test guidelines does not need to be fulfilled.
2) Modified and enhanced screening test methods and pass criteria to demonstrate ready biodegradation	T1) "Ready Biodegradability" (OECD TG 301 B, C, D, F) (OECD, 1992a). T2) "Ready Biodegradability – CO_2 in sealed vessels (Headspace Test)" (OECD TG 310) (OECD, 2014). T3) "Biodegradability in Seawater" (OECD TG 306) (OECD, 1992b).	60% mineralisation measured over 60 days as consumed O_2 (T1 and T2 only) or evolved CO_2.	The test duration can be extended up to 60 days and larger test vessels used. The 10-day window requirement mentioned in T1 and T2 test guidelines does not need to be fulfilled.
3) Screening test method and pass criteria to demonstrate inherent degradation	T4) "Inherent Biodegradability: modified MITIT Test (II)" (OECD TG 302 C) (OECD, 2009).	>70% mineralisation measured as consumed O_2 or evolved CO_2 within 14 days	The pre-adaptation of the inoculum mentioned in the T4 guideline shall not be allowed.
4) Screening test methods and pass criteria to demonstrate degradation relative to a reference material.	T5) "Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium – Method by analysis of evolved carbon dioxide." (EN ISO 14852:2021) (ISO, 2021b). T6) "Determination of the ultimate aerobic biodegradability of plastic materials in an aqueous medium – Method by measuring the oxygen demand in a closed respirometer." (EN ISO 14851:2019) (ISO, 2019b). T7) "Plastics – Determination of aerobic biodegradation of non-floating plastic materials in seawater/ sediment interface – Method by	Ultimate degradation of >90% relative to the degradation of the reference material within: 6 months in aquatic tests, or, 24 months in soil, sediment, or water / sediment interface tests.	The specifications laid down in ISO 22403:2020 "Plastics – Assessment of the intrinsic biodegradability of materials exposed to marine inocula under mesophilic aerobic laboratory conditions – Test methods and requirements" shall be taken into account when applying T7 and T8. Pre-adaptation of the inoculum shall not be allowed. Result shall be reported as the maximum level of degradation determined from the plateau phase of the degradation curve, or as the highest value if the plateau has not been reached. The form, size, and surface area of the reference material shall be comparable to that of the test material. The following materials may be used as reference materials:

	analysis of evolved carbon dioxide" (EN ISO 19679:2020) (ISO, 2020). T8) "Plastics – Determination of aerobic biodegradation of non-floating plastic materials in seawater/sandy sediment interface – Method by measuring the oxygen demand in closed respirometer" (EN ISO 18830:2016) (ISO, 2016). T9) "Plastics – Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved" (EN ISO 17556:2019) (ISO, 2019c). T10) "Plastics – Determination of the aerobic biodegradation of non-floating materials exposed to marine sediment – Method by analysis of evolved carbon dioxide" (ISO 22404:2019) (ISO, 2019d).		- positive controls: biodegradable materials such as micro-crystalline cellulose powder, ashless cellulose filters, or poly-ß-hydroxybutyrate - negative controls: non-biodegradable polymers such as polyethylene or polystyrene
5) Simulation test methods and pass criteria to demonstrate degradation under relevant environmental conditions	T11) "Aerobic and Anaerobic Transformation in Soil" (OECD TG 307) (OECD, 2002a). T12) "Aerobic and Anaerobic Transformation in Aquatic Sediment Systems" (OECD TG 308) (OECD, 2002b). T13) "Aerobic Mineralisation in Surface Water – Simulation Biodegradation Test" (OECD TG 309) (OECD, 2004b).	The degradation half-life in soil in less than 180 days. The degradation half-life in marine, fresh or estuarine sediment is less than 180 days. The degradation half-life in marine, fresh, or estuarine water is less than 60 days	The required test temperatures shall be 12°C for fresh/estuarine water, fresh/estuarine water sediment and soil, and 9°C for marine water and marine sediment because these are the average temperatures for those compartments in the Union.

1.5. Measuring Biodegradation in Soil – The Mineralised Fraction

Within REACH Annex XVII Appendix 15, there are two specified guidelines for monitoring biodegradation in soil, ISO 17556:2019 and OECD 307 (Table 1.2). ISO 17556:2019 has been specifically designed for the biodegradation of plastics in soils, whereas OECD 307 was designed for testing the biodegradation of chemicals in soil (ISO, 2019c; OECD, 2002a). ASTM D5988-18 (ASTM, 2018) is classed as an equivalent test to ISO 17556:2019, that is often used an alternative reference in the literature (Briassoulis and Mistriotis, 2018; Pires et al., 2022; Sander et al., 2023). Whilst ASTM D5988-18 is not included in REACH due to it being an American standard, because it is considered equivalent to ISO 17556:2019 it is useful to compare the test set-up. ISO 17556:2019 and ASTM D5988-18 measure biodegradation (by either biological O_2 demand (BOD) or evolved CO_2) relative to a positive control reference material that is known to be biodegradable in soil. It is useful to compare ISO 17556:2019, ASTM D5988-18 and OECD 307 as they are all designed for measuring biodegradation in soil (Table 1.3). There is variation between the tests regarding the preparation of soil and test material, as well as the test set-up and execution, which could explain differences in their reproducibility (Briassoulis et al., 2020; Pires et al., 2022).

Table 1.3. Comparison of test specifications between ISO 17556:2019, ASTM D5988-18, and OECD 307 (ASTM, 2018; ISO, 2019c; OECD, 2002a).

Test	ISO 17556:2019	ASTM D5988-18	OECD 307
General Information			
Test Name	Plastics - Determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the O_2 demand in a respirometer or the amount of CO_2 evolved.	Determining Aerobic Biodegradation of Plastic Materials in Soil.	OECD Guideline for the testing of Chemicals: Aerobic and Anaerobic Transformation in Soil.
Test Classification (*Table 1.2*)	Group 4 – Screening.	Group 4 - Screening [*Not listed within REACH, but it is equivalent to ISO 17556:2019*].	Group 5 – Simulation.
Purpose	Determining the ultimate aerobic biodegradability of plastic materials in soil.	Degree and rate of aerobic biodegradation of plastic materials, including formulation additives, in contact with soil.	Designed for evaluating aerobic and anaerobic transformation of chemicals in soil.
Designed specifically for plastic?	Yes.	Yes.	No. The test is applicable to all chemical substances (non-labelled or radiolabelled) for which analytical methods with sufficient accuracy and sensitivity are available.
Measurement Endpoints	CO_2 evolved or biological O_2 demand (BOD). The influence of possible nitrification processes on the BOD needs to be considered.	Primary method is CO_2 production. Alternatively, BOD is possible, however, the influence of possible nitrification processes on the BOD must be considered.	After appropriate time intervals, soil samples are extracted and analysed for the parent substance and for transformation products. Volatile products are also collected for analysis using appropriate adsorption devices. Using ^{14}C-labelled material, the various mineralisation rates of the test substance can be measured by trapping evolved $^{14}CO_2$ and a mass balance, including the formation of soil bound residues can be determined.
Test Material Preparation			
Test Material	Preferably in powder form, but may be introduced in the form of films, fragments, or shaped articles. Test samples may be reduced in size by	It is acceptable for test specimens to be in the form of films, pieces, fragments, powders, or formed articles, or in aqueous solution, in	The purity of the test substance should be at least 95 %. Before carrying out a test on aerobic and

	means of cryogenic milling. Powder should have a particle size distribution with its maximum at 250 µm diameter. If not powder, then the size of pieces of material should not be greater than 5mm x 5mm. Optional: determine the hydrogen, oxygen, nitrogen, phosphorus and sulphur content, as well as molecular mass. Preferably plastic materials without additives should be tested. When additives are present, information on their biodegradability will be needed to assess the biodegradability of the polymeric material.	accordance with ASTM D618 (ASTM, 2021). It is recommended, but not required, to characterize any test specimens in the form of powders as to particle size distribution by sieve analysis.	anaerobic transformation in soil, the following information on the test substance should be available: (a) solubility in water [OECD Guideline 105 (OECD, 1995a)]; (b) solubility in organic solvents; (c) vapour pressure [OECD Guideline 104 (OECD, 2006)] and Henry's law constant; (d) n-octanol/water partition coefficient [OECD Guidelines 107 and 117 (OECD, 1995b, 2022)]; (e) chemical stability in dark (hydrolysis) [OECD Guideline 111 (OECD, 2004a)]; (f) acid dissociation constant (pKa) if a molecule is liable to protonation or deprotonation [OECD Guideline 112 (OECD, 1981)]. 8. Other useful information may include data on toxicity of the test substance to soil microorganisms [OECD Guidelines 216 and 217 (OECD, 2000a, 2000b)].
Test material application rate	For BOD, 100 mg – 300 mg in 100g – 300g of soil. For CO_2 evolution, a higher test amount is needed, e.g. 2500 mg in 200g of soil.	Add 200 mg to 1000 mg carbon for 500g soil and mix thoroughly.	The treatment rate should correspond to the highest application rate of a product recommended.
Reference Materials	Microcrystalline cellulose powder, ashless cellulose filters, or poly-R-3-hydroxybutyrate. If possible, the physical form and size of the reference material should be comparable to that of the test material.	Starch or cellulose.	Reference substances should be used for the characterisation and/or identification of transformation products by spectroscopic and chromatographic methods. [*Note: these reference materials serve a different purpose, and are not used to compare the mineralisation of the test material*]

Soil Preparation and Characteristics			
Soil Sampling	Natural soil collected from the surface layer of fields and/or forests. Alternatively, a standard soil may be used composed of industrial quartz sand, clay, natural soil, and mature compost.	Natural fertile soil collected from the surface layers of fields and forests. Make a laboratory mixture of equal parts (by weight) of soil samples obtained from at least three diverse locations (for example, an agricultural field, a forest, and a pasture or meadow). Soils are preferably used fresh from the field to assure active microbiota. It is preferable to use fertile soil classified as "sandy loam" in accordance with USDA classification, or "silty sand" in accordance with the German DIN classification.	To determine the transformation pathway, a representative soil can be used; a sandy loam or silty loam or loam or loamy sand [according to FAO and USDA classification (USDA, 2024)]. For transformation rate studies at least three additional soils should be used representing a range of relevant soils. The soil should be freshly collected from the field (from the A horizon or top 20 cm layer) with a soil water content which facilitates sieving. Sampling should be avoided during or immediately following long periods (> 30 days) of drought, freezing or flooding.
Soil Sieving	Particles of less than 5mm, preferably less than 2mm. Remove plant material, stones, other inert materials, and organic solids.	Less than 2mm. Remove plant material, stones, or other inert materials.	Remove vegetation, larger soil fauna and stones, then pass through a 2mm sieve.
Soil Storage	Store in a sealed container at 4°C ± 2°C.	Store in a sealed container at 4°C ± 1°C for a maximum of one month.	4°C ± 2°C for a maximum of three months to maintain microbial activity.
Pre-incubation	The soil may be pre-conditioned but, normally, pre-exposed soil should not be used, especially when biodegradation behaviour in natural environments is being simulated. Pre-conditioning is defined as: "pre-incubation of soil under the conditions of the subsequent test in the absence of the chemical compound or organic matter under test, with the aim of improving the performance of the test by acclimatization of the microorganisms to the test conditions"	NA	Prior to use in the test, soils should be pre-incubated to allow the microbial metabolism to equilibrate after being sampled and stored. Pre-incubation should be between 2 and 28 days, in the temperature and moisture conditions of the test. Storage and pre-incubation time should not exceed three months.

Measurement of Soil Characteristics	It is recommended that the soil is characterised for at least total water holding capacity, pH, and organic matter content.	Determine moisture holding capacity, pH, moisture content, and total nitrogen.	All soils should be characterised, at least, for texture (% sand, % silt, % clay), pH, cation exchange capacity, organic carbon, bulk density, water retention characteristic and microbial biomass.
pH	6.0 – 8.0. Measured by ISO 10390 (ISO, 2021a).	6.0 – 8.0.	5.5-8.0.
Soil Moisture	40 – 60% of the total water holding capacity, measured by ISO 11274 (ISO, 2019a).	80 – 100% of the moisture holding capacity if determined by test method ASTM D425 (ASTM, 2017a). 50-70% of the moisture holding capacity if determined by test method ASTM D2980 (ASTM, 2017b).	Adjusted to and maintained at pF 2.0 and 2.5. Moisture content recommended for optimal microbial growth range from 40-60% water holding capacity and 0.1-0.33 bar. 0.1-0.33 bar is equivalent to a pF range of 2.0-2.5.
Carbon to Nitrogen (C:N)	At least 40:1.	Between 10:1 and 20:1 by weight. Adjust by the addition of ammonium phosphate solution.	Not specified. Organic carbon content should be 0.5-2.5%.
Test Set Up and Execution			
Test Conditions	Dark or diffused light.	Dark.	Dark.
Test Temperature	20-28°C, maintained to within ±2°C. Preferably 25°C.	20-28°C, maintained to within ±2°C.	A constant temperature representative of the climatic conditions where use or release will occur. 20±2°C is recommended for temperature climates.
Test System	Triplicates are needed for test material, blank control, and positive control.	Triplicates are needed for technical control, soil blanks, positive reference material, and each test material.	Duplicate incubation flasks for removal at appropriate time intervals
Soil Moisture Maintenance	If the biodegradation rate is considered to have slowed down because the test soil has dried out during the test, stop the measurements and remove the flasks from the respirometer or CO_2-free-air production system. Weigh the flasks and add a suitable amount of water to the test soil to bring its water content back to its initial value. Reconnect the flasks to the system and restart measurement of the O_2 consumed or CO_2 evolved.	At the time of removal of the traps, weigh the vessel to monitor moisture loss from the soil. Add distilled or deionized water back periodically to the soil to maintain the initial weight of the vessel. Traps should be removed every 3-5 days for the first 2-3 weeks, then every 1-3 weeks after.	Soil moisture content should be regularly controlled (e.g. in 2-week intervals) by weighing of the incubation flasks and water losses compensated by adding water.

Test Completion	When a constant level of BOD or CO_2 evolution is attained (plateau phase reached) and no further biodegradation is expected, the test is considered completed.	The cumulative CO_2 evolution reaches a plateau when all the accessible carbon has been oxidized. The incubation is continued until no net CO_2 production is noted between consecutive measurements taken four weeks apart, from both the positive reference material and test material vessels.	Test should ideally not exceed 120 days. Termination is possible after 120-days, or when at least 90% of the substances is transformed, but only if at least 5% CO_2 is formed.
Quantification of Test Material Remaining	Optionally at the end of the test, the residual test material may be extracted from the soil with a suitable solvent (if this is possible) and weighed.	Optionally at the end of the test, extract any residual test material from the soil with an appropriate solvent and quantify.	Test material is extracted with appropriate solvents of different polarity throughout the test at appropriate time intervals. Soil samples are analysed for the test substance and/or transformation products.

1.5.1. Test Purpose and Measurement Endpoints

First, it is important to consider the purpose of the test: both ISO 17556:2019 and ASTM D5988-18 were designed specifically to monitor the aerobic biodegradation of plastics in soil, with ISO 17556:2019 designated as screening test, and, as ASTM D5988-18 is considered to be equivalent, it would be considered a screening test also. Therefore, the purpose would be to optimise the test conditions (such as the nutrient availability, soil water content, temperature) within the biodegradation tests to ensure that microbial activity is not limited, and that the biodegradability of a test material under optimal conditions can be determined (Chinaglia et al., 2024; Krzan et al., 2006). OECD 307 is a simulation test, which aims to determine the mechanism and transformation products of biodegradation under specified soil environmental conditions (Hughes et al., 2022; OECD, 2002a). In screening tests, the result is compared to the biodegradation of the reference material, which must reach 60% to meet the validity criteria of the test. Biodegradation is measured by the non-specific methods of O_2 consumed or CO_2 evolved, so they are designed to measure the ultimate biodegradation in aerobic environments to CO_2, water and biomass. The pass criteria is the ultimate biodegradation of ≥90% relative to the degradation of the reference material within 24 months in soils. In contrast, OECD 307 looks at the disappearance of the parent test material, requiring at least 90% of the substance to be transformed into metabolites.

The measurement endpoint is important to consider between the tests. ISO 17556:2019 and ASTM D5988-18 measure mineralisation, which is the ultimate biodegradation of a test material. OECD 307 does also measure mineralisation but incorporates additional analysis by requiring the extraction and analysis of soil samples throughout the test for identification of the original (parent) test material, and any transformation products (metabolites) to follow the biodegradation process. Utilising non-specific methods of O_2 consumption and CO_2 evolution to measure the biodegradation of non-labelled test materials means there is uncertainty in the measurement.

Whilst the use of a soil blank is incorporated to account for the uncertainty, recent research into polymer biodegradation highlights that this could be insufficient. The biodegradation of polymers in soil measured by CO_2 evolution has been studied with the use of isotopically labelled polymers, with results demonstrating that not all measured CO_2 evolved is from the biodegradation of a polymer, but that part of it is due to the induced degradation of soil organic matter (known as a priming effect) (Huo et al., 2024; Yang et al., 2023; Zhang et al., 2023). The biodegradation of polymers in soil measured by O_2 demand has additional concerns, as the recorded O_2 could be used for other processes, such as nitrification (Ardisson et al., 2014). It is important to consider the usefulness of mineralisation measurements for polymer biodegradation and understand what could influence the measured result to ensure accuracy in the determination of a specific pass level for polymer biodegradation.

1.5.2. Test Material Considerations

When considering chemical persistence testing, test materials that are difficult to test include those that are poorly soluble, sorbing, volatile, toxic to micro-organisms, ionisable, complexing, unstable, or chiral (Hughes et al., 2022). Therefore, polymers would be classified as difficult to test materials as they are poorly soluble and have the potential to be toxic to micro-organisms.

The test material can be tested in any form in ISO 17556:2019 and ASTM D5988-18, including powder, films, fragments or shaped articles. ASTM D5988-18 also allows the testing of test material in an aqueous solution. ISO 17556:2019 preferentially tests a polymer in powder form, suggesting that the powder size distribution should be a maximum of 250 µm, whereas the ASTM D5988-18 recommends but does not require that the particle size distribution is characterised. If a powdered material is not tested, then ISO 17556:2019 requires pieces to not be greater than 5 mm x 5 mm, but ASTM D5988-18 does not specify a size. It has been demonstrated that the mineralisation of a biodegradable plastic in soil is correlated with the surface area of the test material, and that if a plastic were tested at the scale of a nanoplastic (100nm diameter) it could

theoretically biodegrade with 15-20 days (Chinaglia et al., 2018b; Tosin et al., 2019). This trend was also observed by Huo et al. (2024), who found that smaller microplastics (<250 μm) caused greater overall mineralisation than larger microplastics (250- 4750 μm) in a soil biodegradation test. This suggests that the test material form and surface area to volume ratio are important characteristics of the test material, that influence the biodegradation and should be properly controlled within a biodegradation test.

Generally, in comparison to OECD 307, there are fewer requirements for the complete characterisation of a test material in both ISO 17556:2019 and ASTM D5988-18, and no requirement is set for the purity of the initial polymer whereas OECD 307 states the tested material should be at least 95% purity. ISO 17556:2019 states that preferably plastic materials without additives should be tested, and that the biodegradability of the additives should be determined separately. In contrast ASTM D5988-18 covers the aerobic biodegradation of plastic materials including formulation additives. Plastic products when released to the open environment will be a blend of polymers and additives, as additives enhance the functionality of the product. Previous research into the biodegradation of plastic mulching films found that the recorded biodegradation value can be influenced by additive content (Yabannavar and Bartha, 1994). When PE films with additives were incubated, measured CO_2 evolved indicated that biodegradation had occurred. However, further chemical analysis highlighted that the polymer additives were degraded, not the polymer itself (Yabannavar and Bartha, 1994). It is important to consider the impact that additives might have on the biodegradation rate of polymers in the environment (Harrison et al., 2018; Kyrikou and Briassoulis, 2007). Additives can leach from plastics into the soil environment (Maddela et al., 2023; Viljoen et al., 2023), and there has been extensive research into the effects of organic additives on soil biota, with adverse effects on seed germination, plant growth, oxidative stress and reduced organism growth (Cao et al., 2023). In addition, additives can impact soil physicochemical properties, for example, phthalic acid esters

(PAEs) have been found to promote soil particle aggregation and reduce soil porosity and permeability, which can impact soil quality, nutrient cycling and the microbial community (Cao et al., 2023). Therefore, the leaching of additives has the potential to impact the environmental degradation of the polymer, through effects on soil biota and properties. There should be a requirement for toxicity testing of the relevant additives and any polymer degradation products, to ensure that toxicity to soil microorganisms does not occur that could hinder further biodegradation.

The recommended application amount of test material differs between ISO 17556:2019 and ASTM D5988-18. Specifically, ISO 17556:2019 expresses the test material application in milligrams (mg) of test material per gram (g) of soil, whereas ASTM D5988-18 expresses it as mg of carbon per g of soil. For example, cellulose has a carbon content of 44.4%. ISO 17556:2019 suggests an application rate of 2500 mg of test material in 200g of soil, then the carbon content for cellulose would be 1110 mg carbon in 200g soil. However, as ASTM D5988-18 suggests that 200 mg to 1000 mg carbon should be applied for 500g of soil, this equates to 450.45 mg and 2252.25 mg of cellulose in 500g soil. Therefore, these application rates are not equivalent which could have consequences for biodegradation rate. In ISO 17556:2019, different application rates are suggested dependent on the analytical endpoint used, with a greater amount of test material required for the measurement of evolved CO_2 than for O_2 demand, due to differences in the analytical detection limits of the methods. In OECD 307, the application rate of a test material is required to be set to the highest application rate of the product, which is considered to be more environmentally relevant. There has been no direct research conducted into if the amount of polymer added can influence the results of a biodegradation test. However, previous research highlighted that the concentration of plastic is a significant factor in explaining the measured ecotoxicological impacts for soil plants, biota, and microorganisms (Huo et al., 2022). Therefore, the potential impacts of the application rate of the test polymer need to be considered further.

1.5.3. Reference Material Considerations

ISO 17556:2019 and ASTM D5988-18 test the biodegradation of a polymer test material with reference to a positive control reference material. ISO 17556:2019 suggests the use of microcrystalline cellulose powder, ashless cellulose filters, or poly-R-3-hydroxybutyrate (PHB) as a reference material, whilst ASTM D5988-18 suggests the use of starch or cellulose. Multiple factors regulate how carbon is processed by soil microbial biomass, with carbon being used either for anabolic processes such as the growth and maintenance of microbes, or catabolic processes such as respiration. The use of a positive reference material as a control is designed to account for the incorporation of carbon by microbial biomass. However, this relies on the assumption that the carbon use efficiencies of the reference material and the test material are the same (Sander et al., 2023). Carbon use efficiency (CUE) is the proportion of a test material's carbon that microbes use for growth compared to the rate of total carbon uptake, which can vary with the tested material (Islam et al., 2023; Manzoni et al., 2012; Sinsabaugh et al., 2013). There is a lack of consensus on the main environmental drivers of CUE, with soil nutrient availability, pH, and moisture all potential drivers. CUE is also likely to depend on the polymer chemistry, and their accessibility for microbes (Sander et al., 2023). Relative CUE of polymers has not been verified to be comparable between polymers and could result in the underestimation of polymer biodegradation (Sander et al., 2023). If CUE values were known, it would enable an understanding of the level of uncertainty by using respirometric analytical methods. Additionally, understanding how CUE changes between polymer type and environmental conditions could provide useful information for the development of biodegradable polymers, to maximise the CUE and therefore have faster biodegradation of polymers. In contrast to ISO 17556:2019 and ASTM D5988-18, OECD 307 does not have a comparable reference material that is used in this same way. In OECD 307 the degradation of the test material is measured through the identification of specific degradation products that are then compared with standard reference materials for the degradation products.

A key difference is that in OECD 307 the reference material is not used to compare degradation rates between different test materials or prove test validity.

1.5.4. Soil Source, Preparation and Characteristics Considerations

Soil is a biological media that provides a permanent source of experimental variability within biodegradation tests (Davenport et al., 2022; Hughes et al., 2022). The test guidelines set out several requirements for soil preparation, as well as the determination of key soil characteristics. Both natural and standard (i.e. artificial) soils are allowed to be used in ISO 17556:2019 with the composition of a standardised soil specified. Soil type has been highlighted to influence biodegradation, with fine soil textures having higher biodegradation rates than coarse soil textures (Briassoulis and Mistriotis, 2018; Hamarashid et al., 2010). However, information on soil texture is not required to be characterised in ISO 17556:2019 or ASTM D5988-18, whereas this is required in OECD 307. Previously, the ISO 17556:2019 standardised soil has been compared to natural soils, and the lowest level of biodegradation was noted in the standardised soil, whilst results were most reproducible when natural soils were used (Briassoulis et al., 2020). Natural soils in ISO 17556:2019 are to be taken from a single source, whereas ASTM D5988-18 recommends the mixing of natural soils from three sources, which would improve the diversity of the soil microbial community but is not representative of environmental conditions. OECD 307 does not allow the use of artificial soils, only natural soils.

There are different recommendations given regarding a pre-incubation period, ASTM D5988-18 does not mention a pre-incubation but says it is preferable to use soil fresh from the field to ensure active microbiota. ISO 17556:2019 says that pre-conditioning may occur. Pre-conditioning/pre-incubation are when soil is added to the test vessels, then placed under test conditions to allow the microbial community to adapt to the testing environment before the test material is added. This is different to a pre-exposure/pre-adaptation of the soil, which would be the pre-incubation with the presence of the test material. Pre-exposure/pre-adaptation aims to enhance

biodegradation of the test material by a pre-adaptation of the soil microbial community, this is not allowed as stipulated within the Appendix 15 of REACH (European Union, 2023). OECD 307 considers a pre-incubation to be important, suggesting it should be between 2-28 days in the temperature and moisture conditions to allow the microbial metabolism to equilibrate after sampling and storage. How the soil sample is sampled, stored and treated prior to use in an experiment is known to affect the soil microbial activity, with storage followed by drying then rewetting causing an increase in microbial respiration rates (Birch, 1958; Schroeder et al., 2021). Given that the non-specific methods of O_2 demand and CO_2 evolution are based on the soil microbial respiration, it would be important to have a specific approach to a pre-incubation period to ensure that the initial increase in respiration rate is accounted for within the measured biodegradation of the test material.

ISO 17556:2019 requires the soil to be characterised for water holding capacity (WHC), pH, and the organic matter content. The soil moisture should be adjusted to between 40-60% of total WHC for ISO 17556, and between 50-70% of total WHC for ASTM D5988-18. OECD 307 also suggests a moisture content of 40-60% of the WHC, and states this is equivalent to a pF (field capacity) of 2.0-2.5. Soil moisture is a well characterised driver of soil respiration (Orchard and Cook, 1983). Microbial respiration has a linear relationship with soil water content (SWC), whereby respiration increases with increasing water content up until a saturation point, and then decreases as soil dries out (Cook and Orchard, 2008; Orchard and Cook, 1983). Soil drying reduces microbial metabolic activity both by slowing down enzymatic reactions and limiting the diffusion of nutrients in soil pores (Wilson and Griffin, 1975; Curiel Yuste et al., 2007). Chinaglia et al. (2024) observed increasing biodegradation rates of cellulose when SWC was raised from 55% to 80% WHC. The wide range of allowed SWC can therefore result in variable test results.

The availability of nutrients such as carbon, nitrogen, and phosphorus in soils is important for the functioning of the soil microbial community and influences microbial activity. When added to soils, polymers become a source of carbon themselves, and as they generally contain little nitrogen or phosphorus, the addition of a polymer widens the carbon to nitrogen (C:N) ratio (Rillig et al., 2021). Therefore, it is important to consider the availability of nitrogen and other nutrients in soil biodegradation tests. This is important for ISO 17556:2019 and ASTM D5988-18 where conditions are to be optimised for microbial activity. OECD 307 does not allow the alteration of the soil's nutrient availability, however both ISO 17556:2019 and ASTM D5988-18 suggest alterations can be made to the C:N ratio by the addition of nitrogen. The definition of the C:N ratio has been noted to be ambiguous meaning it is not clear when additional nutrients are required (Briassoulis and Mistriotis, 2018; Briassoulis et al., 2014). ISO 17556:2019 specifies that the C:N should be adjusted to at least 40:1, whereas ASTM D5988-18 specifies between 10:1 and 20:1 by weight. Biodegradation of polymers has been noted to be dependent on nitrogen availability with the addition of 0.1g nitrogen suggested to improve the reproducibility of the test (Briassoulis and Mistriotis, 2018). Additionally, the biodegradation of poly(butylene succinate-co-adipate) (PBSA) has been noted to be dependent on nitrogen availability, with nitrogen addition increasing the mineralisation of PBSA to CO_2 (Guliyev et al., 2023). Given that the nitrogen content has a clear impact on the biodegradation of polymers, it is important to consider that the range of recommended C:N ratios could be a significant driver of variations within the test. ISO 17556:2019 does not provide a suggested methodology for measuring the nitrogen in soil, whilst ASTM D5988-18 suggests that total nitrogen should be measured following the test method ISO 11261:1995 "Soil quality – determination of total nitrogen" (ISO, 1995). This would measure all forms of nitrogen, however not all forms are easily available to soil microbes. Nitrogen is available to soil microbial communities in inorganic forms such as nitrate, nitrite, and ammonium. However, the organic forms of nitrogen are not readily available and would need to be transformed

to inorganic forms first. The presence of nitrogen fixing bacteria has also been observed to increase microbial biodegradation of polymers in soils (Tanunchai et al., 2022). Therefore, it could be useful to consider the availability of the forms of nitrogen in addition to defining the soil C:N ratio.

1.5.5. Test Set-Up and Execution

ISO 17556:2019 and ASTM D5988-18 both suggest a temperature of 20-28°C maintained within 2°C. All microbes have an optimal temperature range, with mesophilic microorganisms favouring growth between 20-45°C (Bher et al., 2022; Schiraldi and De Rosa, 2014). The temperature range allowed is wide, and previous work has shown that cellulose mineralisation increases as the temperature increased from 20 to 28°C, which can increase the variability and reproducibility of the test (Pischedda et al., 2019).

As previously described, the soil moisture content is important to maintain the soil microbial community activity, however the maintenance of the soil moisture throughout the test is not consistent between the guidelines. ISO 17556:2019 uses ambiguous language and does not clearly state that the moisture should be maintained throughout the test, but only that it should be re-moistened if the biodegradation rate slows. ASTM D5988-18 and OECD 307 both do state that the soil moisture should be maintained throughout the test. ASTM D5988-18 suggests that moisture lost should be monitored gravimetrically at the time of removal of the traps and then readjusted. OECD 307 states the moisture should be controlled regularly in two-week intervals by weighing and remoistening. A lack of available water impacts soil microbial activity, and the rewetting of soils can result in a burst of microbial activity, which influences the test measurement (Birch, 1958; Chinaglia et al., 2018b; Orchard and Cook, 1983; Thomson et al., 2010). Therefore, is important to ensure the moisture content is maintained throughout a biodegradation test, but it should also be taken into account that the removal of test vessels from the system for moisture

maintenance can interrupt the measurement of O_2 demand and CO_2 evolved (Bahmani et al., 2018; Chinaglia et al., 2024).

The biodegradation test is considered complete when the plateau phase has been reached, meaning there is a constant level of biological O_2 demand (BOD) or evolved CO_2 reached. ISO 17556:2019 is ambiguous in this description, whereas ASTM D5988-18 specifies the incubation should continue until no net CO_2 is produced after 4 consecutive weeks. Early termination of a test has been noted previously to cause differences in the total biodegradation recorded, with shorter test lengths generally resulting in the lowest biodegradation (Briassoulis et al., 2020). This can significantly influence the measured total biodegradation, and it has previously been suggested that the wording in the test guideline should be amended to provide clarity (Briassoulis et al., 2020).

At termination the test is considered complete within ISO 17556:2019 and ASTM D5988-18. Both test guidelines state that optionally the residual test material may be extracted with a suitable solvent and quantified/weighed. In OECD 307, the test material must be extracted throughout the test by solvents, and the soil sample analysed for the test substance and/or transformation products. Overall, there has been minimal research conducted into the quantification of polymer from soils after a biodegradation test. Recent work has utilised solvent extraction to isolate residual polybutylene succinate (PBS) from soils, finding that between 34-40% of the originally added PBS remained in the soil at the end of a biodegradation test and had not been mineralised (Nelson et al., 2022). This work demonstrated that most of the non-mineralised polymer remained in the soil and had not been taken up by the microbial biomass, highlighting the importance of the inclusion of the analysis of the soil sample for the test substance and transformation products at the end of a biodegradation test.

1.6. Measuring Biodegradation in Soil – The Non-Mineralised Fraction

Respirometric methods for measuring polymer biodegradation only provide information about the mineralised portion of the polymer. No information is gained regarding the environmental fate of the non-mineralised polymer carbon, which could be remaining in the soil as residual polymer, be partially degraded, or have been taken up by soil microbes (Nelson et al., 2020, 2022; Sander et al., 2023; Zumstein et al., 2018). The specified pass criteria is the ultimate degradation of ≥90% relative to the degradation of the reference material, with the reference material needing to reach 60% for the test to be considered valid. Therefore, a large portion of the test material can be unaccounted for when a test is deemed complete. OECD 307 is a simulation test designed for measuring the biodegradation of chemicals in soil, and within this there is a requirement to monitor the degradation of the parent test material and metabolite formation during the test, alongside mineralisation measurements (OECD, 2002a). To develop an understanding of the process of polymer biodegradation in soils, the application of this approach to polymers would advance the understanding of the mechanism of biodegradation, including if persistent microplastics are formed, and their environmental fate. However, there are a lack of analytical methods to isolate and identify biodegradable polymers in soils. Therefore, it would be important to develop methods that can isolate the test polymer from the soil matrix, and monitor the polymer degradation process, to quantify the non-mineralised fraction and determine if it has been taken up by microbial biomass, or if it remains in the soil as a microplastic. Whilst research studies have considered the impacts of microplastics in soils on soil microbial community structure and abundance (e.g. through phospholipid fatty acid (PLFA) profiling, Brown et al. (2023)), less research has delved into methodologies to quantify the amount of a polymer that is bioassimilated by soil microbes (Sander et al., 2023). Isotopic labels such as ^{13}C have been used to demonstrate the incorporation of polymer carbon from polybutylene adipate-co-terephthalate (PBAT) into the soil microbial biomass (Zumstein et al., 2018). However, this research area is

generally understudied, and more in-depth mechanistic studies utilising stable isotopes would be required to understand the process (Goudriaan et al., 2023; Montazer et al., 2020; Obrador-Viel et al., 2024; Sander et al., 2019). It is important to develop methodologies that can distinguish if polymer carbon has been taken up by soil microbes, or if it remains within the soil as residual polymer. Whilst the quantification of microbial uptake is more limited, there has been significant research into the identification of polymers in soil, mostly for the identification of microplastics. The uptake of polymers by soil microbes is out of the scope of this research, with the focus on the identification of residual polymer in soil.

Currently there are two general approaches utilised to isolate MNPs from a complex soil sample matrix. One approach is to remove MNPs from the matrix via solvent extraction. Whilst solvent extraction allows for effective removal and can provide quantitative mass measurements, it also results in the loss of the solid state, meaning information on particle size, shape, and number are lost, which are relevant information for risk assessment (Schrank et al., 2022; de Souza Machado et al., 2018b, 2018a). The second approach that is more commonly used, involves the removal of the matrix through a series of sample purification steps, whilst leaving MNPs particles intact. Both approaches will be discussed in the context of identifying biodegradable polymers from soil samples.

Solvent extraction is a suggested approach by both ISO 17556:2019 and ASTM D5998-18 to enable the quantification of the residual test material remaining in the soil at the end of a biodegradation test (Table 3). However, both test methods state this is optional, and limited details are given for a methodology to do this. General approaches include Soxhlet extraction and accelerated solvent extraction (ASE). Pfohl et al. (2022a) utilised Soxhlet extractions with hexafluoroisopropanol (HFIP) for thermoplastic polyurethanes (TPU) to quantify the residual mass after a biodegradation study. TPU was successfully dissolved in HFIP, and the mass in this

extract was then quantified with nuclear magnetic resonance (NMR) spectroscopy. Two studies have also looked at a solvent extraction approach to quantify biodegradable polymers in soils (Nelson et al., 2020, 2022). Nelson et al. (2020) compared the use of Soxhlet extractions, ASE, and ultrasonication with a chloroform/methanol co-solvent mixture as extraction methods for PBAT mulch films in soil, prior to quantification by quantitative proton NMR spectroscopy (q-^{1}H NMR). Effective recovery of PBAT via ASE was determined in six soils, irrespective of the form of PBAT (film vs particles), verifying that ASE was a suitable method for extraction of PBAT. The effectiveness of ASE for PLA was also verified, and the applicability of ASE in chloroform was suggested for other biodegradable polyesters (Nelson et al., 2020). Nelson et al. (2022) later expanded upon this work, applying a Soxhlet extraction using a methanol/chloroform cosolvent followed by q-^{1}H NMR spectroscopy to quantify residual PBS in soil at the end of a biodegradation incubation. In this work a stable carbon isotope (^{13}C) label was used to monitor the mineralisation of PBS-^{13}C during a soil biodegradation incubation, and then the Soxhlet extraction was used as a complementary analysis to extract the non-mineralised PBS for subsequent quantification by q-^{1}H NMR spectroscopy. The mass quantification could then be used in parallel with ^{13}C measurements to calculate the amount of polymer carbon that had been taken up by soil microbes. This work has significantly advanced the field, by providing evidence that residual polymer accounts for most of the non-mineralised polymer, rather than uptake into biomass. Therefore, this provides clear evidence of the need to include additional analyses to quantify non-mineralised polymers in biodegradation tests.

However, solvent extraction completely dissolves the polymer, which may not always be appropriate depending on the research question. Recent studies have identified biodegradable MNPs in the environment, including soils, raising the question of if complete biodegradation will occur in environmental conditions (Kaur and Chauhan, 2024; Lara-Topete et al., 2024; Liao and Chen, 2021; Qin et al., 2021; Shen et al., 2020; Sintim et al., 2019; Sintim and Flury, 2017; Wei et

al., 2022). It is therefore of interest to know the form and environmental fate of the non-mineralised residual polymer, to identify if persistent MNPs are inadvertently formed due to incomplete biodegradation, or if further biodegradation would occur. Therefore, it has been proposed that the breadth of methodologies currently used to isolate and quantify conventional MNPs by removal of the sample matrix, could be applied to identify biodegradable polymers (Fojt et al., 2020).

A significant body of research has been dedicated to identifying suitable extraction methods for MNPs (of conventional non-biodegradable polymers) from all environmental matrices, including soils (Elkhatib and Oyanedel-Craver, 2020; Junhao et al., 2021; Lusher et al., 2020; Möller et al., 2020; Qiu et al., 2016). Soil samples are complex, with inorganic and organic fractions that require extensive pre-treatments prior to the analysis of plastic particles (Möller et al., 2020). Whilst there is not a single standardised method for this, there is a general protocol that combines a density separation (e.g. using a high-density salt solution) to separate out inorganic material, and a chemical digestion step to remove the organic material. Examples of pre-treatments to remove organic material are oxidisation with hydrogen peroxide or Fenton's reagent, and enzymatic digestion methods (Hurley et al., 2018; Löder et al., 2017; Möller et al., 2020, 2022). It is important to have effective pre-treatments that remove background matrix materials to allow subsequent analyses.

A range of analytical methods are utilised to identify MNPs in environmental samples, with a range of detection limits (Pinto da Costa and Duarte, 2020; Silva et al., 2018; Wirnkor et al., 2019). Visual identification with microscopy is commonly used as it is a relatively simple method and the accuracy can be easily improved with the use of fluorescent dyes such as Nile Red and the inclusion of a strict detection criteria (Kukkola et al., 2023; Maes et al., 2017; Nel et al., 2021). Microscopy can be complemented by vibrational spectroscopic methods (e.g. Raman or Fourier

transform infrared (FTIR) spectroscopy), which enable the accurate identification of polymer type, and measurements of particle number, size, and shape (Araujo et al., 2018; Lusher et al., 2020; Prata et al., 2021). Chromatographic methods such as pyrolysis gas chromatography mass spectrometry (Py-GC-MS), and thermal extraction desorption gas chromatography mass spectrometry (TED-GC-MS) have been increasingly used in use in recent years to analyse MNPs in environmental sample, as no lower size limits apply and they generally require less extensive sample pre-treatments than microscopy and spectroscopic methods (Fischer and Scholz-Böttcher, 2017; Okoffo et al., 2021, 2022; Picó and Barceló, 2020; Steinmetz et al., 2020). Chromatographic methods allow for the mass-based quantification of plastics in samples, but they are destructive methods meaning no subsequent analyses are possible, nor the measurement of any particle related size/shape characteristics.

The pre-treatments required to enable successful isolation and measurement of MNPs in environmental samples are extensive, so it is important to know that the extraction protocol does not induce degradation of the plastic particles that could result in inaccurate measurements of MNPs. This has been considered in the literature regarding conventional non-biodegradable polymers. The impact of strong acid and alkali pre-treatments has been considered, with induced degradation observed in several polymers such as polyethylene terephthalate (PET), polycarbonate (PC), polyamide (PA), polyethylene (PE), and polypropylene (PP) and so their use in sample pre-treatment has been discouraged (Hurley et al., 2018; Munno et al., 2018; Schrank et al., 2022). The impact of microplastic extraction protocols on biodegradable polymers has rarely been considered. Möller et al. (2022) developed an enzymatic-oxidative digestion protocol, with high removal efficiency of soil organic material, and tested the impact on multiple polymers including the biodegradable polymer PLA, finding that this protocol resulted in a recovery of >100% (based on particle number) due to significant physical changes and degradation that led to the fragmentation of the polymers. The PLA particles were noted to only show signs of

degradation after exposure to a protease enzyme (Möller et al., 2022). A further study by Pfohl et al. (2021) found that after exposure to an alkaline digestion there was significant degradation of the biodegradable polymers PLA, PBAT, and PBS. Degradation was also noted for PLA, PBAT, and PBS after exposure to Fenton's reagent with a reduction in their average particle size of up to 11.7%. Biodegradable polymers are likely to be more susceptible to degradation by pre-treatment steps due to their chemical structures, for example, the presence of easily hydrolysable bonds., It is important to understand the potential degradation caused by extraction methods, and the mechanisms behind it. The development of a soil microplastic extraction method that does not induce degradation of biodegradable polymers would be a critical step towards improving the detection and quantification of biodegradable microplastics in soils. This would also enhance future polymer biodegradation testing, allowing the identification of any residual or persistent biodegradable microplastics, and inform future environmental risk assessments.

Due to the uncertainty regarding the impact of extraction methods on biodegradable polymers, it is important to explore analytical methods that are less reliant on extensive pre-treatments for sample analysis. Py-GC-MS is a promising technique that can identify and quantify polymers in environmental samples (Steinmetz et al., 2020). Recent studies have demonstrated the application of Py-GC-MS for the identification of biodegradable polymers in soil (Okoffo et al., 2022). Py-GC-MS is a thermal degradation method, that can provide valuable insights into polymer composition by the identification of pyrolysis products (De Falco et al., 2023). Recent work has highlighted the potential for Py-GC-MS to determine the difference between pristine and degraded samples of PLA, based on changes in the relative ratio of pyrolysis products (Arrieta et al., 2013, 2014; De Falco et al., 2023; Khabbaz et al., 2000). Py-GC-MS can provide detailed information on polymer structure without the need for isotopic labels; therefore, it is considered worthwhile for further exploration into the mechanisms of polymer biodegradation. Generally, the development of additional methodologies that would develop the understanding of the

mechanisms of polymer biodegradation in soils is important. Utilising such methodologies alongside the respirometric methods of CO_2 evolution or O_2 demand would provide greater insights into biodegradation and assist in determining the suitability of respirometric endpoints for measuring polymer biodegradation in soils.

Currently measuring the biodegradation of polymers in soil relies on respirometric endpoints such as O_2 demand and evolved CO_2. However, there is uncertainty regarding the appropriateness of these methods for determining environmental biodegradation of polymers in soils. The standard test methods allow significant variations within their set-up and execution, that can reduce the reproducibility and reliability of the test. This is further complicated by the fact that the physical and chemical properties of polymers result in a wide range of degradation mechanisms. There is therefore a significant research gap regarding best practice methodologies that can applied for identifying biodegradable polymers and their degradation products in soil. It is important to determine if mineralisation is a sufficient endpoint to quantify biodegradation or if further measurements are needed to provide evidence of the biodegradation of polymers in soils to meet legislative requirements.

1.7. Thesis Aims and Structure

The aim of this research is to assess the suitability of standard test methods that utilise respirometric endpoints for measuring the biodegradation of polymers in soil, and to investigate the potential for additional analytical methods that could be applied alongside respirometric methods.

Research Hypotheses and Aims:

It is hypothesised that currently used respirometric methods might be unsuitable for measuring polymer biodegradation in soil, and that they have poor reproducibility due to both methodological test set-up, and variations in soil properties. Therefore, this research aimed to implement and assess two respirometric methods (biological O_2 demand and CO_2 evolution) to measure the biodegradability of polymers in soils. It also aimed to identify the potential causes of poor reproducibility in respirometric polymer biodegradation tests across soil types, to identify the main drivers of poor reproducibility.

Further, it is hypothesised that respirometric methods alone do not provide enough information for assessing polymer biodegradation, and that additional information can be obtained through the inclusion of additional analyses of polymer biodegradation throughout a standardised test. It is hypothesised that methods which are commonly used to extract non-biodegradable polymers (i.e. microplastics) from soil would be unsuitable for use with biodegradable polymers, as they are likely to induce degradation. Therefore, the research aimed to determine the impacts of specific soil extraction methods on exemplar biodegradable polymers. It is hypothesised that alternative analytical methods such as Py-GC-MS can be used to directly identify non-labelled biodegradable polymers in soil without the need for comprehensive soil extractions. Therefore, this research aimed to assess the suitability of Py-GC-MS alongside respirometric measurements

to develop a deeper understanding of the degree and mechanism of polymer biodegradation in soil.

Thesis Structure:

Chapter 1 provides a comprehensive literature review of the state of knowledge regarding the suitability of standard test methods that utilise respirometric endpoints for measuring polymer biodegradation in soil, and current methodologies used to identify polymers in soils.

Chapter 2 outlines the initial experimental work undertaken to set-up and validate the use of two respirometric methods (biological O_2 demand and CO_2 evolution) for measuring the biodegradation of polymers in soils.

Chapter 3 compares the reproducibility of the two respirometric methods for measuring the biodegradation of the biodegradable polymer microcrystalline cellulose across three soils (two natural agricultural soils and an artificial soil) and examines the potential causes for poor reproducibility.

Chapter 4 evaluates the suitability of an extraction method designed for the recovery of non-biodegradable polymers from soil for biodegradable polymers and specifically considers the potential impacts on the physicochemical properties of the polymers.

Chapter 5 measures the biodegradation of polymers in soil by the respirometric endpoint of evolved CO_2 alongside the direct analysis of polymers in soil by pyrolysis gas chromatography-mass spectrometry to identify the potential insights into the mechanism of polymer biodegradation that additional analytical methods could provide.

Chapter 6 discusses and integrates the research findings from all chapters and outlines future research needs.

1.8. References

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Chapter 2. Validation of Respirometric Biodegradation Testing Methods

2.1. Introduction

Persistence is an environmental concern, especially regarding plastics due to their potential negative impacts for the environment and human health (Krause et al., 2024; Rahman et al., 2021; de Souza Machado et al., 2018a, 2018b; Wright and Kelly, 2017; Zhou et al., 2020). Therefore, testing their biodegradation is important to minimise environmental persistence. Plastics are a type of polymer, and there is interest in the applicability of respirometric biodegradation testing methods for polymers. Polymers have a more complex chemical structure with a carbon-carbon backbone that is resistant to microbial degradation. Generally, polymers require initial abiotic (e.g. chemical hydrolysis, mechanical, thermal, photo) and/or biotic degradation (e.g. by extracellular enzymes), to increase their accessibility for bio assimilation by soil microbes prior to complete mineralisation (Bher et al., 2022). Whilst several studies have monitored the biodegradation of polymers such as poly(butylene adipate-co-terephthalate) (PBAT), poly(butylene succinate) (PBS), poly(butylene succinate-co-adipate) (PBSA), polylactic acid (PLA), and polyhydroxyalkanoates (PHA) in soil using stable carbon isotopes or radiolabelled carbon (e.g., (Guliyev et al., 2023; Huo et al., 2023; Nelson et al., 2022; Zhang et al., 2023; Zumstein et al., 2018)), this is generally considered an expensive and complex option to obtain representative sample amounts for large scale biodegradation testing (McDonough et al., 2023; Menzies et al., 2023). Therefore, there is a need for reliable methods that utilise indirect measurements of biodegradation, such as the commonly used respirometric methods of measuring carbon dioxide (CO_2) evolution, or oxygen (O_2) demand.

Respirometric methods allow determination of biodegradation by measuring O_2 demand or CO_2 evolved during microbial respiration. There are three distinct phases of microbial activity: first, when a carbon source is added there is a short lag phase, as the microbial community adapt to

the new substrate; second, there is an exponential growth phase where microbial activity rapidly increases as the substrate is utilised as a carbon source (this is often referred to as the biodegradation phase); finally, a plateau phase is reached, where microbial activity slows down because the carbon substrate is depleted (Zwietering et al., 1990). Measured biodegradation will rarely reach 100% because during biodegradation, microbes will utilise part of the carbon substrate for growth and maintenance, resulting in incomplete mineralisation. Recorded biodegradation values exceeding 100% sometimes occur, however this is attributed to nitrification in O_2 demand methods or soil priming effects that stimulate further soil organic carbon decomposition (Ardisson et al., 2014; Zhang et al., 2023). Measuring biodegradation using non-specific respirometric methods makes it is difficult to differentiate between these processes and true polymer biodegradation.

Respirometric methods are commonly used in the OECD testing guidelines for the measurement of the biodegradation of chemicals, particularly in the OECD 301 test for Ready Biodegradability (OECD, 1992). OECD 301 requires 60% biodegradation to occur within 28-days, and it is a screening test where the test substance is applied at concentrations much greater than environmentally relevant. OECD 301 tests are conducted in aqueous media with low levels of microbial inoculum, and the results do not simulate environmental conditions. Polymers that are designed to be biodegradable are unlikely to show such a fast rate of biodegradation as chemicals because of their carbon-carbon backbone, and the requirement for initial abiotic/biotic degradation that results in a longer lag phase prior to bioassimilation. Therefore, several standard test methods have been designed to measure the biodegradation of polymers (see Section 1.4.2 for an overview of all test methods). Specifically, of relevance for soil environments is the standard test ISO 17556:2019 "*Plastics – determination of the ultimate aerobic biodegradability of plastic materials in soil by measuring the oxygen demand in a respirometer or the amount of carbon dioxide evolved.*" (ISO, 2019).

ISO 17556:2019 suggests that either O_2 demand or CO_2 evolution can be used to measure the biodegradation of plastics in soil. ISO 17556:2019 has been criticised due to the poor reproducibility of the results (Briassoulis and Mistriotis, 2018). Variability in soil characteristics can influence the biodegradation of polymers, such as soil water content, carbon and nitrogen availability, pH, temperature, and soil texture (Briassoulis and Mistriotis, 2018; Briassoulis et al., 2020; Chinaglia et al., 2018, 2024; Pischedda et al., 2019; Tosin et al., 2019). Differences are also allowed within the experimental test set-up and measurement method, which could be a potential contributor to the variability seen. Previous literature has focused on using CO_2 as an analytical endpoint for the biodegradation of plastics in soil following ISO 17556:2019 (Briassoulis and Mistriotis, 2018; Briassoulis et al., 2020; Chinaglia et al., 2024; Pischedda et al., 2019; Tosin et al., 2019). However, O_2 demand has been used less frequently to monitor the biodegradation of polymers in soil. O_2 demand is commonly measured within OECD 301, with different respirometer types that either provide a continual supply of O_2 or are in closed vessels. The suitability of closed vessel respirometric methods for measuring the biodegradation of polymers is less certain (McDonough et al., 2023). Recent work utilised O_2 demand to measure the biodegradation of polymers in an aqueous matrix of sewage sludge following OECD 301 (McDonough et al., 2017, 2023; Menzies et al., 2023). McDonough et al., (2023) found that intra-laboratory variation was low and concluded that microcrystalline cellulose was a suitable reference material for polymers that rapidly biodegrade. While this work verifies that O_2 demand methods are suitable for measuring polymer biodegradation in aqueous media, such as sewage sludge, further investigation was deemed necessary to verify test efficacy for measuring polymer biodegradation in soil. Within a closed bottle approach, the O_2 availability will be limited by the headspace in the test vessel. It is expected that polymer biodegradation will occur slowly, with a 6-month test duration allowed and possible extension to 2 years (ISO, 2019). Therefore, it is important to ensure sufficient O_2 would be available throughout a biodegradation test to prevent

the formation of an anaerobic environment during the lengthy test duration required for a polymer to reach 60% biodegradation.

This chapter aimed to validate the use of CO_2 and O_2 test methods for measuring polymer biodegradation in soil. A series of experiments were performed following the ISO 17556:2019 study design. First the baseline soil respiration was determined using both CO_2 and O_2 test methods, with the aim of i) determining the length of a pre-incubation period and ii) facilitating the calculation of the headspace requirements for O_2 demand tests. Second, the biodegradation of sodium benzoate (a chemical known to rapidly biodegrade within 28-days) was measured in both O_2 and CO_2 test systems to validate the experimental set-up and identify potential issues within a short time scale. Third, as the O_2 demand method has been less frequently used for measuring polymer biodegradation in soil, it was further tested within two additional experiments. The first additional experiment aimed to determine the limit of detection of the system for polymers, by measuring the biodegradation of the polymer microcrystalline cellulose at different concentrations in soil. The second additional experiment aimed to determine the suitability of the system for measuring the biodegradation of polymers over a longer experiment length of 84-days within a closed vessel system by measuring the biodegradation of sodium benzoate, glucose, cellulose, and polyhydroxybutyrate (PHB). Overall, the work presented in this chapter outlines the initial experimental work completed to validate the use of both CO_2 and O_2 methods for measuring polymer biodegradation in soil.

2.2. Methods

2.2.1. Materials

All biodegradation tests were undertaken using an agricultural soil, 18 Acres, UK (18A, 51°27'16.2828''N, 0°43'49.232''W, Section 2.1.1). Soil was sampled in November 2022 and characterised prior to use by Agvise (Northwood, ND, USA, Table 2.1).

Table 2.1. Soil characteristic data for 18 Acres soil used in the method validation tests.

Soil Characteristic	18 Acres
USDA textural class	sandy loam
Sand / Silt / Clay (%)	59 / 22 / 19
Bulk Density (g/cm^3)	1.09
Cation Exchange Capacity (meq/100g)	14.5
Water Holding Capacity (%)	53.1
Moisture at 10 kPa (%)	30.2
Organic Carbon (%)	2.8
Organic Matter (%)	4.8
pH	6.1
Total Nitrogen (%)	0.24
C:N ratio (C/N)	11.67

Sodium benzoate was obtained as pellets (ThermoFisher Scientific). Microcrystalline cellulose, PHB, and glucose were obtained as powders (Sigma Aldrich). Test material properties are given in Table 2.2. The chemical formula, molecular weight, and solubility information were obtained from the supplier. The particle size was measured for the cellulose and PHB using a Mastersizer 3000 fitted with an Aero S to disperse dry powder (Malvern Panalytical). The theoretical O_2 demand (ThOD) and theoretical CO_2 evolved ($ThCO_2$) were calculated (see Section 2.2.2). Sodium hydroxide (NaOH) (ThermoFisher Scientific), and soda lime (Sigma Aldrich) were obtained in pellet form. Barium hydroxide ($Ba(OH)_2$, 0.0125mol/L) was obtained as a solution (Sigma Aldrich). Potassium hydroxide (KOH) was obtained in pellet form (Thermo Fisher Scientific). Hydrochloric acid (HCl) was obtained as a solution (Thermo Fisher Scientific) and diluted to form a 0.25N solution. Phenolphthalein indicator was obtained as a powder (Thermo Fisher Scientific). All solutions were filtered through glass microfibre filters with a pore size of 2.7 µm (Whatman, Sigma Aldrich) prior to use.

Table 2.2. Properties of the test materials used in the validation of the soil biodegradation test set-ups.

	Sodium benzoate	**Microcrystalline cellulose**	**Poly-(R)-3-hydroxybutyric acid**	**Glucose**
Chemical Formula	$C_7H_5NaO_2$	$(C_6H_{10}O_5)_n$	$(C_4H_6O_2)_n$	$C_6H_{12}O_6$
Molecular Weight (g/mol)	144.10	$(162.14)_n$	400,000-500,000	180.16
Form	Pellets	Powder	Powder	Powder
Solubility	Water soluble	Insoluble	Insoluble	Water soluble
Particle Size (µm) (DT50)*	–	19.9	26	–
ThOD (mg O_2/mg)	1.665	1.184	1.672	1.066
ThCO_2 (mg CO_2/mg)	2.139	1.629	2.046	1.467

2.2.2. Biodegradation Test Systems

Biodegradation tests to monitor the biodegradation of plastic in soil were set-up following the standard test ISO 17556:2019 where respirometric techniques were used to quantify either evolved CO_2 or biological O_2 demand (ISO, 2019). All biodegradation tests took place in aerobic conditions at a constant temperature of 20°C in the dark.

