Interactions between plastic pollution, microorganisms and invertebrates in freshwater systems

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This is the sweet spot in time. As never before, and maybe as never again, there is a chance to protect the natural systems that keep us alive.

~ Sylvia A. Earle

Abstract

Plastic pollution is ubiquitous throughout the environment and there is widespread concern over its impacts on aquatic ecological systems. Whilst work to understand the risks of plastic has traditionally focused on the physical and chemical characteristics of materials, the biological factors that may alter the impacts and fate of plastics have been largely overlooked, particularly within freshwater environments. Therefore, this thesis explored interactions between plastic litter, microbial communities and aquatic invertebrates within UK freshwater. Work to investigate the composition and metabolic functionality of microbial communities attached to plastic surfaces was conducted. The attraction of benthic invertebrates to microbially colonised plastic and their influence on the fate and potential impacts of plastic litter in the environment was also examined.

Plastic surfaces hosted significantly distinct microbial communities compared to a non-plastic surface, and harboured communities with the potential to be pathogenic and to alter xenobiotic and biogeochemical cycling within the wider environment. These plastic-associated communities were also found to produce the odorous compound dimethyl sulfide. Given the role of this compound as a foraging cue in marine systems, this could significantly enhance the interactions which occur between plastic litter and aquatic organisms. Work to examine the influence of invertebrates on the fate of plastic in the environment found no significant interactions between microbially-colonised plastic and the amphipod *Gammarus pulex*. In contrast, the caddisfly larvae *Agrypnia* sp. rapidly fragmented plastic films (maximum diameter 6 mm) into hundreds of microplastics between 35.61 – 927.86 µm (maximum diameter), which is likely to have considerable implications for the bioavailability and fate of plastic litter in the environment.

These findings demonstrate that to build an accurate and comprehensive understanding of the risks of plastic pollution in freshwater environments a better working knowledge of the biological factors which influence the fate and impacts of plastics is now urgently needed.

299/300 abstract word limit

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Declaration

I declare that this thesis is a presentation of original work, completed by myself as a PhD student at the University of York. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references. This research was funded mainly by the NERC ACCE doctoral training program, with DNA sequencing work funded by the Center for Ecology and Hydrology, Wallingford.

Chapter 3, 4 and 5 of this thesis have been either submitted for publication in international peer-reviewed journals, or have been prepared for submission, and have had contributions from other co-authors. These papers have been re-worked, so they are presented in a consistent style within the thesis. Contributions from co-authors are outlined below.

- **Chapter 2**: Due to COVID 19 restrictions, a small portion of practical work for this chapter was carried out by my supervisor Lindsay Newbold at the Center for Ecology and Hydrology, Wallingford. Work carried out by Lindsay Newbold included the transfer of extracted and PCR-amplified DNA to the Illumina MiSeq instrument and the processing of resulting data through the DADA2 pipeline. All other practical work and data analysis was conducted by myself.
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Chapter 1

Introduction, literature review and thesis aims

1.1 Introduction

The first household use of plastics was in the early 20th century (Thompson et al., 2009). The use of plastic then remained relatively small-scale until the end of World War II, after which the mass production and widespread use of plastic took hold – giving rise to the beginning of the "Plastic Age" in the 1950s (Thompson et al., 2009). Plastics are now used extensively throughout society, with applications in sectors such as packaging, electronics, consumer items, agriculture and construction (Plastics-Europe, 2021). Very little of our lives remain untouched by plastics and they have undoubtedly shaped the development of modern society (Napper & Thompson, 2020). This widespread use is unsurprising given that plastics offer immense benefits, for example in health care, food packaging and construction (Napper & Thompson, 2020; Plastics-Europe, 2021), where they have improved health and hygiene, reduced food waste, and lowered energy consumption due to their light-weight nature (Andrady & Neal, 2009). However, there are also significant environmental problems associated with plastics. These problems largely stem from our over-reliance on these materials, particularly for single-use items, and the improper management of waste plastic at the end-

of-life stage (Napper & Thompson, 2020). Huge volumes of plastic are released into the environment every year, where they are known to elicit far-reaching effects on individual organisms and natural ecosystems (MacLeod et al., 2021). Consequently, scientific and public concern for the impacts of environmental plastic litter is now tremendous (Bellou et al., 2021), and targets for plastic reduction are included in the United Nations 2030 Sustainable Development Goals (United-Nations, 2015). This chapter reviews our current knowledge of the presence and effects of plastic pollution in aquatic environments; it examines the interactions which are known to occur between plastic litter, microbial communities, and larger aquatic organisms, and identifies the key knowledge gaps which are yet to be addressed or require further attention.

1.2 Plastic litter in the environment: abundance and implications

1.2.1 Abundance of plastic in the environment

Although often referred to as a single material, the term plastic encompasses a vast range of polymers with different chemical structures and physical properties (Andrady, 2017) and therefore represents a diverse mixture of broadly similar but distinct materials. In 2020, approximately 367 million tonnes of plastic was produced globally and production is only forecast to increase in coming years (Plastics-Europe, 2021). Much of this plastic is intended for single or short-term use, and between 1950 and 2050 it is estimated that just over 2.5 billion tonnes of plastic waste will have been generated (Geyer et al., 2017). Although steps are taken to manage plastic waste at the end-of-life stage, such as through landfilling, recycling and incineration, significant leakage along these waste collection and disposal pathways results in the release of large quantities of plastic to the environment (Sharma et al., 2019). For example, countries such as the UK and the USA ship thousands of tonnes of plastics, intended for recycling, to developing countries, despite the knowledge that these countries do not have the infrastructure to contain and appropriately manage this plastic, with large quantities being illegally dumped (Comolli, 2021). It has been estimated that by 2026 the mismanagement of plastic waste will lead to 156 – 266 million tonnes of plastic waste being emitted into the environment every year (Lebreton & Andrady, 2019; Zhang et al., 2021), resulting in the continued pollution of

terrestrial, freshwater and marine ecosystems. Due to the relatively inert nature of plastic it is thought to take hundreds to thousands of years to breakdown (Barnes et al., 2009), leading to its significant accumulation in the environment. During this breakdown process, many smaller plastic fragments are released, a process first described by Thompson et al. (2004) who coined the term 'microplastics' to describe these smaller plastic particles, and in a 2008 NOAA workshop the term microplastic was more formally defined as plastic particles smaller than 5 mm (Arthur et al., 2008; Verschoor, 2015). Following from this, the term 'macroplastic' is now generally used to refer to plastics larger than 5 mm, and 'nanoplastic' is used to describe particles smaller than 1 µm. However, there is no internationally agreed recognition of these size classifications and definitions can vary between different studies (Hartmann et al., 2019). Care and clarification is therefore required when interpreting the findings of studies and reports which use these different terminologies.

The discharge of plastics from land is known to be the greatest contributor to plastic pollution in aquatic systems (He et al., 2022). Once in the aquatic environment the breakdown of plastic into smaller pieces occurs through environmental weathering, involving a mixture of photooxidation from exposure to UV radiation, photothermal oxidation, mechanical abrasion, hydrolysis and surface biodegradation (Song et al., 2017). The presence of plastic fragments in the ocean was first noted in 1972 (Carpenter et al., 1972; Carpenter & Smith, 1972), since which, field-based research has provided clear evidence that both larger pieces of plastic litter and smaller microplastics have become ubiquitous throughout practically all aquatic environments (Castro-Castellon et al., 2022). Modelling-based techniques estimate that in 2010 there was around 4.9×10^5 tonnes of plastic (< 5 mm) floating in the surface ocean, with a potential for this value to increase up to 1.3×10^8 tonnes by the year 2100 (Everaert et al., 2018). The same study also estimated that by the year 2100 there could also be up to 8050 plastic particles ($<$ 5mm) per kg of sediment present in intertidal areas and up to 373 particles per kg in deep sea sediment. Plastic litter has even reached the deepest point in the ocean, 10898 meters below the surface in the Mariana Trench (Chiba et al., 2018), as well as Antarctic ecosystems (Waller et al., 2017), demonstrating the ability of these materials to reach even the most remote locations.

Whilst plastic litter in the marine environment has received extensive research attention, freshwater

systems such as rivers and lakes have been less studied, with 87 % of identified plastic litter studies published between 1980 and 2018 focusing on marine environments and only 13 % on freshwater environments (Blettler et al., 2018). Despite being less studied, plastic litter is similarly widespread throughout natural freshwater systems, as well as man-made systems such as reservoirs and waste water treatment plants, and has an abundance which is comparable to or higher than the marine environment (Klein et al., 2015; Luo et al., 2019; Wang et al., 2022). For example, the microplastic ($<$ 5mm) concentration of various environmental freshwater compartments across different continents is shown in Figure 1.1 where it can be seen that the microplastic concentration of sediment in the Pearl River in China can reach up to 9597 particles/kg. The most common forms of microplastic particles found in these freshwater systems are fragments, films and fibres (Lu et al., 2021; Yang et al., 2021b) and larger plastics litter items ($>$ 5 mm) are also highly abundant. For example, between 426 and 120,632 large identifiable plastic litter items per system were found across nine different European lakes and rivers with food wrappers, bottles and bags being the most commonly reported items (Winton et al., 2020). Similar reports have since been made from a separate study of freshwater rivers in Greece, where drinking bottles, single-use bags and product packaging were the most common items of plastic litter found, with authors identifying the need for better waste management to improve the situation (Aslam et al., 2022). The dominance of these items demonstrates the high pollution potential of single-use plastics. Furthermore, the materials these items are commonly made from is consistent with the types of microplastic polymers most often reported in freshwater - polypropylene, polyethylene, polystyrene and polyethylene terephthalate (Figure 1.2) (Andrady, 2011; Lu et al., 2021; Yang et al., 2021b). This is a further indication that the breakdown of these larger single-use plastic items is directly contributing to microplastic contamination of freshwater systems.

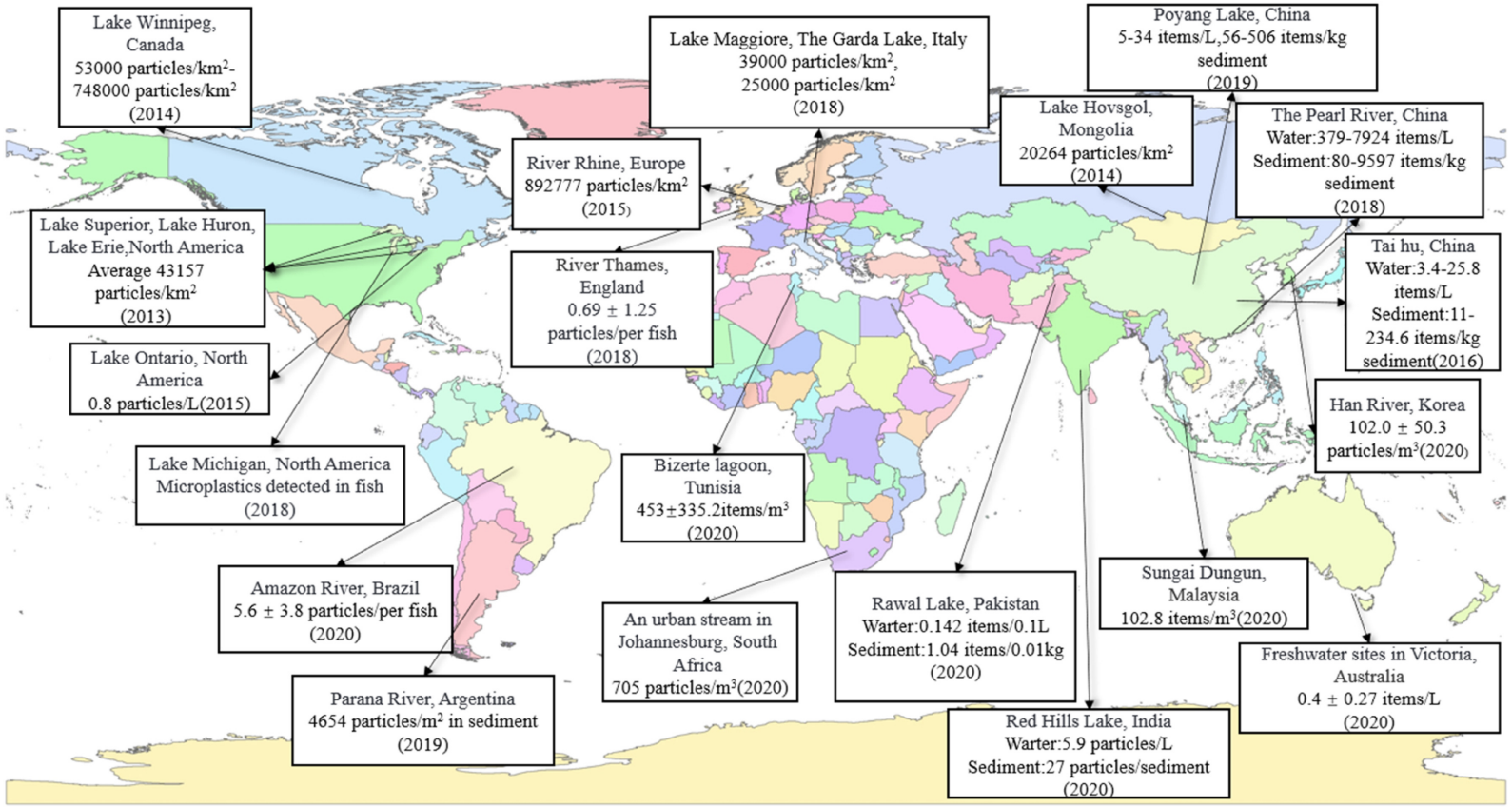


Figure 1.1: Examples of microplastic abundance ($<$ 5 mm) found within different freshwater systems across different continents. Reproduced with permission from Wang et al. (2022).

Rivers expedite the transport of thousands of tonnes of plastic litter from the land into the ocean each year (González-Fernández et al., 2021; Mai et al., 2020; van Emmerik et al., 2022) and understanding riverine plastic pollution is therefore important for building our knowledge of plastic levels and impacts in the marine environment. However, rivers are far more than just a transport mechanism for plastic, they are often the first recipients of plastic emissions (Vaid et al., 2021) and there is growing evidence to suggest that many rivers may act as plastic reservoirs, with a considerable portion of plastic which enters them remaining trapped within the sediment, banks, plants and infrastructure of the riverine system itself (van Emmerik et al., 2022). The impacts of plastic on freshwater systems are thought to be immense, however our knowledge is less developed than for marine systems (Castro-Castellon et al., 2022). Therefore, although the number of freshwater plastic studies has increased in recent years (He et al., 2022; Vaid et al., 2021) much more work is still needed to better understand the dynamics, impacts and most effective management options for plastic pollution in freshwater systems.

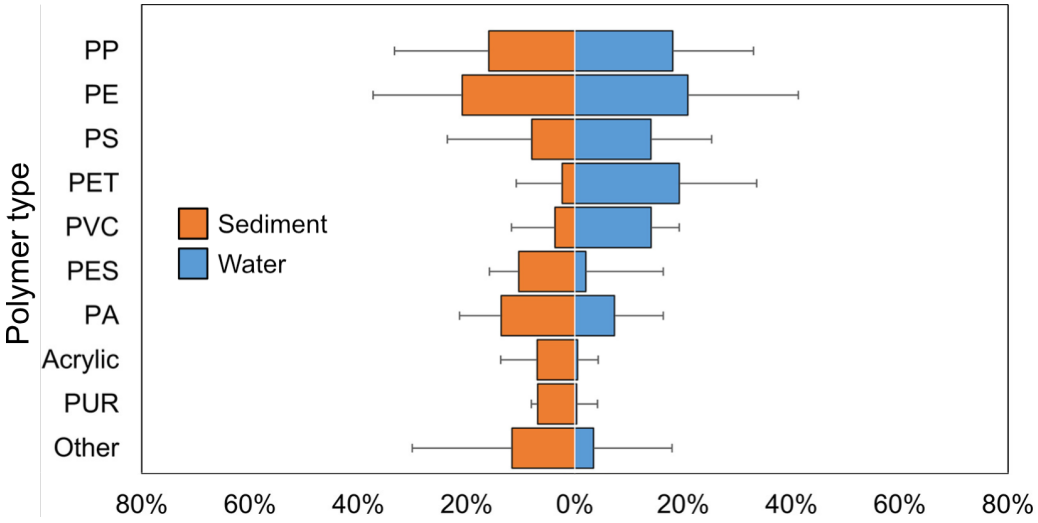


Figure 1.2: Relative proportion of different microplastic types (< 5mm) found throughout freshwater systems globally, gathered from the systematic review of 131 peer-reviewed studies. Reproduced with permission from (Lu et al., 2021). PP: Polypropylene; PE: Polyethylene; PS: Polystyrene; PET: Polyethylene terephthalate; PVC: Polyvinyl chloride; PES: Polyester; PA: Polyamide; PUR: Polyurethane; Other: Polyacrylonitrile (PAN), Polyvinyl alcohol (PVA), Alkyd, Rayon, Poly(methyl methacrylate) (PMMA).

1.2.2 Impacts of plastic - why should we be concerned?

Many organisms ingest and interact with plastics in the environment, which can result in wide ranging effects for organisms and ecosystem health (MacLeod et al., 2021). The ingestion of whole smaller microplastics is the most well studied route of exposure and can occur either by direct ingestion or through trophic transfer (Parker et al., 2021). Plastic uptake has been documented in many marine species, with a recent study reporting the occurrence of plastic in 386 species of marine fish and a significant positive correlation between microplastic occurrence in organisms and microplastic concentration in the surrounding water (Savoca et al., 2021). Other studies report uptake by an even broader range of species; with Santos et al. (2021) showing plastic uptake by around 700 marine fish species, as well as by roughly 230 bird, 220 invertebrate, 80 mammal and 10 reptile marine species. Although research is less comprehensive, plastic uptake is also widespread throughout freshwater species. For example: Parker et al. (2021) describe plastic uptake in various freshwater fish species across six different continents, with an average of up to 19 plastic particles per individual; Azevedo-Santos et al. (2021) found plastic ingestion in around 160 freshwater fish species, along with one mammal, and several species of freshwater invertebrates including crustaceans, rotifers, cnidarians, platyhelminths, annelids, molluscs and arthropods; in a European river 48.5 % of microbenthic invertebrate community taxa showed evidence of plastic ingestion, with collector-gathers and predators showing considerably higher levels of microplastic uptake than filter feeders, shredders and scrapers (Bertoli et al., 2022).

Other plastic-organism interactions besides uptake are also known to occur. For example: in marine systems several benthic octopus species have been found sheltering in plastic litter and carrying it around (Freitas et al., 2022); hermit crabs have been discovered living inside pieces of plastic instead of shells (e.g. Barreiros & Luiz, 2009); and some isopods and polychaetes are known to burrow into and live inside large blocks of polystyrene foam (Davidson, 2012; Jang et al., 2018). Similar interactions occur within freshwater systems, with Wilson et al. (2021) identifying a large number of invertebrates living on plastic litter in UK streams, with two of these species (black fly larvae and a ramshorn snail) found to be exclusively associated with flexible plastic or fabric litter and

not with natural rocks or other material. Artru and Lecerf (2019) also found a range of different invertebrates to be associated with biodegradable plastic films submerged in a freshwater stream. Other interesting interactions include the use of plastic particles by freshwater caddisfly larvae to construct their protective cases (Ehlers et al., 2019; Tibbetts et al., 2018), however, these types of organism-plastic interactions are particularly understudied in freshwater, with little understanding of the extent to which they are occurring and the impacts they may have.

It is common knowledge that interactions with and ingestion of plastic by aquatic species can result in a huge variety of negative impacts. These impacts act at different levels of biological organisation. For example, microplastic exposure can cause damage at the cellular level; this then alters the functionality of whole tissues and organs, such as by causing gut dysbiosis and altered metabolic activity, and ultimately the entire individual organism can become impacted with alterations to overall health and fitness (Bucci et al., 2020; Parker et al., 2021). Examples of the types of individual-level effects induced by microplastics in different aquatic organisms are out outlined in Table 1.1.

Table 1.1: Examples of the impacts of microplastics on different marine and freshwater organisms. (1) Athey et al. (2020), (2) Qiang and Cheng (2019), (3) Wright et al. (2013), (4) Yin et al. (2018), (5) Murphy and Quinn (2018), (6) Redondo-Hasselerharm et al. (2018), (7) Watts et al. (2015), (8) Yu et al. (2018).

Organism	**Concentration**	**Impact**	**Reference**
Larval freshwater fish *Menidia beryllina*	5 x 10^5 beads mL^{-1}	Reduced weight gain after exposure to 10 – 20 µm polyethylene beads	(1)
Larval freshwater zebrafish *Danio rerio*	1.91 x 10^5 beads mL^{-1}	Reduced swimming distance and speed after exposure to 1 µm polystyrene beads	(2)
Marine polychaete *Arenicola marina*	0.5 - 5.0 % of exposure sediment by weight	Reduction in feeding activity and energy reserves after exposure to 130 µm unplasticised polyvinylchloride particles	(3)
Marine fish *Sebastes schlegelii*	1 x 10^3 beads mL^{-1}	Decrease in feeding activity and swimming speed after exposure to 15 µm polystyrene beads	(4)

Freshwater cnidarian *Hydra attenuata*	0.01 - 0.08 grams particles mL^{-1}	Reduction in feeding rate and changes to morphology after exposure to polyethylene particles <400 µm	(5)
Freshwater amphipod *Gammarus pulex*	0.1 - 40 % of exposure sediment by weight	Reduced growth rate after exposure to 20 – 500 µm polystyrene fragments	(6)
Marine crab *Carcinus maenas*	0.3 - 1.0 % of feed by weight	Reduced food consumption and available energy for growth after exposure to 1 – 5 mm polypropylene fibres	(7)
Marine crab *Eriocheir sinensis*	0.04 - 40 µg beads mL^{-1}	Reduced growth and weight gain after exposure to 5 µm polystyrene beads	(8)

As well as the harmful effects of plastic ingestion, other interactions such as entanglement and entrapment in larger plastic debris also occur and often have severe consequences for the organisms involved. Entanglement in discarded plastic-based fishing gear has been shown to occur in over 350 different marine species (Høiberg et al., 2022), and entrapment in beached plastic debris is thought to cause the death of between 61,000 and 508,000 hermit crabs each year on two remote islands in the South Pacific and Indian Ocean (Lavers et al., 2020). Similar incidences occur within freshwater, for example, many species of fish have been found, often dead, extensively entangled in plastic litter such as discarded fishing gear, plastic bottles and plastic bags in rivers across South America (Blettler & Mitchell, 2021). These individual-level effects elicited by plastic can also scale up to influence the wider environment, with potential implications for whole communities, as well as the functioning, general health, and resilience of wider ecosystems (Parker et al., 2021). For example, the exposure of a freshwater benthic macroinvertebrate community to polyethylene microplastics led to a significant change in community structure after eight days, due to a reduction in deposit-feeders and grazers (Silva et al., 2022). Exposure to microplastics has also been found to result in a significant reduction in leaf litter decomposition by detritivores within a stream microcosm (López-Rojo et al., 2020), and significant modifications to the invertebrate-mediated nitrogen cycling activity in freshwater sediment (Huang et al., 2021). Other studies also suggest a wider general trend of reduced primary productivity

and carbon sequestration in ecosystems as a result of microplastic exposure (Sridharan et al., 2021).

Whilst entanglement and entrapment in larger plastic debris is likely to always be detrimental for organisms, the overall effects of microplastic uptake are less clear. Along with the multitude of studies which report negative effects of plastic exposure on aquatic organisms, there are many others which report little or no effects and findings in the literature are highly contrasting. For example, no adverse effects were seen in the marine mussel *Perna perna* after exposure to polystyrene beads for 21 days (Santana et al., 2018), or in the freshwater crustacean *Gammarus pulex* after exposure to polyethylene terephthalate fragments for 48 days (Weber et al., 2018). Furthermore, the systematic analysis of 43 different studies of fish and invertebrate groups found that, whilst microplastic exposure often leads to reduced food intake, there is relatively weak evidence to suggest consistent negative effects on growth, reproduction or survival of organisms (Foley et al., 2018). Other recent reviews similarly suggest that, although there is strong evidence of microplastic toxicity in some species under certain conditions, there is a high level of uncertainty over whether microplastics cause significant long-term negative impacts on the environment (Castro-Castellon et al., 2022).

These inconsistent conclusions of plastic exposure studies are likely to be due to the highly complex nature of microplastic particles, which can differ in their size, shape, polymer type, surface properties and chemical load (Paul-Pont et al., 2018; Potthoff et al., 2017). For example, despite being similar sizes, the shape of microplastics (fibre, bead or fragment) was found to significantly influence the number of particles which were ingested by marine zooplankton; with the copepod *Calanus helgolandicus* ingesting more fragments, the copepod *Acartia tonsa* ingesting more fibres, and the lobster larvae *Homarus Gammarus* ingesting more beads (Botterell et al., 2020). The size of particles can have a similar effect. For example, the marine reef fish *Acanthochromis polyacanthus* was found to uptake a maximum of just five 1-2 mm diameter microplastic fragments per individual after one week of exposure, however, when the same types of fragments were $<$ 300 µm in diameter, up to 2102 microplastics per individual was recorded (Critchell & Hoogenboom, 2018). The general experimental design of exposure studies also differs hugely. With no currently accepted standardised framework for the design of microplastic exposure experiments (Adhikari et al., 2022; Heinrich et al., 2020) factors such as microplastic concentration, exposure time, particle distribution and bioavail-

ability vary dramatically and can have significant impacts on the results of these studies (Paul-Pont et al., 2018). Furthermore, methods of measuring and reporting experimental conditions such as microplastic concentration can vary greatly between different studies (e.g. Table 1.1), making comparisons between different studies tricky or impossible. The concentration of microplastics used in laboratory exposures is a particularly important factor in the outcome of studies (Adhikari et al., 2022), with some authors highlighting that the concentrations of microplastics used in laboratory studies are orders of magnitude higher than the concentration found in the environment, and hence may not represent the actual effects of plastic in natural systems (Burns & Boxall, 2018). For example, although some studies outlined in Table 1.1 do include environmentally relevant microplastic concentrations (references 3, 6, 7 and 8) in their study design, others use far higher concentrations, with some (references 1,2 and 4) using a concentration between 10,000 and 5 million times higher than the highest microplastic concentrations reported in natural waters (Burns & Boxall, 2018). There are also other discrepancies between the plastic particles found in the natural environments and those typically used in experimental exposure studies (Heinrich et al., 2020). For example, one factor which adds considerable further complexity to the nature of plastics in the environment is the attachment and growth of microorganisms on plastic surfaces. This microbial colonisation can lead to significant changes to the risk and fate of plastics in the environment (Rummel et al., 2017). Furthermore, the presence of these microorganisms is thought to disguise plastics as a nutritional food source to organisms in a 'Trojan horse effect' (Fabra et al., 2021), and has been shown to stimulate microplastic uptake in some species (e.g. Vroom et al., 2017). Despite this, the role of microbial colonisation in the overall risks of plastic in the environment has been traditionally overlooked (Paul-Pont et al., 2018), and the pressing need to increase our general understanding of the interactions between plastic and microbial communities has recently been highlighted (Castro-Castellon et al., 2022).

1.3 Colonisation of plastic in the environment

1.3.1 Microbial attachment and biofilm formation

In natural aquatic systems, such as rivers, lakes and the ocean, submerged surfaces quickly become colonised by a diverse assortment of microorganisms including bacteria, algae and fungi (Mora-Gómez et al., 2016). Once attached to a surface, many microorganisms produce extracellular polymeric substances (EPS) which are comprised of various biopolymers (Flemming & Wingender, 2010). EPS surrounds and immobilizes cells, resulting in the formation of a complex three-dimensional 'biofilm' matrix over the substrate surface, providing mechanical stability for microorganisms and creating a habitat with a distinct mode of life from the free-living planktonic environment (Flemming & Wingender, 2010; Mora-Gómez et al., 2016). This biofilm environment offers advantages to microbial communities such as strong nutrient retention, along with protection from desiccation, harmful xenobiotics and grazing predators (Flemming & Wingender, 2010). The density of microorganisms within biofilms is often substantially higher than in the surrounding planktonic environment and these communities can therefore be highly influential on the overall health and functionality of aquatic ecosystems (Mora-Gómez et al., 2016; Weitere et al., 2018).

Biofilm formation occurs in several stages and is a micro-scale rendition of traditional ecological succession (Cowles, 1899). Dang and Lovell (2000) previously described this for biofilms formed over 72 hours in a saltmarsh and outlined it to be a process involving the following steps. Firstly, upon submersion in the water a conditioning film almost instantaneously forms on the material surface. This conditioning film consists of various ions, as well as organic molecules such as proteins, lipids and polysaccharides; it has been previously referred to as the 'ecocorona' and can alter surface characteristics of the material from their virgin state (Galloway et al., 2017; Lorite et al., 2011). Just after formation of the conditioning film, the first pioneering microbial colonisers begin to attach to the surface and form an organic layer of cells and cellular excretions. These organisms can attach in as little as 15 minutes after surface submersion and are often species of bacteria, as well as photosynthetic eukaryotes such as diatoms and brown algae (Latva et al., 2022). There is then

progressive recruitment of other microbial organisms (secondary colonisers) and a gradual build-up of biofilm mass, followed finally by maturation of the biofilm through competitive interactions between community members, driven by various factors such as the surrounding water conditions (Lang, 2015; Oberbeckmann et al., 2018).

1.3.2 Plastic-associated biofilms - does a true 'Plastisphere' exist?

The ubiquity and persistence of plastic debris in aquatic environments provides an ideal and abundant substrate for microbial colonisers, offering access to high light and nutrient conditions (Reisser et al., 2014). The presence of microorganisms on plastic debris was documented during the first reports of plastic litter in the ocean in 1972 when samples of plastic were collected from the Sargasso Sea (Carpenter et al., 1972; Carpenter & Smith, 1972). Despite this early discovery, the presence of plastic-associated biofilms was largely overlooked when investigating aquatic plastic pollution until Dang and Lovell (2000) studied the growth of biofilms on different synthetic polymers in a controlled experiment (Oberbeckmann et al., 2014). Research on plastic biofilms in the marine environment began to accelerate significantly after Zettler et al. (2013) captured interest by using molecular techniques and electron microscopy to reveal the complex microbial world living on the surface of plastic debris collected from the North Atlantic – coining the term 'Plastisphere' to specifically describe communities associated with plastic surfaces. Examples of plastic-associated biofilms captured from similar studies are shown in Figure 1.3. The use of these methods to characterise plastic-associated biofilms in freshwater followed shortly after (McCormick et al., 2014), however, the freshwater 'Plastisphere' did not begin to gain significant attention until 2019 (Barros & Seena, 2021) and consequently less is understood about these biofilms compared to those in marine systems.

It has been suggested that early biofilm formation is a stochastic process, with microorganisms from the surrounding water attaching indiscriminately to surfaces; however, the ability and inclination of microorganisms to attach to surfaces does vary (Besemer, 2015) and there is evidence that many factors can influence the formation and species composition of plastic-associated biofilms (Rummel et al., 2017). These factors (outlined in Table 1.2) can include material and surface

specific characteristics, such as polymer type and surface roughness, as well as wider environmental influences such as water conditions and locational factors. The hydrophobicity of surfaces is one material-specific factor thought to mediate the ability of initial colonising organisms to attach to it, with some studies showing that more hydrophobic surfaces favour microbial attachment (Artham et al., 2009) and other studies reporting strong links between surface hydrophobicity and biofilm community composition (Ogonowski et al., 2018). Similarly, material texture is thought to drive microbial cell attachment, with rougher surfaces shown to provide more surface area, increase cell attachment (Anselme et al., 2010; Song et al., 2015) and provide better protection from shear stress felt from the surrounding water (Renner & Weibel, 2011). The influence of plastic surface texture on colonisation has been demonstrated in the bacteria *Vibrio crassostreae*, which readily colonised irregularly shaped textured polystyrene particles for six days, but only remained attached to smooth spherical polystyrene particles for up to ten hours and did not establish a complex biofilm (Foulon et al., 2016). Other material factors, such as particle size (microplastics *vs.* larger macroplastic) and abiotic weathering, of plastic have also been shown to influence biofilm composition (Huang et al., 2022; Laverty et al., 2020; Mughini-Gras et al., 2021; Nguyen et al., 2021). Nevertheless, it should be noted that many other studies have found no effect of these material-specific factors and it has been suggested that they may only be important in the very early stages of biofilm formation, with subsequent colonisation becoming disassociated from these material properties as the biofilm begins to accumulate over time (Rummel et al., 2017).

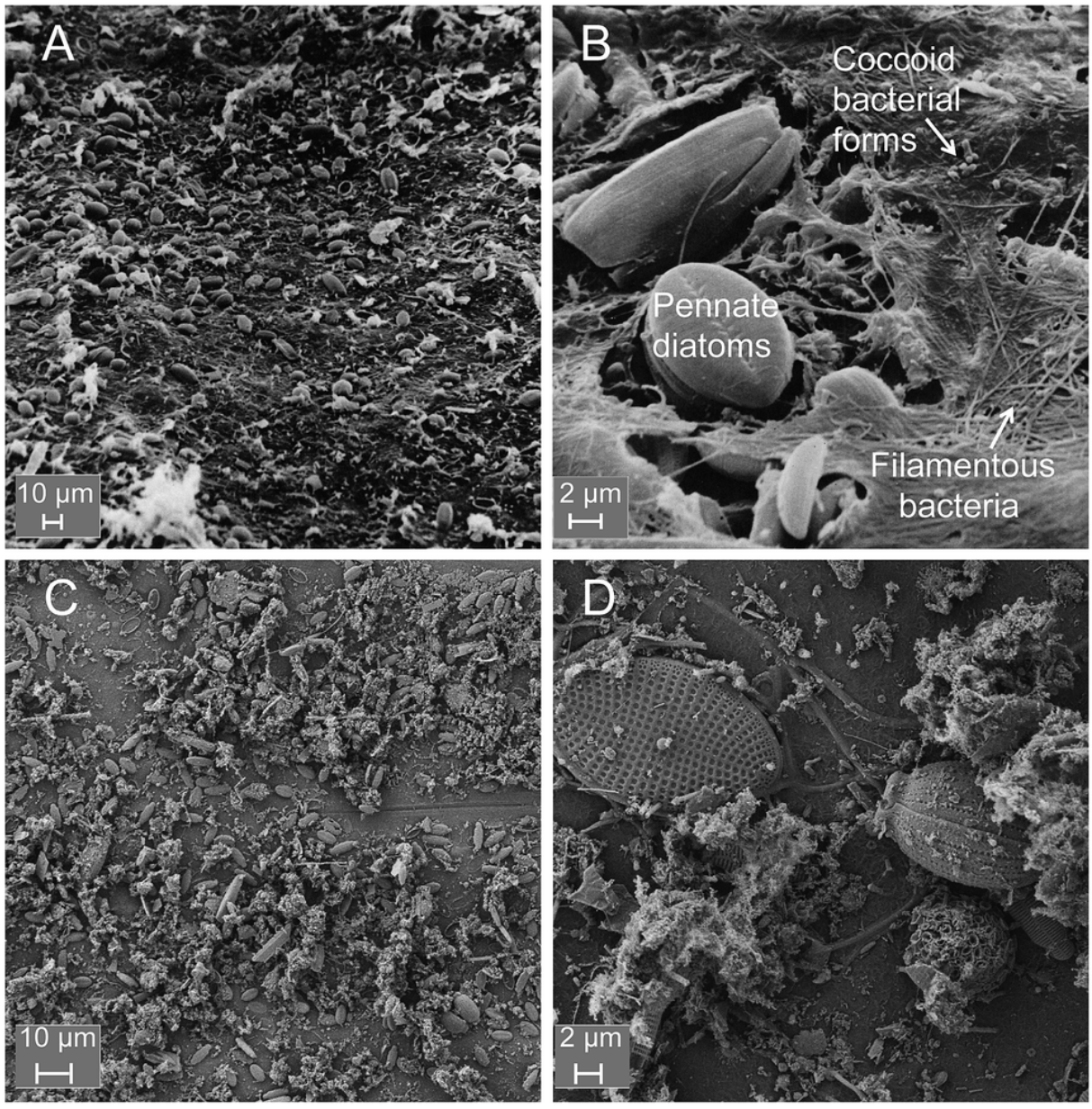


Figure 1.3: Example of microbial biofilms formed on the surface of plastic after submersion in the marine environment. (A) and (B) show the biofilm community attached to the surface of a discarded bleach bottle. (C) and (D) show biofilm communities attached to polyethylene samples. Reproduced with permission from De Tender et al. (2017)

Table 1.2: Factors known to influence the composition of plastic-associated microbial biofilms with examples of each. Partially based on and adapted from work by Harrison et al. (2018). PP: Polypropylene; PE: Polyethylene; PS: Polystyrene; PET: Polyethylene terephthalate; PVC: Polyvinyl chloride; LDPE: low-density polyethylene; HDPE: high-density polyethylene. (1) Qiang et al. (2021), (2) Wen et al. (2020), (3) Huang et al. (2022), (4) Mughini-Gras et al. (2021), (5) Ogonowski et al. (2018), (6) Foulon et al. (2016), (7) Lanning et al. (2019), (8) Amaral-Zettler et al. (2021a), (9) Pinnell and Turner (2020), (10) Nguyen et al. (2022), (11) Yang et al. (2021a), (12) Pinto et al. (2019), (13) Kesy et al. (2019), (14) Harvey et al. (2020), (15) Song et al. (2022), (16) Amaral-Zettler et al. (2015).

Type of factor	**Factor**	**Example**	**Reference**
Material-specific property	Polymer type	Significant differences in biofilm bacteria communities between HDPE, PET and PS particles.	(1)
	Chemical additives	Different coloured polyethylene particles, with different chemical additives, promoted different distinct bacteria biofilm compositions.	(2)
	Plastic age and weathering	Bacteria community on LDPE film differed significantly between virgin plastic and plastic which had been subject to photo-aging.	(3)
	Particle size	Bacteria biofilm communities differed significantly between microplastics in different size ranges – becoming more similar to water communities as they got smaller.	(4)
	Hydrophobicity	Variation in bacterial community structure was strongly linked to the hydrophobicity of glass, cellulose, PS, PP and PE surfaces.	(5)
	Roughness and texture	*Vibrio crassostreae* formed long-lasting biofilms on rougher textured PS and not on smoother PS beads.	(6)

Biological	Successional development	Bacteria biofilm diversity increased over time on HDPE, PET, PP, PS and PVC. Significant shifts in dominant taxa between one and seven days of submersion.	(7)
	Inter-specific competition and interactions	Significant causal effects found within and between microbial domains (prokaryotes and eukaryotes) of plastic biofilms.	(8)
Environmental conditions	Temperature	Bacteria biofilm diversity on PET and presence of plastic-discriminant species significantly influenced by temperature.	(9)
	Oxygen	Bacteria biofilm communities on PE and PS clustered according to the dissolved oxygen concentration of the water.	(10)
	Nutrients	Nitrate and nitrite concentration was found to be a significant driver of plastic biofilm bacteria community in riverine water.	(11)
	Light	Light levels experienced by submerged plastic significantly affected the bacteria community composition which formed on glass, HDPE, LDPE, PP and PVC.	(12)
	Salinity	Differences in salinity significantly explained variation in bacteria biofilm communities on plastics sampled from the Baltic Sea coastline.	(13)
	pH	Bacteria composition on PET bottles was significantly influenced by the surrounding water pH caused by submersion proximity to a volcanic seep.	(14)
	Transport between habitats	Bacteria communities on HDPE and tyre particles underwent huge turn over in species composition when travelling from freshwater rivers out into coastal environments. Although some species could successfully raft from freshwater to marine systems.	(15)

Biogeography	Geographic location	Bacteria community composition of plastic differed significantly between the North Pacific and North Atlantic sub-tropical gyres. Clear correlation between bacterial richness and latitudinal gradient.	(16)

Wider environmental factors, such as the salinity, nutrient content, temperature and pH of the water in which biofilms form are also known to contribute considerably to community structure. For example, salinity was the most significant influence on biofilm composition associated with polyethylene and polystyrene in the Baltic Sea (Kesy et al., 2019), and organic nutrient content, salinity and dissolved oxygen were found to significantly shape plastic biofilm communities in freshwater mesocosms (Nguyen et al., 2022). Parameters such as alkalinity, total organic carbon and dissolved nitrates have also been shown to be the main drivers of biofilm composition on microplastics sampled across several freshwater river locations (Yang et al., 2021a), and pH can similarly be a major influence on bacterial biofilm assemblages in marine systems (Harvey et al., 2020). Some studies have identified that plastic biofilms can exhibit large-scale biogeographic patterns, with Amaral-Zettler et al. (2015) demonstrating that the community composition of plastic debris differed significantly between the North Pacific and North Atlantic sub-tropical gyres, and that there was a clear correlation between bacterial richness and latitudinal gradient. Although not specifically addressed by the authors in this study, the seawater salinity differs considerably between the Pacific and Atlantic regions surveyed in this study (NASA, 2022) and this may therefore have contributed to the observed community differences. These distinct biogeographic patterns, likely based on trends in water parameters, indicate the potential for plastic communities to present unique compositions and risks depending on their surrounding conditions, and Amaral-Zettler et al. (2015) concluded that more regional-based plastic pollution management efforts may therefore be appropriate.

As well as abiotic factors, ecological interactions are thought to be as equally important in shaping the formation of plastic-associated biofilms. Similar to other microbial biofilms, plastic-associated communities can be regarded as a micro-scale functional ecosystem, for which many traditional community ecology concepts are applicable (Delacuvellerie et al., 2022; Lang, 2015). Within biofilms, cells live in very close proximity to each other (Flemming & Wingender, 2010), with direct and indirect interactions such as competition, predation, herbivory and symbiosis occurring between and within species - resulting in biotic control over community composition and structure (Lang, 2015). For example, photosynthetic autotrophs are thought to support heterotrophic activity by providing a larger carbon and nutrient source than the surrounding environment (Arias-Andres et al., 2018a), and predators such as ciliates have been shown to feed on biofilm bacteria associated with plastics

(Foulon et al., 2016). Some community members can form symbiotic relationships, for example ecostosymbiotic bacteria have been found growing on stalked ciliates within plastic biofilms (Zettler et al., 2013), and encrusting bryozoans on plastic debris were shown to host a unique diatom-dominated microbial assemblage (Reisser et al., 2014). Using network analysis techniques, recent studies have statistically confirmed significant causal effects within and between microbial domains of plastic biofilms; for example Alphaproteobacteria was found to strongly influence the structure of photosynthetic diatoms, chlorophytes and dinoflagellates (Amaral-Zettler et al., 2021a).

Whilst many studies support the idea of true plastic-specific communities (the 'Plastisphere'), many do not, with substantial disagreement in the literature over whether plastic is capable of hosting unique microbial assemblages distinct from other surrounding hard surfaces such as wood, glass or rock, or even communities specific to certain polymer types. For example, significant differences in bacteria and micro-eukaryotic communities between plastic and natural surfaces (wood and pebble) were found for samples incubated in lake water for 35 days (Miao et al., 2021). Kelly et al. (2020) found that microplastics supported bacterial assemblages distinct from tile surfaces after 34 days in river water, and an *in situ* experiment in coastal waters found that bacterial assemblages on polyvinyl chloride differed significantly to those on three other polymers and glass after one, four and eight weeks of submersion (Pinto et al., 2019). In contrast, no differences were reported in the bacterial communities found on plastic, glass, tile and aluminium after incubation in an artificial stream for 52 days (Hoellein et al., 2014) or on different polymers of microplastics collected from various sites of an estuary (Wu et al., 2020). Similarly, no differences in bacterial or eukaryotic assemblages were reported between glass and plastic samples incubated in the North Sea for one month (Oberbeckmann et al., 2016). From the literature it seems likely that many factors interact to influence biofilm communities. Whilst certain combinations of material properties, biological activity and environmental conditions lead to unique assemblages on plastic, other combinations do not. Indeed, Oberbeckmann et al. (2018) found that plastic supported distinct bacterial assemblages under high salinity low nutrient conditions, but not in water with lower salinity and a high nutrient concentration.

Overall, these findings indicate that the ability of plastic to host microbial assemblages unique from

surrounding hard surfaces is highly location and context dependent and further studies are therefore needed to gain a deeper understanding of areas which promote the presence of plastic-specific communities. As discussed, the plastic biofilm communities of freshwater systems are considerably less well understood than those in the marine environment (Barros & Seena, 2021), and of particular note, is the lack of knowledge surrounding plastic biofilm communities throughout UK freshwater. Of the studies identified by this thesis which characterise plastic-associated microbial communities sampled from or incubated in freshwater (n = 68), 69 % are based in non-European freshwater systems, mainly East-Asia and North America, whilst 25 % take place in European freshwater, with just one study (2 %) based within a UK freshwater systems, albeit within water from a waste water treatment facility. The other 4 % of studies were conducted under highly controlled laboratory conditions in completely artificial freshwater. A list of these identified studies is given in Appendix A.1. Given that large-scale location factors have been shown to mediate the composition of plastic biofilms (e.g. Amaral-Zettler et al., 2015), work to characterise these plastic communities across a broad range of environments is important and areas lacking in information, such as the UK, should therefore be targeted.

1.3.3 Importance of understanding plastic-associated biofilms: role of the biofilm in mediating the risks of plastics pollution

Biofilm formation can significantly alter the fate and impacts of plastics (examples are outlined in Table 1.3), and understanding the composition and metabolic functionality of these biofilms is therefore fundamental to our ability to determine the relative risks of plastic within the environment (Jahnke et al., 2017; Rummel et al., 2017; Amaral-Zettler et al., 2020). For example, biofilm formation can alter physical characteristics of plastic such as buoyancy - which dictates the environmental compartment in which the plastic resides, and therefore which organisms will interact with it. The weight of the biofilm can alter plastic density, causing normally positively buoyant plastics to sink, and facilitating the transport of plastic from the air-water interface into the sediment (Amaral-Zettler et al., 2021b). However, this is a complex process, and some particles can subsequently regain buoyancy after reaching the sediment, or show unpredictable sinking velocities due to patchy and irregular

biofilm distribution (Amaral-Zettler et al., 2021b; Jalón-Rojas et al., 2022).

Table 1.3: Examples of the ways in which plastic biofilms can alter the fate and impacts of plastic pollution in aquatic environments. PP: Polypropylene; PE: Polyethylene; PS: Polystyrene; PES: polyester.(1) Amaral-Zettler et al. (2021b), (2) McCormick et al. (2014), (3) Chen et al. (2020), (4) Bhagwat et al. (2021), (5) Huang et al. (2022), (6) Murano et al. (2021), (7) Kirstein et al. (2016), (8) Sun et al. (2022).

	Factor	**Example**	**Reference**
Fate	Buoyancy	Microbial colonisation of PE particles in coastal environment increased their density and caused buoyant plastic to sink.	(1)
	Biodegradation potential	*Pseudomonas* genus (known to contain key plastic-degrading bacteria species) were fifteen times more abundant on plastics sampled from a river compared to suspended organic matter.	(2)
Ecosystem-wide impacts	Alterations to biogeochemical cycling	PP plastics, that had been microbially colonised in a freshwater pond, significantly increased ammonia oxidation, nitrate oxidation and denitrification when added to a freshwater mesocosm.	(3)
Toxicity	Sorption kinetics of aqueous pollutants	Presence of microbial biofilm increased the adsorption of lead (Pb) and the aqueous pollutant perfluorooctane sulfonate on PE, PP, PES and nylon microplastic fibres.	(4)
	Transfer of animal pathogens	*Flavobacterium* genus, known to contain common fish pathogens, was found on biofilms on LDPE films after exposure to river water, and had a higher relative abundance compared to biofilms on wood surfaces.	(5)
	Increased immune reaction	Exposure to microbially colonised PS microplastics induced significantly more catalayse and total antioxidant activity in sea urchin *Paracentrotus lividus* compared to virgin PS particles.	(6)

Human health	Transfer of human pathogens	Presence of human pathogen *Vibrio parahaemolyticus* on PE, PP and PS microplastics sample from the North and Baltic Sea.	(7)
	Hotspot for antibiotic resistance	After 30 days of incubation in mangrove sediment the total abundance of antibiotic resistance genes was significantly higher on microplastics compared to the surrounding sediment at two of three sites examined.	(8)

The presence of microorganisms can also lead to changes in the chemical and physical surface of plastic, leading to alterations in the surface roughness and degradation of the polymer. Microbial colonisation is generally thought to increase surface roughness and is tightly linked with the physical degradation of the plastic surfaces (Hossain et al., 2019). Bacteria which belong to plastic-degrading groups, such as *Exiguobacterium*, *Pseudomonas* and *Ideonella*, have been documented in many marine and freshwater plastic biofilms and can be significantly more abundant on plastic than on non-plastic surfaces under the same conditions (Dong et al., 2021; McCormick et al., 2014; Morohoshi et al., 2018; Yang et al., 2021a). The ability of eukaryotic algae to age plastic and induce fractures in the surface has also recently been demonstrated (Kiki et al., 2022). These microorganisms can lead to fragmentation of plastic, particularly for some polymers specifically designed to be 'degradable', resulting in the creation of ever-smaller particles, and increasing the microplastic concentration in the environment (Jahnke et al., 2017; Lott et al., 2020). Interestingly, the presence of a biofilm can also reduce the amount of UV light which reaches plastic and may therefore act to shield plastic and reduce degradation from abiotic processes (Mincer et al., 2016; O'Brine & Thompson, 2010). However, the overall result of these opposing effects are not well understood (Andrady, 2022).

Zhao et al. (2021) estimated that marine plastic biofilms could harbour between 1500 and 11,000 tonnes of carbon mass in the open ocean. Given that freshwater biofilms are known to be as large or even larger than those formed in marine environments (Shan et al., 2022), total plastic biofilm mass in freshwater is also likely to be substantial. A number of studies have documented significant variation in the functional profile of plastic biofilms compared to the surrounding water, and the

large quantity of these biofilms has led to questions over the ability of biofilms to disrupt the natural cycling of nutrients, carbon and xenobiotics within aquatic systems (Mincer et al., 2016). For example, metabolic functions such as nitrification, denitrification and sulfate respiration were found to be strongly enriched in plastic communities sampled from a river, and microbial communities formed on polypropylene surfaces in a freshwater pond significantly influenced the overall nitrogen and phosphorus cycling of an aquatic system (Chen et al., 2020; Xue et al., 2020). Recent studies further confirm the ability of plastic biofilms to exhibit high levels of denitrification and nitrous oxide production in estuarine environments (Su et al., 2022), and the potential for plastic biofilms to contribute to the inventories of carbon dioxide and nitrous oxide in the surface ocean layer has also been demonstrated (Cornejo-D'Ottone et al., 2020). Other studies report the enrichment of aromatic compound degradation, pharmaceutical transformation and mercury metabolism functionality on plastics (Bowman et al., 2021; Hu et al., 2021; Li et al., 2021; Porter et al., 2020). Whilst these processes can influence the overall carbon, nutrient and xenobiotic dynamics of the wider environment, biofilms can also mediate the sorption kinetics of harmful pollutants onto plastic surfaces, and may therefore affect the organism-level impacts of the plastic if ingested. Plastic can adsorb a complex mixture of compounds, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls and various metals, which are known to cause harmful effects to aquatic organisms (Rochman, 2015; Rochman et al., 2013), and the effect of biofilms on plastic sorption kinetics appears to be complex. Whilst biofilms can significantly increase the adsorption of some compounds, such as lead (Pb) and the persistence organic pollutant perfluorooctane sulfonate (Bhagwat et al., 2021), the adsorption of other compounds is significantly inhibited by the biofilm (Zhang et al., 2022a). Furthermore, the biofilm can influence compound desorption rate from plastics and may therefore have implications for the transfer of compounds into organisms after ingestion (Bhagwat et al., 2021; Liu et al., 2022).

It is also becoming increasingly clear that plastic biofilms can harbour harmful microorganisms. Studies frequently report the presence of various potential pathogens, such as *Vibrio* sp., *Flavobacterium* sp., *Aeromonas* sp. and *Pseudomonas* sp., some of which have been found to be significantly enriched on plastic surfaces (Huang et al., 2022; Kesy et al., 2019; Kirstein et al., 2016; Viršek et al., 2017). The presence of these pathogens may have significant consequences for organisms which interact with plastic. For example, the *Flavobacterium* genus contains common fish pathogens which

cause severe complications with gill functionality and general fish fitness (Gong et al., 2019), and *Vibrio* species are known to cause significant disease and mortality in important aquaculture species such as oysters, mussels, and scallops (Bowley et al., 2020). Human *Vibrio* pathogens, which can cause septicaemia and food poisoning can also occur within plastic-associated biofilms (Kirstein et al., 2016) and the ability of plastic biofilms to host human viruses has also recently been shown (Li et al., 2022; Moresco et al., 2022) - demonstrating that disease transfer between plastic pollution and humans is a further potential risk. Moreover, the ability of plastic to act as a hotspot for bacterial antimicrobial resistance (AR) is becoming an increasing concern. AR occurs when over-exposure to antimicrobial drugs causes bacteria to become genetically resistant to them, and was thought to be associated with around 4.6 million human deaths and directly responsible for around 1.3 million deaths world-wide in 2019 (Murray et al., 2022). The close contact of bacteria within biofilms creates an optimal environment for the spread of AR through horizontal gene transfer, and AR genes are commonly detected within biofilms from a range of different environments (Yao et al., 2022). They have also been detected on the surface of plastics, often with a distinct gene composition from the surrounding environment (Liu et al., 2021). For example, the *sul 1* AR gene was significantly enriched on plastic surfaces compared to the surrounding water or natural rock in a freshwater lake (González-Pleiter et al., 2021). These findings, coupled with the high abundance of plastic litter throughout the environment have led to concerns over the implications of the presence of these pathogenic organisms for ecological and human health (Sooriyakumar et al., 2022).

Although it is unknown whether pathogenic species are the cause, the toxicological effects of microbially colonised plastic have also been directly demonstrated for various aquatic organisms. For example, the mussel *Mytilus galloprovincialis* showed a significantly higher expression of the *HRG* gene (associated with immune response) when exposed to microbially-colonised microplastics compared to sterilized ones (Bandini et al., 2021). Similar findings were reported for sea urchins, which showed an enhanced immune response after ingestion of biofilm-covered microplastics compared to virgin ones (Murano et al., 2021). Furthermore, other studies have found that the growth and fecundity of freshwater snails can be significantly impacted when these organisms feed on plastic-associated biofilms compared to glass-associated biofilms formed under the same conditions (Michler-Kozma et al., 2022; Vosshage et al., 2018) and together these findings indicate a clear need to better

understand how plastic-associated biofilms contribute to the toxicity and risks of plastic pollution.

Within the plastic-biofilm research field the presence and impacts of plastic-associated bacteria typically receive a large amount of attention, whereas eukaryotic and fungal groups are far more often overlooked (Miao et al., 2021). Despite this, these groups may be highly important within plastic biofilms (Miao et al., 2021), for example, fungi are highly abundant and important within freshwater systems and some species have been implicated in their ability to biodegrade plastic (Amobonye et al., 2021; Grossart et al., 2019). Furthermore, fungi are known to be a highly important dietary component for many freshwater invertebrates such as detritivores (Graça et al., 1993), and therefore have the potential to couple plastic biofilms with higher trophic levels. Various metazoan eukaryotes are also known to comprise and be associated with plastic biofilms. For example bryozoans, barnacles and nematodes have been found associated with marine plastics, and heavy fouling of plastic by insect larvae was observed on plastics after submersion in freshwater systems (Delacuvellerie et al., 2022; Imam et al., 1992; Ryley et al., 2021). The role of these invertebrates is thought to be important for mediating biofilm structure; for example by driving top-down control over microbial populations, increasing light penetration to deeper layers of the biofilm and influencing nutrient cycling within the biofilm (Burns & Ryder, 2001). They can also contribute to a rapid loss in plastic buoyancy (Fazey & Ryan, 2016; Kaiser et al., 2017) and may catalyse the release of microbial metabolites from the biofilm matrix (Gaudes et al., 2006). The presence of these complex multi-domain biofilms has also been linked with an increased uptake of plastic by organisms, which is thought to occur due to enhanced palatability and attractivity of the plastic induced by the biofilm (e.g. Polhill et al., 2022). Given the potential importance of these groups within plastic communities and the current lack of knowledge surrounding them, further research efforts should be dedicated to unpicking the role and relative importance of these groups in mediating the impacts of plastic in the environment.

1.4 The interlinked interactions between microbial biofilms, plastic, and aquatic organisms

In the environment, complex interlinked interactions between plastic, microbial biofilms and larger aquatic organisms can occur. For example, biofilms can modify plastic palatability, which then influences subsequent interactions between plastic and larger organisms. These larger organisms may then even further modify plastic, such as by altering particle shape, size, or buoyancy, and potentially cause additional changes to plastic behaviour, bioavailability, and fate in the environment. Unpicking and understanding these complex interactions is therefore key for developing our knowledge of plastic within aquatic environments and its long-term impacts on biological ecosystems.

1.4.1 Biofilm influence on plastic-organism interactions

Many organisms are known to ingest plastic or preferentially associate with it over natural materials (section 1.2), however reasons for this are often unclear. Over recent years the role of biofilms in mediating these interactions between plastic and aquatic organisms has become better understood, and offers some explanation to these observations. Many organisms, such as copepods and anemones, select prey for ingestion through touch detection, and reject particles not deemed to be edible (Weideman et al., 2020; Xu et al., 2022). The presence of an organic biofilm layer over plastic is thought to disguise its inert non-nutritional nature, leading to increased ingestion and the creation of a 'Trojan horse effect' (Fabra et al., 2021). This has been observed in numerous marine species with different feeding modes, as well as a few freshwater filter-feeding species (Table 1.4). For example, both copepods and anemones have been shown to ingest significantly more plastic with a microbial biofilm over its surface, compared to clean plastic (Vroom et al., 2017; Weideman et al., 2020). Similar findings have been reported in other species, such as the sea urchin *Paracentrotus lividus*, which ingested significantly more biofilm-coated polyethylene microbeads - formed from plastic incubation in seawater for ten days, compared to clean microbeads (Murano et al., 2021). This effect has also been observed in a number of terrestrial soil-dwelling invertebrates (Helmberger et al., 2022), and the role of biofilms on particle palatability therefore seems to be far reaching throughout

metazoan taxa. Interestingly, whilst biofilms generally increase plastic palatability and ingestion, some studies do report either no effect or a reduced palatability effect of the biofilm. For example, the copepod *Temora longicornis* showed no differentiation between clean or microbially-colonised microbeads, rejecting both equally (Xu et al., 2022), and the coral *Astrangia poculata* ingested significantly more clean microplastic fragments, compared to those which had been incubated in seawater for one week (Allen et al., 2017). The high variability in biofilm composition (section 1.3.2) is likely to be responsible for these different outcomes, and therefore understanding both the composition of plastic biofilms and the resulting influence on organism interactions is important.

Table 1.4: Studies demonstrating interactions between plastic, microbial communities and larger aquatic organisms. (1) Allen et al. (2017), (2) Savoca et al. (2017), (3) Vroom et al. (2017), (4) Corona et al. (2020), (5) Pfaller et al. (2020), (6) Weideman et al. (2020), (7) Fabra et al. (2021), (8) Murano et al. (2021), (9) Sucharitakul et al. (2021), (10) Joppien et al. (2022), (11) Xu et al. (2022), (12) DeMott (1986), (13) Polhill et al. (2022), (14) Davidson (2012), (15) Watts et al. (2015), (16) Saborowski et al. (2019), (17) Dawson et al. (2018), (18) Jang et al. (2018), (19) Cau et al. (2020), (20) Torn (2020), (21) Immerschitt and Martens (2020), (22) Mateos-Cárdenas et al. (2020), (23) Hodgson et al. (2018), (24) Porter et al. (2019).

Organism	**Ecosystem**	**Material type**	**Plastic size**	**Main findings**	**Reference**
Biofilm influence on plastic-organism interactions					
Stony coral polyps (*Astrangia poculata*)	Marine	Polystyrene, low-density polyethylene, high-density polyethylene fragments (clean or biofouled)	125 – 1000 µm	$\sim$ 4.6 times more clean plastic was ingested compared to biofouled plastic	(1)
Anchovy (*Engraulis mordax*)	Marine	'Odour solution' formed from incubation with biofouled plastic or clean plastic	n/a	Biofouled plastic odour stimulated foraging behaviour response, clean plastic odours did not	(2)
Copepods (*Calanus finmarchicus*, *Acartia longiremis*)	Marine	Polystyrene spheres (clean or biofouled)	15 µm	Higher rate of ingestion of biofouled plastic compared to clean plastic	(3)
Stony coral (*Danafungia scruposa*)	Marine	Polyethylene spheres	212 – 1000 µm	Likelihood of ingesting and retaining plastic was higher for biofouled plastic than clean plastic	(4)

Loggerhead turtle (*Caretta caretta*)	Marine	Odour from clean or biofouled plastic bottle	n/a	Significantly more foraging behaviour when exposed to biofouled plastic odour than clean plastic odour	(5)
Anemone (*Bunodactis reynaudi*)	Marine	High-density polyethylene film (clean or biofouled)	9 cm x 20 cm	Significantly higher ingestion of biofouled plastic than clean plastic	(6)
European oyster (*Ostrea edulis*)	Marine	Polymethyl methacrylate spheres (clean or biofouled with *Escherichia coli* bacteria)	20 – 77 µm	$\sim$ 3.7 times more uptake of biofouled plastic compared to clean plastic	(7)
Sea urchin (*Paracentrotus lividus*)	Marine	Polystyrene spheres (clean or biofouled)	45 µm	Higher ingestion rate of biofouled plastic compared to clean plastic	(8)
Jelly fish larvae (*Aurelia coerulea*)	Marine	Polystyrene spheres (clean, or biofouled in either the light or the dark)	5 µm	Generally higher ingestion of biofouled plastic formed under light conditions, compared to clean or dark-formed biofouled plastic	(9)
Large foraminifera (*Amphistegina gibbose*)	Marine	Polyethylene terephthalate particles (clean or biofouled)	150 – 300 µm	Higher ingestion of biofouled plastic compared to clean plastic	(10)
Copepod (*Temora longicornis*)	Marine	Polyethylene spheres (clean or biofouled)	20 µm	No significant difference between ingestion or biofouled and clean plastic	(11)

12 different zooplankton species: mixture of copepods, diplostracas and rotifers	Freshwater	Polystyrene spheres (clean or 'flavoured' with algae solution	6.5 ± 1.4 µm	Significantly higher ingestion of 'flavoured' plastic compared to virgin plastic by 5 species (2 copepod, 2 diplostraca and 1 rotifer species).	(12)
Diplostraca (*Daphnia magna*)	Freshwater	Polyethylene spheres (clean or biofouled)	63 – 75 µm	∼ 7 times higher ingestion of biofouled plastic compared to clean plastic	(13)
Organism modification of plastic					
Boring isopods (*Sphaeroma* sp.)	Marine	Expanded polystyrene (clean when deployed)	Large blocks	Isopods extensively burrowed into plastic and created millions of microplastics from the process	(14)
Crab (*Carcinus maenas*)	Marine	Polypropylene fibres (clean)	5 mm in length	Fibres became smaller (surface area of 1.5 – 2.0 mm^2 became <1 mm^2) and amalgamated into balls during passage through the crabs	(15)
Atlantic shrimp (*Palaemon varians*)	Marine	Polyacrylic wool fibres and polystyrene spheres (clean)	Fibres: 236 ± 176 µm and Spheres: 9.9 µm	Observation of gut material found small (<100 µm) fragments of microfibres	(16)
Antarctic krill (*Euphausia superba*)	Marine	Polyethylene spheres (clean)	32 µm	Internal fragmentation of plastic spheres – fragmented plastic reduced to 6.0 ± 5.0 µm	(17)

Polychaete (*Marphysa sanguinea*)	Marine	Expanded polystyrene (clean when deployed)	Large blocks/buoys	Specimens burrowed into plastic and created many microplastics from the process	(18)
Norwegian langoustine (*Nephrops norvegicus*)	Marine	Mixture of environmental plastics	n/a	Microplastic found in sampled specimens significantly smaller in intestine than those in gut. Evidence of plastic fragmentation through digestion	(19)
Crab (*Rhithropanopeus harrisii*)	Marine	Polypropylene fibres, polyethylene terephthalate fragments	Fibre: 0.2 – 0.5 mm and fragments: 0.013 – 0.25 mm	Fibres became knotted into tight balls in the stomach	(20)
Dragonfly larvae *Anax imperator*	Freshwater	Polyethylene fibres	Length = 8 - 10 mm, diameter = 1 mm	Fragmented plastic fibres found in 16 % of larvae faeces, fragmented fibres were between 0.5 mm and 3.5 mm in length	(21)
Amphipod (*Gammarus duebeni*)	Freshwater	Polyethylene spheres	10 – 45 µm	Fragmentation of spheres into smaller irregular particles within midgut and hindgut. Some nanoplastics (<1 µm) also present	(22)
Biofilm influence on plastic-organism interaction AND organism modification of plastic					
Amphipod (*Orchestia gammarellus*)	Marine	High-density polyethylene, undefined 'degradable' and 'biodegradable' plastic films (clean or biofouled)	1 cm^2	Shredding and fragmentation of plastic films using feeding appendages, production of microplastics. Significantly more biofouled plastic fragmented than clean plastic.	(23)

Sea urchin (*Paracentrotus lividus*)	Marine	Polyethylene trays (clean or biofouled)	Unknown (large macro litter size)	Grazing on and fragmentation of plastic, significantly higher fragmentation of biofouled than clean plastic	(24)

As well as touch detection many organisms use longer-distance olfaction to locate food, and the odours produced by microbially-colonised plastic litter have been shown to drive foraging behaviour in marine organisms (Table 1.4). For example, odours extracted from biofouled plastic elicited significant foraging behaviour in anchovy compared to odours from clean plastic (Savoca et al., 2017). Sea turtles also displayed significantly greater food-search behaviour when exposed to airborne odours from biofouled plastic compared to clean plastic. Furthermore, in this study turtle response to biofouled plastic was comparable to the response observed for food odours (shrimp and fish meal) – indicating clear food-driven behaviour (Pfaller et al., 2020). Volatile organic compounds (VOCs) are widely important odorous infochemicals in both terrestrial and aquatic environments, they can mediate complex predator-prey interactions (Fink, 2007) and are likely to be partly responsible for these observed odour responses. One VOC widespread throughout the marine environment is dimethyl sulfide (DMS), which is produced in large quantities by photosynthetic algae. DMS is known to be a highly important foraging stimulant for a wide range organisms and has been implicated in its ability to drive plastic-organism interactions in marine systems (Savoca, 2018). This hypothesis is supported by work which has shown that microbially colonised marine plastic can consistently produce a clear DMS signal (Savoca et al., 2016), and that the infusion of microplastics with synthetic DMS significantly increases their uptake by copepods and lobster larvae compared to non-infused control microplastics (Botterell et al., 2020; Procter et al., 2019). These findings indicate that a mixture of both odours and other chemosensory cues are likely to be important for driving the response of aquatic organisms to plastic debris, and that different biofilm compositions and species-specific feeding traits (Scherer et al., 2017) may interact to determine the overall outcome of these interactions.

VOCs are known to be important in freshwater systems, for example, in mediating the ability of invertebrates to locate algal grazing patches (Fink et al., 2006a). Despite this, whilst the influence of biofilms on marine plastic-organism interactions has received moderate attention, similar studies are severely lacking for freshwater systems and represents a significant knowledge gap. To the authors knowledge, only two studies to date have explored the effects of microbial colonisation on plastic ingestion for freshwater species. The first of these studies, conducted in 1986, demonstrated that five out of twelve tested zooplankton species (two copepod, two diplostraca and one rotifer species) had a significantly higher ingestion rate of polystyrene microbeads that had been incubated

with algae cultures to 'flavour' them, compared to clean beads (DeMott, 1986). The one other study (Polhill et al., 2022) found the ingestion of microbially colonised polyethylene microbeads by *Daphnia magna* to be seven times higher than clean microbeads. Interestingly though, no significant differences between clean and biofouled plastic were seen for *D. magna* during investigations by DeMott (1986), demonstrating that similar to marine species, there is complexity in these biofilm-mediated plastic-organism responses. Given that these studies demonstrate the ability for biofilms to modify plastic-organism interactions in freshwater in a similar way to marine systems, further work in this area is warranted.

1.4.2 Organism modification of plastic

Whilst biofilm-forming microorganisms can influence plastic characteristics and interactions with larger organisms, the ability of these larger organisms to further modify plastic during these interactions is also becoming increasing clear. For example, the ingestion of plastic by aquatic organisms is known to mediate particle buoyancy by encapsulation in faecal pellets (Cole et al., 2016; Katija et al., 2017; Pérez-Guevara et al., 2021). Therefore, as well as a direct influence on plastic buoyancy (section 1.3), plastic biofilms may therefore also indirectly influence the vertical transport of plastic from surface waters to the benthos, by increasing particle palatability, and consequently ingestion and egestion by aquatic organisms. However, the relative importance and scale of these mechanisms is not well understood, particularly in freshwater, and therefore requires further attention (Liu et al., 2020).

Interactions between plastic and larger organisms can also result in significant alterations to plastic shape and size, a process which has recently been termed 'biofragmentation', and recognised as a potentially important but understudied pathway for microplastic creation (So et al., 2022). Biofilms have also been shown to directly influence the degree of plastic modification which occurs from these interactions. For example, the marine amphipod *Orchestia gammarellus* was shown to use its feeding appendages to fragment pieces of plastic film; this fragmentation resulted in the creation of hundreds of microplastics, and plastic film with a microbial biofilm was fragmented significantly more than

clean material (Hodgson et al., 2018). Similar findings were reported for the sea urchin *Paracentrotus lividus*, which grazed on clean and biofouled polyethylene trays resulting in their fragmentation into many smaller pieces, with the largest amount of fragmentation occurring for biofouled trays (Porter et al., 2019). Soil invertebrates have also been shown fragment polystyrene which was 'flavoured' with a soil suspension to a significantly greater extent that clean plastic (Helmberger et al., 2022).

Many biofilm-independent plastic-modification interactions have also been documented, with internal plastic modification in aquatic crustaceans being the most widely reported. This has been found to occur in several species (Table 1.4) and is thought to be facilitated by the mechanical grinding that occurs within crustacean digestive systems (Mateos-Cárdenas et al., 2020; Watts et al., 2015). For example, the breakdown of microbeads into irregularly shaped micro and nano particles has been observed (Dawson et al., 2018; Mateos-Cárdenas et al., 2020), and the amalgamation of singular microfibres into tight balls within the digestive system is also common (Murray & Cowie, 2011; Torn, 2020; Watts et al., 2015). This type of internal fragmentation has also been seen in larvae of the dragonfly *Anax imperator*, where the break down of polyester fibres was similarly thought to be facilitated by grinding within the organisms chitinous teeth-lined stomach (Immerschitt & Martens, 2020). Other 'non-feeding interactions' can also modify plastic (So et al., 2022), for example marine isopods and polychaetes were found to create thousands of microplastics from their burrowing behaviour into expanded polystyrene blocks (Davidson, 2012; Jang et al., 2018). Whilst knowledge of biofragmentation processes in marine systems is slowly growing, it remains largely unaddressed in freshwater, with only two studies to date having investigated the ability of freshwater species to alter the shape and size of plastics (Immerschitt & Martens, 2020; Mateos-Cárdenas et al., 2020; So et al., 2022).

1.5 Key knowledge gaps and thesis aims

Plastic litter is a widespread pollutant throughout aquatic systems globally. Research to determine the impacts of plastic in these systems has traditionally focused on the chemical and physical aspects of plastics. However, biological factors such as the growth of microbial biofilms can add further

complexity to the characteristics and dynamics of plastics in the environment; for example through altering plastic buoyancy, harbouring pathogenic species, influencing biogeochemical functionality, and disguising plastic as nutritional food. Additionally, the interactions which occur between plastic litter and larger aquatic organisms can facilitate further alterations to the physical characteristics of plastic. Within marine systems, research has demonstrated that these interactions between plastic litter, microbial communities and larger organisms can have a significant influence on the overall fate and impacts of plastic within a system. For example, the effects of plastic may be mediated through: the ability of biofilms to emit odours compounds which actively attract organisms to plastic and increase the likelihood of ingestion; the increased toxicological response of organisms to plastic when a microbial biofilm is present; and the ability of plastic-organism interactions to result in plastic fragmentation and microplastic creation, altering the bioavailability and fate of plastic litter in the environment. Whilst there is still more to be learnt, our understanding of these processes in marine systems is beginning to develop, but is in its infancy for freshwater systems where we have a severely limited understanding of these processes. Given the recognised importance of these interactions in the marine environment, and the fact that freshwater systems are the initial receptor for many plastic materials and exhibit some of the highest levels of plastic pollution, work is urgently needed to understand the plastic-biofilm-organism interactions occurring in freshwater environments.

Our current state of knowledge around these interactions within freshwater has been summarised in a conceptual diagram (Figure 1.4), which highlights our current understanding of plastic-biofilm-organism dynamics in freshwater environments, alongside key knowledge gaps where no research has yet been conducted or is in its inception. Areas which were identified to be the most lacking in information compared to the marine environment are: i) the structure and composition of plastic-associated microbial biofilms, with knowledge particularly deficient around eukaryotic and fungi communities, and plastic biofilm communities generally in UK freshwaters; ii) the attraction of freshwater organisms to microbially colonised plastic, with no studies which address this for benthic organisms; iii) the potential for organisms to physically modify the shape and size of plastic litter, specifically, their ability to accelerate plastic breakdown in the environment and increase microplastic contamination.

Taking these knowledge gaps together, the overarching aim of this thesis is therefore to build our knowledge of the dynamics between plastic, microbial communities, and larger organisms within freshwater, in order to better understand the specific relative risks posed by plastic pollution in these ecosystems. To address this aim, the first objective of this thesis is therefore to determine the importance of plastic-associated biofilms within UK rivers, and their role in mediating the impacts of plastic on freshwater ecosystems. The second objective of this thesis is to ascertain whether interactions between plastic and larger freshwater organisms, such as invertebrates, can result in the significant modification and fragmentation of plastic in a similar manner to that seen by marine and terrestrial species. This thesis is therefore centred around the two following broad hypotheses:

1. *'Plastic litter can provide a unique substrate for the formation of biofilms that can enhance interactions between plastic and freshwater organisms in UK rivers'*
2. *'Interactions between plastic and larger freshwater organisms can alter the fate and potential impacts of plastic litter in the environment'*

Work performed to address the objectives and test the hypotheses is described in four experimental chapters. A final discussion chapter brings together the findings and places the results in a broader context:

Chapter 2 describes work to characterise the composition and development of biofilms on plastic litter in a freshwater river. This is to determine the potential for plastic in UK rivers to host communities which are distinct from other surrounding non-plastic surfaces, and make inferences about the ability of plastic to host biofilms which may possess, ecologically significant, unique metabolic functionality. Bacterial, eukaryotic and fungal communities are analysed to expand our knowledge of these communities within plastic biofilms.

Chapter 3 investigates whether microbially colonised plastic can produce ecologically important odorous compounds in UK rivers, and compares this ability to surrounding non-plastic surfaces to understand the relative risk of plastic within the area. To determine the role of local water conditions in this, two river locations with different characteristics and water conditions are investigated.

Chapter 4 examines the ability of microbially colonised plastic to attract a model freshwater benthic invertebrate under environmentally relevant conditions and determines if interactions with this invertebrate can lead to significant plastic modification.

Chapter 5 explored the utilization and modification of plastic litter by another model benthic invertebrate and examines the potential implications of these interactions.

Chapter 6 brings together the knowledge gained from the preceding chapters into a general discussion and makes recommendations for the most effective way to build on this work and continue the advancement of our understanding in this area.

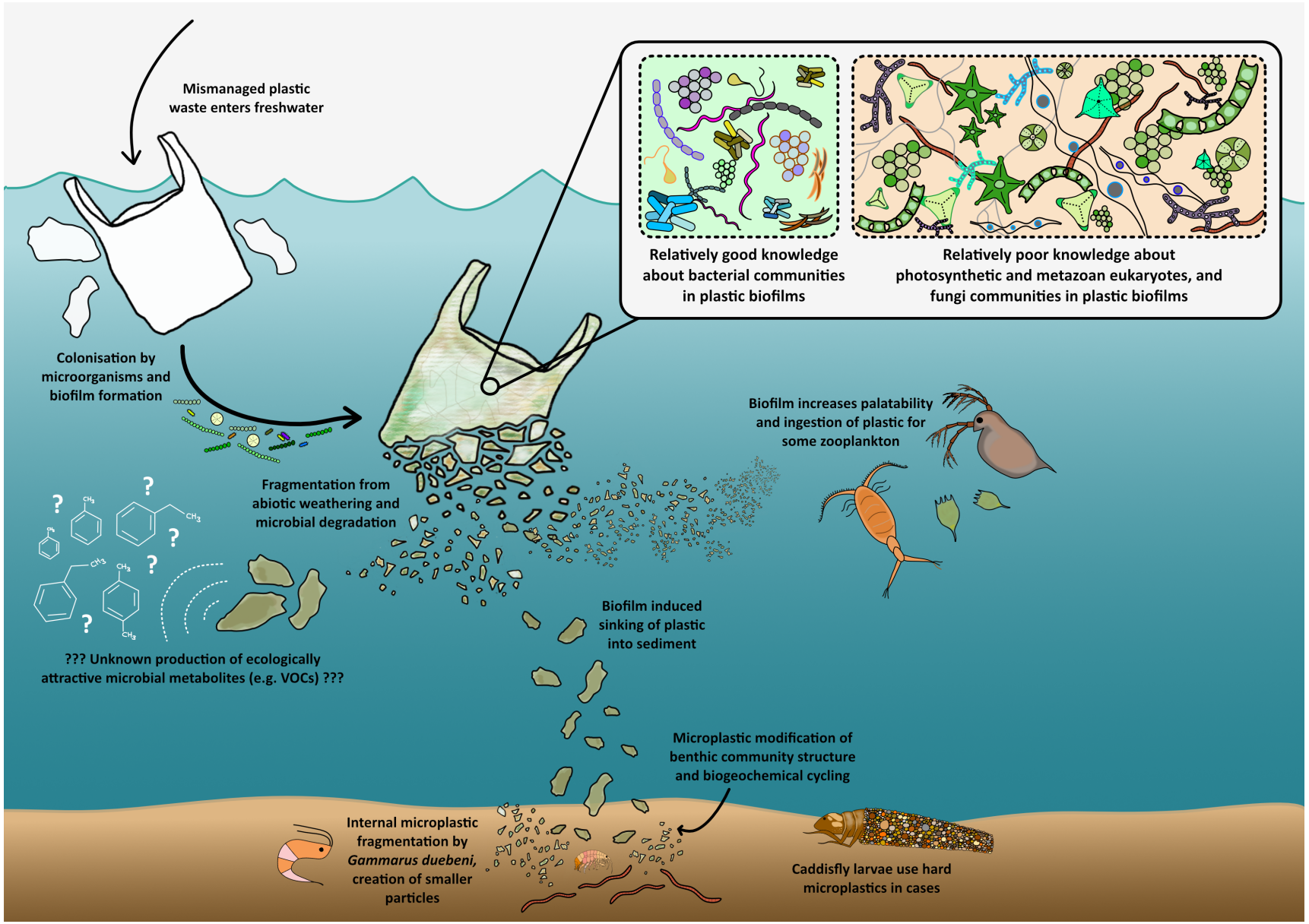


Figure 1.4: Conceptual diagram highlighting our current knowledge around plastic-biofilm-organism interactions in freshwater and areas where a better understanding is needed.

1.6 Study materials

Low-density polyethylene (LDPE) and polylactic acid (PLA) films were chosen as representative study plastics for this thesis and are used in experimental studies throughout. Plastic films were considered an environmentally relevant study material because of their extensive use in single-use packaging and their known high abundance throughout rivers and other freshwater systems (Calcar & Emmerik, 2019; Kabir et al., 2022; Treilles et al., 2021; van Emmerik et al., 2020; Wilson et al., 2021; Winton et al., 2020). LDPE has a density of 0.91 – 0.93 g/cm^3 (Lambert & Wagner, 2017) and its virgin state is therefore buoyant in both marine and freshwater. LDPE is used extensively in food and other consumer packaging, plastic bags and agricultural films, and with a market demand of 8.57 million tonnes, it was the second most desired polymer across Europe in 2020 (Plastics-Europe, 2021). Furthermore, polyethylene is one of the most commonly detected polymer types in freshwater (Kabir et al., 2022; Laju et al., 2022; Lu et al., 2021; Wang et al., 2022).

PLA is a bio-based polyester traditionally marketed as a biodegradable alternative to conventional plastic and was chosen as a second study material. The density of PLA is around 1.25g/cm^3 (Farah et al., 2016) and it is therefore negatively buoyant in its virgin state. Its "eco-friendly" status has led to its increased use in many sectors such as single use food packaging and agricultural films (Akhir & Mustapha, 2022; Ncube et al., 2020). Although its production is currently much less than traditional plastic (approximately 400,000 tonnes globally in 2020) its production is expected to increase significantly in coming years (B-Fortune-Business-Insights, 2021; European-Bioplastics, 2020). However, despite being biodegradable in industrial compositing systems, in aquatic environments PLA degradation is reported to be extremely slow and it is therefore thought to pose a similar pollution risk as conventional plastics (Ncube et al., 2020). Given the increasing demand and use of this material, understanding its behaviour and impacts within the environment will be important, and it was therefore chosen as a relevant bio-based plastic to develop a better understanding of.

Throughout this thesis, glass was chosen as a representative inert non-plastic control surface to make comparisons against when experimentally necessary. Given that surface roughness can strongly influence biofilm composition (Foulon et al., 2016), to eliminate this as a factor when trying to asses

the role of material in biofilm composition, the use of natural materials such as rock was avoided. Instead, plain, smooth glass slides were used. Although the microscopic surface roughness of glass is generally somewhat less than LDPE and PLA films ($<$ 1 nm RA for glass and around 17 - 34 nm for plastics) (Abdulkareem et al., 2021; Garner et al., 2014; Suresh et al., 2011), larger scale cracks and crevices seen on rocks are not present, and this difference is considered to be relatively small compared to other surfaces such as ceramic tile which has a surface roughness orders of magnitude higher than plastic (Wang et al., 2002). Many other studies have also used glass slides to compare plastic and non-plastic biofilms (e.g. Hoellein et al., 2014; Pinto et al., 2019; Rummel et al., 2021; Zhao et al., 2021), and glass slides were therefore considered suitable for this purpose.

Chapter 2

Composition and development of plastic-associated biofilms in a UK freshwater river

2.1 Introduction

Plastic litter is widespread throughout all aquatic environments and can have tremendous impacts on individual organisms and the wider ecosystem (MacLeod et al., 2021). It is also becoming clear that the microbial biofilm which forms on plastic after submersion in natural water can play a significant role in mediating the impacts of plastic. For example, reports of pathogenic bacteria, antibiotic resistance genes and even harmful viruses within plastic communities are common (Lai et al., 2022; Li et al., 2022; Moresco et al., 2022; Wu et al., 2022). Plastic biofilms have been linked to their ability to transport species involved with harmful algal blooms (Amaral-Zettler et al., 2021a; Masó et al., 2003), influence the absorption kinetics of toxic pollutants onto plastic surfaces (Qi et al., 2021), and significantly alter biogeochemical cycling within environments (Bowman et al., 2021; Wang et al., 2021b). Furthermore, plastic-associated communities can alter the attraction and palatability of plastic to larger organisms in the environment by disguising the inert nature of the

plastic material (Polhill et al., 2022; Porter et al., 2019). Having a comprehensive understanding of these communities and their distinctiveness compared to surrounding non-plastic surfaces is therefore fundamental for developing our understanding of the risks of plastic in aquatic systems.

There is reasonable agreement in the literature that the composition of plastic biofilm communities differs significantly from the surrounding free-living community of the water in which they are submerged (e.g. Di Pippo et al., 2020; Kettner et al., 2017; McCormick et al., 2014). However, the ability of plastic to host communities distinct from other surfaces, such as glass, rock and wood is less well understood, with conclusions around whether plastic can do so differing enormously between studies. These inconsistent findings are likely to be due to the wide range of substrate and environmental factors which can drive biofilm composition and formation (Chapter 1), and it is therefore clear that findings cannot be generalised too broadly, particularly in environments such as freshwater rivers where water conditions can vary hugely (Rahman et al., 2021). Understanding the ability of plastic to host unique communities is important for assessing the specific relative risk of plastic litter within a given area, and efforts must therefore be made to characterize and better understand these communities across various habitats and conditions.