Measurement of evolved carbon dioxide

Biodegradation test flasks were connected by gas impermeable tubing and supplied with CO_2 free air. Soil (100g dry weight) was placed into a test vessel, and the soil moisture was adjusted to pF 2 (10 kPa) as this is within the 40-60% of water holding capacity required by ISO 17556 and maintained throughout the test. Test vessels were pre-incubated for one week, then the test material was added (1250 mg / 100g soil). CO_2-free air was continuously supplied to the test vessels, and CO_2 produced in the test vessels was captured in subsequent CO_2 absorption flasks containing KOH, which were periodically removed. Upon removal, fresh KOH was added to allow continual capture of evolved CO_2. CO_2 absorbed in KOH was measured by titration with HCl, using a phenolphthalein indicator.

Measurement of biological oxygen demand

O_2 demand was measured using the manometric respirometers "OxiTop®-IDS" (Xylem Analytics, Germany). OxiTop pressure sensor heads were added to a closed bottle test vessel, that contained soil, the test material, and a CO_2 absorber (soda lime pellets). During the test, soil microbial respiration occured, where O_2 was consumed, and CO_2 was produced. CO_2 was then absorbed by the soda lime resulting in a drop in pressure with O_2 consumption that was monitored by the pressure sensor. The amount of O_2 consumed was calculated from the pressure measurement by Equation 2.1 where BA is soil respiration (mg O_2/kg), M_R (O_2) is the molar mass of oxygen (32000 mg/mol), V_{fr} is the free gas volume (L), R is the general gas constant (83,14 L mbar mol^{-1} K^{-1}), T is the measuring temperature (K, kelvin), m_{Bt} is the mass of dry soil substance in the measuring preparation (kg), and Δp is the change in pressure of the measuring preparation (mbar).

$$BA = \frac{M_R(O_2)}{R \times T} \times \frac{V_{fr}}{m_{Bt}} \times \Delta p$$

[Equation 2.1]

Percent Biodegradation

Percent biodegradation (%) was calculated by comparing the measured CO_2 evolved or O_2 demand with the theoretical amounts that would be produced if the test material were completely biodegraded. The theoretical amount of evolved CO_2 ($ThCO_2$) is calculated by Equation 2.2, where m is the mass of test material introduced into the test system, in milligrams, w_c is the carbon content of the test material, expressed as a mass fraction, 44 is the relative molecular mass of CO_2, and 12 is the relative molecular mass of carbon.

$$ThCO_2\,(mg) = \frac{44}{12} \times m \times w_c$$

[Equation 2.2]

The theoretical O_2 demand (ThOD) is calculated based on the elemental composition of a substance of $C_cH_hCl_{cl}N_nS_sP_pNa_{na}O_o$ with relative molecular mass M_r by either Equation 2.3 for ThOD without nitrification, or Equation 2.4 for ThOD with nitrification.

$$ThOD_{NH_3}\ (mg/mg) = \frac{16[2c + 0.5(h - cl - 3n) + 3s + 2.5p + 0.5na - o]}{M_r}$$

[Equation 2.3]

$$ThOD\ _{NO_3}(mg/mg) = \frac{16[2c + 0.5(h - cl) + 2.5n + 3s + 2.5p + 0.5na - o]}{M_r}$$

[Equation 2.4]

Once $ThCO_2$ and ThOD are calculated for the concentration of test material added, then the percent biodegradation can be calculated using Equation 2.5.

$$\text{Biodegradation (\%)} = \left(\frac{\text{CO}_2\text{ evolved (mg)}}{\text{ThCO}_2} \times 100\right) = \left(\frac{\text{O}_2\text{ demand (mg)}}{\text{ThOD}} \times 100\right)$$

[Equation 2.5]

2.2.3. Individual Experimental Designs

A series of experiments were conducted, with the results informing the experimental design for the subsequent study.

Determination of basal soil respiration and pre-incubation period

It is well known that soil sample handling and adjustment of soil moisture can affect soil microbial activity (Schroeder et al., 2021). Specifically, the Birch effect is of concern, as this causes a pulse in mineralisation of soil organic carbon and microbial respiration after the rewetting of dry soils (Birch, 1958). Therefore, it is important to determine a suitable pre-incubation period for the test

system and soil of interest, which is the time when the soil is placed into the test vessels with the soil water content adjusted, and then incubated under the test conditions (dark, 20°C) to allow the soil microbial community to become adjusted. Basal soil respiration was measured by CO_2 evolution and O_2 demand to determine the rate of CO_2 evolved and O_2 demand, and to identify a suitable pre-incubation period. Soil (100g dry weight) was incubated in a CO_2 evolution test (n=5) and a O_2 demand test (n=3) (Table 2.3). Measurements started from the moment the soil was placed into the test system with the moisture adjusted, to capture any increases in respiration that were due to sample handling.

Table 2.3. Test set-up for determination of the basal soil respiration rate in both CO_2 and O_2 biodegradation test methods.

	CO_2 Evolution: Soil-Blank	**O_2 Demand: Soil-Blank**
Vessel Size (L)	0.4	0.25
Soil (g)	100	100
Test Material	NA	NA
Test Material (mg)	NA	NA
Replicates	5	3

Measured basal soil respiration (mg O_2/g soil/day) then allowed the predicted O_2 demand to be calculated for a soil incubation experiment with a known soil mass (S, grams) over a specified duration (T, days) (Equation 2.6).

$$Predicted\ O_2(mg\ O_2/S\ /T\ ) = Basal\ Soil\ Respiration \times S\ \times T$$

[Equation 2.6]

As the ThOD (mg O_2 / mg test material) had been calculated (Equations 2.3, 2.4), the predicted O_2 demand for a specific experimental design was determined by combining the predicted O_2 demand for basal soil respiration for a set time and the ThOD for a set amount of test material. Then the headspace volume required to meet the total predicted O_2 demand was approximated,

given that the partial pressure of O_2 in the air is approximately 0.21atm (atmospheric pressure) (Equation 2.7) (WTW, 2010).

$$Approximated\ Predicted\ Headspace\ Volume\ (L) = \frac{Predicted\ O_2\ Demand}{0.21}$$

[Equation 2.7]

Utilising these calculations allowed the design of subsequent O_2 experiments with sufficient O_2 availability for the test duration, to prevent anaerobic test conditions.

CO_2 Test Method Validation

The biodegradation of sodium benzoate in soil was measured by CO_2 evolution to validate the CO_2 test set up, prior to its use for polymer biodegradation studies. Soil (100g dry weight) was added to test vessels and pre-incubated under test conditions for two weeks. Soil blanks (n=3) contained soil only, and soil-sodium benzoate (n=3) contained soil treated with 1250 mg of sodium benzoate (Table 2.4).

Table 2.4. Test set-up for the soil biodegradation test measured by CO_2 evolution of sodium benzoate.

	Soil-Blank	**Soil-Sodium Benzoate**
Soil (g)	100	100
Test Material	NA	Sodium benzoate
Test Material (mg)	NA	1250
Replicates	3	3

O_2 Test Method Validation

Three separate experiments were conducted using the O_2 test method to validate it and assess its suitability for use with polymers in soil incubations. All experiments were set up with soil (50g dry weight) added to each test vessel, the soil moisture was adjusted to pF 2 (10 kPa), and soils were pre-incubated for one week prior to test material addition. Soil blanks contained soil only, and test vessels contained soil treated with the specific test material. All sample types were measured in triplicate where possible, however due to equipment limitations, triplicates were not always

possible, resulting in duplicates used for samples where previous data supported high replicate repeatability (e.g. soil blanks). Three individual experiments were run, with experiment specific test materials and concentrations.

O_2 Experiment 1 – Method validation with sodium benzoate

The biodegradation of sodium benzoate in soil was measured by O_2 demand over 28-days to validate the experimental set up prior to its use for polymer biodegradation studies. Soil blanks (n=3) contained soil only, and test vessels contained soil treated with either 25 mg (n=3) or 50 mg (n=3) of sodium benzoate (Table 2.5).

Table 2.5. Test set-up for the soil biodegradation test measured by O_2 demand of sodium benzoate at two test concentrations.

	Soil-Blank	**Soil-Sodium Benzoate (25mg)**	**Soil-Sodium Benzoate (50mg)**
Vessel size (L)	1	1	1
Soil (g)	50	50	50
Test Material	NA	Sodium benzoate	Sodium benzoate
Test Material (mg)	NA	25	50
Replicates	3	3	3

O_2 Experiment 2 – Application of the positive reference biodegradable polymer cellulose, and determination of analytical detection limits.

O_2 demand has been used less frequently to measure polymer biodegradation in soil, therefore a further experiment was carried out using the polymer cellulose to determine the suitability of the method for polymers and the analytical detection limit of the pressure sensor. Cellulose was chosen as it is a well-known biodegradable polymer in soil that is used as a positive reference material in ISO 17556: 2019 (ISO, 2019). The biodegradation of cellulose in soil was measured by O_2 demand over 28-days at four concentrations to determine the analytical detection limit of the O_2 method for a polymeric material. Soil blanks (n=2) contained soil only, and test vessels contained soil treated with either 25 mg (n=3), 10 mg(n=3), 5 mg (n=3), or 1 mg (n=3) of microcrystalline cellulose (Table 2.6).

Table 2.6. Test set-up for the soil biodegradation test measured by O_2 demand using microcrystalline cellulose at four concentrations.

	Soil-Blank	Soil-Cellulose (1 mg)	Soil-Cellulose (5 mg)	Soil-Cellulose (10 mg)	Soil-Cellulose (25 mg)
Vessel size (L)	1	1	1	1	1
Soil (g)	50	50	50	50	50
Test Material	NA	Microcrystalline cellulose	Microcrystalline cellulose	Microcrystalline cellulose	Microcrystalline cellulose
Test Material (mg)	NA	1	5	10	25
Replicates	2	3	3	3	3

O_2 Experiment 3 – Application to polymers for an extended test period.

The final O_2 experiment measured the biodegradation of polymers during an extended test period of 84-days. The O_2 pressure sensor utilised in this work is generally used for ready biodegradability tests, where the test duration is 28-days. However, it has previously been suggested that the test vessels can be opened to replenish the O_2 availability to allow for a longer test duration (WTW, 2010). As ISO 17556:2019 suggests a test duration of 6 months (and a possible extension to 2 years), it is important that a suitable O_2 method is chosen that enables biodegradation measurements for extended test periods greater than 28-days. In this experiment, the biodegradation of sodium benzoate, glucose, cellulose, and PHB was measured by O_2 demand for 84-days. Sodium benzoate acted as a positive reference material in this test to confirm soil viability. Glucose was measured because it is a monomer of cellulose. Cellulose and PHB were measured as they are both suggested positive reference materials in ISO 17556:2019. Soil blanks (n=2) contained soil only, test vessels were treated with 50 mg of four different test materials; sodium benzoate (n=2), glucose (n=3), cellulose (n=2), and PHB (n=3) (Table 2.7). To facilitate the test extension, on days 28 and 56 the test vessels were opened to allow influx of fresh air containing O_2 and the pressure measurements were restarted.

Table 2.7. Test set-up for the soil biodegradation test measured by O_2 demand of sodium benzoate, glucose, microcrystalline cellulose and poly-(R)-3-hydroxybutyric acid.

	Soil-Blank	Soil-Sodium Benzoate	Soil-Glucose	Soil-Cellulose	Soil-PHB
Vessel size (L)	1	1	1	1	1
Soil (g)	50	50	50	50	50
Test Material	NA	Sodium benzoate	Glucose	Microcrystalline cellulose	Poly-(R)-3-hydroxybutyric acid
Test Material (mg)	NA	50	50	50	50
Replicates	2	2	3	2	3

2.2.4. Data Analysis and Statistics

All data were analysed and visualised in RStudio (Version 4.3.1, R Core Team, 2024). Biodegradation percent was determined based on a comparison of the measured CO_2 evolved / O_2 demand with the theoretical CO_2 evolved / O_2 demand. To obtain the O_2 demand data all recorded pressure data were downloaded from the measuring heads at the end of the study and imported into Microsoft Excel for pre-processing. Pressure data was converted to O_2 demand, following the relevant calculations detailed above.

Statistical analyses were performed on CO_2 evolved, O_2 demand, and biodegradation data to determine the significance of results at relevant individual time points. The significance level was set at $p = 0.05$. Data were assessed for normality using a Shapiro Wilk test, and heterogeneity of variance using Levene's test. When the assumptions of normality and homogeneity of variance were met, parametric tests were conducted. For the CO_2 evolved data, the difference between soil blank and soil sodium benzoate respiration was compared using a Welch's one sample t-test. For O_2 experiment 1 with sodium benzoate, the O_2 demand was compared between the three test groups using a one-way ANOVA followed by a Tukey post hoc test. Differences in biodegradation percent were compared between the two test concentrations of sodium benzoate using a Welch's t-test. For O_2 experiment 2 with different concentrations of cellulose, a one-way ANOVA followed

by a Tukey post hoc test was used to determine differences in O_2 demand between five test groups. For O_2 experiment 3 with different test materials, a one-way ANOVA followed by a Tukey post hoc test was used to determine differences in O_2 demand between five test groups, and differences in biodegradation percent between four test groups. If the data did not meet the assumptions of normality and homogeneity of variance, then the equivalent nonparametric tests were used, a Mann-Whitney U test instead of a Welch's t-test, or a Kruskal-Wallis test followed by Dunn's post hoc test instead of a one-way ANOVA followed by a Tukey post-hoc test. The *p*-values from all statistical analyses are presented in Appendix B.

2.3. Results and Discussion

2.3.1. Soil Basal Respiration Rates to Determine the Pre-Incubation Period and the Headspace Requirements for the O_2 Demand Method

Soil basal respiration rate was initially determined using both methods. Soil respiration rate was measured by CO_2 evolution, with measurements commencing after the addition of soil to test flasks, with no pre-incubation period. The CO_2 evolved initially increased from days 1 to 5, and the slope of the curve then flattened from day 5 to 8, with a slight steady increase noted from days 8 to 12 (Figure 2.1). Therefore, a suitable pre-incubation period of minimum one week was identified, which fits with the suggestion of a preincubation period of 2-28 days in OECD 307 (OECD, 2002).

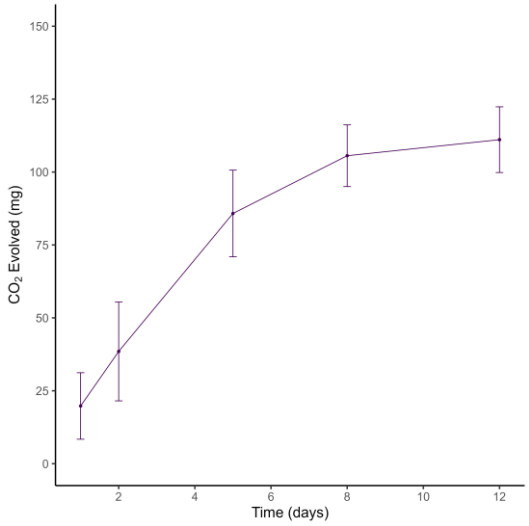


Figure 2.1. CO_2 evolution from 18 Acres soil for the initial 12-days after addition into test flasks, with no pre-incubation period (n=5 ± standard deviation).

Soil respiration rate was also measured by O_2 demand. Raw data was recorded as pressure (hPa), and then converted to O_2 demand (Figure 2.2). The replicate soil-blank-3 recorded erroneous pressure results (Appendix B), consistent with the pressure head not being sealed correctly, and therefore it was not included in the displayed average calculations.

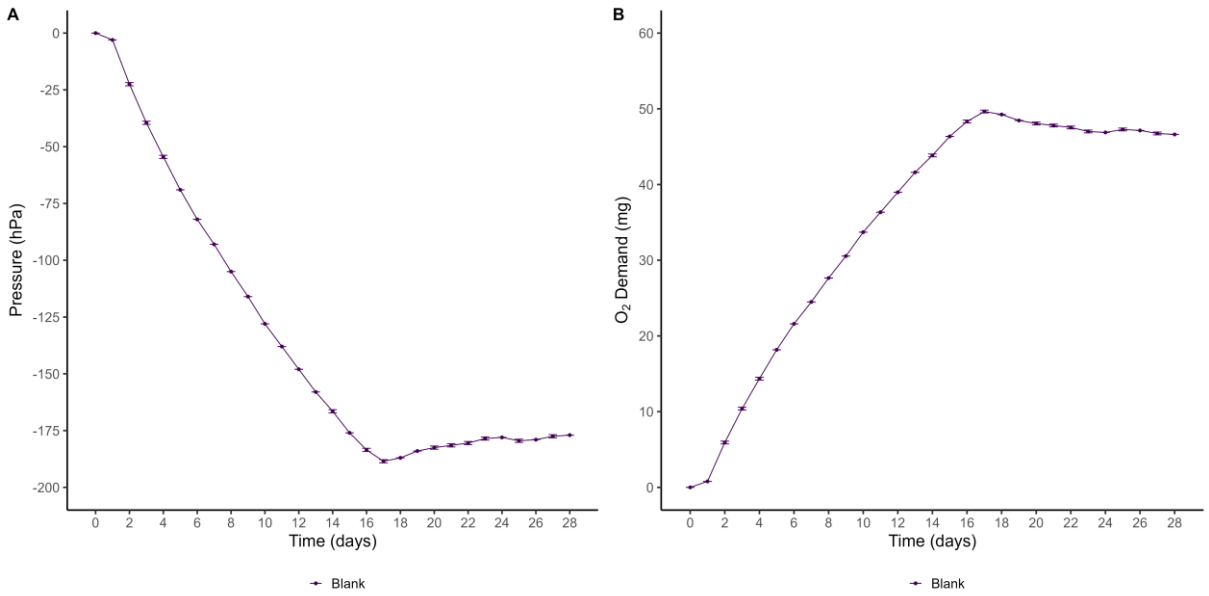


Figure 2.2. Recorded pressure (hPa) (A) and O_2 demand (B) measurements for 18 Acres soil in an OxiTop®-IDS system for 28-days. Data is displayed as the mean (n=2) ± standard deviation.

O_2 demand reached the maximum recorded value of 49.6 mg O_2/100g soil within 17 days (Figure 2.2B), then there was a slight decrease. The measurement heads have a maximum level of detection of a pressure change up to 1250 hPa, and in this experiment the maximum recorded pressure value was 188.5 hPa (Figure 2.2A). Therefore, it was concluded that the maximum recorded value of 188.5 hPa was reached due to a lack of available O_2 in the test vessel headspace, rather than being the result of exceeding the detector sensitivity.

Based on the recorded measurements from the first 17-days, the basal soil respiration rate was calculated to be 0.029 mg O_2/g soil/day. This enabled subsequent experiments to be planned based on the predicted O_2 headspace requirements for daily soil respiration per gram of soil, and the ThOD of the test material. Utilising this information, the predicted headspace requirement was then determined for each of the subsequent O_2 experiments in this chapter, to facilitate in their experimental design (Table 2.8, 2.9, 2.10). Based on the predicted O_2 demand, the test vessel volume was increased to 1L, to ensure that sufficient O_2 was available through the experiments.

Table 2.8. Calculation of the predicted O_2 demand for O_2 Experiment 1 with sodium benzoate at two concentrations.

	Sodium benzoate (25 mg)	**Sodium benzoate (50 mg)**
Soil (g dry weight)	50	50
Time (day	28	28
Calculated Soil Respiration (mg O_2 / 50g soil / 28 days)	40.6	40.6
Test Substance ThOD (mg / mg)	1.665	1.665
Test Substance Application (mg)	25	50
Test Substance ThOD (mg / 50 mg)	41.625	83.25
Total Predicted O_2 Demand (mg)	82.225	123.85
Predicted Headspace Volume Requirement (mL)	391.548	589.762

Table 2.9. Calculation of the predicted O_2 demand for O_2 Experiment 2 with microcrystalline cellulose at four concentrations.

	Cellulose (1 mg)	Cellulose (5 mg)	Cellulose (10 mg)	Cellulose (25 mg)
Soil (g dry weight)	50	50	50	50
Time (days	28	28	28	28
Calculated Soil Respiration (mg O_2 / 50g soil / 28 days)	40.6	40.6	40.6	40.6
Test Substance ThOD (mg / mg)	1.184	1.184	1.184	1.184
Test Substance Application (mg)	1	5	10	25
Test Substance ThOD (mg / 50 mg)	1.184	5.92	11.84	29.6
Total Predicted O_2 Demand (mg)	41.784	46.52	52.44	70.2
Predicted Headspace Volume Requirement (mL)	198.971	221.524	249.714	334.286

Table 2.10. Calculation of the predicted O_2 demand for O_2 Experiment 3 with sodium benzoate, glucose, cellulose, and PHB.

	Sodium benzoate	Glucose	Cellulose	PHB
Soil (g dry weight)	50	50	50	50
Time (days	84	84	84	84
Calculated Soil Respiration (mg O_2 / 50g soil / 84 days)	121.8	121.8	121.8	121.8
Test Substance ThOD (mg / mg)	1.665	1.066	1.184	1.672
Test Substance Application (mg)	50	50	50	50
Test Substance ThOD (mg / 50 mg)	83.25	53.3	59.2	83.6
Total Predicted O_2 Demand (mg)	205.05	175.1	181	205.4
Predicted Headspace Volume Requirement (mL)	976.429	833.810	861.905	978.095

2.3.2. CO_2 Test Method Validation Using Sodium Benzoate

The biodegradation of sodium benzoate in soil was measured by CO_2 evolution (Figure 2.3). There was a statistically significant difference in CO_2 evolution after sodium benzoate addition, with CO_2 evolved being significantly higher than in the soil blank between days 7 and 72 of the incubation (t-test: p>0.05, Appendix B). A clear biodegradation curve is observed with distinct lag (days 1-7), biodegradation (days 7-28), and plateau (days 28-72) phases (Figure 2.3B). Sodium benzoate reached 60% biodegradation by day 17, therefore this met the validity requirement meaning

sufficient biodegradation had occurred. An average of 76.31±1.86% biodegradation was reached in the first 28-days, with the plateau phase then extended until test termination at 84.40±2.07% biodegradation on day 72.

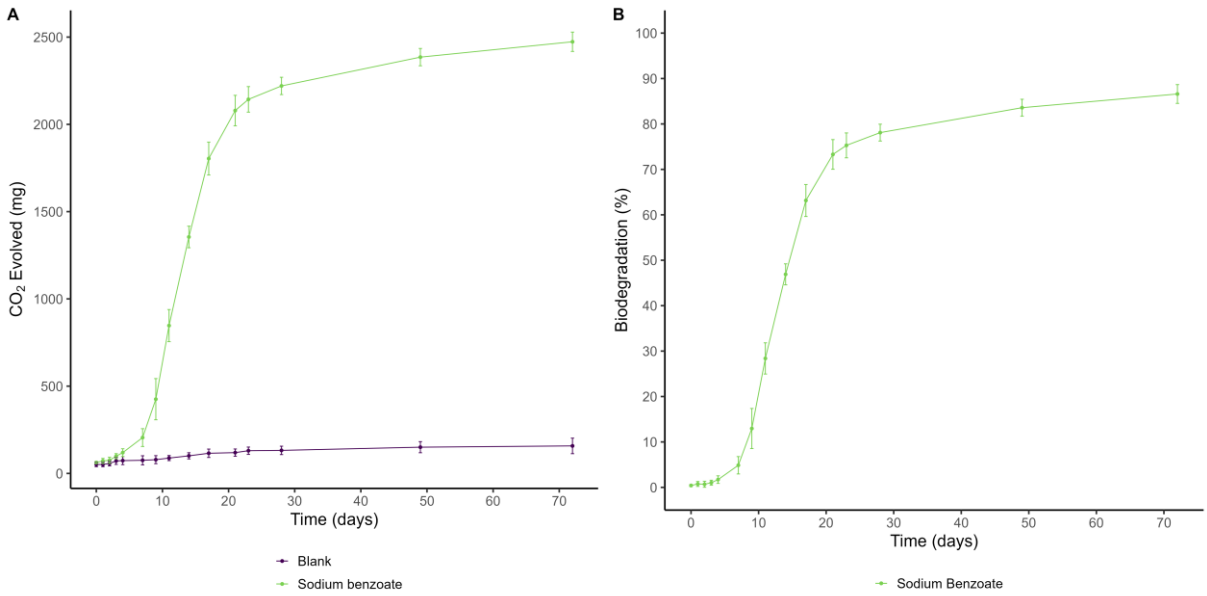


Figure 2.3. (A) CO_2 evolved from soil-blank (100g) and soil-sodium benzoate (1250 mg/100g) test vessels measured over 28-days. (B). Biodegradation (%) data for sodium benzoate measured over 28-days. Data is presented as the mean (n=3) ± standard deviation.

Sodium benzoate has previously been used to validate experimental set-ups where biodegradation is measured in soil, with the observed biodegradation allowing confirmation that the test method is set up correctly (Kaakinen et al., 2007; Strotmann et al., 2004). Whilst the purpose of ISO 17556:2019 is to measure the biodegradation of plastic in soil, this experiment utilised the test set-up specified in ISO 17556:2019 to measure the biodegradation of sodium benzoate in soil. The obtained results (Figure 2.3) therefore provide confirmation that this test set-up can successfully detect differences in CO_2 evolution between blank soil and test soil to allow calculation of biodegradation percent. CO_2 evolution methods for measuring the biodegradation of polymers in soil have been previously demonstrated in the literature (Briassoulis and Mistriotis,

2018; Briassoulis et al., 2020; Chinaglia et al., 2018, 2024; Pischedda et al., 2019; Tosin et al., 2019). Therefore, based on the prior use of CO_2 evolution methods in the literature and the validation that this specific test method was set up correctly with sodium benzoate in this experiment, it was concluded that this test set-up would be appropriate for subsequent biodegradation of polymers in soils.

2.3.3. O_2 Test Method Validation Using Sodium Benzoate, Glucose, Cellulose and PHB

In contrast to CO_2 evolution as an analytical endpoint, less work has looked at the use of O_2 demand to measure polymer biodegradation in soil. Therefore, three experiments were conducted using O_2 demand measurements for assessing biodegradation in soil.

Sodium benzoate biodegradation was measured at two different concentrations in soil (25 mg / 50g, and 50 mg / 50g). There was a significant difference in the measured O_2 demand between the soil blanks and soil treated with sodium benzoate (Figure 2.4A). The cumulative O_2 demand measured in soil-sodium benzoate replicates was significantly greater than in the soil-blanks between days 3 and 28 of the incubation (one-way ANOVA and Tukey post-hoc tests: $p<0.05$, Appendix B). The biodegradation of sodium benzoate showed high reproducibility, with both concentrations reaching the 60% pass requirement by day 7 (Figure 2.4B). There was no significant difference in biodegradation between either concentration throughout the test duration (t-test: $p>0.05$, Appendix B). Since sodium benzoate has previously been used to validate biodegradation test methods (Kaakinen et al., 2007), these results provided sufficient evidence that the O_2 method set-up is suitable to measure biodegradation in soil.

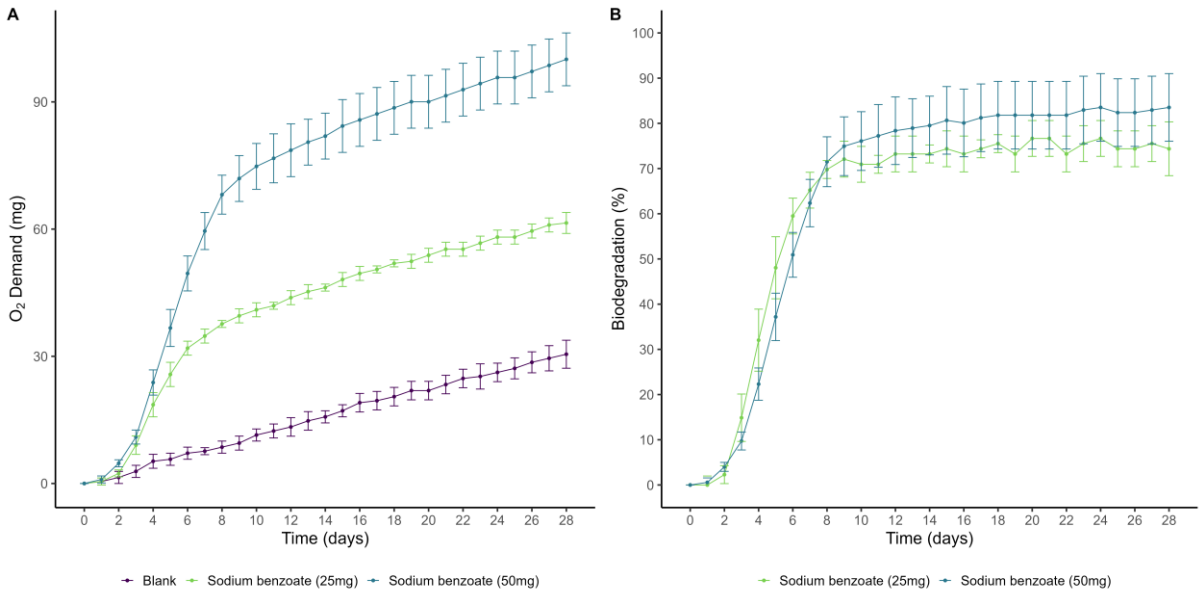


Figure 2.4. (A) O_2 consumption (mg/50g) of soil-blank and soil-sodium benzoate at two concentrations (25 mg / 50g, 50 mg / 50g) measured over 28-days. (B). Biodegradation (%) data for sodium benzoate at two concentrations measured over 28-days. Data is presented as the mean (n=3) ± standard deviation.

In the second O_2 experiment, the biodegradation of cellulose in soil was determined at four different concentrations to investigate the detection limit of the OxiTop system to polymer biodegradation at different application rates (Figure 2.5). There was no significant difference between the O_2 demand of the soil blank and soil cellulose at concentrations 1 mg, 5 mg, and 10 mg for any test day (Figure 2.5A, Figure 2.6, one-way ANOVA, Appendix B). Cellulose-25 mg had greater levels of O_2 demand than the soil blank (Figure 2.6), however overall, this was a non-significant trend (one-way ANOVA, Appendix B). Therefore, there is a lack of sensitivity of the O_2 demand method at lower sample concentrations of cellulose. The inability to accurately distinguish between O_2 demand associated with biodegradation of the test material versus the background soil respiration, results in the inability to accurately and confidently measure biodegradation. This is reflected when the O_2 demand is converted to biodegradation, as it produced values in the range of -100 to 300% (Figure 2.5B, Figure 2.6). Therefore, these results indicate that whilst the OxiTop system does have high sensitivity in detecting differences between

blank soil and test soil, the pressure sensor cannot accurately detect differences at such low application rates of polymer.

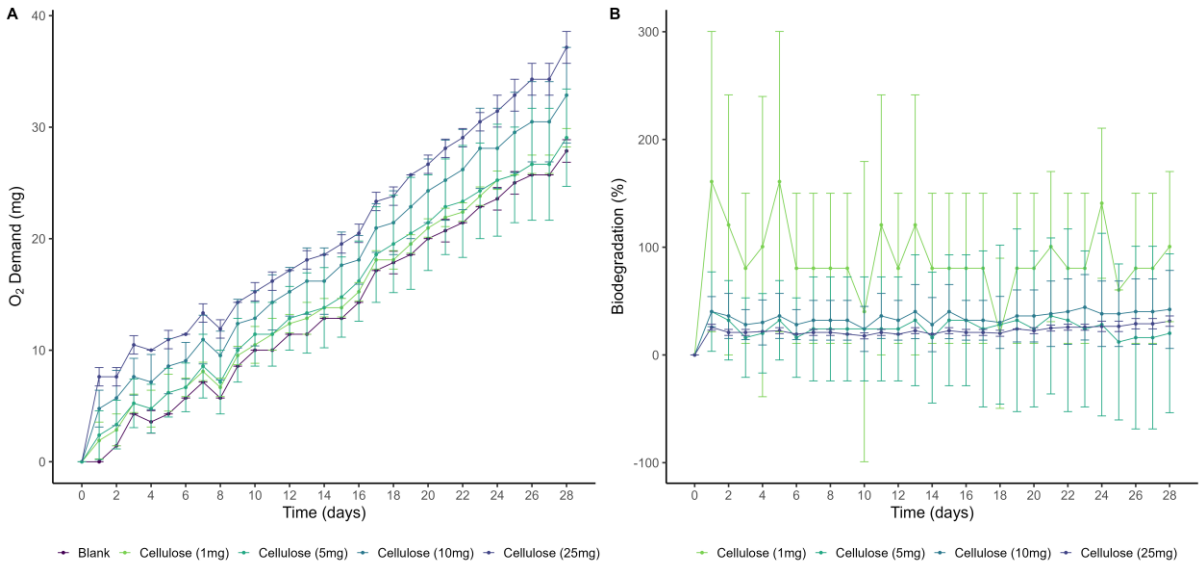


Figure 2.5. (A) O_2 consumption (mg / 50g soil) of soil-blank and soil-cellulose at four concentrations (1 mg / 50g, 5 mg / 50g, 10 mg / 50g, 25 mg / 50g) measured over 28-days. (B). Biodegradation (%) for cellulose at four concentrations (1 mg / 50g, 5 mg / 50g, 10 mg / 50g, 25 mg / 50g) measured over 28-days. Data is presented as mean ± standard deviation, the soil-cellulose (all concentrations) replicates are n=3, and the soil-blank replicates are n=2.

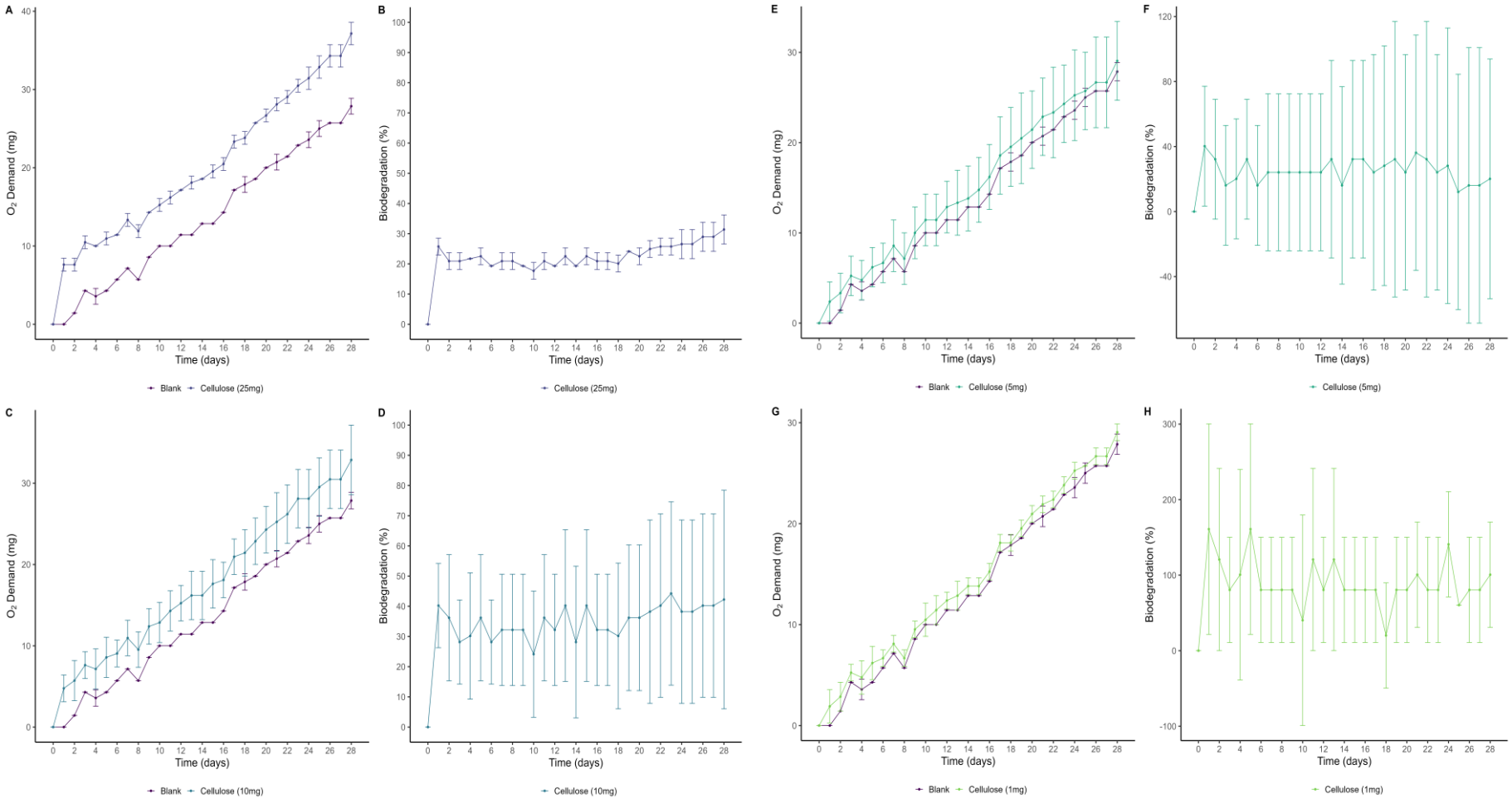


Figure 2.6. (A) O_2 consumption (mg / 50g soil) of soil-blank and soil-cellulose-25 mg. (B). Biodegradation (%) for cellulose-25 mg. (C) O_2 consumption (mg / 50g soil) of soil-blank and soil-cellulose-10 mg. (D). Biodegradation (%) for soil-cellulose-10 mg. (E) O_2 consumption (mg / 50g soil) for soil-blank, and soil-cellulose-5 mg test vessels. (F) Biodegradation (%) for soil-cellulose-5 mg. (G) O_2 consumption (mg / 50g soil) for soil-blank, and soil-cellulose-1 mg test vessels. (H) Biodegradation (%) for soil-cellulose-1 mg. All data is measured over 28 days and is presented as mean ± standard deviation. Soil-cellulose replicates are n=3, and soil-blank replicates are n=2.

This experiment was conducted to investigate if a lower application rate would be possible within an O_2 demand system. Currently ISO 17556:2019 specifies high application rates to increase the difference between soil respiration and the biodegradation of a test material. However, this can result in environmentally unrealistic levels of application to soil. In OECD 307 for testing of the biodegradation of chemicals in soil, the application rate is specified to correspond to the highest application rate of a relevant product, to ensure environmental relevance (OECD, 2002). This is because high application rates could influence soil microbial functions (OECD, 2002). The potential influence from plastics and plastic additives on soil microbial function and how they may affect the test performance remains largely unexplored, making it of note for future research into biodegradability testing (Dong et al., 2024; Rillig et al., 2024). As this experiment found that the detection limit was insufficient at lower concentrations, it was decided to maintain the application rate at 1 mg test material per 1 mg of soil for subsequent O_2 demand tests in keeping with ISO 17556:2019 (ISO, 2019).

In contrast with sodium benzoate, cellulose did not reach 60% biodegradation in 28-days. Cellulose is a known biodegradable polymer, acting as a positive reference material in ISO 17556:2019, therefore, it is expected to biodegrade in soils. However, as this test was run for just 28-days, the low observed biodegradation of cellulose is expected because the lag phase is longer for polymers as abiotic and/or biotic degradation need to initially occur to increase the availability of lower molecular weight monomers that would be available for bioassimilation by soil microbes (Bher et al., 2022).

Based on the previous results for measuring biodegradation in soil by O_2 demand (Figures 2.4 and 2.5), confidence in this test-up was increased, but further investigation was deemed necessary to utilise it for measuring biodegradation of polymers in soil. Critically, the test length needed to be extended. As the OxiTop-IDS system was designed for use with the OECD 301 test series,

generally they are used for a test length of 28-days. The O_2 demand and subsequent biodegradation were measured for sodium benzoate, glucose, cellulose, and PHB were measured for 84-days (Figure 2.7).

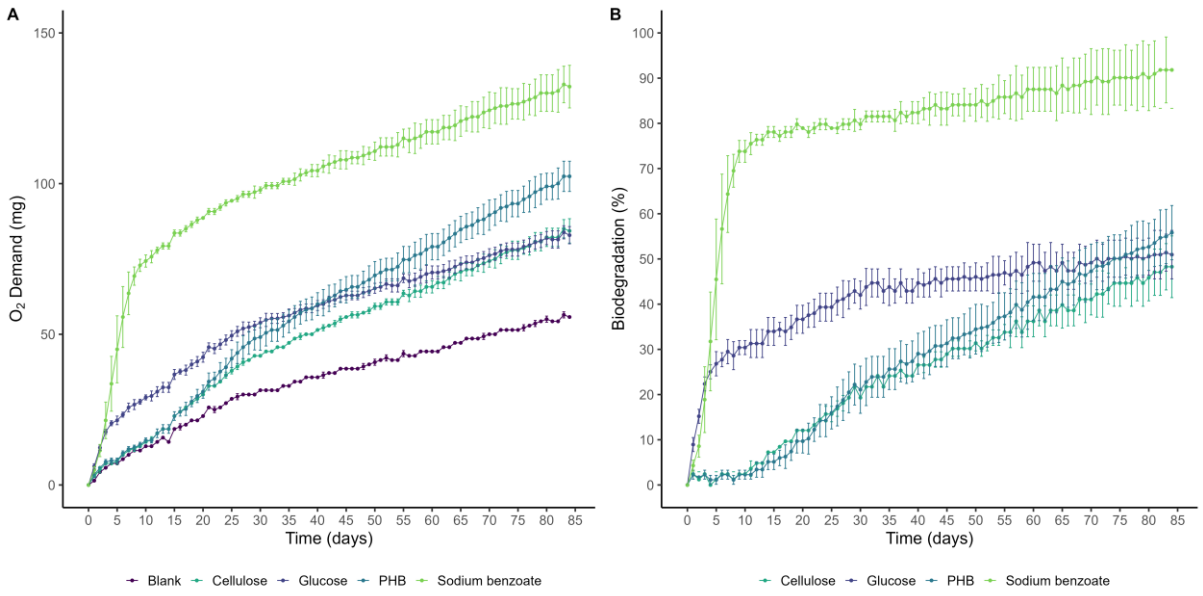


Figure 2.7. (A) O_2 consumption (mg / 50g soil) of soil-blank, soil-sodium benzoate, soil-glucose, soil-cellulose, and soil-PHB measured over 84-days. (B). Biodegradation (%) of soil-sodium benzoate, soil-glucose, soil-cellulose, and soil-PHB measured over 84-days. Data is presented as mean ± standard deviation. The soil-glucose and soil-PHB replicates are n=3, and the soil-blank, soil-cellulose, and soil-sodium benzoate replicates are n=2.

All tested materials had significantly higher O_2 demand than the soil blank by test termination at day-84 (one-way ANOVA, $p<0.05$, Appendix B). However, the rate of increased O_2 demand, and thereby inferred microbial uptake by soil microbes differed between the materials. There were significant differences in measured biodegradation between all tested materials for the duration of the test (one-way ANOVA, $p<0.05$, Appendix B). Sodium benzoate O_2 demand rapidly increased and was significantly higher than all other test materials and the soil blank from day 4 to day 84 (Tukey multiple comparisons test: $p<0.001$, Appendix B). Sodium benzoate biodegradation was also significantly higher than all other test materials from day 4 to day 84 (Tukey multiple

comparisons test: $p<0.001$, Appendix B). All sodium benzoate replicates surpassed 60% biodegradation by day 8 of the test, with the plateau phase starting by day 12 at 76.8 ± 1.2% biodegradation, and extending until test termination on day 84 at 91.8 ± 8.5% biodegradation. The rapid biodegradation seen for sodium benzoate is consistent with results seen in the first O_2 experiment (Figure 2.4) and confirms that the soil is biologically active.

Glucose had significantly higher O_2 demand than the soil blank for the duration of the test (Tukey multiple comparisons test: $p<0.001$, Appendix B). Glucose biodegradation was initially fast, reaching 26.8 ± 2.7% biodegradation by day 5, however biodegradation then slowed down, with the test terminating on day 84 at only 50.9 ± 5.4% biodegradation. Therefore, approximately half of the measured biodegradation of glucose occurred during the first 5 days of the soil incubation. This was an unexpected result, because glucose mineralisation has been extensively studied in soil, with it known to be easily accessible to soil microorganisms (Boddy et al., 2007; Fischer et al., 2010; Hill et al., 2008). Additionally, the use of glucose has been seen to have a priming effect on the mineralisation of soil organic matter, where the addition of glucose stimulates the biodegradation of soil organic matter (Shen and Bartha, 1997). The increased decomposition of soil organic matter due to a priming effect would be expected to increase soil respiration and thereby O_2 demand.

It is important to note that the results seen in previous research are derived from following the mineralisation of carbon to CO_2, whereas in this experiment O_2 demand was used to monitor the biodegradation of glucose. Therefore, it is not possible to directly compare the results with those in the literature. Previous research into glucose mineralisation in soil has highlighted that the rate of carbon mineralisation is affected by the microbial carbon use efficiency (CUE), which is the amount of a substrates carbon that microbes use for growth compared to the rate of total carbon uptake (Hill et al., 2008; Manzoni et al., 2012; Sinsabaugh et al., 2013). Glucose has been

extensively studied with relation to microbial CUE, and a recent meta-analysis suggests that soil clay content is a significant driver of glucose CUE, and that the amount of glucose substrate applied can also impact the CUE (Islam et al., 2023). A suggested explanation for this is that increased clay content can promote greater adsorption of substrates, reducing substrate availability to microbes (Islam et al., 2023; Jagadamma et al., 2012; Porras et al., 2018). If this was the case, then adsorption could reduce glucose availability for microbial decomposition and be a potential explanation for the reduced O_2 demand and incomplete biodegradation in this study. However, glucose is a neutrally charged sugar meaning that sorption to soil would be expected to be lower, and studies have shown it does not sorb to soil (Hill et al., 2008; Kuzyakov and Jones, 2006). Additionally, glucose is expected to have rapid microbial uptake within less than two days, which would outcompete sorption to soil (Fischer et al., 2010; Geyer et al., 2019). This is a very short time span, which could contribute to the unexpected result, as the limit of detection by O_2 demand could be insufficient for the rate of glucose uptake by microbes. The simultaneous measurement of both O_2 demand and CO_2 evolved has been suggested to be more useful as it allows the calculation of a respiratory quotient, which can improve the understanding of the relationship between O_2 and CO_2 during biodegradation (Taok et al., 2007).

Both cellulose and PHB had similar initial O_2 demand patterns, for days 1-15 there was no significant difference from the soil-blank (one-way ANOVA, Tukey multiple comparisons test, Appendix B). This is consistent with a lag phase, and fits with the current understanding that initially polymeric materials are inaccessible to soil microbes, and so initial abiotic and/or biotic degradation is required to allow bioassimilation by soil microbes. From day 15 to the end of the test, both cellulose and PHB had significantly higher O_2 demand than the soil blank, which is consistent with the biodegradation phase. However, the O_2 demand between the polymers diverged from day 37-84, with PHB then having a significantly higher O_2 demand than cellulose (Tukey multiple comparisons test: $p<0.05$, Appendix B). Despite differences in measured O_2

demand, there was no significant difference in the biodegradation of cellulose and PHB at any time point during the test (one-way ANOVA, Tukey multiple comparisons test, Appendix B). The maximum biodegradation recorded at the end of the test for cellulose and PHB was 48.27±6.83% and 55.82±6.00% respectively. Therefore, within the 84-day test period, neither polymer reached the 60% pass level. However, the rate of biodegradation is still constantly increasing, and the plateau phase has not started. Therefore, it is likely that if the experiment had continued the 60% pass level would be met, especially as both cellulose and PHB are suggested for use as positive reference materials in ISO 17556:2019 (ISO, 2019). Due to the considerable length of time of 6 months to 2 years allowed in ISO 17556:2019 to complete a biodegradation experiment, and the time constraints of completing subsequent biodegradation experiments for the thesis (detailed in Chapters 3 and 5), the decision was made to not extend the experiment further. The results obtained in this test extension achieved the purpose of demonstrating that the O_2 method was suitable for use in measuring the biodegradation of slowly degrading polymers in soil with sufficient detection limits to distinguish the O_2 demand from background soil respiration.

2.4. Conclusion

This experimental work validated the test set up for the CO_2 and O_2 test methods. O_2 demand methods have been less frequently used in the literature to monitor polymer biodegradation in long term tests. It is important to determine the required headspace volume to ensure sufficient O_2 demand throughout the duration of a test, to prevent anaerobic conditions. O_2 can be used for different processes (e.g. nitrification) in soils, meaning there can be uncertainty regarding the measurement, however there are significant benefits (e.g. time, cost) to using automated measurements. The reproducibility of both CO_2 and O_2 test methods are further explored in Chapter 3.

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Chapter 3. A comparative analysis of the reproducibility of respirometric methods for measuring the biodegradation of the polymer microcrystalline cellulose in different soils.

This chapter has been prepared as manuscript for journal submission.

Authors and Affiliations

Grace Davies[1], Samantha Marshall[2], Massimiliano Mascelloni[3], Stefan Krause[1,4], and Iseult Lynch[1,5].

[1]School of Geography, Earth, and Environmental Sciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

[2]Product Safety, Jealott's Hill International Research Centre, Syngenta Limited, United Kingdom

[3]Product Technology and Engineering, Jealott's Hill International Research Centre, Syngenta Limited, United Kingdom

[4]Birmingham Institute for Sustainability and Climate Action, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

[5]Centre for Environmental Research and Justice, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

CRediT author statement

Grace Davies: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing Original Draft, Writing Review and Editing. **Samantha Marshall**: Conceptualisation, Methodology, Writing Review and Editing, Supervision. **Massimiliano Mascelloni**: Conceptualisation, Methodology, Supervision. **Stefan Krause**: Conceptualisation, Supervision. **Iseult Lynch**: Conceptualisation, Writing Review and Editing, Supervision.

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Abstract

Biodegradable polymers are considered to be more environmentally sustainable than conventional polymers, as complete environmental biodegradation will prevent the formation of environmentally persistent micro and nano plastics. Current standard test methods for measuring the biodegradation of polymers utilise respirometric endpoints of biological oxygen demand and carbon dioxide evolution. However, respirometric methods were developed for testing the biodegradation of chemical and small molecules, and their suitability for testing the biodegradation of polymers is unknown. Biodegradability in soil is a desirable trait for agricultural applications, as the recovery of small micro and nano plastics from soil is difficult. To be classified as soil biodegradable, polymers must pass a standard test method, such as ISO 17556:2019 which determines the ultimate aerobic biodegradability of plastic in soils. The biodegradation of a test material is compared with a reference material, such as microcrystalline cellulose. However, biodegradation tests have been criticised for their poor reproducibility, and their potential unsuitability for polymers. In this study, the biodegradation of cellulose was measured in three soils by two test set ups that measured either biological oxygen demand or carbon dioxide evolution. Results were variable between all three soils and both test methods. Soil texture, nutrient availability and water content were identified as key factors that contributed to poor reproducibility. Additionally, the variation noted between measurement endpoint highlights how indirect respirometric methods introduce uncertainty into the results. Critically, a key concern is whether the measurements are representative of polymer biodegradation or altered soil respiration induced by the addition of the polymer to soil. Therefore, this highlights the need for more mechanistic studies to ascertain the suitability of indirect respirometric methods.

3.1. Introduction

Plastic waste is a major environmental pollutant, with microplastics (particles, <5mm) of concern due to their prevalence in all environments (Allen et al., 2022; Borrelle et al., 2020; Geyer et al., 2017; Rillig and Lehmann, 2020; de Souza Machado et al., 2018; Xu et al., 2020). In the European Union (EU), a restriction on the use of synthetic polymer microparticles (i.e. microplastics) was brought into force in October 2023 under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (European Union, 2023). (Bio)degradable polymers are derogated from this restriction, and are proposed as alternatives to conventional polymers, due to their proposed increased sustainability (Hofmann et al., 2023). However, the environmental sustainability of biodegradable polymers is of concern, with questions regarding their degradability under realistic environmental conditions, and the associated potential risk of the increased generation of biodegradable micro and nano plastics (Hofmann et al., 2023; Qin et al., 2021; Sintim and Flury, 2017; Yu and Flury, 2024).

Plastic usage in agriculture is high, with 12.5 million tonnes used annually (FAO, 2021). Plastics are utilised to improve crop production, with examples including plastic mulching films, greenhouses, polytunnels, protective nets, and the use of polymer coated fertilisers and plant protection products (FAO, 2021). The use of plastic in agriculture is beneficial, for example plastic mulching films represent approximately 50% of agricultural plastic use, and they have significant benefits such as the maintenance of soil moisture and temperature, and enhanced nutrient uptake, which reduces demand for water and nutrients and increases crop yields (Hofmann et al., 2023; Kasirajan and Ngouajio, 2012). Polymer encapsulation technology used in fertilisers and pesticides contributes to improved environmental and human safety, by controlling the release and efficiency of chemicals, and minimising losses to the environment via agricultural runoff (Pinto et al., 2022; Shirley et al., 2001; Sikder et al., 2021; Sinha et al., 2019). However, all plastic uses can result in the formation of micro and nano plastic pollution in soils, which can arise

indirectly through fragmentation of macro plastics, such as mulching films, or directly by the application of polymer encapsulated fertilisers and plant protection products (Accinelli et al., 2019; European Union, 2023; Langlet et al., 2024; Lian et al., 2021; Pirzada et al., 2020). With the global population expected to rise from 8 to 10 billion by 2050, pressure on food production systems will increase, making it critical to retain the product functionality associated with the use of plastics in agriculture to maintain high yields (Hofmann et al., 2023). Therefore, biodegradable polymers in agricultural applications are considered a viable solution to reduce microplastic pollution whilst retaining key product functionality. However, the biodegradation of polymers in soil needs to be proven to ensure they are not persisting in the environment.

Biodegradable polymers are expected to be assimilated by microbial action, and the polymer carbon should be converted to microbial biomass, carbon dioxide (CO_2) (under aerobic conditions), and methane (CH_4) (under anaerobic conditions) (Hofmann et al., 2023; Sander et al., 2023; Yu and Flury, 2024). The process of biodegradation is dependent on both the physicochemical polymer properties, and the environmental conditions (Bher et al., 2022; Briassoulis and Mistriotis, 2018; Chinaglia et al., 2024; Sander et al., 2023). Several standard test methods have been developed to measure polymer biodegradability in different environments, with aerobic biodegradation measured by following oxygen (O_2) consumption or CO_2 evolution. Respirometric methods to monitor the biodegradation of chemicals are well established by the OECD (Organisation for Economic Co-operation and Development). In the REACH regulation, standardised OECD and ISO (International Organization for Standardization) tests are proposed for assessing polymer biodegradability, which are based on the principles of small molecule testing (European Chemicals Agency, 2019; European Union, 2023). However, polymer properties such as crystallinity, high molecular weight and insolubility in water can have a greater impact on the accessibility of polymers to microbial degradation compared to small molecules (Bher et al.,

2022). Therefore, the suitability of respirometric tests for measuring polymer biodegradation is still uncertain.

ISO 17556:2019 is a proposed standard test to measure the biodegradation of plastics in soil (ISO, 2019). The biodegradation of a test polymer is measured with respect to a reference material, with microcrystalline cellulose, ashless cellulose filters or poly-R-3-hydroxybutyrate (PHB) suggested as appropriate reference materials (ISO, 2019). The test is only considered valid if the reference material reaches >60% degradation at the plateau phase or by the end of the test. Additionally, REACH Annex XVII sets out that the test material needs to measure 90% of the biodegradation relative to the reference material (European Union, 2023). Therefore, the measured biodegradation of the reference material is of critical importance. However, considerable variability in the biodegradation of the reference material microcrystalline cellulose has been observed (see ISO 17556:2019, Annex G). Previously, an interlaboratory test was carried out with six laboratories to compare the biodegradation of cellulose and a starch-poly(butylene adipate-co-butylene terephthalate) (PBAT) blend, but all laboratories measured the evolution of CO_2 only, and O_2 demand was not considered (ISO, 2019). Considerable variability was observed in the rate and maximum biodegradation of cellulose in both natural and standard (i.e. artificial) soils. Furthermore, biodegradation values were observed of greater than 100% for cellulose by one laboratory, and these measurements were not considered further or explained.

ISO 17556:2019 allows for variances across environmental conditions and measurement approaches that can lead to poor reproducibility and as such has been criticised in the literature (Briassoulis and Mistriotis, 2018; Briassoulis and Degli Innocenti, 2017). Soil is a complex media, with soil water content, soil structure and texture, nutrient availability, temperature, pH, and microbial composition all examples of soil properties that cause variability in biodegradation rates (Ardisson et al., 2014; Briassoulis and Mistriotis, 2018; Briassoulis et al., 2020; Chinaglia et

al., 2024; Fernandes et al., 2020; Al Hosni et al., 2019; Mistriotis et al., 2019; Pischedda et al., 2019). Additionally, the measurement endpoint is critical to consider, as whilst respirometric methods are widely applied for small molecules, there are concerns that indirect methods are not appropriate for polymers (McDonough et al., 2023).

In this study, the standard test method ISO 17556:2019 was followed to measure the biodegradability of the positive reference polymer microcrystalline cellulose in three soil types. Biodegradation was measured by the respirometric endpoints of CO_2 evolution and O_2 demand. The aim of the work was to investigate the potential causes of poor reproducibility in biodegradation rates between methodological approaches and soil types.

3.2. Methods

3.2.1. Test Materials

Microcrystalline cellulose (cellulose) was obtained in powdered form (Sigma Aldrich). Particle size data was measured using a Mastersizer 3000 fitted with an Aero S to disperse dry powder (Malvern Panalytical, Table 3.1). Theoretical O_2 demand (ThOD) and theoretical CO_2 evolved ($ThCO_2$) were calculated based on the chemical formula (Table 3.1). Barium hydroxide ($Ba(OH)_2$, 0.3N) was obtained as a solution (ThermoFisher Scientific), potassium hydroxide (KOH) was obtained in pellets (ThermoFisher Scientific), hydrochloric acid (HCl, 2N) was obtained as a liquid (ThermoFisher Scientific) and soda lime pellets were obtained in granular form with an ethyl violet indicator that changes from white to purple as CO_2 is absorbed (ThermoFisher Scientific).