Whilst biofilms formed on marine plastics have received considerable attention, those in freshwater systems are significantly less well understood (Barros & Seena, 2021), with information around plastic communities in UK freshwater particularly lacking. Furthermore, many studies tend to characterise only the bacterial species within plastic biofilms, with the structure of eukaryotic and fungi communities being far more overlooked (Miao et al., 2021). To date, only two studies have simultaneously analysed the bacterial, eukaryotic and fungal communities associated with plastic in a freshwater system. Although useful, one of these studies, conducted in an Arctic Lake, did not compare the development of plastic communities to those on other surfaces over the same time period (González-Pleiter et al., 2021), and relative differences therefore remain unknown. The other of these studies, conducted within the Chicago River, made interesting comparisons between different plastic and non-plastic surfaces, but did not include the occurrence of non-photosynthetic eukaryotes in their analysis (Chaudhary et al., 2022).

Despite receiving less attention, eukaryotes and fungi may be important members of plastic biofilms, and previous work has recognised the need to consider their presence alongside bacteria in holistic, multi-domain studies (Amaral-Zettler et al., 2021a). Fungi are for example, highly important in freshwater systems; they play a significant role in the decay of organic material and have been found to comprise 90 – 95 % of the microbial biomass associated with leaf litter decomposition (Grossart et al., 2019). Many fungi species are also capable of utilising plastic as their primary carbon source and show a considerable ability to biodegrade a broad range of plastics (Amobonye et al., 2021). Furthermore, compared to a single species in isolation, plastic biodegradation is highest when exposed to a mixture of several fungi species (Amobonye et al., 2021), making microbial biofilms a potential hotspot for this activity. Given that fungi are an important dietary component for many freshwater shredder invertebrates, such as amphipods, isopods and caddisfly larvae (Arsuffi & Suberkropp, 1985; Graça et al., 1993), their presence could alter the palatability of plastic to these organisms. Many shredders also show strong feeding preferences towards particular species of fungi, with different species also exhibiting different preferences (Arsuffi & Suberkropp, 1985); understanding fungi species composition may therefore be important for discerning these plastic-organisms interactions for these species. Eukaryotes also play a significant role within aquatic biofilms. Photosynthetic micro-eukaryotes such as diatoms and green algae can be highly abundant, showing significant primary productivity and a substantial influence on ecosystem-level carbon cycling (Serôdio et al., 2020). The ability of some benthic diatoms to produce volatile organic compounds (VOCs), such as oxylipins, may also act as an attractant for grazing invertebrates (Fink et al., 2006a), potentially creating a strong link between plastic litter and metazoans in the ecosystem (Serôdio et al., 2020) and leading to changes in biofilm structure. Small invertebrates living within and in close association with plastic biofilms has also been documented, with previous observations of insect larvae on the surface of plastic films after submersion in a freshwater river and lake (Imam et al., 1992). Despite the known importance of meiofaunal invertebrates for processes such as nutrient cycling, predation (Burns & Ryder, 2001) and their potential importance for the liberation of microbial metabolites (Gaudes et al., 2006) within biofilms generally, little is understood about their role in plastic-associated biofilms.

Given the ability for biofilms to mediate the impacts of plastic pollution within aquatic ecosystems,

and the knowledge gap surrounding the multi-domain communities associated with plastic in freshwater, further studies which work to characterise and understand these biofilms are clearly needed. The aim of this study was therefore to characterise the composition and development of biofilms on plastics in a UK river (where knowledge is particularly lacking), by examining the mass, photosynthetic pigment content and taxonomic composition of biofilms which became associated with plastic during submersion. The bacterial, eukaryotic and fungal communities present were investigated and the ability of plastic to host a unique community assemblage with distinct development compared to surrounding non-plastic surfaces was determined. This chapter partly addresses the first broad hypothesis outlined for this thesis and works to answer the sub-hypothesis '*Plastic litter provides a unique substrate for the formation of biofilms in UK rivers*'.

2.2 Methods

2.2.1 Experimental design

Low-density polyethylene (LDPE) and polylactic acid (PLA) films were used in this study and were derived from commercially available plastic bags. Both films were clear, with a thickness of 50.0 µm and 40.6 µm respectively, and their identity was confirmed using Fourier-transform infrared (FTIR) spectroscopy (Appendix B.1). Glass used in the study was derived from 1 mm-thick non-coated Academy microscope slides. Plastic films were laser cut into 7 mm x 82 mm coupons and glass slides were cut into 13 mm x 76 mm coupons before being mounted on a custom-built raft, consisting of a floating stainless-steel frame which suspended samples around 20 cm below the water's surface. Samples were deployed into the River Derwent, York, UK (53°53'29.9"N; 0°56'41.1"W) (Figure 2.1) on 29/07/2019. After either one, three or six weeks of submersion, sample coupons were removed and maintained in river water in the dark during transportation back to the laboratory. Coupons were handled by the edges using sterile forceps and were rinsed thoroughly with sterile Milli-Q water before analysis to remove loosely attached material. Coupons were then analysed for: i) biofilm weight; ii) biofilm chlorophyll *a* and pheophytin content; iii) taxonomic composition of bacterial, eukaryotic and fungal communities; iv) visual observations of the biofilm and species interactions. At each sampling

point water samples were also collected for molecular analysis from the surface water. For this, water was placed into sterile Duran bottles and stored in the dark at 4°C until processing several hours later. For molecular analysis of water samples, 500 ml of the collected water was filtered through 47 mm Whatman GFA (1.6 µm) filters into sterile glass beakers. Around 250 ml of this filtrate was then passed through 47 mm Durapore PVDF 0.22 µm membrane filters. Both filters were transferred to sterile centrifuge tubes and stored at -80°C until analysis. To minimise contamination, water filtration was performed in a biosafety cabinet sterilised with 70 % ethanol. For molecular analysis of coupons, three replicates per treatment were transferred to sterile centrifuge tubes and stored at stored at -80°C.

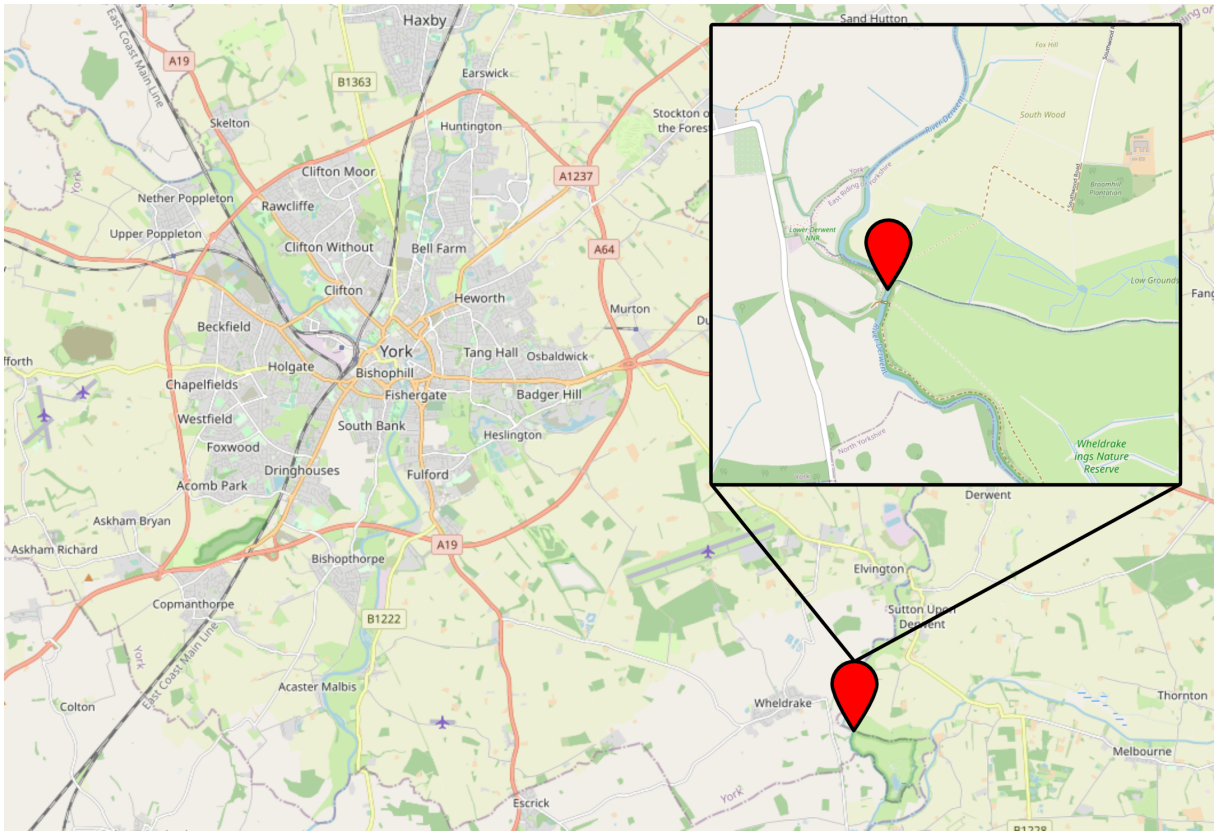


Figure 2.1: Map of the river location where plastic and glass samples were submerged.This map was produced with resources from ©OpenStreetMaps under the Open Data Commons Open Database Lisence.

2.2.2 Biofilm weight and photosynthetic pigment content

Separate coupons from each sampling point were used to quantify the amount of biofilm present on each material ($n = 3$), along with the biofilm's chlorophyll *a* and pheophytin content ($n = 3$). The weight of biofilm was measured by drying coupons and attached biofilm for three hours at 30°C before weighing with an analytical balance. The biofilm was then completely removed using a soft natural bristle brush and Milli-Q water before drying and re-weighing the clean coupons to determine mass loss. Biofilm removal and drying times were previously validated with preliminary work (Appendix B.2). It should be noted that throughout sample processing, no changes to the structural integrity of PLA or LDPE films were evident, with no observed biodegradation of the PLA samples within the river.

To measure the chlorophyll and pheophytin content of biofilms, coupons were placed in 90 % HPLC grade acetone (Fisher Scientific, UK). LDPE and PLA were extracted using 23.5 ml of solvent, while glass was extracted in 35 ml, and pigment extraction was carried out by incubating samples at 4°C for 24 hours. The chlorophyll and pheophytin content of acetone were then measured with a Turner Designs Trilogy fluorometer using the acidification method procedure outlined in the user manual (Turner-Designs, 2019) to obtain Raw Fluorescence Unit (RFU) values. The RFU response was related to pigment concentration by creating an external calibration curve. To do this, around 1 mg of chlorophyll *a* powder was dissolved in 90 % acetone. The resulting solution concentration was determined by measuring the absorbance at 664 nm using a Shimadzu UV-1800 spectrophotometer and the equation given by Jeffrey and Humphrey (1975). A series of solutions were then made with 90 % acetone and measured in the fluorometer to create a calibration curve which was used along with equations provided by Turner-Designs (2019) to calculate chlorophyll *a* and pheophytin content of biofilm samples (Appendix B.3).

2.2.3 DNA extraction and sequencing of biofilm communities

Biofilms were obtained from plastics, water and glass for all timepoints. However, due to differences in biofilm growth, it was necessary to adjust the amount of biofilm extracted to ensure optimal biological material for DNA extraction. Therefore, biofilm from the entire coupon was used for extraction of samples from weeks one and three, whereas only half of the biofilm material was needed from week six samples. DNA was extracted from samples using Qiagen DNeasy 96 PowerSoil Pro Kit using the manufacturers recommended protocol with the following amendments: Glass biofilms were thoroughly scraped from each coupon using a scalpel blade into a sterile 5 ml centrifuge tube, suspended in 2 ml of phosphate buffered saline and transferred to a new tube. The homogenate was then centrifuged, and the resulting pellet was used for DNA extraction. Plastic coupons, one quarter of GFA filters and one half of Durapore filters were cut into smaller pieces and placed into separate 2 ml centrifuge tubes. For all samples, 800 µL of Lysis buffer CD1 and 20 µL of proteinase K (20 mg/L) was added before samples were incubated in a water bath for one hour at 56°C. Samples were then transferred into homogenisation plates and the manufacturers protocol was followed from step 7. Three extraction blanks and three PCR-negative blanks were run alongside samples to control for contamination. DNA extraction was verified using gel electrophoresis and quantified using a nanodrop 8000 UV–Vis spectrophotometer. Sample DNA was normalised to 10 ng/L using molecular grade water to use as a template for PCR. Bacterial, eukaryotic and fungal communities were assessed using rarefied sequence abundance of the genetic regions encoding for 16S and 18S small subunit ribosomal RNA (16S and 18S rRNA) and the internal transcribed spacer region 2 (ITS2) - targeting bacteria, eukaryotes and fungi respectively. Amplicons were generated under a 2-step amplification approach using Illumina TruSeq tagged primers based upon the primers from Andersson et al. (2008) (16S-F), Arenz et al. (2015) (16S-R), Medlin et al. (1988) (18S-F and 18S-R), Ihrmark et al. (2012) (ITS-F), White et al. (1990) (ITS-R), with the addition of a unique custom barcode combination corresponding to each sample (Kozich et al., 2013). Primer sequences and PCR conditions are given in Appendix B.4. PCR Products were normalised using Sequalprep normalisation plates (Invitrogen, CA, USA). Pooled amplicon libraries were vacuum concentrated and gel purified. Resultant libraries were quantified using a Qubit dsDNA HS Assay kit (Invitrogen) and

the pool sequenced at a concentration of 6 pM with 10 % addition of Illumina generated PhiX control library. Sequencing was performed on an Illumina MiSeq platform using V3 chemistry (Illumina Inc., CA, USA).

2.2.4 Bioinformatics

Sequences were trimmed, quality filtered, de-replicated, and amplicon sequence variant (ASV) tables were generated using the DADA2 (Callahan et al., 2016) pipeline in R V.3.0.17 (R-Core-Team, 2019). Following the guidance given in the DADA2 Pipeline tutorial 1.16, DADA2 was used to trim, quality filter, merge, denoise and assign taxonomies – settings for this process are outlined in supplementary Appendix B.5. After quality filtering a total of 1,517,106 bacterial (16S rRNA), 2,820,505 eukaryotic (18SrRNA) and 2,230,072 fungal (ITS2) sequences were used in the analysis. Downstream analysis of sequencing data was performed in R studio using the packages: phyloseq (McMurdie & Holmes, 2013), vegan (Oksanen et al., 2017) and ggplot2 (Wickham, 2016). Each taxa domain was processed separately, and to account for the effect of sequencing bias the resultant ASV tables were rarefied without replacement to an even depth of 6455 (16S), 3323 (18S) and 6175 (ITS2). For 16S all samples were retained after rarefaction, whereas for 18S and ITS data rarefaction resulted in the loss of one sample each (a glass-week 6 replicate) due to a low read number.

2.2.5 Physical biofilm structure and species interactions

Scanning electron microscopy (SEM) was performed to visualise the three-dimensional structure of biofilms and observe potential inter-domain interactions occurring between biofilm members. Smaller subsections of coupons 13 mm x 7 mm were used for imaging with two replicates per treatment. Given that SEM analysis was only used for qualitative observations in this study, two replicates were considered to be sufficient. After rinsing, coupons were fixed by immersing them in 2.5 % SEM-grade glutaraldehyde for two hours. Glutaraldehyde was then removed and three 30-minute washes with 0.05 M phosphate buffer (pH 7.4) were performed. Samples were fixed for one hour with 1 % osmium tetroxide, followed by another three phosphate buffer washes. Samples were dehydrated for

30 minutes each in an ethanol series of 25 %, 50 %, 70 %, 90 %, and twice in 100 %, followed by 30 minutes in hexamethyldisilazane. Coupons were placed in a glass desiccator to dry before being mounted on a Ø 12.7 aluminium SEM pin and stored under cool dry conditions until imaging. Coupons were imaged with a Jeol JSM-6490LV scanning electron microscope (University of York, Imaging and Cytometry Laboratory), using a voltage of 8 - 10 kV, a spot size of 27 and a working distance of 10 - 17 mm. Images were captured at varying magnification to examine and represent the biofilm structure at different levels.

2.2.6 Water parameters

The concentration of nitrogen and phosphorus, as well as the temperature, pH, alkalinity, dissolved oxygen, and conductivity were measured in the river throughout the six weeks. Nitrogen levels (measured as total oxidisable nitrogen) ranged between 4.33 and 5.62 mg/L over the study and phosphate-phosphorus ranged from 0.04 to 0.10 mg/L. All parameter values measured at each week, along with details of the equipment used are given in Appendix B.6.

2.2.7 Data analysis

A detailed description of all statistical tests and transformations carried out for the study are given in Appendix B.7. Differences in biofilm weight, total photosynthetic pigment content (chlorophyll *a* + pheophytin), and pheophytin proportion were analysed using a two-way ANOVA with week and material as factors. All data were tested for normality and equal variance using a Shapiro-Wilk and Levene test and statistical transformation (square root or natural logarithm) was applied when necessary to achieve this.

The alpha diversity of biofilms was assessed by calculating the Shannon-Wiener index, which was then statistically analysed to evaluate differences between samples (using a two-way ANOVA or non-parametric aligned ranks ANOVA). Beta diversity was visualised using nonmetric multidimensional scaling (NMDS) plots, calculated using the Bray-Curtis dissimilarity index. To confirm that free-living

water communities differed from biofilms, all samples were first grouped into 'biofilm' and 'water' groups, an NMDS plot was constructed and analysed for statistical differences using PERMANOVA on the Bray-Curtis distance matrix. After this, water samples were removed and NMDS plots and PERMANOVA analyses were subsequently carried out on biofilm samples only. Within each taxa domain, an overall PERMANOVA was first carried out on all biofilm samples to establish the presence of overarching and interacting effects; data was subsequently subset by week and material and PERMANOVA analyses were applied to examine differences between weeks for each material, and differences between materials for each week. For all PERMANOVA tests a beta-dispersal analysis was also conducted to understand the potential contribution of replicate variation to the results (Appendix B.7. To identify any taxa specifically enriched on certain samples a linear discriminant analysis size effect (LEfSe) was carried out on all treatments for which significant community differences were found. This was performed online using the Galaxy Huttenhower lab portal (Segata et al., 2011) with individual ASVs as the input. Alpha values were set to 0.05, the LDA score threshold for a discriminative feature was set as 3.0 and an all-against-all strategy was applied.

To visualise taxonomic composition, the most abundant fifteen identified bacterial and eukaryotic orders across all samples were calculated and the average relative abundance for each treatment was plotted, along with the relative abundance of all eleven identified fungal phyla. Decisions to calculate and present these top most abundant taxa were based on similar presentation and processing methods carried out by previous studies (e.g. Dudek et al., 2020; McCormick et al., 2014; Shi et al., 2022; Wallbank et al., 2022; Weig et al., 2021). For these plots, visual identification of interesting changes was followed up with statistical analysis using a two-way ANOVA. Based on reports of particularly interesting groups of taxa encountered within the literature, such as pathogenic or plastic degrading taxa, the abundance of these groups was investigated and those found to be significantly enriched on any material were reported. A list of taxa investigated and the literature which motivated the search is given in supplementary Appendix B.8. Due to the documented production of dimethyl sulfide (DMS) by plastic-associated communities in marine systems (Chapter 1), for further interest the presence of taxa linked with the production of DMS was also assessed (Appendix B.8).

2.3 Results

2.3.1 Biofilm weight and photosynthetic pigment content

The weight and photosynthetic pigment content of biofilms showed variation across material type and time (Figure 2.2, Appendix B.9); with a significant interaction effect present between material and week for biofilm weight ($F_{4,17} = 18.11$, $p < 0.001$), total pigment content (chlorophyll *a* + pheophytin) ($F_{4,17} = 163.2$, $p < 0.001$) and biofilm pheophytin proportion ($F_{4,17} = 8.738$, $p < 0.001$). There was no significant change in biofilm weight or total pigment content between week one and three for any material ($p > 0.05$ for all), whereas between week three and six all materials showed a highly significant increase in biofilm weight ($p < 0.001$ for all) and a highly significant decrease in total pigment content ($p < 0.001$ for all). Whilst there was no significant change in pheophytin percentage between week one and three for PLA or glass, pheophytin percentage of LDPE increased significantly ($p = 0.010$). Between weeks three and six pheophytin percentage showed a significant increase for all materials ($p < 0.01$ for all), becoming higher than the chlorophyll *a* content for LDPE and PLA in week six. The large increase in biofilm weight over time is also visually evident with the three-dimensional build-up of material observed through SEM (Figure 2.3).

When comparing material type within each week, there was no significant differences in the biofilm weight between materials after one or three weeks in the river ($p > 0.05$ for all). After six weeks however, biofilm weight differed significantly between all materials ($p < 0.05$ for all) with PLA possessing the highest weight, followed by LDPE and glass (Figure 2.2). Within week one and week six the total pigment content differed significantly between all materials ($p < 0.01$ for all) with glass consistently possessing the highest, followed by LDPE and PLA. Within week three, the total pigment content of LDPE and PLA did not differ significantly from each other but were both significantly lower than glass ($p < 0.001$). Within week one and week three, the pheophytin percentage did not differ significantly between any materials ($p > 0.05$ for all). Whereas within week six, pheophytin percentage was significantly lower on glass than LDPE and PLA ($p < 0.01$ for both) (Appendix B.9).

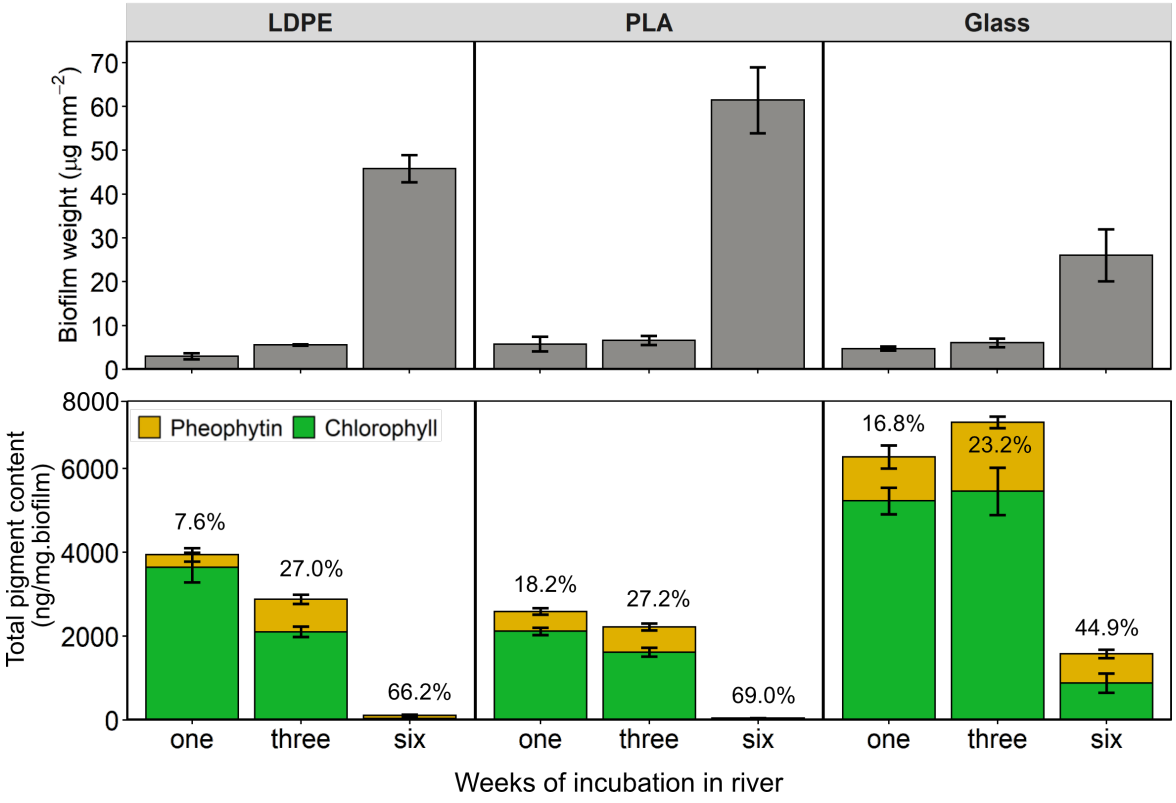


Figure 2.2: Biofilm weight and photosynthetic pigment content (chlorophyll *a* and pheophytin) of biofilms attached to low-density polyethylene (LDPE), polylactic acid (PLA) and glass samples after *in situ* incubation in a river for one, three or six weeks (mean ± S.D). Percentage values above pigment bars indicate the proportion of total pigment which was pheophytin.

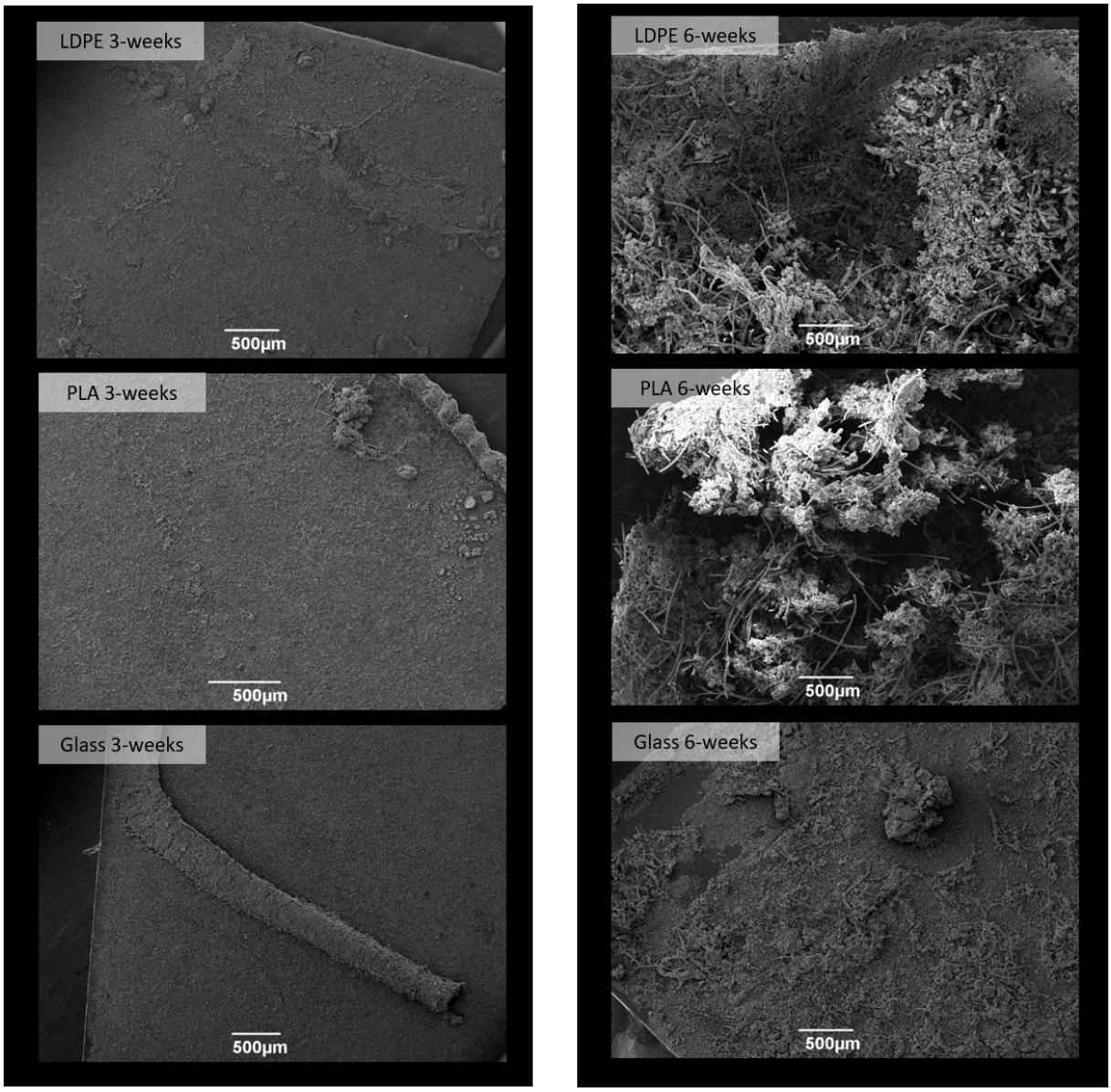


Figure 2.3: Scanning electron microscope images of the biofilm associated with glass, low density polyethylene and polylactic acid films after either three or six weeks of submersion in a river.

2.3.2 Differences in taxonomic community composition

The free-living water community composition showed a clear and significant separation from the biofilm community for bacterial, eukaryotic and fungi (Appendix B.10), and water samples were therefore excluded from subsequent analyses to allow focus on biofilm community characteristics.

The Shannon-Wiener diversity of biofilms varied notably across time but little between material types (Figure 2.4). For bacteria there was a significant main effect of week ($F_{2,18} = 6.21$, $p = 0.009$), with diversity first decreasing significantly between week one and three and then significantly increasing between week three and six ($p < 0.05$ for all). Eukaryotes showed a significant interaction effect between material and time ($F_{4,17} = 5.31$, $p = 0.006$). Whilst there were no significant differences in eukaryotic diversity between materials within any weeks ($p > 0.05$ for all), PLA showed a significant increase in diversity between week one and three ($p = 0.031$) whilst LDPE and glass did not. Between week three and week six all materials showed a significant decrease in eukaryotic diversity ($p < 0.001$ for all). For fungal communities a significant main effect of week was present ($F_{2,17} = 7.10$, $p = 0.006$) with a significant decrease in diversity between week one and three ($p < 0.01$) but no significant changes between week three and six. The p-values for all contrasts are given in Appendix B.11

Differences in biofilm communities between samples (beta-diversity) were visualised with NMDS plots (Figure 2.5), which show communities clearly clustered by week, and to a lesser extent by material type. PERMANOVA analyses identified a significant interaction effect between time and material on the community composition for bacterial, eukaryotic and fungal taxa ($p < 0.05$ for all). Within each material type a significant effect of time on community composition was found for all taxa ($p < 0.05$ for all) and clustering by week can be seen in plots with communities separated along both the x and the y axes.

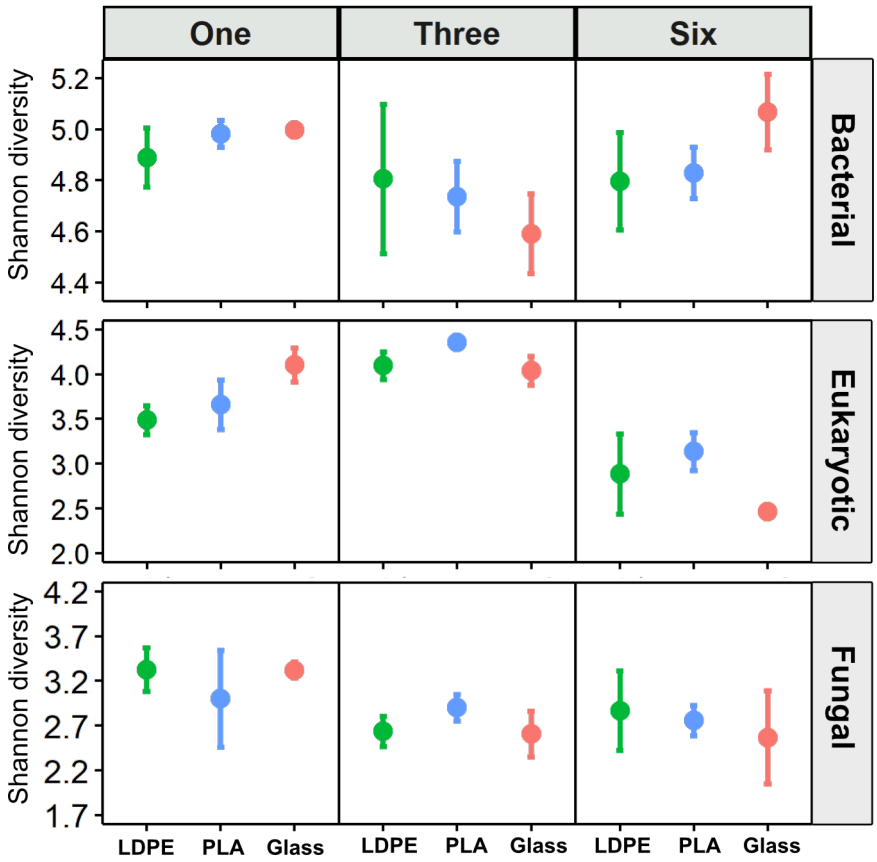


Figure 2.4: Shannon-Wiener diversity index (mean ± S.D) for bacterial, eukaryotic, and fungal communities present on low-density polyethylene (LDPE), polylactic acid (PLA) and glass surfaces after either one, three or six weeks of *in situ* incubation in a UK river.

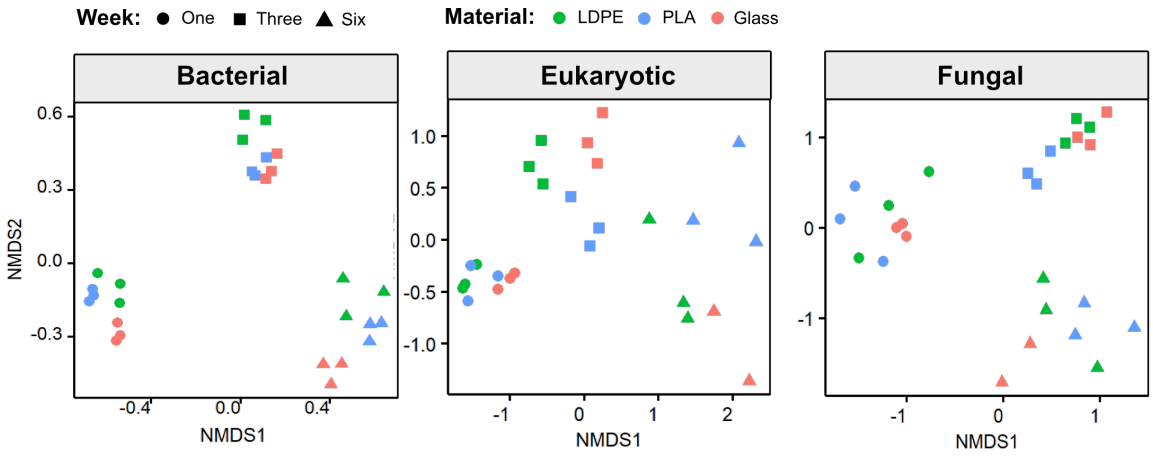


Figure 2.5: Non-metric multidimensional scaling plots for the bacterial, eukaryotic, and fungal taxa of biofilms sampled from low-density polyethylene (LDPE), polylactic acid (PLA) and glass surfaces after either one, three or six weeks of *in situ* incubation in a UK river. Stress of plots: bacterial = 0.079, eukaryotic = 0.091, fungal = 0.100

Within week one and three, a significant difference between materials was found for bacteria and eukaryotic communities ($p < 0.05$ for both). Within week six the significant difference between materials remained for the bacteria community ($p < 0.01$), whereas no significant differences were found in the eukaryotes ($p > 0.05$). For fungal communities there was a significant effect of material after three weeks ($p < 0.05$) but not after one or six weeks ($p > 0.05$). Within treatments where significant community differences are present, glass samples can be seen either clustered away from both LDPE and PLA, or one plastics type is clustered out away from the other samples – demonstrating the ability of plastic to harbour unique communities under these conditions.

2.3.3 Organisms driving community composition differences

The average relative abundance of taxa is shown in Figure 2.6 and the number of significantly discriminant ASVs identified through LEfSe analysis are given in supplementary Appendix B.12. Despite large differences in the bacteria community at the individual ASV level (Figure 2.5), differences at the order level across time and material were less clear. This indicates that whilst substantial shifts in lower-level taxonomy took place, there was greater consistency at the higher taxonomic levels, nevertheless, some interesting differences were present. For example, Flavobacteriales became significantly less abundant between week one and three ($p < 0.001$) and were significantly enriched on LDPE and PLA compared to glass for all time points ($p < 0.01$ for both). Similarly, the predatory bacteria group Myxococcales increased significantly in abundance from week three to week six on LDPE and PLA ($p < 0.001$ for both) but not on glass, and in week six they had a significantly higher abundance on LDPE and PLA compared to glass ($p < 0.01$ for both). In contrast, Rhizobiales increased significantly in abundance between week three and six on glass ($p < 0.001$) but not on LDPE or PLA, and were significantly more abundant on glass than plastics in week one and week six ($p < 0.05$ for all) whereas plastics did not differ from each other. These higher-level trends were supported by the LEfSe analyses, which identified the significant enrichment of Flavobacteriales ASVs on LPDE at week three and six and on PLA in week one. Similarly, ASVs of Myxococcales were significantly enriched on LDPE at week three and PLA at week six, and Rhizobiales ASVs were significantly enriched on glass after six weeks.

At the genus level, genera known to contain compound-degrading bacteria (*Ideonella* sp., *Xenophilus* sp., *Varivorax* sp., and *Polaromonas* sp.) as well as a genus known to contain fish pathogens (*Flavobacterium* sp.) were found on materials and had a significantly higher abundance on at least one plastic treatment compared to glass samples (Figure 2.7). The genus *Aquabacterium* sp., which has previously been documented as a core member of plastic communities, was also significantly enriched on LDPE in week one. Significant differences between treatments are annotated in Figure 2.7. The presence of three bacterial genera, which have been linked to their potential ability to produce the odorous compound dimethyl sulfide, were also detected on glass and plastics, and generally had a higher abundance on glass surfaces (Appendix B.13).

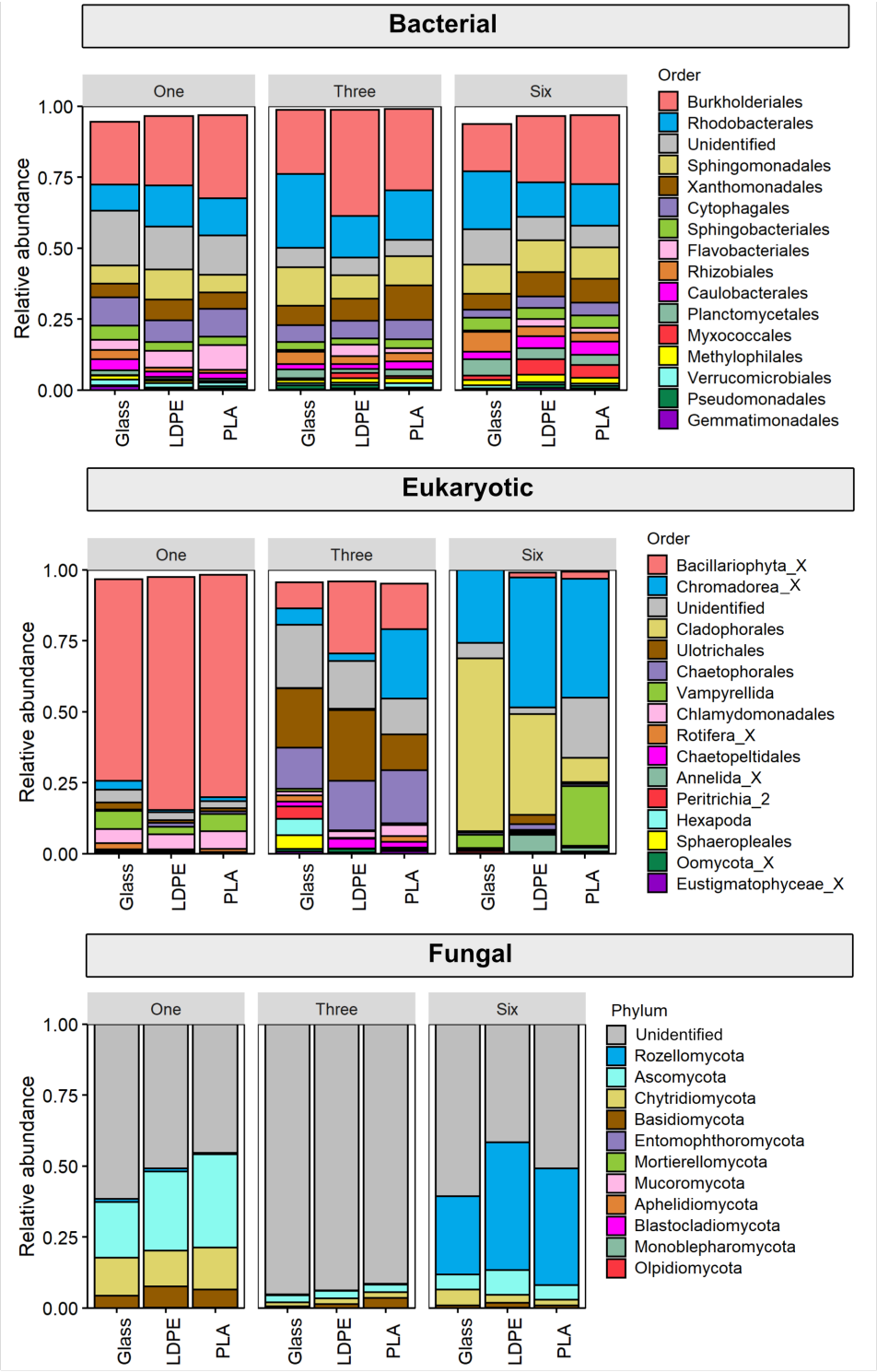


Figure 2.6: Average relative abundance of top 15 identified most abundant bacterial and eukaryotic orders and all 11 fungal phyla across all weeks and materials. The group 'unidentified' contains all taxa which remained unassigned at the order level, and therefore these may belong to various Phyla. Groups are presented in the order of their overall abundance across all samples, with the overall most abundant at the top and overall least abundant at the bottom.

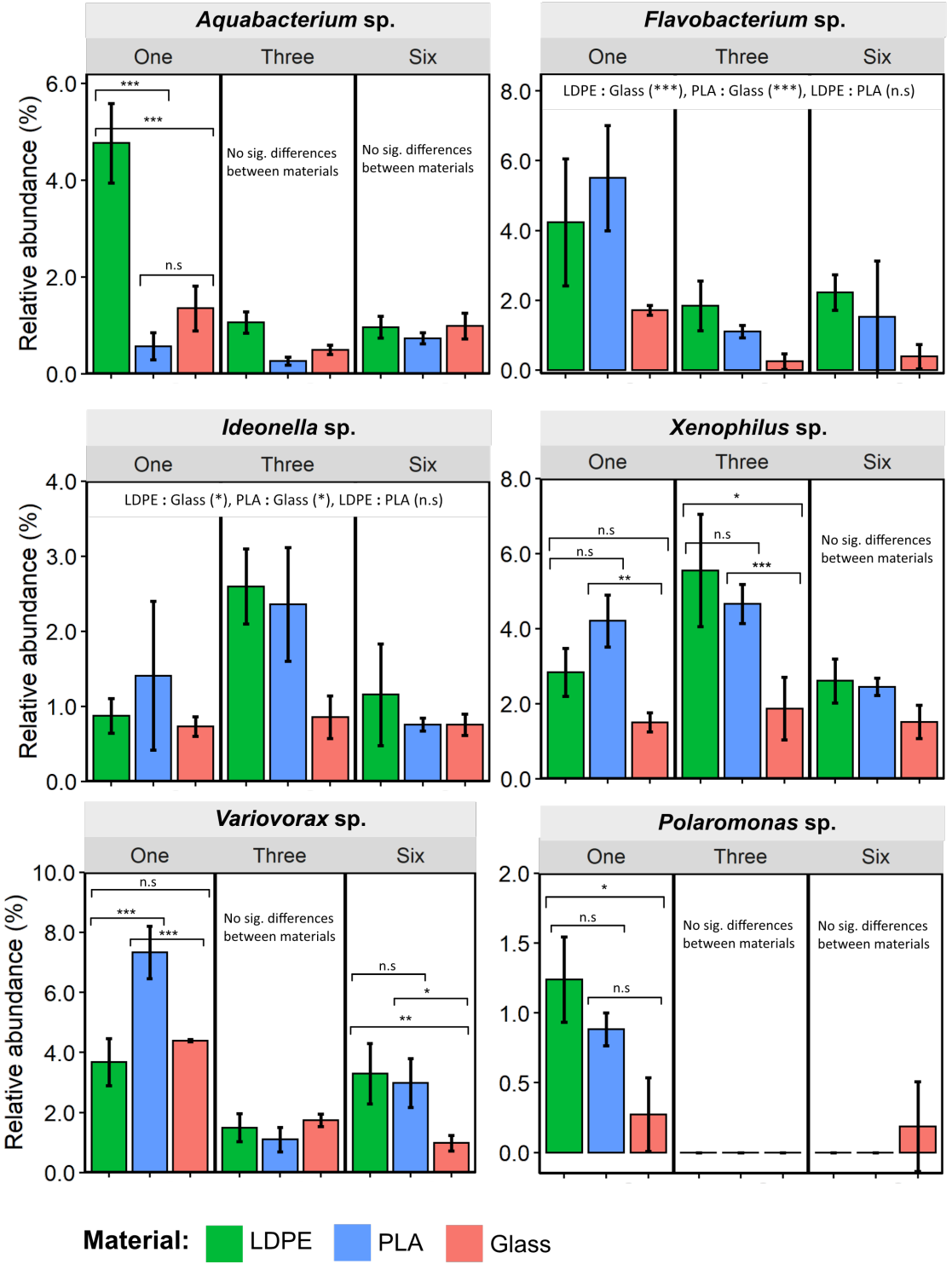


Figure 2.7: Relative abundance (mean ± S.D) of specific bacterial genera of interest in biofilms on low-denisty polyethylene, polylactic acid and glass. Results of two-way ANOVA analyses are shown within plots to show differences between materials. For *Ideonella* sp. and *Flavobacterium* sp. no interaction effect was found so results just show the over all significance of material type. (*) = $p < 0.05$, (**) = $p < 0.01$, (***) = $p < 0.001$.

The most striking trend in the eukaryotic community was the dominance of Bacillariophyta (diatoms) on all materials in week one, with an abundance of 67.0 – 86.5 % across samples (Figure 2.6). This was followed by a dramatic decline in week three and almost complete disappearance in week six. In total, 29 species of diatoms were detected, with many visually dominant in biofilm SEM images of week one samples (Figure 2.8). After six weeks, SEM images show the degradation of diatom cells (Figure 2.8 F) and the increased abundance of other organisms (Figure 2.8 B & D). These changes were reflected in the LEfSe analysis, which identified significant enrichment of several diatom ASVs in week one and three but not in week six. Although less abundant than diatoms, the flagellated green algae Chlamydomonadales also decreased from week one to week three for all materials before almost completely disappearing in week six. After three weeks, the green algae groups Chaetopeltidales, Chaetophorales and Ulotrichales increased considerably in abundance on all materials before decreasing again after six weeks. Over time, nematodes (Chromadorea_X), comprised mainly of the species *Punctodora ratzeburgensis* and to a lesser extent the genus *Eumonhystera*, became considerably more abundant on all materials, but with a very high variability between replicates (25.6 $\pm$ 21.4 % on glass, 45.7 $\pm$ 28.0 % on LDPE and 41.9 $\pm$ 30.4 % on PLA in week six). Some differences in eukaryotic orders between materials were present. For example, there was an overall significant effect of material on diatom abundance, with LDPE differing significantly from glass ($p = 0.023$); this can be seen particularly strongly in week three where diatom abundance is 25.3 $\pm$ 5.9 % on LDPE compared to on glass 9.2 $\pm$ 2.5 %. However, overall, the Eukaryotic community was far more heterogenous, with high variability between replicates compared to the bacterial community. For example, in week six an increase in the green algae Cladophorales was seen for all materials but showed extreme variation between replicates - ranging from 0.1 % to 69.2 % relative abundance. Other taxa similarly showed high variability; in week three the order Hexapoda had an abundance of 16.9 % on one sample and 0.0 – 0.3 % across all other samples, and the order Peritrichia showed an abundance of 10.9 % on sample and 0.0 – 1.3 % on all others.

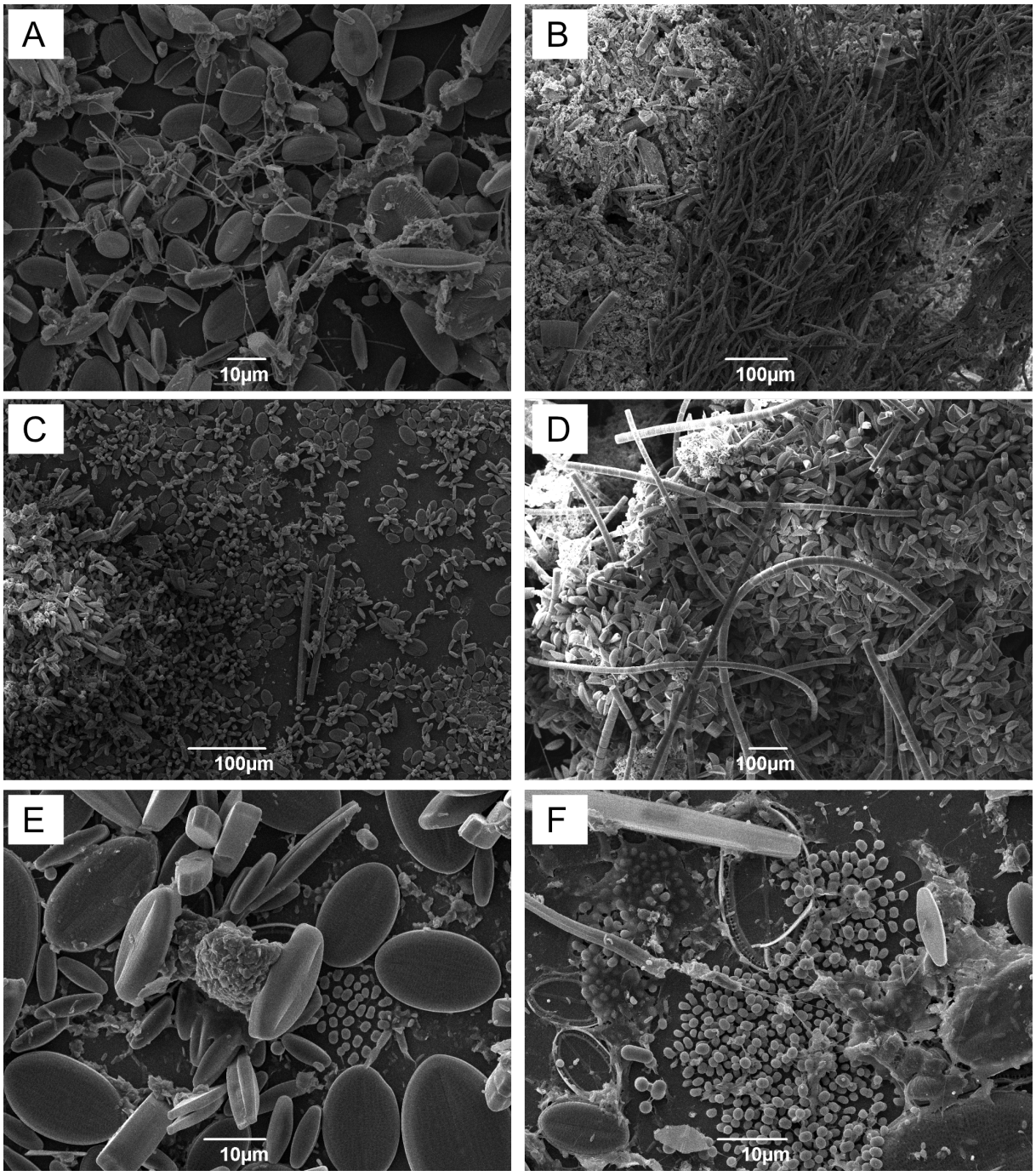


Figure 2.8: Scanning electron microscope images of low-denisty polyethylene (LDPE), polylactic acid (PLA) and glass surfaces after submersion in a UK river. A: LDPE after one week, B: LDPE after six weeks. C: PLA after one week, D: PLA after six weeks. E: glass after one week, F: glass after six weeks.

For fungal communities the majority of taxa were unidentified even at the phylum level. Although conclusions about the absolute abundance of bacterial, eukaryotic and fungal communities in relation to each other cannot be drawn from this data, there is strong indications that fungi comprised only a very small percentage of the total biofilm community and may not be as important as bacterial and eukaryotic taxa. Evidence for this comes from the number of fungal *vs.* eukaryotic reads detected with ITS and 18s primers, which often cross detect these groups. Taxa identified with 18s primers were comprised of only 0.9 – 1.8 % fungal reads, whereas taxa identified with ITS primers, which are designed to specifically target fungal groups, were comprised of 62.9 – 76.3 % of the eukaryotic green plant group Viridiplantae. A low presence of fungal organisms comprised of only a small number of individuals may explain these dramatic phylum-level shifts observed and the high levels of unidentified taxa.

2.4 Discussion

A comprehensive understanding of biofilm formation on plastics in aquatic systems is important for progressing our knowledge of the fate and impacts of plastic litter in the environment. Whilst the composition of marine plastic biofilms has been extensively investigated, freshwater systems have received far less attention, with knowledge around eukaryotic and fungal communities particularly lacking. To address this knowledge gap, LDPE and PLA films were submerged in a UK river alongside a glass control, for one, three and six weeks. The biofilms which formed were examined to determine the ability of plastic to harbour a unique community composition and alter biofilm development. The importance of considering bacterial eukaryotic and fungal taxa together was also determined, along with the potential ability for plastic biofilms to mediate interactions with larger organisms in the environment.

2.4.1 General trends and the influence of time on the biofilm

The distinction between biofilm and free-living communities was in line with expectations and similar to previous observations (Kettner et al., 2017; Xue et al., 2020). This is unsurprising given that biofilms support a distinct mode of life compared to free-living communities (Mora-Gómez et al., 2016), and reaffirms that research should now focus on determining whether plastic litter harbours communities unique from other surrounding non-plastic surfaces and the resulting relative risk of plastic pollution within aquatic ecosystems.

During submersion in the river, all plastic and glass samples accumulated a distinct biofilm on their surface, with biofilm weight increasing over time but photosynthetic pigment decreasing. Biofilm accumulation is in line with other studies which found a steady increase in biofilm mass during submersion in estuaries (Li et al., 2019) and the marine environment (Zhao et al., 2021). The significant drop in photosynthetic pigment seen on all materials in week six is comparable to previous studies which report a decrease in chlorphyll *a* (Chaudhary et al., 2022) and gross primary productivity (GPP) (Hoellein et al., 2014) on plastic and non-plastic surfaces. The drop in pigment content in the present study was accompanied by a drop in eukaryotic diversity and a reduction in diatom abundance and green algae taxa, indicating that photosynthetic microeukaryotes became less abundant over time and that biofilms shifted towards heterotrophy, with the bacterial community and non-photosynthetic eukaryotes becoming more dominant. The increased abundance of the predatory bacteria Myxococcales over time also supports this. The increase in pheophytin (a breakdown product of chlorophyll) seen in week three and week six is indicative of the physical breakdown of photosynthetic taxa, which is likely due to the die-off of old cells and lack of replacement with new ones. The increased abundance of the nematode *Punctodora ratzeburggensis*, which graze extensively on diatoms and other algal species (Schroeder et al., 2013) is also likely to have accelerated cellular breakdown and subsequent pheophytin production.

A strong influence of time was also observed for the taxonomic community composition of bacterial, eukaryotic and fungal taxa in this study, which is in keeping with previous findings for plastic-associated communities (Chaudhary et al., 2022; Lanning et al., 2019; Laverty et al., 2020; Tourova

et al., 2021) as well as broader well-defined biofilm successional patterns (Burns & Ryder, 2001; Dang & Lovell, 2000). The higher-level composition of biofilms was largely consistent with previous findings which report the dominance of Alpha- Beta- and Gamma-proteobacteria in plastic biofilms, as well as diatoms and green algae (Besemer, 2016; Shan et al., 2022). Interestingly, the high proportion of unidentified fungal taxa also appears to be common when examining plastic associated fungi communities (González-Pleiter et al., 2021; Wallbank et al., 2022; Wang et al., 2021c), and is likely to be due to the lack of research aquatic fungi have received (Grossart et al., 2019). Whilst some studies do report higher levels of fungal identification in plastic biofilms (Ashar et al., 2020; Kettner et al., 2017) biofilms in these studies were formed in highly urbanised environments or near waste water treatment plants, which are the best described fungal habitats (Grossart et al., 2019). This would explain the higher abundance of identifiable taxa and demonstrate a high variability in biofilm communities between different areas. In some freshwater environments fungi can comprise $>$ 50 % of eukaryotic reads, and they are known to be importance colonisers of submerged organic material such as wood and leaf litter (Besemer, 2016; Grossart et al., 2019; Kettner et al., 2017). In contrast, this study found only 0.9 – 1.8 % of eukaryotic (18S) sequence reads belonged to fungi and similar reports were made by Kettner et al. (2017) and González-Pleiter et al. (2021) where fungi comprised only 7.9 % and 3.8 % of total 18s eukaryotic reads. These findings indicate that in contrast to organic surfaces fungi may be less important members of biofilm communities on plastics and other hard smooth surfaces, potentially due to an inability of fungi hyphae to penetrate deeply into harder surfaces as they do with organic material (Artru and Lecerf, 2019). However, much uncertainty surrounds this and further research is therefore needed to quantitatively evaluate the relative abundance and importance of different taxa domains within plastic biofilms.

2.4.2 Influence of material type on biofilm composition and development

After one and three weeks in the river, the amount of biofilm on samples did not differ between materials, whereas after six weeks plastics accumulated significantly more biofilm than glass. Larger biofilms on plastics compared to glass and natural rock have similarly been reported by other studies (Miao et al., 2021; Zhao et al., 2021) and could be due to material specific characteristics such

as surface hydrophobicity, or potential differences in the hydrodynamic interactions between sample surfaces and the water column (Lau & Liu, 1993; Rummel et al., 2017; Shan et al., 2022). The photosynthetic pigment content also differed between materials, with glass-biofilms consistently possessing a higher pigment content than LDPE and PLA – indicating higher levels of heterotrophy within plastic biofilms. Hoellein et al. (2014) similarly found glass to have a higher GPP than plastic biofilms formed under controlled mesocosm conditions, however, the same study also found no differences between materials sampled from a pond, and a higher GPP of plastic compared to glass for materials sampled from a river. Chaudhary et al. (2022) also report no significant differences in the chlorophyll content of plastic and tile biofilms, but interestingly, did find the respiration rates of polystyrene foam and tile to be significantly higher than polyvinyl chloride and low-density polyethylene. Under the river conditions tested in the current study, plastic films supported biofilms which differed in size and photosynthetic profile to glass, and likely supported a biofilm with higher levels of heterotrophy. However, taken together with the reports from previous literature it is clear that many factors may influence this, and that these findings should not be generalised too broadly.

Whilst time was the largest influence on biofilm taxonomic community composition in this study, material type also exerted a significant influence, and the test hypothesis for this experimental work can therefore be accepted. Material showed a sustained significant effect on bacteria composition throughout week one, three and six, consistently explaining 50 – 56 % of bacteria community differences. For eukaryotes, material drove differences in week one and three but not in week six, and fungal communities only differed across materials in week three - indicating that whilst bacteria populations remained distinct, eukaryotes and fungi merged into more general non-specific communities. It is logical that substrate properties would influence initial microbial colonisation, but become less important over time when newly recruited organisms are only interacting with the organic layer already established (Oberbeckmann et al., 2016). This is supported by previous findings, such as those from Pinto et al. (2019) who found that bacteria communities showed less differences across materials with increasing submersion time. However, in contrast, the sustained importance of material for bacteria in the current study indicates that connectivity between the substrate surface and bacteria remains for at least six weeks under the conditions examined, and similar findings were reported in mature 15-month old biofilms formed in the marine environment, where significant dif-

ferences between plastic and glass were found for bacteria but not eukaryotes (Kirstein et al., 2018). Interestingly, as seen in the present study, Kirstein et al. (2018) also report high heterogeneity of the eukaryotic community, leading the authors to conclude that whilst bacteria communities are strongly connected to material characteristics, eukaryotes may be far more stochastic and driven more by the bacteria community present.

At the order level Myxococcales became more abundant over time and were also significantly more enriched on both plastics than on glass in week six. Myxococcales are heterotrophs which actively predate on a variety of other microorganisms; they can be important for regulating bacteria community composition (Wang et al., 2020a) and their higher abundance on plastics supports the greater levels of heterotrophy thought to have occurred within LDPE and PLA biofilms compared to glass. The ability of plastic to support different levels of heterotrophy compared to other surfaces could have implications for the carbon cycling within the river system (Wang et al., 2021b) and future studies to directly measure this should be undertaken. In contrast, Rhizobiales were significantly depleted on plastic at all time points compared to glass in this study, and have also been found in higher abundance on glass compared to microplastics after submersion in waste water effluent (Martínez-Campos et al., 2021). Rhizobiales are known for their involvement in nitrite reductase activity within biofilms (Pang & Liu, 2007) and may therefore similarly contribute to a different metabolic functional profiles between glass and plastic biofilms.

Flavobacteriales decreased in abundance from week one to week six and maintained a significantly higher abundance on at least one plastic compared to glass. At the genus level, in all weeks *Flavobacterium* sp. was significantly more abundant on LDPE and PLA compared to glass. Members of this genus are known to be major fish pathogens, causing issues related to gill health, and have previously been identified within the community of plastic-associated biofilms from other studies (Gong et al., 2019). Given the common interaction with and ingestion of plastics by fish (Roch et al., 2020), the enrichment of *Flavobacterium* sp. on plastics may be a considerable threat to fish in natural populations and in aquaculture, and the ability of these bacteria to cause disease from fish-plastic interactions should be investigated. Many other pathogenic taxa, as well as antibiotic resistance markers, have also been found to be significantly enriched on plastics (Li et al., 2019; Morohoshi

et al., 2018), however the conditions that promote this enrichment and the subsequent consequences for the surrounding ecosystem remain unknown and further research is therefore needed to quantify these potential impacts.

Other taxa of interest that were enriched on plastics in this study were: *Ideonella* sp., which contains species known to be highly effective at degrading plastics (Bornscheuer, 2016; Morohoshi et al., 2018), as well as *Xenophilus*. sp., *Variovorax* sp., and *Polaromonas* sp. which contain species linked to the degradation of plasticizers and other xenobiotics such a benzene and pyrene (Bai et al., 2020; Eriksson et al., 2002; Posman et al., 2017). Previous studies have also found the enrichment of aromatic compound-degrading species on plastics in marine and freshwater environments (Li et al., 2021), indicating that this may be common. The plastic-specific enrichment of these groups may have interesting implications for the fate of plastic that becomes submerged in the river and has the potential to influence the biotransformation of xenobiotics within the riverine system. Bacteria of the genus *Aquabacterium* were also significantly more abundant on LDPE than PLA or glass in this study, with a > 4 % relative abundance. This genus has been consistently noted to be enriched in biofilms associated with plastics compared to non-plastic surfaces (Kelly et al., 2020) and may therefore be a core member of freshwater plastic communities. Reasons for this, such as its ability to utilize plastic or its additives as a carbon source should therefore be investigated, as it may hold potential as a previously undocumented plastic-degrading bacteria.

The identification of significant community differences between materials in this study both agrees and contrasts with previous literature findings, which encompass a broad range of results. For example, whilst some studies find significant differences in the bacteria and eukaryotic community between plastic and natural substrates (e.g. González-Pleiter et al., 2021; Kettner et al., 2019; Miao et al., 2021; Miao et al., 2019), as well as a significant influence of finer scale factors such polymer type and polymer colour on bacteria communities (Qiang et al., 2021; Wen et al., 2020), other studies report few differences in bacterial and eukaryotic communities between plastics, wood, rock and glass surfaces (Hoellein et al., 2014; Hu et al., 2021; Laverty et al., 2020; Wu et al., 2019). For fungal communities, differences are generally found between plastic and natural surfaces such as wood and rock but not between polymer types (González-Pleiter et al., 2021; Kettner et al., 2017;

Wang et al., 2021c). A detailed examination of factors known to drive community composition is discussed in section 1.3. Findings from this study demonstrate that plastic pollution in UK rivers can support biofilms with a distinct composition compared to other non-plastic surfaces (i.e. glass), and that plastic could therefore pose unique risks. However, taken together with existing literature it is clear that this effect is likely to be highly dependent on location and surrounding environmental conditions, such as nutrient concentrations. Further work to determine the conditions which enhance this unique composition and potential metabolic functionality are therefore required.

2.4.3 Importance of considering plastic-associated communities as a whole

Environmental biofilms are by nature, highly complex multi-species communities and observing just one taxonomic group in isolation therefore limits understanding of the community and its holistic functionality, potentially resulting in incorrect conclusions being drawn. In this study, the differences seen between substrates were distinct for bacteria, eukaryotes and fungi, demonstrating that all these communities must be investigated to gain a complete picture of plastic biofilms. Inter-domain interactions are also known to occur within the biofilm and were observed between diatoms and bacteria cells in this study on LDPE and PLA surfaces (Appendix B.14). These interactions often involve the mutualistic exchange of metabolites and essential micronutrients; they can have ecosystem-scale influences on the biogeochemistry of an environment (Amin et al., 2012; Seymour et al., 2017) and are also therefore likely to influence the overall community structure of the biofilm.

Whilst the presence of invertebrates on marine plastics is reported periodically (Enrichetti et al., 2021; Ma et al., 2022), similar observations are much more limited in freshwater. In this study, algal-grazing and deposit-feeding nematodes were detected from DNA sequencing and became prominent members of the biofilm community by week six. Similar observations were made in marine plastic biofilms (Kirstein et al., 2018) and nematodes may therefore be an important member of plastic communities across a broad range of environments. Grazing invertebrates such as nematodes can be a key influence on community composition and dynamics within aquatic biofilms, and can therefore affect successional development and biofilm functionality (Burns & Ryder, 2001; Mora-Gómez et al.,

2016) - for example through top-down control on bacteria and algae (Majdi & Traunspurger, 2015). Furthermore, invertebrate grazing in the biofilm is thought to significantly disrupt the physical biofilm structure and catalyse the release of key biological metabolites, that would have otherwise remained locked within the biofilm matrix (Gaudes et al., 2006). This may have implications for the release of ecologically important metabolites, such as certain VOCs known to drive the attraction of grazing organisms (Fink, 2007). Nematodes are also thought to contribute to ecosystem wide benthic-pelagic coupling; for example, they are predated on by specialised fungi, larger macroinvertebrates and even fish, therefore connecting the microbial production of plastic biofilms to higher trophic levels (Majdi & Traunspurger, 2015). Furthermore, nematodes are thought to play a major role in nutrient and carbon cycling within the biofilm and therefore have the potential to influence these processes within the wider environment (Hölker et al., 2015).

Although not present in sequenced samples, there was additional qualitative evidence, from non-sequenced replicates in the main study and samples from preliminary investigations, of other metazoan eukaryotes associated with plastic biofilms. For example, flies, preliminary identified as non-biting midges (Chironomids), were observed on samples from the main study. Several of these adult insects emerged from plastic and glass samples, which had been removed from the river at week six and stored at room temperature in sealed vials with sterile filtered river water. The presence of many tube structures were present on plastic and glass samples and were thought to belong to chironomid larvae which often construct tubes from silk, detritus and surrounding algae (Hölker et al., 2015). Furthermore, during preliminary investigations, a chironomidae larvae (Dobson, 2012) was observed actively feeding within the biofilm attached to LDPE. Other invertebrates such as black fly pupa (Simuliddae) and snails (Physidae) (Dobson, 2012) were also associated with plastic samples from preliminary work in the same river. Images and links to videos of these interactions are given in Appendix B.15. These observations demonstrate the variety of organisms that can become associated with plastic in freshwater and support previous associations reported between invertebrates and plastic litter in rivers and lakes (Artru & Lecerf, 2019; Imam et al., 1992; Wilson et al., 2021). The wider implications of these interactions between invertebrates and plastic for the wider food web, biofilm functionality, and impacts of plastic litter remain unclear, but may be important (Wright et al., 2020), and should therefore be considered in future research. Along with previous literature, findings from

this study demonstrate that considering all community taxa, including metazoan invertebrates, is key for comprehensively understanding the structure and functionality of plastic-associated communities.

2.4.4 Conclusions

This study is the first to comprehensively examine, in parallel, the composition of bacteria, eukaryotic and fungi taxa associated with plastic in a UK freshwater river and make comparisons with a non-plastic surface. Under the river conditions investigated, material type exerted a significant influence on the amount and composition of biofilm which formed, with significant differences between plastic and non-plastic surfaces. The structure of all taxa domains was significantly influenced by material type and confirms the need to consider all community taxa when examining plastic-associated biofilms in order to draw accurate conclusions. However, bacteria communities were most strongly affected with continued substrate-based differences after six weeks of submersion in the river, whilst eukaryotic and fungal communities became more general as the biofilm matured and appeared to be more heterogenous than bacteria communities. Additionally, there were indications that unlike on organic surfaces, fungi may be a less important component of plastic biofilms compared to bacteria and eukaryotes, but further work is needed to confirm this.

Over time, biofilms were thought to become more heterotrophic, with plastics supporting a higher level of heterotrophy than the representative non-plastic control - based on the disappearance of photosynthesis pigments and taxa, and the appearance of predatory bacteria. Plastic was also significantly enriched with genera known to contain pathogenic, plastic-degrading, and xenobiotic-degrading species, and significantly depleted in taxa involved with the degradation of nitrates. These findings give strong indications that the composition of biofilms on plastic in this study may have given rise to unique metabolic functionality compared to surrounding surfaces. Furthermore, the presence of nematodes and other grazing invertebrates indicate the potential for the physical disruption of the biofilm, which may expedite the release of microbial metobolites and other compounds from the biofilm matrix. The presence of invertebrates is also likely to have shaped community structure and may mediate benthic-pelagic coupling processes between plastic surfaces and the wider environment.

Under the conditions investigated in this study, it was shown that plastic pollution can support a unique biofilm community in UK rivers. However, taken together with previous literature it is clear this may be influenced by many factors, and future work to determine the conditions in which plastic is most likely to harbour unique microbial communities is therefore needed. Further efforts to directly determine the impacts of these unique assemblages, such as the transfer of pathogenic species and the potentially unique metabolic functioning of plastic-associated communities, is also required in order to unpick the impacts of these complex interactions occurring between plastic litter, microorganisms, and the wider aquatic ecosystem. Given the importance of the odorous compound DMS as a foraging cue in the marine environment, and the identification of putative DMS-producing species within the present study, the next chapter of this thesis will therefore explore the metabolic functionality of freshwater plastic-associated biofilms by investigating their ability to produce DMS.

Chapter 3

The smell of our trash: plastic litter can acquire a distinct dimethyl sulfide signature in UK freshwater rivers

3.1 Introduction

Shortly after submersion in natural waters, bacteria, algae, fungi and small invertebrates accumulate on plastic surfaces, forming a distinct biofilm layer of living organisms within a matrix of extracellular polymeric substances and other particulate matter (Carpenter & Smith, 1972; Zettler et al., 2013). These biofilms release chemical metabolites, such as volatile organic compounds (VOCs) like sulphurous compounds, polyunsaturated aldehydes and cyclic hydrocarbons, which function as foraging cues for larger organisms in the environment (Fink, 2007). For example, the freshwater gastropod *Radix ovata* is known to show significant attraction to VOCs released from certain diatom and green algae species, and is even able to differentiate between high and low quality food using only these odours (Fink et al., 2006a; Fink et al., 2006b; Moelzner & Fink, 2014). Odorous compounds are thought to mediate the interactions between plastic litter and larger organisms, with a growing number of studies demonstrating that the presence of a microbial biofilm on plastic significantly

increases its ingestion by both marine and freshwater organisms, compared to the same plastic in its virgin state (DeMott, 1986; Polhill et al., 2022; Sucharitakul et al., 2021; Vroom et al., 2017). It has therefore been proposed that biofilm attachment to plastic and VOC production by microbial community members may mask the inedible nature of the plastic. This is thought to create a trojan horse effect, increasing the probability that organisms will interact with and ingest plastic particles (Botterell et al., 2020; Fabra et al., 2021; Procter et al., 2019) and therefore also the wider impacts of plastics within the ecosystem.

The VOC dimethyl sulfide (DMS) is produced by plastic-associated biofilms formed in the marine environment (Savoca et al., 2016). This compounds is naturally present in the atmosphere and aquatic systems globally and plays an important role in climate regulation and the global sulfur cycle (Campen et al., 2022; Lomans et al., 2002; Shaw, 1983). DMS is also widely recognised as a key infochemical and foraging cue in marine systems (Owen et al., 2021; Savoca & Nevitt, 2014), with responses to aqueous and air-borne DMS documented in Procellariiform seabirds, seals, penguins, whale sharks, logger head turtles, reef fish larvae, phytoplankton and zooplankton (Amo et al., 2013; Botterell et al., 2020; Dove, 2015; Endres & Lohmann, 2012; Foretich et al., 2017; Kowalewsky et al., 2006; Nevitt et al., 1995; Procter et al., 2019; Savoca et al., 2016; Shemi et al., 2021; Wright et al., 2011). Furthermore, a significantly higher ingestion rate of microplastics infused with DMS, compared to virgin microplastics, has been seen in marine copepods and lobster larvae (Botterell et al., 2020; Procter et al., 2019). Whilst the ecological importance of DMS within freshwater remains largely unexplored, one previous study has documented the sensitivity of three freshwater fish species to DMS and identified its ability to induce feeding behavioural responses in these organisms (Nakajima et al., 1989), indicating the potential role of DMS as a foraging cue in freshwater.

In the marine environment most DMS is produced through the bacteria-mediated degradation of dimethylsulfoniopropionate (DMSP) - which is widely synthesized by marine algae, bacteria and some higher plants, and is thought to be an important osmoprotectant and cryoprotectant (Bentley & Chasteen, 2004; Lomans et al., 2002). By contrast, whilst the DMSP-to-DMS pathway does occur in freshwater (Ginzburg et al., 1998) it is generally thought to be less dominant. Instead, most DMS

in freshwater is thought to be produced from a mixture of other microbially mediated mechanisms such as the degradation of methoxylated aromatic compounds and sulfur-containing amino acids, as well as the methylation of methanethiol (Carrión et al., 2015; Lomans et al., 2002). Although not directly measured, the genetic potential of various common bacteria to produce DMS from the methylation of methanethiol in freshwater and terrestrial environments has been identified (Carrión et al., 2015), and the production of DMS by several freshwater photosynthetic species, including cyanobacteria and green algae has been directly observed (Bechard & Rayburn, 1979; Steinke et al., 2018). Despite its importance in marine systems, there is far less research surrounding DMS in freshwater, and only one study to date has examined DMS production by plastic biofilms in a freshwater system (Zink & Pyle, 2019). Although no evidence of production was found in this study, the detection limit (32 nmol/L) was relatively high and the storage of samples before analysis is likely to have led to rapid DMS depletion (Li et al., 2020). Studies which use more specialised and sensitive methods are therefore required.

In Chapter 2, the bacterial, eukaryotic and fungal communities were found to differ significantly between plastic and a representative non-plastic surface (i.e. glass) after submersion in a UK river, and there were indications that these communities may have distinct metabolic functionality. For example, LDPE and PLA films harboured larger biofilms which were thought to be more heterotrophic than glass surfaces. LDPE and PLA were also depleted in bacteria taxa linked with nitrogen cycling and enriched in genera known to contain pathogenic, plastic-degrading and xenobiotic-degrading species. Previous studies have also found evidence of unique metabolic functionality within plastic biofilms and suggest that it could lead to impacts on ecosystem-scale processes such as biogeochemical cycling (Chen et al., 2020; Hu et al., 2021; Mincer et al., 2016; Su et al., 2022; Xue et al., 2020). However, very little is known about how the composition of plastic biofilms may drive metabolic activities which can influence interactions between plastic litter and larger organisms within freshwater environments. Work in Chapter 2 also identified three bacteria genera with the genetic potential to produce DMS (Carrión et al., 2015) within plastic and glass biofilms, with an average relative abundance of up to 3.3% (Appendix B.13). These findings, along with the known importance of DMS in marine systems and ability for some freshwater species to detect this compound, motivated exploratory work, which subsequently led to the preliminary detection of DMS in water surrounding

plastic and glass biofilms formed within a UK river (Appendix C.1).

Considering the existing knowledge around DMS, and the findings made so far in this this thesis, the aim of the current experimental study was to use sensitive methodology, highly adapted to sulfuric-VOC detection (LOD 0.015 nmol/L), to establish the ability of biofilms which form on plastic pollution within UK rivers to produce the odours compound DMS. Comparisons with surrounding non-plastic surfaces were also made in order to ascertain the potential relative risk of DMS production by plastic within the river. This chapter partly addresses the first broad hypothesis outlined for this thesis and works to answer the sub-hypothesis '*Biofilms associated with plastic litter in UK rivers can produce odorous metabolites which may enhance interactions between plastic and freshwater organisms*'. As identified in Chapter 1 and 2, plastic biofilm communities are thought to be strongly shaped by surrounding location conditions, such as nutrient levels, temperature, and salinity, which can also drive the specificity of communities for plastic. Therefore, to evaluate the role of location in DMS production, materials were analysed after submersion at either a rural or an urban river location, and the mass and photosynthetic pigment content of biofilms was evaluated to identify broad differences in biofilm characteristics driven by location.

3.2 Methods and Materials

3.2.1 Materials

The same low-density polyethylene (LDPE) film, polylactic acid (PLA) film and glass microscope slides used in Chapter 2 were also used in this study. Plastic was laser cut into 7 x 82 mm coupons and glass slides were cut into 26 mm x 38 mm coupons before materials were secured inside a 240 mm x 240 mm x 130 mm custom-built stainless steel woven mesh (3.5 mm aperture) cages for deployment in the field.

3.2.2 Sample incubation

Cages containing plastic and glass samples were deployed at one of two river locations (rural or urban) just under the surface of the water, with coupons orientated vertically within the water column allowing both sides of the coupon to be colonised. Materials were then sampled after either three or six weeks of submersion, with the deployment and sampling regime staggered by one week between locations. Rural river samples were deployed on 10/08/2021 in the River Ouse upstream of York city centre, UK (54°00'30.7"N 1°11'28.7"W). One week later, urban river samples were deployed into the River Foss within York city centre, UK (53°58'35.4"N 1°04'26.6"W). River locations were categorised using the 2011 England Rural-Urban Classification system (Government Statistical Service, 2011; Office for National Statistics, 2020), with the River Ouse location classified as a 'rural hamlet and isolated dwellings' area and the River Foss location classified as an 'urban city and town' area. These locations are shown in Figure 3.1. Deployment in the urban river was logistically restricted to a more shaded area; light flux above cages in the rural location was 95 μmol/s/m^2 and for urban samples it was 22 μmol/s/m^2. The water depth at river sites was 3 m at the rural location and $<$ 1 m at the urban location.

After three or six weeks, materials were removed and transported back to the laboratory and rinsed thoroughly with Milli-Q water to remove loose material before the next stage of analysis. At each sampling point the pH, dissolved oxygen, conductivity, and temperature of the river water were measured. The nitrate and phosphate levels of rivers were also determined by analysing surface water samples (filtered to 0.2 μm with Sartorius surfactant-free cellulose acetate filters) with API colorimetric testing kits. At the six-week sampling point for each location, surface water samples were also collected in gas tight glass vials for measurement of background DMS levels in the river, due to logistical constraints these measurements could not be made for the week-three sampling point.

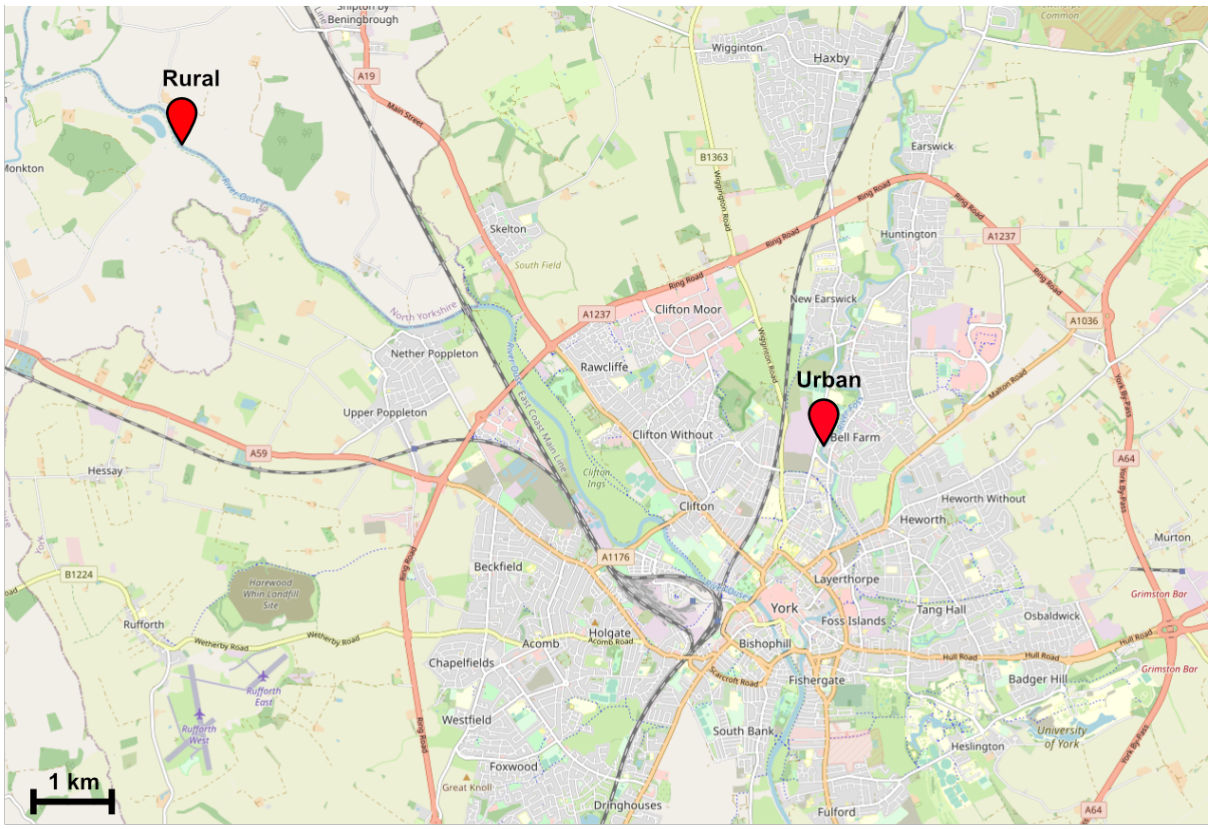


Figure 3.1: Locations of the rural and urban river sites where plastic and glass samples were submerged. This map was produced with resources from ©OpenStreetMaps under the Open Data Commons Open Database Lisence.

3.2.3 Biofilm composition and characteristics

Separate coupons from each sampling point were used to quantify the amount of biofilm present on each material along with the biofilm chlorophyll-*a* and pheophytin content, with six replicates per treatment for each endpoint. Biofilm weight and pigment content were analysed using the same methods described in Chapter 2, with a new pigment calibration curve which is given in Appendix C.2.

3.2.4 Measurement of DMS production

Plastic and glass coupons were placed in 35 ml clear glass vials filled with filter sterilized (0.22 µm) artificial river water (ARW) (Naylor et al., 1989). Vials were capped with EPA gas-tight lids and incubated for 44 hours at 15°C under a light intensity of 120 µmol/s/m^2 and a 14:10 light-dark cycle. For each sampling point there were four replicates per material; each LDPE and PLA replicate vial contained five 64 mm x 7 mm coupons (4480 mm^2 colonised material) and each glass vial replicate contained three 35 mm x 23 mm coupons (4830 mm^2 colonised material). DMS concentrations were subsequently normalised to 1 cm^2 of material surface area. For each sampling point, blank control samples consisting of only ARW were also analysed. Three blanks were analysed prior to the 44-hour incubation and three were analysed after - alongside the treatment samples. To verify that any DMS detected was being produced by the biofilm, rather than the material it was attached to, a separate virgin plastic control analysis was also performed. For this, material from the same source/batch as treatment samples was analysed using the same methodology, however these samples were clean and had never been previously exposed to the environment ($n = 3$ for each material).