Table 3.1. Properties of microcrystalline cellulose

	Microcrystalline cellulose
Chemical Formula	$C_6H_{10}O_5$
Form	Powder
Particle Size (µm) (DT50)	19.9
ThOD (mg O_2/mg)	1.184
$ThCO_2$ (mg CO_2/mg)	1.629

3.2.2. Soil Properties

Three soils were utilised in this study. Two were natural agricultural soils; 18 Acres, UK (18A, 51°27'16.2828''N, 0°43'49.232''W) and Gartenacker, Switzerland (GAR, 46°19'57.72''N, 6°55'30.24''E). The third soil was a standardised test soil, composed of quartz sand (70%, Melcourt Industries), kaolinite clay (10%, Sigma Aldrich), natural soil (16%, 18 Acres, Syngenta), and mature compost (4%, Melcourt Industries) and prepared in accordance with ISO 17556:2019 (Table S3.1). All soils were characterised by Agvise Laboratories (Northwood, ND, USA), with measurements recorded for the percentage of sand, silt, and clay, bulk density, cation exchange capacity, water holding capacity, organic carbon content, organic matter content, total nitrogen content, and pH (Table 3.2). Soils were sampled in March and May 2023, and were used within 3 months of collection to ensure adequate biological activity (OECD, 1992). Soils sampled in March were used in the CO_2 evolution test, and soils sampled in May were used in the O_2 demand test.

Table 3.2. Soil characteristic data for all soils used in the study. 18 Acres, Gartenacker, and Standardised soils sampled/created in March 2023 were used in the CO_2 evolution methodology. Soils sampled in May 2023 were used in the O_2 demand methodology.

Soil Characteristic	18 Acres March 2023	Gartenacker March 2023	Standardised March 2023	18 Acres May 2023	Gartenacker May 2023	Standardised May 2023
USDA textural class	sandy clay loam	silt loam	loamy sand	sandy clay loam	silt loam	loamy sand
Sand / Silt / Clay (%)	58 / 21 / 21	36 / 51 / 13	80 / 11 / 9	57 / 22 / 21	35 / 54 / 11	84 / 6 / 10
Bulk Density (gm/cc)	1.13	0.94	1.28	1.12	0.96	1.34
Cation Exchange Capacity (meg/100g)	14.2	8.3	5.4	15.6	8.8	4.7
Water holding capacity (%)	48.8	60	27.2	45	62.1	27.3
Moisture at 10 kPa (%)	30.8	44.2	14	24.2	47.5	12.7
Organic Carbon (%)	1.7	2	0.88	2.8	2.4	0.94
Organic Matter (%)	3	3.5	1.5	4.7	4.1	1.6
pH	5.8	7.1	6.4	5.3	7.4	6.4
Total Nitrogen (%)	0.17	0.25	0.07	0.24	0.26	0.03
C:N ratio (C/N)	10	8	12.57	11.67	9.23	31.33

3.2.3. Soil Biodegradation Test

The biodegradation of cellulose was measured by evolved CO_2 or biological O_2 demand following the standard test ISO 17556:2019 (ISO, 2019). Both tests were performed at 20°C, under continuous dark conditions. Prior to use, all soils were sieved to 2mm, and their moisture content adjusted by addition of deionised water to within 40-60% of the water holding capacity (WHC), based on ISO 17556:2019 requirements (ISO, 2019). The standardised soil had an aqueous nutrient mix added at the time of water content adjustment, as required by ISO 17556:2019 (ISO, 2019) (Table S3.2). Test vessels were set-up, and soil was added (Table 3.3). Then the soils were pre-incubated in the test vessels under test conditions for one week after moisture adjustment. Test vessels were then treated with cellulose, that was applied as a solid powder and homogenously mixed with a spatula to distribute throughout the soil. Blank soil vessels were also homogenously mixed with a spatula to mimic soil disturbance caused by the addition of cellulose. Test measurements then commenced, with the soil moisture content maintained throughout the test by gravimetric measurements.

Table 3.3. Experimental test vessel setup for the measurement of the biodegradation of cellulose in soil by carbon dioxide evolution or biological oxygen demand.

	CO_2 evolution	**O_2 demand**
Soils	18 Acres (18A) Gartenacker (GAR) Standardised soil (STS)	18 Acres (18A) Gartenacker (GAR) Standardised soil (STS)
Soil (g)	100	50
Cellulose (mg)	1250	50
Vessel Setup	3x 18A-soil-blank 3x 18A-soil-cellulose 3x GAR-soil-blank 3x GAR soil-cellulose 3x STS -soil-blank 1x STS-soil-cellulose*	2x 18A-soil-blank** 3x 18A-soil-cellulose 2x GAR-soil-blank** 3x GAR soil-cellulose 2x STS -soil-blank** 3x STS-soil-cellulose
Test Duration (days)	18A - 313 GAR - 313 STS - 134	18A - 245 GAR - 180 STS - 150

(*Only one replicate ran to completion for the standardised soil in the CO_2 evolution test. ** Only two replicates were run for the O_2 demand soil blanks based on previous high agreement of soil blank data (Chapter 2) and to maximise the experimental set up with the limited number of OxiTop pressure sensor available.).

Determination of amount of evolved carbon dioxide

CO_2 evolution was monitored in a soil incubation system following the set up in ISO 17556:2019 Annex B (ISO, 2019). CO_2 free air was continuously passed through a series of connected test flasks at a controlled flow rate. Soil (100g dry weight) was placed into individual test vessels (0.4 L). Soil blanks contained soil only, whilst test vessels contained soil that was treated with 1250 mg of cellulose. Three CO_2 traps containing 50 ml of 0.5 N KOH were subsequently placed after the test vessel to capture evolved CO_2. The CO_2 absorption flasks were periodically removed, and the evolved CO_2 was measured by titrating aliquot KOH samples with 0.05 mol/l HCl. Titration measurements were recorded on days 1, 5, 8, 14, 21, and 30 in the first month when initial biodegradation was expected to be high. Measurements reduced in frequency to twice a month for the next 5 months, and then to once a month until test completion at day 313 (Table S3.3).

Determination of biological oxygen demand

O_2 demand was measured via a manometric respirometer in closed test vessels fitted with a pressure sensor head (OxiTop®-IDS, Xylem Analytics (WTW, 2010)), and soda lime pellets

as a CO_2 absorber. Soil (50g dry weight) was placed into individual test vessels (1 L). Soil blanks contained soil only, whilst test vessels contained soil that was treated with 50 mg of cellulose. Throughout the test, soil microbes consume O_2 and produce CO_2, which was absorbed by the soda lime pellets, therefore resulting in a drop in pressure within the closed system, which was automatically recorded daily throughout the test duration. O_2 demand was then calculated by the recorded change in pressure (Equation S3.1).

Biodegradation Calculation

Biodegradation (%) for both CO_2 evolved and O_2 demand methods was calculated by comparing the net amount of CO_2 or O_2 measured with the $ThCO_2$ and ThOD (Equation 3.1). Calculations for determining $ThCO_2$ and ThOD are given in the Supplementary Material (Equations S3.2, S3.3, S3.4).

$$\text{Biodegradation (\%)} = \left(\frac{\text{CO}_2 \text{ evolved (mg)}}{\text{ThCO}_2} \times 100\right) = \left(\frac{\text{O}_2 \text{ demand (mg)}}{\text{ThOD}} \times 100\right)$$

[Equation 3.1]

3.2.4. Comparison of CO_2 evolution and O_2 demand

Respiration data for CO_2 evolved and O_2 demand are measured in mg CO_2 and mg O_2 respectively. CO_2 and O_2 have different molar masses, resulting in different scales of respiration. To enable comparison, it is useful to convert the respiration values into comparable units (See Supplementary Information Section 3.5.3). Biodegradation (%) allows comparison between the differing test set-ups and is the main information required to distinguish between a test material passing or failing a biodegradation test.

3.2.5. Statistical Analyses

All statistical analyses were conducted using R version 4.3.1 (R Core Team, 2024). Statistical analyses were performed on CO_2 evolved, O_2 demand, and biodegradation data

to determine the significance of results at individual time points. The significance level was set at $p = 0.05$. Data were assessed for normality using a Shapiro Wilk test, and homogeneity of variance using Levene's test. When the assumptions of normality and homogeneity of variance were met, parametric tests were conducted. For the CO_2 evolved data, the standardised soil could not be compared statistically due to the lack of replicates. Therefore, statistical analyses were completed on the two natural soils 18A and GAR. The difference between respiration and biodegradation between both natural soils was compared using a Welch's one sample t-test. For the O_2 demand, statistical analyses were carried out to compare all three soils for days 1-150. A one-way analysis of variance (ANOVA) followed by a Tukey multiple comparisons post-hoc test was used to determine both respiration and biodegradation between all three soils. An additional Welch's one sample t-test was also used to determine the statistical significance of differences in O_2 demand and biodegradation for the soils 18A and GAR up to day 180. All statistical results tables are presented in Appendix C.

3.3. Results and Discussion

Cellulose biodegradation was measured in three soils in two separate test systems with contrasting analytical endpoints of CO_2 evolution and O_2 demand. Cellulose biodegradation was measured by CO_2 evolution for 313 days for the two natural soils 18A and GAR, and for 134 days for the STS (Figure 3.1A). The two natural soils (18A, GAR) were comparable throughout the CO_2 test, with no significant difference in the amount of CO_2 evolved or percent biodegradation (Figure 3.1B, t-test, $p>0.05$, Appendix C). Both natural soils (18A, GAR), reached 60% biodegradation by day 223, and the test was then terminated on day 313 with 74.89±10.7% biodegradation in 18A, and 72.01±11.3% biodegradation in GAR. In contrast to the natural soils, only one test vessel for soil-cellulose in STS ran successfully, resulting in the inability to compare the STS data statistically. CO_2 evolved in STS initially rapidly increased, then slowed. Cellulose biodegradation was greater than 40%

by day 76, which was approximately twice the level of biodegradation recorded in either natural soil (18A = 23%, GAR = 17%). However, at this point cellulose biodegradation in STS reached a plateau, and no further biodegradation was observed for a further 58 days, resulting in the decision to terminate the test vessel at 46.4% biodegradation by day 134. Therefore, in the CO_2 evolution study, cellulose would not be considered biodegradable in the standardised soil as it did not reach 60% biodegradation which is the validity threshold.

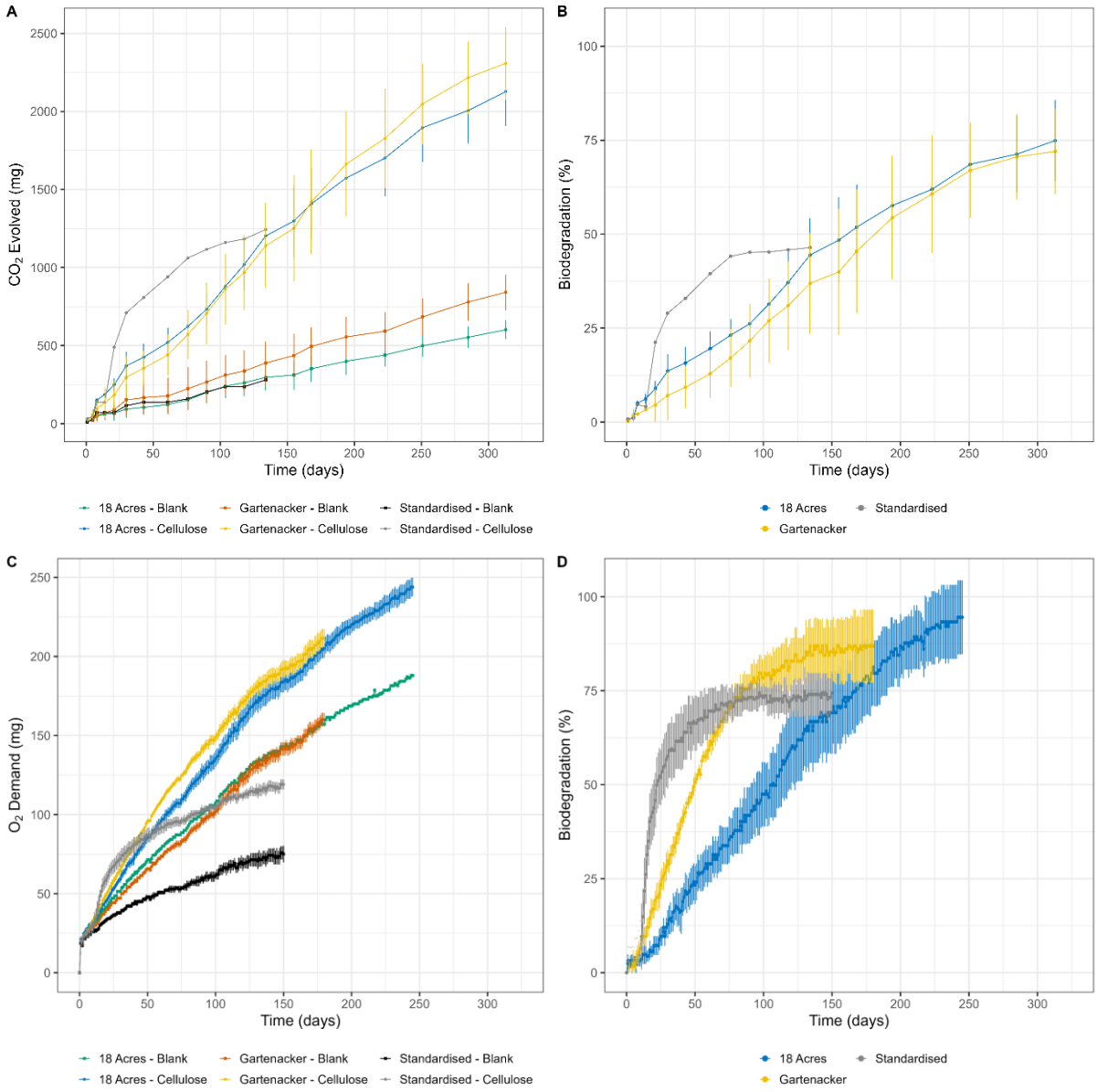


Figure 3.1. (A) Cumulative total CO_2 evolved in soil blanks and soil spiked with cellulose in three soils. (B) Biodegradation of cellulose in three soils measured by CO_2 evolution. For A and B, the three soil types are: 18 Acres (313-days, n=3), Gartenacker (313-days, n=3), and Standardised (134-days, n=1). (C) Cumulative total O_2 demand in soil blanks and soil spiked with cellulose in three soils. (B) Biodegradation of cellulose in three soils measured by O_2 demand. For C and D, the three soil types are: 18 Acres (245-days), Gartenacker (180-days), and Standardised (134-days). Data is represented as mean ± standard deviation. Differences in test durations were due to the early termination of test vessels once a clear biodegradation phase had been reached.

In contrast to the CO_2 evolution study, cellulose biodegradation surpassed 60% in all three soils when measured by O_2 demand, and there was a clear divergence in the biodegradation rate between soils (Figure 3.1C, 3.1D). Biodegradation was measured for up to 245 days, with test termination based on the observation of a clear plateau phase and lack of further

biodegradation. In contrast to the CO_2 evolution study, the biodegradation of cellulose varied between all three soil types. STS had a significantly higher total biodegradation of cellulose than either 18A or GAR from day 13 to day 54 (Figure 3.1D, one-way ANOVA, $p<0.05$ Appendix C), with a significantly faster rate of biodegradation (one-way ANOVA, $p<0.05$, Appendix C). STS reached 67.6±6.1% biodegradation at day 54, then the rate of biodegradation slowed to <0.25%/day, with the plateau phase occurring before test termination at 74.4±5.1% biodegradation on day 150. Therefore, in the O_2 demand test, cellulose was considered biodegradable in STS unlike in the CO_2 evolution test. There were also differences observed between the two natural soils when measured by O_2 demand: GAR had significantly higher total cellulose biodegradation than 18A for days 13-129 (one-way ANOVA, $p<0.05$, Appendix C), and a quicker rate of biodegradation than 18A, reaching a plateau of 86.9±9.7% biodegradation by day 180 (Figure 3.1D). Unlike GAR and STS, 18A had a steady rate of cellulose biodegradation throughout the test at around 0.5%/day for days 15-125, before slowing slightly to around 0.25%/day until test termination (Appendix C). 18A reached 94.5±9.8% biodegradation at test termination on day 245 (Figure 3.1D).

The biodegradation of cellulose varied between both soil type and test method. STS had poor reproducibility of results, both within each test system and between the CO_2 and O_2 test methods. In both CO_2 and O_2 tests, STS biodegradation curves exhibited different characteristics to those of both natural soils, with a faster initial rate of biodegradation and a lower total biodegradation. Cellulose did not reach 60% in the STS measured in the CO_2 test, so would not be classed as biodegradable, but did reach 60% in the O_2 test. The rate and total biodegradation of cellulose was also not reproducible between the natural soils in either test method, and the standard deviation between replicates was high in all soils in both test methods. It is thus important to consider both the influence of soil properties and the differences in the test methods themselves that could be drivers of this variation.

In this study, total cellulose biodegradation in STS reached 46.4% in the CO_2 test, and 67.6% in the O_2 test, which was lower than in either of the natural soils. Briassoulis et al. (2020) also found lower biodegradation of polymers in a standard soil with the same composition as used in this study, than in natural soils (Open-Bio, 2017). Additionally, cellulose disintegration has previously been compared between soil types by Briassoulis and Mistriotis (2018) who observed faster rates of disintegration in finer textured clay and clay loam soils, than in loamy sands. In this study, STS was characterised as a loamy sand, with a composition comprising 70% quartz sand, meaning that STS had a coarser texture than either natural soil, with 18A being a sandy loam, and GAR being a silt loam. Additionally, the biodegradation of plant residue has been monitored by Hamarashid et al. (2010), who observed significantly higher rates of CO_2 mineralisation in finer textured soils than coarser soils. Soil texture is known to affect the decomposition of leaf litter in soil and can influence the composition of the microbial community, therefore this is a plausible explanation for the lower biodegradation of cellulose seen in the STS as presented here (Angst et al., 2021; Seaton et al., 2020).

Fine textured soils are typically characterised by a higher clay content, with the addition of clay minerals (kaolinite, montmorillonite) to soils previously found to increase CO_2 mineralisation from non-biodegradable polymers such as polypropylene and polyethylene (Chen et al., 2022). 18A had the highest clay content of all three soils in this study, with 21% clay content, and had the highest total biodegradation in the O_2 test. Previously Huo et al. (2024) measured CO_2 evolved from a biodegradable mulch film in three different soils at 20°C, also finding that the soil with the highest clay content had the greatest extent of biodegradation. Higher clay mineral content increases the available surface area for microbial growth (Chen et al., 2022) and stabilises the soil organic carbon, reducing its accessibility for degraders, therefore the organic carbon would be less susceptible to mineralisation by microbes (Chen et al., 2022; Kleber et al., 2021; Schweizer et al., 2021;

Torn et al., 1997). This could potentially result in alternative sources of carbon being utilised, such as biodegradable polymers, explaining the higher total biodegradation observed in 18A in the O_2 method. This suggests that soil texture could be an important driver of the measured biodegradation, however, soil texture is not a required characteristic to be measured or reported for soil used within ISO 17556:2019. As soil texture can result in significant variability in biodegradation, it would be useful to require this to be characterised within the standard test method.

There were observed differences in the rate of biodegradation of cellulose between the three soils, as well as in the total biodegradation. In both tests, cellulose had a much quicker rate of biodegradation in STS than in either natural soil, and then in the O_2 test, cellulose had a quicker rate of biodegradation in GAR than in 18A. The rate of polymer biodegradation has previously been linked to the temperature at which the test was performed, with temperature increases linked to an increased rate of biodegradation until a temperature optimum (Pischedda et al., 2019; Sander et al., 2023). However, as the temperature in this study was held constant at 20°C, it is unlikely to be the reason behind the different rates of biodegradation observed. Other environmental factors that can influence biodegradation rate are the availability of nutrients and soil water content.

The availability of nutrients such as carbon, nitrogen, and phosphorus influence soil microbial activity and the ability of microorganisms to biodegrade polymers (Bher et al., 2022; Briassoulis and Mistriotis, 2018; Sander et al., 2023; Tanunchai et al., 2022). Typically, microorganisms in the soil can be described as carbon limited, due to the inaccessibility of soil organic carbon (Soong et al., 2020). In this study, cellulose was added as a source of carbon: as a biodegradable polymer it is assumed this provides a labile source of carbon that is easily accessible to soil microbes, thereby reducing carbon limitation. Therefore, at the start of the biodegradation tests, carbon is not limited but other nutrients, such as

nitrogen, could be. The soils' nitrogen content is thus important to consider because most polymers have a high carbon content and low nitrogen content, meaning that when they are added to soil, they widen the C:N ratio (Rillig et al., 2021).

A larger C:N ratio can reduce the soil microbial activity, because the soil microbes would be N limited. In both the CO_2 and O_2 tests, 18A and GAR had greater organic carbon and nitrogen available than STS (Table 3.2). Nitrogen content was the highest in GAR with 0.25-0.26%, then 18A with 0.17-0.24%. In contrast, STS had much lower nitrogen content with 0.03-0.07% recorded. Due to the initial low nitrogen content of STS, a nutrient mix was then added during the adjustment of soil water content at the start of the biodegradation incubations. The nutrient mix contained sodium nitrate, urea, and ammonium chloride (Table S3.2), which means that the availability of nitrogen will have increased at the start of the test. The addition of nitrogen has previously been shown to increase the initial rate of decomposition of leaf litter, but not to influence the total decomposition (Knorr et al., 2005; Soong et al., 2020). Therefore, the initial high availability of nitrogen in STS due to the addition of the nutrient mix can explain the initial faster rate of cellulose biodegradation seen in both tests.

In all three soils, the rate of biodegradation slowed down over time, suggesting that microbial activity is being limited. As cellulose is utilized by soil microbes, the available carbon would reduce which could mean that the microbes become carbon limited (Frey et al., 2003; Soong et al., 2020). Additionally, as the available nitrogen is used, it would become limiting. This is an important consideration for STS, as it had a low initial nitrogen content, suggesting it would have been reliant on the addition of nitrogen from the nutrient mix. Therefore, the lower total biodegradation seen in STS in comparison to 18A and GAR could be caused by a nitrogen limitation. Nitrogen fixing bacteria are known to be important for the microbial biodegradation of polymers and the addition of nitrogen has been shown

to increase the biodegradation of polymers (Guliyev et al., 2023; Tanunchai et al., 2022). Additionally, the addition of 0.1g nitrogen has been shown to be beneficial in improving the reproducibility of polymer biodegradation tests in soil (Briassoulis and Mistriotis, 2018). However, the addition of nutrients in the form of nitrogen is expressed as a ratio of carbon to nitrogen within polymer biodegradation tests, which has previously been noted as being unclear regarding if this is the polymer carbon content, or the soil carbon content, or a combination of these (Briassoulis and Mistriotis, 2018; Briassoulis et al., 2014). Moreover, the suggested appropriate C:N ratio is at least 40:1 in ISO 17556:2019, which allows a significant range of nitrogen content which likely contributes to the observed variability in biodegradation.

Generally, the biodegradation rate was slower in the CO_2 test than in the O_2 test, with cellulose biodegradation in GAR for example reaching 60% in 223 days in the CO_2 test, but passing this in just 61 days in the O_2 test. Soil water content has previously been shown to have a significant impact on the rate on microbial respiration, with increased mineralisation rates of cellulose in soil as the soil water content was increased from 55% to 80% WHC (Chinaglia et al., 2024). Whilst all three soils in this study have varying WHC, the soil water content was set to be comparable between them at 50-55% WHC. However, the CO_2 and O_2 test methods involved different test set-ups that could have influenced the soil water content throughout the test. In the O_2 method, the test material is in a closed vessel, that minimised moisture loss throughout the study, with minimal change in the moisture throughout or at the end of the test (Table S3.4). However, in the CO_2 method, the supply of O_2 came from a constant flow of air passing through the test vessels, which can cause to soil drying. To minimise this effect, air is first passed through a deionised water trap to moisten it. However, moisture losses were consistently observed throughout the CO_2 evolution test, resulting in the soil requiring remoistening throughout the test and recorded moisture loss at the end of the test (Table S3.5). The ISO guideline is ambiguous in

describing when to remoisten the soil, stating: "*If the biodegradation rate is considered to have slowed down because the test soil has dried out during the test, stop the measurements and remove the flasks from the respirometer or CO_2-free-air production system. Weigh the flasks and add a suitable amount of water to the test soil to bring its water content back to its initial value*" (ISO, 2019). There is no clear way to know when soil moisture is the main cause of a slower biodegradation rate, which means it is unclear to know when to remoisten the soil. Soil moisture content is a well-known driver of soil respiration, and soil drying can reduce microbial activity (Cook and Orchard, 2008; Curiel Yuste et al., 2007; Orchard and Cook, 1983; Wilson and Griffin, 1975). Therefore, the greater moisture loss observed in the CO_2 test could be an explanatory factor for the slower observed biodegradation of cellulose than in the O_2 test. Additionally, as the test vessels must be opened to remoisten the soil, there is a chance for the loss of evolved CO_2, which adds an additional source of variation between the test methods. The use of a closed vessel set-up (e.g. such as the one suggested in ASTM D5988-18, (ASTM, 2018)) could minimise this effect for evolved CO_2 measurements. Additionally, as soil water content is critical to ensure soil microbial activity, it would be beneficial to explicitly define how to maintain the soil water content.

Both CO_2 and O_2 measurements are indirect, which can result in uncertainty regarding the measurements. In the O_2 test in this study, cellulose biodegradation in 18A was slow and steady, with the average biodegradation at the end of the test reaching 94.5±9.8%. One replicate of 18A reached >100% biodegradation, with a value of 105% biodegradation at test termination (Figure 3.1D). Biodegradation values greater than 100% are generally associated with a soil priming effect. Soil priming can occur when a source of labile carbon is added to soil that results in the stimulation of the soil microbial community which alters the rate of decomposition of organic matter and can increase the evolved CO_2 (Bastida et al., 2019; Chen et al., 2014; Guliyev et al., 2023; Huo et al., 2024; Kuzyakov et al., 2000;

Sander, 2019; Shen and Bartha, 1996; Yang et al., 2023; Zhang et al., 2023). This priming effect can result in either an increase (positive priming) or decrease (negative priming) of soil organic carbon mineralisation, and thus can cause uncertainty in the measurement of polymer mineralisation (Bastida et al., 2019; Chen et al., 2014; Kuzyakov et al., 2000; Shen and Bartha, 1996; Yang et al., 2023; Zhang et al., 2023). Recent research has observed soil priming in soils after the addition of biodegradable polymers by utilising isotopic labels such as ^{13}C to identify soil-derived versus polymer-derived carbon (Guliyev et al., 2023; Huo et al., 2023, 2024; Zhang et al., 2023). Guliyev et al. (2023) observed a strong priming effect in nitrogen poor soils after the addition of ^{13}C poly(butylene succinate-co-adipate). However, the main causes of priming are still unclear, highlighting the need to be able to accurately quantify the strength and direction of a soil priming effect to better understand how it might impact the use and interpretation of respirometric methods.

CO_2 is the main endpoint that has been measured regarding the soil priming effect. In this study the observed value of >100% was measured in the O_2 demand test. O_2 demand was measured indirectly by changes in pressure, therefore several assumptions are made regarding the conversion into a value for O_2 demand. O_2 is utilised for several reasons by soil microbes during respiration, that may not all be accounted for by the soil blank. Nitrification for example needs to be considered as it can influence the recorded biological O_2 demand as "*1g of organic nitrogen released as ammonium is equivalent to 4.57g* O_2" (Reuschenbach et al., 2003). The measurement of biological O_2 demand has not been as widely used for polymer biodegradation when compared to CO_2 evolved. It is likely that more mechanistic studies are required to verify if O_2 demand and CO_2 evolution can be considered interchangeable measurement methods for measuring the biodegradation of polymers in soil.

3.4. Conclusion

Cellulose is often used as a positive reference material to prove the validity of biodegradation tests, with the biodegradation of test materials determined with respect to the measured biodegradation of cellulose. Cellulose biodegradation varied across soil type and test method, with the biodegradation measured by O_2 demand resulting in faster rates and higher total biodegradation than the CO_2 evolution method. However, the O_2 demand is less frequently used for polymer biodegradation in comparison to CO_2 evolution, and whilst there was evidence of a potential priming effect, it is difficult to discern the reasons for this with the respirometric endpoint alone. This study highlighted how variability in soil properties and test setup can influence the reproducibility of the biodegradation of cellulose in soil. Biodegradation rate and extent are not independent of soil type, but further work is needed to identify the main causes of variability, which should be considered for future refinement of standard test methods to measure polymer biodegradation. To develop a comprehensive understanding of polymer biodegradation in soil and improve confidence in the respirometric endpoints of O_2 and CO_2 as proxies for polymer biodegradation, mechanistic studies with labelled polymers are required that would enable the tracking of polymer carbon throughout a biodegradation test. Furthermore, it would be critical to be able to develop methods that can accurately identify non mineralised polymer carbon in the microbial biomass and soil at the end of biodegradation tests. An improved mechanistic understanding of polymer biodegradation in soil would reduce the assumptions and remove the sources of irreproducibility from the current test guidelines, resulting in the development of more robust and reliable standard test methods.

3.5. Supplementary Materials

3.5.1. Supplementary Tables

Table S3.1. The composition of the standard soil as outlined in ISO 17556:2019.

Constituent	Dry mass (g/kg)	Supplier
Quartz sand	700	Melcourt Industries
Kaolinite clay	100	Sigma Aldrich
Natural soil	160	18 Acres, Syngenta
Mature compost	40	Melcourt Industries

Table S3.2. The composition of the salts added to the standardised soil at the time of water content adjustment as outlined in ISO 17556:2019.

Constituent	Molecular Formula	g/kg of soil
Potassium dihydrogenphosphate	KH_2PO_4	0.2
Magnesium sulphate	$MgSO_4$	0.1
Sodium nitrate	$NaNO_3$	0.4
Urea	$CO(NH_2)_2$	0.2
Ammonium chloride	NH_4Cl	0.4

Table S3.3. Sampling dates for measurements of evolved carbon dioxide across three soils.

Date	Sampling Day
13.04.23	1
17.04.23	5
20.04.23	8
26.04.23	14
03.05.23	21
12.05.23	30
25.05.23	43
12.06.23	61
27.06.23	76
11.07.23	90
25.07.23	104
08.08.23	118
24.08.23	134
14.09.23	155
27.09.23	168
23.10.23	194
21.11.23	223
19.12.23	251
22.01.24	285
19.02.24	313

Table S3.4. Sample weights recorded at the start and end of the biodegradation test (oxygen demand measurements), reflective of the change in soil moisture loss.

Soil	Sample	Replicate	Start Weight (g)	End Weight (g)	Difference (g)	Percentage Change (%)
18 Acres	Blank	1	62.94	62.14	0.80	-1.27
18 Acres	Blank	2	63.90	63.05	0.85	-1.33
18 Acres	Cellulose	1	63.00	62.11	0.89	-1.41
18 Acres	Cellulose	2	62.97	62.12	0.85	-1.35
18 Acres	Cellulose	3	63.13	62.28	0.85	-1.34
Gartenacker	Blank	1	67.73	66.84	0.89	-1.31
Gartenacker	Blank	2	67.91	67.01	0.90	-1.33
Gartenacker	Cellulose	1	68.99	68.23	0.76	-1.11
Gartenacker	Cellulose	2	67.92	67.06	0.86	-1.26
Gartenacker	Cellulose	3	68.71	67.7	1.01	-1.48
Standardised	Blank	1	56.10	55.88	0.22	-0.39
Standardised	Blank	2	57.07	57.59	-0.52	0.91
Standardised	Cellulose	1	57.28	57.04	0.24	-0.42
Standardised	Cellulose	2	56.72	56.44	0.28	-0.49
Standardised	Cellulose	3	57.30	56.97	0.33	-0.58

Table S3.5. Sample weights recorded at the start and end of the biodegradation test (carbon dioxide evolved measurements), reflective of the change in soil moisture loss.

Soil	Sample	Replicate	Start Weight (g)	End Weight (g)	Difference (g)	Percentage Change (%)
18 Acres	Blank	1	124.45	122.17	2.28	-1.83
18 Acres	Blank	2	124.65	125.88	-1.23	0.99
18 Acres	Blank	3	124.07	120.81	3.26	-2.63
18 Acres	Cellulose	1	125.36	119.64	5.72	-4.57
18 Acres	Cellulose	2	125.51	120.71	4.80	-3.83
18 Acres	Cellulose	3	125.66	120.95	4.71	-3.75
Gartenacker	Blank	1	134.54	130.49	4.05	-3.01
Gartenacker	Blank	2	134.41	130.29	4.12	-3.07
Gartenacker	Blank	3	133.11	128.02	5.09	-3.82
Gartenacker	Cellulose	1	136.24	126.87	9.37	-6.88
Gartenacker	Cellulose	2	137.28	131.35	5.93	-4.32
Gartenacker	Cellulose	3	136.09	141.64	-5.55	4.08
Standardised	Blank	1	111.80	110.74	1.06	-0.95
Standardised	Blank	2	114.19	113.03	1.16	-1.02
Standardised	Blank	3	113.42	110.22	3.20	-2.82
Standardised	Cellulose	1	112.93	110.66	2.27	-2.01
Standardised	Cellulose	2	111.93	108.42	3.51	-3.14
Standardised	Cellulose	3	113.72	112.45	1.27	-1.12

3.5.2. Supplementary Calculations

Calculation of biological oxygen demand

Oxygen (O_2) demand is calculated as the soil respiration, at a specific measuring period and incubation temperature by Equation S3.1. Where BA is soil respiration (mg O_2/kg of test soil), M_R (O_2) is the molar mass of oxygen (32000 mg/mol), V_{fr} is the free gas volume (L), R is the general gas constant (83,14L mbar mol^{-1} K^{-1}), T is the measuring temperature (K, kelvin), m_{Bt} is the mass of dry soil substance in the measuring preparation (kg) and Δp is the reduction in pressure of the measuring preparation (mbar).

$$BA = \frac{M_R(O_2)}{R \times T} \times \frac{V_{fr}}{m_{Bt}} \times \Delta p$$

[Equation S3.1]

Calculation of the theoretical amount of evolved carbon dioxide

The theoretical amount of carbon dioxide evolved from biodegradation of a substance can be calculated by Equation S3.2, where m is the mass of test material introduced into the test system (milligrams, mg), w_c is the carbon content of the test material, expressed as a mass fraction, 44 is the relative molecular mass of carbon dioxide, and 12 is the relative molecular mass of carbon (ISO, 2019).

$$ThCO_2\,(mg) = \frac{44}{12} \times m \times w_c$$

[Equation S3.2]

Calculation of the theoretical oxygen demand

The theoretical oxygen demand (ThOD) of a substance can be calculated if the elemental composition is known (ISO, 2019).
For a substance of $\boldsymbol{C_cH_hCl_{cl}N_nS_sP_pNa_{na}O_o}$ with relative molecular mass $\boldsymbol{M_r}$
ThOD can be calculated without nitrification by Equation S3.3, or with nitrification by Equation S3.4.

$$ThOD_{NH_3}\,(mg/mg) = \frac{16[2c + 0.5(h - cl - 3n) + 3s + 2.5p + 0.5na - o]}{M_r}$$

[Equation S3.3]

$$ThOD_{\,NO_3}(mg/mg) = \frac{16[2c + 0.5(h - cl) + 2.5n + 3s + 2.5p + 0.5na - o]}{M_r}$$

[Equation S3.4]

3.5.3. Comparison of CO_2 and O_2 data

To confirm that the presentation of the respiration data as percent biodegradation accounts for the inherent differences in the set-ups (mass of soil and ratio of soil to polymer) between the two methodological approaches (CO_2 production versus O_2 loss), it is useful to convert the respiration data into comparable units.

Currently respiration is measured in mg of O_2 or CO_2. O_2 and CO_2 have different molar masses (g/mol). O_2 has a molar mass of 31.99 g/mol, CO_2 has a molar mass of 44.01 g/mol. The O_2 test has 50g (dry weight) of soil with 50 mg of polymer, whilst the CO_2 test has 100g (dry weight) of soil with 1250 mg of polymer.

Example Calculations

Oxygen

Measured O_2 (mg/50g soil) (minus blank resp) = 1.43

Divide by 50 to get it to 1g of soil

Measured O_2 (mg/g soil) = 1.43/50 = 0.029

Divide by the molar mass of O_2 (31.999) to convert into moles,

Then multiply by 1000 to get to µmoles

Measured O_2 (µmoles/g soil) = 0.029 / 31.999 * 1000 = 0.893

Divide by the ratio of polymer to soil to correct for the polymer amount added

Measured O_2 (µmoles/g soil/mg polymer) = 0.893 / 1

Carbon Dioxide

Measured CO_2 (mg/100g soil) (minus blank resp) = 25.67

Divide by 100 to get it to 1g of soil

Measured CO_2 (mg/mg soil) = 25.67 / 100 = 0.26

Divide by the molar mass of CO_2 (44.01) to convert into moles,

Then multiply by 1000 to get to µmoles

Measured CO_2 (µmoles/g soil) = 0.26 / 44.01 * 1000 = 5.83

Divide by the ratio of polymer to soil to correct for the polymer amount added

Measured CO_2 (µmoles/g soil/mg polymer) = 5.83 / 12.5 = 0.467

Visualisation of the conversions

Initially the respiration data for the CO_2 and O_2 tests are not on a comparable scale (Figure S3.1 and Figure S3.2).

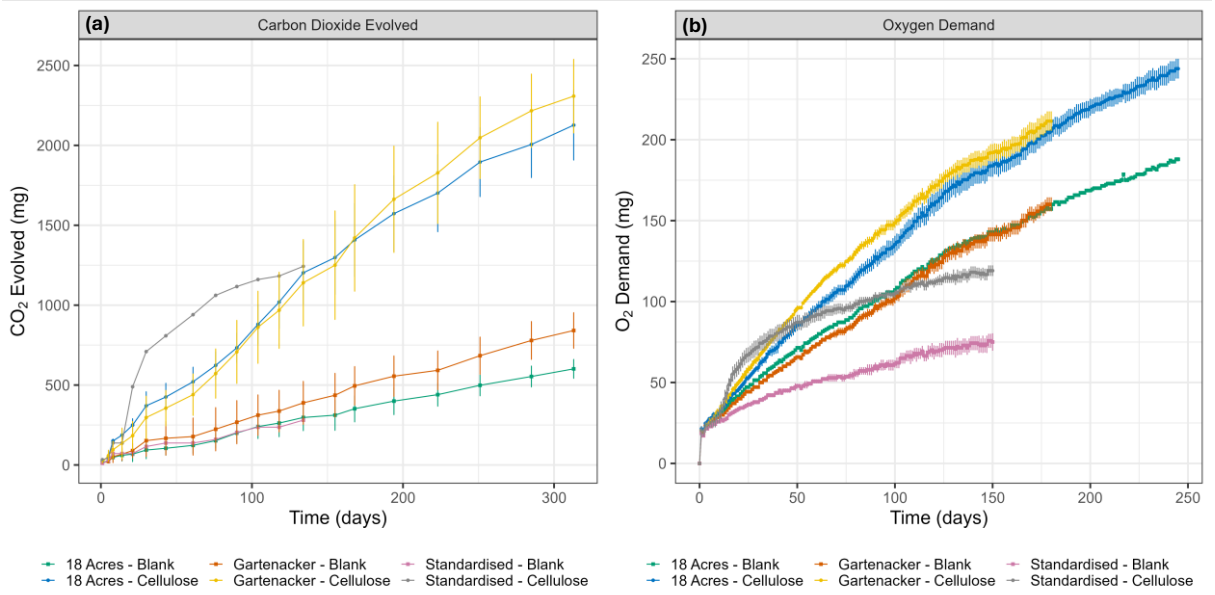


Figure S3.1. Initial respiration data for (a) carbon dioxide evolved, and (b) oxygen demand.

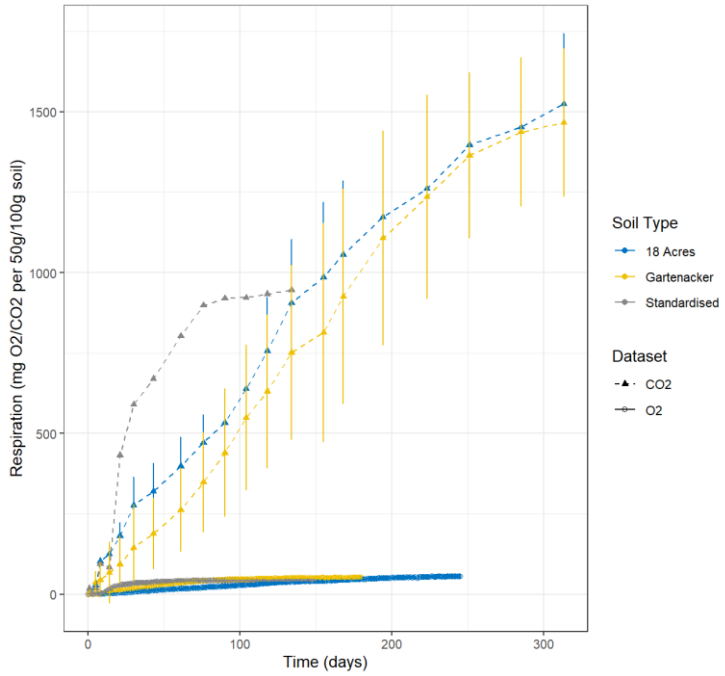


Figure S3.2. Initial respiration data for soil cellulose (blank corrected) measured by both CO_2 and O_2 tests.

To improve the comparability, the data were scaled to 1g of soil (Figure S3.3), then to µmoles per g of soil (Figure S3.4), and then by the amount of polymer added (Figure S3.5).

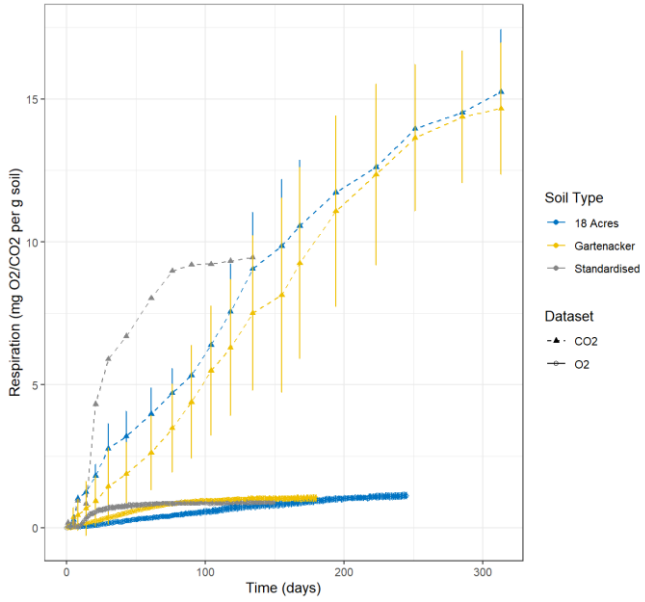


Figure S3.3. Respiration for soil cellulose (blank corrected) in mg of O_2 and CO_2 per g of soil.

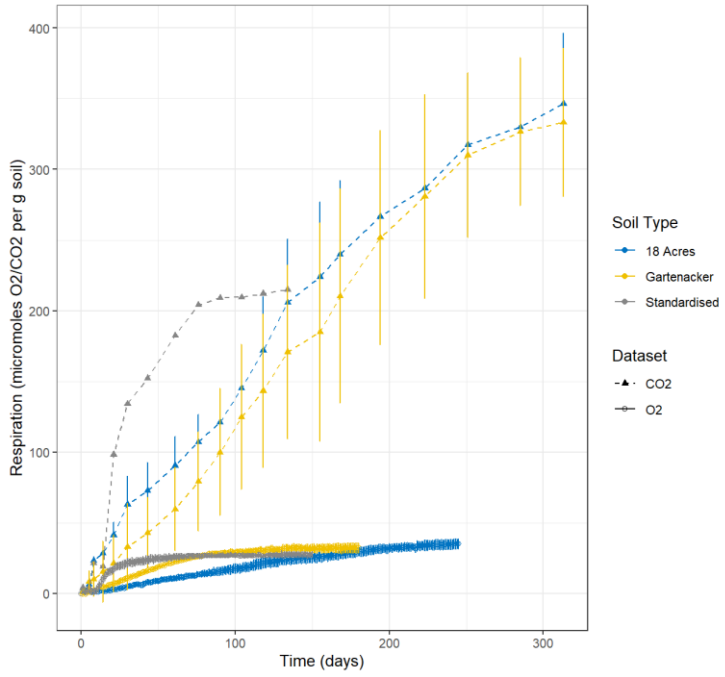


Figure S 3.4. Respiration for soil cellulose (blank corrected) in µmoles of O_2 and CO_2 per g of soil

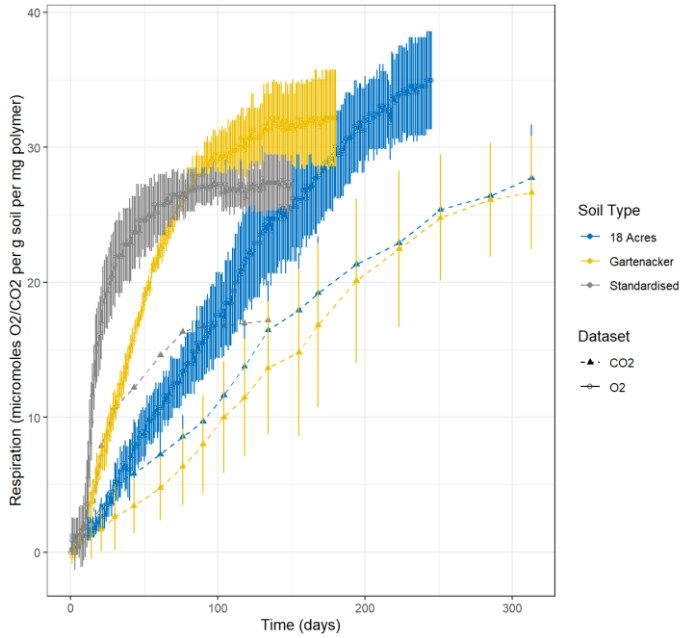


Figure S3.5. Respiration for soil cellulose (blank corrected) in µmoles of O_2 and CO_2 per g of soil per mg of polymer.

This has scaled the data to make them comparable. An alternative way to scale the data, that is commonly used and required to determine the passing level of the test, is to convert the respiration into the biodegradation (%) where the actual amount of CO_2 or O_2 is compared to the theoretical amounts if 100% of all the polymer added were to be degraded (Figure S3.6).

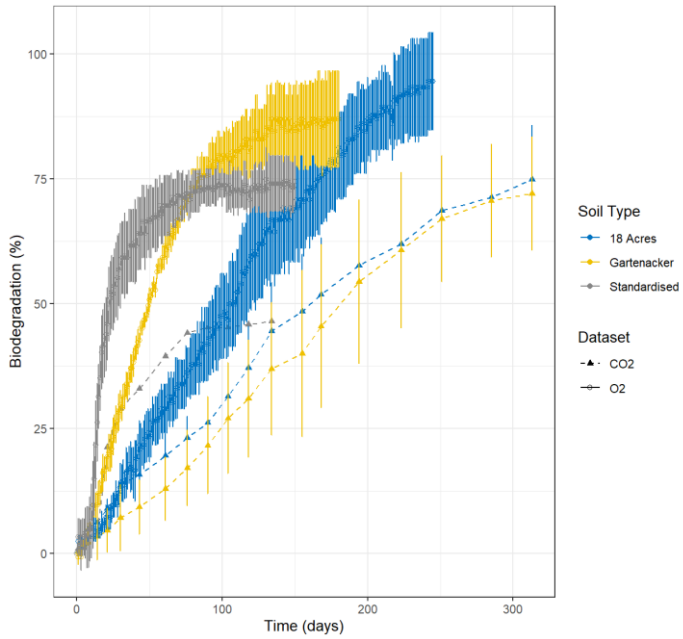


Figure S3.6. Biodegradation (%) of cellulose in 18 Acres, Gartenacker, and Standardised soils measured by both CO_2 and O_2 tests.

3.6. References

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Chapter 4. Determination of the impacts of common microplastic extraction methods from soil matrices on the biodegradable polymers polylactic acid and polyhydroxybutyrate.

This chapter has been prepared as a manuscript for journal submission.

Authors and Affiliations

Grace Davies[1], Sarmite Kernchen[2], Martin Löder[2], Louisa Brenninkmeijer[3], Christian Laforsch[2], Stefan Krause[1,4], and Iseult Lynch[1,5].

[1]School of Geography, Earth and Environmental Sciences, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

[2]Animal Ecology I, Universität Bayreuth, Germany

[3]School of Chemistry, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

[4]Birmingham Institute for Sustainability and Climate Action, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

[5]Centre for Environmental Research and Justice, The University of Birmingham, Edgbaston, Birmingham, United Kingdom

CRediT author statement

Grace Davies: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing Original Draft, Writing Review and Editing, Funding Acquisition. **Sarmite Kernchen**: Methodology, Supervision, Writing Review and Editing. **Martin Löder**: Methodology, Supervision, Writing Review and Editing. **Louisa Brenninkmeijer**: Investigation (DSC), Writing Review and Editing. **Christian Laforsch**: Supervision. **Stefan Krause**: Conceptualisation, Writing Review and Editing, Supervision **Iseult Lynch**: Conceptualisation, Writing Review and Editing, Supervision.

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Abstract

The increasing use of biodegradable polymers in agricultural applications, driven by restrictions on conventional plastic use and concerns regarding their degradation into microplastics, results in the need for reliable methods to identify the environmental fate of biodegradable polymers in soils. If environmental biodegradation is incomplete, there is the potential for the formation of microplastics from biodegradable polymers. To identify the presence of biodegradable microplastics in soils, reliable extraction methods are needed that do not induce further degradation during sample handling which could cause inaccurate estimations. In this study, polylactic acid (PLA) and polyhydroxybutyrate (PHB) films were exposed to an exemplar microplastic soil extraction process of density separation with zinc chloride and enzymatic-oxidative digestion. Polymer degradation was assessed by comparison of polymer properties before and after exposure to each reagent used in the soil extraction method, and to the complete workflow. Treatment-induced degradation of both PLA and PHB films was observed, with enzymatic treatments causing clear changes to the polymers. PLA films became opaque, with reduced molecular weight and increased polydispersity. Both PLA and PHB showed increases in crystallinity after exposure to specific enzymatic treatments. Zinc chloride and Fenton's reagent had minimal impact on the polymers but there is a need to consider how environmental degradation could increase polymer susceptibility to sample processing. This study highlights the need for appropriate polymer specific sample extraction methods to minimise extraction-induced degradation and ensure accurate measurements of biodegradable polymers. Given the complexity of environmental samples, it is important to understand the impacts of the treatments on a wide range of polymer types and blends and thus to optimise the process applied to minimise impacts. This is critical to allow future assessment of the environmental fate of biodegradable polymers, and their potential contribution to microplastic pollution.

4.1. Introduction

Global plastic production reached 400.3 million tonnes (Mt) in 2022 (Plastics Europe, 2023), and concerns around mismanaged plastic waste are increasing. Micro (<5 mm) and nano (<1 µm) sized plastic particles pose a risk to the environment and human health, driving demand for new legislation to limit plastic pollution (Krause et al., 2024; Li et al., 2023; Mitrano and Wohlleben, 2020; de Souza Machado et al., 2018, 2019). In the European Union the European Chemicals Agency (ECHA) recently amended the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation to restrict the use of intentionally produced synthetic polymer microparticles (microplastics) across all sectors (European Union, 2023). The use of biodegradable polymers is exempt, based on the consideration that complete environmental biodegradation should not contribute to microplastic pollution.

Biodegradable polymers have considerable potential for agricultural applications. Agricultural plastic usage is globally estimated to be 12.5Mt annually, with plastic used in a variety of applications including mulching films, polytunnels, irrigation, seed coatings, and the polymer encapsulation of fertilisers and pesticides (FAO, 2021). Plastic usage in these applications is beneficial, contributing to increased crop yield and resilience, efficient water and nutrient usage, and reduction of runoff and erosion risk (FAO, 2021; Gao et al., 2019; Gil-Ortiz et al., 2020; Mansoor et al., 2022; Somanathan et al., 2022; Zanino et al., 2024). However, many agricultural plastic applications are single-use and poorly recoverable from soil, leading to persistent environmental contamination (Hofmann et al., 2023). Moving towards the use of biodegradable plastics that completely breakdown in soils to microbial biomass, water, and carbon dioxide under aerobic conditions is therefore a promising solution, as complete biodegradation will reduce the formation of persistent micro and nano plastics (Yu and Flury, 2024).

Polymer biodegradation is a complex process that is dependent on both the intrinsic chemical properties of the plastic and its end-of-life environment (Sander et al., 2023). Initial abiotic degradation is often required, with fragmentation often being a first step that can result in the formation of interim microplastic fragments prior to the polymer becoming accessible for bio-assimilation by microorganisms (Pfohl et al., 2024; Wohlleben et al., 2023, 2024, Karthika et al., 2019; Samir et al., 2022; Singh and Sharma, 2008). Therefore, it is expected that biodegradable polymers will be present in soils as microplastics for a certain duration before their complete biodegradation (Liao and Chen, 2021; Qin et al., 2021; Sintim et al., 2019; Wei et al., 2022; Weinstein et al., 2020).

A deeper understanding of the environmental fate of biodegradable polymers is needed to verify complete biodegradation and identify the potential risks associated with the production of interim microplastic fragments. This requires the analysis of plastics, including microplastic particles in soils. Soils are complex mixtures of organic and inorganic particles that will interfere with subsequent analytical methods, such as spectroscopy (Möller et al., 2022). Therefore, a suitable pre-treatment is required to isolate the polymeric sample from the environmental matrix (Möller et al., 2020). Isolation of the plastic from the matrix can be done via solvent extraction of the plastic, which is an effective way to analyse the total mass of specific polymeric substances in soils (Thomas et al., 2020). However, this method can be subject to matrix interference, and it destroys the solid state of the plastic meaning that solvent extractions provide no information on polymer particle number, size, or shape (Schrank et al., 2022). Particle size and shape are ecologically relevant characteristics that are important to consider for risk assessments (Koelmans et al., 2023; de Ruijter et al., 2020). Therefore, it is critical to obtain this information for future environmental risk assessments of biodegradable polymers. The removal of the environmental matrix allows information to be obtained pertaining to particle number, size, and shape (Möller et al., 2020; Thomas et al., 2020). There is no single

standardised methodology for extracting microplastics from environmental samples, with existing methods being non-targeted, and usually following a sequence of sieving, density separation of inorganic materials, and then removal of organic material with a digestion process (Junhao et al., 2021; Lusher et al., 2020a; Möller et al., 2020; Radford et al., 2021; Thomas et al., 2020). Soil samples typically have high concentrations of organic material, that is only incompletely removed by oxidative digestion. Incomplete matrix removal results in interference with subsequent analytical techniques, especially vibrational spectroscopic methods (e.g., Fourier Transform Infrared (FTIR), Raman). Enzymatic digestion in combination with oxidisation has been proposed as an effective method with high removal efficiency that is compatible with subsequent spectroscopic analyses to allow the identification of particle number, size, and shape (Möller et al., 2022). Specific enzymes are suggested to ensure effective removal of the soil organic matrix; with protease used to hydrolyse insoluble protein structure into soluble peptides, followed by a sequence of pectinases to break down pectin commonly found in plant cell walls, then hemicellulases and cellulases to target the removal of lignin and cellulosic structures (Möller et al., 2022).

Whilst the impact of microplastic sample processing and extraction methods has been considered for conventional polymers (Cole et al., 2014; Hurley et al., 2018; Munno et al., 2018; Schrank et al., 2022), much less is known regarding their impacts on biodegradable polymers. It is likely that biodegradable polymers will be more susceptible to induced degradation caused by extraction methods, because of their chemical and structural properties that are designed to facilitate their biodegradation (Pfohl et al., 2021). Previously, the use of Fenton's reagent was observed to reduce the particle size distribution of polylactic acid (PLA) based polymer blends (Pfohl et al., 2021). Additionally, a comprehensive enzymatic-oxidative digestion that effectively removes the soil matrix was noted to cause degradation of PLA particles, with a protease enzyme suspected to be the main driver of this degradation (Möller et al., 2022). Therefore, suitable methodologies are

required that can isolate biodegradable microplastics from environmental matrices whilst retaining polymer integrity. As the use of biodegradable polymers increases, it is important to consider the reliability and impact of methodologies used to extract and separate microplastics for analysis in soil and determine their suitability for biodegradable polymers.

Commonly used biodegradable polymers include PLA, polyhydroxyalkanoates (PHAs), polybutylene succinate (PBS), polybutylene adipate terephthalate (PBAT), polycaprolactone (PCL), and starch blends (FAO, 2021). Plastic mulch films are frequently used in agriculture, and to ensure that the films have desired traits (e.g., strength, flexibility), most films are used as blends of polymer types and contain additives such as plasticisers and stabilisers (Mansoor et al., 2022). PLA and poly-3-hydroxybutyrate (PHB) are widely used as blends within agricultural films, with PHB also used in polymer encapsulation technologies (Mansoor et al., 2022; Zytner et al., 2023).

In this work, PLA and PHB polymer films were used as representative biodegradable polymers for testing the impacts of a soil microplastic extraction method on the physico-chemical characteristics of the polymers, and therefore on the reliability of the microplastic extraction method for environmental samples. The enzymatic oxidative digestion protocol by Möller et al., (2021) was tested due to the high removal efficiency of the organic matrix, that enables subsequent spectroscopic analyses. Polymer films were assessed for evidence of degradation by microscopy, mass and thickness measurements, and changes in polymer chemistry were measured by gel permeation chromatography (GPC), differential scanning calorimetry (DSC), and attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. We hypothesised that enzymatic-oxidative digestion steps would induce degradation, and that the degree of degradation would differ depending on the polymer type and treatment step. The aim of this work is to understand the level of

degradation induced by soil extraction methods on exemplar biodegradable polymers to inform appropriate future method development and application.

4.2. Methods

Key method information is detailed below, additional information regarding the experimental design can be found in Schrank, Möller et al., 2022 and Möller et al., 2021.

4.2.1. Generation of Polymer Films

PLA and PHB were obtained from Sigma Aldrich (GF45989881, 29435-48-1). Following the procedure described in Schrank, Möller et al. (2022), polymer films were made to increase the surface area and enable visual integrity of the films to be examined. Powdered polymer material was pressed into a film using a hydraulic press (P/O/Weber TRG1, Germany). Films were cut into 10 mm x 10 mm squares (Figure 4.1) with a thickness of ~100 µm, to ensure a high surface area to volume ratio, then stored in pre-cleaned glass beakers covered with aluminium foil to avoid contamination.

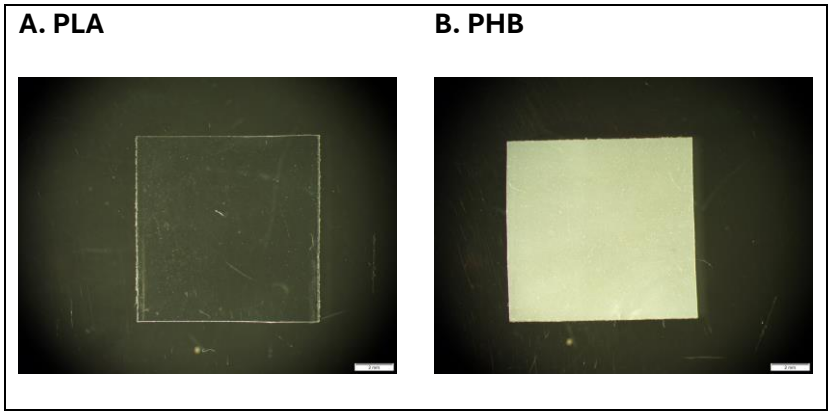


Figure 4.1. Images of the produced polymer film samples (10mm x 10mm) that were used in the study. PLA (a) started as a transparent film and PHB (b) started as an opaque film.

4.2.2. Setup of Experimental Treatments

Polymer films were exposed in triplicate to each of the specific reagents used in soil microplastic extractions, to determine the impact of each step in the protocol on the polymer film. Before and after treatment the polymer films were rinsed with deionised water, then vacuum dried (0.001 kPa) in glass vials covered with aluminium foil at 50°C

(Vacutherm vacuum oven, Thermo Scientific) for 24h. Polymer films were characterised before and after treatment exposures (Figure 4.2) by several analytical methods.

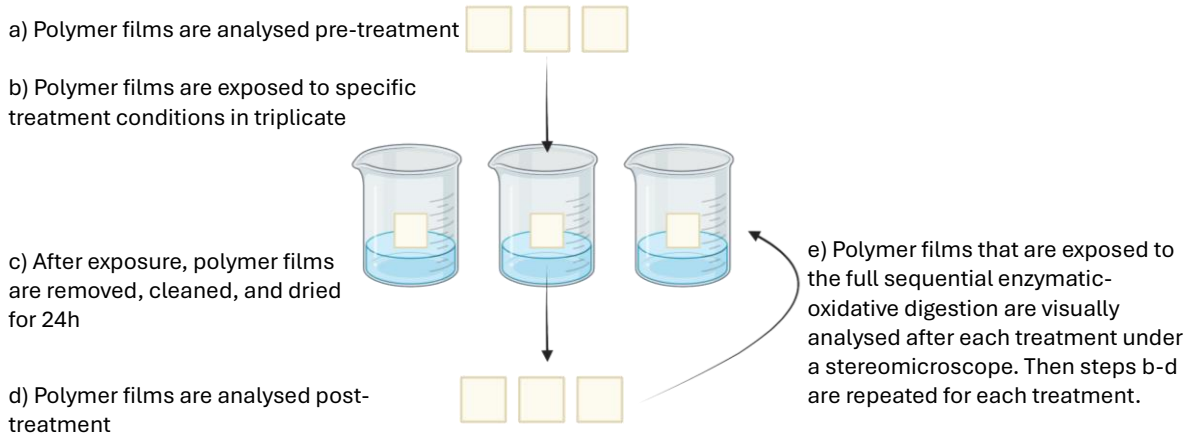


Figure 4.2. Workflow of the experimental procedure, highlighting the steps at which analyses occurred. Created with Biorender.com.

Control samples were incubated with 30 mL of ultrapure water at 20°C for the same duration as the longest treatment studied, i.e., the full treatment sequence. To simulate exposure to a density separation, polymer films were incubated with 30 mL of 1.8g cm^3 $ZnCl_2$ (Carl Roth, Germany), a commonly used salt for density separations, at 20°C for 48h. The individual steps in the enzymatic-oxidative digestion (i.e., sodium dodecyl sulphate (SDS), Fenton's reagent, protease, pectinase, viscozyme and cellulase) were examined by individual exposures under the relevant conditions (Table 4.1, Table S4.1, Möller et al., 2021). To simulate exposure to the full sequential treatment that would occur for an environmental sample, the polymers were exposed to the sequential treatment of $ZnCl_2$ and then the enzymatic-oxidative digestion. For the sequential exposure, the films were removed after each step, cleaned with ultrapure water, photographed under a stereomicroscope, and then added back into the vial to undergo the next step in the treatment sequence.

Table 4.1. Reagents and conditions polymer films were exposed to during the enzymatic-oxidative digestion protocol described in Möller et al., 2021. Table is adapted from Möller et al., 2021.

Step	Reagent	Buffer	Volume (mL)	Incubation Time (h)	Incubation Temperature (°C)	pH
1	Sodium dodecyl sulphate (SDS) 10%		30	48	50	
2	Fenton's reagent		30	1	40	
3	Protease	Tris HCl 0.1M	5 + 25	12	50	9
4	Pectinase	NaOAc 0.1M	5 + 25	48	50	5
5	Viscozyme L	NaOAc 0.1M	1 + 25	48	50	5
6	Cellulase	NaOAc 0.1M	5 + 25	24	40	5

4.2.3. Analytical Methods

Visual analysis, determination of mass, and thickness

Polymer films were analysed and photographed with a stereomicroscope (Leica M50, Leica Microsystems, coupled with an Olympus DP 26) to visually assess potential signs of degradation. Mass loss was determined by measuring film weight (Sartorius Analytical Balance) and thickness before and after treatment exposures (Mitutoyo ABS Digimatic Indicator ID-C equipped with a thickness gauge).

Gel permeation chromatography (GPC)

Polymer films were weighed (Ohaus Discovery DV215CD, Ohaus Corporation, USA) and the corners of the triplicate films were cut and pooled. Molecular weight measurements were carried out by GPC on pooled corners from triplicate samples of the original film, and then on the pooled corners from triplicate samples post treatment, as described in Schrank, Möller et al. 2022. Polymer molecular weight distribution of the PLA films was analysed by gel permeation chromatography (GPC) using an Agilent 1200 with a refractive index detector (Agilent Technologies, 1260 Infinity) and a single 4-styrene divinylbenzene column (SDV linear XL 5 µm (separation range 100 – 3,000,000 Dalton (Da)), Polymer Standards Service, Germany). Chloroform (HPLC grade, Carl Roth, Germany) was used as a solvent, and

Toluene (HPLC grade, Carl Roth, Germany) was used as an internal standard. The sample was dissolved in chloroform and then toluene (2 mg/mL concentration) was added. The sample was then filtered through a 0.22 µm PTFE filter before analysis. Samples were injected (20 µL) using an autosampler (Agilent Technologies,1200 Series), and the flow rate was 0.5 mL/min at room temperature. PHB films were unable to be dissolved in a solvent due to the high molecular weight of the original polymer (550 kDa, Sigma Aldrich), and therefore the molecular weight of PHB could not be assessed by GPC analysis.

Differential scanning calorimetry (DSC)

The thermal characteristics of the polymer films were determined using differential scanning calorimetry (DSC) (STARe system DSC3, Mettler Toledo) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles. The glass transition temperature (T_g) was determined from the inflection point in the second heating cycle of DSC. Melting temperature (T_m) was determined from the peak minimum in the second heating cycle of DSC. The enthalpy of fusion (ΔH_f) was determined from the integration of the exothermic transition in the second heating cycle of DSC for the PHB polymer films. PLA films underwent cold crystallisation in the first heating cycle of DSC (TA Instruments, 2007); therefore, ΔH_f was determined from exothermic transition in the first heating cycle of DSC to enable a comparison between the degradation induced by the treatment exposures. The percent crystallinity of the polymers was calculated using Equation 4.1, where X_c is the percent crystallinity, ΔH_m is the melting enthalpy of fusion, and ΔH_0 is the melting enthalpy for a 100% crystalline polymer:

$$X_c = \frac{\Delta H_m}{\Delta H_0} \times 100$$

[Equation 4.1]

100% crystalline PLA has a ΔH_f of 93.6J/g and 100% crystalline PHB has a ΔH_f of 146J/g (Davaritouchaee et al., 2023; Song et al., 2021).

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

Polymer surface chemistry of the films was determined by attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy (Bruker Optik, Germany). Replicate measurements were averaged (n=3) and data were normalised to account for instrument variability between runs. The normalised spectra were compared between reference films and the films exposed to each treatment to identify changes in molecular bonds.

4.2.4. Statistical Analysis

All data visualisation and statistical analyses were conducted in R (R Core Team, 2024). Polymer mass and thickness data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test (Fox and Weisberg, 2019; R Core Team, 2024). A significance level of p=0.05 was used for all tests. Data met the assumptions of normality and homogeneity of variance. A paired t-test was used to assess for statistical significance in the differences in polymer mass and thickness before and after treatment.

4.3. Results

4.3.1. Changes in polymer properties determined visually and by mass and thickness measurements

Visual assessment

All films were visually assessed after exposure to each treatment (Tables S4.2 to S4.5). PLA films exposed to the individual treatments control, $ZnCl_2$, Fenton's reagent, and cellulase had no visible differences pre and post treatment, with the films remaining clear and no fragmentation occurring (Table S4.2). However, PLA films that were exposed to the individual treatments SDS, protease, pectinase, and viscozyme all became opaque (Figure 4.3, Table S4.2). Additionally, after the protease treatment, fragmentation of the polymer film was observed (Table S4.2). PLA films exposed to the full treatment sequence had notable differences compared to the untreated samples. All replicate films became opaque after the SDS treatment (Figure 4.4A, Table S4.3). The protease step then resulted in varied results, with one replicate no longer being recoverable (complete loss of film), whereas

others showed varying degrees of fragmentation (Figure 4.4). Despite fragmentation during the protease treatment, the fragments did not alter further throughout the subsequent treatment steps. PHB films started as an opaque film, and in contrast to PLA there were no visually observable differences for the PHB films from any individual treatment (Table S4.4) or from the full treatment sequence (Table S4.5).

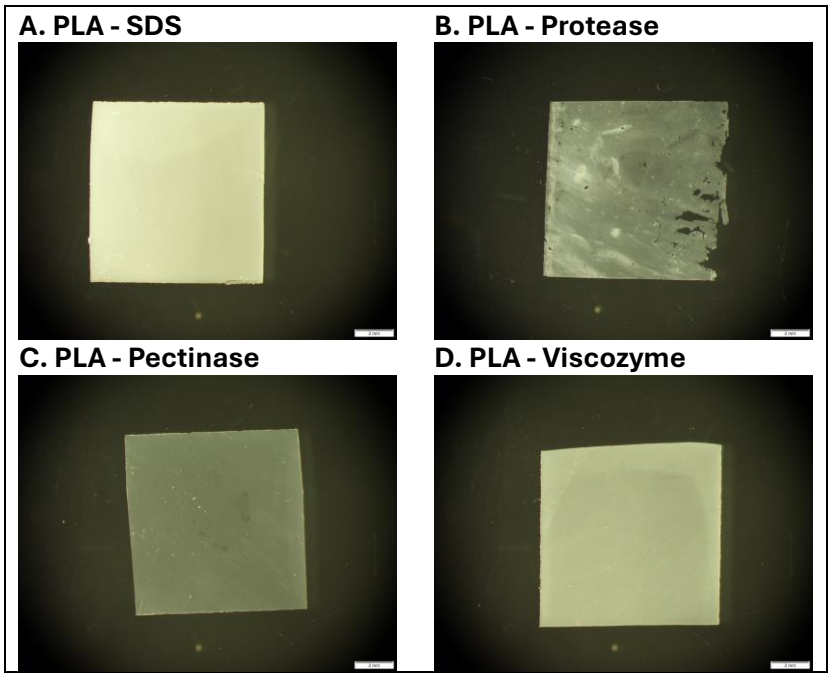


Figure 4.3. Example images of PLA films after exposure to specific steps in the enzymatic oxidative digestion process, specifically SDS (A), protease (B), pectinase (C), and viscozyme (D).

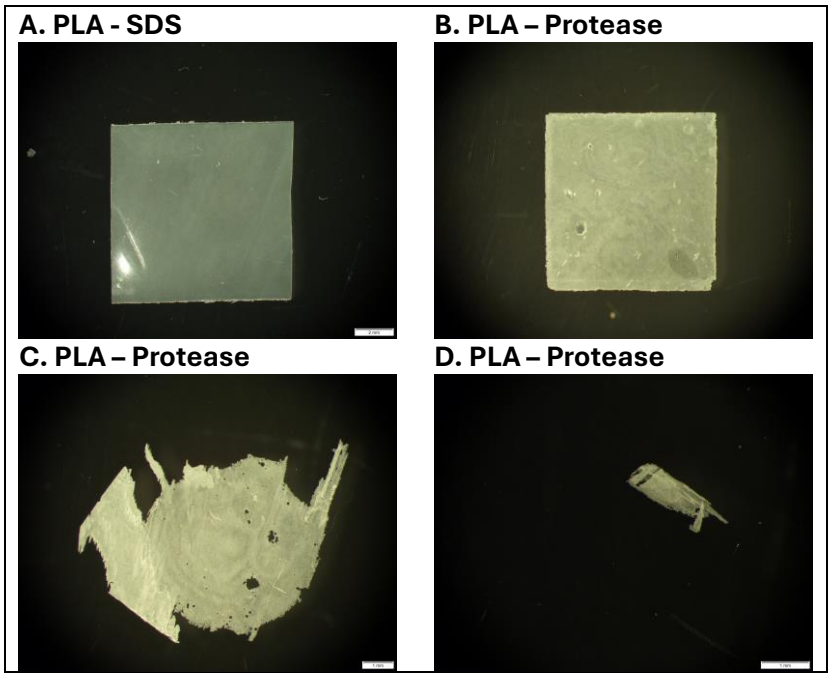


Figure 4.4. Example images of PLA films after exposure to specific steps in the sequential enzymatic digestion protocol. (A) PLA films became opaque after exposure to SDS. (B) One replicate is opaque but appears visually intact after protease exposure except for the bottom right corner where there is evidence of fragmentation. Another replicate had clear evidence of fragmentation, with larger (C) and smaller (D) fragments being clearly visible after exposure to protease.

Mass and Thickness Measurements

PHB films had a significant reduction in mass after exposure to pectinase (Paired t-test: t = 1.04, df = 2, p<0.01), and to the full treatment sequence (Paired t-test: t = 1.04, df = 2, p<0.01). There was no significant difference in the mass of the PHB films after exposure to any other treatments (Figure 4.5A). PLA films had a significant reduction in the mass after exposure to protease (Paired t-test: t = 1.26, df = 5, p<0.001), and to the full treatment sequence (Paired t-test: t=1.49, df = 4, p<0.001). There was no significant difference in the mass of the PLA films after exposure to any other treatments (Figure 4.5A). There was no significant difference in the thickness of PHB after exposure to any treatments (Figure 4.5B). PLA films had a significant reduction in thickness after exposure to protease from 100 µm to 46 µm (Paired t-test: t = 5.73, df = 5, p<0.01). PLA thickness also significantly reduced after the full treatment sequence from 100 µm to 41 µm (Paired t-test: t = 9.45, df = 4, p<0.001) (Figure 4.5B).

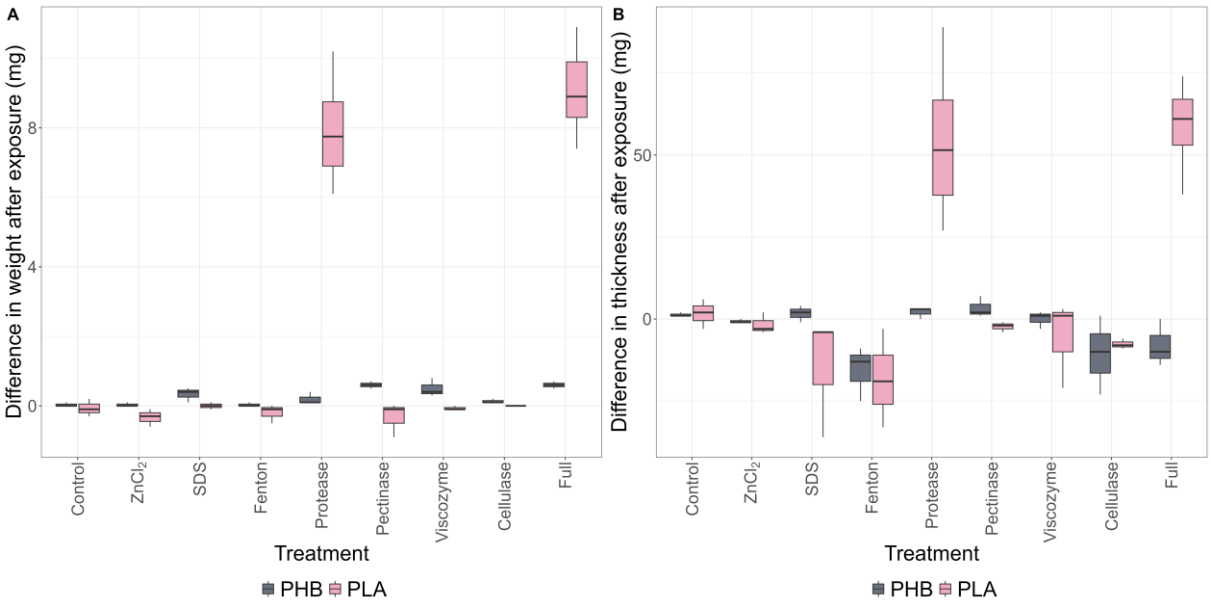


Figure 4.5. Polymer mass (A) and thickness (B) measurements displayed as the difference between pre-treatment and post-treatment measurements. Data is presented as mean ± standard deviation.

4.3.2. Changes in polymer properties determined by GPC, DSC, and ATR-FTIR

Polymer properties were assessed after exposure to all treatments. The molecular weight distribution was measured by GPC for PLA films after exposure to all treatments. The number average molecular weight (M_n) of PLA decreased from 98500g/mol to 30400g/mol after exposure to the full sequential treatment, and the dispersity increased (Figure 4.6, Table 4.2). Additionally, the treatments SDS, protease, pectinase, and viscozyme caused a slight decrease in M_n (Figure 4.6, Table 4.2). Dispersity increased after exposure to the individual treatments SDS, pectinase, and cellulase (Table 4.2).

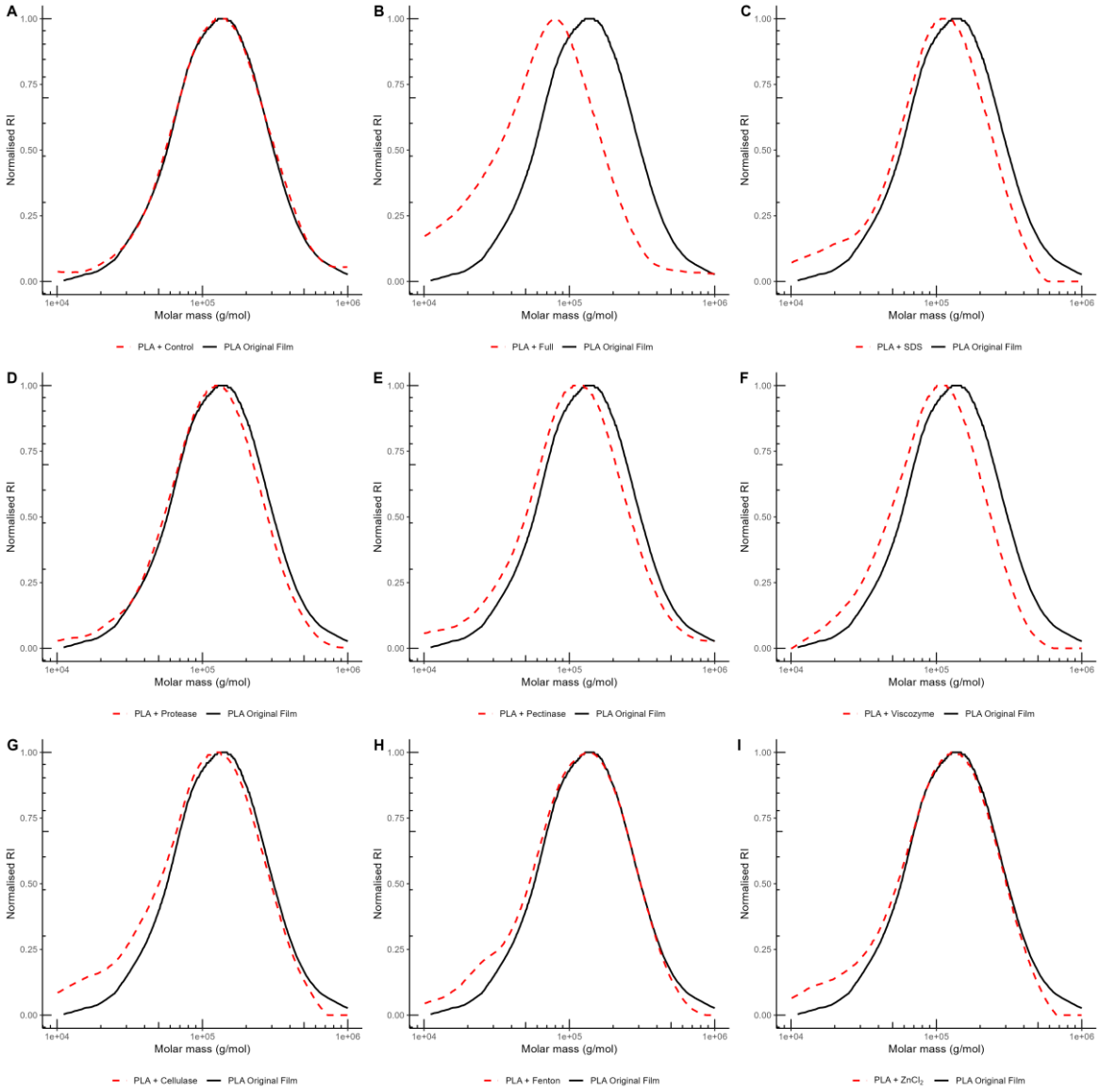


Figure 4.6. Molecular weight distribution measured by gel permeation chromatography of the original PLA film compared to the PLA films after exposure to the following treatments: (B) full treatment sequence, (C) SDS, (D) protease, (E) pectinase, (F) viscozyme, (G) cellulase, (H) Fenton's reagent, and (I) $ZnCl_2$.

Table 4.2. Polymer molecular weight distribution of polylactic acid (PLA) polymer films measured by gel permeation chromatography after exposure to specified treatments, as described by the number average molecular weight (M_n), the weight average molecular weight (M_w), and dispersity (Đ).

Sample Treatment	**M_n (g/mol)**	**M_w (g/mol)**	**Đ**
Original Film	98424	169000	1.72
Full Treatment Sequence	30422	81123	2.67
$ZnCl_2$	76339	151130	1.98
SDS	62458	128290	2.05
Fenton	86463	148030	1.71
Protease	89015	147990	1.66
Pectinase	63857	135570	2.12
Viscozyme	69447	124650	1.80
Cellulase	66606	145070	2.18

Thermal transitions were measured after all treatments by DSC (Figure 4.7, Table 4.3, Table S4.6, Appendix D). After exposure to the full treatment sequence, the T_m of PHB reduced from 167.41°C, to 136.54°C with a second melting peak formed at 148.46°C, which is indicative of degradation (Figure 4.7H). No other changes in T_g and T_m were noted for PHB films. The ΔH_f of PHB was similar to the control after most treatments, except for the pectinase enzyme and full treatment sequence. The intrinsic crystallinity of PHB increased from 42% to 49% after exposure to pectinase, and to 51% after exposure to the full treatment sequence. For the PLA films, there were no changes in T_g and T_m noted. There were large changes in the ΔH_f of PLA after multiple treatments, with the crystallinity increasing relative to the control (13%) after exposure to the full treatment sequence (20%) and to the individual treatments SDS (29%), protease (24%), pectinase (31%), and viscozyme (32%).

Table 4.3. Normalised enthalpy and percent crystallinity values for the polymer films PLA and PHB after exposure to different treatments.

Treatment	Normalised Enthalpy (Jg^{-1})	Crystallinity (%)
PHB		
Control	-61.53	42.14
Full Treatment Sequence	-75.89	51.98
$ZnCl_2$	-63.06	43.19
SDS	-63.92	43.78
Fenton	-59.45	40.72
Protease	-64.95	44.49
Pectinase	-71.77	49.16
Viscozyme	-62.16	42.58
Cellulase	-57.91	39.66
PLA		
Control	-12.5	13.35
Full Treatment Sequence	-19.33	20.65
$ZnCl_2$	-5.75	06.14
SDS	-27.6	29.49
Fenton	-16.96	18.12
Protease	-22.75	24.31
Pectinase	-29.12	31.11
Viscozyme	-30.28	32.35
Cellulase	-14.79	15.80

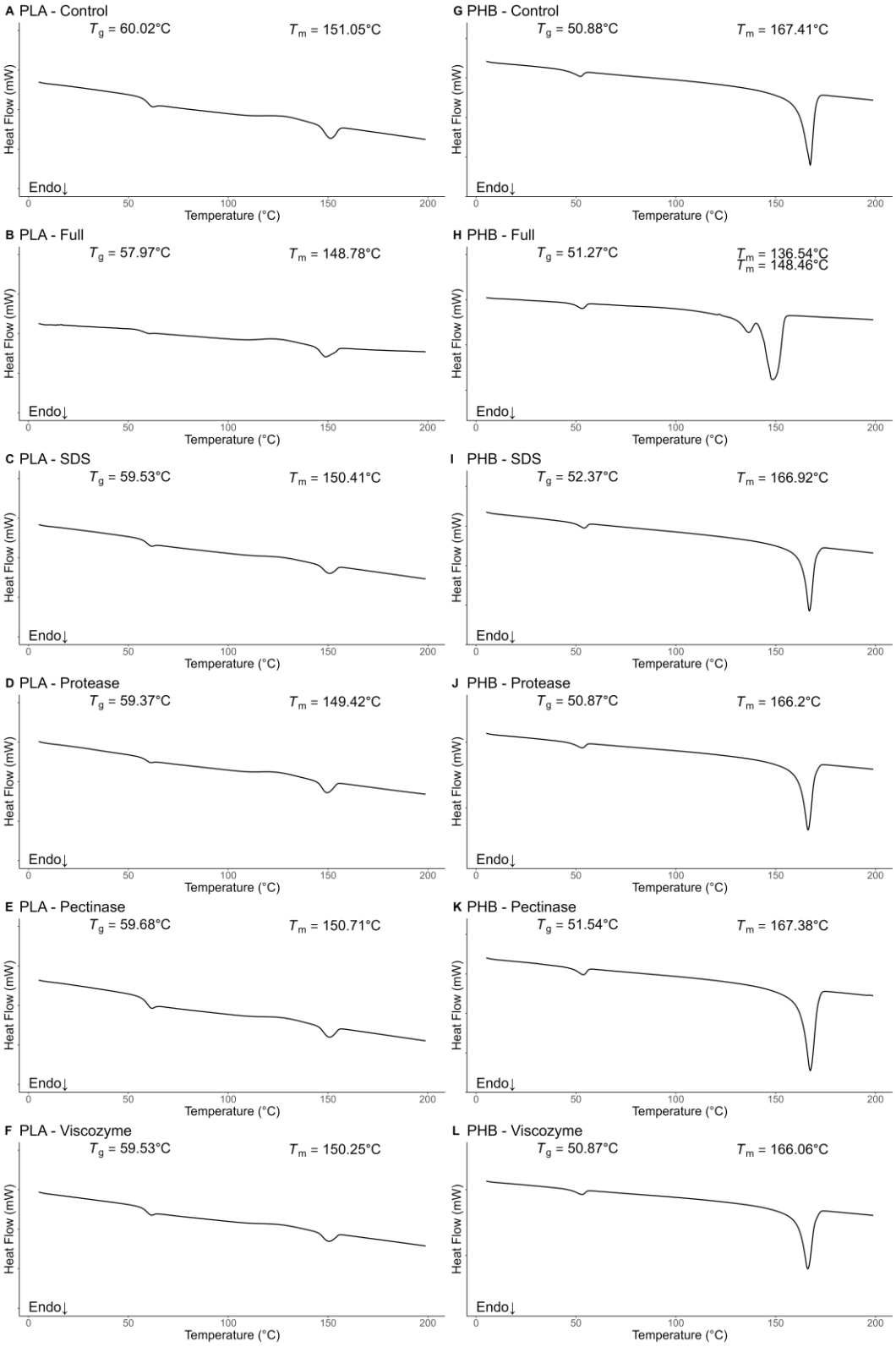


Figure 4.7. Heat flow curves measured by differential scanning calorimetry in the second heating cycles for PLA films (A-F) and PHB films (G-L) exposed to the treatments: control, full treatment sequence, SDS, protease, pectinase, and viscozyme. The glass transition temperature (T_g) and melting temperature (T_m) are displayed.

Polymer films were also analysed by ATR-FTIR spectroscopy to identify changes in the chemistry of the films, such as oxidation or the loss/addition of functional groups. Minimal differences were noted for PLA films, with no distinct additional peaks or functional groups formed after most of the individual treatments (Figure S4.1). However, after exposure to the full treatment sequence (Figure 4.8A), a distinct shoulder formed in the region 1500-2000cm^{-1}. The main peak identified at 1751cm^{-1} arises from the ester C=O bond. Additional peaks formed at 1652cm^{-1} and 1532cm^{-1}, arising from the C=O and C-OH bonds, which are associated with carboxylic acid, which is a degradation product of PLA. For the PHB films, the full treatment sequence (Figure 4.8B) also formed a distinct shoulder in the region 1500-2000cm^{-1}. Again, the main peak identified at 1718cm^{-1} is a C=O bond associated with the ester bond, and an additional peak formed at 1623cm^{-1}, associated with carboxylic acid, which is a degradation product of PHB. This additional peak was also observed within PHB films after exposure to pectinase and viscozyme (Figure S4.2).

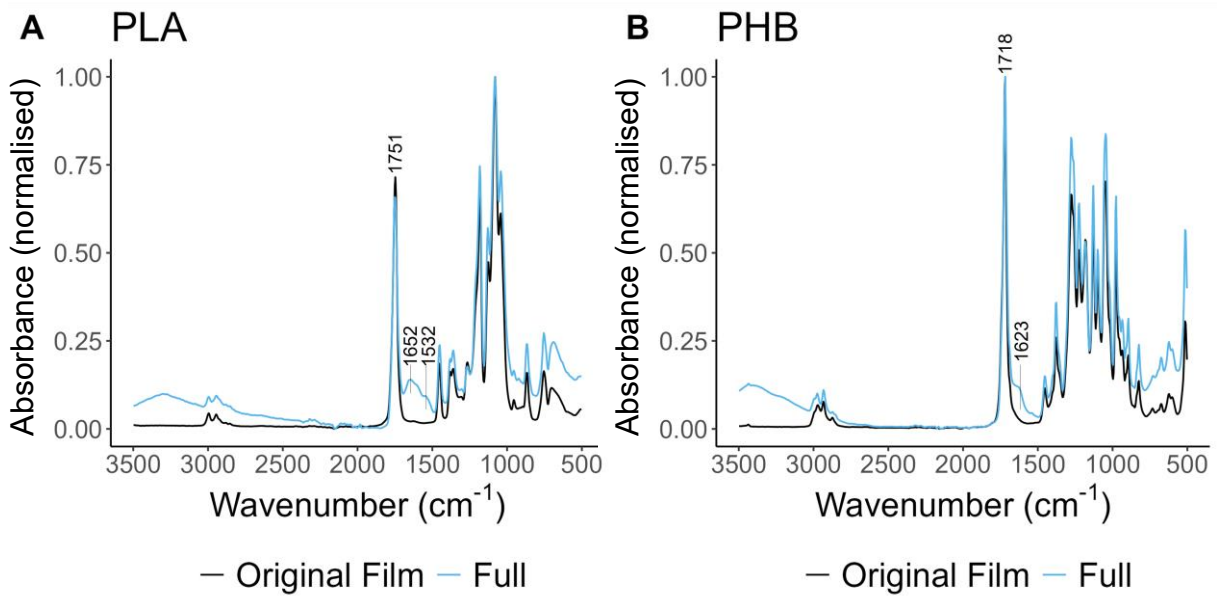


Figure 4.8. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of the PLA (A) and PHB (B) films both as the original film, and after exposure to the full treatment sequence.

4.4. Discussion

Distinct differences in degradation between PLA and PHB were observed after exposure to the soil microplastic extraction treatments. PLA was significantly degraded by the treatments SDS, protease, pectinase, viscozyme, and the full treatment sequence. Visual degradation and loss of mass/thickness were noted, as well as a reduction in molecular weight and increased polymer crystallinity. PHB was also degraded after exposure to the full treatment sequence, as well as by the pectinase and viscozyme treatments. In contrast to PLA, the degradation was not visually apparent for PHB, but significant mass losses were noted after the pectinase treatment and the full treatment sequence. Additionally, chemical changes were observed in PHB, such as increased crystallinity, and the increased presence of –COOH groups.

Consideration of how the different treatments impact the polymer structures and lead to degradation is important for future selection of soil microplastic extraction methods. PLA and PHB are both susceptible to hydrolytic and enzymatic degradation (Arrieta et al., 2014; Bher et al., 2022, 2023). The hydrolysis kinetics are dependent upon their molecular weight (Bonartsev et al., 2012; Speranza et al., 2014). PHB samples with higher molecular weights (e.g., 300-1000kDa) have previously been found to be more stable, and less susceptible to hydrolytic degradation than PHB samples with a lower molecular weight (e.g., 150kDa) (Bonartsev et al., 2012). The molar mass of the PHB used in this study was 550kDa (Sigma Aldrich), indicating it is a more stable form of PHB, that is less susceptible to degradation. Generally, previous research has shown that PLA has poorer stability than PHB (Bonartsev et al., 2012; Speranza et al., 2014), and as the PLA used in this study had a molar mass of 98kDa, it would be susceptible to degradation. This is an important consideration for future applications aimed at isolating biodegradable polymers from complex matrices.

The impacts of $ZnCl_2$, and Fenton's reagent were considered due to their prevalence in microplastic extraction methods (Cutroneo et al., 2021; Junhao et al., 2021; Lusher et al., 2020b; Möller et al., 2020; Radford et al., 2021). Neither treatment resulted in evidence of degradation, suggesting that both treatments are suitable for use with PLA and PHB and would not cause damage to the films, which agrees with previous literature (Möller et al., 2022; Schrank et al., 2022). However, it is important to note that both this study and Schrank et al. (2022) looked at the impact on films finding no effects, while previous work by Möller et al. (2022) and Pfohl et al., (2021) looked at the impact of Fenton's reagent on particles, with contrasting results. In the study by Möller et al. (2022), no effects were noted for the biodegradable PLA after exposure to Fenton's reagent, whereas Pfohl et al. (2021) found that after exposure to Fenton's reagent, there was a reduction in particle size of 11.7% for the biodegradable polymers PLA, PBAT, and PBS. Specifically, the PLA particles <100 µm in size disappeared suggesting they were degraded. Möller et al. (2022) used PLA particles that ranged in size from 200 µm – 1 mm, whereas Pfohl et al. (2021) used PLA particles <500 µm. As the surface area to volume ratio plays a critical role in polymer degradation rates, this difference in PLA particle size could explain these contrasting results (Chinaglia et al., 2018). This highlights the importance of considering polymer form, shape, and size for the selection of appropriate microplastic extraction methods, as they can influence susceptibility to degradation.

The impact of SDS was assessed because it is a surfactant that has been used in microplastic extraction processes to solubilise lipids and proteins prior to subsequent enzymatic digestion (Löder et al., 2017; Mbachu et al., 2021; Möller et al., 2020). However, surfactants are also often used to reduce polymer surface tension and enhance wettability, which will increase the hydrophilicity of polymers, thus making them more susceptible to degradation through hydrolysis (Feng et al., 2021; Michael et al., 2017). No changes were noted for PHB after exposure to SDS. However, PLA films became opaque, there was a

reduction in M_n and an increase in polydispersity and crystallinity. There were no changes noted in the film mass and thickness, or the in the chemical bonds at the surface of the PLA films after SDS treatment. Opacity occurrence is associated with polymer crystallinity increasing and can occur without significant mass loss (Fukushima et al., 2011; Paul et al., 2005). The hydrolytic degradation of PLA is well known, with the degradation rate being greater in the bulk of the material rather than at the surface (Grizzi et al., 1995; Hakkarainen, 2002; Paul et al., 2005). Therefore, the increased opacity and polydispersity (Đ increased to 2.06) suggest that the PLA has been degraded though chain scission in the amorphous regions, decreasing the average molecular weight, which correlated with the observed reduction in M_n of PLA after treatment with SDS from 98,500g/mol to 62,400g/mol (Fukushima et al., 2011; Paul et al., 2005).

PLA was degraded by multiple treatments in this study, with increased opacity seen after exposure to the enzyme's protease, pectinase, and viscozyme. Four distinct enzymes were assessed due to their use in soil microplastic extractions; protease is used to hydrolyse insoluble protein structures in soils, followed by a sequence of pectinase, viscozyme, and cellulase that degrade lignocellulosic structures (Möller et al., 2022). In this study, fragmentation of PLA was observed after the protease treatment, but not for any other treatments. Fragmentation of PLA by protease was also previously observed by Moller et al. (2022). Protease is a proteolytic enzyme that can catalyse the hydrolysis of ester bonds (RCOOR', where R and R' are alkyl groups) (Hanphakphoom et al., 2014; Möller et al., 2022; Shalem et al., 2024; Zaaba and Jaafar, 2020). Proteinase K is a well-known protease enzyme that has been used extensively to degrade PLA and PLA blends (Shalem et al., 2024). PLA also increased in crystallinity after exposure to protease. However, it is important to consider that the other enzyme treatments used a pH 5 buffer, whereas the protease enzyme used a pH 9 buffer, which is a more alkaline environment. Alkaline treatments degrade conventional plastics including polycarbonate (PC) and polyethylene

terephthalate (PET) (Dehaut et al., 2016; Hurley et al., 2018; Schrank et al., 2022), through the hydrolysis of the ester bond (Dave et al., 1987; Tagaya et al., 1999). PLA is known to hydrolyse in alkaline environments (Shirahase et al., 2006), and previous work focusing on the mass loss of PLA at different pHs (without enzyme treatments) found that at pH 10 PLA mass loss continuously increased with time. Whereas in acidic conditions (e.g., pH 2, 3) and neutral conditions (pH 7.4) there was no significant mass loss (Vaid et al., 2021). The stability of PLA based polymer blends was also assessed by exposure of powdered PLA based polymers to an alkaline oxidation process at pH 10 at 70°C, with observed surface degradation, reduction in PLA content, reduced molar mass distributions, and reduced particle size (Pfohl et al., 2021). However, as the T_g of PLA is 55-60°C (Chen et al., 2003; Zhang et al., 2022), and PLA is known to be compostable at 55-60°C (Fogašová et al., 2022; Iovino et al., 2008; Sedničková et al., 2018), it is plausible that in the study by Pfohl et al. (2021) the increased temperature is influencing the PLA degradation seen, rather than the pH. In the present study the protease treatment was carried out at 50°C, this temperature is below the T_g of PLA meaning temperature is not considered a main cause of the observed degradation and thus the alkaline environment could be a likely driver of the fragmentation that occurs during the protease treatment.

Pectinase and viscozyme induced degradation in both PLA and PHB. PLA became opaque, with a reduction in M_n and increased crystallinity. PHB increased in crystallinity after exposure to pectinase, but not to viscozyme, whilst both enzymes resulted in the formation of a shoulder in the ATR-FTIR spectra, indicating the presence of carboxylic acid which is a degradation product of PHB. The presence of a shoulder is due to a low intensity bond which is associated with the crystalline C=O bond stretching (Guo et al., 2010), and has previously been observed in PHB after composting, therefore is indicative of degradation (Arrieta et al., 2014). It has previously been suggested that the initial degradation of the amorphous region leaves behind the crystalline portion of the polymer (Arrieta et al., 2014), which fits with the

increases in crystallinity observed in this study. Cellulase reduced PLA M_n but had no noted effects on PHB. Complex polysaccharides have previously been observed to be significantly decomposed by enzyme mixtures including pectinase, viscozyme and cellulase, with long chain oligomers being reduced to shorter chains, and the cleaving of ester bonds noted (Liu et al., 2024), therefore these are potential mechanisms behind the observed degradation of PLA and PHB in this study.

Degradation was observed for both PLA and PHB films after exposure to the full treatment sequence where all individual treatments were applied sequentially, as would occur during sample processing for microplastics extractions from soil. PHB thermal transition characteristics altered after exposure to the full treatment sequence, with a reduction in T_m, and the formation of a second melting peak. The formation of a double melting peak has previously been seen after degradation of PHB films in a soil incubation, along with the decrease in T_m and is suggestive of the formation of different crystalline regions in the polymer (Kim et al., 2023). The crystallinity of the PHB also increased after the full treatment from 42% to 51%, suggesting that the amorphous regions were degraded first (Pantani and Sorrentino, 2013; dos Santos et al., 2018). Whilst the molecular weight of the PHB in this study could not be measured, due to solubility issues, previous research has observed lower T_m, and increased crystallinity of PHB alongside a reduction in the molecular weight (Kim et al., 2023).

The observed degradation of PLA after the full treatment sequence varied, with the complete loss of one sample, and varying degrees of fragmentation in the others. The molecular weight distribution altered significantly, with decreased M_w and M_n, and a greater increase in polydispersity compared to any individual treatment, which is indicative of random chain scission. PLA crystallinity also increased in the full treatment, although this was relatively less than the increase in the enzymatic treatments individually. Changes in

the ATR-FTIR spectra were evident after the full treatment sequence, with the formation of a shoulder as the C=O bond became broader, typical of carboxylic acid. As PLA degrades the number of carboxylic acid end groups in the polymer chain increases, resulting in the formation of the associated bonds (Arrieta et al., 2014).

In addition to the polymer structure, it is essential to consider the environmental conditions that microplastics will have been exposed to when applying extraction protocols to environmental samples. This will be important for applications like agricultural mulching films that will potentially have been subject to photodegradation. PLA biodegrades slowly in soil, primarily limited by temperature, but it is susceptible to photodegradation, which increases fragmentation and decreases stability, which will likely make it more susceptible to further degradation induced by the extraction method (González-López et al., 2020; Zaaba and Jaafar, 2020). Therefore, microplastic extraction methods must avoid inducing degradation, as this can lead to inaccurate estimations of biodegradable microplastics occurrence.

4.5. Conclusion

The extraction of microplastics from soils is complex, due to significant background matrix interference that require multiple removal steps. Where extraction is a basis for quantification of microplastics, there is a need to determine the effects of the extraction process on the physico-chemical characteristics of the polymer to ensure that the extraction itself does not confound the results. Whilst enzymatic-oxidative digestion successfully removes the matrix interference, the process is lengthy and degrades both PLA and PHB, particularly the enzymatic digestion steps. It is unlikely that one extraction method will be appropriate for all biodegradable polymers, which raises doubt about the efficacy of non-target microplastic analyses in soils. This study looked at pure polymers, but assessing the impact of the extraction methods is likely to be further complicated for

polymer blends and the addition of polymer additives. Future methods should consider the chemical structure of biodegradable polymers and their known degradation mechanisms to minimise the induced effects of a chosen microplastic extraction method. Utilising methodologies that are known to not degrade biodegradable polymers will improve the reliability of environmental monitoring of biodegradable plastics and allow further research into their environmental fate.

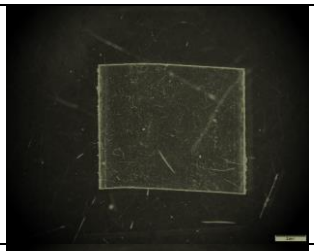
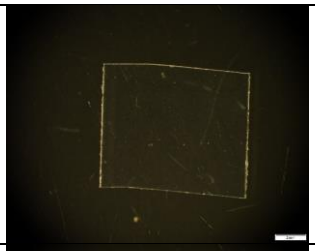
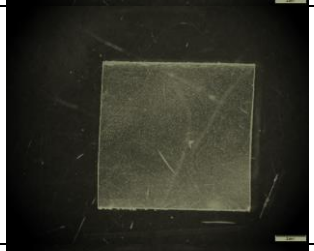
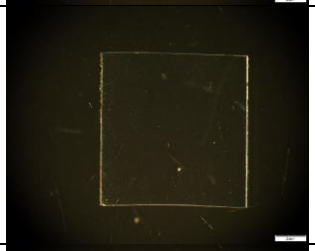
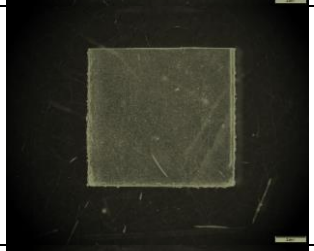
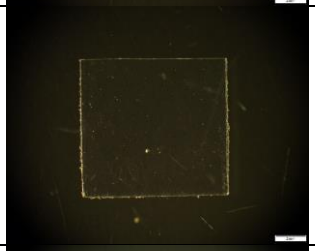
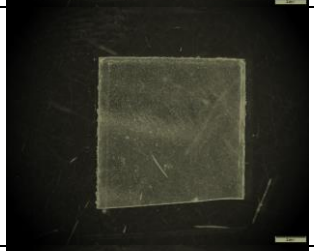
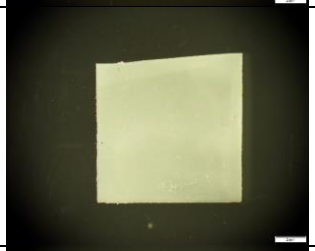
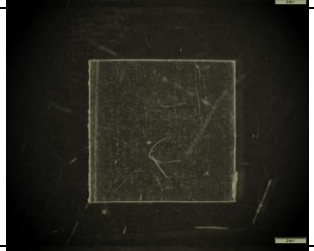
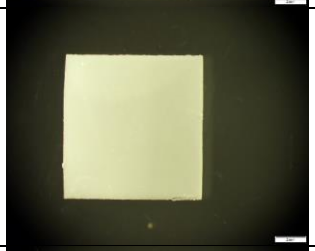
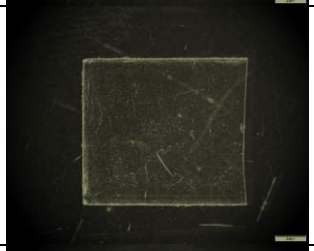
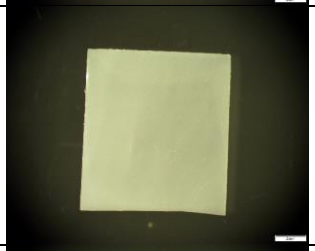
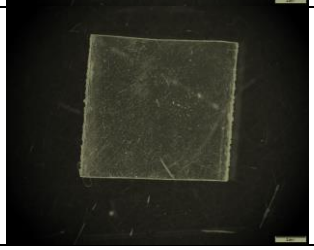
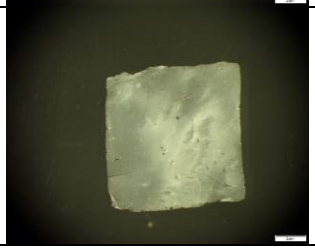
4.6. Supplementary Information

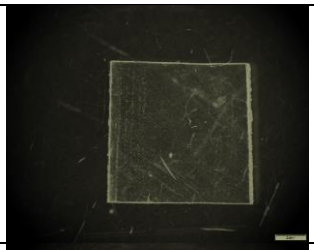
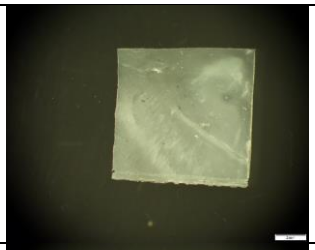
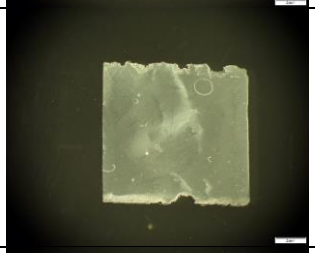
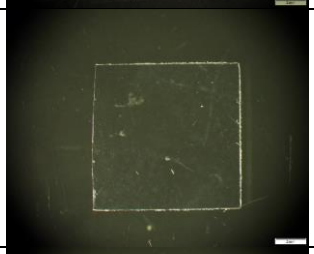
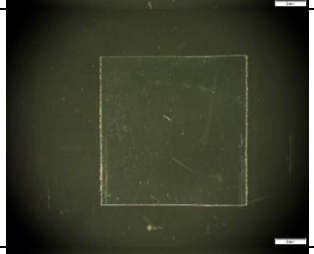
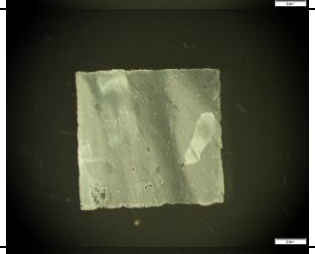
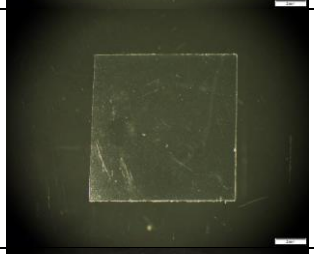
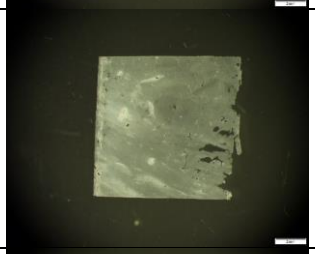
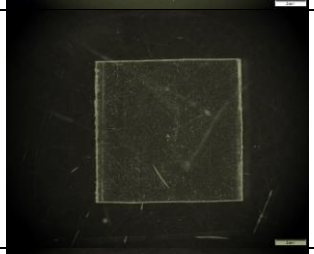
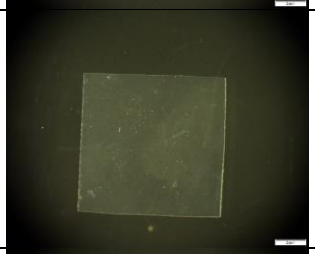
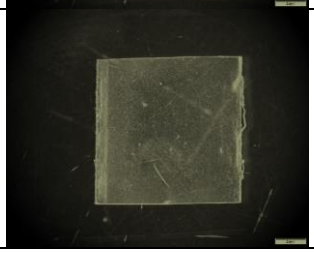
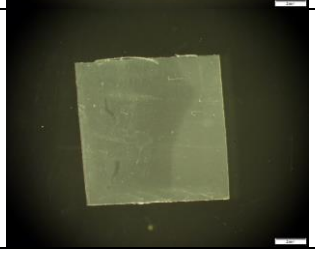
4.6.1. Supplementary Tables

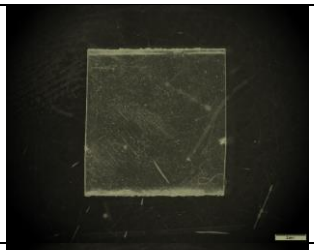
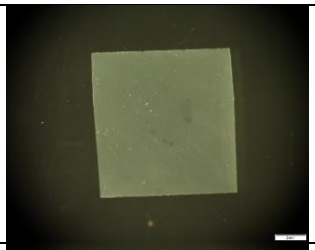
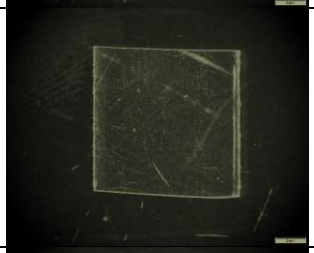
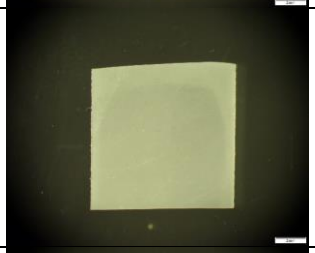
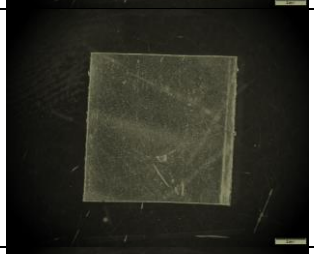
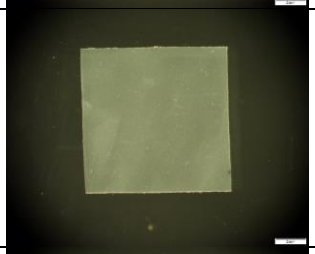
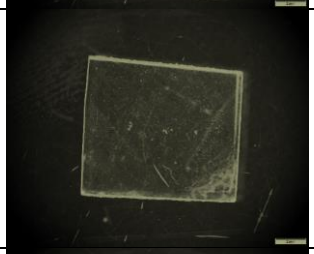
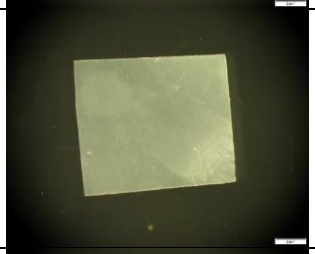
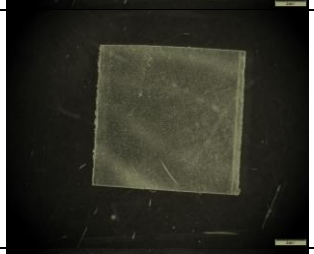
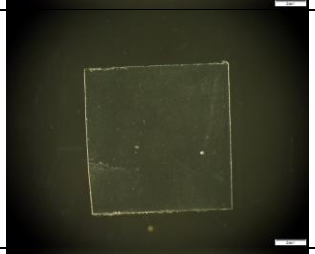
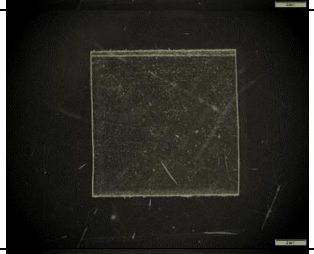
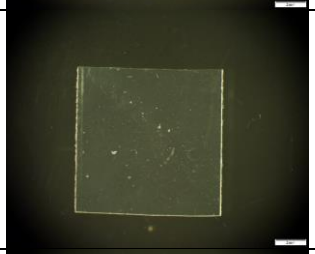
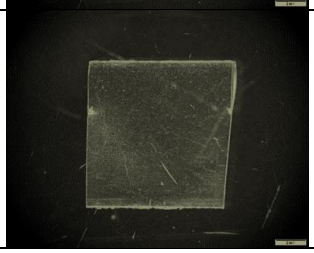
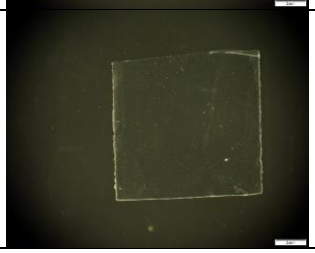
Table S4.1. Reagent and enzyme supplier details, adapted from Schrank, Möller et al., 2022 and Möller et al., 2021.

Reagent	Details
Sodium dodecyl sulphate SDS	Carl Roth GmbH & Co. KG, Germany
Hydrogen peroxide	VWR International GmbH, Germany
Protease A-01	(subtilisin, EC 3.4.21.62, enzymatic activity 1.100 U mL^{-1}; ASA Spezialenzyme)
Pektinase L-40	(polygalacturonase, pectin depolymerase, EC 3.2.1.15; ASA Spezialenzyme)
Viscozyme L	(endo-beta-glucanase, V2010; Novozymes)
Cellulase TXL	(endo-1,4-beta-glucanase, EC3.2.1.4; ASA Spezialenzyme)

Table S4.2. Microscope images of PLA polymer films pre and post treatments. Samples were assessed in triplicate, with individual films visually compared before and after treatments.