After 44 hours of incubation, the DMS concentration of ARW samples was measured using a custom-built purge-and-trap system coupled with an Agilent 7890B gas chromatograph (GC) and Agilent 5977A mass spectrometer (MS). ARW samples were inverted several times before 20 ml of liquid was drawn into a gas tight glass syringe and spiked with 20 µL of internal standard. The sample was then injected through a sterile 0.45 µm Sartorius surfactant-free cellulose acetate filter into a glass purging tube and nitrogen gas was bubbled through the sample at a rate of 20 ml/minute, for ten minutes. The gas stream was dried using two in-line Nafion driers filled with 4A molecular sieves, and gases were collected in a PTFE loop trap cooled to -150°C with liquid nitrogen and an in-house built liquid nitrogen boiler. After ten minutes, the trap was rapidly heated to 100°C by submerging the loop in boiling water for one minute to inject gases into the GCMS system. All tubing for the purge-and-trap system was polytetrafluoroethylene (PTFE) and sample-contact fittings were polyether ether ketone (PEEK). Gases were separated on an Agilent DB-VRX column (length: 60 m, inner diameter: 320 µm, film thickness: 1.8 µm). Injection of samples was splitless with injector temperature set at 250°C

and 11.8 psi. Helium was used as a carrier gas with a constant flow of 2 ml/min. The GC oven temperature regime was: initial temperature of 32°C held for 5 minutes; temperature ramp to 110°C at 20°C/min; temperature ramp to 160°C at 40°C/min held for one minute. The transfer line from GC to MS was held at 280°C and MS source temperature was 230°C with an electron ionisation of 70 eV. A solvent delay of two minutes was in place. Between samples the glass purging tube was thoroughly rinsed with Milli-Q water five times, and frequent checks confirmed that no cross contamination of samples occurred.

Instrument response was calibrated to analyte concentration using a DMS standard from Sigma Aldrich, which was diluted to a tertiary working standard solution using HPLC-grade methanol. Deuterated DMS (DMS-d_6) from Sigma Aldrich diluted in methanol was used as an internal standard and was spiked into all samples, blanks and calibration standards at a concentration of 655 pmol/L. Single ion monitoring mode was used to look for four ions belonging to DMS and DMS-d_6 which were determined from the NIST MS spectra database. For DMS, ions with an m/z of 62 and 47 were measured and for DMS-d_6, ions with an m/z of 68 and 50 were measured. Analyte response was normalised to internal standard response to account for small-scale variations in analyte recovery. Calibration curves were created (Appendix C.3) and the equation of the regression line was used to relate sample response to DMS concentration. The limit of detection (LOD) for these methods was 14.86 pmol/L, the limit of quantification (LOQ) was 45.03 pmol/L and percentage recovery was 101.4 % These values were calculated from the analysis of ten replicate ARW samples spiked with DMS standard to a concentration of 65.7 pmol/L and internal standard. LOD was defined as $3.3 \times \sigma/S$ and LOQ was defined as $10 \times \sigma/S$, where σ is the standard deviation of the calculated concentration of the ten samples and S is the slope of the calibration curve (Shrivastava & Gupta, 2011).

3.2.5 Statistical analysis

The successional changes of microbial biofilms are well documented in the wider literature (Eich et al., 2015; Pinto et al., 2019) and in Chapter 2 of this thesis, therefore direct statistical comparisons of

biofilm changes over time was not of interest in this study. Instead, differences between material types and locations were focused on, with endpoints (biofilm weight, pigment content and DMS production) analysed using a two-way ANOVA, or non-parametric aligned ranks ANOVA, with material type and river location as factors. Statistical analysis and figure construction was carried out in R-studio version 1.2.1335 using packages ggplot2, car and ARTool. All data were tested for normality and equal variance using a Shapiro-Wilk and Levene test. Data which did not meet these assumptions were transformed or an aligned ranks ANOVA was carried out when assumptions were still not met. Details of each test and transformation performed are given in Appendix C.4. The significance level for this study was set at 0.05.

3.3 Results

3.3.1 Surrounding water conditions

Conductivity and pH levels were similar between the two river locations. Water temperature was also relatively similar between locations, although a notable drop in the urban river was seen at six weeks - likely caused by the sensitivity of shallow urban systems to rapid temperature fluctuations from events such as precipitation (Croghan et al., 2019). In contrast, at the urban location nitrate and phosphate concentrations were considerably higher and dissolved oxygen levels were lower compared to the rural river (Table 3.1). This indicates that the shallower urban river was a more eutrophic environment than the deeper rural river. The average DMS concentration of river water from the rural location at the six-week sampling point was 577 $\pm$ 5.79 pmol/L, which was significantly lower than river water from the urban location ($t_{(4)}$ = -34.64, $p < 0.001$) where it was 2680 $\pm$ 105.02 pmol/L (mean $\pm$ SD).

Table 3.1: Environmental parameters of river water measured at each of the sampling points, $n = 3$ for each parameter at each time point. Nutrient values given represent the closest value identified on the colorimetric chart consistently given by all three water samples, or are the range of the values obtained from the three tested water samples.)

	Three weeks		**Six weeks**	
Parameter	**Rural**	**Urban**	**Rural**	**Urban**
Conductivity (µs/cm)	541	823	574	664
[Salinity (ppt)]	[0.26]	[0.40]	[0.28]	[0.32]
Temperature (°C)	16.4	18.1	16.4	13.8
pH	7.88	7.43	7.74	7.04
Dissolved oxygen (mg/L)	8.14	5.77	8.39	4.27
NO_3^- (mg/L)	$\sim$ 10.0	$\sim$ 80.0	5.0 – 10.0	$\sim$ 80.0
PO_4^{3-} (mg/L)	$\sim$ 0.25	0.5 – 1.0	0 – 0.25	0.5 – 1.0

3.3.2 Biofilm composition and characteristics

All samples at both river locations had a detectable biofilm on their surface after both three and six weeks of submersion. The amount of biofilm attached to materials varied considerably between locations, ranging between 0.007 – 1.2 mg/cm^2 and 0.165 – 1.3 mg/cm^2 after three and six weeks of submersion respectively (Figure 3.2 A). Example images of these samples and the attached biofilm can be seen in Appendix C.5. After three weeks a significant interaction effect between material and location was present ($F_{2,29} = 8.71$, $p = 0.001$) with glass possessing the lowest biofilm weight in rural samples (0.70 mg/cm^2) but the highest amongst urban samples (0.01 mg/cm^2). A significant and dramatic difference between biofilm weight was present between materials from different locations, with biofilm weight on rural materials around two orders of magnitude higher than on urban materials after three weeks of submersion. Interestingly, after six weeks, no significant effect of material or interaction effect was present ($F_{2,30} < 1.8$, $p > 0.05$) but, similarly to week three, there was a strong and significant effect of location ($F_{1,30} = 58.35$, $p < 0.001$) with biofilm weight on rural material around five times higher than on urban material.

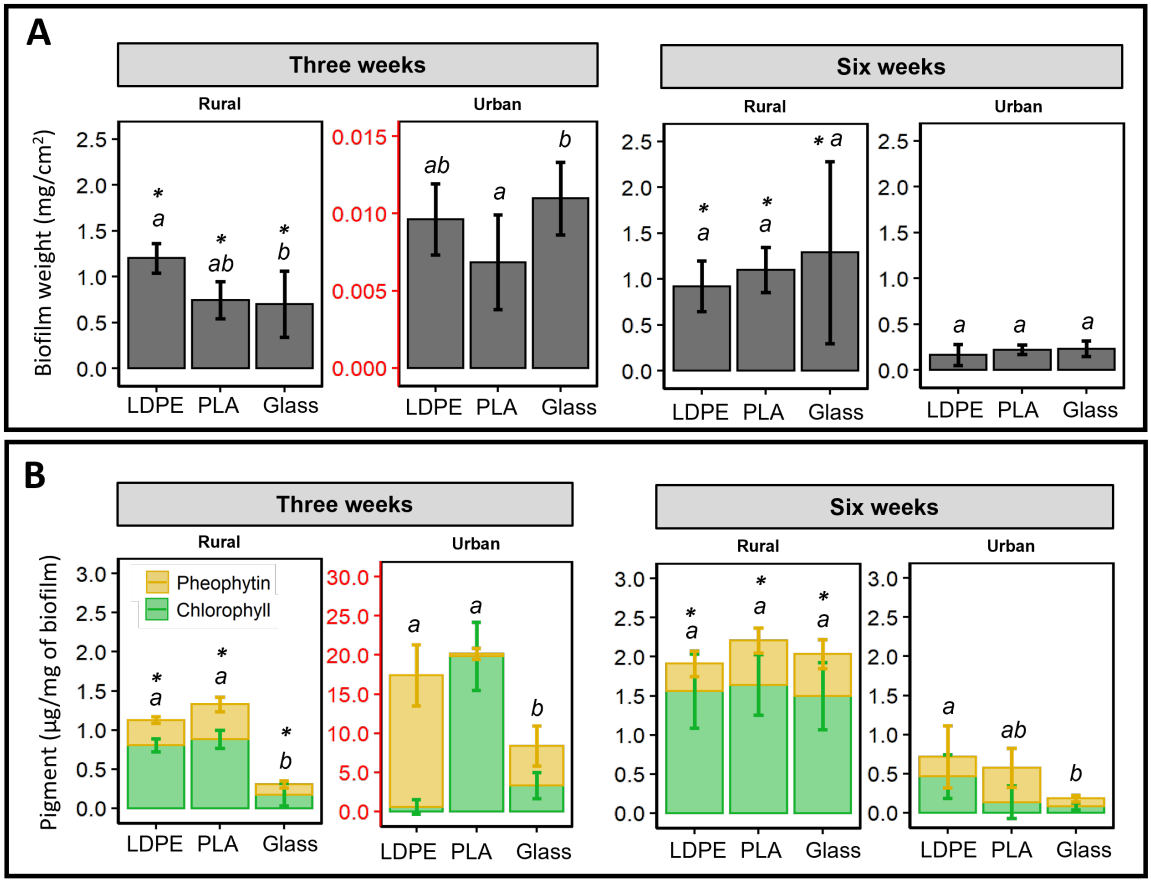


Figure 3.2: (A) Weight of biofilm attached to low-density polyethylene (LDPE), polylactic acid (PLA) or glass, expressed as mg/cm^2 of material. (B) Photosynthetic pigment content (chlorophyll *a* and pheophytin) normalised to biofilm weight, expressed as µg/mg. All values are the mean ± standard deviation. For both plots: note the scale of axes on week-3 urban plots (highlighted with red axes). Letters above bars indicate significant differences between materials within each individual subplot only, asterisk (*) above bars in rural plots indicates that the amount of DMS produced by these materials differed significantly from the amount produced by the same material in the urban location after the same incubation time. Letters and asterisks on plot (B) indicate statistical analysis results for the total pigment content (chlorophyll *a* + pheophytin).

To better understand biofilm composition, the total photosynthetic pigment content of biofilms (chlorophyll *a* + pheophytin) was normalised to the average weight of attached biofilms for each treatment condition (ug/mg.biofilm). Similar to biofilm weight, pigment content varied across both material type and location (Figure 3.2 B), with a significant interaction effect between material and location present after three ($F_{2,30} = 13.58$, $p < 0.001$) and six weeks ($F_{2,10} = 4.28$, $p = 0.023$). After three weeks, the pigment content of glass was significantly lower than LDPE and PLA at both locations ($p < 0.01$ for all), however, the scale of difference varied with plastic possessing around four and two times more pigment than glass at rural and urban locations respectively. The pigment content of all urban materials after three weeks was around an order of magnitude higher than for rural materials, with significant differences across locations present for all ($p < 0.001$). After six weeks, whilst the pigment content between rural materials (1.9 – 2.2 µg/mg) did not differ significantly ($p > 0.05$ for all), for urban materials, glass had significantly less pigment than LDPE ($p = 0.002$), but not PLA ($p = 0.09$). In contrast to week three, the pigment content of all urban materials (0.18 – 0.72 µg/mg) was significantly lower than rural materials ($p < 0.05$ for all).

Pheophytin is a breakdown product of chlorophyll and can therefore indicate the health of a photosynthetic community. Therefore, the proportion of total pigment that was pheophytin was also investigated (Figure 3.2B and Appendix C.6). A significant interaction effect between material and location was found after three ($F_{2,30} = 118.40$, $p < 0.001$) and six ($F_{2,30} = 4.36$, $p = 0.02$) weeks of incubation. After three weeks, rural glass had a significantly higher proportion of pheophytin compared to rural LDPE ($p < 0.001$) and urban materials all differed significantly from each other ($p < 0.05$ for all) with PLA possessing a considerably low pheophytin proportion. Between locations LDPE and PLA differed significantly ($p < 0.001$ for both), whereas glass did not. After six weeks, rural material had an average pheophytin content of 19 – 26 % with no significant variation between materials ($p > 0.05$ for all). Amongst urban materials LDPE had a significantly lower pheophytin content than PLA and glass ($p < 0.05$ for both), however the standard deviation for LDPE and PLA was remarkably high. Between locations pheophytin proportion did not differ significantly for any materials after six weeks.

3.3.3 DMS production

All materials at both locations consistently produced a detectable DMS signature after three and six weeks of submersion, with an average DMS production between 0.14 – 1.46 pmol per cm^2 of surface area (Figure 3.3). No DMS production from virgin materials or blank artificial river water (ARW) samples was detected, confirming that DMS was produced from the biofilms associated with samples. After three weeks of incubation, a significant interaction effect between material type and location was found ($F_{2,15} = 5.47$, $p = 0.017$), with LDPE producing significantly more DMS than glass at the rural location ($p = 0.005$) but no significant differences between materials at the urban location ($p > 0.05$ for all). DMS production by glass at the rural site (0.39 pmol/cm^2) did not significantly differ from glass at the urban site after three weeks (0.46 pmol/cm^2) ($p = 0.059$), whereas significant differences were present for LDPE and PLA ($p < 0.05$ for both) with DMS production being higher for rural materials. After six weeks of submersion similar patterns in DMS production were seen at both locations, with a significant effect of material ($F_{2,17} = 39.93$, $p < 0.001$) and location ($F_{1,17} = 14.046$, $p = 0.002$) but no interaction effect. LDPE and PLA produced significantly more DMS than glass at both locations ($p < 0.01$ for all) and rural materials produced between 1.2 and 2.9 times more DMS that the same material from the urban location.

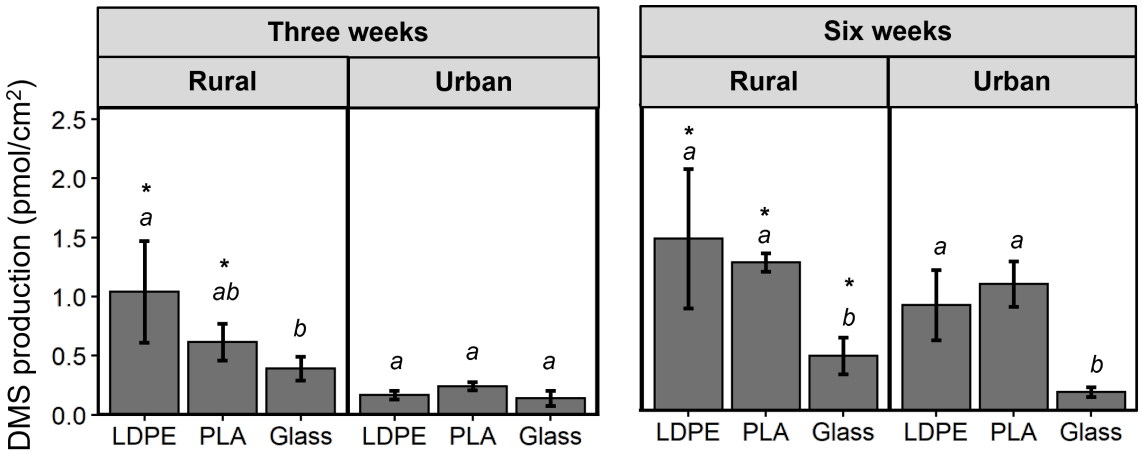


Figure 3.3: Dimethyl sulfide (DMS) produced per cm^2 of low-density polyethylene (LDPE), polylactic acid (PLA) and glass material, after they were incubated in a rural and urban river for either three or six weeks (mean $\pm$ S.D). Week three and week six data were analysed separately. Letters above bars indicate significant differences between materials within each individual subplot only, asterisk (*) above bars in rural plots indicates that the amount of DMS produced by these materials differed significantly from the amount produced by the same material in the urban location after the same incubation time.

To further investigate differences in biofilm composition, DMS production was normalised to the average biofilm weight for each treatment and expressed in pmol/mg.biofilm (Figure 3.4). This analysis also identified considerable differences across material type and locations. After three weeks, there was an overall significant interaction between material and location ($F_{2,15} = 10.67$, $p = 0.001$). Whilst rural materials produced an average of 0.83 – 1.58 pmol/mg and did not differ significantly from each other ($p > 0.05$ for all), amongst urban materials, the normalised DMS production of PLA (35.38 pmol/mg) was significantly higher than LDPE (17.44 pmol/mg) and glass (12.94 pmol/mg) ($p < 0.001$ for all). Notably, DMS production of all urban materials was around an order of magnitude higher than samples from the rural river, with significant difference across locations for all materials ($p < 0.001$ for all). A significant interaction between material and location was also present after six weeks ($F_{2,17} = 9.68$, $p = 0.002$). Normalised DMS production was significantly lower for glass compared to LDPE amongst rural samples ($p = 0.008$), and significantly lower than LDPE and PLA amongst urban samples ($p < 0.001$ for both). Furthermore, differences between plastics and glass were considerably larger for urban material, where plastics produced around seven times more DMS/mg than glass. Similar to week three, the normalised DMS production of LDPE and PLA from the urban location was significantly higher compared to materials at the rural location ($p < 0.001$ for both). However, no significant differences were present between locations for glass ($p = 0.70$).

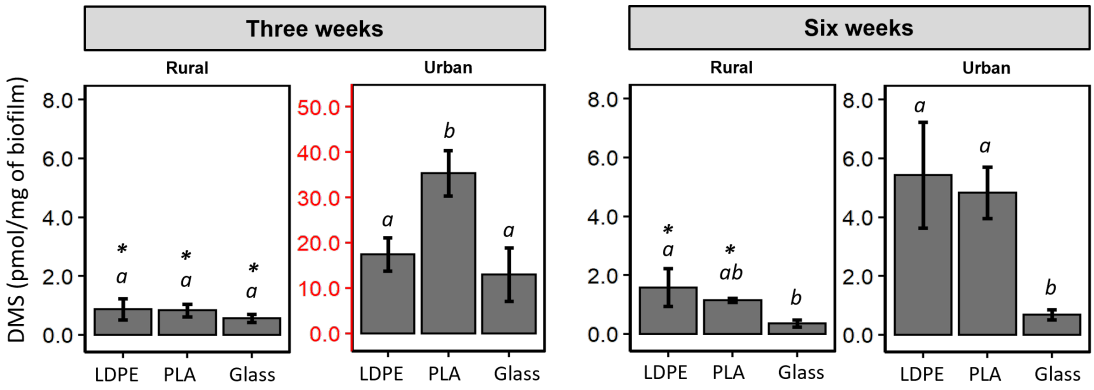


Figure 3.4: Dimethyl sulfide (DMS) production normalised to average biofilm weight for each treatment condition, expressed as pmol/mg (mean ± S.D). Note the scale of axes on week-3 urban plots (highlighted with red axes). Letters above bars indicate significant differences between materials within each individual subplot only, asterisk (*) above bars in rural plots indicates that the amount of DMS produced by these materials differed significantly from the amount produced by the same material in the urban location after the same incubation time.

3.4 Discussion

The odorous compound DMS is an important foraging cue in the marine environment (Savoca & Nevitt, 2014); it has been shown to be produced by microbially colonised plastic from marine systems (Savoca et al., 2016) and is known to increase the ingestion of microplastics in some species (Botterell et al., 2020; Procter et al., 2019). However, despite previous studies also demonstrating the sensitivity of some freshwater species to the compound (Nakajima et al., 1989) little is known about the DMS production by plastic pollution in freshwater. Furthermore, the only previous study to address this (Zink & Pyle, 2019) did not detect the presence of DMS on plastics from freshwater. In contrast, using specialised and sensitive methodology, this study demonstrates for the first time that plastic pollution can consistently acquire a distinct DMS signature after submersion in two different types of UK rivers.

3.4.1 Material and location differences in DMS production and biofilm composition

In this study, all plastic and glass materials acquired an evident biofilm layer over their surface after three and six weeks of submersion in rivers. Community members of this biofilm were consistently found to produce the odorous compound DMS, leading to acquisition of a clear DMS signature by materials. The hypothesis for this experimental work can therefore be accepted. For all but one treatment (urban, 3 weeks) plastic films produced significantly more DMS (per cm^2 of material) than glass under the same conditions. The DMS signature also differed between the same material at different river locations, indicating that the development of a DMS signature by materials in freshwater is influenced by multiple factors. After three weeks in the rural river, variation in the DMS signature between materials was accompanied by similar differences in biofilm weight, whilst normalised DMS production (pmol per mg of biofilm) did not differ. This suggests that under these conditions most variation in the DMS signature was driven simply by the amount of biofilm attached to samples, which is in line with previous studies which have noted that differences in substrate properties can mediate the amount of biofilm which accumulates (Li et al., 2019; Miao et al., 2020). For all other treatments, normalised DMS production showed significant variation between materials in the same treatment. Of particular note, was the differences seen between materials submerged in the urban river for six weeks, for which the weight of biofilms differed very little between materials, whilst the DMS signature and normalised DMS production was significantly and substantially lower for glass than for LDPE and PLA. Furthermore, excluding glass after six weeks, there were significant differences between the normalised DMS production of materials at rural and urban locations. These findings suggest that the taxonomic composition and metabolic functionality of biofilms differed between material type and location, and there is also evidence that river location influenced the relative differences between materials under each treatment. These results are in keeping with previous work which has found plastic biofilm composition to be shaped by surrounding water conditions (Nguyen et al., 2022; Yang et al., 2021a), as well as studies which have found distinct differences in community functioning between plastic and natural surfaces within freshwater systems (Hu et al., 2021). The capacity for the surrounding water conditions to influence the ability of plastic-specific biofilms to form have also been previously noted (Oberbeckmann et al.,

2018). Interestingly, despite normalised DMS production being higher for urban materials compared to rural ones, their DMS production per cm^2 of material was lower. Given that rural materials also had a higher biofilm weight, the degree of DMS signature acquired by materials in this study was likely to have been driven by both the amount and the composition of the associated biofilms.

The significant variability observed in the photosynthetic pigment content of biofilms further supports the notion of differences in community composition between materials and locations. Besides materials after six weeks in the rural river, the total pigment content of biofilms on LDPE and PLA was consistently higher than on glass, and may indicate different levels of heterotrophy (Hoellein et al., 2014). Whilst differences have previously been observed in the photosynthetic capacity between glass and plastic biofilms (e.g. Hoellein et al., 2014), these findings are in direct contrast to those in Chapter 2 of this thesis, where glass was found to consistently possess the highest photosynthetic pigment content. The pigment content of biofilms also differed between the same materials at different locations for all treatments.

Whilst the existence of a true plastic-specific microbiome remains controversial, interactions between substrate properties, such as roughness and hydrophobicity, with surrounding environmental conditions, such as nutrient concentration and temperature, are likely to determine biofilm composition and substrate specificity (Chapter 1). In the present study, conditions of the urban river were more eutrophic than in the rural location, with higher nutrients and lower dissolved oxygen, and are therefore likely to have contributed to the differences in biofilm composition and metabolic functioning observed. Interestingly though, normalised DMS production and pigment content (indicative of biofilm composition) differed most between materials in the urban river, which is in contrast with findings from Oberbeckmann et al. (2018) who report the largest differences between materials under conditions with lower nutrient levels. However, many other factors are likely to shape these outcomes, and this further highlights the complexity of plastic-associated biofilms in freshwater systems. As well as influencing biofilm composition, the trophic status of the river may have also driven the amount biofilm which attached to materials. The lower biofilm weight seen on urban samples is in keeping with previous studies which found a significantly higher biofilm mass on microplastics in oligo-trophic freshwater lakes compared to eutrophic ones (Arias-Andres et al., 2018b). Furthermore, this appears

to be a known phenomenon, whereby lower nutrient concentrations trigger substrate attachment and biofilm formation in microorganisms and higher ambient nutrient levels reduce this drive with more cells remaining planktonic (Du et al., 2022; Stanley & Lazazzera, 2004). It should however be noted, that whilst the trophic conditions of the rivers are likely to have driven many biofilm differences, some variation could have been due to the light levels under which the samples were incubated, which were higher at the rural location. Nevertheless, given that the photosynthetic pigment content of urban biofilms was much higher than rural ones after three weeks, the role of light is probably negligible.

As well as surrounding water conditions, the biofilm differences observed may have additionally been driven by river depth and the proximity of samples to the river sediment. Although all samples were submerged just below the water surface, samples in the rural river were sitting above around 3 meters of water, whereas samples in the urban river were in $<$ 1 meter of water. High levels of DMS production are known to take place in freshwater sediments, with the DMS concentration of overlying water decreasing with increasing distance from the sediment (Lomans et al., 1997). Given that a recent study found that the core bacterial microbiome of microplastics was most strongly derived from the core microbiome of nearby sediment (Zhang et al., 2022b), there may have been some connectivity between DMS producing species in the river sediment and those which colonised experimental materials, contributing to the differences in biofilm composition and DMS production between locations. This may also explain the large difference in the DMS concentration observed in water samples from both locations.

Overall, the normalised DMS production and pigment content of biofilms observed in this study further demonstrates the importance of surrounding water and location conditions in shaping the plastic biofilm composition and functionality, as well as the occurrence of distinct plastic-specific communities.

3.4.2 Drivers of DMS production within the biofilm

Although several common freshwater photosynthetic species are known to produce DMS (Bechard & Rayburn, 1979; Steinke et al., 2018), the normalised DMS production of biofilms in this study was

not consistently and convincingly explained by either their total pigment content or their pheophytin proportion. For example, after three weeks, normalised DMS production did not differ significantly between rural materials, whereas total pigment content and pheophytin percentage did. Conversely, after six weeks in the rural river, normalised DMS differed significantly between materials, but total pigment content and pheophytin precentage did not. Similarly, there was also no discernible links between normalised DMS production and pigment for materials submerged in the urban river for three weeks. On the other hand, after six weeks in the urban river, differences in normalised DMS values between materials were mirrored by the total pigment content, and, although highly variable, LDPE samples possessed both a significantly higher normalised DMS and a significantly lower pheophytin percentage. Comparisons between locations also show a similarly unclear pattern; after three weeks urban materials had a higher normalised DMS production and total pigment content than rural ones, however, after six weeks urban materials unexpectedly showed a higher normalised DMS production but a lower total pigment content. Therefore, despite previous studies finding a correlation between DMS production and chlorophyll *a* concentration in freshwater (Steinke et al., 2018), the present data suggest that a mixture of DMS-production pathways were likely to have occurred in this study, and that whilst photosynthetic organisms may have played a role, they were unlikely to have been the only driver. Instead, bacteria communities may have produced the majority of DMS observed, with taxa such as *Hyphomicrobium*, *Pirellula* and *Novosphingobium*, which were found within biofilms in Chapter 2, potentially being responsible. Putative DMS-producing bacteria have also been found within other biofilms found on plastic in freshwater (González-Pleiter et al., 2021; Huang et al., 2022), adding further evidence to this hypothesis. Interestingly though, despite DMS production being higher on plastics in the present study, in Chapter 2 the relative abundance of the three putative DMS-producing bacteria was generally higher on glass surfaces than on LDPE or PLA, or showed little differences (Appendix B.13). Given that materials were incubated in a different river location in Chapter 2 this provides further indications for the need to link location conditions with biofilm composition and functionality. Grazing invertebrates such as nematodes and chironomid larvae were also identified in plastic and glass biofilms in Chapter 2, and tube structures thought to belong to chironomid larvae (Brennan & McLachlan, 1979; Caudata-Culture, 2008) were similarly observed on samples in the present study (Appaendix C.5). Given that these types of invertebrates are thought

to influence the release of metabolites from the biofilm matrix through physical disturbance (Gaudes et al., 2006), their presence may have enhanced the flux of DMS from these samples, highlighting the role and importance of invertebrates within the biofilm.

3.4.3 Wider implications of DMS production by plastic pollution in freshwater

Whilst many freshwater species are known to interact with plastic pollution, reasons for these interactions often remain unclear. For example, a specific preference for anthropogenic litter compared to natural substates by freshwater invertebrates has been demonstrated by Wilson et al. (2021), who found that *Limnophora* spp. and *Bathyomphalus contortus* were exclusively associated with flexible fabric and plastic in UK urban rivers. However, reasons for these preferences could not be discerned in the study. Other recent work has demonstrated the increased ingestion of microbially colonised plastic by *Daphnia magna* compared to virgin plastic, with the specific factors which drove this response remaining unknown (Polhill et al., 2022). Understanding the processes which drive the attraction to, and ingestion of plastic is an important step in making accurate risk assessments of plastic pollution in the environment. It may also help to identify certain types of plastic or areas that could be particularly high risk, based on the composition of biofilms which form. Furthermore, a better understanding of these attraction processes can help to increase the environmental relevance of laboratory-based ecotoxicological studies.

DMS is a known key foraging cue in marine systems, and can increase the ingestion of microplastics by certain marine species (Botterell et al., 2020; Procter et al., 2019; Savoca & Nevitt, 2014). Furthermore, although the sensitivity of freshwater species to DMS is largely unexplored, DMS has been shown to increase feeding behaviour responses in three species of freshwater fish and stimulate the olfactory nerve response of one species (Nakajima et al., 1989). However, a DMS concentration of 1×10^3 pmol/L was used to test this olfactory response and a concentration of 1×10^9 pmol/L was used to test feeding responses, therefore, the sensitivity of these fish species to DMS concentrations produced by plastic in the present study remains unclear. Nevertheless, given its importance in marine systems and the evidence of detection by some freshwater species, the production of DMS by

plastic-associated biofilms certaintly has the potential to influence interactions between plastic litter and larger organisms within UK rivers. Furthermore, after only six weeks of submersion, LDPE and PLA films produced significantly more DMS per cm^2 of material compared to glass, and plastic litter may therefore host biofilms which particularly enhance interactions between organisms and plastics, compared to biofilms on other surrounding surfaces.

Although the DMS concentrations produced by plastics in this study (0.24 - 1.16 nmol/gram.plastic) were considerably lower than those previously found for marine plastics (9.7 - 450 nmol/gram.plastic) (Savoca et al., 2016), the methodology used in the present study quantified DMS production from living biofilms, and is therefore likely to closely reflect the flux of DMS occurring from plastics within the environment. In comparison, the study of marine plastic involved freezing samples before analysis, which can increase cell lysis and may artificially enhance DMS flux. Furthermore, the lower levels of DMS found within freshwater generally may mean that freshwater organisms possess a higher sensitivity to the compound and are able to detect it at lower concentrations. If freshwater taxa do detect and respond to DMS production from plastic litter, the overall effects of this are also likely to be dependent on DMS concentration of the surrounding water. For example, in areas with higher DMS levels, such as the urban river in the present study, the DMS signal from plastic may not contrast strongly against the background signal. Whereas in water with lower DMS levels, plastic may act as a hotspot for DMS production, enhancing the attraction of organisms. There is also likely to be a temporal element to this, which should further be considered. Additionally, DMS is also only one of a multitude of different VOCs that are produced by freshwater microorganisms. For example, geosmin and 2-methylisoborneol are produced widely by freshwater algae and bacteria, sometimes in concentrations high enough to interfere with the odour and taste of drinking water (Lee et al., 2017). Work to understand the ability of freshwater organisms to detect and respond to environmentally relevant levels of DMS, and its relative importance compared to other VOCs, is therefore now needed in order to elucidate the role of DMS in mediating interactions between plastic and freshwater species.

3.4.4 Conclusions

The findings of this study demonstrate, for the first time, that the formation of microbial biofilms on plastic surfaces can result in the acquisition of a distinct DMS signature by plastic pollution in UK rivers, which could have implications for the attraction of freshwater organisms to plastic litter. Materials in this study consistently acquired a DMS signature in an urban and a rural river after both three and six weeks of submersion, indicating that this could be occurring across freshwater environments widely. Furthermore, material type and river conditions appeared to interact to influence the composition and DMS production capacity of these plastic associated biofilms. The DMS signal acquired by plastic was found to be significantly stronger than non-plastic surfaces under most conditions, and may therefore result in an increased attraction of aquatic organisms to plastic, further enhancing its impacts within the environment. The trophic status and water depth of river locations was also thought to mediate the strength of DMS signature acquired by materials by influencing the composition and amount of biofilm which formed. Materials in the deeper and less eutrophic rural river showed a lower DMS production per unit weight of biofilm, but a larger biofilm weight and stronger overall DMS signature per cm^2 of material. Location conditions also influenced the relative differences observed in DMS production between materials in each river.

Whilst the production of DMS by biofilms in this study may have been partially driven by phototrophic organisms, such as cyanobacteria and green algae, the data suggests that heterotrophic organisms, such as bacteria, played a more major role. Further studies which combine the measurement of DMS production with taxonomic characterisation and identification of DMS producing genes would be useful to determine the main producers of DMS in plastic-associated biofilms. Overall, it is clear that DMS signature acquisition by plastic in freshwater is highly dynamic and the strength of the attained signal is context dependent. More plastic types, water conditions and temporal factors should therefore be investigated to identify further influences on biofilm DMS production, and to determine which combinations of material and water conditions result in the strongest signal. The sensitivity of freshwater organisms to DMS also remains largely unknown, and further work to determine this for environmentally relevant DMS concentrations is therefore required in order

to understand the potential ecological impacts of this DMS production by microbially colonised plastic. The relative importance of DMS compared to other VOCs emitted by biofilms should also be examined. Finally, to enhance our knowledge of the wider relative risks of plastic pollution within natural ecosystems the overall influence of biofilms on the attraction of freshwater taxa to plastic in the environment is urgently needed. Therefore, in the next chapter of this thesis, the interactions between microbially-colonised plastic and benthic invertebrates are explored under environmentally relevant conditions.

Chapter 4

Interactions between plastic, microbial biofilms and *Gammarus pulex*: An initial investigation

4.1 Introduction

The presence of a complex multi-species biofilm on the surface of plastics (Zettler et al., 2013) is one factor known to stimulate interactions and uptake of plastics by a variety of marine species (Corona et al., 2020; Fabra et al., 2021; Joppien et al., 2022; Sucharitakul et al., 2021; Vroom et al., 2017; Weideman et al., 2020) and may therefore enhance the toxicological impacts of plastic exposure for organisms. An attraction to microbially colonised plastic may also distract organisms from their natural food, for example, the sea urchin *Paracentrotus lividus* actively chewed and ingested biofouled plastic even when natural food was replete (Porter et al., 2019), and could therefore have longer-term implications for organism energy budgets. The influence of plastic biofilms on organism interactions is thought to be partly driven by odorous metabolites produced by microbial members of the biofilm - which can modify the chemosensory signature of plastic (Fabra et al., 2021; Savoca et al., 2016). For example, the foraging infochemical dimethyl sulfide (DMS) is known to be produced by marine

plastic-associated biofilms (Savoca et al., 2016) and can stimulate plastic uptake by marine copepods and lobster larvae (Botterell et al., 2020; Procter et al., 2019).

Although the attraction to plastic biofilms is seen in a variety of marine species, similar studies are scarce within freshwater systems, with only two studies to date exploring this, both of which focused on filter feeding species. In these studies, freshwater zooplankton (including copepods, diplostraca and rotifers) showed a significant preference and uptake of microbially colonised microplastic beads (DeMott, 1986; Polhill et al., 2022), demonstrating the attractivity and palatability of plastic biofilms to freshwater organisms. Chapter 3 of this thesis evidenced the ability of plastic-associated biofilms, formed within different UK rivers, to produce the odorous compound DMS. Furthermore, DMS production from these plastic biofilms was significantly higher than from biofilms on non-plastic surfaces under most conditions, indicating the potential for plastic to act as a hotspot for DMS production within rivers. Findings from this thesis so far, taken together with previous literature and the known ability for some freshwater species to detect DMS (Nakajima et al., 1989) indicate the urgent need to explore the overall risk of microbially colonised plastic in the environment, along with the ability of biofilms to influence specific interactions between plastics and freshwater organisms. Given that biofilm formation can induce the sinking of plastics in freshwater (Jalón-Rojas et al., 2022; Semcesen & Wells, 2021), the probability that benthic organisms will encounter plastic is considerable, and interactions between benthic freshwater organisms and microbially colonised plastic is therefore of particular interest and concern.

Striking interactions between plastic films and the common benthic marine amphipod *Orchestia gammarellus* have previously been documented (Hodgson et al., 2018). In this study amphipods readily fragmented 1 cm^2 pieces of polyethylene and biodegradable films using their mandibles; this resulted in considerable microplastic creation and organisms demonstrated significantly higher amounts of shredding on microbially colonised films than those which were clean. However, a choice of other natural food was not present in any treatments in this study, and the ability of microbially colonised plastic to induce this behaviour under environmentally relevant conditions is therefore unclear. Despite the fragmentation of plastics being reported in several marine and terrestrial species, knowledge of the occurrence of this type of behaviour in freshwater taxa is severely lacking (So et al.,

2022). In freshwater environments, benthic amphipods in the *Gammarus* genus have been found to significantly preferentially associate with plastic litter over other anthropogenic materials and natural surfaces (Wilson et al., 2021). They have also been found to associate with biodegradable films within small streams, and, whilst no data or methodological details are available, there have been suggestions that *Gammarus* species may chew on the edges of plastic films in a similar manner to *O. gammarellus* under mesocosm conditions (Artru & Lecerf, 2019). *Gammarus* are shredding detritivores; they use their toothed mandibles to shred and consume food such as leaves and other plant material (Mateos-Cárdenas et al., 2020) and show a strong dietary preference for material which has a microbial biofilm on its surface (Bärlocher & Kendrick, 1975; Bloor, 2011). It is even thought that *Gammarus* sp. may feed on plant material only as a means to access the nutritional microorganisms on its surface (Nelson, 2011) and in behaviour experiments they have demonstrated a clear attraction to the chemosensory signature of microbial biofilms (Lange et al., 2005).

The aims of this experimental study were therefore, firstly, to evaluate whether microbially colonised plastic is attractive to freshwater *Gammarus* amphipods under environmentally relevant conditions (when other natural food is available), and understand if plastic biofilms can act as a distraction or disruption to the normal feeding behaviour of the organism. The second aim was to determine whether *Gammarus* will biofragment plastic films in a similar way to marine amphipods, and if this behaviour is influenced by the presence of a microbial biofilm on plastic surfaces.

This chapter partly addresses the first and second broad hypotheses of this thesis, and based on the existing information in the literature the two sub-hypotheses were formed: i) *'Microbially colonised plastic will be attractive to Gammarus sp. and distract organisms from their normal feeding activity'*. ii) *'Gammarus sp. will fragment plastic films in a similar manner to marine amphipods when no other food is present, and fragmentation will be higher for microbially colonised plastics'*. *Gammarus pulex* was chosen as the model species for this study as it is widespread in rivers across Europe and is a common model organism for ecotoxicology studies (Weber et al., 2018).

4.2 Methods and Materials

4.2.1 Plastic materials and biofilm formation

White low-density polyethylene (LDPE) and poly lactic acid (PLA) films, derived from commercially available bags were used in this experiment. LDPE film was 45 µm thick and cut from plastic sold as carrier bags. PLA film was 40 µm thick and cut from plastic sold to hold food items. The FTIR spectra of these materials was obtained using a Nicolet iS10 spectrometer (Appendix D.1) and used to confirm their polymer identity. To prepare plastic for microbial colonisation, sheets of plastic were cut and attached inside a 240 x 240 x 130mm custom-built stainless-steel woven mesh (0.57 mm aperture) cage. Healthy *Acer pseudoplatanus* leaves were obtained from trees in a semi-rural location (53°47'01.6"N 1°21'59.4"W) and air-dried for three weeks before being attached inside cages with the plastic. Cages were placed in the surface water of the River Ouse upstream of York city centre, UK, in the same location as rural materials from Chapter 3 (54°00'30.7"N 1°11'28.7"W). After three weeks, the cages were removed from the river, plastic and leaves were rinsed with milli-Q water and cut into 3 cm diameter discs. During these three weeks, virgin discs of LDPE and PLA of the same size were soaked in sterile milli-Q water in the dark at 15°C in order to control for any changes that may have occurred to the plastic due to water absorption. Five discs from each of the treatments were used to quantify the weight of biofilms attached to the plastic and three discs from each treatment were imaged under a microscope to visualize the plastic surface and biofilm – details of these methods are given in Appendix D.2.

4.2.2 Test organisms

G. pulex were collected from a small stream in Bishop Wilton, UK (53°59'07.9"N 0°47'08.6"W) using a kick sampling method and their identity was confirmed with an taxonomic key (Dobson, 2012). They were transported back to the laboratory and maintained at 15°C under a 12:12 diurnal cycle in an aerated 50 L glass acclimation tank containing river water for at least one week prior to experimental testing. During this time, they were fed *ad libitum* with commercially available Tetra®

crustacean food. The river water was collected from the same site in the River Ouse where cages were placed; this water was used unfiltered for the acclimation, but before its use in experiments was freshly collected and filtered to 0.7 µm with Whatman glass fibre filters.

4.2.3 Experimental design

For this study two separate feeding experiments and one short-term behaviour experiment were conducted. For all experiments, amphipods were a mixed population of males and non-egg-bearing females larger than 10 mm in length and were starved for 48 hours prior to the start of all experiments.

For the two feeding experiments each organism was placed in an individual glass jar containing 150 ml of filtered river water and maintained under the same temperature and light conditions as during acclimation. Jars were aerated with a glass pipette for 15 minutes each day to maintain a high dissolved oxygen concentration in the water. The first feeding experiment was designed to determine the ability of microbially colonised plastic to act as a distraction and influence the natural feeding behaviour of *G. pulex*. For this experiment there were three treatment groups, with amphipods given either: a single leaf disc; a leaf disc and a colonised LDPE disc; a leaf disc and a colonised PLA disc ($n = 9$ for each group). Ten control jars containing one leaf disc but no amphipod were also run in parallel. The second feeding experiment addressed the ability and inclination of *G. pulex* to fragment plastic films when no other food was available and evaluated the role of the microbial biofilm in this behaviour. To do this, one plastic disc was placed in a jar with one amphipod for five days. This experiment consisted of four treatment groups: LDPE colonised; LDPE virgin; PLA colonised and PLA virgin ($n = 10$ for each group). Ten control jars for each treatment were also set up with the same conditions but without *G. pulex*. Both virgin and colonised LDPE material were positively buoyant and therefore remained at the air-water interface. All PLA material was negatively buoyant and sat at the bottom of the jar. At the end of both feeding experiments plastic and leaf discs were removed, rinsed, and stored at -80°C until they were imaged to quantify their surface area. Plastic discs were scanned using an Epson ET-2720 scanner and leaf discs were imaged using an Olympus TG-5 camera. The surface area of plastic and leaf discs was then calculated using ImageJ version

1.53a using the thresholding tool. Plastic discs were examined under a stereo microscope to look for bite marks or other visual evidence of plastic shredding.

The behaviour experiment was performed to understand the relative short-term attraction of *G. pulex* to microbially colonised plastic when natural food was present in the environment. To do this, a single amphipod was transferred to a 10.5 cm diameter low form cylindrical glass beaker filled with 600 ml of aerated river water, placed in a 15°C environmental cabinet under dim light and allowed to acclimate for one hour. After this, one plastic and one leaf square (1 x 1 cm), held with a metal clip, were placed at either end of the glass beaker around 3 cm apart and the behaviour of the amphipod was video recorded for fifteen minutes with an iPhone 6 camera set around 30 cm above the beaker. Treatment groups for this experiment consisted of: leaf and colonised LDPE; leaf and colonised PLA; a control treatment which consisted of one leaf square and an empty clip holding no material (n = 9 for each treatment). The amount of time that amphipods spent on each material type and the number of visits they made to each material was determined. The time that amphipods spent not in contact with any material was also recorded and is referred to as 'swimming time'.

4.2.4 Statistical analysis

Statistical analysis and plot construction were carried out in R studio Version 1.2.1335. All data was examined for normality and homogeneity of variances with either parametric or non-parametric tests carried out based on the outcome. The surface areas of plastic discs were analysed using a Welch's t-test, leaf disc areas were compared using a Kruskal-Wallis test followed by a post hoc Wilcoxon test with a Benjamini-Hochberg adjustment, and the time and number of leaf visits in the behaviour experiment were analysed using either a Kruskal-Wallis test or a Wilcoxon signed rank test. Further details of each test and the justifications for their use are given in Appendix D.3. The significance level was set at 0.05.

4.3 Results and Discussion

The average weight of biofilm attached to LDPE and PLA material was found to be 120 ± 44.72 and 73.3 ± 22.36 µg cm^{-2} (mean ± S.D) respectively. Imaging under the microscope clearly showed the presence of a biofilm compared to controls, with organisms such as diatoms and green algae attached to the surface (Appendix D.4 and D.5). It should also be noted that, as with materials in Chapter 2 and 3, no changes to the structural integrity of PLA or LDPE films were observed and their was no evidence of PLA biodegradation within the river.

4.3.1 Attraction of G. pulex to microbially colonised plastic and the influence of natural food

In the behaviour experiment there was a substantial amount of variability in the response of amphipods between replicates (Figure 4.1). Between the three treatment groups (leaf only, LDPE *vs.* leaf, and PLA *vs.* leaf) no significant differences were found in the time that amphipods spent in contact with leaf material ($p = 0.335$), with an average (mean ± S.D) of 455 ± 374, 513 ± 439 and 679 ± 295 seconds spent on leaf squares for leaf-only, LDPE-choice and PLA-choice treatments respectively. The average number of leaf visits also did not differ significantly between these groups ($p = 0.792$), or the amount of time spent swimming ($p = 0.284$). For treatments where colonised plastic was presented alongside the leaf, the contact of organisms with plastic squares showed similarly high variation between replicates, with an average (mean ± S.D) of 30 ± 88 seconds and 12 ± 20 seconds contact time for LDPE and PLA treatments and no significant difference found between the time spent on each plastic type ($p = 0.379$) or the number of visits to each plastic type ($p = 0.543$).

In the first feeding experiment, where natural food was present, there was a visually obvious consumption of leaf discs by amphipods for all treatment groups where *G. pulex* was present, and no noticeable changes for the control group (Figure 4.2). These observations were reflected in the surface area measurements, with a significant difference in disc area found between groups ($p < 0.001$). Post-hoc statistical comparisons showed that the larger surface area of control discs (786

$\pm$ 24 mm^2) (mean $\pm$ S.D) differed significantly from the leaf-only treatment (672 $\pm$ 53 mm^2), the leaf *vs.* LDPE treatment (696 $\pm$ 36 mm^2) and the leaf *vs.* PLA treatment (667 $\pm$ 87 mm^2) with all p values < 0.001. There were no significant differences found between leaf-only treatments and the LDPE-choice ($p = 0.556$) or PLA choice ($p = 0.743$) treatments, or between the two choice treatments ($p = 0.743$). Furthermore, there was no evidence that any plastic fragmentation had taken place in this experiment.

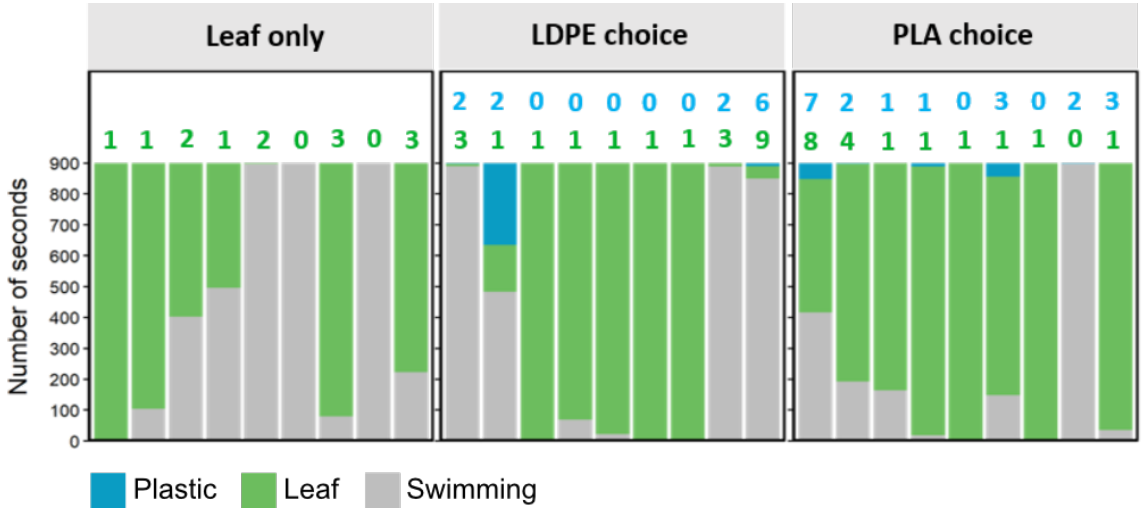


Figure 4.1: Total amount of time that *G. pulex* spent in contact with leaf, LDPE or PLA, or swimming over the fifteen-minute (900 second) observation period. Each bar represents one replicate for the given treatment. Numbers above bars indicate the number of separate visits that the amphipod made to each material (leaf visits in green, plastic visits in blue) for the given replicate.

These results demonstrate that under the conditions investigated there was no significant attraction of *G. pulex* to microbially colonised plastic when natural food was present. Additionally, organisms were not distracted by plastic biofilms and no interference with their consumption of natural food was evidenced. The first hypothesis of this study can therefore be rejected. The clear preference for leaf material seen in this study is similar to previous work with other Gammarid species, which also exhibited strong preferences for some food types over others (e.g. Pellan et al., 2016). The composition and related odours of biofilms associated with the materials may be responsible for the observed preference for leaf material. For example, *G. pulex* are known to show a strong attraction to fungi-dominated biofilms and odours, which are often in high abundance on organic plant material (Grossart et al., 2019; Lange et al., 2005). *Gammarus sp.* also show a preference for certain species

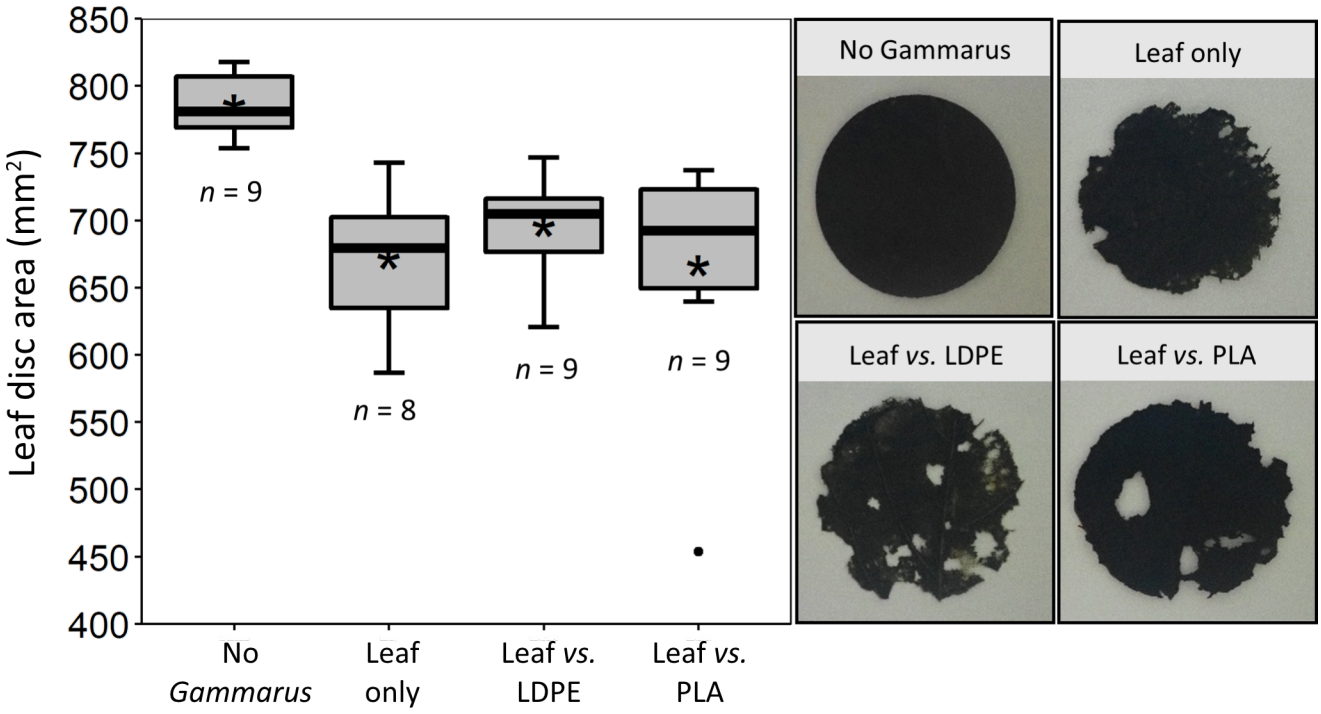


Figure 4.2: Plot on left shows the surface area of leaf disc after three days in each treatment. The 'No *Gammarus*' treatment was the control treatment to compare against other experimental treatments. Asterisks (*) show the average for each treatment, outliers are represented by black circles and the number of replicates for each treatment is shown below each box. Images on the right show examples of a typical leaf disc for each treatment after three days.

of fungi (Nelson, 2011). Given that the fungi composition has been shown to differ significantly between organic wood material and microplastics in freshwater rivers (Kettner et al., 2017), and that results from Chapter 2 of this thesis alluded to a relatively low abundance of fungal biomass on plastic films after submersion in a UK river, the microbial composition and resulting metabolites released from plastic biofilms may have simply not being attractive or chemically palatable to *G. pulex* compared to those on leaf material. Although the actual toughness values were not measured in this study, colonised leaf material was observed to be much softer, and pulled apart with little mechanical force compared to colonised LDPE and PLA, which remained physically robust when handled. It is therefore also possible that the physical toughness of the plastic also reduced its overall palatability

to the organisms. A mixture of these two effects could also be present, and further studies which present a choice of biofilm odours without allowing physical access to the material, in a similar manner to Lange et al. (2005), would help to distinguish which of these factors is the main driver of the results seen in this study.

These findings suggest that the associations observed between *Gammarus sp*. and plastic materials in rivers (Artru & Lecerf, 2019; Wilson et al., 2021) is likely to be due to the ability of these materials to provide physical shelter, rather than a food-driven attraction to the attached biofilms. They also indicate that the presence of microbially colonised plastic macro-litter in freshwater rivers is unlikely to negatively impact the health and fitness of *G. pulex*. Nevertheless, given the large variation in biofilm composition which can occur between different freshwater environments (Yang et al., 2021a), it is possible that the formation of biofilms on plastic in different locations than this study could support microbial community members which are more attractive to *G. pulex*. Furthermore, previous literature has demonstrated that plastic-biofilm-organism interactions are not clear cut even within the same species. For example, DeMott (1986) report no significant effect of biofilms on the ingestion of microplastics by *Daphnia magna*, whereas Polhill et al. (2022) demonstrated that the presence of a microbial biofilm increased microplastic ingestion in this species by up to seven times. It is well documented that *G. pulex* generally feeds by shredding and consuming the substrate and attached biofilm together, this is in contrast to organisms such as *Asellus aquaticus* which scrapes microbial biofilms off the surface of material to feed (Graça et al., 1993). The ability or tendency for *G. pulex* to feed directly on the biofilm in this way without shredding the associated substrate is unknown, and it should therefore be noted that organisms could have accessed the biofilm in this way in the study without it being documented. Although only anecdotal, individuals from four of the nine LDPE replicates and seven of the nine PLA replicates in this study did make visits to the plastic at some point during the behaviour exposure period, and in one of the LDPE-choice replicates the amphipod spent over four minutes in contact with the plastic with its feeding appendages orientated onto the plastic-biofilm surface. For one of the PLA-choice replicates the amphipod repeatedly went back and forth between the PLA and the leaf before finally settling on and feeding on the leaf. Although it remains unclear whether these interactions were indicative of food-searching behaviour or if the amphipods were simply exploring materials until they felt safe enough to begin feeding,

some interactions with the plastic biofilm were observed.

4.3.2 Ability of G. pulex to biofragment plastic and the role of the biofilm

In the second feeding experiment no significant differences were found in the surface area of plastic discs between treatment groups (*G. pulex* present) and control groups (*G. pulex* absent) for either virgin or colonised plastics (Figure 4.3): virgin LDPE ($p = 0.152$), colonised LDPE ($p = 0.103$), virgin PLA ($p = 0.191$), colonised PLA ($p = 0.949$). Averages and standard deviations of treatments are given in Appendix D.6. Examination of discs under the microscope also showed no evidence that amphipods had shredded or bitten at the edges of plastic discs. Due to the lack of fragmentation observed in this experiment, the ability of the microbial biofilm to increase organism interactions compared to virgin plastic could not be evaluated, which is an unfortunate limitation of the study.

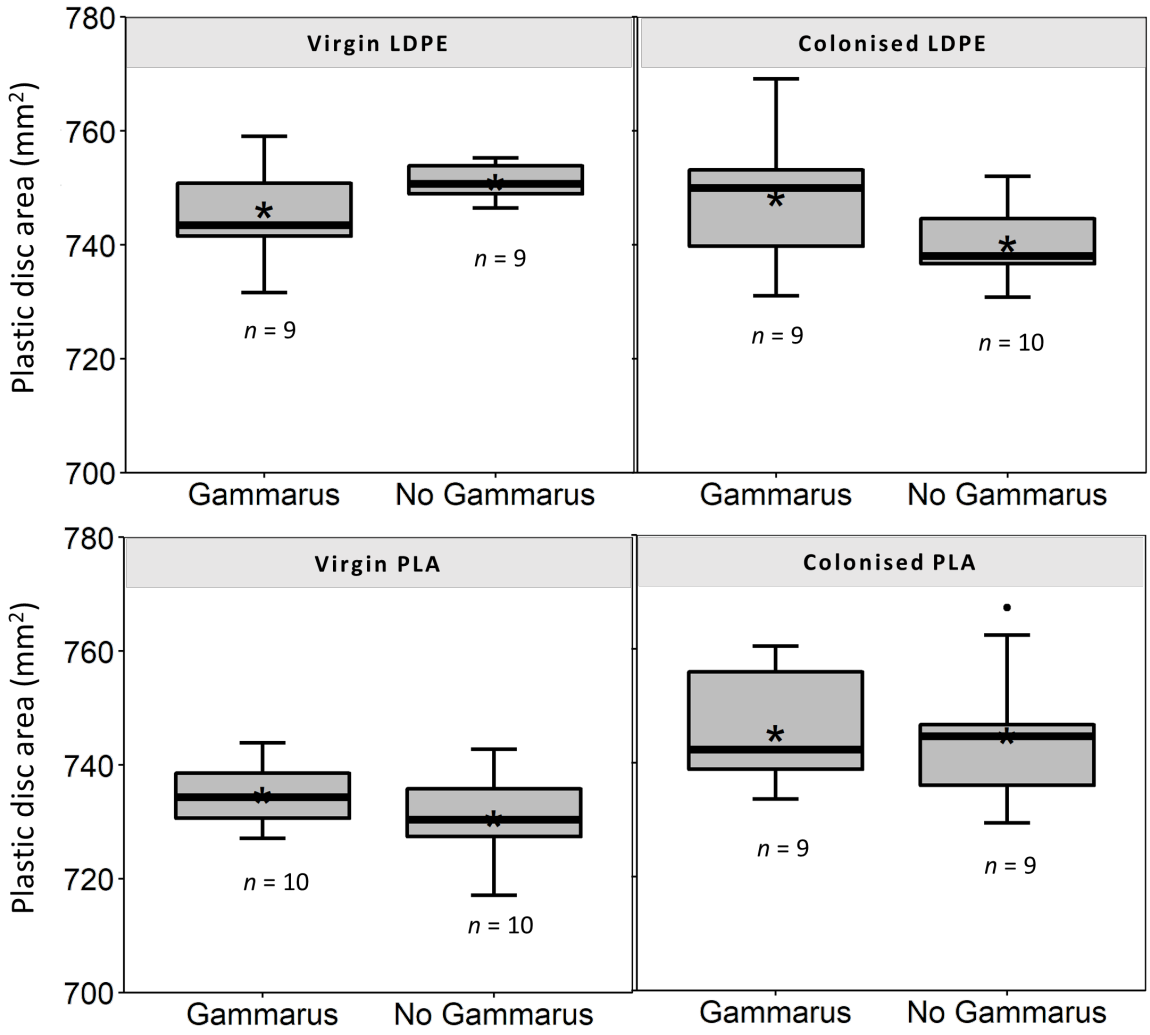


Figure 4.3: Surface area of virgin and microbially-colonised LDPE and PLA discs after five days under treatment conditions. Box plots show control treatments where no *G.pulex* was present compared to experimental treatments where plastic was exposed to *G.pulex*. Asterisks (*) symbols represent the mean average for each treatment with the number of replicates shown below each box.

The absence of any plastic fragmentation even when no other food sources were present, strongly indicates that these types of macro-litter plastic films which pollute the benthos of freshwater systems (Wilson et al., 2021) are unlikely to be fragmented by *G. pulex* in the same manner that they are in marine systems by the amphipod *O. gammarellus* (Hodgson et al., 2018), and the second hypothesis of this study can therefore also be rejected. However, it should be considered that these conclusions may only be valid for plastic with a toughness and thickness the same or greater than that of the tested materials. Previous studies have found a significant correlation between leaf toughness and *G. pulex* feeding rate – with tougher leaves being consumed at a lower rate than softer ones (Foucreau et al., 2013). It is therefore possible that *G. pulex* was physically unable to shred these LDPE and PLA films with its mandibles. In natural environments plastic is subjected to various weathering processes. These include photooxidation, mechanical abrasion, hydrolysis and biodegradation, and over extended periods (months-years) these processes can result in physical alteration of the plastic with it often becoming more brittle and easily fragmented (Song et al., 2017). To tease apart whether it was the physical toughness of plastic which prevented fragmentation, or simply a lack of palatability, it may be interesting for future studies to determine the ability and inclination of *G. pulex* to fragment less tough plastic films - such as those weakened by prolonged environmental weathering. Interestingly, Artru and Lecerf (2019) make suggestions that some fragmentation of PLA-based films was observed by *Gammarus* species, indicating that shredding behaviour may take place in some species, or under certain conditions. Internal biofragmentation of microplastics within the digestive system of *G. duebeni* is also known to occur (Mateos-Cárdenas et al., 2020), and given that there are around 204 *Gammarus* species (Costa et al., 2009), most of which are freshwater, further investigation of plastic interactions within the *Gammarus* genus may be warranted.

4.3.3 Conclusions

Under environmentally relevant conditions *G. pulex* did not display significant attraction to plastic biofilms in this study and they did not distract it from its natural food consumption; therefore, the presence of these plastic films in natural systems is likely to have a minimal effect on the health and fitness of *G. pulex* populations. Furthermore, findings from this study indicate that, unlike behaviour

observed in marine amphipods, structurally intact microbially colonised macro-plastic films present in freshwater benthic environments are unlikely to undergo biofragmentation by *G. pulex*. These results represent findings for two types of plastic film in their virgin form and in one stage of microbial colonisation, and therefore further questions regarding different material types and toughness, and biofilm compositions do remain. Nonetheless, this study provides a broad initial exploration of the interactions between plastic, microbial biofilms and a benthic freshwater invertebrate, which until now, have remained unexamined. These findings set an initial point of reference for future research to build on to address outstanding questions, such as the role of plastic biofilm composition in influencing *G. pulex* interactions, and whether there is a link between the physical toughness of plastic and the occurrence of biofragmentation behaviour. Furthermore, exploration of other *Gammarus* and freshwater amphipod species is warranted. From a wider perspective, this study represents only one species of freshwater benthic organism, and, given the largely unknown ability of freshwater organisms to influence the impacts and fate of plastic in the environment, further work to document interactions between microbially colonised plastic and other types of freshwater taxa is also still required. Similar to *G. pulex*, the benthic larvae of caddisfly (order Trichoptera) are another group of freshwater taxa known to associate with several different types of plastics in the natural environment (e.g. Artru & Lecerf, 2019; Ehlers et al., 2019; Wilson et al., 2021). The next chapter of this thesis will therefore investigate the nature of interactions which occur between plastics and these organisms, and determine the ability of these interactions to influence the risks of plastic within the environment.

Chapter 5

Caddisfly larvae are a pathway for plastic litter breakdown and microplastic formation in freshwater environments

5.1 Introduction

The majority of microplastics found in freshwater systems are secondary microplastics - resulting from the gradual degradation and fragmentation of larger plastic items in the environment (Vaid et al., 2021; Wong et al., 2020; Yang et al., 2021b). Abiotic factors which result in this fragmentation, such as mechanical stress, photodegradation, thermal stress and chemicals degradation, along with biodegradation by microorganisms living on plastic surfaces are well-documented (Hossain et al., 2019; Shah et al., 2008; Zettler et al., 2013; Zhang et al., 2021). In contrast, an emerging but poorly understood mechanism of plastic fragmentation is its breakdown by larger organisms, such as invertebrates, through their biting, chewing and digestive alteration of plastic (So et al., 2022;

Zhang et al., 2021).

The break down of plastic pollution by larger organisms is referred to as biofragmentation and has been observed in a variety of marine species. For example, the marine amphipod *Orchestia gammaerellus* and the sea urchin *Paracentrotus lividus* have been shown to degrade plastic materials into smaller fragments using their feeding appendages (Hodgson et al., 2018; Porter et al., 2019). Marine polychaetes and isopods are known to create plastic fragments from their burrowing behaviour into expanded polystyrene (Davidson, 2012; Jang et al., 2018) and evident bite marks in plastic litter washed up on beaches is a common occurrence (Carson, 2013). Biofragmentation can also occur internally within an organism's digestive system and is predominantly seen in aquatic crustaceans. For example, the amphipod *Gammarus dubeni*, Antarctic krill (*Euphausia superba*), Atlantic ditch shrimp (*Palaemon varians*), the lobster (*Nephrops norvegicus*) and the crab (*Carcinus maenas*) can internally degrade larger plastics into smaller particles, including micro- and nanoplastics (Cau et al., 2020; Mateos-Cárdenas et al., 2020; Murray & Cowie, 2011; Saborowski et al., 2019; Torn, 2020; Watts et al., 2015). Small amounts of internal plastic fiber fragmentation has also been documented in larvae of the freshwater dragonfly *Anax imperator* (Immerschitt & Martens, 2020). Some terrestrial insects have also recently gained attention for their ability to rapidly shred plastic films and foams, resulting in microplastic formation, and have been investigated for their bioremediation potential (e.g. Billen et al., 2020; Bombelli et al., 2017; Brandon et al., 2018; Helmberger et al., 2022; Wang et al., 2020b). Given the taxonomically broad range of organisms known to fragment plastic, biofragmentation may be an important contributor to the break down of plastic in the environment and the formation of secondary microplastics, and may therefore substantially influence the fate and impacts of plastics in the environment. However, research into the biofragmentation of plastic is in its infancy, and studies which address this process in freshwater species are particularly lacking (So et al., 2022) despite rivers being one of the largest contributors of microplastics to marine systems (González-Fernández et al., 2021; Lebreton et al., 2017). As plastic often becomes negatively buoyant within freshwater systems and is often deposited in or on the sediment (Egessa et al., 2020; Jalón-Rojas et al., 2022) the interactions between plastic litter and benthic freshwater organisms is of particular interest and relevance.

Interactions between plastic litter and the benthic larvae of caddisfly (order Trichoptera) have been recently noted, for example they have been found to associate with macro-plastic litter in freshwater rivers and streams (Artru & Lecerf, 2019; Wilson et al., 2021). Many caddisfly larvae build portable protective cases from hard particles such as mineral grains, or organic material like leaves and other plant material, which they attach together using self-produced silk (Holzenthal et al., 2015). Microplastics have been found within these hard mineral-grain cases of caddisfly sampled from several rivers (Alvarez Troncoso et al., 2022; Ehlers et al., 2019; Gallitelli et al., 2021; Gallitelli et al., 2020; Tibbetts et al., 2018) and the active incorporation of hard plastic particles into their cases has since been confirmed in laboratory studies with three different caddisfly species (Ajiboye, 2021; Ehlers et al., 2020; Gallitelli et al., 2021). Caddisfly which build with flexible organic material, such as leaves, often first fragment the material into the desired size and shape using highly-sclerotized mandibles (Wiggins, 1960). To date, studies have only examined the interactions between caddisfly and hard plastic particles; they have also mainly focused on caddisfly species which build from hard mineral grains and have never observed interactions between caddisfly and softer flexible plastic films. The ability of some caddisfly to fragment material, along with the documented interactions of caddisfly with plastic litter and the discovery of the rapid degradation of plastics by terrestrial insect larvae, therefore raises two questions: 1) '*do caddisfly larvae utilize and fragment the flexible plastic film they encounter in the environment?'* and as a result 2) *'are they a potential important contributor to microplastic formation in freshwater systems?*'.

Therefore, this study explores the interactions between the freshwater caddisfly larva *Agrypnia* sp. and microbially colonised polylactic acid (PLA) film. *Agrypnia* sp. is common in still and slow running freshwater systems throughout Europe and North America (GBIF-Backbone-Taxonomy, 2021) and builds its case from pieces of fragmented flexible organic material (Appendix E.1). PLA film was selected for this study as it is negatively buoyant even with no microbial biofilm attached, and therefore is likely to be encountered by benthic species. The first aim of this study was to establish whether the benthic larval invertebrate *Agrypnia sp.* would actively utilize PLA film to build a protective case. The second aim of this study was to determine whether interactions between caddisfly larvae and plastic films would lead to biofragmentation and microplastic formation. This experimental study addresses the second broad hypothesis outlined for this thesis and addressed the following two

sub-hypotheses: 1) '*Agrypnia sp. will use microbially colonised plastic films to build their protective cases*' 2) '*The interactions between Agrypnia sp. and plastic films will result in significant plastic modification and microplastic formation*'.

5.2 Methods and Materials

Throughout this study, plastic particles with a maximum Feret diameter between 1 and 10 mm are defined as meso-plastics and those between 1 and 1000 µm are defined as micro-plastics, as recommended by Hartmann et al. (2019).

5.2.1 Test organisms and materials

Agrypnia sp. larvae were obtained from the online retailer Blades Biological (Kent, United Kingdom) and their identification was confirmed using a taxonomic key (Wallace et al., 2003). Experimental work with caddisfly larvae was approved by the Environment and Geography Department Ethical Review Committee at the University of York. Upon arrival, organisms were maintained at 15°C under a 12:12 diurnal light cycle in a glass aquaria containing 10 L of artificial pond water (APW) (Naylor et al., 1989) and fed *ad libitum* with algal pellets comprising nutrient agar, cellulose and chlorella powder (Kampfraath et al., 2012), full recipe is given in Appendix E.2.

The plastic film used was derived from white, commercially available food-contact-grade PLA bags 45 µm in thickness, and polymer identify was confirmed using a Nicolet iS10 spectrometer (Appendix D.1). The PLA film had a density of 1133 kg/m^3 and was therefore negatively buoyant. Previous studies report higher levels of interaction between invertebrates and plastic when the plastic is first microbially conditioned (Hodgson et al., 2018; Porter et al., 2019). Therefore, microbially colonised PLA film was used throughout this study and was formed using the same methods and during the same time as colonised material used in Chapter 4 of this thesis. The weight and visual images of the biofilm which formed on PLA are given in Appendix D.2 and D.5).

Oak (*Quercus robur*) leaves were used as a representative natural flexible building material. Leaves were sun-dried green leaves from an organic woodland (Hanging wood, North Downs, Surrey, UK) and were obtained online. Leaves were soaked in milli-Q water for two days at 4°C to rehydrate them. Previous efforts to form a biofilm on the surface of leaf material, through *in situ* incubation in a river with the same methods used for plastic (Chapter 4), was found to substantially alter the structural integrity of the material, making it possible but highly challenging to handle and perform surface area measurements of the material. For this reason, to ensure accurate measurements could be performed, *in situ* microbial conditioning of leaf material was not carried out in the present study. Although some biofilm formation may have occurred on leaf material during the experimental exposure period (Artigas et al., 2011), the work of other studies reports that very little growth of bacterial and fungal colonies occurs on *Q. robur* leaves before around eight days of submersion in natural water (Sampaio et al., 2001). The experimental design of this study therefore represents a choice between plastic film already present in the environment and leaf litter which has recently entered the environment after falling from surrounding trees. For experimental use plastic and leaf material were cut into 6 mm x 3.4 mm pieces. The microbially colonised PLA weighed 51.2 $\pm$ 0.91 µg/mm^2 and rehydrated leaf material weighed 175.5 $\pm$ 20.8 µg/mm^2.

5.2.2 Experimental design

Preliminary trials showed that larvae used around 26 (6 mm x 3.4 mm) pieces of material (leaf or plastic) to build a new case, and that case building was most successful when leaf material was also available, rather than plastic alone. The study therefore consisted of two treatment groups: a 'Material limited' (ML) group where larvae were given 13 PLA and 13 leaf pieces - to determine whether larvae would use PLA if doing so was the only way to build a complete new case; a 'Material Replete' (MR) group - which was more environmentally relevant, and larvae were given 26 PLA and 26 leaf pieces to determine if they would use PLA even if there was sufficient organic building material available for case construction. Each treatment group contained twelve replicates. Twelve control replicates per treatment group, which consisted of an identical set up without the larvae were also run in parallel.

Before being placed into experimental jars, larvae were gently removed from their original cases using a blunt glass pipette. Larvae were randomly assigned to treatments so that there was no significant difference in the original case length of larvae between the ML and MR treatments ($t = 1.03$, $df = 22$, $p = 0.31$). The experimental treatments ran for six days with larvae placed in glass jars containing 150 ml APW, building material and a 0.95 g algae pellet, and were maintained under the same light and temperature conditions used for organism acclimation. During this time, all jars were covered with aluminium foil and continuously aerated using an airline and hypodermic needle. To maintain high water quality a 90 % water change was made after two and four days and water from these changes was stored. After six days, larvae were removed from their new cases and sacrificed by storing at -80°C. Newly built cases were stored at -20°C along with any meso- plastic and leaf material remaining in the jar which was recoverable by hand using steel forceps. The remaining water was collected, added to that from the water changes and stored in a Duran bottle at 4°C until analysis.

5.2.3 Material analysis

Cases were measured, photographed, and deconstructed to separate out plastic and leaf pieces. Plastic and leaf pieces from the cases and those recovered by hand from the jar were then imaged using an EPSON ET-2720 scanner and their surface area was quantified using Image J version 1.53a using a thresholding technique. These pieces were compared visually to control pieces to determine whether they were intact or if they showed evidence of chewing or fragmentation. To determine if micro-PLA fragments had been created, firstly, all exposure water samples were made up to 800 ml volume with milli-Q water (filtered to 0.2 µm). To minimise microplastics adhesion to each other and the bottle sides, 1 ml of a 10 % (v/v) TWEEN®20 solution in milli-Q water was added to each bottle. Each sample was shaken before around 50 % of the sample was passed through black Cyclopore 0.2 µm polycarbonate filters using a glass vacuum filtration pump. The exact volume of water filtered was determined by weighing water samples before and after samples were decanted for filtration. The filter was then imaged using a Zeiss axio zoom V16 microscope coupled with an axio zoom 105 colour camera and Zen (version 2.0) software. Images were processed in Image J, with white

micro-PLA particles identified visually within each image and measured using a thresholding-based technique for each individual particle. The number of counted micro-PLA particles was subsequently scaled to the full sample exposure water volume.

5.2.4 Quality control and assurance

Throughout the study, external microplastic contamination was minimised wherever possible by rinsing all apparatus and glassware three times with 0.2 µm-filtered milli-Q water and keeping all samples and apparatus closed to the air or covered in aluminium foil. Further quality control steps were taken to ensure that the micro-PLA count data were accurate. Firstly, micro-Fourier transform infrared spectroscopy (µFTIR) analysis of two treatment samples was performed and confirmed that the visually observed white microparticles were PLA - details of µFTIR methodology are given in Appendix E.3. Analysis of one control sample using µFTIR found no PLA particles present. Secondly, to further account for any white particles which still may have been misidentified as PLA, exposure water of control samples was filtered and visually analysed in the same way as treatment samples. In the exposure water of control replicates the average scaled number of particles that would have been visually identified as PLA was 0.3 in ML controls ($n = 10$) and 1.2 in MR controls ($n = 11$). This value was deducted from the scaled number of particles counted in each treatment replicate.

5.2.5 Statistical analysis

Statistical analysis of data and figure construction was performed in R-studio Version 1.2.1335 with packages ggplot2, car and FSA. Data was analysed to compare the amount of plastic and leaf used within and between treatments, along with the proportion of intact and chewed meso-PLA pieces, and the number and size of micro-PLA particles formed. All data residuals were tested for normality using a Shapiro-wilk test and for equal variance with either a Levene or a Breusch-Pagan test. When data did not meet these assumptions, it was either transformed or a non-parametric test was carried out if assumptions were still not met after transformation. Details of the tests and transformations carried out for each of the endpoints are outlined in Appendix E.4. The significance level for this

study was set at 0.05.

5.3 Results

5.3.1 Incorporation of plastic film into cases

After six days all larvae had used the provided material to construct a new case (Figure 5.1). Except for three larvae in the material limited (ML) and one in the material replete (MR) treatment, which built their case against the side of the glass jar, all larvae built complete portable cases. There was no significant difference in the length of new cases (U = 55.5, p = 0.35) or total surface area of the material (leaf + plastic) (t = -0.68, df = 21, p = 0.51) used to build them across treatments. There was also no significant difference in the wet weight of larvae between treatments at the end of study (t = -0.04, df = 22, p = 0.97). All twelve larvae in the ML treatment and eleven of the twelve larvae in the MR treatment incorporated PLA into their new case. Overall, larvae tended to favour leaf material over plastic. This preference was present when natural material was limited (cases on average contained 41 % plastic and 59 % leaf) and became clearer when natural material was replete (cases on average contained 17 % plastic and 83 % leaf) and in both treatments cases consisted of significantly more leaf than PLA ($t_{(ML, MR)}$ = 3.36, 7.65, $df_{(ML, MR)}$ = 10, 11, $p_{(ML, MR)}$ = 0.01, 9.93E^{-6}) (Figure 5.1). The amount of each material available that was used by larvae differed significantly across treatments, with larvae in ML treatment incorporating significantly more PLA into their new case compared to larvae in the MR treatment (t = 2.64, df = 21, p = 0.02). In contrast, significantly more leaf was used to build cases by larvae in the MR treatment compared to the ML treatment (t = -3.84, df = 14.14, p = 1.76E^{-3}). Of particular note was that of the eleven larvae that used PLA in the MR treatment, nine of them still had unused pieces of leaf material remaining loose in the exposure jar at the end of the study.

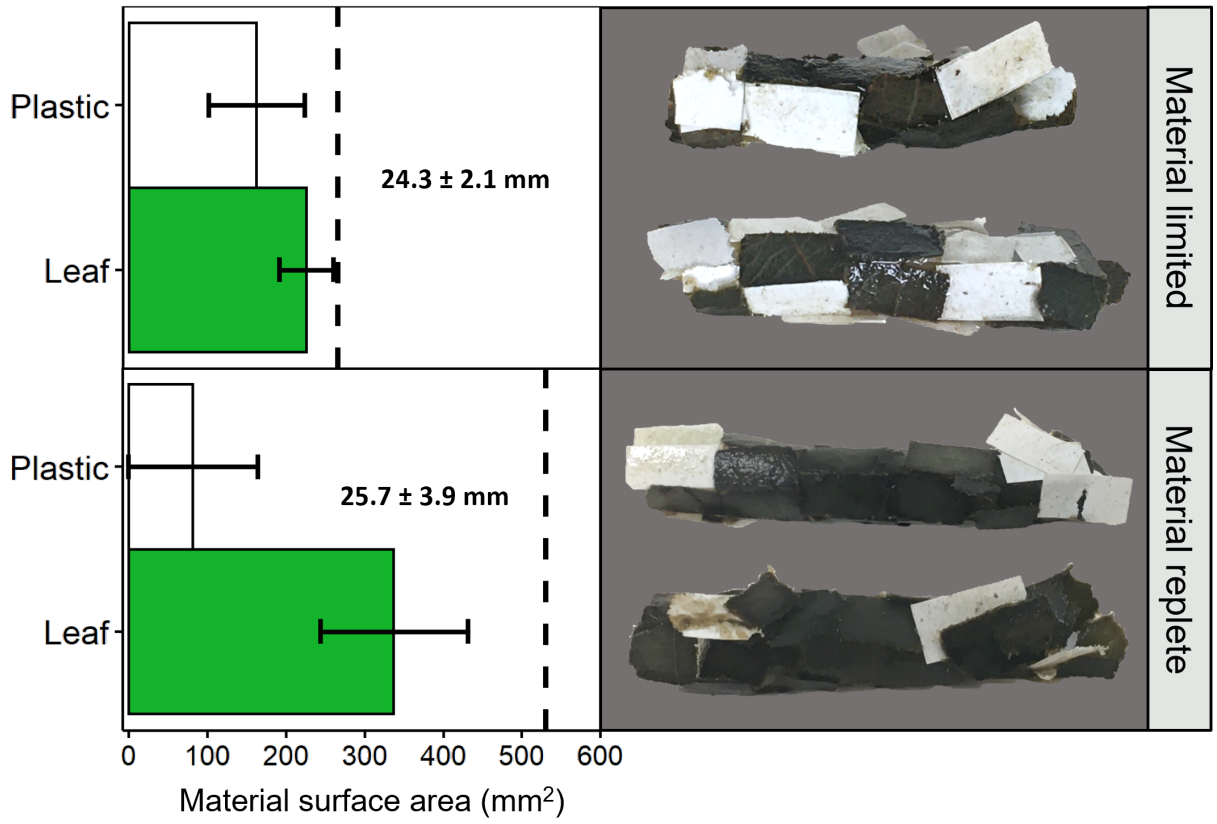


Figure 5.1: (Left) Average amount of leaf and PLA material used by caddisfly larvae to construct new cases for material limited and material replete treatments, error bars show standard deviation. The text on each plot is the average length $\pm$ SD of the newly built cases. Dashed line on each plot indicates the total amount of plastic and the total amount of leaf available to the larvae in each treatment. For material limited treatment ($n = 11$), for material replete ($n = 12$). (Right) Examples of newly constructed cases made from leaf and plastic material by larvae in each treatment group; dark green parts are oak leaf and white parts are PLA film.

5.3.2 Plastic fragmentation and microplastic formation

Chewing and fragmentation of PLA film by *Agrypnia* sp. larvae was clearly evident. In both treatments many of the meso-PLA pieces incorporated into cases, as well as those remaining in the exposure jar, showed clear signs of chewing which was not observed in pieces from control treatments (Figure 5.2). In the ML treatment 52 ± 31 % (mean ± SD) of the provided meso-PLA pieces remained intact, with no visual signs of chewing. This was significantly less (t = -2.87, df = 20 p = 0.01) than in the MR treatment where 85 ± 14 % remained intact. The extent of chewing on PLA pieces was highly variable, with some pieces extensively chewed and broken into small fragments and others only slightly chewed around the edges. Across all treatment and control groups the size (mean ± SD) of intact meso-PLA pieces was 20.3 ± 0.8 mm^2, chewed pieces in the ML treatment were 14.3 ± 6.5mm^2 and 16.4 ± 5.7mm^2 in the MR treatment. A significant difference was seen between the size of intact, ML-chewed and MR-chewed PLA pieces (H = 138.76, $p = 2.2E^{-16}$) and a post hoc test revealed significant differences between all three of these groups ($p < 0.001$ for all). Further evidence for the plastic-fragmentation behaviour of *Agrypnia* sp. larvae, and the use of their mandibles to perform plastic fragmentation, was seen outside of the main study when a larva involved in preliminary investigations was captured in a video recording chewing, fragmenting and incorporating into its case pieces of macro-PLA film (Appendix E.5).