Sample ID	Pre-treatment	Post-treatment
PLA-control-1		
PLA-control-2		
PLA-control-3		
PLA-SDS-1		
PLA-SDS-2		
PLA-SDS-3		
PLA-protease-1		

PLA-protease-2		
PLA-protease-3		
PLA-protease-4		
PLA-protease-5		
PLA-protease-6		
PLA-pectinase-1		
PLA-pectinase-2		

PLA-pectinase-3					
PLA-viscozyme-1					
PLA-viscozyme-2					
PLA-viscozyme-3					
PLA-cellulase-1					
PLA-cellulase-2					
PLA-cellulase-3					

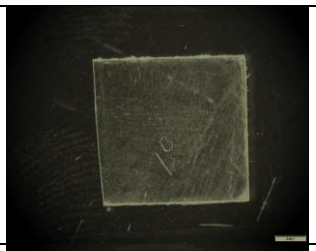
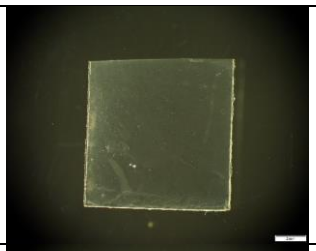
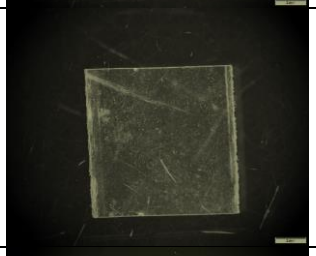
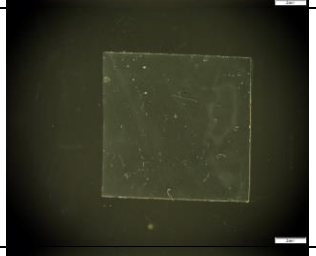
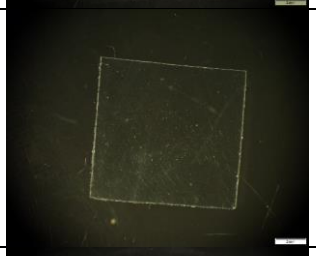
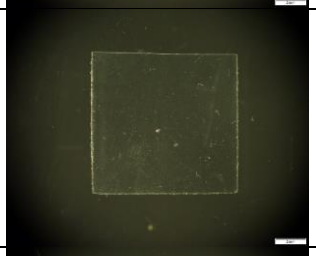
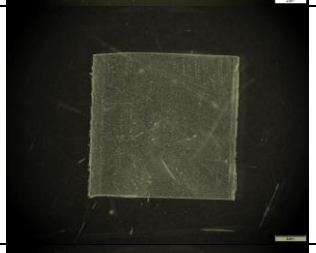
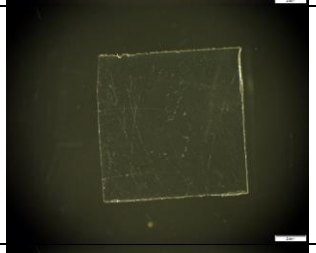
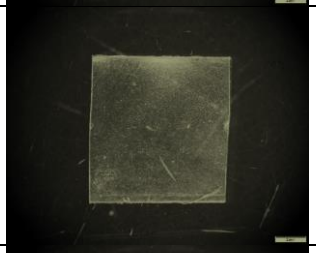
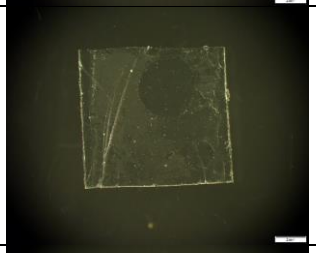
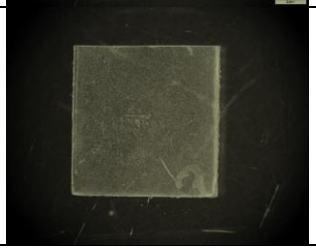
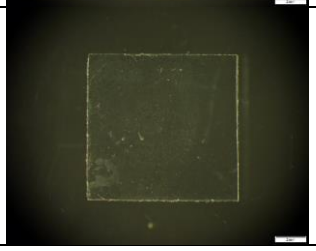
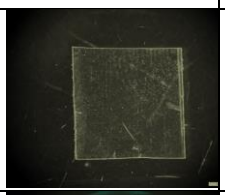
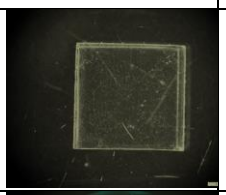
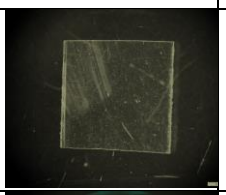
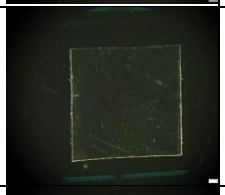
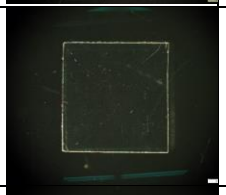
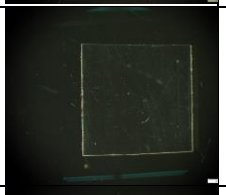
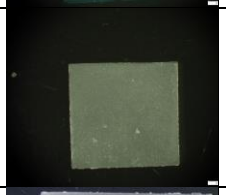
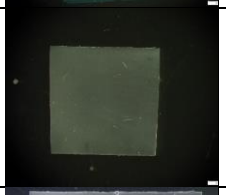
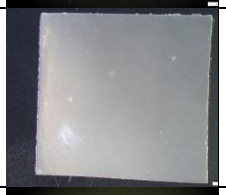
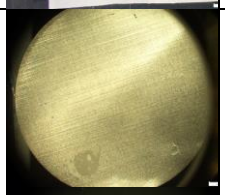
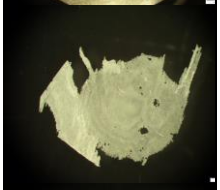
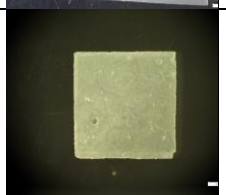
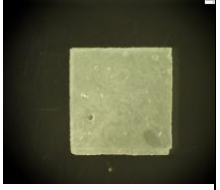
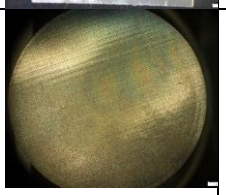
PLA-fenton-1		
PLA-fenton-2		
PLA-fenton-3		
PLA-$ZnCl_2$-1		
PLA-$ZnCl_2$-2		
PLA-$ZnCl_2$-3		

Table S4.3. Microscope images of replicate PLA polymer films pre and post each treatment step in the full treatment sequence. Samples were assessed in triplicate, with individual films visually compared before and after treatments.

Sample ID	Replicate 1	Replicate 2	Replicate 3	Notes
PLA-full-start				
PLA-full-post-$ZnCl_2$				No visible impact
PLA-full-post-SDS				Films have turned opaque
PLA-full-post-Fenton				Films remain opaque
PLA-full-post-protease				Replicate 1 has considerable disintegration. Replicate 2 has become cloudy and there are some surface irregularities. Replicate 3 has completely gone.

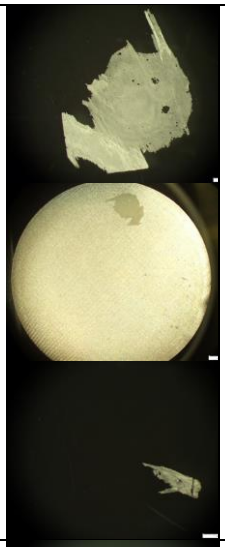
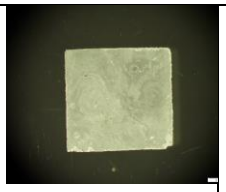
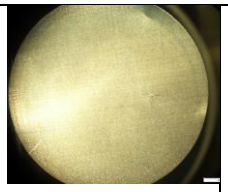
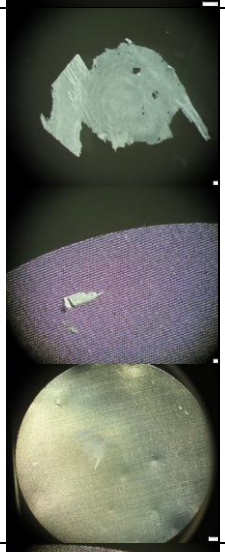
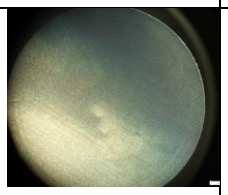
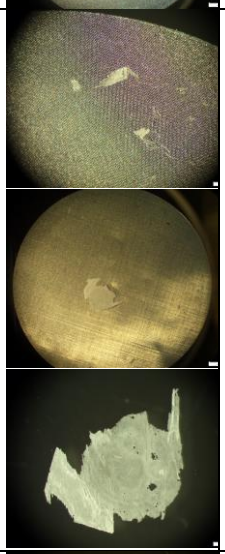
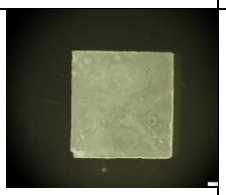
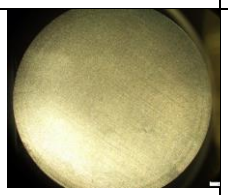
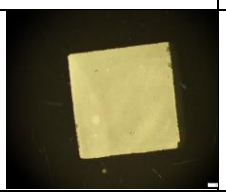
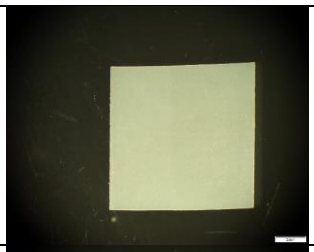
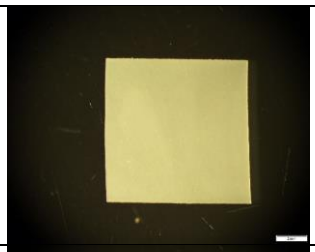
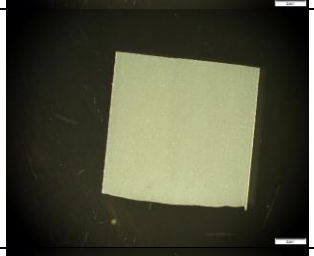
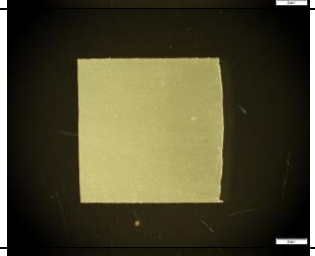
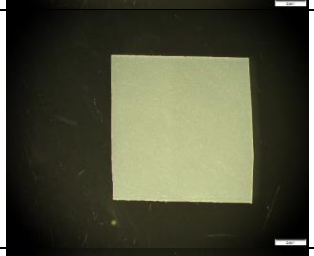
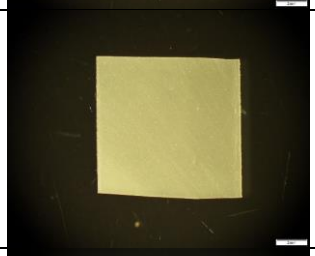
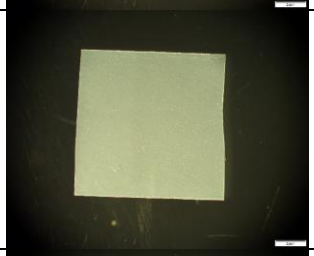
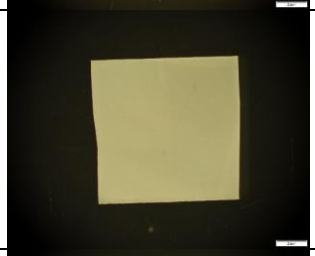
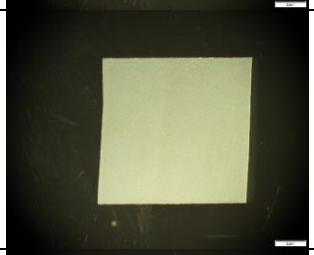
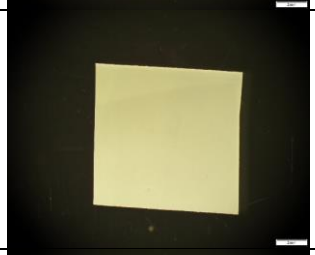
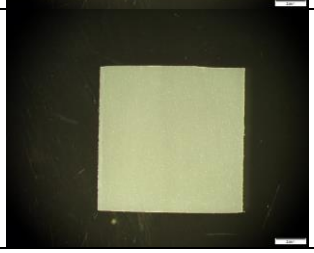
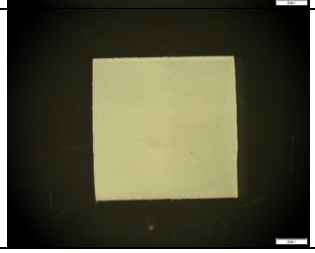
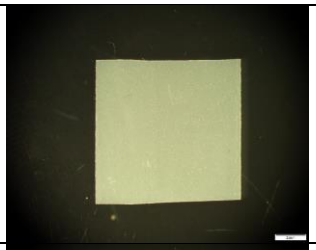
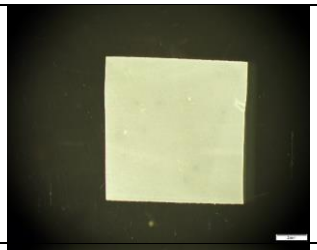
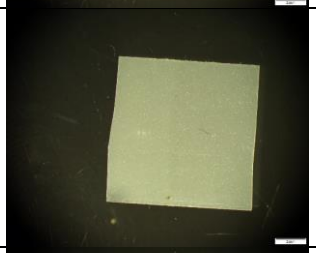
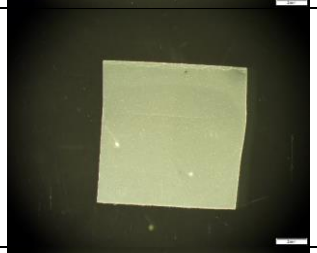
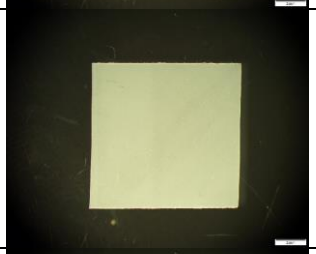
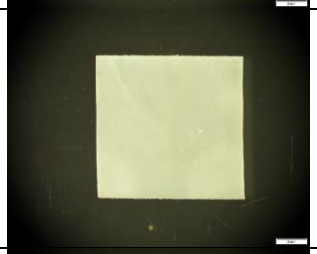
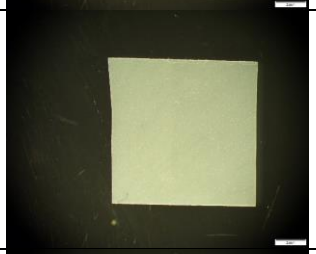
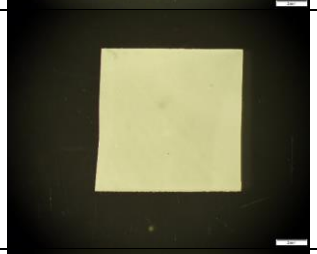
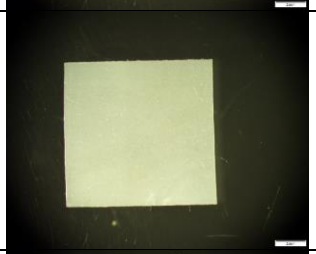
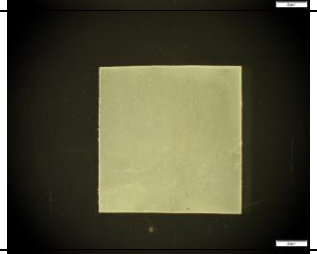
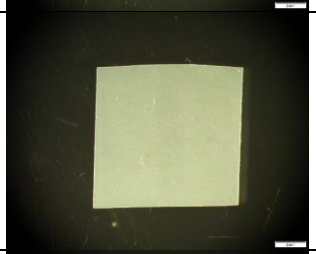
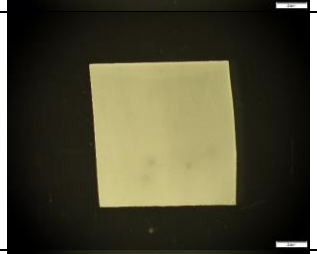
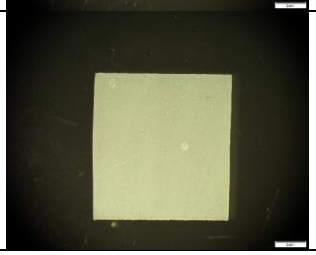
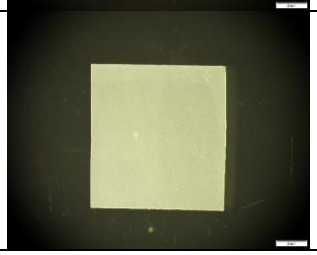
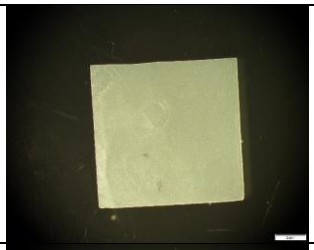
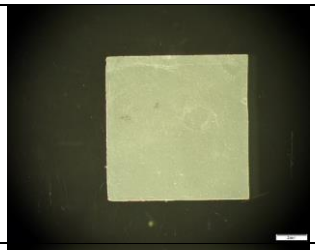
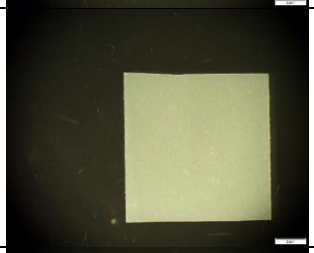
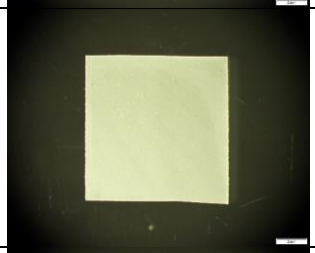
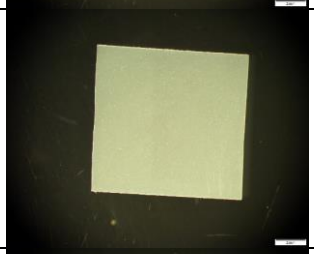
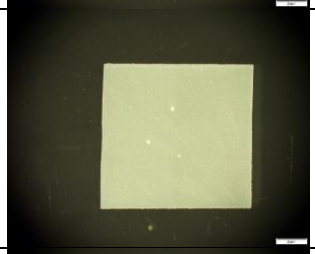
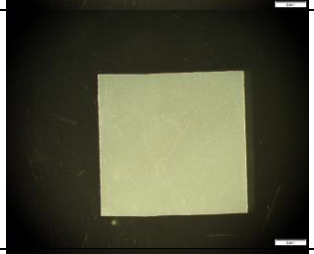
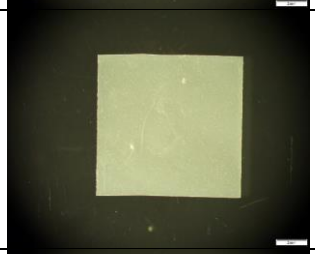
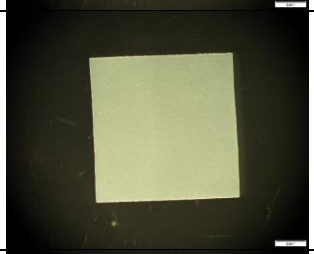
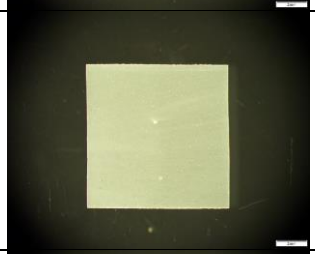
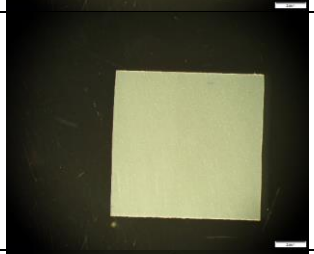
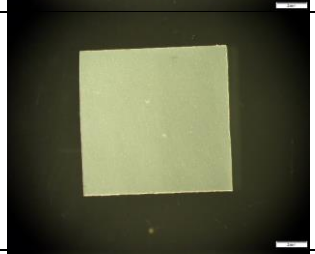
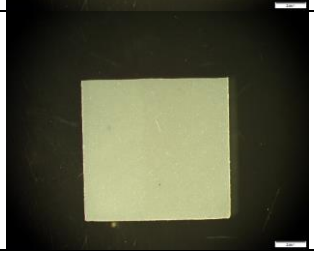
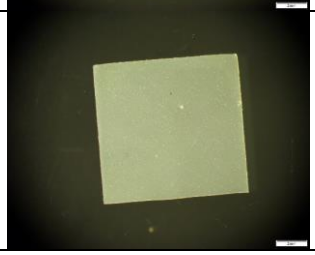
PLA-full-post-pectinase				Samples remain the same as previous step of protease. There does not seem to be any further visual changes to the polymer films.
PLA-full-post-viscozyme				No further visual changes.
PLA-full-post-cellulase				Does not seem to be any further changes to the polymer films.
PLA-full-post-oven-END				No further visual changes.

Table S4.4. Microscope images of all replicate PHB polymer films pre and post treatments. Samples were assessed in triplicate, with individual films visually compared before and after treatments.

Sample ID	Pretreatment	Post treatment
PHB-control-1		
PHB-control-2		
PHB-control-3		
PHB-SDS-1		
PHB-SDS-2		
PHB-SDS-3		

PHB-protease-1		
PHB-protease-2		
PHB-protease-3		
PHB-pectinase-1		
PHB-pectinase-2		
PHB-pectinase-3		
PHB-viscozyme-1		

PHB-viscozyme-2		
PHB-viscozyme-3		
PHB-cellulase-1		
PHB-cellulase-2		
PHB-cellulase-3		
PHB-fenton-1		
PHB-fenton-2		

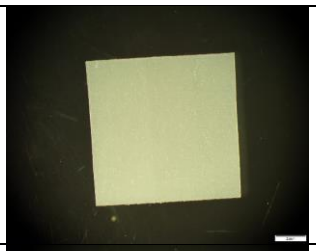
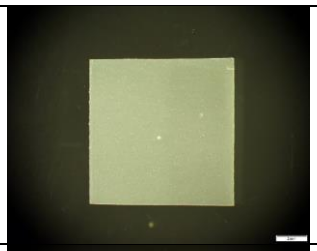
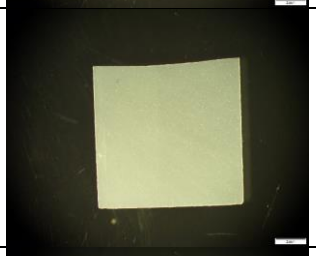
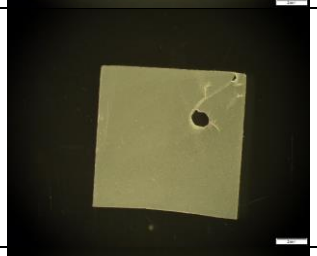
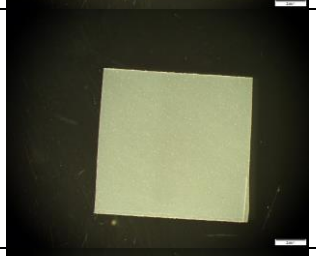
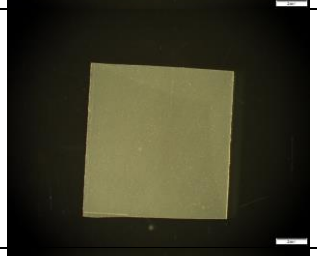
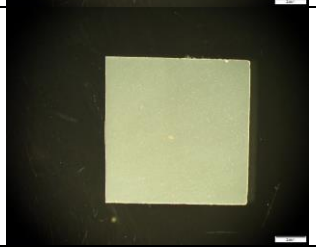
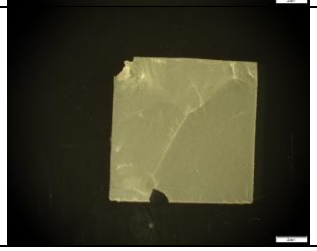
PHB-fenton-3		
PHB-$ZnCl_2$-1		
PHB-$ZnCl_2$-2		
PHB-$ZnCl_2$-3		

Table S4.5. Microscope images of replicate PLA polymer films pre and post each treatment step in the full treatment sequence.

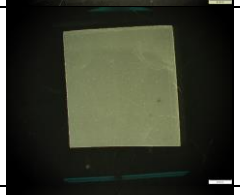
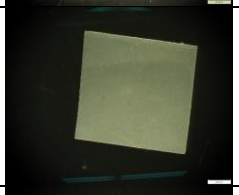
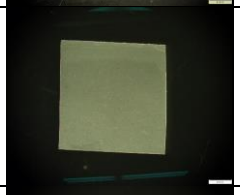
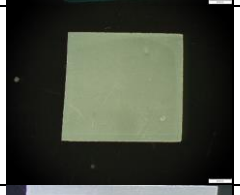
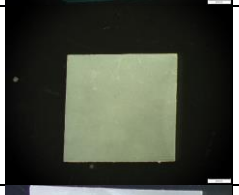
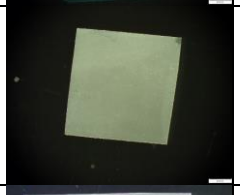
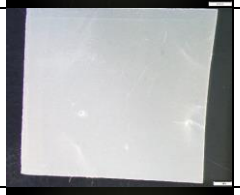
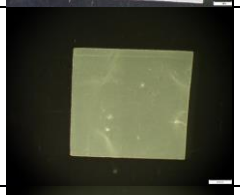
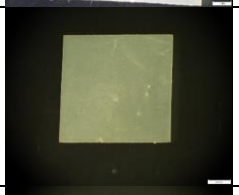
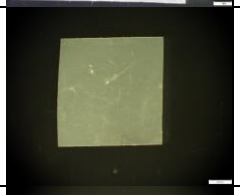
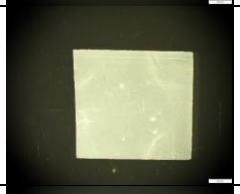
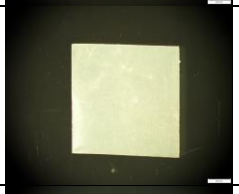
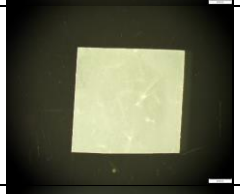
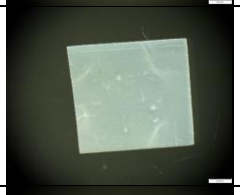
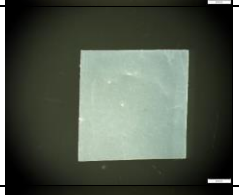
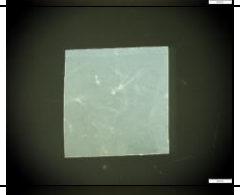
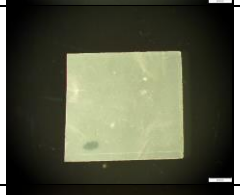
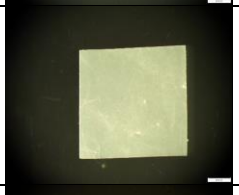
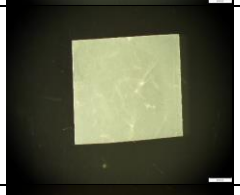
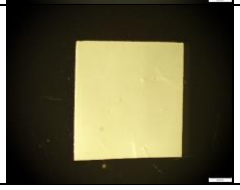
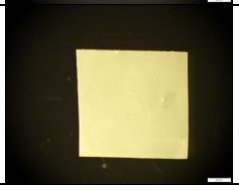
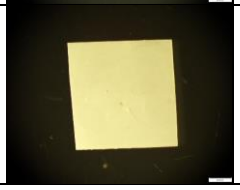
Sample ID	Replicate 1	Replicate 2	Replicate 3	Notes
PHB-full-start				
PHB-full-post-$ZnCl_2$				No visual changes observed.
PHB-full-post-SDS				No visual changes observed.
PHB-full-post-Fenton				Images taken with a different microscope. No visual changes observed.
PHB-full-post-protease				No visual changes observed.
PHB-full-post-pectinase				No visual changes observed.
PHB-full-post-viscozyme				No visual changes observed.
PHB-full-post-cellulase				No visual changes observed.
PHB-full-post-oven-END				No visual changes observed.

Table S4.6. Glass transition temperature (T_g) and melting temperature (T_m) of PLA and PHB films after exposure to different treatments, measured by differential scanning calorimetry in the second heating cycle.

Treatment	Tg (°C)	Tm (°C)
PHB		
Control	50.88	167.41
Full Treatment Sequence	51.27	136.54, 148.46
$ZnCl_2$	49.37	165.04
SDS	52.37	166.92
Fenton	49.54	165.72
Protease	50.87	166.20
Pectinase	51.54	167.38
Viscozyme	50.87	166.06
Cellulase	50.70	166.42
PLA		
Control	60.02	151.05
Full Treatment Sequence	57.97	148.78
$ZnCl_2$	59.53	150.24
SDS	59.53	150.41
Fenton	59.53	150.24
Protease	59.37	149.42
Pectinase	59.68	150.71
Viscozyme	59.53	150.25
Cellulase	59.51	150.55

4.6.2. Supplementary Figures

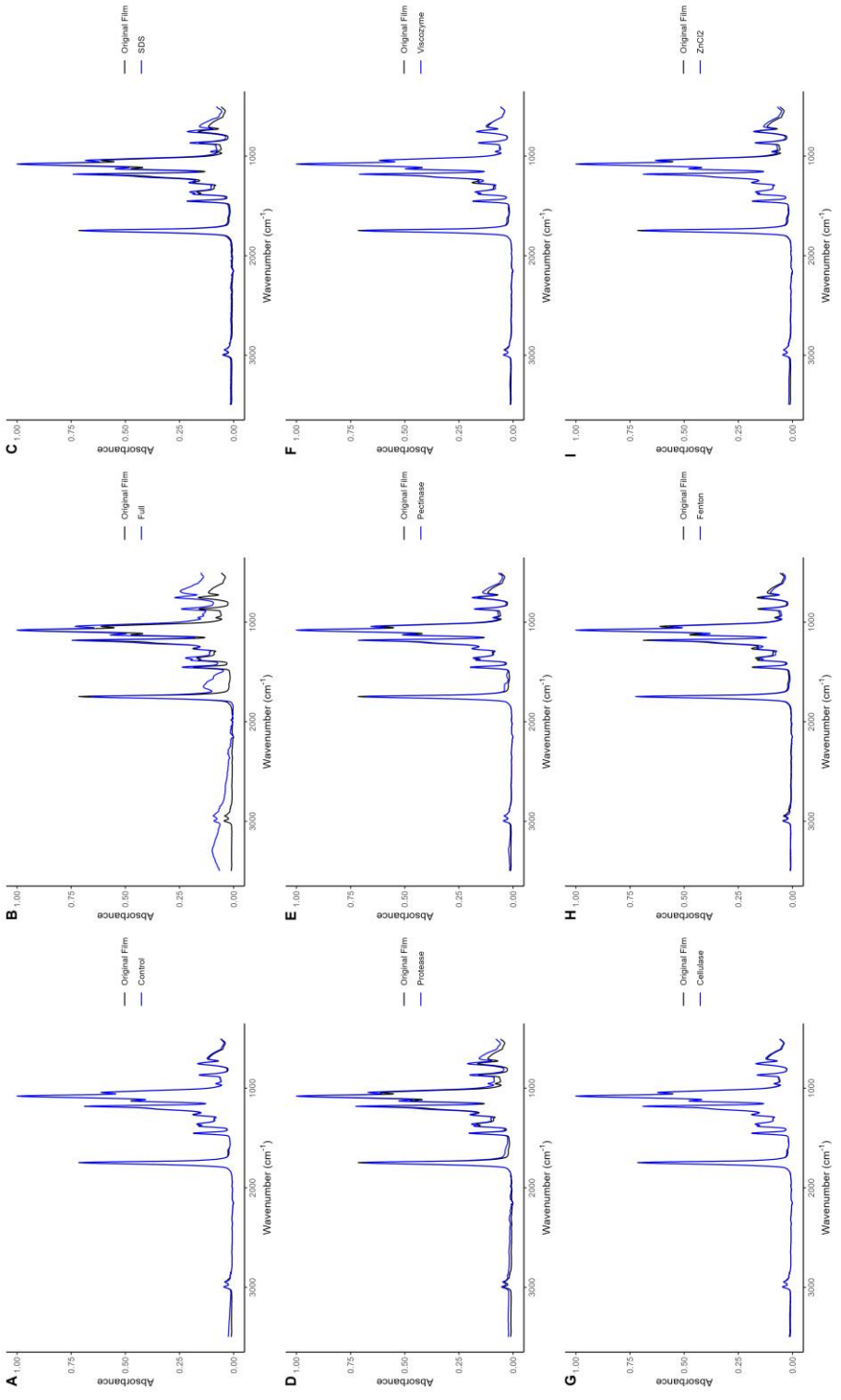


Figure S4.1. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of the original PLA film compared to the PLA films after exposure to the following treatments: (A) control, (B) full treatment sequence, (C) SDS, (D) protease, (E) pectinase, (F) viscozyme, (G) cellulase, (H) Fenton's reagent, and (I) $ZnCl_2$.

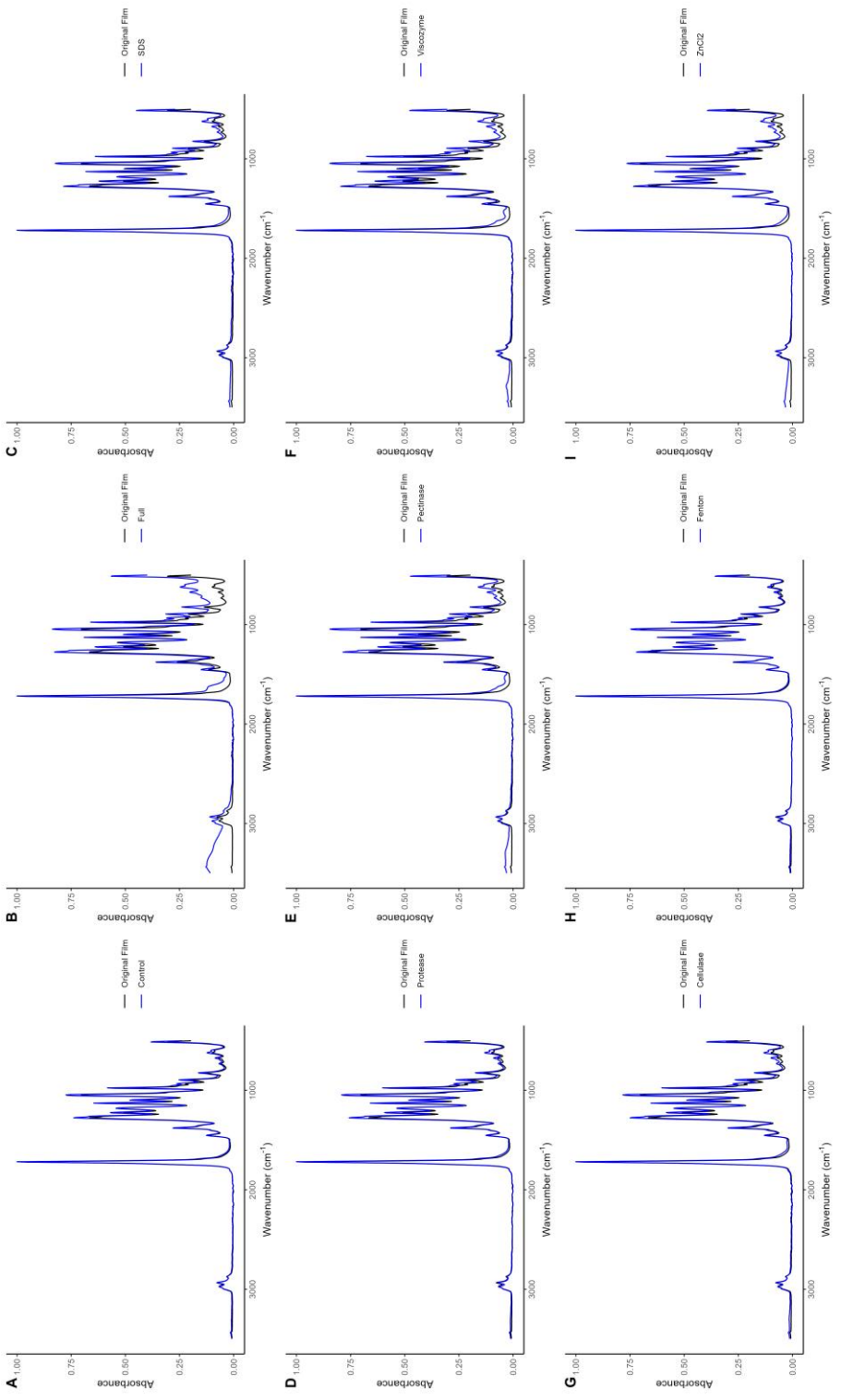


Figure S4.2. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra of the original PHB film compared to the PHB films after exposure to the following treatments: (A) control, (B) full treatment sequence, (C) SDS, (D) protease, (E) pectinase, (F) viscozyme, (G) cellulase, (H) Fenton's reagent, and (I) $ZnCl_2$.

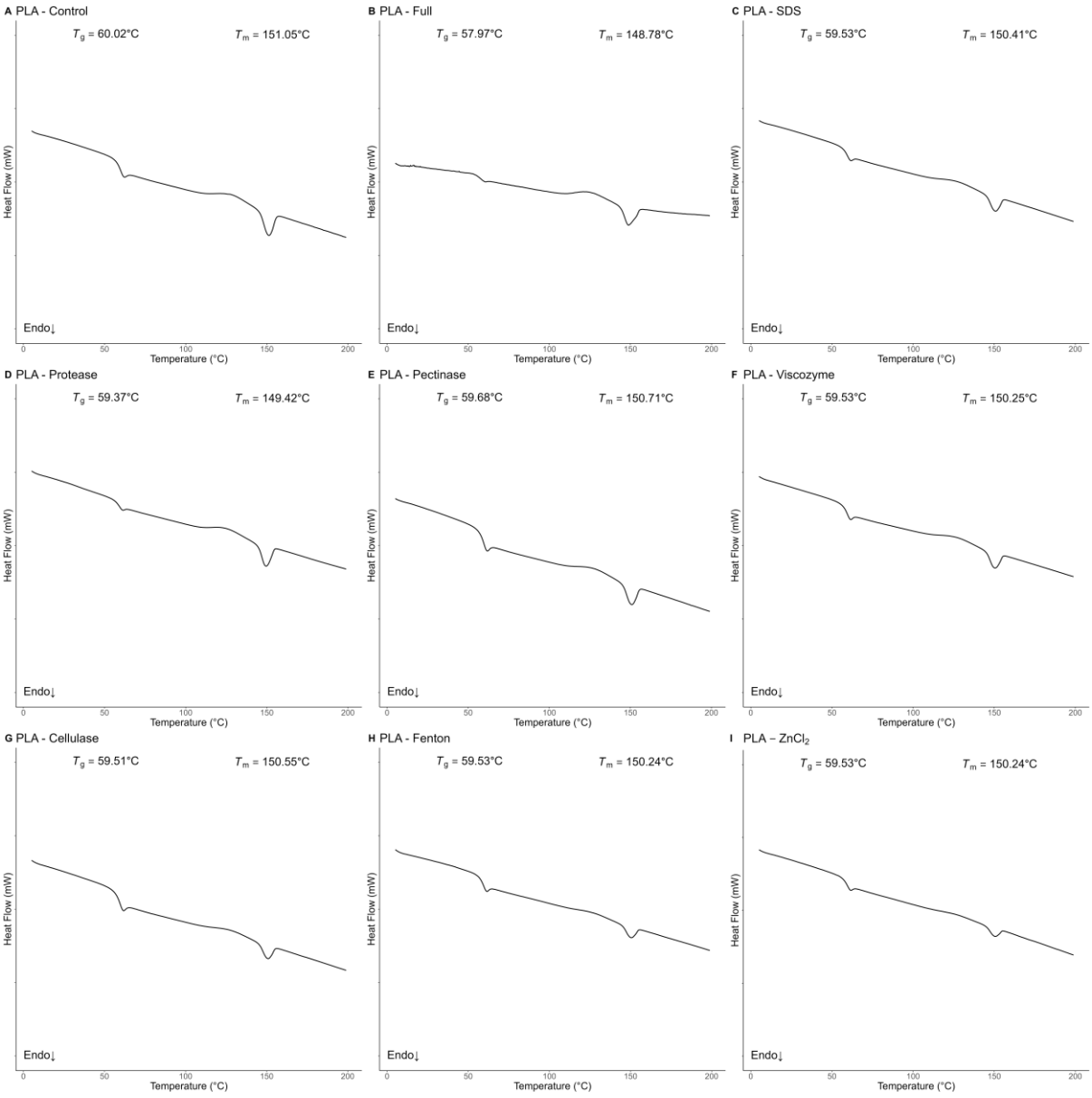


Figure S4.3. Heat flow curves measured by differential scanning calorimetry on the 2nd heat cycle of the PLA films after exposure to the following treatments: (A) control, (B) full treatment sequence, (C) SDS, (D) protease, (E) pectinase, (F) viscozyme, (G) cellulase, (H) Fenton's reagent, and (I) $ZnCl_2$.

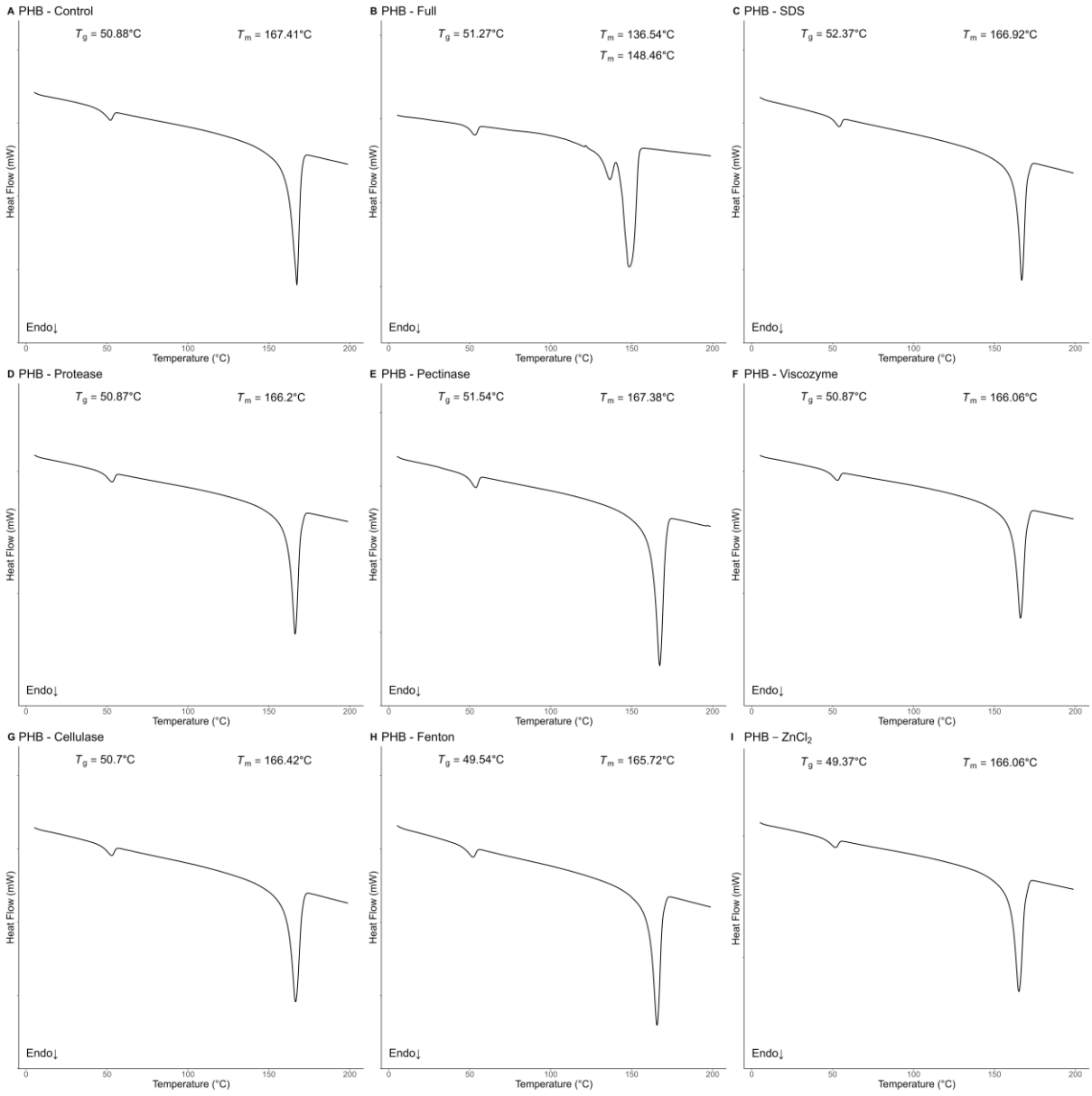


Figure S4.4. Heat flow curves measured by differential scanning calorimetry on the 2nd heat cycle of the PHB films after exposure to the following treatments: (A) control, (B) full treatment sequence, (C) SDS, (D) protease, (E) pectinase, (F) viscozyme, (G) cellulase, (H) Fenton's reagent, and (I) $ZnCl_2$.

4.7. References

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Chapter 5. Investigating the use of pyrolysis gas chromatography-mass spectrometry alongside respirometric measurements of evolved carbon dioxide to provide insights into the mechanisms of polymer biodegradation in soil.

This chapter has been prepared as manuscript for journal submission.

Authors and Affiliations

Grace Davies[1], Michaela K. Reay[2], Samantha Marshall[3], Charlotte Lloyd[2,4], Stefan Krause[1,5], and Iseult Lynch[1,6]

[1]School of Geography, Earth, and Environmental Sciences, The University of Birmingham, United Kingdom

[2]Organic Geosciences Unit, School of Chemistry, The University of Bristol, United Kingdom

[3]Product Safety, Jealott's Hill International Research Centre, Syngenta Limited, United Kingdom

[4]School of Geography, The University of Bristol, United Kingdom

[5]Birmingham Institute for Sustainability and Climate Action, The University of Birmingham, United Kingdom

[6]Centre for Environmental Research and Justice, The University of Birmingham, United Kingdom

CRediT author statement

Grace Davies: Conceptualisation, Methodology, Investigation, Formal Analysis, Visualisation, Writing Original Draft, Writing Review and Editing, Funding Acquisition. **Michaela Reay**: Methodology, Investigation, Formal Analysis, Visualisation, Writing Review and Editing. **Samantha Marshall**: Conceptualisation, Supervision. **Charlotte Lloyd**: Supervision. **Stefan Krause**: Conceptualisation, Funding Acquisition, Supervision. **Iseult Lynch**: Writing Review and Editing, Supervision.

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Abstract

Biodegradable polymers are increasingly being used in agricultural applications, and their biodegradability in the environment needs to be confirmed. Currently, standardised testing methods utilise respirometric endpoints such as evolved carbon dioxide (CO_2) as a proxy for biodegradation. However, respirometric methods were originally developed for small molecules, and so their suitability for the assessment of polymer biodegradation in soils in uncertain. Alternative analytical approaches are required to provide further insights into polymer biodegradation and the mechanisms involved. In this study, the biodegradation of polylactic acid (PLA) and polyhydroxybutyrate (PHB) was investigated by measurement of the CO_2 evolved, and in parallel, the polymers were extracted from soil sampled across the duration of the experiment and directly analysed by pyrolysis gas chromatography-mass spectrometry (Py-GC-MS). PLA did not biodegrade under the standardised test conditions, and this was confirmed by both CO_2 and Py-GC-MS analyses. PHB showed evidence of biodegradation, as confirmed by measured CO_2 evolution, and through direct identification of a reduction in the relative monomers over time by Py-GC-MS. Results demonstrated that Py-GC-MS could be useful as an additional method to identify polymer degradation within standard biodegradation studies, however there are limitations due to the small sample sizes required for Py-GC-MS. Overall, this work highlights the importance of verifying biodegradation as measured by respirometric endpoints, and the need to confirm the presence or absence of residual polymers after incomplete or complete biodegradation, respectively.

5.1. Introduction

Plastic is extensively used in agricultural applications, with an estimated 12.5 million tonnes used annually globally (FAO, 2021). However, most agricultural plastics are difficult to recover from the soil, resulting in significant soil pollution. Microplastics (particles <5mm) are of particular concern due to their numerous negative impacts on the environment and human health (Krause et al., 2024; Rahman et al., 2021; Rochman, 2018; de Souza Machado et al., 2018a, 2018b, 2019). Microplastics are either intentionally produced for use in agriculture (e.g., for the polymer encapsulation of plant protection products or fertilisers), or they are formed from the breakdown of larger plastics in the environment (e.g., plastic mulching films). Global concerns regarding plastic pollution have increased, as evidenced by recent efforts to form a global plastics treaty, and the recent wide-ranging restriction across multiple sectors on the use of intentionally added microplastics in the European Union (European Union, 2023; United Nations, 2024).

Biodegradable polymers have been proposed as alternatives to conventional non-biodegradable polymers, especially for agricultural plastics where poor recoverability results in soil being the end-of-life environment for plastic products. Biodegradation is the process whereby a material is metabolised by microbes to carbon dioxide (CO_2), water and microbial biomass under aerobic conditions (methane is formed under anaerobic conditions) (Chinaglia et al., 2018; Sander et al., 2023). The conversion of carbon from a polymer to carbon in CO_2 is termed mineralisation, however mineralisation and biodegradation are often utilised interchangeably in the literature. Polymer biodegradation is dependent on both the degradation environment and the intrinsic polymer structure (Sander et al., 2023; Suzuki et al., 2020). Intrinsic polymer properties that control biodegradation include molecular weight, chemical structure, degree of crystallinity, cross-linking, and the presence of polymer blends/additives. Environmental parameters influencing biodegradation include temperature, pH, soil water content, nutrient availability

and the presence of microbial communities of appropriate degraders (Emadian et al., 2017; Kumari et al., 2022, 2024). It is unlikely that all optimum conditions will be met in the environment, therefore, biodegradable polymers will not fully degrade to the expected products of microbial biomass, water and CO_2 under aerobic environmental conditions. Incomplete biodegradation can potentially result in the accumulation of biodegradable polymer-derived microplastics in soil (Fojt et al., 2020; Sintim et al., 2019). Recent work has shown the presence of microplastics derived from biodegradable polymers across environments, including soils (Liao and Chen, 2021; Qin et al., 2021; Sintim et al., 2019; Wei et al., 2022; Weinstein et al., 2020). Therefore, there are concerns regarding the environmental persistence and safety of biodegradable polymers (Agarwal, 2020; Lara-Topete et al., 2024; Sintim and Flury, 2017).

Whilst considerable research has been conducted into polymer degradation mechanisms in lab settings (e.g., from hydrolysis (Zaaba and Jaafar, 2020)), there has been comparatively little work into understanding the mechanisms of polymer degradation in environmentally relevant soil conditions. Understanding of the mechanisms of polymer (bio)degradation in soil, and the development of appropriate analytical methods to quantify biodegradation, will contribute to understanding if biodegradable polymers are likely to form persistent microplastics, or whether they will completely biodegrade in the soil environment.

Currently plastics are classed as “soil biodegradable” if they pass a standard test method, under specified conditions within a set time. ISO (International Organisation for Standardisation) 17556:2019 is a standard test for measuring the biodegradation of plastic in soil, ASTM (American Society for Testing and Materials) D5988-18 is also often used, and is considered an equivalent method (ASTM, 2018; Briassoulis and Mistriotis, 2018; Briassoulis and Degli Innocenti, 2017; ISO, 2019). These test methods utilise respirometric methods, by following either biological oxygen demand or evolved CO_2 to quantify

mineralisation as a proxy for biodegradation. However, the suitability of respirometric tests, which were developed and validated for small molecules, for measuring the biodegradation of polymers in environmental matrices has been criticised (Chapter 3).

Respirometric methods are commonly used to measure chemical biodegradation, using the OECD (Organisation for Economic Cooperation and Development) test guidelines, such as OECD 301 for ready biodegradability, or OECD 307 for measuring biodegradation in soils (OECD, 1992, 2002). These methods are generally used to measure the biodegradation of water soluble, low molecular weight molecules (Kowalczyk et al., 2015; McDonough et al., 2023). Polymers typically have a high molecular weight, are insoluble in water, and are less accessible for microbial degradation. The mechanisms of polymer biodegradation in soils are unknown, and the use of non-specific respirometric endpoints does not provide information about the degradation mechanisms, as it only measures mineralisation. However, a portion of the polymer will likely remain unmineralized, and could be bioassimilated by microbes, or remain in the soil as residual polymer. To become accessible for microbial uptake, polymers need to first be reduced in size through initial abiotic and/or biotic degradation. Therefore, it is not clear if all non-mineralised polymer will be bioassimilated, and so the fate of the residual polymer needs to be determined.

Recent work by Nelson et al. (2020) has demonstrated that a significant amount of polymer carbon remains in the soil as residual polymer, rather than being taken up by soil microbes, which cannot be determined from respirometric analyses alone. Additionally, there are concerns, especially for soil biodegradation tests, that the addition of a polymer will stimulate the decomposition of soil organic carbon (i.e., induce a soil priming effect) or alter microbial community activity through changes in nutrient or moisture availability. This may increase the soil-derived CO_2 produced, potentially resulting in the mis-estimation of polymer biodegradation when measured by respirometric endpoints alone. Priming effects

have been noted to be induced in several studies that have utilised a form of labelled carbon (e.g., ^{13}C or ^{14}C) which allows the source of the evolved CO_2 to be identified (Chen et al., 2014; Huo et al., 2023, 2024; Zhang et al., 2023; Zumstein et al., 2018). Therefore, there are concerns that respirometric methods measuring mineralisation alone are insufficient for quantification of polymer biodegradation. Furthermore, as only the end product of degradation is quantified (i.e., CO_2), there is no insight into the mechanisms of polymer biodegradation in soil, including the relative uptake of polymer carbon by soil microbes versus how much remains as a residual polymer that could be classified as a persistent microplastic.

The inclusion of additional direct analysis of polymer degradation in soils would improve the understanding of polymer biodegradation in the environment and increase confidence in the results from respirometric tests. Pyrolysis gas chromatography-mass spectrometry (Py-GC-MS) is a destructive thermoanalytical technique that can detect and quantify polymeric material via their characteristic pyrolysis products in different environmental matrices, including soil, with limited pre-treatment required (Picó and Barceló, 2020). Py-GC-MS has frequently been used for the identification and analysis of conventional polymers (e.g., polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC)) from environmental samples. Recent studies by Okoffo et al. (2022) and Fojt et al. (2022) have confirmed the effective identification of biodegradable polymers from soil using Py-GC-MS. Py-GC-MS has also been used as an analytical tool for the identification of the structure and thermal degradation products of biodegradable polymers (e.g., polylactic acid (PLA), polyhydroxyalkanoate (PHA), polycaprolactone (PCL), polybutylene adipate terephthalate (PBAT)) (Aoyagi et al., 2002; Arrieta et al., 2013; Khabbaz et al., 2000; Llana-Ruíz-Cabello et al., 2017; Okoffo et al., 2022; Sato et al., 2005; Westphal et al., 2001). However, there is little research into whether Py-

GC-MS would be a suitable method for investigating the degradation mechanisms of polymer biodegradation by microbial action in soils.

Py-GC-MS has been utilised in several studies with conventional polymers to investigate thermal and/or photo degradation, due to its high sensitivity allowing the observation of structural differences after degradation. Parres et al. (2007) investigated the degradation of PS caused by temperature and ultraviolet (UV) radiation using Py-GC-MS and found that the styrene trimer to monomer ratio decreased as exposure to UV increased. Therefore, during pyrolysis, the relative abundance of monomers formed increased compared to that from the unexposed PS (Parres et al., 2007). This same pattern has also been observed for PS when looking at the influence of molecular weight on the formation of thermal degradation products, with the trimer to monomer ratio decreasing as molecular weight decreased (Faravelli et al., 2001). UV degradation has also been studied in low density polyethylene (LDPE), high density polyethylene (HDPE), PP, and PS. Ainali et al. (2021) found that with prolonged UV exposure, the relative abundance of low molecular weight molecules (e.g., monomers) increased compared to higher molecular weight compounds (e.g., oligomers) following pyrolysis. Additionally, a study by Westphal et al. (2001) highlighted that Py-GC-MS can be used to identify indicators of degradation for the biodegradable polymer PLA; as after biological hydrolysis, the ratio of *meso*-lactide to *D,L*-lactide altered because microbes preferentially utilise *L*-lactide, rather than *D*-lactide. Therefore, as degradation occurs, the amount of *meso*-lactide will increase relative to *D,L*-lactide (Arrieta et al., 2014a; De Falco et al., 2023; Khabbaz et al., 2000; Reeve et al., 1994). Thus, the ratio of *meso*-lactide to *D,L*-lactide can be used as an indicator of the degradation of PLA (Arrieta et al., 2013, 2014c; Khabbaz et al., 2000; Westphal et al., 2001).

It is hypothesised that Py-GC-MS is a suitable method that can identify biodegradable polymers extracted from soil samples and quantify relative ratio changes that are indicative

of degradation, to provide greater insights into the mechanisms of polymer biodegradation in soils. PLA and polyhydroxybutyrate (PHB) are biodegradable polymers of interest, because they are both commonly used biodegradable polymers in agriculture, resulting in a requirement for their biodegradation in environmentally relevant soil conditions. PLA and PHB are used in plastic mulching films, whilst PHB is also used in plant protection products and fertilisers because it is suitable for polymer encapsulated controlled release systems (Boyandin et al., 2016; Kasirajan and Ngouajio, 2012a; Volova et al., 2016).

This work investigates the suitability of Py-GC-MS as an additional analysis that can be used alongside traditional respirometric measurements of biodegradation to improve our understanding of the mechanisms of polymer biodegradation in soils. The biodegradation of PLA and PHB in soil was measured by evolved CO_2 following the standard test method ISO 17556:2019 (ISO, 2019). Destructive soil samples were also removed throughout the study, for analysis of residual polymers by Py-GC-MS. It was hypothesised that Py-GC-MS would be able to directly identify non-labelled biodegradable polymers in the soil without the need for a complex extraction process. Evolved CO_2 and the relative ratios of pyrolysis products were analysed, and it was hypothesised that Py-GC-MS could provide information regarding the polymer structure which would improve the mechanistic understanding of the process of polymer biodegradation in soil. This study aimed to identify whether Py-GC-MS can facilitate confirmation of biodegradation of polymers in soil alongside respirometric methods during a standard test.

5.2. Methods

5.2.1. Materials

The tested polymers, microcrystalline cellulose (cellulose), poly-(R)-3-hydroxybutyrate (PHB), and polyethylene (PE), were supplied in powdered form by Sigma Aldrich. Polylactic acid (PLA) was supplied in powdered form by Magerial. Particle size was measured using a

MasterSizer 3000 fitted with an Aero S to disperse dry powder (Malvern Panalytical, Table 5.1). Theoretical CO_2 evolved ($ThCO_2$) was determined based on the polymer chemical formulae (Table 5.1, Equation S5.1).

Table 5.1. Measured characteristics of polymers utilised in the soil biodegradation test.

Polymer	Chemical Formula	Particle Size (µm) (D50)	$ThCO_2$ (mg CO_2 / mg)
Cellulose	$C_6H_{10}O_5$	19.9	0.444
Poly-(R)-3-hydroxybutyrate (PHB)	$C_4H_6O_2$	26	0.558
Polylactic acid (PLA)	$C_3H_4O_2$	217	0.5
Polyethylene (PE)	C_2H_4	40.8	0.856

Zinc chloride ($ZnCl_2$) was obtained as a powder (>98%, Alfa Aesar). High performance liquid chromatography (HPLC) grade solvents dichloromethane (DCM), methanol (MeOH), and hexane were purchased from Rathburn Chemicals Ltd. Standards of anthracene-d_{10}, fluorenone, androstane, and pyrene-d_5 were purchased from Sigma Aldrich, with >99% purity, to use as internal standards to normalise for any between-run variability. The deuterated standards (anthracene-d_{10} and pyrene-d_5) were 98 atom% D. Tetramethylammonium hydroxide (TMAH) 25 wt.% in MeOH was purchased from Sigma Aldrich.

5.2.2. Soil Biodegradation Test

Agricultural soil was sampled in November 2022 (18 Acres, 51°27'16.2828''N, 0°43'49.232''W). Soil characteristic measurements were recorded for percent sand, silt, and clay, bulk density, cation exchange capacity, moisture, organic carbon, organic matter, total, nitrogen, and pH by Agvise Laboratories (Table 5.2, Northwood, ND, USA).

Table 5.2. Soil (18 Acres) characterisation measurements for the biodegradation test.

Soil Characteristic	18 Acres

USDA textural class	sandy loam
Sand / Silt / Clay (%)	59 / 22 / 19
Bulk Density (g / cm^3)	1.09
Cation Exchange Capacity (meq / 100g)	14.5
Moisture at 10 kPa (%)	30.2
Organic Carbon (%)	2.8
Organic Matter (%)	4.8
pH	6.1
Total Nitrogen (%)	0.24
C:N ratio (C/N)	11.67

Polymer biodegradability was measured following the test guideline ISO 17556:2019 (ISO, 2019). Soil was sieved (2 mm) and then 100g (dry weight) aliquots were placed into test vessels, soil moisture was adjusted within 40-60% of the maximum water holding capacity. Soil vessels were then left to pre-incubate in the test conditions (20°C, complete dark), for two weeks. After the preincubation period, all soils were treated with 1250 mg of the relevant test material (Table 5.3). Test materials were applied using a spatula and mixed into the soil, the test then immediately started and evolved CO_2 was measured following ISO 17556:2019 for a test duration of 132 days (ISO, 2019). CO_2 evolution from all treatments were measured in triplicate throughout the test duration (on days 1, 3, 7, 10, 14, 21, 30, 37, 44, 59, 91, 105, 120, 132). Measurements were taken frequently during the first month when biodegradation was anticipated to be highest, then reduced in frequency.

A further 24 test vessels were set up for destructive sampling throughout the test duration, they contained soil treated with either PLA (n=12) or PHB (n=12). The destructive samples were removed in duplicate at specific timepoints (days 7, 14, 30, 58, 91, 120, 132) throughout the test for analysis by Py-GC-MS. Destructive samples were immediately frozen upon removal to prevent further biodegradation of polymers through microbial activity. All destructive samples were analysed at the same time, after completion of the biodegradation test. Cellulose and PE were not analysed destructively by Py-GC-MS because they acted as a positive and negative control, respectively, for biodegradation in the test system.

Table 5.3. Test vessel set up for a soil biodegradation test following ISO 17556:2019 with the inclusion of destructive samples.

Test Vessel	Soil (g)	Test Material	Amount of Test Material (mg)	CO_2 Evolution Replicates	Destructive Sampling Replicates
Blank	100	NA	0	3	0
Soil activity check	100	Cellulose	1250	3	0
Test material	100	Poly-(R)-3-hydroxybutyrate (PHB)	1250	3	12
Test material	100	Polylactic acid (PLA)	1250	3	12
Negative control	100	Polyethylene (PE)	1250	3	0
Technical control	0	NA	0	2	0

5.2.3. Pyrolysis Sample Preparation

All quartz pyrolysis tubes and quartz wool for pyrolysis analyses were furnaced at 1000°C for 2h before use to remove any potential organic contamination. Soil samples treated with PLA and PHB were prepared for analysis with Py-GC-MS by a density separation with $ZnCl_2$ (Supplementary Materials). Preliminary testing was completed to confirm recovery of polymers from the soil matrix. The sample processing steps required to isolate PHB from the soil matrix resulted in the aggregation of PHB. Therefore, the PHB samples were pyrolyzed as two parts, with a size fraction <500 µm and ≥500 µm. PLA samples did not aggregate and so were pyrolyzed as a single complete sample. Extracted particles were filtered onto glass fibre filters (2.2 µm, Whatman GF/D) and then transferred into a quartz pyrolysis tube for analysis. Aliquots were weighed (50 µg) into pre-cleaned quartz pyrolysis tubes using an analytical mass balance (Mettler Toledo) and covered with quartz wool to prevent sample loss. Four internal standards were added to monitor instrument performance and to normalise for potential variability between pyrolysis runs. The internal standard anthracene-d_{10} was prepared in hexane (0.1 mg ml^{-1}). The other internal standards, fluorenone, androstane, and pyrene-d_5, were prepared as a mixture in methanol (each at 0.1 mg ml^{-1}). Both internal standard solutions (4 µL each) were added to the quartz pyrolysis

tubes. PHB samples additionally had 2 µL of 25% TMAH in MeOH added as a thermochemolysis reagent for the methylation of pyrolysis products, with excess solvent evaporated at room temperature before analysis.

5.2.4. Pyrolysis Gas Chromatography Mass Spectrometry (Py-GC-MS)

Polymer identification was performed using a pyrolyzer equipped with an autosampler (Pyroprobe 6200, CDS Analytical), coupled to a gas chromatograph (GC) (TRACE 1310 Gas Chromatogram, Thermo Scientific) and a single quadrupole mass spectrometer (MS) (ISQ 7000 Mass Spectrometer, Thermo Scientific). The GC was fitted with a HP1 silica column (60 m x 0.32 mm inner diameter, 0.25 µm film thickness. 19091z-416, Agilent). All samples were pyrolyzed in single shot analysis, at a set temperature for each polymer of interest (Table 5.4).

Table 5.4. Pyrolysis conditions adopted for the Py-GC-MS analysis of the polymer samples.

Sample	Temperature (°C)	Duration (seconds)	Autosampler	Fan	Trap
Polylactic acid (PLA)	500	40	Y	Y	Y
Poly-(R)-3-hydroxybutyrate (PHB)	500	40	Y	Y	N

The inlet was held at 300°C with a split flow rate of 40 mL/minute and ratio of 20:1. The carrier gas was helium operated in constant flow mode at 2 mL/min. The GC programme started at 40°C for a 6-minute hold, then ramped up to 310°C at 20°C/min, and was then held at 310°C for 15 minutes. The MS transfer line and ion source temperature were set to 310°C with an ionisation voltage of 70 eV. MS range settings were 50-650 amu and the scan time was 0.2 seconds, with a delay time of 4 min.

PLA and PHB were analysed using different pyrolysis methods, due to previous method optimisation. PLA pyrolysis was tested at three temperatures (400°C, 500°C and 610°C); 500°C provided the optimal balance of pyrolysis to short chain and cyclic oligomers. Therefore, PLA was pyrolysed at 500°C, with a rate of 20°C/millisecond for 40 seconds,

operated in trap mode. Two different pyrolysis ramps were tested for PHB, 20°C/second and 20°C/millisecond; the slower ramp aided complete derivatisation of pyrolysis products by TMAH. Therefore, PHB samples were pyrolysed at 500°C, ramping at 20°C/second, then held for 40 seconds in direct mode.

The data acquisition and analysis were conducted using Xcalibur, version 4.1.31.9 (Thermo Fisher Scientific Ltd). Specific pyrolysis products were identified using pure reference samples for PLA and PHB, analysed under the same conditions by Py-GC-MS. The pyrolysis products were identified based on previous literature (De Falco et al., 2023; Okoffo et al., 2022), with the most abundant compounds being selected as indicators for the analysis. To determine changes in the abundance of different pyrolysis products, selected ions were extracted from the total ion chromatogram and integrated to calculate changes in the relative abundance using the ratio of pyrolysis products.

5.2.5. Contamination Controls

Laboratory contamination is critical for studies focusing on microplastic and polymer analyses, as contamination is a known issue. To minimise contamination sources, all equipment used for sample preparation and extraction was glass or metal. For the density separations, all glassware was thoroughly cleaned, then rinsed with DI water and 98% ethanol prior to use. All equipment was covered with aluminium foil to minimise airborne contamination. Where possible all steps were undertaken within a laminar flow hood (Purair LF Series, Air Science, UK). Samples were processed prior to Py-GC-MS analysis in a lab containing an air filter (Zero S, Winix, Europe) to minimise airborne contamination. Blank samples were run throughout the process, following each step in the density separation process to account for background contamination. To minimise contamination all pyrolysis sample preparation glassware was furnaced at 450°C for 2h prior to usage, the quartz pyrolysis tubes, and quartz wool were furnaced at 1000°C for 2h.

5.2.6. Statistical Analyses

All statistical analyses were conducted using R version 4.3.0 (R Core Team, 2024). A significance level of $p = 0.05$ was used for all tests. Data were assessed for normality, and for homogeneity of variance. The evolved CO_2 data were compared at each measurement time point. The data were log transformed to ensure normality, then analysed using the parametric one-way analysis of variance (ANOVA) followed by a Tukey post-hoc test to compare evolved CO_2 between treatments. Changes in the relative ratios of indicator ions measured by Py-GC-MS over time were assessed for statistical significance using a linear model.

5.3. Results

5.3.1. Polymer biodegradation measured by evolved carbon dioxide

Evolved CO_2 was measured for all polymer treatments and the soil blanks, and then the percent biodegradation was calculated (Figure 5.1). There was a statistically significant difference in the amount of CO_2 evolved between all treatments throughout the duration of the test (one-way ANOVA, $p<0.05$, Table S5.1).

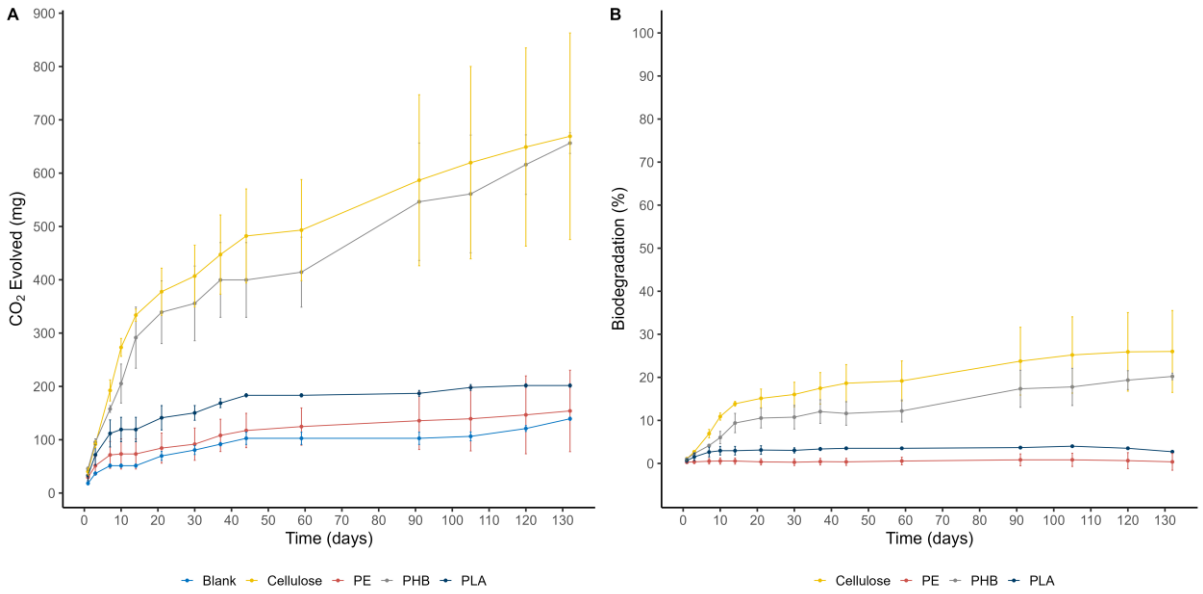


Figure 5.1. (A) CO_2 evolved from soil-blank, soil-cellulose, soil-PE, soil-PHB, and soil-PLA test vessels in 18 Acres soil. (B) Biodegradation of cellulose, PE, PHB, and PLA in 18 Acres soil. Data were collected over 132 days and is presented as the mean (n=3) standard deviation.

Evolved CO_2 was lowest in the soil-blank and soil-PE throughout the test, with no significant difference between these at any time point (Tukey post hoc, $p>0.05$, Table S5.1). The evolved CO_2 in the soil-cellulose and soil-PHB samples was the highest throughout the test, with both having significantly higher CO_2 evolution than the soil blank, and there was no significant difference between these polymer treatments throughout the test (Tukey post hoc, $p>0.05$, Table S5.1). At the end of the test (day 132), PHB had reached 20.22±0.62% biodegradation, which was not significantly different to cellulose biodegradation (26.01±7.77%) (Tukey post hoc, $p>0.05$, Table S5.2). CO_2 evolved from soil-PLA was not significantly different than the negative control soil-PE for days 1-14 of the incubation (Tukey post-hoc, $p>0.05$, Table S5.1). CO_2 evolved from soil-PLA was significantly higher than the soil-blank for days 1-59 of the incubation (Tukey post-hoc, $p<0.05$, Table S5.1). However, this could be attributed to the initial rapid increase in evolved CO_2 during the first two weeks. When calculating biodegradation from the evolved CO_2 values, PLA is recorded to have reached a maximum of 4% biodegradation in this study. PLA reached 3% biodegradation within the first 21-days of the test, and then took a further 84-days to reach 4%. PLA did not

have significantly different biodegradation from the negative control PE for the duration of the test (Tukey post-hoc, $p>0.05$, Table S5.2).

5.3.2. Polymer degradation measured by Py-GC-MS

Pyrolysis products

Specific pyrolysis products were selected for both PLA and PHB based on previous literature, and initial preliminary experimental work. The degradation products of PLA analysed by pyrolysis were previously identified (Table 5.5). Thermal degradation of PLA via intramolecular transesterification results in the main pyrolysis product of lactide ions (De Falco et al., 2023; Kopinke et al., 1996). The characteristic ions of the lactide isomers are *m/z* 43, 45, 56, and 144 (De Falco et al., 2023; Khabbaz et al., 2000; Tsuge et al., 2011). In this study, the ion *m/z* 56 was used as the characteristic ion for the *meso*-lactide and *D,L*-lactide isomers eluting at retention times of 12.85 and 13.10 minutes respectively (Figure 5.2).

Table 5.5. Pyrolysis products produced by the polymers PLA and PHB

Polymer	Derivatisation reagent	Pyrolysis Products	Molecular Ion	*m/z*
PLA	None	3,6-dimethyl-1,4-dioxane-2,5-dion (*meso* form) 3,5-dimethyl-1,4-dioxane-2,5-dion (*DL* form)	144	43, 45, 56, 144
PHB	TMAH	methyl 3-butenoate methyl cis-2-butenoate methyl trans-2-butenoate	100	41, 59, 69
		4-methoxy-4-oxobutan-2-yl bu-3-enoate 4-methoxy-4-oxobutan-2-yl trans-but-2-enoate 4-methoxy-4-oxobutan-2-yl cis-but-2-enoate	186	41, 59, 67, 69, 85, 117, 127, 154

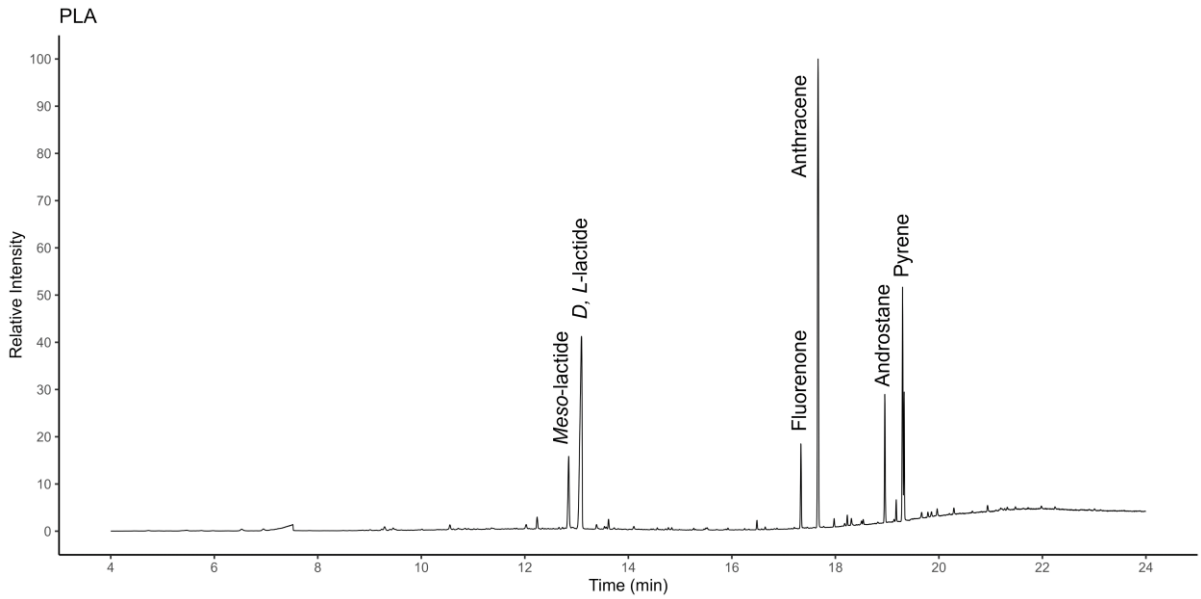


Figure 5.2. Py-GC-MS chromatogram at 500°C of polylactic acid (PLA), with the inclusion of internal standards (fluorenone, anthracene, androstane, and pyrene) to monitor instrument performance.

PHB samples were measured by pyrolysis with TMAH addition for methylation of pyrolysis products. The main degradation product in pyrolysis is methyl butenoate, with a molecular ion of *m/z* 100. There are three possible isomers: methyl 3-butenoate, methyl cis-2-butenoate, and methyl trans-2-butenoate, which all have a molecular ion of *m/z* 100, and fragment ions of *m/z* 69, 59, and 41 (Hocking, 2011; Sato et al., 2005; Tsuge et al., 2011). In this study, the ion *m/z* 69 was used as the characteristic ion associated with methyl 3-butenoate, methyl cis-2-butenoate, and methyl trans-2-butenoate isomers occurring at retention times of 6.16, 6.63, and 7.30 minutes respectively (Figure 5.3). The *m/z* 67 ion was used as the characteristic ion associated with the PHB dimers, with 4-methoxy-4-oxobutan-2-yl bu-3-enoate, 4-methoxy-4-oxobutan-2-yl trans-but-2-enoate, and 4-methoxy-4-oxobutan-2-yl cis-but-2-enoate eluting at retention times of 14.79, 15.08, and 15.35 minutes respectively (Figure 5.3). Structures of pyrolysis products are presented in Appendix E.

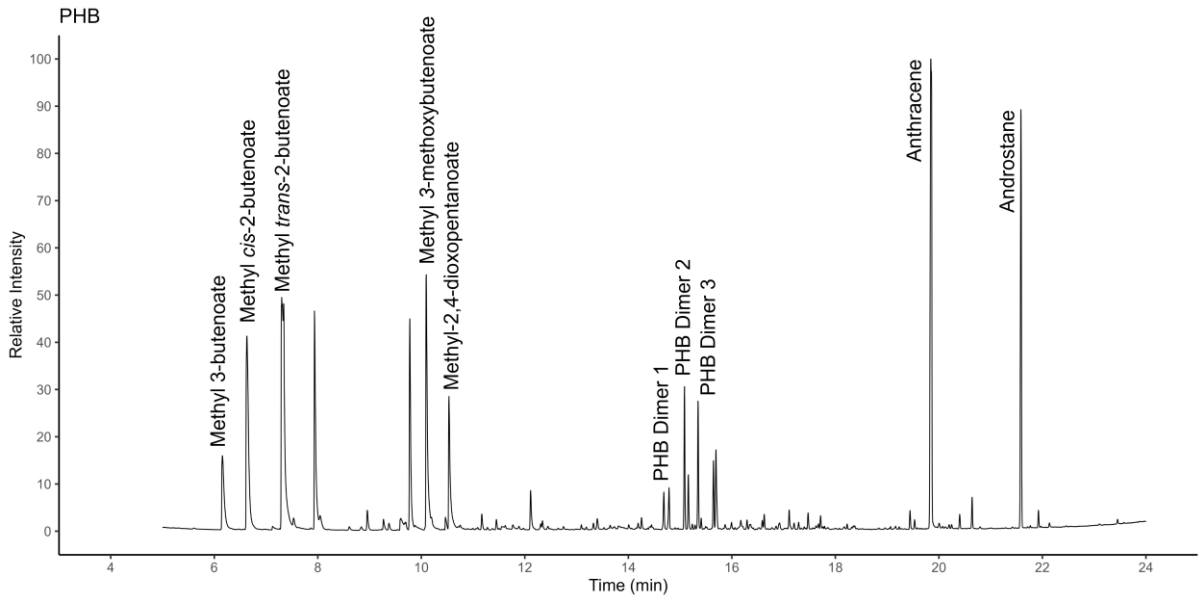


Figure 5.3. Py-GC-MS chromatogram at 500°C of polyhydroxybutyrate (PHB) with tetramethylammonium hydroxide (TMAH) addition, and with the inclusion of internal standards (anthracene and androstane) to monitor instrument performance. PHB dimers are (1) 4-methoxy-4-oxobutan-2-yl bu-3-enoate, (2) 4-methoxy-4-oxobutan-2-yl trans-but-2-enoate, and (3) 4-methoxy-4-oxobutan-2-yl cis-but-2-enoate.

Indicator compound analysis can be subject to background matrix interference if there is co-elution of characteristic *m/z* ions that are used for quantification. Therefore, blank soil samples were also processed and pyrolyzed, results were analysed for all relevant indicator compounds, and no background interference was noted.

Relative ratio comparisons of Py-GC-MS products

The ratio of *meso*-lactide to *D,L*-lactide in PLA samples was compared between time points, which has previously been shown to be indicative of PLA degradation (Khabbaz et al., 2000; Westphal et al., 2001). The ratio of *meso*-lactide to *D,L*-lactide did not significantly change throughout the duration of the incubation experiment, therefore no degradation of PLA was evident (Figure 5.4, linear model: $F_{1,12} = 3.804$, $p = 0.0749$).

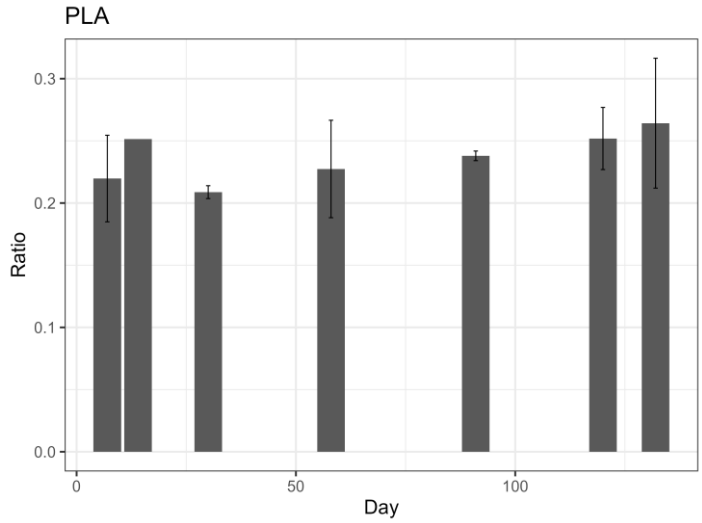


Figure 5.4. Average ratio of *meso*-lactide to *D,L*-lactide measured throughout the soil incubation period in the destructive soil samples. Data is presented as mean ± standard deviation, for day 132 (n=3), Day 14 (n=1), all other days are n=2.

In comparison to PLA, there is significantly less literature focused on Py-GC-MS pyrolysis products of PHB, and their potential to monitor degradation. Therefore, the ratio of monomers to oligomers in PHB samples was compared to determine if there was a relative change over time, analogous to previous studies for conventional polymers (Faravelli et al., 2001; Parres et al., 2007). The ratio of monomers to oligomers measured for the >500 µm size fraction did significantly change throughout the duration of the incubation experiment (Figure 5.5, linear model: $F_{1,12} = 8.24$, $p < 0.05$). Therefore, the number of oligomers is increasing relative to the number of monomers during the soil incubation.

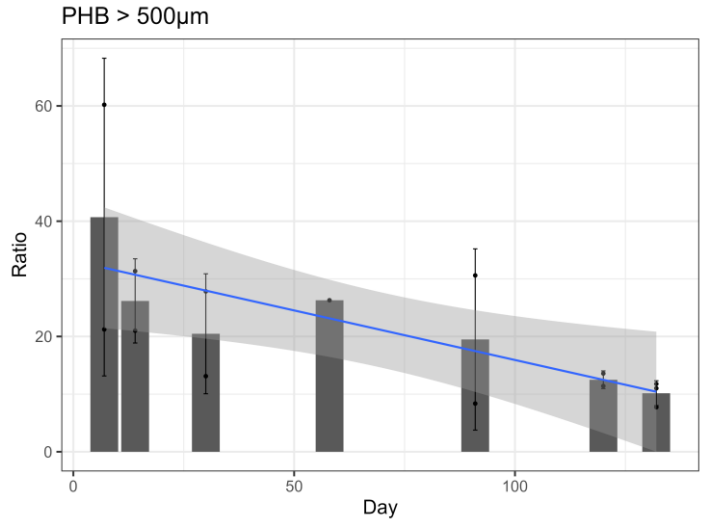


Figure 5.5. Average ratio of PHB monomers to oligomers measured throughout the soil incubation in the destructive soil samples (size fraction: >500 µm). Data is presented as mean ± standard deviation, for day 132 (n=3), Day 58 (n=1), all other days are n=2.

The ratio of monomers to oligomers measured for the <500 µm size fraction did not significantly change throughout the duration of the incubation experiment (Figure 5.6, linear model: $F_{1,12} = 1.535$, $p = 0.239$).

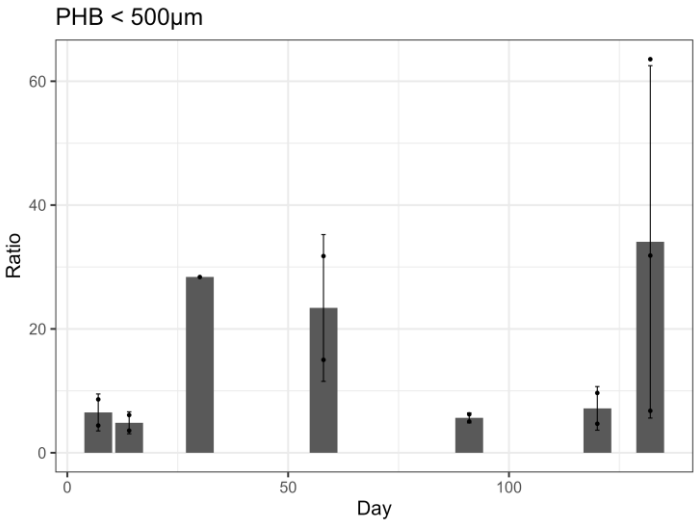


Figure 5.6. Average ratio of PHB monomers to oligomers measured throughout the soil incubation in the destructive soil samples (size fraction: <500 µm). Data is presented as mean ± standard deviation, for day 132 (n=3), Day 30 (n=1), all other days are n=2.

5.4. Discussion

5.4.1. Minimal biodegradation of PLA was observed due to environmental conditions, and evolved CO_2 measurements can be influenced by the soil priming effect

Minimal biodegradation of PLA (4%) was observed via the evolved CO_2 measurements, and there were no changes in the relative ratio of lactide stereoisomers throughout the duration of the test meaning that no degradation of the PLA polymer was noted. The ratio of *meso*-lactide to *D,L*-lactide has been used as a measure of PLA degradation by Py-GC-MS in several studies, where the relative quantity of *D,L*-lactide has been found to reduce with respect to the *meso*-lactide, which is attributed to microbes preferentially degrading the *L*-lactide form (Arrieta et al., 2013, 2014a, 2014c, 2014b; Khabbaz et al., 2000; Westphal et al., 2001). However, it is critical to note the environmental conditions under which previous studies were conducted because biodegradation is controlled by both the environment and polymer structure. Westphal et al. (2001) found that the relative amount of *D,L*-lactide decreased compared to *meso*-lactide after exposure of PLA to biological hydrolysation in a salt medium with addition of microorganisms from compost incubated at 30°C. Arrieta et al. (2014a) looked at the disintegration of PLA and PLA-PHB nanocomposite film blends under composting conditions at 58°C, also finding a significant change in the ratio of *meso*-lactide and *D,L*-lactide stereoisomers.

PLA is known to be biodegradable under composting conditions, where initial abiotic hydrolysis occurs, and temperature is an important factor controlling biodegradation (Castro-Aguirre et al., 2017; Stloukal et al., 2015). The microbial community in compost is more biologically active than that of soil, with a different range of microbes that are suited to higher temperatures (Castro-Aguirre et al., 2017). As the present study was performed at a constant temperature of 20°C, with a microbial community sourced from natural agricultural soil, the environmental conditions are therefore considered unfavourable for

the biodegradation of PLA. Direct analysis of the polymer structure by Py-GC-MS indicated that minimal microbial degradation occurred. However, the evolved CO_2 measurements suggest that 4% of the PLA biodegraded. Given that the soil environmental conditions do not promote PLA biodegradation, and limited degradation was confirmed by the direct polymer analysis with Py-GC-MS, it is important to consider the accuracy and reliability of the evolved CO_2 measurements.

In this study the measurement of evolved CO_2 is an indirect measurement as non-labelled polymers are used; this means that the source of the carbon in the evolved CO_2 cannot be distinguished between polymer-derived and soil-derived. Therefore, the evolved CO_2 measured can also arise from background soil carbon mineralisation. Whilst the inclusion of a soil blank control is supposed to account for this due to the blank correction of evolved CO_2 measurements prior to the calculation of biodegradation percent, recent research suggests that this is insufficient. The addition of a carbon substrate to soil can result in a change in the decomposition of soil organic matter, known as the priming effect, whereby soil microbial activity can be either stimulated (positive priming) or reduced (negative priming) (Bastida et al., 2019; Kuzyakov et al., 2000; Liu et al., 2020; Shen and Bartha, 1996). The priming effect has previously been experimentally measured with respect to microplastics, where the addition of degradable microplastics to soil resulted in a positive priming effect (Guliyev et al., 2023; Huo et al., 2023, 2024; Zhang et al., 2023). Whilst in this study non-labelled polymers were used, the inclusion of the known non-biodegradable polymer PE acts as a negative control, representing the changes that occur due to the addition of a substrate. Throughout the test the measured biodegradation did not significantly differ between PLA and PE, and the evolved CO_2 measured was not significantly different during the first two weeks of the incubation test. However, both showed a different pattern of evolved CO_2 compared to the soil blank, with a rapid increase in CO_2 emissions at the start of the test. As PE was the negative control, that is not

biodegradable, it can be concluded that PLA did not biodegrade either during this test. Therefore, the increase in evolved CO_2 is likely due to a soil priming effect induced by the addition of the plastics. As the Py-GC-MS analysis provides a direct measurement of the lack of chemical structural changes, this is further evidence to support the conclusion that PLA did not biodegrade under the experimental conditions used in this study.

This is an important finding to highlight, as PLA is included within many agricultural products e.g., plastic mulching films (Briassoulis and Giannoulis, 2018; Jeszeová et al., 2018; Kasirajan and Ngouajio, 2012b; Monkley et al., 2024). However, as PLA biodegradation is reliant on specific environmental conditions if these are not met, then PLA could instead form persistent biodegradable microplastics. Biodegradable microplastics have been found to have both particle toxicity effects and toxicity associated with the release of additives (Tao et al., 2024). Whilst research into the toxicity of biodegradable plastics is less comprehensive than conventional plastics, several studies found evidence of negative impacts of microplastics from biodegradable plastics on organisms and ecosystems, including within soils (Sun et al., 2023; Tao et al., 2024; Wang et al., 2023; Yang and Gao, 2022; Yu et al., 2023)

5.4.2. Insights into PHB biodegradation mechanisms by Py-GC-MS

PHB biodegradation was measured by CO_2 evolution and was comparable to the biodegradation of the positive control cellulose in this test study. The Py-GC-MS analysis of PHB was conducted on two separate size fractions for each sample (<500 µm and ≥500 µm) due to the aggregation of PHB particles during sample preparation for analysis. For PHB ≥500 µm, there was a significant decrease in the ratio of monomers to oligomers over time, whereas the pattern was less clear for PHB <500 µm, with highly variable monomer to oligomer ratios observed. This suggests that the degradation was not homogenous.

In contrast to PLA, there is less research into the biodegradation of PHB determined by Py-GC-MS in soil environments. PHB is an aliphatic polyester that is produced by biological organisms, and it is susceptible to microbial biodegradation (Altaee et al., 2016; Martínez-Tobón et al., 2018; Savenkova et al., 2000). Julinová et al. (2023) conducted a biodegradation test for PHB in soils following ISO 17556:2019 (the same test as used in this study) and complemented this by the inclusion of multiple chemical analyses (microscopy, gel permeation chromatography (GPC), differential scanning calorimetry (DSC), and X-ray diffraction (XRD)). Based on multiple analyses, Julinová et al. (2023) concluded that PHB follows a multistage biodegradation, with polymer crystallinity being a key determining factor that controlled the rate and extent of biodegradation, especially at the initial stages, with the amorphous regions being preferentially degraded. Tomasi et al. (1996) also found that crystallinity was a controlling factor of biodegradation, with the rate decreasing as crystallinity increases. Whilst this study did not measure polymer crystallinity after biodegradation, the initial crystallinity of the polymer was high and in previous work of this thesis, the PHB increased in crystallinity after exposure to biological enzymes (Chapter 4).

Recent research has considered the mechanisms of PHB degradation, with the average molecular weight of PHB noted to increase after a 60-day soil incubation, therefore suggesting that soil microbes were able to use chain-end low molecular weight compounds, but that higher molecular weight fractions were more inaccessible (Julinová et al., 2023). The same result was found by Park et al. (2022), where the lower molecular weight fractions of PHB biodegraded faster than higher molecular weight fractions in a marine environment (Park et al., 2022). In this study, the ratio of monomers to oligomers decreased over time, which could have been caused by the relative decrease of lower molecular weight polymers. This is consistent with previous findings, where monomers are more likely to be biodegraded by soil microbial communities, because they are more easily assimilated and thus more accessible for biodegradation than oligomers (Julinová et al., 2023). This result

highlights how direct analyses of polymers in soil can improve our understanding of polymer properties and (bio)degradation mechanisms.

5.4.3. Limitations and Future Work

ISO17556:2019 has previously been considered to have poor reproducibility, with variations allowed in the testing conditions that can result in differences in the rate and amount of biodegradation (Chapter 3, Briassoulis and Mistriotis, 2018; Chinaglia et al., 2024, 2018; Pischedda et al., 2019; Tosin et al., 2019). In this study, whilst the biodegradation of cellulose and PHB was consistent, neither reached the validity level of 60% specified in the ISO 17556:2019 guideline. However, as this study was designed to facilitate the collection of subsequent samples for further analysis, it was not run for the period of six months suggested by ISO 17556:2019. Previous research utilising the same soil (18 Acres) demonstrated a similar slow rate of cellulose biodegradation measured by CO_2 evolved and 60% degradation was achieved by 223 days (Chapter 3), which is significantly longer than the 132-days that the biodegradation was measured in this study. Therefore, it is expected that cellulose and PHB would have reached 60% biodegradation if the test had continued.

The biodegradation of polymers in soil is a complex process, and current standardised testing methods have several limitations. Firstly, respirometric methods such as the measurement of evolved CO_2 only provide information on the amount of carbon that has been mineralised by microorganisms. This does not confirm that the mineralised carbon comes from the polymer source. It would only be possible to directly attribute the CO_2 to the polymer if a form of labelled carbon was used (Nelson et al., 2022; Sander et al., 2019, 2023). Additionally, polymer biodegradation is a complex and lengthy process, where a significant amount of non-mineralised polymer carbon can either remain in the soil as a residual polymer or utilised by soil microbes (Nelson et al., 2020; Zumstein et al., 2018). Further work is required to determine the relative distribution of the polymer carbon, as

residual polymer versus in soil microbes, especially in the context of persistent soil pollution with microplastics.

Additionally, the recommended amount of sample (12.5 mg test polymer per 1 g of soil) is thought to be higher than would be expected to be found in the environment. PLA mineralisation has previously been assessed by source-partitioning of evolved CO_2 using ^{13}C-PLA at different concentrations (0.1%, 1%, and 5%) in different soils, with cumulative CO_2 emissions increasing with increased PLA concentration (Yu et al., 2024). Yu et al. (2024) found that measurements of mineralisation by evolved CO_2 alone mis-estimated PLA mineralisation compared to using ^{13}C-labelling approaches as evolved CO_2 measurements did not consider the soil priming effect (Yu et al., 2024). Carbon mineralisation in soils has also been observed to be size dependent, with smaller microplastics (<250 μm) causing greater overall mineralisation than larger microplastics (250 – 4750 μm) in a soil biodegradation test (Huo et al., 2024). Additionally, it is important to consider how the addition of a biodegradable polymer to soils would impact the soil microbial community. Microbial activity in soils is typically carbon limited, and the addition of available carbon can therefore cause changes in the soil microbial community activity (Soong et al., 2020; Zhou et al., 2021). Microplastic addition has also been noted to alter the soil structure for example through aggregate formation and can influence the composition of soil bacteria (Han et al., 2024; Zhou et al., 2021).

It is important therefore to determine if complete biodegradation has occurred in soils, and if it has not, how much residual polymer remains as persistent microplastics, or how much has been partially degraded and to identify what the degradation products are. However, there is still a significant knowledge gap in understanding how to effectively isolate biodegradable polymers from soil matrices for subsequent analyses, as most extraction methodologies have been developed for conventional polymers and can induce

degradation in biodegradable polymers (Chapter 4). Therefore, the inclusion of direct analysis methods of microplastics could be important to improve the understanding of the mechanisms of polymer biodegradation. In this work Py-GC-MS has been explored as a potential analytical method to do this. A limitation of Py-GC-MS is that it does not allow for large sample sizes, within this study 50 µg sub-samples were analysed, therefore, this makes any quantifications unreliable for extrapolation. The small sample size could explain the observed variability in the PHB samples between the size fractions. Future work would benefit from an increased number of pseudo-replicates to determine within sample variability. Furthermore, duplicate samples were destructively sampled for analysis by Py-GC-MS, it would be important to increase this to at least triplicate in future studies. Additionally, the PHB samples were analysed as >500 µm and <500 µm fractions, as the initial protocol for separating polymers from the soil resulted in the aggregation of PHB particles. The focus of this work is not to develop an effective extraction protocol for the isolation of biodegradable MPs from soil, but to explore if Py-GC-MS can be used to compare the relative ratio of pyrolysis products to quantify the degree of polymer degradation and the mechanism(s) of degradation by a direct analysis of the residual polymer. Therefore, the aggregation is noted but further exploration of this, and how to overcome it, was beyond the aims of this work. However, this is an important consideration for future work with PHB which may require pre-treatment steps.

Overall, respirometric methods provide insight into the degree of mineralisation of biodegradable polymers but do not provide information regarding the mechanisms of microbial biodegradation of polymers in soil. Pyrolysis represents an approach to determine the degree of degradation for residual polymers, although it cannot be upscaled to provide a percentage degradation at this stage, due to sub-sample variation. Further work should explore whether differing conditions results in different degradation patterns observed via pyrolysis to shed further light on the mechanisms of polymer biodegradation.

Both techniques (evolved CO_2 and Py-GC-MS) yield different insights into the degradation of biodegradable polymers, and it is suggested that a multiple-approach strategy may be required to explore the degree and mechanisms of degradation.

5.5. Conclusion

Ensuring the complete biodegradation of polymers in soil is important for the effective replacement of conventional plastic products to reduce microplastic pollution in soils. However, current methods based on respirometry do not provide a complete understanding of whether a polymer has biodegraded fully and does not account for potential residual polymer in the soil. Respirometric methods are also subject to interference from priming effects, that can lead to misestimation of the degree of biodegradation. The inclusion of additional analyses is needed to determine if persistent microplastics remain in the soil especially under real environmental conditions, and to understand the mechanism of polymer biodegradation in soil in more depth. Py-GC-MS has been explored as a potential method for the direct analysis of the fate of biodegradable polymers in soils. Py-GC-MS can detect alterations in the relative ratios of different pyrolysis products that can be used to indicate the degree of degradation a polymer undergoes; however, it is likely that this needs to be used in conjunction with other analytical methods in the future to further develop our understanding of polymer biodegradation in soils.

5.6. Supplementary Materials

5.6.1. Supplementary Calculations and Statistics

Calculation of the theoretical amount of evolved carbon dioxide

The theoretical amount of carbon dioxide evolved from biodegradation of a substance can be calculated by Equation S5.1, where m is the mass of test material introduced into the test system (milligrams, mg), w_c is the carbon content of the test material, expressed as a mass fraction, 44 is the relative molecular mass of carbon dioxide, and 12 is the relative molecular mass of carbon (ISO, 2019).

$$ThCO_2\,(mg) = \frac{44}{12} \times m \times w_c$$

[Equation S5.1]

Calculation of percentage biodegradation (%)

The percentage biodegradation, D_t, for each test flask can be calculated from the amount of carbon dioxide evolved during each measurement interval by Equation S5.2, where $\sum m_T$ is the amount of carbon dioxide, in milligrams, evolved in the test flask between the start of the test and time *t*, $\sum m_B$ is the amount of carbon dioxide, in milligrams, evolved in the blank control flask between the start of the test and time *t*, $ThCO_2$ is the theoretical amount of carbon dioxide, in milligrams, evolved by the test material.

$$Dt\,(\%) = \frac{\sum m_T - \sum m_B}{ThCO_2}\; x\; 100$$

[Equation S5.2]

Table S5.1. Statistical significance of one-way ANOVA Tukey post hoc tests for the comparisons of CO_2 evolved from soil-blank, soil-PE, soil-cellulose, soil-PHB, and soil-PLA at each of the measurement days during the respirometric biodegradation test.

Day	1	3	7	10	14	21	30	37	44	59	91	105	120	132
PE-blank	0.123	0.479	0.427	0.419	0.427	0.855	0.966	0.827	0.924	0.734	0.748	0.823	0.976	1.000
PLA-blank	<0.05	<0.05	<0.01	<0.01	<0.01	<0.05	<0.05	<0.01	<0.05	<0.05	0.067	0.072	0.175	0.425
PHB-blank	<0.01	<0.01	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00
cellulose-blank	<0.01	<0.01	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00

PLA-PE	0.972	0.351	0.094	0.074	0.077	<0.05	<0.05	<0.05	<0.05	0.091	0.378	0.331	0.378	0.489
PHB-PE	0.123	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PLA-PHB	0.290	0.435	0.246	0.058	<0.01	<0.01	<0.01	<0.001	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PE-cellulose	0.370	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PHB-cellulose	0.928	0.999	0.748	0.465	0.908	0.952	0.884	0.909	0.685	0.749	0.998	0.994	1.000	1.000
PLA-cellulose	0.691	0.554	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.01	<0.01

Table S5.2. Statistical significance of one-way ANOVA Tukey post hoc tests for the comparisons of percent biodegradation for PE, cellulose, PHB, and PLA at each of the measurement days during the respirometric biodegradation test.

Day	**1**	**3**	**7**	**10**	**14**	**21**	**30**	**37**	**44**	**59**	**91**	**105**	**120**	**132**
PE-cellulose	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.001	<0.001
PHB-cellulose	1.000	0.765	<0.05	<0.01	<0.05	<0.05	0.052	0.081	<0.05	0.052	0.369	0.330	0.392	0.502
PHB-PE	<0.05	<0.01	<0.01	<0.001	<0.001	<0.001	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05	<0.01	<0.01
PLA-cellulose	0.288	0.083	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.01	<0.01
PLA-PE	0.440	0.093	0.050	0.082	0.192	0.270	0.401	0.454	0.488	0.563	0.868	0.863	0.881	0.932
PLA-PHB	0.296	0.314	0.181	<0.05	<0.01	<0.01	<0.01	<0.01	<0.05	<0.05	<0.05	<0.05	<0.05	<0.01

5.6.2. Preliminary Experiment: Density separation of PLA and PHB from soil, and subsequent analysis by pyrolysis gas chromatography-mass spectrometry (Py-GC-MS)

A preliminary experiment was conducted, which is described in detail here. First the general methodology for the density separation step is described (5.6.2. i). Then the initial calibrations that were completed to be used for PLA and PHB quantification by Py-GC-MS are outlined (5.6.2. ii). Then, these methods were used in a preliminary recovery experiment (5.6.2. iii and 5.6.2. iv).

5.6.2. i) Density separation with zinc chloride

All sample processing and filtration steps were carried out under a laminar flow hood (Purair LF Series, Air Science, UK). Zinc chloride ($ZnCl_2$) solution (1.8g cm^3) was prepared, then

filtered through a 2.2 µm glass microfibre filter (Whatman) prior to use. Soil samples were then wet sieved with deionised water through a sieve cascade with mesh sizes of 2 mm and 500 µm into a clean 1L glass beaker. Material >2 mm was discarded, material >500 µm was retained for further analysis. Material <500 µm was kept in the clean 1L glass beaker, covered with aluminium foil, and left overnight to facilitate sedimentation. After a 12h sedimentation, the soil settled at the bottom of the beaker, leaving a supernatant. The supernatant was then removed by filtration over a 10 µm stainless steel filter (Rolf Körner, Germany). This filter was retained and stored in a clean glass jar covered with aluminium foil, with the same filter being used per sample throughout all subsequent filtration steps.

$ZnCl_2$ (200 mL) was then added to the 1L glass beakers containing the sedimented soil, then using a stainless-steel spoon the mix was agitated to resuspend all soil. A further addition of $ZnCl_2$ (200 mL) occurred, and the mixture was stirred on a magnetic stir plate for 5 minutes. After stirring, the sample was then transferred into a 1L glass separatory funnel, and a further 400 mL of $ZnCl_2$ was added. The sample was continuously stirred on addition to the separatory funnel to facilitate suspension of particles. Samples were then left for 24 hours to sediment. After 24 hours, the separatory funnels were slowly opened to remove the sedimented material from the bottom of the funnel and collected into a clean beaker. This is then transferred into a second density separation funnel for a second density separation. Removal of the sedimented material is done slowly to not resuspend any sediment; only the upper fraction of $ZnCl_2$ where microplastic particles will be suspended is retained. After the removal of all sedimented material, the upper fraction of $ZnCl_2$ was then filtered (10 µm metal filter) to retain suspended particles. The final sample was then transferred by filtration onto GF/D filters (pre cleaned in a furnace oven at 550°C for 4 hours) and weighed, then stored in a fridge at 4°C prior to Py-GC-MS analysis.

5.6.2.ii) PLA and PHB Calibrations for Quantification with Py-GC-MS

Prior to sample analysis, a series of test calibration samples were run to determine if Py-GC-MS could be used to quantify the polymers. PLA was dissolved in 50 mL dichloromethane (DCM) to make a 1.08 mg ml^{-1} solution. The PLA solution was then added into quartz pyrolysis tubes in triplicate volumes of 2, 4, 8, 12, 16, and 24 µL. Pyrolysis can vary between runs, therefore internal standards were added at a known quantity to standardise. The internal standard anthracene-d_{10} was prepared in hexane (0.1 mg ml^{-1}). The other internal standards, fluorenone, androstane, and pyrene-d_5, were prepared as a mixture in methanol (each at 0.1 mg ml^{-1}). Both internal standard solutions (4 µL each) were added to the quartz pyrolysis tubes. All the PLA calibration samples were then pyrolyzed at 500°C. Samples were analysed for specific peaks for lactide and the internal standards (Table S5.3).

Table S5.3. Specific pyrolysis products that were used within the calibrations and the relevant *m/z* ions and retention time.

Product	***m/z***	**Retention Time (min)**
Lactide	56	12
PHB	69	6
Anthracene	188	16.5
Androstane	245	17
Pyrene	212	18

The peak area was recorded, and then the ratio of polymer to each internal standard was calculated to determine which calibration was most appropriate. Anthracene gave the best fit (Figure S5.1); therefore, this was decided to be the internal standard to use for quantification of PLA samples.

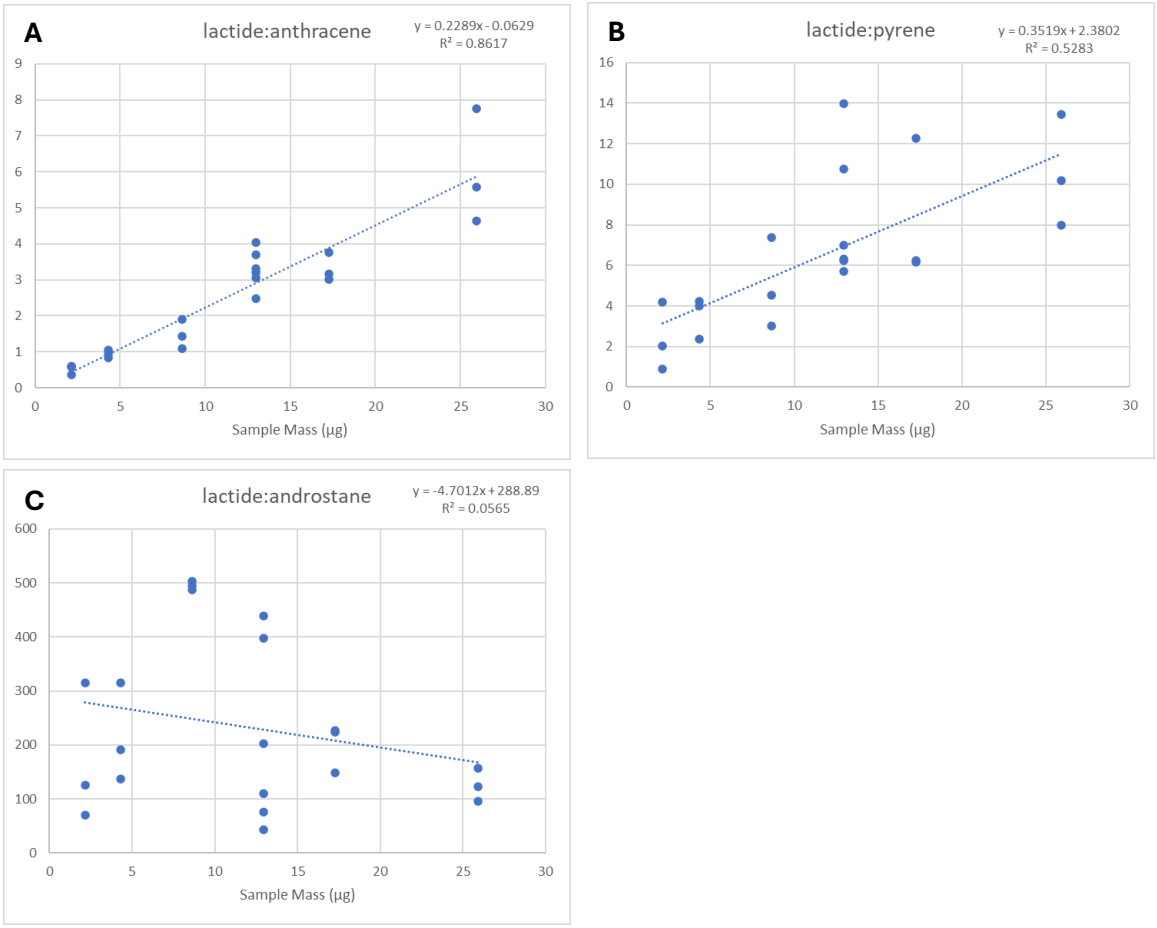


Figure S5.1. Calibration curves for PLA in DCM, with the ratio of lactide to the internal standards (A) anthracene, (B) pyrene, and (C) androstane presented.

PHB samples additionally had 2 µL of 25% TMAH in MeOH added as a thermochemolysis reagent for the methylation of pyrolysis products, with excess solvent evaporated at room temperature before analysis. PHB was initially attempted to be calibrated in the same way as PLA, however, the PHB had poor solubility in DCM. Therefore, an initial calibration was run with PHB in chloroform with triplicate samples at 4, 8, 12, 16, and 25 µL, but PHB did not stay in solution and the resulting calibrations were poor (Figure S5.2).

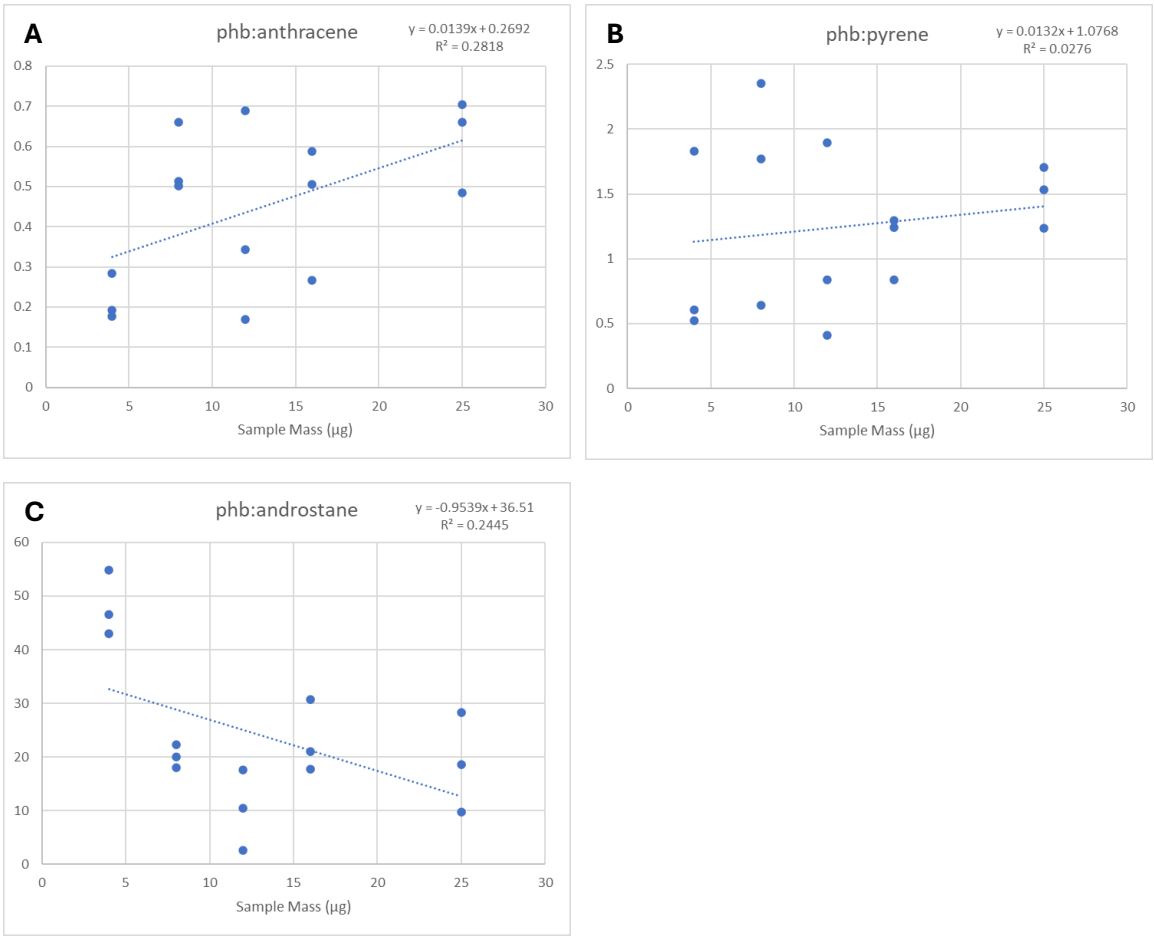


Figure S5.2. Calibration curves for the ratio of PHB in chloroform to the internal standards (A) anthracene, (B) pyrene, and (C) androstane.