Analysis of exposure water revealed that the fragmentation of PLA film by *Agrypnia* sp. larvae led to the formation of micro-PLA particles (< 1 mm in diameter). The number of micro-PLA particles found in the filtered exposure water varied considerably between replicates in both treatments (Figure 5.3). Larvae in the ML treatment created 225 ± 269 (mean ± SD) micro-PLA particles throughout the study which did not statistically differ to the 134 ± 133 particles created by larvae in the MR treatment (t = 0.58, df = 20, $p = 0.57$). As would be expected, the total surface area of meso-PLA pieces recovered from the case and exposure jar of each replicate was a significant predictor for the number of micro-PLA particles found for both the ML ($R^2 = 0.87$, $F_{1,7} = 46.93$, $p = 2.42E\text{-}4$) and MR ($R^2 = 0.63$, $F_{1,9} = 15.04$, p 3.74E-3) treatments, with a higher number of micro-PLA particles found in replicates from which a lower amount of meso-PLA surface area was recovered (Appendix

E.6). Although the size of larvae did not differ significantly between the two treatments, small amounts of natural variation in their size across all individuals was present. Interestingly however, the size of caddisfly was not a significant predictor for the number of microplastics formed in either of the treatments, ML ($R^2 = 0.24$, $F_{1,8} = 2.47$, $p = 0.15$), MR ($R^2 = 0.22$, $F_{1,9} = 2.53$, $p = 0.15$).

The size (maximum feret diameter) of visually identified micro-PLA particles formed by larvae ranged between 35.61 – 927.86 µm. FTIR analysis to a resolution of 6.25 µm did not identify any PLA microparticles below this size range, however, it should be noted that whilst unlikely, it is possible that micro-PLA particles which fell below 6.25 µm could have been generated. For both treatments, the most common size of micro-PLA particle was 100 – 300 µm (Figure 5.3) with 68.7 % and 66.3 % of particles measuring between 100 – 300 µm for ML and MR treatments respectively. Although the average particle size for ML (244.1 $\pm$ 121.2 µm) and MR (228.6 $\pm$ 142.1 µm) treatments was very similar, a significant statistical difference in particle size across treatments was detected ($U = 697142$, $p < 0.001$). However, interpretation of the size-frequency distribution and the descriptive statistics suggests that the large number of particles used for the analysis may have led to high power and sensitivity of statistical analysis, which exaggerated small differences that are unlikely to be biologically meaningful.

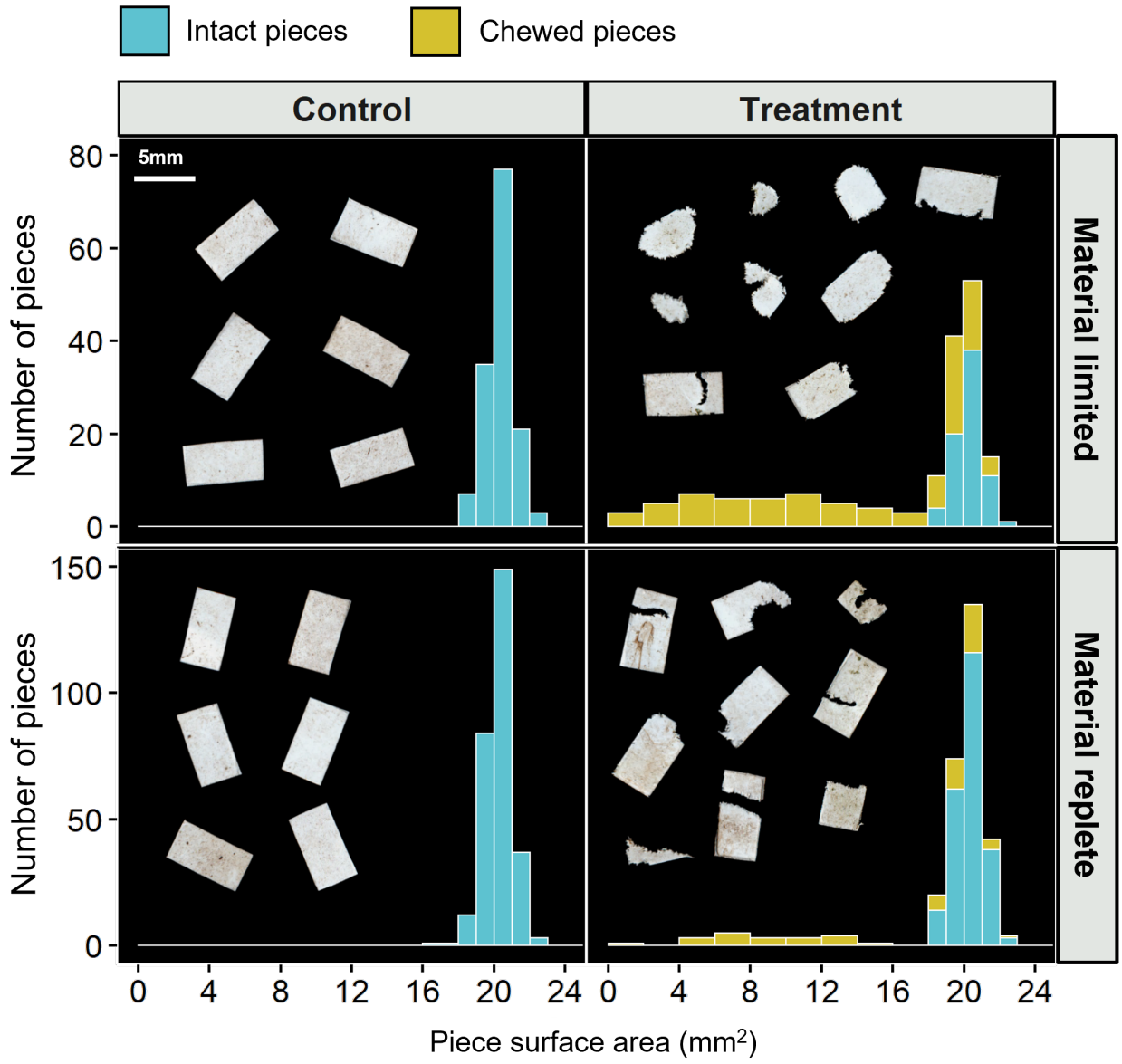


Figure 5.2: Size distribution of meso-PLA pieces ($>$ 1 mm) recovered from caddisfly cases and exposure water in material limited and material replete treatments, displayed as a stacked histogram. Colour coding denotes the number of pieces within each bin which were either intact or had visual evidence that chewing had occurred, $n = 11$ for both treatment and control groups. Inlet into control plots are example images of intact meso-PLA pieces and inlet into treatment plots are examples of chewed pieces from each treatment group. Scale bar = 5 mm for all PLA piece images.

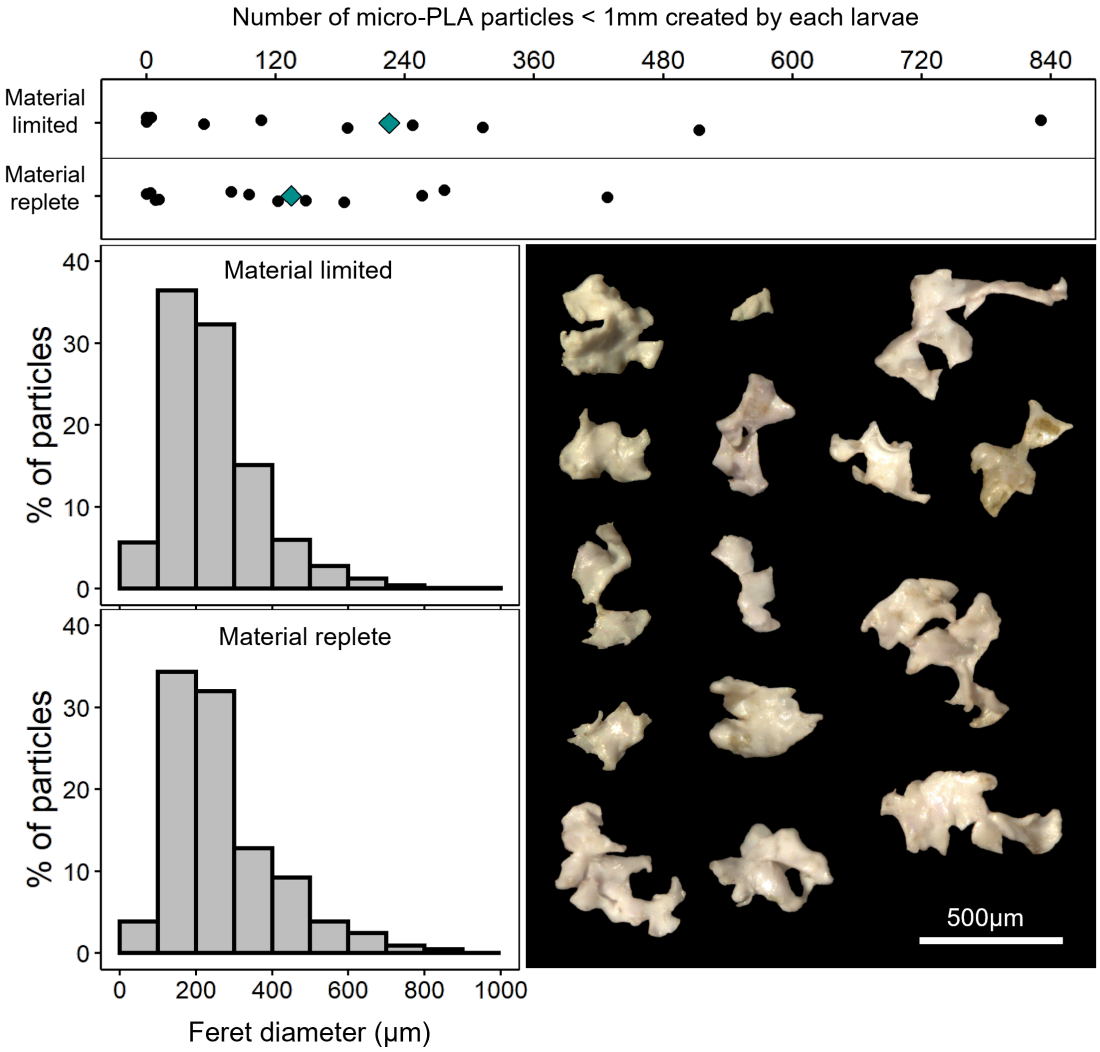


Figure 5.3: (Top) Scaled number of PLA microplastic fragments ($<$ 1 mm) formed by larvae over the six-day study. Black dots show the number of PLA microparticles for each replicate in either material limited (n = 10) or material replete (n = 12) treatment. Blue diamond's show the average for each treatment. (Left) normalised size distribution of all microplastic fragments found in the exposure water of replicates from either material limited ($n = 1440$) or material replete ($n = 1054$) treatments. (Right) example images of microplastic fragments from both treatments.

5.4 Discussion

The biofragmentation of plastic by larger organisms (such as macroinvertebrates) is beginning to be recognised as a potentially important pathway for plastic litter break-down and microplastic formation (So et al., 2022). However, data on the occurrence of biofragmentation in freshwater systems is severely limited. For the first time, this study evidenced the active biofragmentation of plastic films by a benthic freshwater invertebrate - which led to the formation of hundreds of microplastic particles. Throughout the study *Agrypnia* sp. larvae consistently interacted with pieces of microbially colonised flexible PLA film (6 mm maximum diameter); they used their mandibles to fragment the plastic, and along with leaf material used it to build a new protective case.

5.4.1 Larvae interaction with and use of plastic films

The use of plastic to build protective cases is in line with previous studies with other caddisfly species, which found that larvae incorporated both natural and plastic particles into their cases when a mixture was available (Ajiboye, 2021; Ehlers et al., 2020; Gallitelli et al., 2021). In the present study *Agrypnia* sp. larvae generally showed a preference for leaf material, however, their use of PLA still occurred under environmentally relevant conditions when leaf material was replete, and many larvae which had incorporated PLA into their case still had unused leaf material remaining. The first hypothesis of this study can therefore be accepted. Whilst it could be that the larvae's use of plastic was random, with larvae including material as they encountered it, PLA is likely to have possessed both desirable and undesirable properties as a building material - resulting in a trade-off between the benefits and drawbacks if its use. For example, a desirable property of PLA could have been its apparent smoothness compared to leaf material (Appendix E.7), as some caddisfly show a strong preference for smoother materials as they can more easily attach their silk to the surface (Okano & Kikuchi, 2009; Okano et al., 2012). In order to reduce the chance that their case will be consumed by other shredding organisms, caddisfly are also known to favour material which has a lower palatability and perceived nutritional quality (Moretti et al., 2009; Rincón & Martínez, 2006). Despite the PLA in this study being microbially conditioned for three-weeks and the leaf material

not, caddisfly often feed on leaf detritus, including *Quercus* sp. (González & Graça, 2003), and may therefore have still perceived the PLA as less palatable than the leaf. Whilst the smoothness and lack of palatability may therefore have encouraged the use of plastic in case construction, in contrast, the higher toughness of PLA compared to *Q. robur* leaves (Campanella et al., 2013; Jeong et al., 2018; Mirkhalaf & Fagerström, 2021; Vanstrom, 2012) is likely to be an undesirable property of PLA, as larvae would need to expend more time and energy fragmenting it into the desired shape and size compared to leaf material. After initially interacting with PLA and determining its toughness, this may therefore have been a deterrent to its use. This is supported by the meso-PLA pieces found loose in the exposure jar – many of which had been partially chewed or fragmented before being abandoned. As discussed, the design of this study represented a choice between plastic film already present in the environment and leaf litter which had recently entered the environment after falling from surrounding trees. Given that the leaf material in this study was not microbially conditioned in the same way as plastic, it should be noted that whilst the results presented here may be applicable for caddisfly interactions with organic and plastic material more generally, further experimental work to test the preferences of caddisfly between plastic and leaf litter which has been submerged in the environment for longer would be needed to confirm this. Furthermore, other types of leaf material from different tree species, as well as PLA film which has been submerged in the environment for longer or shorter periods, could elicit a different response, and additional studies to determine the role of these factors in the behaviour of caddisfly would also be useful.

Macro- and meso- sized plastic films are already abundant in freshwater systems (Blettler et al., 2017; Lahens et al., 2018; Winton et al., 2020) and the increasing use of PLA specifically, in applications such as agriculture mulching films (Akhir & Mustapha, 2022) and food packaging (European-Bioplastics, 2020), means that its presence in the environment is only likely to grow. Caddisfly have previously been found to be associated with biodegradable plastics in a riparian stream (Artru & Lecerf, 2019) and the presence of microplastics in caddisfly cases collected from the field (Alvarez Troncoso et al., 2022; Ehlers et al., 2019; Gallitelli et al., 2021; Gallitelli et al., 2020; Tibbetts et al., 2018) further confirms that caddisfly do interact with plastic in their natural environment. These previous findings, coupled with the tendency of larvae to interact with, use, and fragment plastic even when natural material was abundant in the present study therefore indicates

that this behaviour is likely to occur when caddisfly encounter plastic films in their natural habitat. Furthermore, evidence from this study suggests that even if larvae do not ultimately incorporate plastic into their cases, they may still interact with and 'test out' material by beginning to fragment it.

5.4.2 Potential impacts of caddisfly-plastic interactions

The consequences for *Agrypnia* sp. individuals of interacting with plastic film are unclear, but there is the potential for several favourable and adverse effects. Caddisfly are common prey for larger organisms and the design of their cases can alter the extent to which they are predated on (Ferry et al., 2013; Johansson, 1991; Nislow & Molles, 1993). For example, visibility is an important factor for the rate at which they are attacked by fish (Otto & Svensson, 1980) and the inclusion of plastic litter, which is often brightly coloured (Lu et al., 2021; Manikanda Bharath et al., 2021), may increase their vulnerability to visual predators. Furthermore, the structural strength of mineral-grain cases is known to be reduced by the inclusion of microplastics (Ehlers et al., 2020) which can decrease the chance of survival when larvae are attacked (Otto, 1987). Nevertheless, the toughness and lower susceptibility of plastic film to microbial degradation compared to leaf material, has the potential to improve the robustness of organic cases and therefore provide increased protection for the larvae.

Plastic inclusion in cases has also been shown to increase case buoyancy due to the lower density of plastic compared to mineral grains (Ehlers et al., 2020). In the present study PLA film was less heavy than leaf material and its inclusion would therefore have made cases lighter than those constructed only from leaf. Many other plastic films, such as low-density polyethylene and polypropylene are less dense than PLA, and their inclusion in larvae cases would likely result in even larger differences in case weight. A lighter case may have both negative and positive consequences for the larvae; whilst heavier cases can provide greater stability in water currents (Delgado & Carbonell, 1997; König & Waringer, 2008), successful prey capture was found to be considerably higher in larvae inhabiting lighter cases (Otto, 1987).

Determining plastic ingestion by larvae was beyond the scope of this study, however, given the extent

of chewing, the large number of microplastics created by some larvae, and the documented ingestion of microplastics by certain caddisfly species (López-Rojo et al., 2020; Windsor et al., 2019; Winkler et al., 2022), a degree of plastic ingestion would not be unexpected and has the potential to causes considerable adverse effects (López-Rojo et al., 2020). Furthermore, even if microplastic ingestion did not occur, interactions with harmful chemicals often associated with plastics, such as phthalates, persistent organic pollutants and toxic metals (Rochman, 2015), could pose an exposure risk to larvae during plastic fragmentation and during their prolonged interaction with the plastic after its incorporation into their cases.

Caddisfly are important members of freshwater ecosystems, with an important role in food webs as well as and carbon and nutrient cycling (Morse et al., 2019), and their fitness is known to influence key ecosystem process such as leaf litter decomposition (López-Rojo et al., 2020). Any impacts to their health caused by these plastic interactions may therefore also impact the wider ecological community. Furthermore microplastics are ingested by a wide range of organisms (Horton et al., 2018; Hurley et al., 2017; Klein et al., 2021; Scherer et al., 2017) and can cause considerable negative effects on individual organisms (Guimarães et al., 2021; Karami et al., 2016; Ziajahromi et al., 2018) as well as wider community structures (Rauchschwalbe et al., 2022). Given that the biofragmentation behaviour observed in this study is likely to increase the bioavailability of macro-plastic litter in the environment, the impacts of this microplastic influx should also be considered, and further research to determine the overarching ecological effects of caddisfly interactions with plastic films is now needed.

5.4.3 Implications of caddisfly behaviour for the fate of plastic litter

The significant link between the amount of meso-PLA recovered and the number of microplastics found, confirms that *Agrypnia* sp. larvae can facilitate the breakdown of meso-plastic into micro-sized particles and are therefore likely to be a previously unrecognised pathway by which microplastics are generated in freshwater systems. The second hypothesis of this study can therefore be accepted. As noted by Mateos-Cárdenas et al. (2020), many studies report a relatively slow break down of plastics from environmental weathering. For example, minimal degradation of polyethylene film was seen after

25 weeks in static freshwater (Julienne et al., 2019). Song et al. (2017) found that plastics could take up to 4.2 years to breakdown in a simulated beach environment, and in salt marshes the release of microplastics from larger plastic pieces only begun after 8 weeks (Weinstein et al., 2016). By comparison, in this study caddisfly facilitated the rapid breakdown of plastic films over just six days, with each larva, which had access to replete natural building material and a high-quality food source, creating an average of 22.3 microplastic per day. Interestingly though, the degree of fragmentation and microplastic formation in this study varied considerably among individual organisms. Given that there was no relationship between larvae size and number of microplastics formed, reasons for the variability remain unclear, however large variation in the case building behaviour between individuals and even within the same individual is common for caddisflies (Hansell, 1968), and therefore this variability is to be expected.

Shredding organisms generally account for around 20 % of macroinvertebrate biomass in temperate streams and rivers, and play a crucial role in the degradation of large organic matter into smaller particles (Cummins et al., 1989). A range of freshwater invertebrates are known to associate with plastic litter in rivers (Artru & Lecerf, 2019; Wilson et al., 2021), and in the present study, preliminary evidence from outside of the main experiment indicated that other caddisfly species, provisionally identified as *Limnephilus* sp., also incorporate plastic into their cases and show the same plastic fragmentation behaviour as *Agrypnia* sp. (Appendix E.8). Many other organisms therefore have the potential to contribute to plastic breakdown and microplastic release in freshwater systems, but have been largely overlooked (So et al., 2022). Various studies work to develop models to map the sources, behaviour, and fate of plastic in the environment (e.g. Kawecki & Nowack, 2019; Liro et al., 2020; Waldschläger et al., 2020), and fragmentation is already considered an important component in models such as the open source "Full Multi" modelling framework for the transport and fate of nano-micro plastics in the environment (Domercq et al., 2022). Active biological fragmentation, such as that demonstrated here for caddisfly larvae, is therefore likely to be an important consideration for the next generation of models which map the fate and transformation of plastic litter in the environment (Harrison et al., 2021). Given the findings of the current study, and the known internal fragmentation of plastic by freshwater amphipods and dragonfly larvae (Immerschitt & Martens, 2020; Mateos-Cárdenas et al., 2020) the role of biofragmentation in macroplastic breakdown and

microplastic release in freshwater systems should now receive greater research attention and be more widely considered by studies which model the dynamics of plastic in the environment. Further work with other polymer types, sizes and concentrations is now needed to assess the potential for biofragmentation in freshwater taxa and the extent to which it may be occurring throughout environmental systems. Mesocosm and *in situ* field-based studies would be particularly useful for identifying key organisms that are most important for plastic fragmentation in freshwater, and for establishing an accurate estimation for plastic litter breakdown rate through biofragmentation pathways.

The most common size of microplastic formed by *Agrypnia* sp. larvae in this study was between 100 and 300 µm. This is similar to those created by the amphipod *O. gammaerellus*, of which the majority were 200 – 600 µm (Hodgson et al., 2018), but generally smaller than the particles formed by the sea urchin *P. lividus* (Porter et al., 2019). Although analytical methods do influence conclusions about the size of microplastics formed, the size and morphology of an organism's feeding appendages are likely to be closely linked with the size of microplastics that are formed. Like other insects, caddisfly larvae have a well-developed mouth, consisting of sclerotized paired mandibles, paired maxillae and associated maxillary palps and galea; all of which function together to manipulate, guide and fragment material (Baptista et al., 2006; Friedrich et al., 2015; Holzenthal et al., 2015). *Agrypnia* sp. larvae in this study had serrated mandibles roughly 600 µm in length, of which the serrated portion was around 200 µm wide (Appendix E.9). The resting width between the maxillary palps was around 200 µm, and the feeding appendages of the larvae therefore appear to be in-keeping with the sizes of microplastic fragments observed. The irregular shape and notched edges of many of the microplastic fragments (Figure 5.3) matched very closely to the serrated area of the larvae mandible, indicating that plastic was likely cut on this part. During future work to explore microplastic formation from biofragmentation, the ability to link the size of organisms feeding appendages with the size and shape of created microplastics would be a valuable tool for further developing our understanding of the types of secondary microplastics being formed in the environment and the ecological risks that they may pose.

5.4.4 Conclusions

To the authors' knowledge, this is the first documented evidence of a freshwater invertebrate using external feeding appendages to actively fragment and physically alter plastic. This work therefore reveals a previously unidentified mechanism of plastic litter breakdown and microplastic formation in freshwater systems - which is likely to alter the bioavailability and fate of plastics within these environments. The interactions observed between caddisfly larvae and plastic films have the potential be either beneficial or detrimental to larvae fitness, and the impacts of the resulting microplastic influx on the wider benthic community remain unclear. Taken together, these findings suggest that to advance our knowledge of the sources, behaviour, fate, and impacts of plastic pollution in freshwater systems, further work to understand the extent of plastic biofragmentation behaviour throughout freshwater taxa, and their relative contribution to secondary microplastic creation, is now needed. Future studies should also address the choices and interactions of caddisfly larvae when presented with natural and plastic material in different stages of microbial colonisation, as well as the tendency of caddisfly to utilize and fragment different plastics types present in the environment.

Chapter 6

Discussion

6.1 Introduction

The plastic debris which pollutes aquatic environments consists of a broad range of plastic materials varying by characteristics such as size, shape, polymer type and associated chemical contaminants. Whilst this already makes the impacts of plastic pollution on the environment challenging to discern, interactions with biological factors further complicates work on impact assessment. Based on research in marine systems, interactions between plastic pollution, microbial communities, and larger aquatic organisms have been shown to be an important influence on the overall risk of plastics in the environment. For example, the formation of biofilms on plastic surfaces can modify the physical and chemical characteristics of plastic, disguise plastic as nutritional particles to larger organisms, increase exposure to pathogenic microorganisms, and alter the wider biogeochemical cycling within an aquatic system (Amaral-Zettler et al., 2021b; Chen et al., 2020; Huang et al., 2022; Vroom et al., 2017). The interactions between plastic and larger aquatic organisms can also further physically modify plastic and alter it fate and impacts within the environment. Furthermore, these biological factors can be interlinked, with the presence of biofilms enhancing interactions with larger organisms (Hodgson et al., 2018; Porter et al., 2019). Despite this knowledge, our understanding around the nature and implications of these types of biological interactions in freshwater systems is severely

lacking.

The aim of this thesis, therefore, was to advance our understanding in this area and build essential groundwork for the future progression of this research field. This thesis focused on three specific areas that are known to be important in marine systems, but where knowledge is particularly lacking for freshwater environments: i) the structure and composition of plastic-associated microbial biofilms, with knowledge particularly deficient around eukaryotic and fungi communities, and plastic biofilm communities generally in UK freshwaters; ii) the attraction of freshwater organisms to microbially colonised plastic, with no studies which address this for benthic organisms; iii) the potential for organisms to physically modify the shape and size of plastic litter, specifically, their ability to accelerate plastic breakdown in the environment and increase microplastic contamination.

These knowledge gaps were addressed in four experimental chapters which: i) examined the characteristics, composition and specificity of plastic biofilms formed in a UK river (Chapter 2); ii) quantified the production of the odorous microbial metabolite dimethyl sulfide by plastic biofilms (Chapter 3); iii) investigated the attraction of the benthic invertebrate *Gammarus pulex* to microbially colonised plastic, and the ability of *G. pulex* to fragment plastics films (Chapter 4); iv) examined the ability of benthic caddisfly larvae to utilize and physically modify plastic in its surrounding environment (Chapter 5). The knowledge gained from this experimental work has been integrated into the conceptual diagram first presented in Chapter 1 and illustrates how these findings inform our wider understanding of the impacts and fate of plastic in freshwater environments (Figure 6.1). This discussion chapter firstly summarises the knowledge gained from the experimental work of this thesis and discusses the findings from different chapters together within the wider context of the two broad hypotheses outlined in Chapter 1. The second part of this chapter addresses the remaining knowledge gaps, future research priorities, and presents a conceptualised framework for the most efficient way to build on work from this thesis and continue to expand our knowledge of the risks of plastic in freshwater environments.

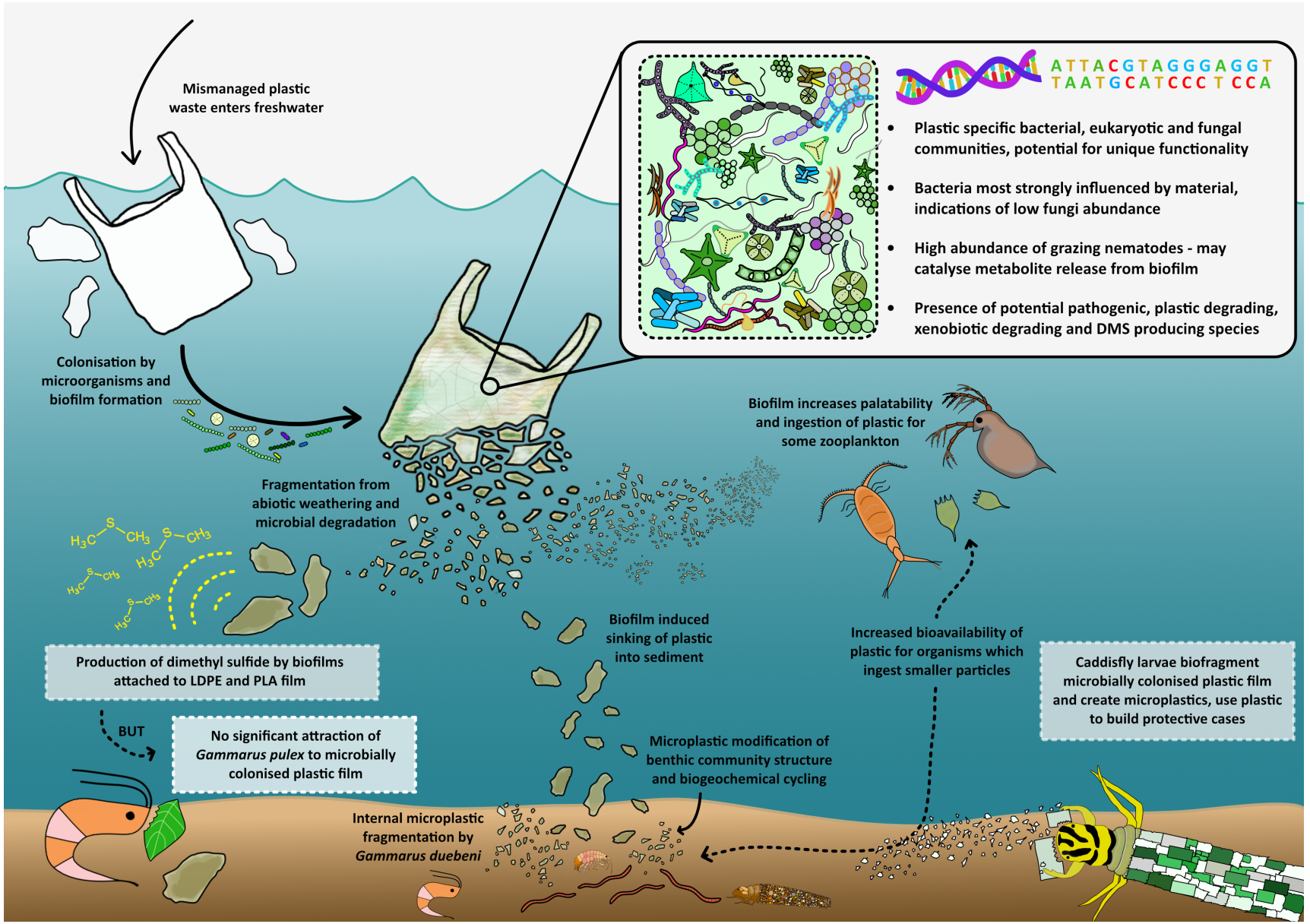


Figure 6.1: Revisited conceptual diagram outlining the key findings from this thesis and how they fit within our wider understanding of the fate and impacts of plastic in freshwater environments.

6.2 Summary of thesis results and general discussion

6.2.1 Hypothesis 1: Plastic litter can provide a unique substrate for the formation of biofilms that can enhance interactions between plastic and freshwater organisms in UK rivers

The first chapter of this thesis examined the ability of LDPE and PLA to host biofilms with a significantly distinct composition compared to non-plastic surfaces in the surrounding area. In this study, as anticipated, time was found to be the dominant influence on biofilm composition, with biofilms showing natural successional patterns (Dang & Lovell, 2000). However, material type also influenced this successional development, with certain taxa becoming significantly more or less enriched on plastic over time. Under the river conditions investigated, plastic supported a unique composition of bacterial, eukaryotic and fungal communities compared to glass. Nevertheless, bacteria communities were most strongly influenced by material type, with continued differences between materials after six weeks of submersion. There were also indications that fungi may have been less abundant than eukaryotes and that their relative importance in plastic biofilms may be low, however the application of quantitative techniques such as real-time polymerase chain reaction are required to verify this. Bacteria genera known to contain pathogenic, plastic-degrading and xenobiotic-degrading species were identified to be significantly enriched on plastic compared to glass and indicated the ability of plastic biofilms to possess unique risk and metabolic functionality compared to other surfaces within the river. Additionally, significant differences in the abundance of taxa linked to carbon and nutrient cycling were found between plastics and glass, indicating further differences in functionality and a potential to alter biogeochemical cycling processes within the wider river (Chen et al., 2020). Given that plastic biofilms formed in marine systems are known to produce the key foraging infochemical dimethyl sulfide (DMS) (Savoca & Nevitt, 2014; Savoca et al., 2016), the occurrence of microorganisms that produce DMS in freshwater was also examined in this chapter. This revealed the presence of bacteria in the genera *Hyphomicrobium*, *Pirellula* and *Novosphingobium*, which have been linked with their potential ability to produce DMS in freshwater and terrestrial environments (Carrión et al., 2015).

The differences in biofilm composition and indications of unique functionality observed in Chapter 2, coupled with previous work which reports evidence of the unique functionality of plastic biofilms (Su et al., 2022; Xue et al., 2020) motivated the examination of biofilm functional processes which may influence plastic-organism interactions occurring within the river. Given the presence of the putative DMS producing bacteria also identified in Chapter 2, preliminary work was carried out which subsequently detected DMS in water which had been in close contact to microbially colonised plastic. Therefore, the production of DMS by plastic litter in UK rivers was comprehensively investigated in Chapter 3, and comparisons were made to glass surfaces to determine the potential relative influence and importance of plastic litter compared to other materials. This study found the significant production of DMS by biofilms associated with LDPE and PLA films after three and six weeks of submersion in two different river locations (one urban and one rural). Furthermore, after six weeks both plastics produced significantly more DMS per cm^2 of material compared to glass. There were also significant differences in the DMS production per mg of biofilm, and this was thought to indicate the presence of a significantly different biofilm composition between materials. This study confirmed unique metabolic functionality of plastic surfaces within UK rivers and, given the known sensitivity of some freshwater species to DMS (Nakajima et al., 1989), demonstrated the potential for this functionality to influence interactions between plastic litter and riverine organisms. Further implications of these findings also include the potential for plastic pollution in freshwater environments to contribute to global DMS budgets and influence the biogenic sulfur cycle (Carrión et al., 2015). However, although macroplastic litter would likely be the largest contributor to this, due to constituting the largest surface area of plastic within riverine systems, our understanding of river macroplastic budgets are poor (Al-Zawaidah et al., 2021), and making estimates of their relative contribution to global DMS budgets is not yet possible.

As discussed within Chapter 3, the chlorophyll *a* and pheophytin content of biofilms in this study indicate, that in contrast to marine systems (Savoca et al., 2016; Simó et al., 2018), photosynthetic taxa were unlikely to have contributed to the majority of observed DMS production. Instead, it is thought that bacteria based DMS production pathways may have dominated. This notion is further supported by results from Chapter 2, which found the convergence of eukaryotic communities after six weeks, but continued differences between glass and plastic for bacterial communities, as well as

the presence of three putative DMS-producing bacteria genera. However, it should be noted that considerable differences in biofilm composition across different studies is common and using results from one of these chapters to inform the other should therefore be carried out with care. Furthermore, several freshwater photosynthetic eukaryotes are known to produce DMS (section 3.1) and their presence and relative abundance should always be considered alongside bacterial communities. The presence of grazing nematodes within biofilms was also documented through DNA sequencing in Chapter 2, along with qualitative observations of various other plastic-invertebrate associations. Tube structures, thought to belong to chironomid larvae, were similarly seen on materials in Chapter 3 (Appendix C.5) and given that grazing invertebrates within the biofilm are thought to enhance the liberation of microbial metabolites (Gaudes et al., 2006), the interactions of these organisms with plastic may therefore have catalysed the release of DMS from biofilms. Findings from these chapters confirm, that in order to unpick the complex ways in which plastic and its associated communities may impact freshwater systems, all taxa which comprise or interact with plastic-associated biofilms must be considered collectively.

The identified production of DMS by plastic biofilms in Chapter 3 and the clear association between freshwater invertebrates and microbially colonised plastic in Chapter 2, demonstrated a need to explore the relative attraction of common larger freshwater invertebrates to plastic under environmentally relevant conditions. Chapter 4 of this thesis therefore explored the interactions between microbially colonised plastic films, and the benthic invertebrate *Gammarus pulex*. Despite the documented production of DMS by plastic biofilms, when presented with a choice between microbially colonised LDPE and PLA films and colonised leaf material, *G. pulex* showed no significant short-term behavioural attraction towards plastics. There was also no evidence that the presence of these microbially colonised films distracted *G. pulex* from its normal feeding activity over a three-day period. Some volatile organic compounds released from microbial communities are known to be important infochemicals and foraging cues in freshwater systems (Fink, 2007; Fink et al., 2006b). However, although some DMS sensitivity has been noted in three freshwater fish species (Nakajima et al., 1989), it is generally unknown if freshwater organisms can detect or are attracted to DMS in the same way as marine species. The ability of *G. pulex* to detect and respond to this chemical is therefore unclear. Moreover, despite plastics in the DMS and *Gammarus* study both being submerged for

three-week periods in the same river location during the same season (August 2020 and 2021), the microbial composition on plastics could have differed, and the actual production of DMS by plastic used in the *Gammarus* study is unknown. As discussed in section 4.3, the composition, and related odours of the biofilm on leaf material may have simply been more attractive to *G. pulex*, potentially due to a higher fungal biomass on leaf material (Grossart et al., 2019), and therefore in the natural environment there is unlikely to be any significant impacts of macro plastic litter on these organisms. Nevertheless, biofilm composition of plastic can vary considerably between different locations (Yang et al., 2021a), and the formation of biofilms on plastic in a river with different conditions may have the potential to be more attractive.

The variability in biofilm composition between locations and the role of location in driving substrate-level differences in the biofilm was in fact a feature also identified throughout this thesis. For example, in Chapter 2, LDPE and PLA were found to have a significantly higher biofilm mass than glass after six weeks of submersion in the river; whereas after six weeks in two different rivers in Chapter 3, no significant differences in the biofilm mass between materials were present. Similarly, the photosynthetic pigment content of plastics was significantly lower than glass after three and six weeks of submersion in Chapter 2, however in Chapter 3, plastic pigment content was significantly higher or no different than glass after the same time period. Whilst these differences may potentially be attributed to the use of slightly different *in situ* submersion methods between these two studies (samples on a raft *vs.* contained within a cage), considerable differences between river locations in Chapter 3, where identical methods were used, were also observed. Most notably, the biofilm mass, pigment content and DMS production differed significantly between the same material type submerged at different locations. This was interpreted as being due to a difference in biofilm composition – most likely driven by the trophic status of the river and the proximity of samples to the river sediment. The relative difference in these parameters between materials also varied between locations, adding further evidence to previous studies which found location conditions to influence the effect of substrate type on biofilm composition (Oberbeckmann et al., 2018).

Overall, findings from this experimental work partly support the first broad research hypothesis of this thesis; they confirm that plastic litter in UK rivers can and does host distinct biofilm communities

which have a unique metabolic functionality with the potential to enhance interactions between plastic pollution and freshwater organisms. However, there was no evidence that the benthic invertebrate *G. pulex* was attracted to microbially colonised plastic under environmentally relevant conditions. Additional work is therefore needed to determine the attraction of other common freshwater taxa to plastic biofilms, along with the relative role of different biofilm members and metabolic compounds in mediating organism interactions. Furthermore, whilst the unique composition and functionality of plastic biofilms was apparent in these studies it was also clear that this is highly likely to depend on factors such as the surrounding water and location conditions, and efforts to systematically determine the circumstances which mediate the formation of plastic-specific communities and functionality should now be made. Studies which compare overall differences between plastic and other surfaces in the environment such as rock and wood would also be of further use in determining the relative risks of plastics compared to other common surfaces.

6.2.2 Hypothesis 2: Interactions between plastic and larger freshwater organisms can alter the fate and potential impacts of plastic litter in the environment

As well as working to determine the relative attraction of *G. pulex* to microbially colonised plastic in the presence of natural food, Chapter 4 of this thesis also examined the ability and tendency of these organisms to biofragment plastic to access the microbial biofilm, in a similar manner to that observed by the marine amphipod *Orchestia gammarellus* (Hodgson et al., 2018). In this study *G. pulex* showed no attempt to biofragment either virgin or microbially colonised plastic, despite other food sources been withheld for seven days. Given that no other food was available this could indicate a complete lack of attraction to the microbial biofilm, or, may indicate an inability of this species to fragment these plastic films with their mandibles. As discussed in Chapter 4, further work to determine whether *G. pulex* shows interest in and an inclination to fragment other plastic films, such as those weakened by environmental weathering, may be of use. Nevertheless, it should be considered that even if biofragmentation of weaker films did occur, if significant attraction was still not present under environmentally relevant conditions, then it would be unlikely to be an important or relevant pathway for plastic breakdown or microplastic formation in the environment.

The final chapter of this thesis worked to explore interactions between plastic and other benthic freshwater invertebrates that may result in the modification and biofragmentation of plastics. Given the documented association of caddisfly larvae with plastic in the environment (Artru & Lecerf, 2019; Ehlers et al., 2019; Wilson et al., 2021) the interactions between these organisms and microbially colonised PLA films was investigated. Whilst work by Artru and Lecerf (2019) mentions the brief examination of interactions between plastic films and Limnephilidae caddisfly larvae, the extent of this investigation is not described and no biofragmentation behaviour was reported. In contrast, Chapter 5 of this thesis found that *Agrypnia* sp. larvae (family Phryganeidae) utilize PLA films to build new protective cases and that this results in the extensive modification of PLA films and microplastic formation. This behaviour occurred under environmentally relevant conditions when there was replete food and a choice of other natural building material available. Furthermore, even if PLA was not eventually incorporated in the larvae's case, there was evidence that these organisms 'tested out' case-building material by shredding slightly at the edges, before abandoning them. Work for this study also found preliminary evidence that this behaviour may also occur in Limnephilidae species (Appendix E.8). Taken together, findings from this study, along with previous reports of plastic usage by caddisfly in the environment (e.g. Ehlers et al., 2019), indicate that biofragmentation of plastic films is likely to occur within natural freshwater systems where these larvae encounter plastic litter. Moreover, as this behaviour was documented in two families of caddisfly (Limnephilidae preliminarily) plastic biofragmentation may occur in other caddisfly species which fragment natural debris to build their cases, as well as other shredding taxa, and may be widespread throughout freshwater systems.