Due to this, a solid calibration was done with PHB powder instead, in which 15 samples were weighed out (0.010-0.080 µg) as solids and the internal standards were added. Three samples were removed as outliers, resulting in a solid calibration being ran with 12 samples. The resulting calibrations were better than the PHB solution, however the calibration was poorer than that of PLA (Figure S5.3). Again, anthracene produced the best calibration curve, and so was decided to be used for quantification.

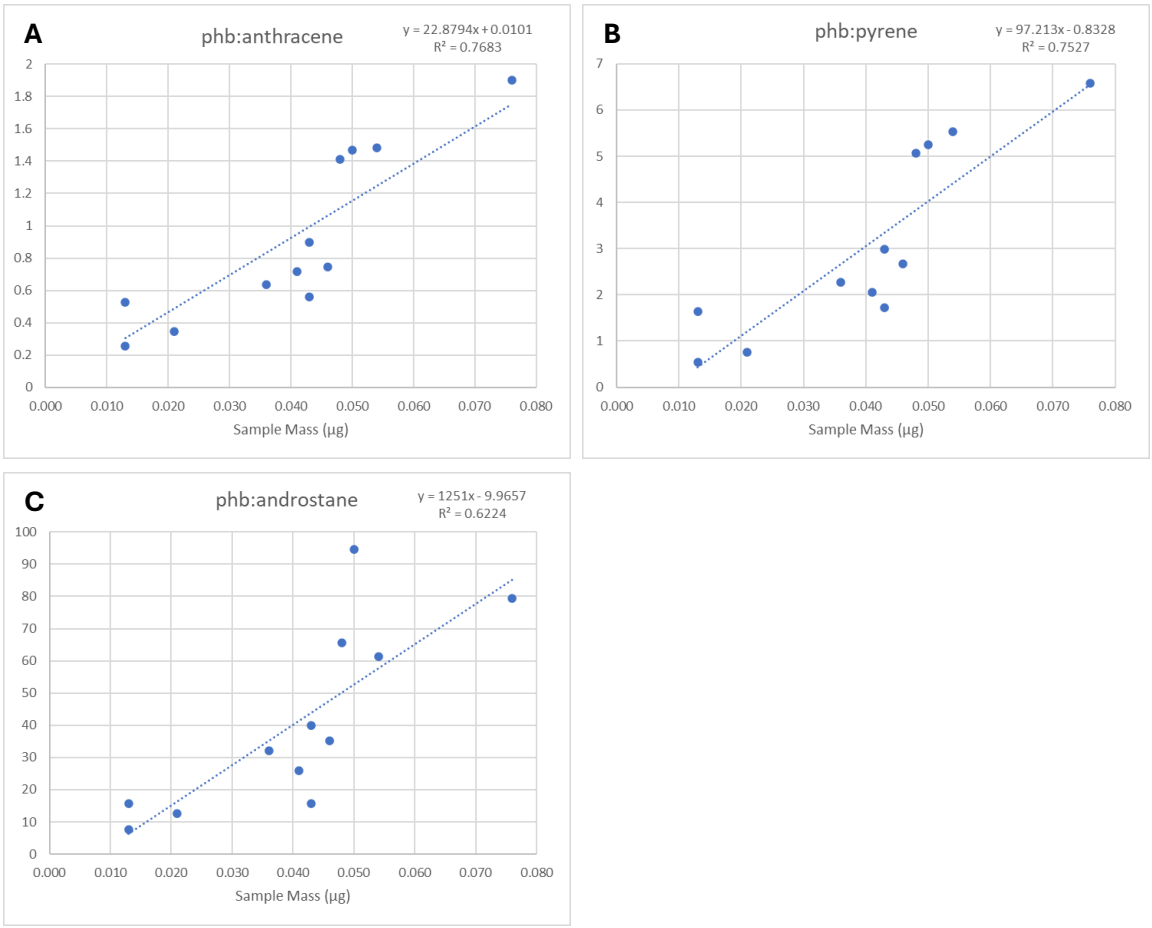


Figure S5.3. Calibration curves for the ratio of PHB powder to the internal standards (A) anthracene, (B) pyrene, and (C) androstane.

5.6.2. iii) Recovery Experiments – Mass Based

18 Acres soil (100g dry weight) was weighed into individual test vessels, and then treated as either a blank soil, or with the addition of the polymers PLA and PHB at three concentrations (1250 mg / 100g soil, 625 mg / 100g soil, and 375 mg / 100g soil) (Table S5.4). Samples were frozen immediately after the addition and mixing of polymer, to prevent degradation by soil microbial activity, and to replicate the sample processing steps taken at the point of sample removal in the soil biodegradation study.

Table S5.4. Soil (100g dry weight) was prepared as either blank soil, or with the addition of the polymers PLA or PHB at different weight concentrations. All treatments were prepared in triplicate for recovery experiments and initial Py-GC-MS method development.

Treatment	Polymer Weight (mg)	Replicates
Soil-Blank	0	3
Soil-PLA	1250	3
Soil-PLA	625	3
Soil-PLA	375	3
Soil-PHB	1250	3
Soil-PHB	625	3
Soil-PHB	375	3

The samples as described in Table S5.4 were density separated following the standard protocol described above (5.6.2. i) developed by Moller et al., 2021 and Kernchen et al., *unpublished*, which has been experimentally validated for conventional microplastics. A recovery experiment was conducted using the biodegradable polymers PLA and PHB. Percent recovery varied between the polymer types but was consistent for each polymer across all concentrations tested (Figure S5.4).

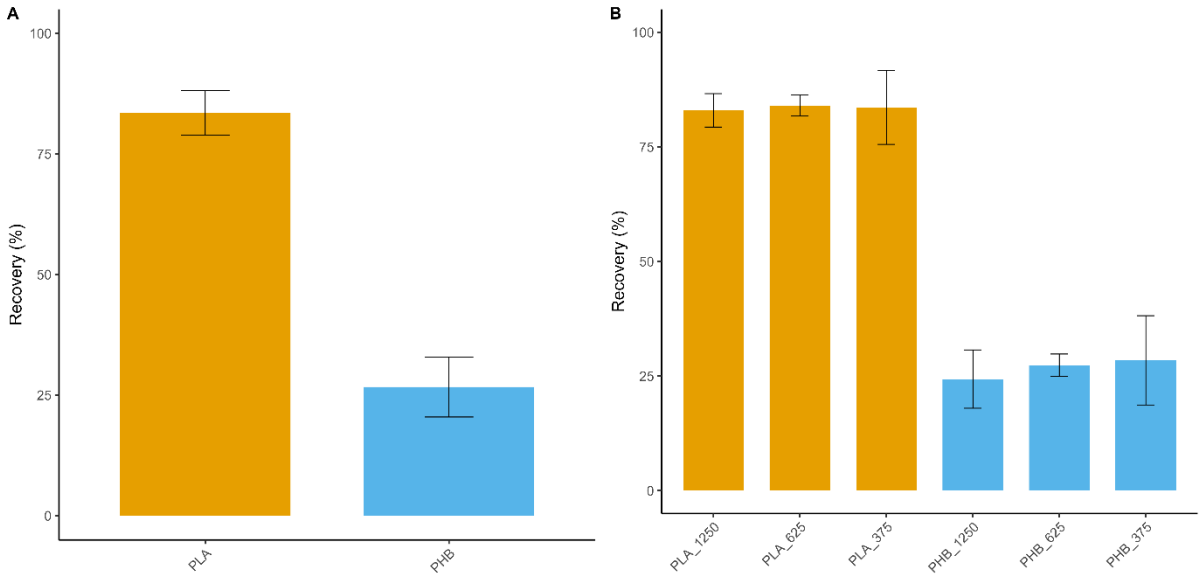


Figure S5.4. Recovery (%) after a density separation step with zinc chloride for the polymers PLA and PHB overall (n=9) (A), and at different application concentrations (375 mg n=3, 625 mg n=3, 1250 mg n=3) (B).

PLA recovery was consistent with recovery percentages given in the literature (Way et al., 2022), however PHB recovery was significantly lower than previously reported. It was observed that PHB recovery was impacted by aggregation of PHB particles after the wet

sieving step with deionised water, resulting in a significant proportion of PHB particles remaining on the sieve after the 500 µm sieving step. Both PLA and PHB are hydrophobic, therefore it is likely that this hydrophobicity could be the cause of the noted aggregation of PHB. Recovery was consistent between all PHB samples; therefore, it was determined to analyse the PHB samples in two size fractions by Py-GC-MS.

6.6.2. iv) Sample Analysis and Quantification

PLA and PHB samples were subsampled for Py-GC-MS analysis. Triplicate sub samples (~50 µg) were taken each individual sample (i.e. three pseudo replicates) and weighed into quartz pyrolysis tubes, then the internal standards (anthracene-d_{10}, fluorenone, androstane, and pyrene-d_5 as described in section 5.6.2. ii) were added to both PLA and PHB. As previously described in section 5.6.2. ii, PHB samples additionally had TMAH (4 µL) added as a methylation agent. Samples were analysed for the relevant pyrolysis products (Table S.5.3), and then the calibration with anthracene was used to quantify the sub-sample. Resulting quantification accuracies using the calibration curves were poor (Figure S5.5). Therefore, it was determined that this method was not appropriate to quantify the mass of polymer in the soil.

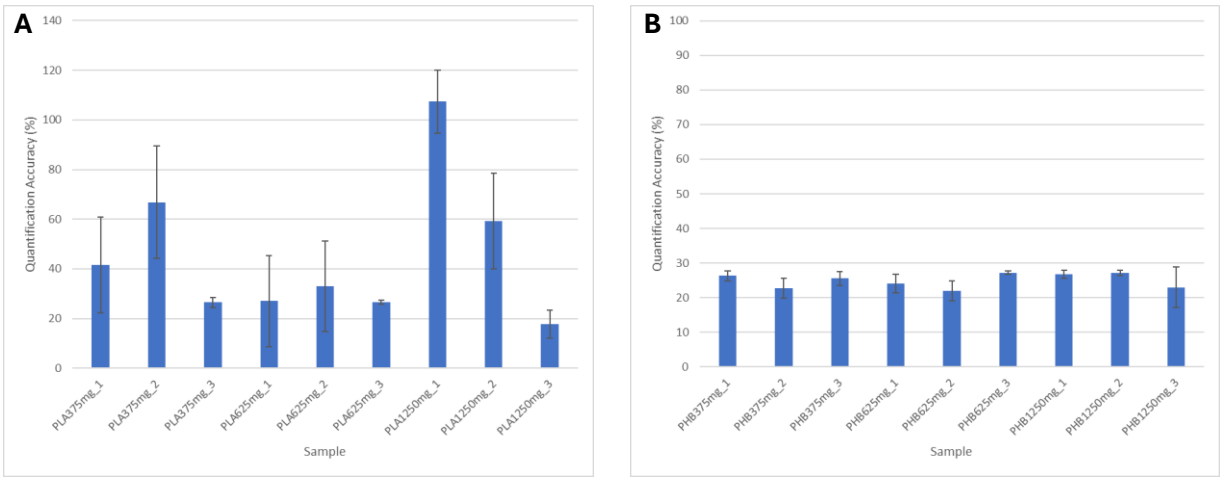


Figure S5.5. Quantification accuracy for (A) PLA and (B) PHB samples using the calibration curves with anthracene determined by Py-GC-MS analysis.

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Chapter 6. General Discussion

6.1. Research Context

Driven by regulatory pressures to reduce plastic pollution, biodegradable polymers are increasingly suggested as alternatives to conventional polymers (Hofmann et al., 2023). It is expected that complete environmental biodegradation should result in no ecological harm. Therefore, providing scientific evidence of complete environmental biodegradation is necessary to ensure that the potential solution to one environmental problem does not inadvertently cause another, thereby preventing regrettable substitutions (Drohmann and Hernández, 2020).

Biodegradation is determined based on standard test methods, with specific standard tests outlined for different environments. However, as current standard test methods were designed for small molecules, there are concerns regarding their suitability for use with polymers (McDonough et al., 2023). A series of test methods have been outlined for plastics in different environments by ISO (International Organisation for Standardisation), which are based on the measurement of respirometric endpoints (biological oxygen demand and evolved carbon dioxide) that are frequently used for small molecules (European Union, 2023). These test methods are designated as screening tests by the European Chemicals Agency's REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation (European Union, 2023). However, there are concerns that these respirometric methods are insufficient to confirm that polymers are fully biodegraded, due to their poor reproducibility, and the lack of insights into or characterization of the breakdown products (metabolites), as well as their lack of environmental realism (Briassoulis and Mistriotis, 2018; McDonough et al., 2023).

Respirometric methods focus on the mineralised portion of a polymer but provide no information regarding the environmental fate of the non-mineralised polymer fraction.

There are concerns regarding whether the non-mineralised polymer is taken up by soil microbes, and so will eventually mineralise, or if it remains in the soil as a persistent microplastic due to incomplete biodegradation. Generally, screening tests are not sufficient when we are not confident of the mechanisms of polymer degradation, especially because we lack information on the residual polymer and transformation products. Biodegradable does not automatically mean safer, and it is important to understand what could be left behind and the associated risks that may occur. Therefore, ensuring the reliability and comprehensiveness of methods to demonstrate environmental biodegradation in soils is important to minimise and quantify associated environmental risk.

6.2. Overview of Research Findings

The research in this thesis examined the suitability of the existing standard test method ISO 17556:2019 to measure polymer biodegradation in soils. This thesis aimed to understand the causes of the poor reproducibility of respirometric methods for measuring polymer degradation, and to investigate alternative analytical methods that could provide more information regarding the environmental fate of the non-mineralised polymer.

Respirometric methods following O_2 demand and CO_2 evolution were set-up to measure polymer biodegradation in soil (Chapter 2). To assess the reproducibility of the current test methods, the biodegradation of cellulose was compared in three soils (two representative agricultural soils and an artificial soil) and measured by both respirometric endpoints (O_2 and CO_2). Significant variability in both the measured rate and total biodegradation of cellulose occurred, highlighting how the standardised test method allows for levels of variability that result in a lack of reproducibility, even for known biodegradable polymers (Chapter 3).

Measurements of mineralisation alone are not sufficient to understand the mechanism of polymer biodegradation. Information regarding the environmental fate of the non-mineralised polymer is important to allow future environmental risk assessment. It is important to distinguish whether the polymer has been taken up by soil microbes or if it remains in the soil as residual polymer material and would be classed as a persistent microplastic. Measuring the uptake into the microbial biomass was out of the scope of this thesis, and the research focused on potential methodologies to identify residual non mineralised polymers within the soil matrix. An exemplary soil extraction method for the identification of microplastics was investigated and several reagents were found to induce degradation in the two exemplary biodegradable polymers tested here, polylactic acid (PLA) and polyhydroxybutyrate (PHB). This could result in misestimations of their presence in environmental samples (Chapter 4). Therefore, it is likely that the analysis of any residual biodegradable polymers will require a method that does not induce degradation and/or can identify polymers within soil despite background soil matrix interference. Pyrolysis gas chromatography-mass spectrometry (Py-GC-MS) is known to be suitable for samples with background matrix interference, therefore it was investigated as a potential method to directly analyse polymers in soil. Py-GC-MS was used as an additional analytical method alongside measurements of evolved CO_2, which highlighted the lack of PLA degradation in soil and the potential for a soil priming effect to occur with the addition of a polymer into soil. However, as Py-GC-MS only allows the analysis of small sample sizes this work was limited and so highlights the need to explore alternative analytical methods that can be used alongside respirometric methods to confirm the biodegradation of polymers.

6.3. Future Research Needs

Overall, the suitability of respirometric methods to measure polymer biodegradation remains unclear. ISO 17556:2019 allows for variability within test parameters, specifically there needs to be clearer guidance on test temperature, the soil water content adjustment

and maintenance throughout the test, and the adjustment of the carbon to nitrogen ratio. This would likely improve the reproducibility of the test and allow greater comparability between studies. ISO 17556:2019 is currently designated as a screening test, however, as the ISO tests are specific to different environments, it could be beneficial to improve the environmental realism of the tests in line with the OECD simulation tests (such as OECD 307 for biodegradation in soil). Additionally, it is important to consider that ISO 17556:2019 is measuring the biodegradation of a test polymer of interest in relation to a reference material. This means that the reference material, such as cellulose, is required to meet 60% biodegradation, and that the test material then must achieve 90% of the measured 60% cellulose biodegradation. This relies on the assumption that the mineralisation by microbes would be similar for all test materials and needs broader consideration (Sander et al., 2023). It is important to consider the carbon use efficiency of different polymer materials, and therefore the relevance of cellulose as a reference material. Cellulose is a natural polymer that is already present in soils, so should be biodegraded easily, and this might occur more easily than for synthetic polymers. Further research is needed to determine if the carbon use efficiency is equivalent between polymers (Sander et al., 2023), as this could impact the determination of whether a material is biodegradable or not according to the standardised test criteria.

To further enhance the usefulness of biodegradability tests for polymers it is important to consider their environmental realism. Currently tests such as ISO 17556:2019 are focused on monitoring the pure polymer, however this is an unrealistic expectation of what will reach the environment. Plastic products that reach the environment are likely to contain blends of polymers and additives. This adds a level of complexity that is not currently accounted for in standardised tests. The addition of polymer blends and additives could change the process of biodegradation. Of concern is the potential release of additives into the soil, as whilst there is evidence of additives leaching into soil, the full ecotoxicological impacts on

the soil microbial community is unclear (Bridson et al., 2021; Cao et al., 2023). There is a need for research that bridges laboratory biodegradation and field studies to advance our understanding of how controlled settings translate into the natural environment. By considering the complexity of plastic products and the interaction with the environment we can develop better predictions of biodegradability in the environment.

A significant concern for the use of non-specific respirometric endpoints is that the measurement is not necessarily a true representation of the biodegradation of polymers. The comparison of cellulose biodegradation across soils resulted in mineralisation values greater than 100%, which could be caused by either soil priming or nitrification. However, as respirometric methods with non-labelled polymers cannot distinguish between the source of evolved carbon (i.e. if it is polymer derived CO_2 or soil derived CO_2), it results in the need for further mechanistic studies to determine the relative quantities, and thus allow for future respirometric tests to account for this within the result. Whilst in this thesis, Py-GC-MS provided evidence suggestive of a priming effect occurring, it could not quantify the relative source of carbon from the polymer versus the soil. Such quantification would only be possible with the use of an isotopically labelled polymer such as ^{13}C or ^{14}C. Recent research has utilised labelled polymer carbon to accurately partition the source of evolved CO_2 and to quantify the magnitude of the priming effect (Guliyev et al., 2023; Huo et al., 2024; Zhang et al., 2023). However, more research is needed to understand if the effects are consistent across polymer and soil types. Whilst it is impractical to suggest that the biodegradation of polymers should always utilise a labelled polymer (due to the associated high cost and need for specialised laboratory facilities), more in-depth mechanistic studies using a set of reference polymers that accurately track the carbon from the polymer being converted to carbon in CO_2 evolved (and potentially soil microbial uptake) would develop our understanding and provide a baseline against which to compare standard biodegradation studies with unlabelled polymers. A more comprehensive overview of

polymer degradation mechanisms would allow for the allowed pass levels to be reassessed. This would ensure that the classification of a polymer as biodegradable based on these standard tests is consistent, in terms of demonstrating that the degree of degradation meets the test requirements and that priming effects are accounted for. Overall, the development of the mechanistic understanding of polymer biodegradation combined with the use of respirometric methods would provide a fuller picture of the process of biodegradation and allow associated environmental impacts to be quantified.

To fully assess if a polymer has biodegraded and to determine the environmental impacts, it would be critical to identify if residual polymer remain at the end of a test. As a simplistic approach, if the pass level is 60% mineralisation and this is met, the polymer would be classed as biodegradable. However, that still leaves 40% of the polymer unaccounted for. In the biodegradation testing of small molecules (which are water-soluble or water-dispersible), it is assumed that this 40% is incorporated into the biomass and that complete biodegradation of the test substance will occur (Gartiser et al., 2017; Martin-Aparicio et al., 2023). However, this same assumption cannot be met for polymers as they are typically higher molecular weight and insoluble in water. Therefore, they are not easily accessible for bioassimilation and will require initial abiotic and/or biotic degradation to increase their availability for bioassimilation by microbes prior to complete mineralisation. Consequently, this may result in partially degraded polymers that could persist indefinitely in soils as microplastics, however this is still uncertain. There is a clear need for methods to be developed that allow identification and quantification to ensure that effective environmental risk assessments can be completed. It would be important to consider alternative methods to extract biodegradable polymers from soil matrices such that degradation is not induced, and the polymers retain their ecologically relevant characteristics. This would generate a more comprehensive understanding of the

environmental fate of biodegradable polymers and accurately measure if persistent microplastics are formed due to incomplete (bio)degradation.

6.4. Conclusion

The research conducted in this thesis was motivated by the recent REACH restriction on intentionally added microplastics, which permits the use of biodegradable polymers on the basis that they are unlikely to persist in the environment. This thesis focused on exploring the reproducibility of respirometric methods for the assessment of the biodegradation of polymers in soil environments, and how these methods could be supplemented with analytical approaches to provide mechanistic insight into the process of polymer degradation. The findings highlight that the current test guidelines have too many assumptions and allow too much variability in the test set up to allow comparability of results and to be confident that polymer biodegradation is actually occurring. Further development of reliable and reproducible standard test methods is critical to ensure complete environmental biodegradation and thus to reduce microplastic pollution. Generally, it can be concluded that current respirometric methods need to be supplemented with additional mechanistic studies that would develop our understanding of polymer biodegradation in soils. This will require an interdisciplinary approach that considers the intrinsic chemical and structural polymer properties as well as the soil environment and soil microbial activity. By reassessing current standards, and conducting detailed mechanistic studies, we can more accurately evaluate the environmental fate of polymers. This would lead to better development and regulation of biodegradable polymers and minimise their potential environmental risk.

6.5. References

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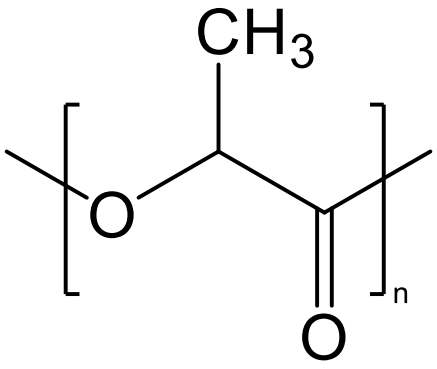
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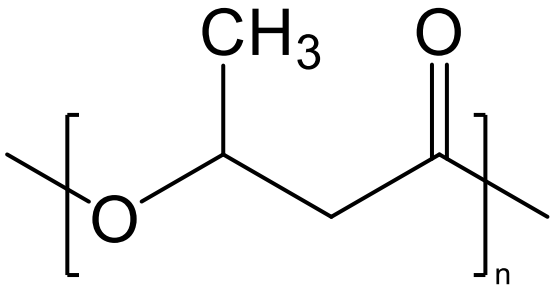
Appendices

Appendix A

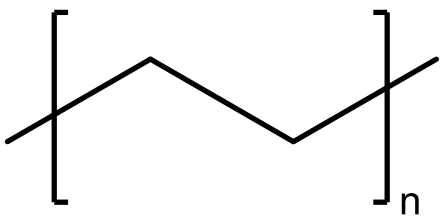
Appendix A provides the chemical structures of the tested materials used throughout the thesis.



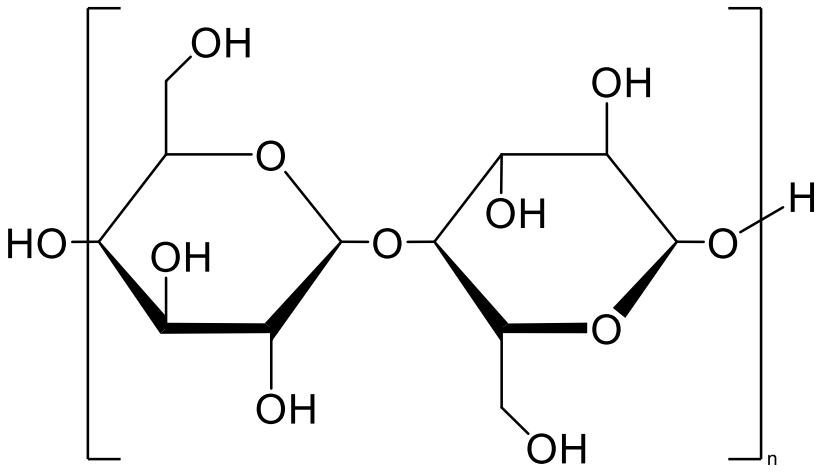
Appendix Figure A.1. Chemical structure of polylactic acid (PLA).



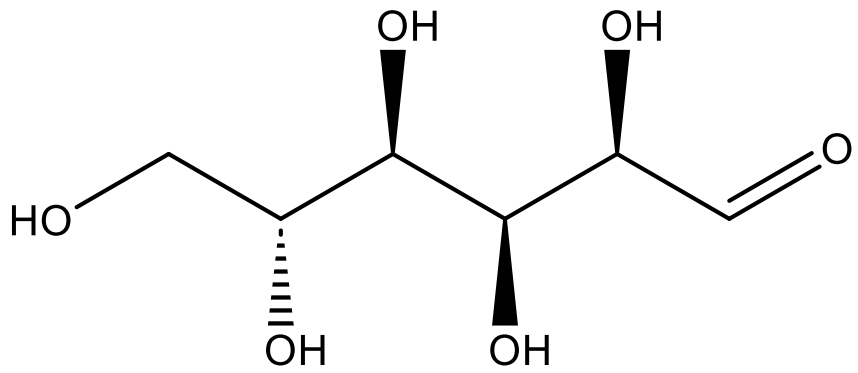
Appendix Figure A.2. Chemical structure of polyhydroxybutyrate (PHB).



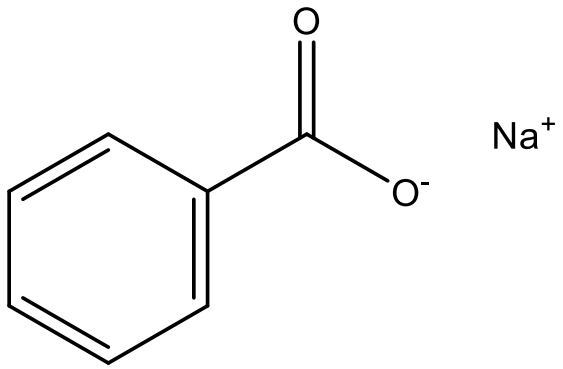
Appendix Figure A.3. Chemical structure of polyethylene (PE).



Appendix Figure A.4. Chemical structure of microcrystalline cellulose.



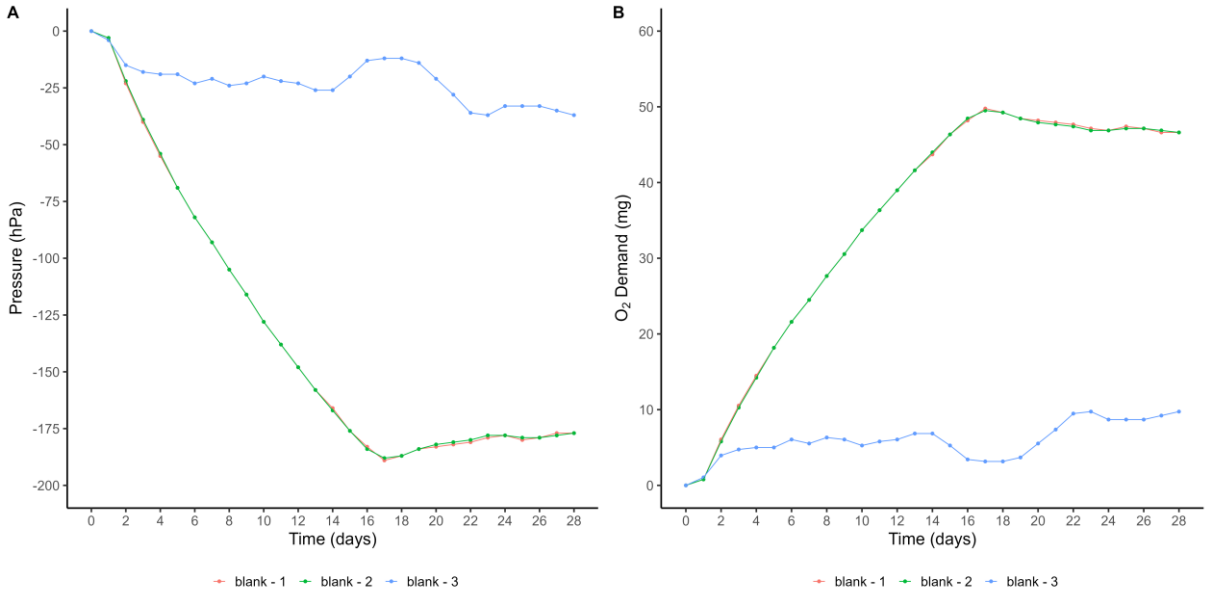
Appendix Figure A.5. Chemical structure of glucose.



Appendix Figure A.6. Chemical structure of sodium benzoate.

Appendix B

Appendix B provides additional information for Chapter 2.



Appendix Figure B.1. (A) Pressure (hPa) data for soil-blank test vessels measured for 28-days. (B) Respiration (O_2 consumption, mg / 100g soil) of soil-blank and soil-sodium benzoate test vessels measured for 28-days. Data is presented as individual replicates. "Blank – 3" has highly variable pressure recordings due to the measurement head being poorly sealed.

Appendix Table B.1. Statistical significance (*p*-value) of cumulative CO_2 evolved between sample types at each timepoint during "CO_2 Experiment 1 – Method Validation with Sodium Benzoate". Results are from a Welch's one sample t test, with the significance level set at $p = 0.05$.

Days	*p*-value
0	0.274
1	0.146
2	0.234
3	0.131
4	0.068
7	<0.05
9	<0.05
11	<0.01
14	<0.001
17	<0.001
21	<0.001
23	<0.001
28	<0.001
49	<0.001
72	<0.001

Appendix Table B.2. Statistical significance (*p*-value) of cumulative O_2 demand between sample types at each timepoint during "O_2 Experiment 1 – Method Validation with Sodium Benzoate". Results are from a one-way ANOVA followed by a Tukey post-hoc test to compare between groups. The significance level was set at $p = 0.05$.

	2	**3**	**7**	**14**	**21**	**28**
sb25-blank	0.551	<0.05	<0.001	<0.001	<0.001	<0.001
sb50-blank	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001
sb50-sb25	0.075	0.441	<0.001	<0.001	<0.001	<0.001

Appendix Table B.3. Statistical significance (*p*-value) of cumulative biodegradation between sample types at each timepoint during "O_2 Experiment 1 – Method Validation with Sodium Benzoate". Results are from a Welch's t-test or Mann-Whitney U test, with the significance level set at $p = 0.05$.

Day	**Statistical Test**	***p*.value**
1	Welch's t-test	0.686
2	Mann-Whitney U	0.302
3	Welch's t-test	0.225
4	Welch's t-test	0.117
5	Welch's t-test	0.100
6	Welch's t-test	0.082
7	Welch's t-test	0.496
8	Welch's t-test	0.653
9	Welch's t-test	0.557
10	Welch's t-test	0.319
11	Mann-Whitney U	0.653
12	Welch's t-test	0.369
13	Welch's t-test	0.276
14	Mann-Whitney U	0.184
15	Welch's t-test	0.287
16	Welch's t-test	0.254
17	Welch's t-test	0.249
18	Welch's t-test	0.280
19	Welch's t-test	0.176
20	Welch's t-test	0.369
21	Welch's t-test	0.369
22	Welch's t-test	0.176
23	Welch's t-test	0.224
24	Welch's t-test	0.254
25	Welch's t-test	0.199
26	Welch's t-test	0.199
27	Welch's t-test	0.224
28	Welch's t-test	0.176

Appendix Table B.4. Statistical significance (*p*-value) of cumulative O_2 demand between sample types at selected representative timepoints during "O_2 Experiment 2 – Application to the positive reference biodegradable polymer cellulose, and determination of analytical detection limits.". Results are from a one-way ANOVA followed by a Tukey post-hoc test to compare between groups. The significance level was set at $p = 0.05$.

	1	**3**	**7**	**14**	**21**	**28**
cell10mg-blank	0.051	0.150	0.216	0.513	0.416	0.418
cell1mg-blank	0.675	0.940	0.974	0.989	0.987	0.991
cell25mg-blank	<0.01	<0.01	<0.05	0.113	0.088	<0.05
cell5mg-blank	0.491	0.940	0.898	0.989	0.903	0.991
cell1mg-cell10mg	0.245	0.305	0.353	0.695	0.585	0.558
cell25mg-cell10mg	0.245	0.175	0.512	0.695	0.704	0.455
cell5mg-cell10mg	0.393	0.305	0.512	0.695	0.815	0.558
cell25mg-cell1mg	<0.01	<0.01	<0.05	0.149	0.115	0.055
cell5mg-cell1mg	0.995	1.000	0.997	1.000	0.992	1.000
cell5mg-cell25mg	<0.05	<0.01	0.057	0.149	0.210	0.055

Appendix Table B.5. Statistical significance (*p*-value) of cumulative O_2 demand between sample types at selected representative timepoints during "O_2 Experiment 3 – Application to polymers for an extended test period.". Results are from a one-way ANOVA followed by a Tukey post-hoc test to compare between groups. The significance level was set at $p = 0.05$.

	1	**4**	**7**	**10**	**16**	**21**	**28**	**56**	**84**
sb-blank	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
gluc-blank	<0.01	<0.05	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01
cell-blank	0.373	1.000	0.984	0.620	<0.05	<0.05	<0.01	<0.01	<0.01
phb-blank	0.124	0.998	0.940	0.309	<0.01	<0.01	<0.001	<0.001	<0.001
sb-gluc	0.449	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
sb-cell	0.113	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
sb-phb	0.195	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
gluc-cell	<0.01	<0.05	<0.01	<0.001	<0.001	<0.001	<0.01	0.503	0.996
phb-gluc	<0.05	<0.05	<0.01	<0.001	<0.001	<0.001	0.122	0.132	<0.01
phb-cell	0.947	0.998	1.000	0.981	1.000	0.890	0.134	<0.05	<0.05

**sb= sodium benzoate, gluc = glucose, cell = cellulose, phb = PHB.*

Appendix Table B.6. Statistical significance (*p*-value) of cumulative biodegradation between sample types at selected representative timepoints during "O2 Experiment 3 – Application to polymers for an extended test period.". Results are from a one-way ANOVA followed by a Tukey post-hoc test to compare between groups. The significance level was set at $p = 0.05$.

	1	**5**	**7**	**10**	**14**	**21**	**28**	**56**	**84**
sb-gluc	<0.05	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01
sb-cell	0.442	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01
sb-phb	0.326	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01
gluc-cell	<0.01	<0.05	<0.01	<0.001	<0.001	<0.001	<0.01	0.079	0.966
phb-gluc	<0.01	<0.01	<0.001	<0.001	<0.001	<0.001	<0.001	0.213	0.793
phb-cell	0.999	1.000	1.000	1.000	0.701	0.862	0.975	0.726	0.603

sb= sodium benzoate, gluc = glucose, cell = cellulose, phb = PHB.

Appendix C

Appendix C provides additional information for Chapter 3.

Carbon dioxide evolved

Appendix Table C.1. Statistical results from the t-test for the difference between the total CO_2 production in the soil-cellulose vessels in the two different soil types 18 Acres and Gartenacker.

Day	t-value	df	p.value
1	1.341641	2.941176	0.273893
5	-0.28222	2.035711	0.803889
8	1.717795	2.17773	0.217673
14	0.906059	2.281109	0.450219
21	1.173566	2.770136	0.331533
30	0.795715	3.457478	0.477228
43	0.862079	3.761735	0.440125
61	0.881588	3.587215	0.433095
76	0.504116	3.136241	0.647474
90	0.203886	2.848949	0.85213
104	0.123307	3.112902	0.909398
118	0.306253	3.57107	0.776422
134	0.32238	3.661681	0.764721
155	0.20084	3.532465	0.851847
168	-0.05494	3.545552	0.95914
194	-0.38031	3.626669	0.724923
223	-0.54998	3.736249	0.613544
251	-0.78709	3.89045	0.476404
285	-1.18143	3.958428	0.303511
313	-0.99054	3.988981	0.378124

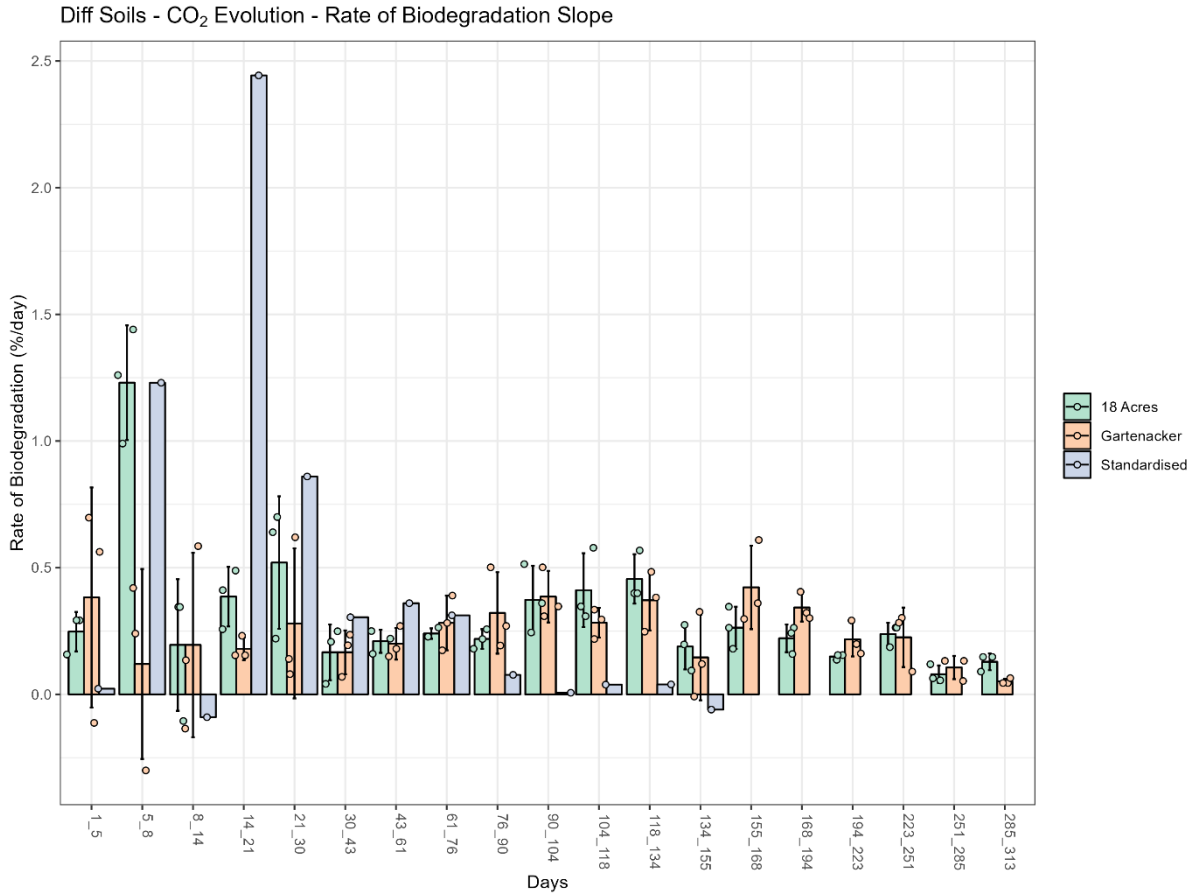
Appendix Table C.2. Statistical results from the t-test for the difference between the total CO_2 production from the soil-blank vessels in the two different soil types 18 Acres and Gartenacker.

Day	t-value	df	p.value
1	1	3.2	0.386809
5	0.426401	3.872	0.692448
8	-0.14704	3.356544	0.891495
14	-0.23408	3.946494	0.826562
21	-0.59264	3.986341	0.585407
30	-0.90007	3.098081	0.432505
43	-0.94737	2.668138	0.421122
61	-0.7533	2.713582	0.511229
76	-0.86817	2.46079	0.461639
90	-0.79796	2.732864	0.488374

104	-0.84227	3.240458	0.457271
118	-0.83761	3.452639	0.456291
134	-1.00851	3.312296	0.381103
155	-1.29671	3.555776	0.272471
168	-1.69726	3.526387	0.174407
194	-1.77392	3.489692	0.161144
223	-1.87192	3.255721	0.150684
251	-2.4034	3.157834	0.091274
285	-2.89272	3.111707	0.060163
313	-3.30614	3.037128	0.044714

Appendix Table C.3. Statistical results from the t-test for the difference between the total biodegradation each day in the two different soil types 18 Acres and Gartenacker.

Day	t-value	df	p.value
1	0.447214	2.941176	0.685598
5	-0.47036	2.035711	0.683685
8	1.832315	2.17773	0.197936
14	1.035496	2.281109	0.397561
21	1.564755	2.770136	0.222961
30	1.432286	3.457478	0.235902
43	1.633413	3.761735	0.182205
61	1.482671	3.587215	0.220193
76	1.206277	3.136241	0.310766
90	0.742727	2.848949	0.514119
104	0.604202	3.112902	0.586913
118	0.754696	3.57107	0.497126
134	0.796468	3.661681	0.474194
155	0.726112	3.532465	0.512936
168	0.557244	3.545552	0.61064
194	0.279414	3.626669	0.795127
223	0.11159	3.736249	0.916883
251	0.170695	3.89045	0.872972
285	0.082186	3.958428	0.938486
313	0.320176	3.988981	0.764904



Appendix Figure C.1. Rate of biodegradation each day of cellulose in three different soils. Bars represent the mean rate per sample type. Circles represent the individual replicate values of each measurement.

Biological oxygen demand

Appendix Table C.4. Statistical significance (*p*-value) of the differences in the total respiration of soil-cellulose each day measured by O_2 demand between measurement days overall (one-way ANOVA), and between the different soil types (Tukey multiple comparisons post-hoc test). Data is for days 0-150. Notation of p-values is as follow: p<0.05; *, p<0.01; **, p<0.001; ***.

Day	anova p.value	GAR-A18 p-value	STD-A18 p-value	STD-GAR p-value
0	NA	NA	NA	NA
1	0.3944	0.3650	0.7511	0.7511
2	0.5008	0.7074	0.4827	0.9135
3	0.2657	0.3231	0.3231	1.0000
4	0.2282	0.3650	0.2339	0.9285
5	0.1866	0.5698	0.1655	0.5698
6	0.2441	0.6324	0.2195	0.6324
7	0.2213	0.7532	0.2030	0.4827
8	0.1147	0.9649	0.1320	0.1827
9	0.2317	1.0000	0.2865	0.2865
10	0.4115	0.9729	0.5386	0.4255
11	0.6622	0.9269	0.8452	0.6408
12	0.8194	0.8097	0.9087	0.9761
13	0.3170	0.8514	0.3000	0.5508
14	0.0875	0.8232	0.0879	0.1897
15	**	0.4827	**	*
16	**	0.4827	**	*
17	**	0.4649	**	*
18	**	0.3858	**	*
19	**	0.2694	**	*
20	**	0.2214	**	*
21	**	0.2137	**	**
22	**	0.1962	**	*
23	**	0.1962	**	*
24	**	0.2587	**	*
25	**	0.1469	**	*
26	**	0.1766	**	*
27	**	0.1525	**	*
28	**	0.1420	**	0.0552
29	**	0.1469	**	*
30	**	0.1655	**	0.0842
31	**	0.1469	**	0.0531
32	**	0.1231	**	0.1231
33	*	0.1159	*	0.1855
34	*	0.1119	*	0.2866
35	*	0.1183	*	0.3199
36	*	0.1183	*	0.3199

37	*	0.0916	*	0.6468
38	*	*	*	0.9217
39	*	*	*	0.9285
40	*	*	0.0601	0.9856
41	*	*	0.0682	0.8232
42	*	*	0.0682	0.8232
43	*	*	0.1278	0.6213
44	*	*	0.2153	0.3431
45	*	*	0.1356	0.2954
46	*	*	0.2658	0.2090
47	*	*	0.5153	0.0998
48	*	*	0.4268	0.1038
49	*	*	0.5756	*
50	*	*	0.7464	*
51	*	*	0.8350	*
52	**	**	0.9746	**
53	**	**	1.0000	**
54	**	*	0.8350	**
55	**	**	0.8350	**
56	**	**	0.6868	**
57	**	**	0.5578	**
58	**	**	0.3491	**
59	***	**	0.3679	***
60	***	**	0.2869	***
61	***	**	0.1300	***
62	***	**	0.1356	***
63	***	**	0.0586	***
64	***	**	0.0586	***
65	***	**	*	***
66	***	***	*	***
67	***	***	*	***
68	***	**	*	***
69	***	**	*	***
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73	***	***	**	***
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97	***	**	***	***
98	***	**	***	***
99	***	**	***	***
100	***	**	***	***
101	***	**	***	***
102	***	**	***	***
103	***	*	***	***
104	***	**	***	***
105	***	*	***	***
106	***	*	***	***
107	***	*	***	***
108	***	*	***	***
109	***	*	***	***
110	***	*	***	***
111	***	*	***	***
112	***	*	***	***
113	***	*	***	***
114	***	*	***	***
115	***	*	***	***
116	***	*	***	***
117	***	0.0554	***	***
118	***	*	***	***
119	***	*	***	***
120	***	0.0554	***	***
121	***	*	***	***
122	***	0.0593	***	***
123	***	0.0811	***	***
124	***	0.0693	***	***
125	***	0.0903	***	***
126	***	0.0970	***	***
127	***	0.0970	***	***
128	***	0.0903	***	***

129	***	0.0842	***	***
130	***	0.1065	***	***
131	***	0.1327	***	***
132	***	0.1443	***	***
133	***	0.1327	***	***
134	***	0.1348	***	***
135	***	0.1348	***	***
136	***	0.1348	***	***
137	***	0.1348	***	***
138	***	0.1778	***	***
139	***	0.1348	***	***
140	***	0.1348	***	***
141	***	0.2055	***	***
142	***	0.1988	***	***
143	***	0.1292	***	***
144	***	0.1554	***	***
145	***	0.1484	***	***
146	***	0.1348	***	***
147	***	0.2055	***	***
148	***	0.1484	***	***
149	***	0.1988	***	***
150	***	0.1988	***	***

Appendix Table C.5. Statistical significance (*p*-value) for the biodegradation of cellulose each day measured by O_2 demand between measurement days overall (one-way ANOVA), and between the different soil types (Tukey multiple comparisons post-hoc test). Data is for days 0-150. Notation of p-values is as follow: p<0.05; *, p<0.01; **, p<0.001; ***.

Day	anova p-value	GAR-A18 p-value	STD-GAR p-value	STD-A18 p-value
0	NA	NA	NA	NA
1	0.2282	0.3650	0.2339	0.9285
2	0.7111	0.9774	0.7074	0.8189
3	0.3476	0.3231	0.6324	0.8085
4	0.7703	0.9285	0.7511	0.9285
5	1.0000	1.0000	1.0000	1.0000
6	0.5787	1.0000	0.6324	0.6324
7	0.9331	0.9291	0.9918	0.9676
8	0.8445	0.8690	0.8690	1.0000
9	0.7727	0.7610	0.9689	0.8829
10	0.6121	0.6631	1.0000	0.6631
11	0.3759	0.4882	0.9810	0.3983
12	0.1007	0.4649	0.4144	0.0870
13	*	0.5508	0.0754	*
14	**	0.4912	*	**

15	***	0.1273	**	***
16	***	0.1273	**	***
17	***	0.1487	**	***
18	***	0.0840	**	***
19	***	0.0523	**	***
20	***	0.0994	**	***
21	***	*	**	***
22	***	*	***	***
23	***	*	***	***
24	***	0.0585	***	***
25	***	*	***	***
26	***	*	***	***
27	***	*	***	***
28	***	*	**	***
29	***	*	***	***
30	***	*	**	***
31	***	*	***	***
32	***	*	**	***
33	***	*	**	***
34	***	*	**	***
35	***	*	**	***
36	***	*	**	***
37	***	**	**	***
38	***	**	**	***
39	***	**	**	***
40	***	**	*	***
41	***	**	**	***
42	***	**	**	***
43	***	**	**	***
44	***	**	**	***
45	***	**	**	***
46	***	**	**	***
47	***	**	*	***
48	***	**	*	***
49	***	**	**	***
50	***	**	*	***
51	***	**	*	***
52	***	**	*	***
53	***	***	*	***
54	***	**	*	***
55	***	***	0.0781	***
56	***	***	0.0948	***
57	***	***	*	***
58	***	***	0.0685	***

59	***	***	0.1300	***
60	***	***	0.0762	***
61	***	***	0.1699	***
62	***	***	0.1356	***
63	***	***	0.1699	***
64	***	***	0.3679	***
65	***	***	0.1293	***
66	***	***	0.2578	***
67	***	***	0.3923	***
68	***	***	0.5276	***
69	***	***	0.4827	***
70	***	***	0.4148	***
71	***	***	0.5698	***
72	***	***	0.8361	***
73	***	***	0.7819	***
74	***	***	0.8690	***
75	***	***	0.9342	***
76	***	***	0.8690	***
77	***	***	1.0000	***
78	***	***	0.9729	***
79	***	***	0.9457	***
80	***	***	0.9767	***
81	***	***	0.9767	***
82	***	***	0.9767	***
83	***	***	0.7008	***
84	***	***	0.9767	***
85	***	***	0.8047	***
86	***	***	0.8514	**
87	***	***	0.7265	***
88	***	***	0.7797	***
89	***	***	0.8420	***
90	***	***	0.8420	***
91	***	***	0.6638	**
92	***	***	0.6638	**
93	***	***	0.4490	**
94	***	***	0.5883	**
95	***	***	0.6583	**
96	**	**	0.5681	**
97	***	**	0.5681	**
98	***	***	0.3909	**
99	**	**	0.5928	**
100	**	**	0.5681	**
101	**	**	0.4582	**
102	***	***	0.4418	**

103	**	**	0.4037	**
104	***	***	0.2811	**
105	**	**	0.6229	*
106	**	**	0.5177	*
107	**	**	0.4246	*
108	**	**	0.4638	*
109	**	**	0.4037	*
110	**	**	0.3698	*
111	**	**	0.3616	*
112	**	**	0.4246	*
113	**	**	0.3617	*
114	*	**	0.2694	0.0685
115	*	*	0.4410	*
116	*	**	0.3256	0.0583
117	*	*	0.2843	0.1065
118	*	*	0.3447	0.0966
119	*	*	0.2253	0.1565
120	*	*	0.2843	0.1750
121	*	*	0.2541	0.1484
122	*	*	0.3140	0.1201
123	*	*	0.2780	0.1762
124	*	*	0.1919	0.1919
125	*	*	0.3584	0.2236
126	*	*	0.3456	0.2546
127	*	*	0.3456	0.2546
128	*	*	0.2843	0.2843
129	*	*	0.2045	0.3411
130	0.0601	0.0511	0.3861	0.3075
131	0.0639	0.0556	0.2546	0.4931
132	0.0848	0.0737	0.3221	0.5094
133	0.0651	0.0556	0.3137	0.4093
134	0.0609	0.0542	0.2130	0.5587
135	0.0970	0.0852	0.3308	0.5587
136	0.0638	0.0542	0.3308	0.3804
137	0.0609	0.0542	0.2130	0.5587
138	0.0833	0.0741	0.2731	0.5854
139	0.0609	0.0542	0.2130	0.5587
140	0.0609	0.0542	0.2130	0.5587
141	0.1170	0.1066	0.3137	0.6825
142	0.1296	0.1276	0.2655	0.8348
143	0.0585	0.0513	0.2218	0.5175
144	0.1050	0.0953	0.2940	0.6635
145	0.1010	0.0903	0.3075	0.6183
146	0.0609	0.0542	0.2130	0.5587

147	0.1317	0.1327	0.2546	0.8665
148	0.0961	0.0903	0.2425	0.7286
149	0.1296	0.1276	0.2655	0.8348
150	0.1375	0.1276	0.3277	0.7416

Appendix Table C.6. Statistical results for the difference between the biodegradation of cellulose each day for 18 Acres and Gartenacker soils for days 0-180 of the O_2 demand test.

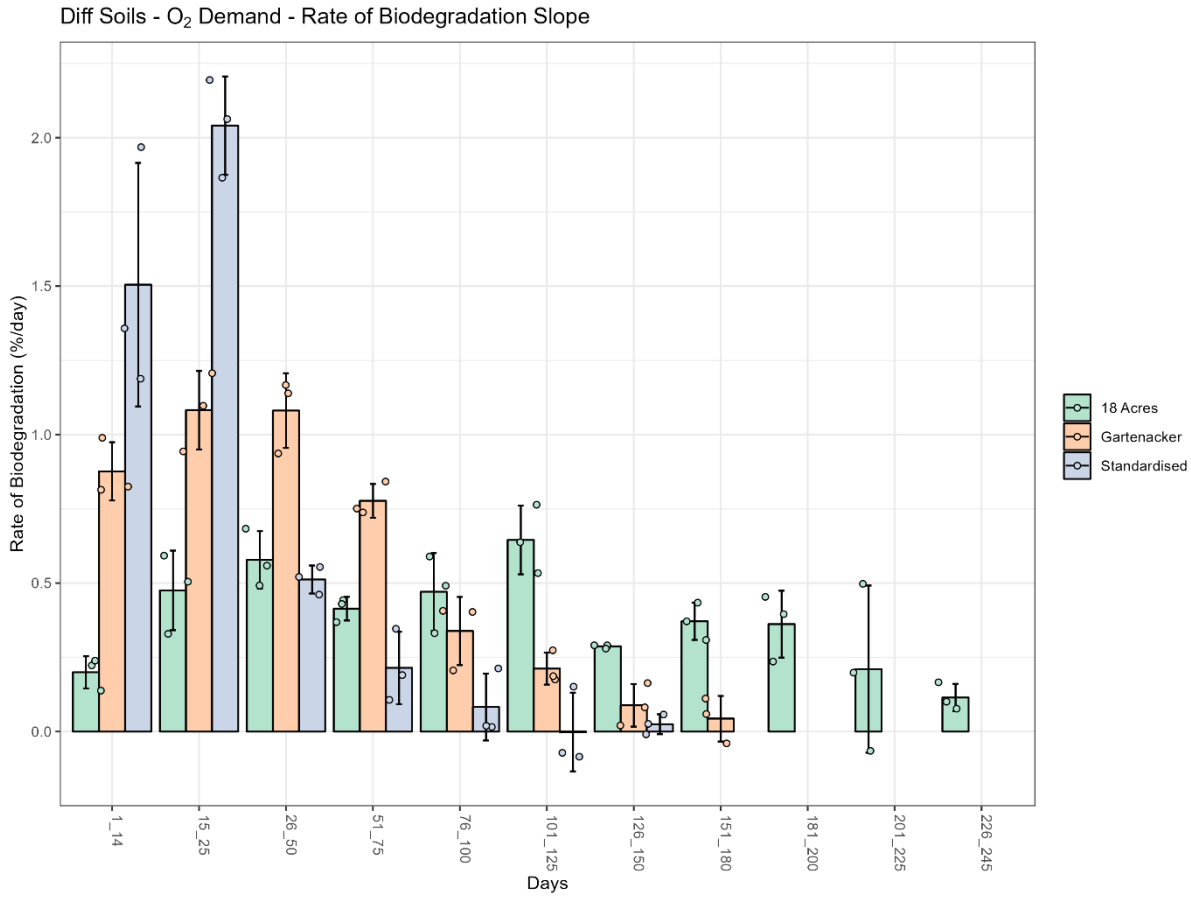
Day	t-value	df	p-value
0	NA	NA	NA
1	2	3.2	0.1336
2	0.353553	4	0.7415
3	3.535534	4	*
4	0.5	3.2	0.6495
5	0	2.941176	1.0000
6	0	4	1.0000
7	-0.61237	4	0.5734
8	-1	3.2	0.3868
9	-1.34164	2.941176	0.2739
10	-2.82843	4	*
11	-3.25	3.2	*
12	-2.44949	4	0.0705
13	-2.44949	4	0.0705
14	-2.44949	4	0.0705
15	-4.75	3.2	*
16	-4.75	3.2	*
17	-4.28661	4	*
18	-4.59619	2.56	*
19	-7	3.2	**
20	-4.89898	4	**
21	-6.12372	4	**
22	-5.05964	3.448276	*
23	-5.05964	3.448276	*
24	-4.52267	3.723077	*
25	-5.37587	3.448276	**
26	-7.18132	3.92	**
27	-5.37587	3.448276	**
28	-8.57321	4	**
29	-6.32456	3.448276	**
30	-6.33174	3.723077	**
31	-5.85021	3.448276	**
32	-6.06218	3.2	**
33	-8.31522	3.92	**
34	-5.5	2.876404	*

35	-6.33174	3.723077	**
36	-6.33174	3.723077	**
37	-5.94212	3.124324	**
38	-6.93375	3.484536	**
39	-6.66973	3.124324	**
40	-6.5964	2.209945	*
41	-8.69626	3.448276	**
42	-8.69626	3.448276	**
43	-7.2111	2.331034	*
44	-8.01784	2.305882	**
45	-10.4298	2.56	**
46	-8.59785	2.331034	**
47	-8.18183	2.331034	**
48	-8.15147	2.305882	**
49	-8.13327	2.941176	**
50	-8.5	2.876404	**
51	-9.4299	2.331034	**
52	-9.01388	2.331034	**
53	-12.1976	2.56	**
54	-9.01388	2.331034	**
55	-10.262	2.331034	**
56	-10.1559	2.305882	**
57	-10.262	2.331034	**
58	-9.84593	2.331034	**
59	-10.0698	2.941176	**
60	-9.68246	2.941176	**
61	-10.4571	2.941176	**
62	-9.875	3.2	**
63	-9.68246	2.941176	**
64	-10.8444	2.941176	**
65	-9.875	3.2	**
66	-10.8444	2.941176	**
67	-10.8444	2.941176	**
68	-8.44483	2.806366	**
69	-8.75761	2.806366	**
70	-10.25	3.2	**
71	-10.8444	2.941176	**
72	-11	3.2	**
73	-11	3.2	**
74	-11	3.2	**
75	-9.8387	2.209945	**
76	-10.625	3.2	**
77	-11	3.2	**
78	-8.96612	2.806366	**

79	-8.62911	3.297561	**
80	-8.62911	3.297561	**
81	-8.62911	3.297561	**
82	-8.62911	3.297561	**
83	-8.62911	3.297561	**
84	-8.62911	3.297561	**
85	-8.62911	3.297561	**
86	-6.48121	3.527807	**
87	-8.62911	3.297561	**
88	-7.0993	2.941176	**
89	-7.0993	2.941176	**
90	-7.0993	2.941176	**
91	-6.71984	3.448276	**
92	-6.71984	3.448276	**
93	-6.71984	3.448276	**
94	-6.72538	3.484536	**
95	-6.245	3.484536	**
96	-5.92857	3.173827	**
97	-6.14286	3.173827	**
98	-6.72538	3.484536	**
99	-5.5	3.173827	*
100	-5.5	3.173827	*
101	-5.71429	3.173827	**
102	-6.08738	3.563474	**
103	-5.27841	3.625434	**
104	-6.48267	3.448276	**
105	-4.8107	3.625434	*
106	-4.67707	3.625434	*
107	-4.54754	3.348298	*
108	-4.87752	3.625434	*
109	-5.01115	3.625434	**
110	-4.67707	3.625434	*
111	-4.67707	3.625434	*
112	-4.00184	3.348298	*
113	-4.12311	3.348298	*
114	-3.81994	3.348298	*
115	-3.90942	3.368856	*
116	-4.00184	3.348298	*
117	-3.57771	3.135718	*
118	-3.42074	3.368856	*
119	-3.3955	3.348298	*
120	-3.2423	3.135718	*
121	-3.42074	3.368856	*
122	-3.45613	3.348298	*

123	-3.30461	3.480449	*
124	-3.4641	3.709199	*
125	-2.90689	3.135718	0.0589
126	-2.90689	3.135718	0.0589
127	-2.90689	3.135718	0.0589
128	-2.90689	3.135718	0.0589
129	-3.0317	3.348298	*
130	-2.84979	3.348298	0.0572
131	-2.6264	3.773674	0.0620
132	-2.50511	3.480449	0.0757
133	-2.6264	3.773674	0.0620
134	-2.66501	3.480449	0.0649
135	-2.34521	3.480449	0.0887
136	-2.66501	3.480449	0.0649
137	-2.66501	3.480449	0.0649
138	-2.42437	3.773674	0.0762
139	-2.66501	3.480449	0.0649
140	-2.66501	3.480449	0.0649
141	-2.17183	3.773674	0.0997
142	-2.02031	3.773674	0.1177
143	-2.66501	3.480449	0.0649
144	-2.29416	3.690735	0.0890
145	-2.29416	3.690735	0.0890
146	-2.66501	3.480449	0.0649
147	-2.02031	3.773674	0.1177
148	-2.29416	3.690735	0.0890
149	-2.02031	3.773674	0.1177
150	-2.02031	3.773674	0.1177
151	-2.04882	3.923607	0.1112
152	-2.02541	3.480449	0.1232
153	-2.02031	3.773674	0.1177
154	-2.29416	3.690735	0.0890
155	-2.29416	3.690735	0.0890
156	-1.86878	3.773674	0.1393
157	-2.04882	3.923607	0.1112
158	-1.73544	3.934114	0.1589
159	-1.61749	3.923607	0.1825
160	-2.04882	3.923607	0.1112
161	-1.72532	3.923607	0.1609
162	-2.07846	3.709199	0.1117
163	-1.95003	3.690735	0.1289
164	-1.77924	3.923607	0.1512
165	-1.56358	3.923607	0.1943
166	-1.42374	3.928264	0.2289

167	-1.42374	3.928264	0.2289
168	-1.40183	3.923607	0.2349
169	-1.21218	4	0.2921
170	-1.272	3.889516	0.2741
171	-1.26918	3.999575	0.2732
172	-1.272	3.889516	0.2741
173	-1.11688	3.999575	0.3266
174	-1.11688	3.999575	0.3266
175	-0.94529	3.996474	0.3981
176	-1	3.99361	0.3740
177	-1.28654	3.957647	0.2684
178	-1.11688	3.999575	0.3266
179	-0.9649	3.957647	0.3898
180	-0.7	3.99361	0.5226



Appendix Figure C.2. Rate of biodegradation of cellulose in three different soils. Bars represent the mean rate per sample type. Circles represent the individual replicate values of each measurement.

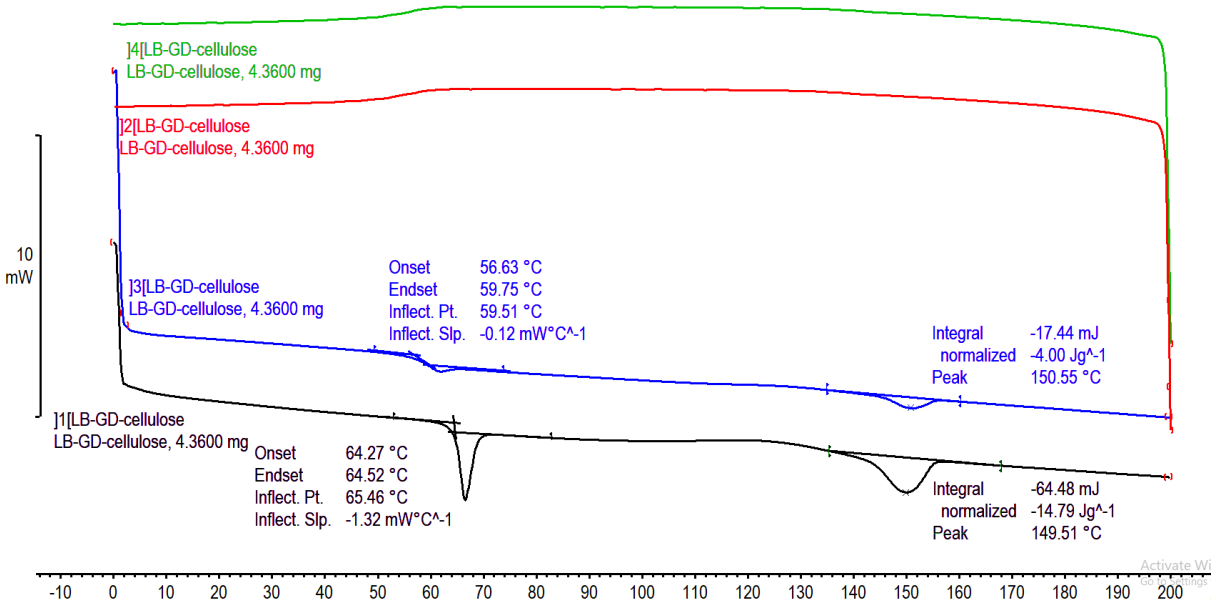
Appendix Table C.7. Statistical significance (*p*-value) of the differences between the rate of biodegradation of cellulose measured by O_2 demand up to day 150 in the three different soils (one-way ANOVA), and between the different soil types (Tukey multiple comparisons post-hoc test). Notation of p-values is as follow: p<0.05; *, p<0.01; **, p<0.001; ***.

Days	anova p-value	GAR-A18 p-value	STD-A18 p-value	STD-GAR p-value
1_14	**	*	**	*
15_25	***	**	***	***
26_50	***	**	0.6916	***
51_75	***	**	0.0529	***
76_100	*	0.4192	*	0.0872
101_125	***	**	***	0.1067
126_150	**	**	**	0.2790

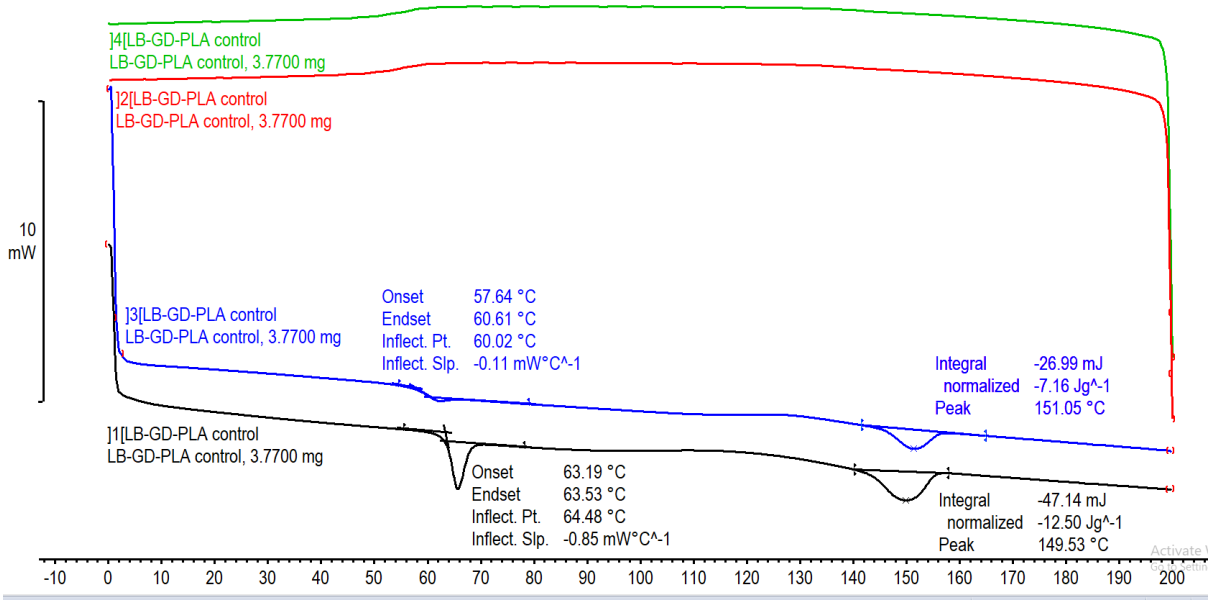
Appendix D

Appendix D provides additional information for Chapter 4.

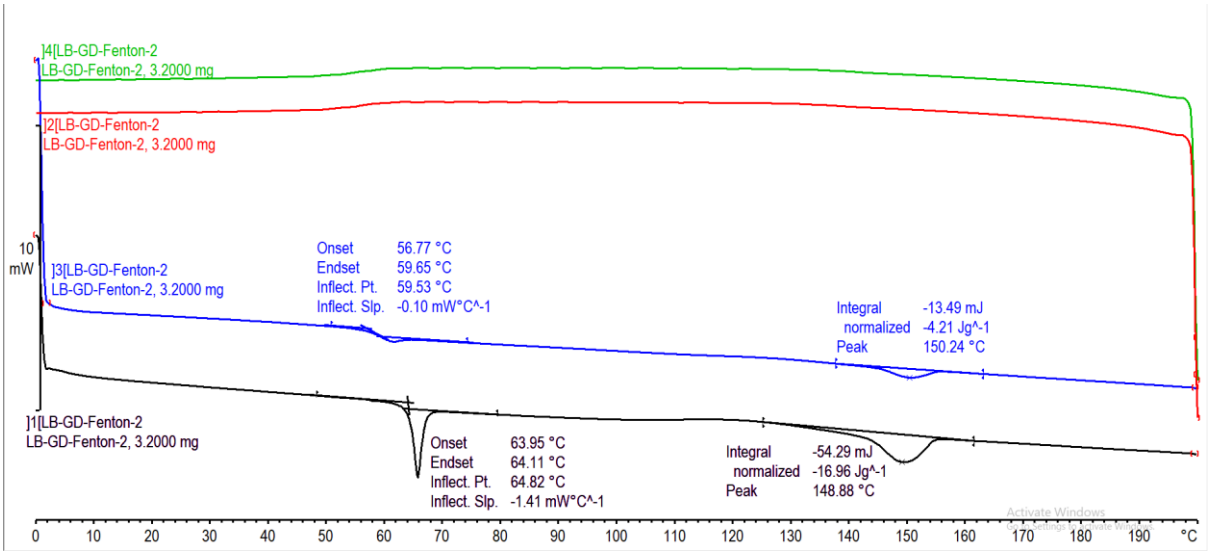
PLA DSC – Heating and Cooling Cycles



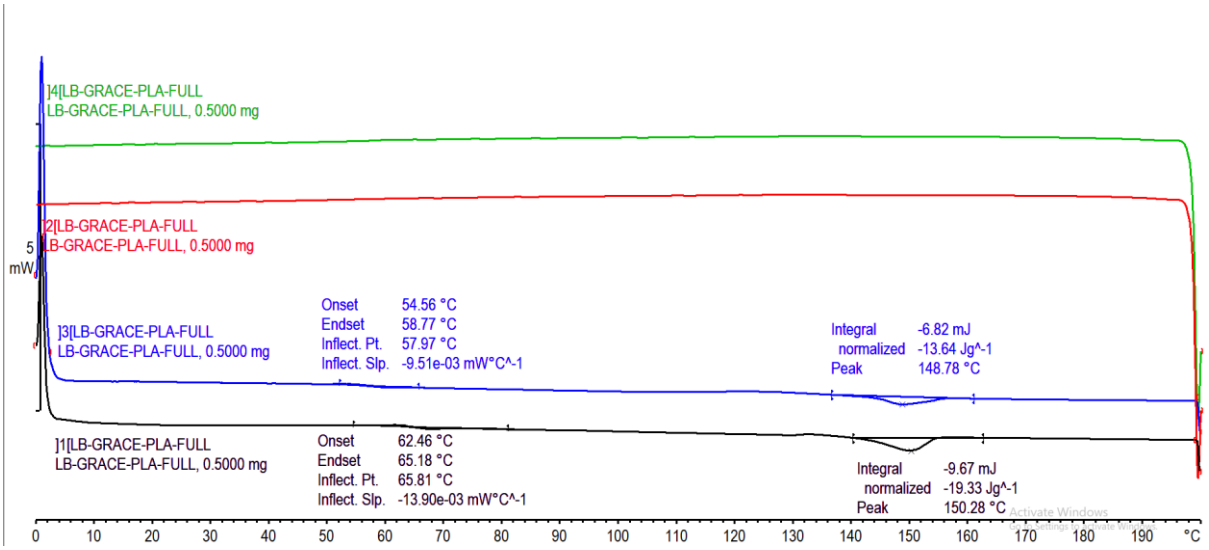
Appendix Figure D.1. Thermal characteristics of PLA after exposure to cellulase, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



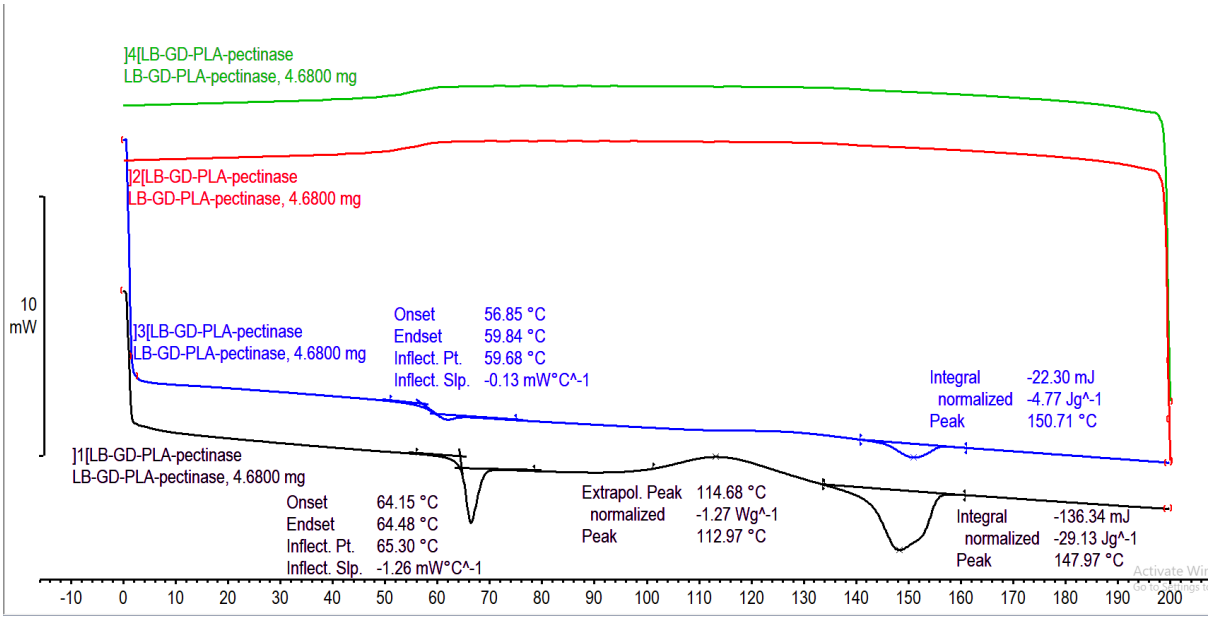
Appendix Figure D.2. Thermal characteristics of PLA after exposure to the control treatment, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



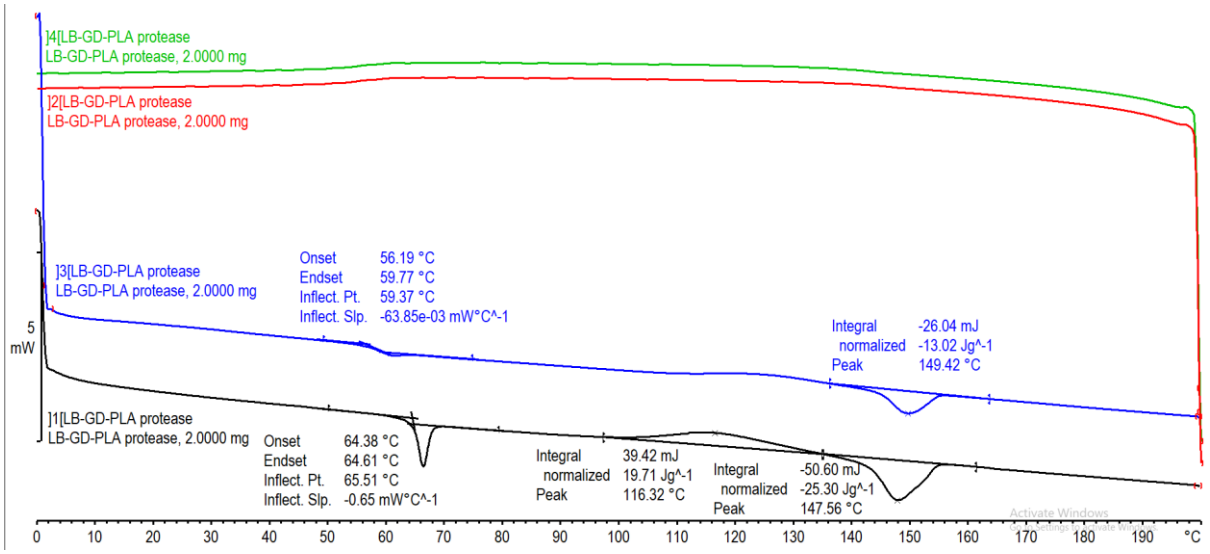
Appendix Figure D.3. Thermal characteristics of PLA after exposure to Fenton's reagent, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



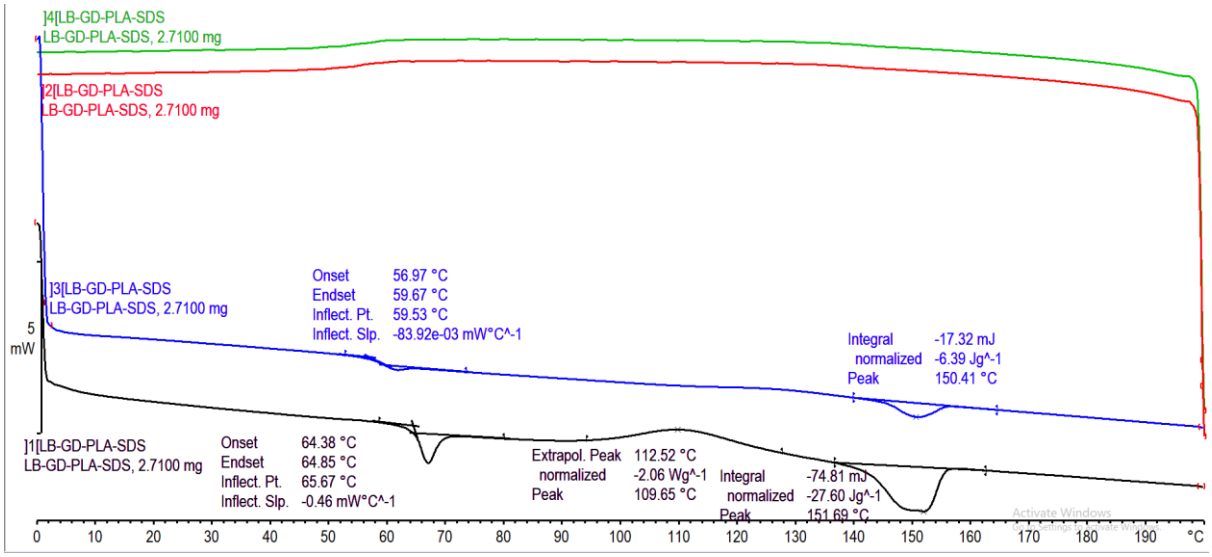
Appendix Figure D.4. Thermal characteristics of PLA after exposure to the full treatment sequence, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



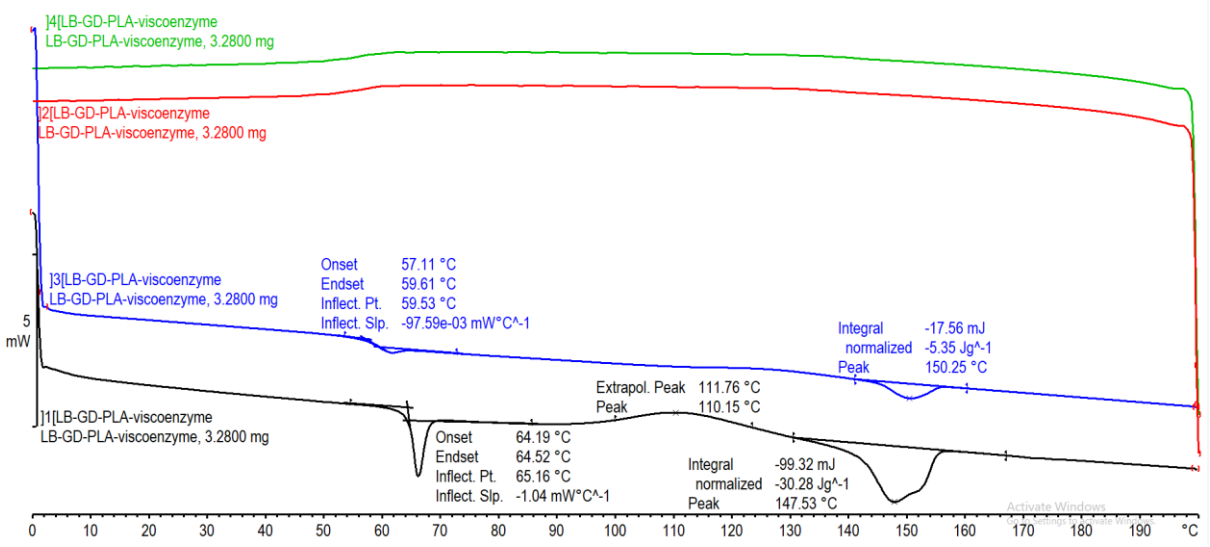
Appendix Figure D.5. Thermal characteristics of PLA after exposure to pectinase, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



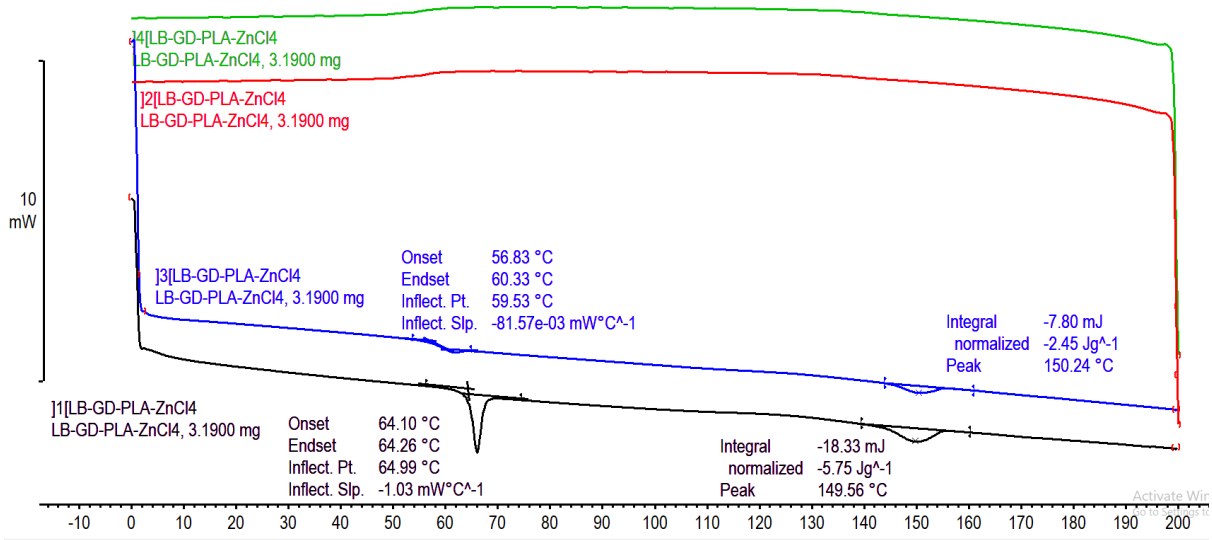
Appendix Figure D.6. Thermal characteristics of PLA after exposure to protease, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



Appendix Figure D.7. Thermal characteristics of PLA after exposure to SDS, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.

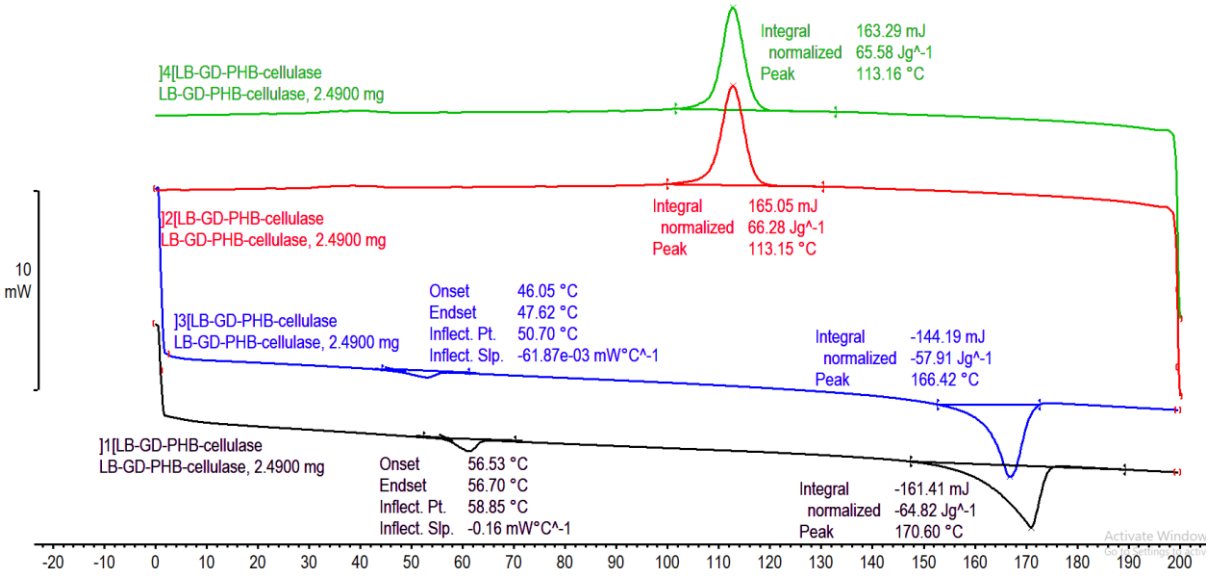


Appendix Figure D.8. Thermal characteristics of PLA after exposure to viscozyme, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.

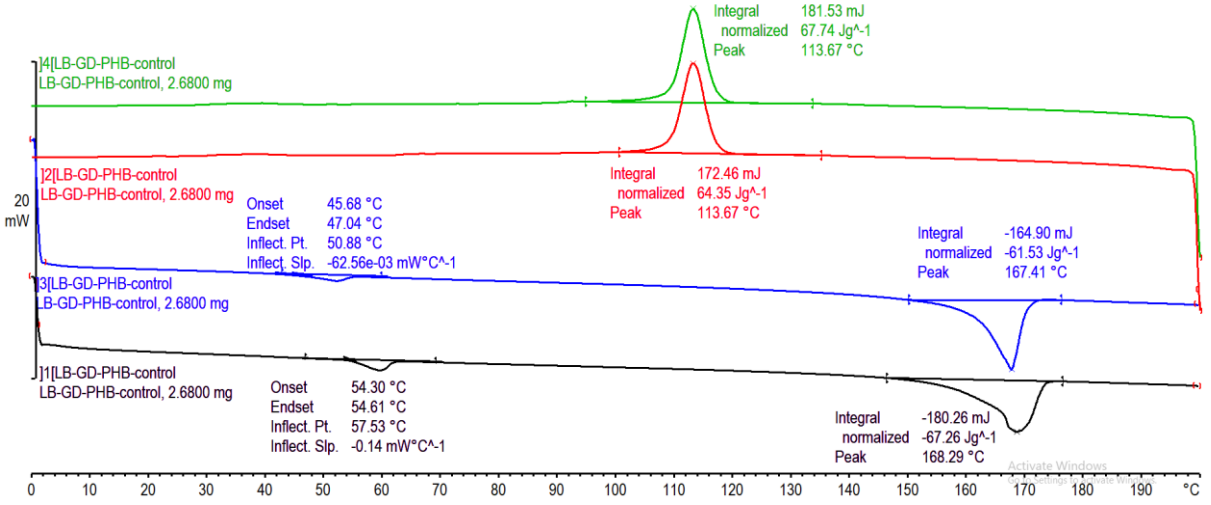


Appendix Figure D.9. Thermal characteristics of PLA after exposure to $ZnCl_2$, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.

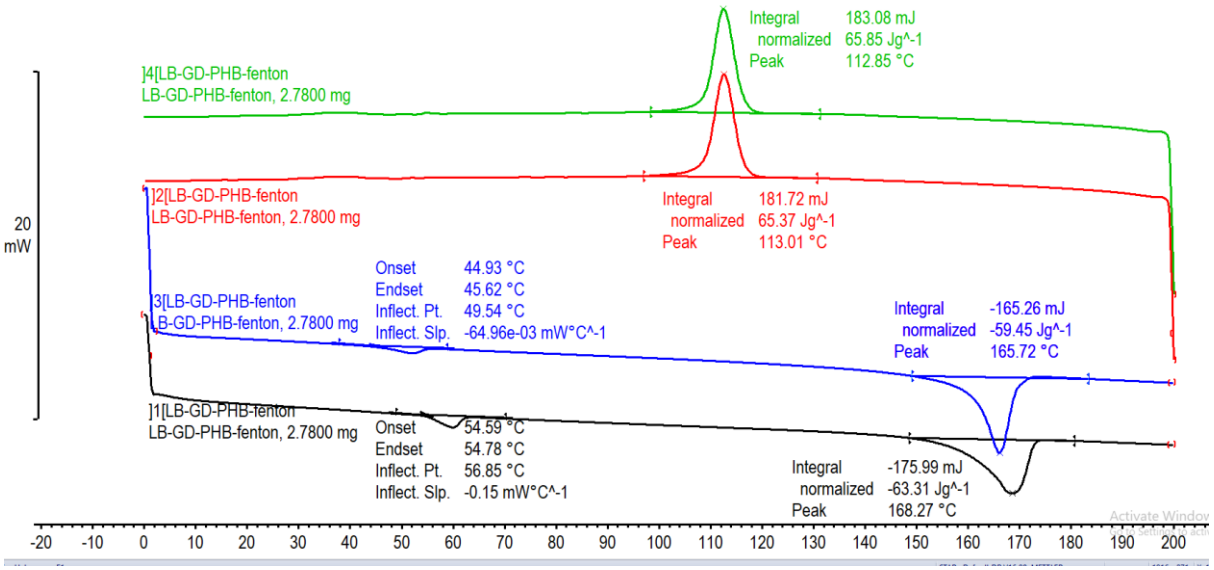
PHB DSC – Heating and Cooling Cycles



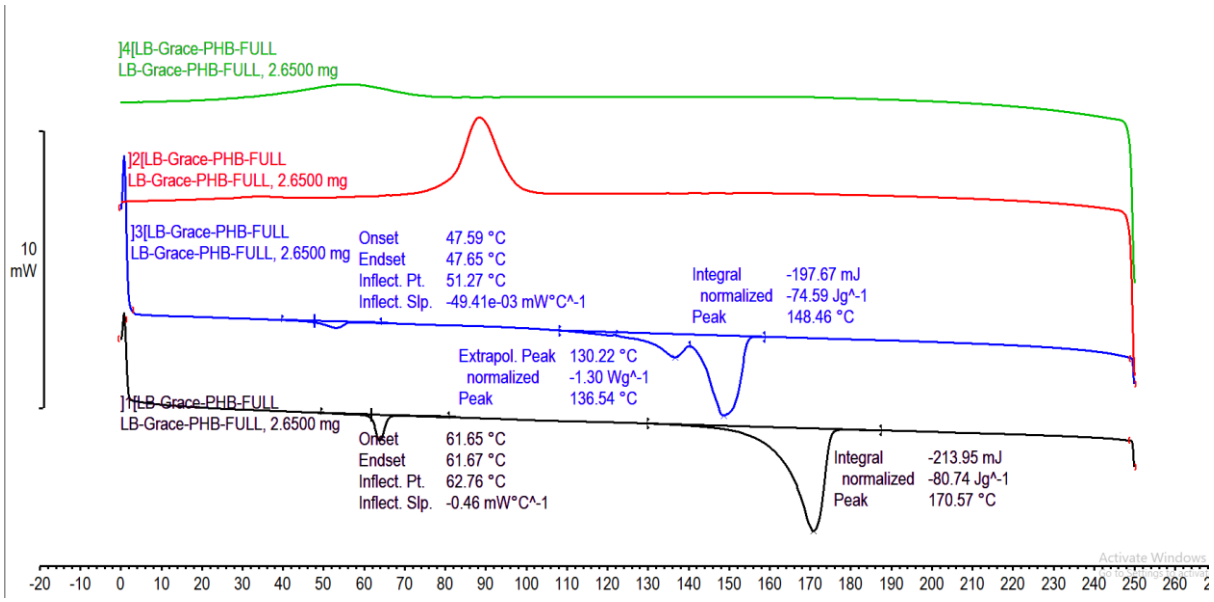
Appendix Figure D.10. Thermal characteristics of PHB after exposure to cellulase, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



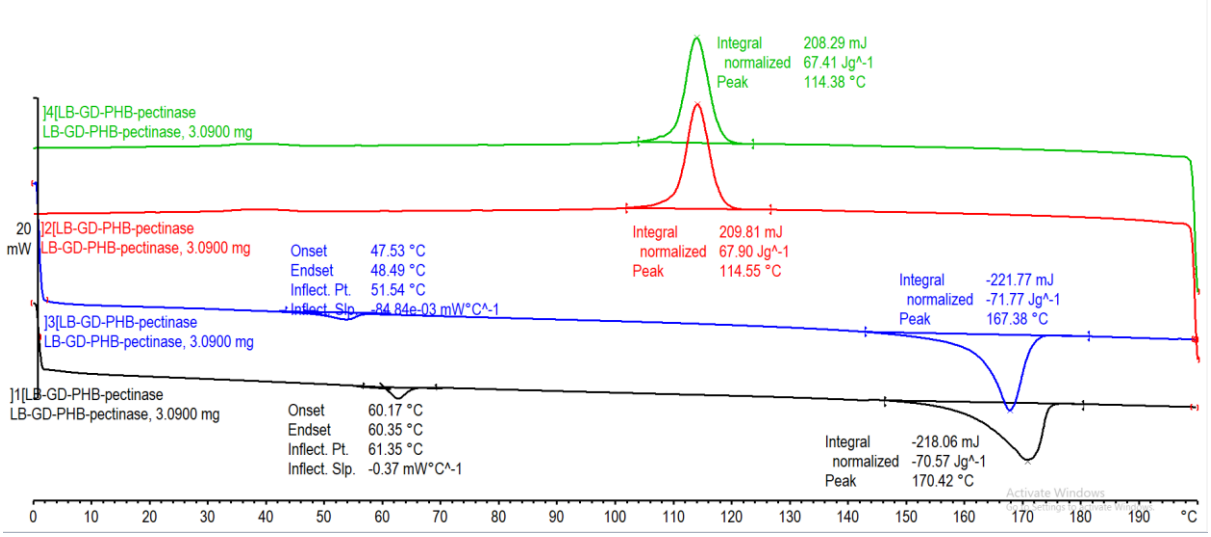
Appendix Figure D.11. Thermal characteristics of PHB after exposure to the control treatment, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



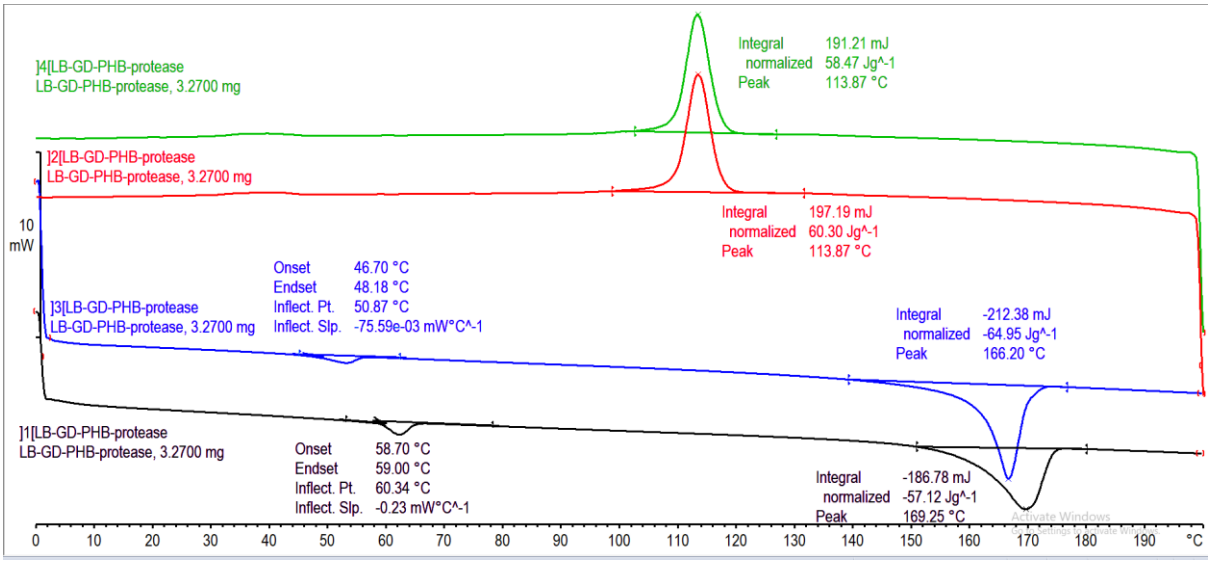
Appendix Figure D.12. Thermal characteristics of PHB after exposure to Fenton's reagent, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



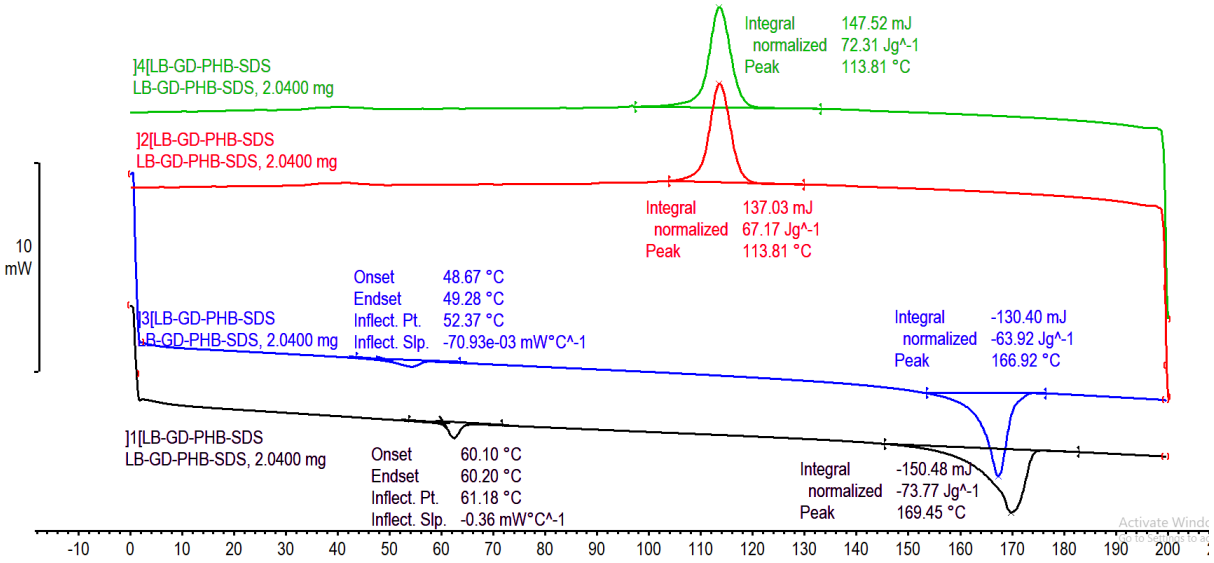
Appendix Figure D.13. Thermal characteristics of PHB after exposure to the full treatment sequence, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



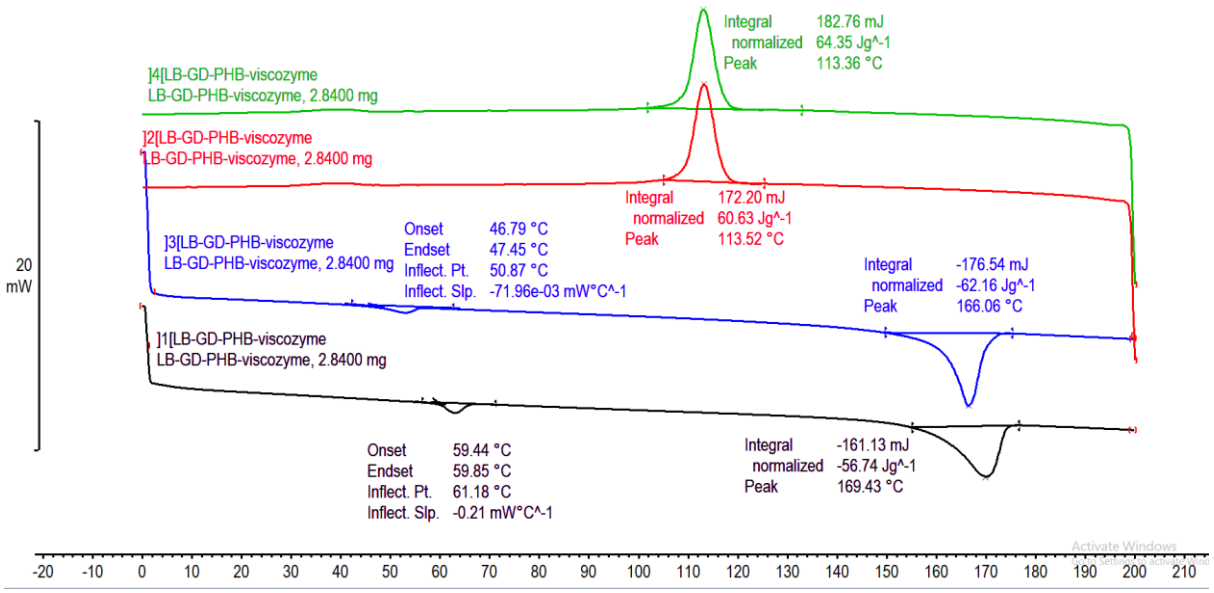
Appendix Figure D.14. Thermal characteristics of PHB after exposure to pectinase, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



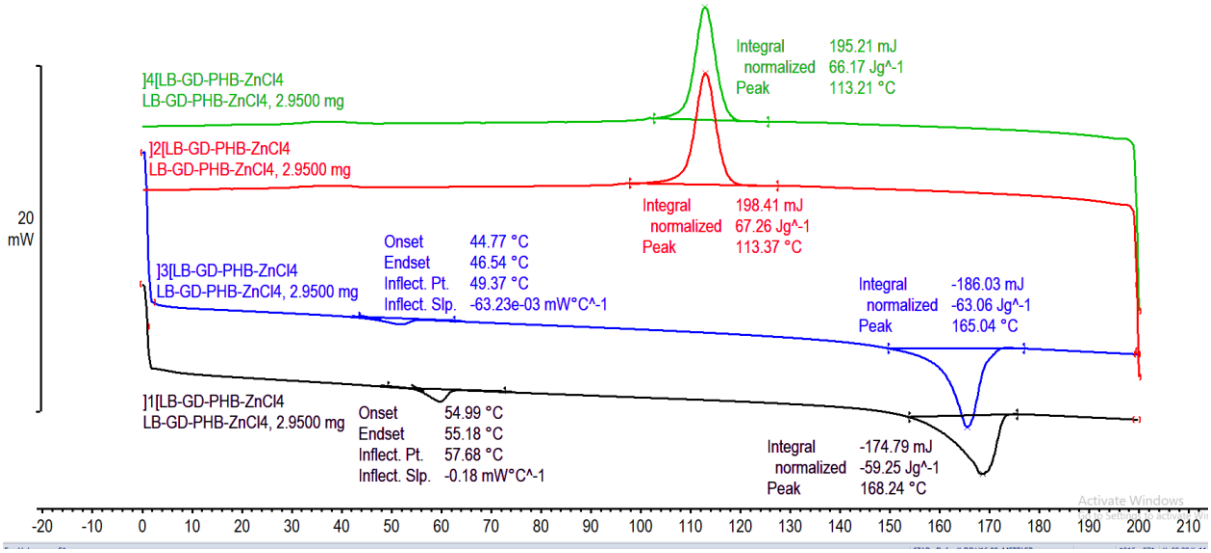
Appendix Figure D.15. Thermal characteristics of PHB after exposure to protease, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



Appendix Figure D.16. Thermal characteristics of PHB after exposure to SDS, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



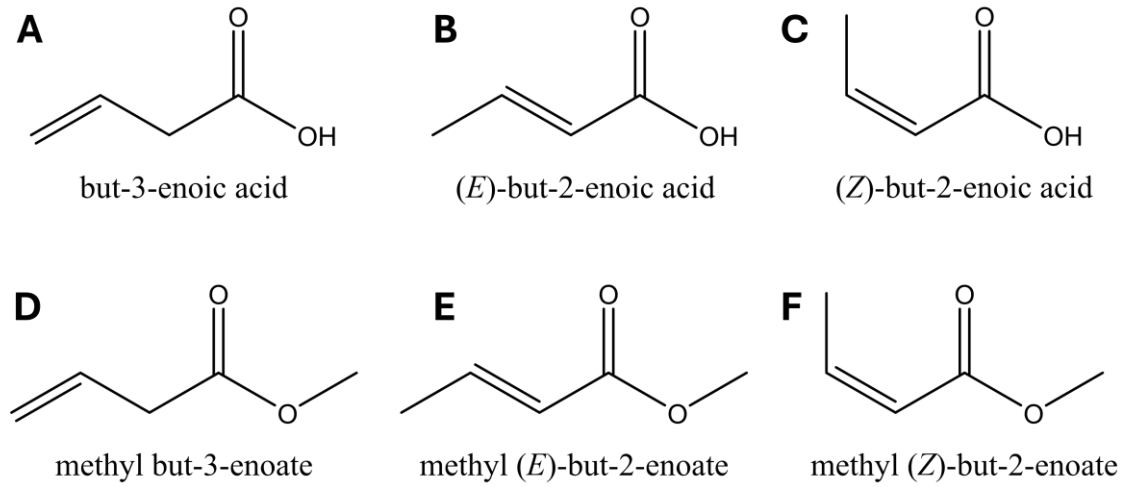
Appendix Figure D.17. Thermal characteristics of PHB after exposure to viscozyme, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.



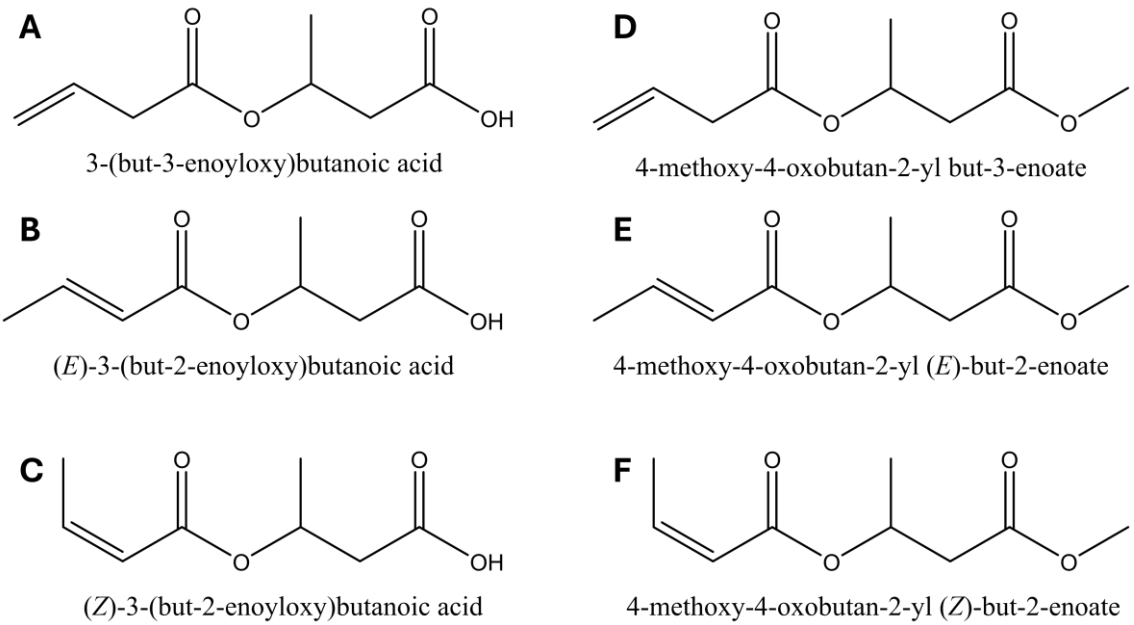
Appendix Figure D.18. Thermal characteristics of PHB after exposure to $ZnCl_2$, determined by differential scanning calorimetry (DSC) from 0–250°C at a heating rate of 10K min^{-1} for two heating/cooling cycles.

Appendix E

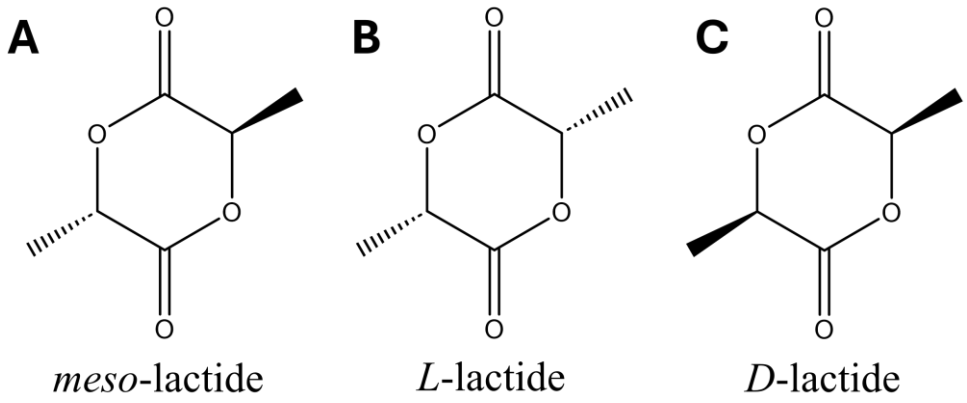
Appendix E provides additional information for Chapter 5.



Appendix Figure E.1. But-3-enoic acid, with all stereoisomers shown in both unmethylated (A-C) and methylated (D-F) forms.



Appendix Figure E.2. 3-(but-3-enoyloxy)butanoic acid, with all stereoisomers shown in both unmethylated (A-C) and methylated (D-F) forms.



Appendix Figure E.3. Lactide with all stereoisomers shown (A-C).

Appendix F

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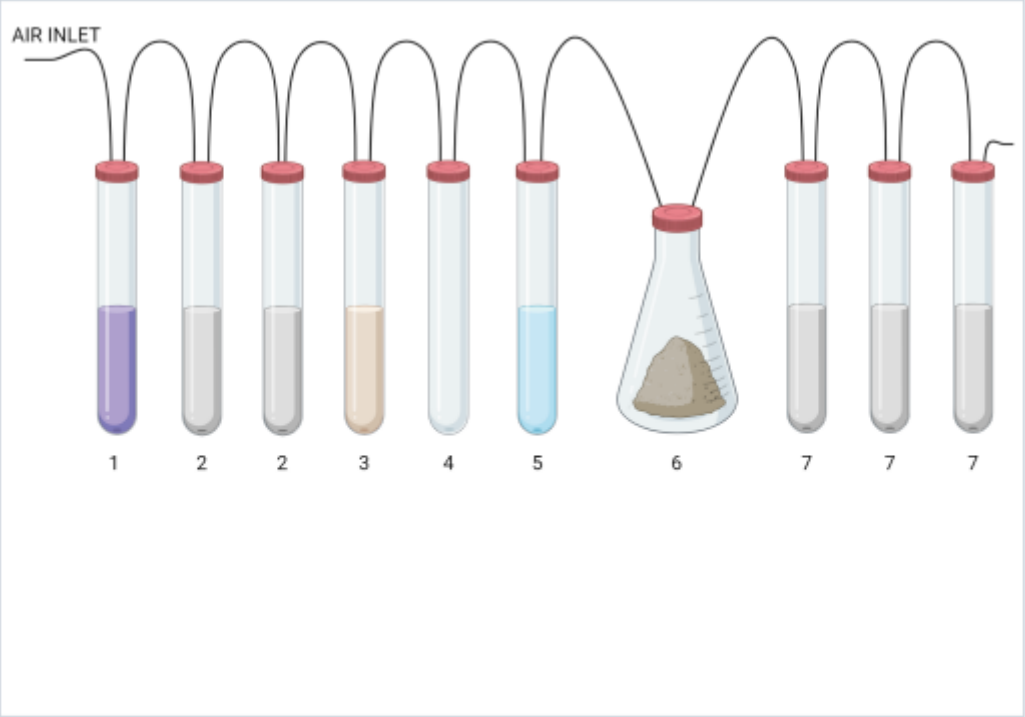
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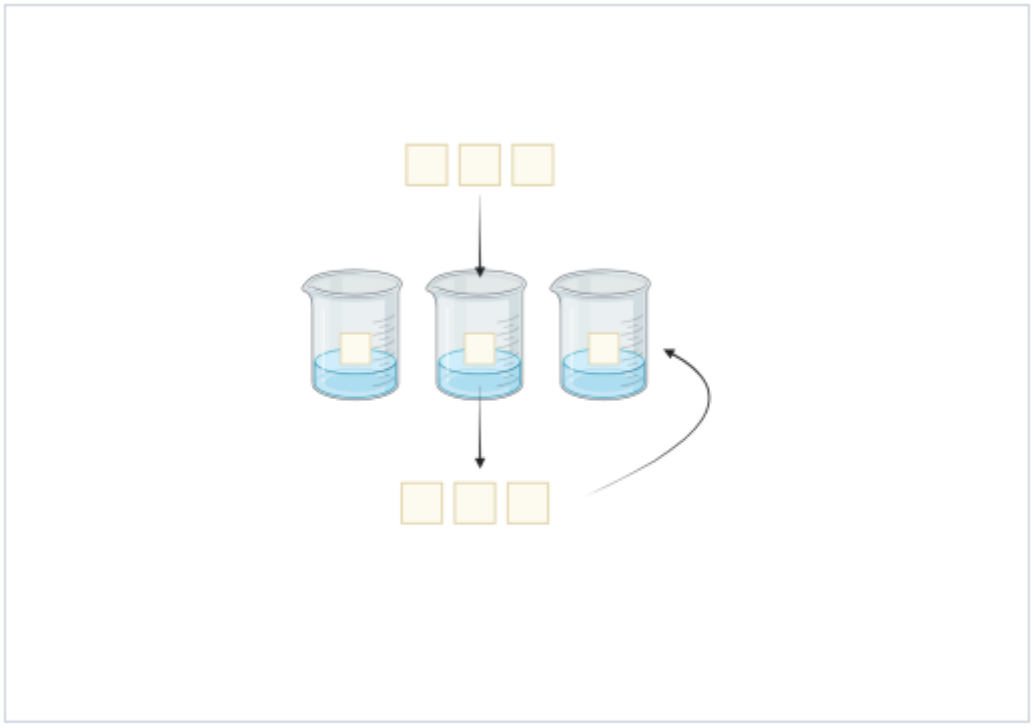
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