Biofragmentation of larger plastic litter will intensify the microplastic contamination load of aquatic systems, as well as increase the bioavailability of plastic for species which ingest smaller whole plastic particles, such as filter feeders and deposit feeders (Botterell et al., 2019; Issac & Kandasubramanian, 2021). The ability of organisms to ingest plastic is mediated by their mouth gape size, and different species show a greater uptake of microplastics within a certain size range based on their overall body size (Botterell et al., 2019). Results from this thesis showed that caddisfly larvae transformed plastic debris measuring 6 mm (maximum diameter) into microplastics measuring between 36 and 928 µm (maximum diameter). This biofragmentation process would therefore make plastic litter more bioavailable to benthic organisms such as asellidae isopods and chironomid larvae, which have

shown a higher ingestion of microplastics < 50 µm (Pan et al., 2021a). Plastic may even become more bioavailable to species such as *G.ammarus duebeni*, which are common amphipods in rivers throughout the UK, and are known to internally fragment 10 – 45 µm microbeads into even smaller fragments (Mateos-Cárdenas et al., 2020) - potentially leading to even further plastic breakdown. As discussed in section 1.2.2, microplastic contamination of sediments can lead to significant changes in the benthic macroinvertebrate community structure, as well as alterations to invertebrate-mediated biogeochemical cycling (Huang et al., 2022; Silva et al., 2022). An understanding of the relative impacts of microplastics created through biofragmentation is now therefore needed. Other implications of biofragmentation include alterations to the fate of plastic in the environment, for example van Emmerik et al. (2022) highlighted that microplastics are likely to behave differently in riverine environments than macroplastic, with a lower chance of entrapment and greater possibility of remobilization into the water column and transportation downstream towards the ocean. Understanding the extent of biofragmentation within rivers, the conditions under which it is most likely to occur, and the quantity of microplastics created through these pathways could therefore also be important for our understanding of plastic pollution with estuarine and marine systems.

During preliminary work, the strong interactions identified between caddisfly and plastic led to a study design to focus on the biofragmentation and microplastic formation behaviour occurring for environmentally representative (microbially colonised) plastic. However, although environmentally relevant, the experimental design of this study focused only on a choice between plastic litter which had been present in the environment for three weeks and leaf litter which had recently entered the water from surrounding trees. It would therefore also be interesting to explore whether the biomass or composition of biofilms associated with materials in the environment can influence the extent of plastic-caddisfly interactions which occur. For example, as discussed in section 5.4, in order to minimise the chance that their case is consumed by other shredding organisms, caddisfly are thought to favour building materials with a lower nutritional quality (Moretti et al., 2009; Rincón & Martínez, 2006), and although caddisfly demonstrated a 'non-feeding interaction' (So et al., 2022) in this study, the relative palatability of PLA compared to leaf material may therefore have influenced their selection of it has a building material. Despite a microbial biofilm being present on PLA, caddisfly still selected it for case construction and this may therefore indicate the biofilm was not palatable to the larvae in

this study. Interestingly, some caddisfly larvae show a food preference for fungal mycelia in a similar manner to *G. pulex* and other shredding detritivores (Arsuffi & Suberkropp, 1988). Therefore, despite the use of subsamples from the same microbially-colonised PLA material in Chapter 4 and Chapter 5, the biofilm composition and potentially low fungal biomass may have reduced interactions between plastic and *G. pulex* but encouraged interactions between plastic and caddisfly larvae. If so, this indicates that the role of the biofilm in plastic-organism interactions in freshwater systems could be highly complex, with variability based on both biofilm composition, and the ecology, traits and biology of the interacting organism. Understanding the factors which drive the use of plastic by caddisfly larvae, including the role of the biofilm and its metabolites such as DMS, and the presence of different types of natural material, will be useful for developing a deeper understanding of the conditions in which plastic is most likely to undergo biofragmentation by these organisms.

Taken together, findings from this experimental work do support the second broad hypothesis proposed by this thesis and confirm that interactions between plastic litter and larger freshwater organisms can significantly alter plastics and have implications for their fate and impacts within the environment. Further work, which continues to explore the extent of this biofragmentation behaviour in other freshwater taxa and quantifies the relative contribution of these pathways to plastic breakdown and microplastic formation is urgently needed.

6.3 Implications of thesis findings for the risk assessment of plastic pollution and recommendations for the future

6.3.1 Is PLA really an "eco-friendly" alternative?

The "eco-friendly" status of PLA has led to its increased use in consumer products and packaging to boost sustainability credentials (Akhir & Mustapha, 2022; B-Fortune-Business-Insights, 2021; Ncube et al., 2020). There is also a clear drive by many G20 countries, such as the United Kingdom, Japan, Brazil and Canada, to actively encourage the development of the bio-plastic market (BBIA, 2022; Fadeeva & Van Berkel, 2021). For example, Japan committed to increase the amount of animal

and plant-derived bio-plastics by around 50 times by the year 2030, using them to replace fossil fuel based plastics (Fadeeva & Van Berkel, 2021). However, there are scientific concerns around this push for bio-based plastics (Wang et al., 2021a) and before a large-scale move towards the replacement of conventional plastics with bio-based alternatives is implemented, it is important to develop a comprehensive understanding of the behaviour of these materials in the environment.

Despite the different chemical composition of LDPE and PLA polymers (Appendix B.1), this thesis consistently found the characteristics of biofilms which formed on plastic surfaces to be notably similar. For example, in chapter two and three, the photosynthetic pigment content of biofilms on PLA generally did not differ significantly from those on LDPE, whilst both plastics showed large and significant differences to the biofilms formed on glass surfaces (section 2.3 and 3.3.2). Taxonomic differences in the biofilm community observed at the order level also exhibited similarities between the two plastics, with bacteria orders Flavobacteriales and Myxococcales both present on LDPE and PLA in a similar abundance, but differing significantly between the plastics and glass. Similarly, the order Rhizobiales increased in abundance over time and was significantly enriched on glass surfaces compared to both PLA and LDPE, where the abundance remained lower. The observed similarity between LDPE and PLA biofilms was also present in the biofilm metabolic functionality, with biofilms on PLA producing similar quantities of DMS as those on LDPE after six weeks submersion in UK rivers, and both plastics producing significantly more DMS than biofilms on glass. Although previous studies do report significant differences between the biofilm composition of PLA and other plastic types (Kirstein et al., 2018), differences were also observed between many other plastic types in this study, and as seen in this thesis, the biofilm characteristics of PLA were most similar to other plastics, whilst showing a stark contrast to glass control surfaces. These findings therefore indicate that the biofilms which form on PLA during its exposure to aquatic environments are relatively similar to those which form on many other commonly used plastic types. The potential risks posed by plastic associated biofilms, such as harbouring pathogenic microorganisms, disguising plastic as nutritional particles and altering biogeochemical and xenobiotic cycling (section 1.3.3), are therefore unlikely to differ much between PLA and other plastics. This should be considered when assessing the impacts of PLA on the environment.

As well as supporting a relatively similar microbial community to traditional plastic types, the physical behaviour of PLA within aquatic environments also does not appear to differ. Despite being marketed as a biodegradable plastic, degradation of this material is most effective under specialised industrial compositing conditions at a temperature of around 50 - 60°C. The cooler conditions of aquatic environments are therefore thought to severely inhibit this (Karamanlioglu et al., 2017; Ncube et al., 2020). This has been confirmed in environmentally relevant conditions, where PLA-based films only showed a 5 % mass loss after 77 days in a riparian stream where significant colonisation by microorganisms and macroinvertebrates had occurred (Artru & Lecerf, 2019). Findings from this thesis also report similar outcomes. As seen for LDPE films in this thesis, the submersion of two types of thin PLA film in UK rivers for up to six weeks did not result in any notable changes to the structural integrity of the material. Additionally, this thesis has shown that interactions with certain benthic invertebrates can lead to the rapid fragmentation of plastic films and release of microplastics. Given that PLA film is negatively buoyant, even before microbial colonisation, it is most likely to reside within the sediment of aquatic systems where it could be encountered by these benthic invertebrates. Despite fragmentation being a key step in plastic degradation (Mateos-Cárdenas et al., 2020) the low temperatures of UK rivers are still unlikely to facilitate biodegradation (Artru & Lecerf, 2019) and these microplastics are therefore likely to behave very similarly to conventional plastic fragments, remaining as contamination within the riverine system, or being transported towards the ocean (Mai et al., 2020; van Emmerik et al., 2022).

Although the carbon footprint of PLA is generally much lower than traditional fossil-fuel based alternatives (Zheng & Suh, 2019), the similarities observed in the surface-associated biofilms and the physical behaviour of PLA compared with LDPE raises concerns over the suitability and benefits of PLA as a replacement bio-plastic material. Its mismanagement and leakage into the environment can still clearly cause significant problems and it should not be viewed as a simple 'cure' for plastic pollution. A better understanding of the potential risks of bio-plastics such as PLA is critical and should be coupled with initiatives to implement better waste management systems to help prevent plastics being released into the environment in the first place. For example, schemes such as 'Close The Loop' by Vegware^{TM}, which recaptures PLA-based post-consumer packaging waste and directs it towards industrial compositing systems (Vegware, 2022), will be an essential component in working

towards reducing the impacts of plastic on the environment.

6.3.2 General recommendations for future work

The ultimate goal of the plastic pollution research field is to achieve an accurate understanding of the overall risks and impacts of plastics in the environment and establish the most effective ways in which these risks can be mitigated. Work which brings together data on the concentration and types of plastic present in the environment, with the known effects of these plastics on organisms and the wider ecological system, is an excellent way to assess these overall risks. However, many studies and risk assessment approaches which do this, almost exclusively consider only the physical and chemical characteristics of plastics. For example, one study which developed a risk assessment framework for the marine environment (Davis et al., 2022) specified that the risk of harm from plastics is related to their physical and chemical characteristics, with no mention of the interactions with microbial communities or other biological factors. A number of other studies (Pan et al., 2021b; Peng et al., 2018; Xu et al., 2018; Yin et al., 2021) which conducted a risk assessment of different freshwater environments made conclusions of risk based only on the concentration of different polymers present and the pre-determined chemical toxicity of theses plastics based on their polymer identity (Lithner et al., 2011). One study, which worked towards a comprehensive ecological risk assessment for microplastics throughout freshwater systems globally, linked the documented microplastic concentration of different areas with the predicted no effect concentration of microplastics determined from 53 ecotoxicity studies (Adam et al., 2019). Although a useful approach, in this study only one of these 53 ecotoxicity studies considered the role of microbial aging in their assessment of plastic toxicity. Furthermore, the one study which did this (Besseling et al., 2014) found that *Daphnia magna* had a mortality rate 4.4 – 6 times higher when exposed to polystyrene nanoplastics which had been aged in an algae culture for five days first, compared to virgin nanoplastics without prior aging. Although the presence of a biofilm on particles in this study was not characterised, the importance of plastic interactions with microbial communities here is clear, and an increased toxicological response to microbially aged plastics compared to virgin plastics has similarly been demonstrated in the sea urchin *Paracentrotus lividus* and the mussel *Mytilus galloprovincialis*. Whilst plastics already possess

a complex array of different chemical and physical characteristics, findings from this thesis and the existing literature demonstrate that the role of the biofilm and other biological factors in mediating the risks of plastic clearly cannot be overlooked. Furthermore, it is clear that the influence of these biological factors cannot be generalised too broadly, and the complexity of biofilm characteristics and the role of larger organisms across different environments must also be captured and addressed within these assessments. Therefore, based on the findings of this thesis, areas for future research which will help us move towards a more accurate and comprehensive understanding of the risks of plastics in the environment have been outlined, and are discussed throughout the rest of this section.

As discussed, the ability of plastic pollution to harbour distinct biofilm communities with unique metabolic functioning, such as DMS production, is thought to be strongly driven by the surrounding location and water conditions. Consequently, the impacts of plastic litter may vary between different freshwater systems. For example, the risks of plastic litter in a large rural river, may differ significantly from the risks posed by the same plastic within a small urban lake, based on the characteristics of the biofilm which forms on the surface. Previous literature has demonstrated the ability of water parameters, including organic nutrient concentration, dissolved oxygen, salinity, alkalinity, and total organic carbon, to significantly shape plastic biofilms (Nguyen et al., 2022; Yang et al., 2021a) and to mediate the influence of substrate type on biofilm composition (Oberbeckmann et al., 2018). Findings from this thesis further confirm this for UK riverine systems, where trophic status was thought to play an important role in biofilm composition. In a similar manner to this thesis, many studies which analyse plastic associated biofilms often target specific and logistically convenient areas for assessment. Whilst this has been a valuable and necessary starting point, to work towards a more comprehensive understanding of plastic communities it is recommended that research efforts should now move away from arbitrarily characterising the biofilm composition of specific individual areas. Instead, systematic efforts to link water conditions to key plastic biofilm characteristics, along with the ability and likelihood of plastic to host unique communities compared to various non-plastic surfaces in the surrounding environment would be far more effective. Data collected from these types of studies could be used to develop computational models to predict the composition and functionality of plastic biofilms in a given area, and their specificity to plastic compared to other surrounding substrates. These types of models have been successfully developed to predict the

effects of sewer water composition on the structure of sewer pipe biofilm communities and their production of hydrogen sulfide and methane gases (Liang et al., 2019; Sun et al., 2018), as well as the influence of different chemical treatments on these endpoints. Similarly, models to predict the biofilm composition and functionality of bioelectric microbial fuel cells and aquaculture biofilters have also been developed (Chen et al., 2006; Lesnik & Liu, 2017), and this approach could therefore be a powerful tool for expediting our understanding of biofilm growth on plastic pollution. Indeed, based on the discovery of biogeographic patterns in plastic biofilm communities, early work by Amaral-Zettler et al. (2015) notes that a regional approach to plastic debris risk management may be most effective.

Different freshwater taxa interact with plastics in different ways and show different responses following exposure (section 1.2.2). The risk of plastic pollution to a given system will therefore also depend on the composition and relative abundance of species within the environment, as well as the concentration and characteristics of plastics present. As explored above, the role of biofilms in mediating this risk remains poorly understood, and although this thesis explored the interactions of two benthic species with microbially colonised plastic under environmentally relevant conditions, these assessments for other common freshwater taxa are urgently needed. Efforts to link plastic biofilm composition and metabolite production with the preferences and sensitivity of freshwater taxa to these microorganisms and compounds would be most effective, and would significantly deepen and increase the environmental relevance of our understanding around the impacts of plastic on organisms.

Finally, some freshwater amphipods and dragonfly larvae are known to internally biofragment microplastics (Immerschitt & Martens, 2020; Mateos-Cárdenas et al., 2020) and this thesis discovered the significant external biofragmentation of plastics films by Phryganeidae caddisfly larvae, as well as preliminary indications of this behaviour in Limnephilidae caddisfly species. Taken together with similar findings in marine and terrestrial species (e.g. Helmberger et al., 2022; Hodgson et al., 2018), these results suggest that biofragmentation may be a considerable source of microplastics to freshwater systems and could represent a previously unrecognised mechanism by which macro-plastic litter become more bioavailable to organisms in freshwater environments. Systematic work to document the ability and inclination of other freshwater taxa to biofragment or otherwise modify plastic lit-

ter is therefore now needed. These studies should be carried out under environmentally relevant conditions, and work to link the abundance of plastic litter within the environment to the rate of biofragmentation observed. The ability of the biofilm to mediate these interactions would also be valuable to include in assessments.

Combining the information and data gained from these recommendations should allow connections to be drawn between the characteristics of a freshwater system and the probability and impacts of plastic-biofilm-organism interactions. Identification of other unique risks plastic might pose, such as an influence on biogeochemical cycling could also be determined. This knowledge could subsequently be used to aid the overall relative risk of plastic pollution within a given freshwater environment and used to inform decision making about areas which should be prioritized to receive clean-up efforts, or be targeted for policy or local behavioural change to reduce plastic pollution emissions. These suggestions have been summarised into a conceptual framework (Figure 6.2), which outlines the data and information future studies should now work to collect, along with the measurements which would need to be taken for a freshwater system undergoing assessment and the outputs and information gained from these applied measurements. Despite their focus on chemical and physical parameters, many risk assessment frameworks currently proposed (e.g. Adam et al., 2019; Brunning et al., 2022; Davis et al., 2022) are highly useful, and the framework proposed here offers specific complementary considerations for role of the biofilm and other biological elements in the risk assessment of plastic pollution. Whilst the recommendations proposed here can be developed around multiple common plastic types, it does not capture the vast complexity of plastic polymers or other environmental processes (Brunning et al., 2022) and does not explicitly consider other factors such as seasonal and other temporal changes which can influence the biofilm (Weig et al., 2021). However, this framework should provide a broad initial outline and direction for the efficient advancement of our understanding of the interactions occurring between plastic pollution, microbial communities and larger aquatic organisms, and the overall risks of plastics within freshwater environments.

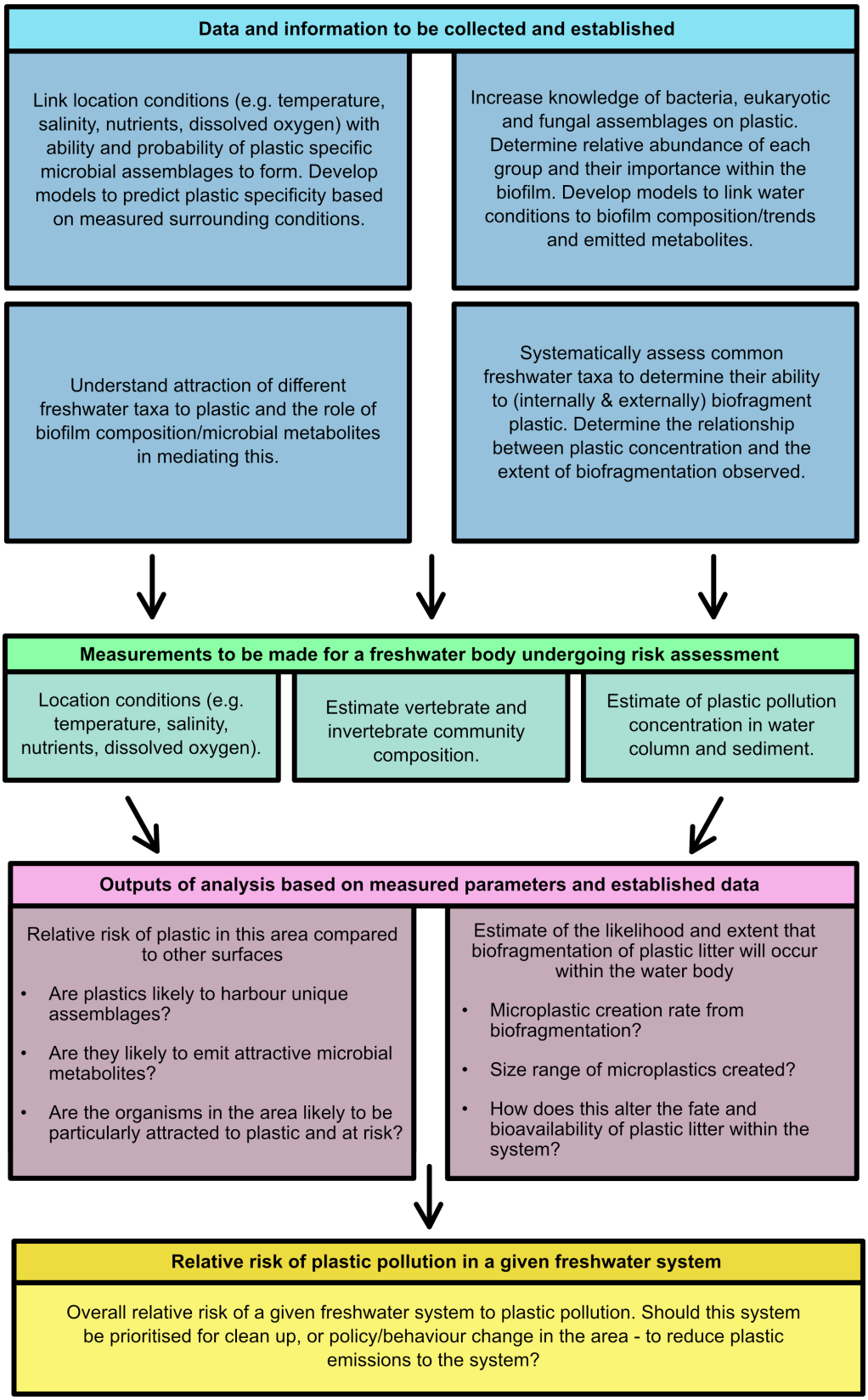


Figure 6.2: A framework for working towards the inclusion of biological factors (role of biofilms and interactions with larger aquatic organisms) in the risk assessment of plastics within freshwater environments.

6.3.3 Specific recommendations and priorities for future work

Based on the specific findings of this thesis, the three following questions have been identified as the most pressing knowledge gaps in this field and are areas that should therefore be prioritised for future investigations:

1. ***'Can we predict the composition and characteristics of plastic biofilms based on the trophic status of a freshwater system?'*** Given the role of nutrient concentration and trophic status identified within this thesis and previous literature, work to connect the trophic status of freshwater systems to plastic biofilm composition is likely to offer important insights. This work could allow a better understanding of areas where plastic is most likely to harbour pathogenic microorganisms, have implications for wider carbon and nutrient cycling within aquatic systems, and host species which may influence interactions with larger aquatic species. Given that the nutrient levels of UK freshwater is already a topic of concern and subject to ongoing monitoring (Water Environment (Water Framework Directive) (England and Wales) Regulations, 2017), data around the trophic status and general water quality of many UK freshwater systems already exists. Being able to link these water conditions with the specific risks posed by plastic litter and associated biofilms in these areas could therefore add further depth to our understanding of the health of these ecological environments. The most effective way to carry out this work is likely to be to develop models using data collected from semi-realistic mesocosm studies, where experimental parameters can be more tightly controlled. This data could then be validated against plastic biofilms formed under *in situ* submersion conditions within the natural environment.

2. ***'Do freshwater taxa show significant sensitivity and attraction to DMS under environmentally relevant conditions?'*** The production of DMS by plastic films in UK rivers, coupled with the known sensitivity of many marine species and a few freshwater species to DMS, indicate an urgent need to understand the attraction of other freshwater taxa to this odorous compound. Work to determine this should be carried out to understand the ability of organisms to detect this compound at similar concentrations to those observed in this thesis,

and their use of the compound as a foraging stimulant. If an ability for species to detect and respond to DMS is found, their relative attraction DMS produced from plastic surfaces should be determined under environmentally relevant conditions, with the presence of other natural substrates and food present.

3. ***'Do other common invertebrate shredders show an ability to biofragment plastic under environmentally relevant conditions?'*** Given that the external biofragmentation of plastic by caddisfly larvae in this thesis significantly altered the bioavailability and potential fate of plastic within freshwater, and that similar behaviour has been observed in marine shredders, other common freshwater shredders should be examined for their ability and tendency to biofragment plastic in the environment. Based on findings from this thesis and reports from other literature, groups of organisms to prioritise for investigation are:

    - **Limnephilidae caddisfly larvae** – many of these species build with plant material which they fragment using sclerotized mandibles, they are common throughout UK freshwaters and preliminary work from this thesis has indicated their ability to utilize and fragment plastic.
    - **Other freshwater amphipods** – given the preliminary indications of Artru and Lecerf (2019) that small amounts of plastic shredding was seen by an unidentified *Gammarus* species, other shredding amphipods present in UK waters, such as *Crangonyx pseudogracilis*, *Dikerogammarus villosus* and *Orchestria cavimana* (same genus as marine amphipods known to biofragment plastic films) should be examined.
    - **Beetles** – recent studies have highlighted the tendency of some terrestrial beetle larvae to biofragment, chew, and eat plastic (Helmberger et al., 2022; Wang et al., 2020b). Given that aquatic and semi-aquatic beetles are also common through the UK freshwater, their interactions with plastic litter should be investigated. Some taxa which may be useful to examine are *Hydraena riparia*, which is a generalist feeder and lives among submerged vegetation and substrates, as well as other species in the Hydraenidae family which are generalist omnivores, feeding on algae, bacteria and decaying vegetation and have well-developed mandibles (Beutel et al., 2003; UK-Beetles, 2022).

6.4 Conclusion

In conclusion, the work from this thesis has demonstrated that the plastic litter which pollutes UK freshwater systems has the potential to host biofilm communities with a distinct composition compared to surrounding non-plastic surfaces, with potential implications for the pathogenicity of plastics and their influence on wider biogeochemical and xenobiotic cycling. The ability of plastic associated biofilms to produce the odours compound dimethyl sulfide was also evidenced here for the first time, and the role of surrounding water conditions in shaping this metabolic functionality and other biofilm characteristics was demonstrated. The biofilms which form on plastic pollution within UK freshwater therefore have the potential to significantly influence the overall risks of plastics in the environments. Further work is now needed to determine the sensitivity and attraction of freshwater species to dimethyl sulfide, and to link the water conditions of different environments to the composition and functionality of biofilms which form on plastic surfaces. This thesis also worked to understand the role of larger aquatic organisms in mediating the fate and impacts of plastic in the environment. Whilst no significant interactions were observed between the benthic invertebrate *Gammarus pulex* and microbially colonised plastic, the benthic caddisfly larvae *Agrypnia* sp. displayed a striking inclination to utilize plastic films to build its protective case, which subsequently resulted in the rapid biofragmentation of plastic and the formation of hundreds of microplastics. These interactions between plastic films and caddisfly larvae have the ability to increase the bioavailability of macro-plastics to smaller organisms in the environment and may alter the fate and transport of plastic within freshwater systems.

Taken together, findings from this work have shown that the interactions which occur between plastic pollution, microbial communities and freshwater invertebrates, can significantly influence the impacts of plastics within freshwater systems and that these biological factors must be considered alongside the physical and chemical aspects of plastic materials. Given than this work is still in its infancy, time and research efforts must therefore now be channelled into this area in order to continue to develop an accurate and holistic understanding of the risks that our plastic-centred lifestyles pose to the natural environment.

Appendix A

Appendix for Chapter 1

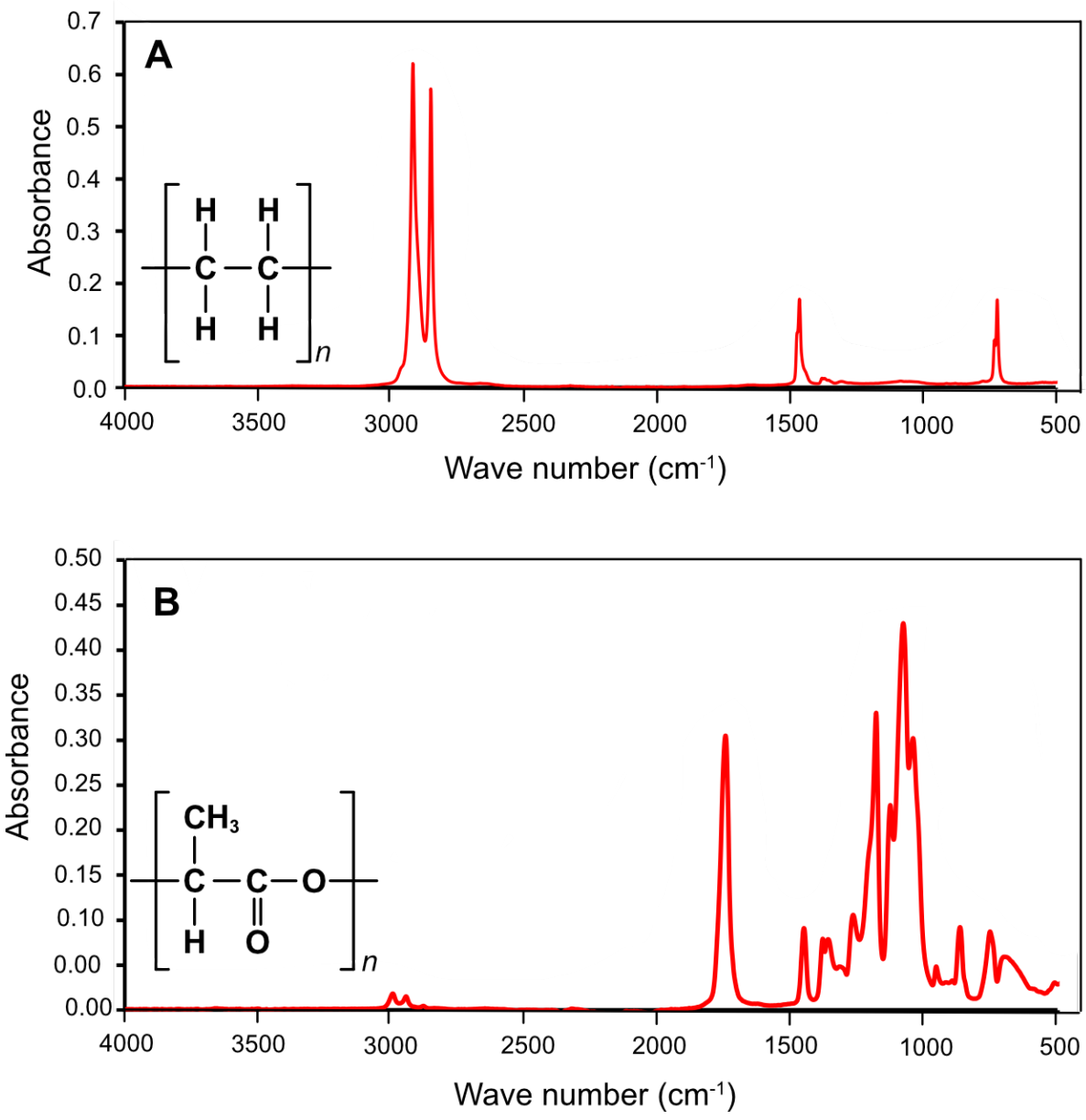
A.1 List of studies identified by this thesis which use DNA/molecular techniques to assess the taxonomic composition of plastic-associated biofilms in freshwater.

List of studies is available at https://figshare.com/s/6de9f6897858c6a91e33

Appendix B

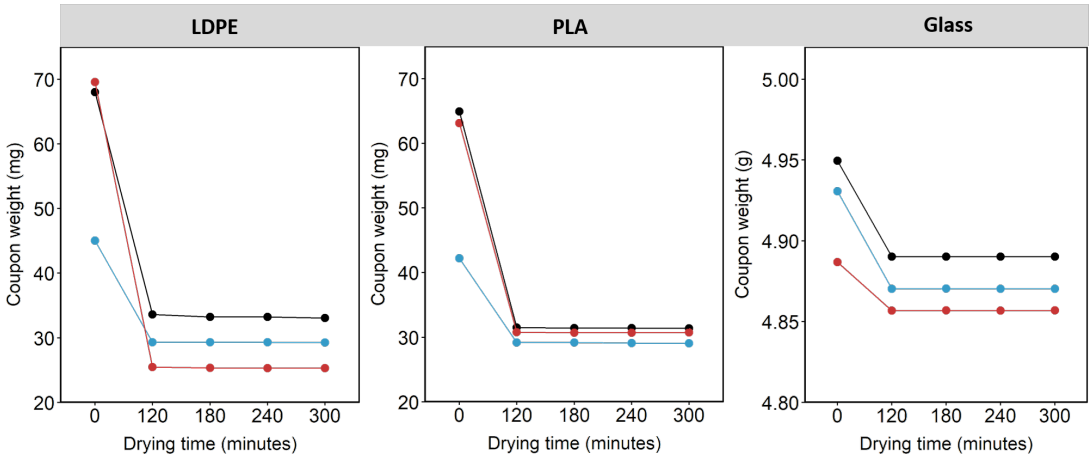
Appendix for Chapter 2

B.1 Fourier transform infrared spectra for plastic film materials used in experimental work



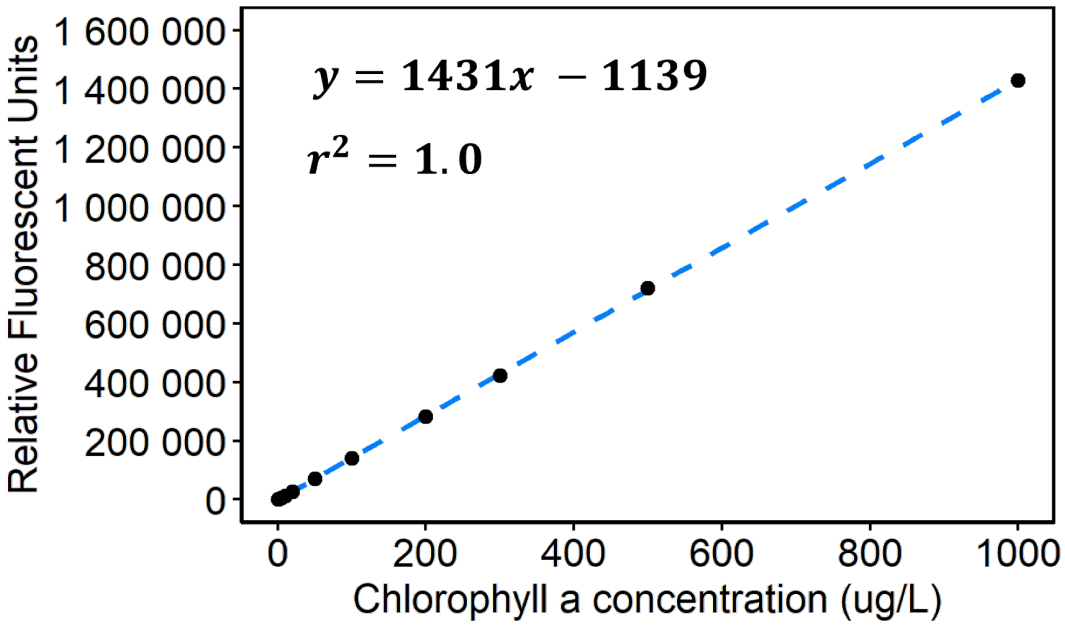
Fourier transform infrared spectroscopy spectra for plastic film materials used in this thesis experimental work. (A) Clear low-density polyethylene film used in Chapter 2 and 3. (B) Clear polylactic acid film used in Chapter 2 and 3. The monomer structure of each polymer is inset into each plot. Spectra were obtained using a Bruker Alpha II platinum ART with a diamond crystal coupled with Bruker OPUS software. Spectra were recorded between 400 and 4000cm^{-1} at a resolution of 4cm^{-1} and 144 scans.

B.2 Oven drying time method validation



Weight of LDPE, PLA and glass coupons with a biofilm attached after different amounts of drying time at 30°C. Different colours represent different replicates which have been displayed separately due to having different starting biofilm weights. After 180 minutes of drying LDPE and PLA coupons showed no more than 0.5 % decrease in weight and glass showed no more than a 0.002 % decrease, three hours was therefore considered an appropriate and practical drying time.

B.3 Calibration curve and equations used to calculate the chlorophyll *a* and pheophytin content of biofilms



Calibration curve used to calculate the chlorophyll *a* and pheophytin content of biofilms

$$\text{Chlorophyll (µg/L)} = \frac{Fm}{Fm-1} \times slope \times (RFU_b - RFU_a)$$

$$\text{Pheophytin (ug/L)} = \frac{Fm}{Fm-1} \times slope \times ((Fm \times RFU_a) - RFU_b)$$

Where:

Fm = Acidification ratio of standard curve

Slope = slope of standard curve

RFU_b = relative fluorescent units before acidification

RFU_a = relative fluorescent units after acidification

B.4 Primer sequences and conditions of the thermocycler used for PCR

DNA sequence of primers used to amplify bacterial (16S), eukaryotic (18S) and fungal (ITS) genes.

Primer	Sequence
16S – Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGATTAGATACCCTGGTA
16S – Reverse	AGACGTGTGCTCTTCCGATCTCGACRRCCARGCANCACCT
18S – Forward	ACACTCTTTCCCTACACGACGCTCTTCCGARCRAACCTGGTTGATCCTGCCAGT
18S – Reverse	AGACGTGTGCTCTTCCGATCTTCTCAKGCKCCYTCTCCG
ITS – Forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCTCCGCTTATTGATATGC
ITS – Reverse	AGACGTGTGCTCTTCCGATCTGTGARTCATCGAATCTTTG

PCR conditions were as follows: 2 minutes at 95°C, 30 cycles of 20 seconds at 95°C, 15 seconds at 57°C, 5 minutes at 72°C, followed by an extension phase for another 5 minutes at 72°C.

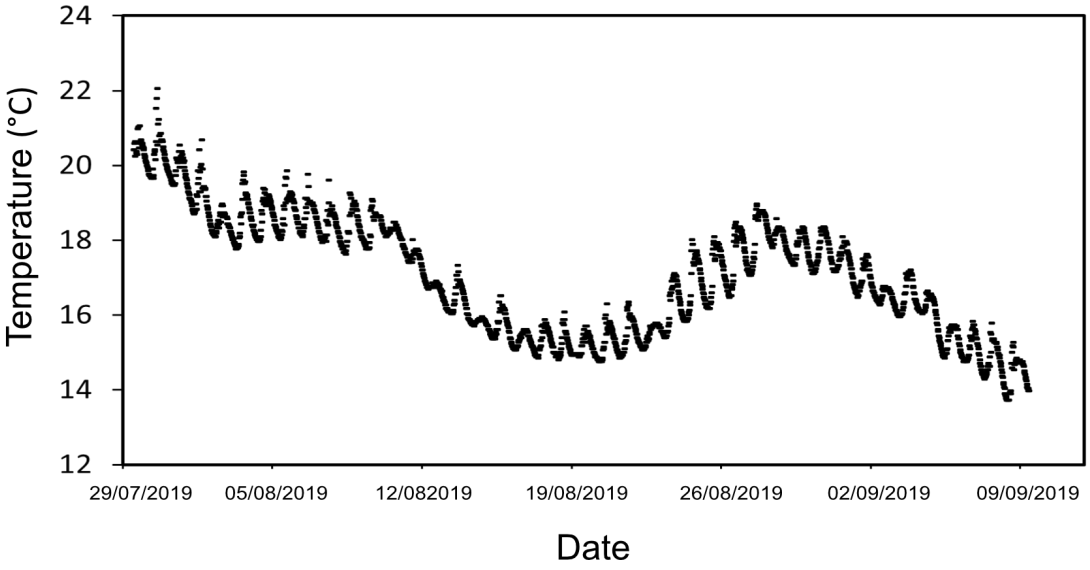
B.5 Settings used to trim, quality filter, merge, denoise and assign taxonomies

16S rRNA amplicon reads were trimmed to 290 and 200 bases, 18S were trimmed to 290 and 205 bases and ITS2 amplicons reads were trimmed to 270 and 220 bases, forward and reverse respectively. Filtering settings were maximum number of Ns (maxN) = 0, maximum number of expected errors (maxEE) = (2, 5). The primer sequences were removed using truncQ = 2. Sequences were dereplicated and the DADA2 core sequence variant inference algorithm 'applied. mergePairs' was used to merge sequences and actual sequence variant (ASV) tables were constructed. Chimeric sequences were removed using 'removeBimeraDenovo' default settings. ASVs were subject to taxonomic assignment using 'assignTaxonomy' at default settings. Training databases were GreenGenes v13.8 (DeSantis et al., 2006), Pr2 v4.14 (Vaulot, 2021) and Unite v7.2 (Kõljalg et al., 2005) for 16S rRNA, 18S rRNA and ITS, respectively.

B.6 Measurement of river water parameters throughout material submersion

Values of parameters measured in the river throughout the submersion period.

Parameter	Week one	Week three	Week six
pH	7.96 ± 0.02	7.79 ± 0.10	7.40 ± 0.12
Alkalinity	159.33 ± 0.58	134.00 ± 1.00	137.67 ± 0.58
Dissolved oxygen (mg/L)	9.10 ± 0.13	9.94 ± 0.24	8.66 ± 0.02
Conductivity (µS/cm)	653.00 ± 1.00	536.00 ± 4.00	641.33 ± 8.62
Nitrate & nitrite-N (mg/L)	4.95 ± 2.11	4.33 ± 2.09	5.62 ± 0.36
Ammonium-N (mg/L)	0.07 ± 0.02	0.04 ± 0.02	0.07 ± 0.02
Phosphate-P (mg/L)	0.10 ± 0.02	0.06 ± 0.02	0.04 ± 0.01



Water temperature recorded in the river throughout the sample submersion period.

Alkalinity was measured using a HANNA Freshwater Alkalinity Colorimeter (HI-775). Conductivity was measured using a Hanna HI9033 Multi Range Meter, pH was measured using a Fisher Scientific accumet AP72 meter and dissolved oxygen was measured using a YSI Pro20 meter. Nutrients were measured using a SEAL continuous flow Autoanalyzer 3 HR. Three replicate measurements were made for each of these parameters at each time point. Temperature was monitored using loggers (HOBO MX2202), which were attached the centre of rafts placed in the river and were configured to log every 30 minutes.

B.7 Statistical analysis

Details of statistical analyses carried out.

Endpoint comparison	Transformation	Test
Biofilm weight/mm^2	Square-root	Two-way ANOVA with material and week as factors
Total pigment content (ng/mg.biofilm)	Natural logarithm	Two-way ANOVA with material and week as factors
% of pigment that is pheophytin	None	Two-way ANOVA with material and week as factors
16s Shannon diversity for biofilm samples	None	Two-way ANOVA with material and week as factors
18s Shannon diversity for biofilm samples	None	Two-way ANOVA with material and week as factors
ITS Shannon diversity for biofilm samples	None	Two-way ANOVA with material and week as factors
16s Shannon diversity for biofilm vs. water	None (normality could not be achieved)	Non-parametric aligned ranks ANOVA with material and week as factors
18s Shannon diversity for biofilm vs. water	None	Two-way ANOVA with material and week as factors

ITS Shannon diversity for biofilm vs. water	None (normality could not be achieved)	Non-parametric aligned ranks ANOVA with material and week as factors
Order level differences in Rhizobiales, Bacillariophyta	None	Two-way ANOVA with material and weeks as factors
Order level differences in Flavobacteriales, Myxoccales	Square-root	Two-way ANOVA with material and weeks as factors
Genus level differences in *Hyphomicrobium*, *Novosphingobium*, *Aquabacterium*, *Ideonella*, *Xenophilius*, *Variovorax*	None	Two-way ANOVA with material and weeks as factors
Genus level differences in *Pirellula*, *Flavobacterium*	Square-root	Two-way ANOVA with material and weeks as factors
Genus level differences in *Polaromonas*	None (normality could not be achieved)	Non-parametric aligned ranks ANOVA with material and week as factors

Results of beta-disperal tests. Values in bold are where significant differences in dispersion between treatments was identified, however, in all of these examples the location (x-y) effect was still visually obvious and it was therefore deemed that the results of these PERMANOVAS could still be interpreted.

	Comparison	beta-dispersal value
Bacteria	Materials within week 1	0.313
	Materials within week 3	0.124
	Materials within week 6	0.421
	Weeks within LDPE	0.856
	Weeks within PLA	0.579
	Weeks within Glass	0.88
Eukaryotes	Materials within week 1	0.74
	Materials within week 3	0.257
	Materials within week 6	0.639
	Weeks within LDPE	0.32
	Weeks within PLA	**0.025**
	Weeks within Glass	**0.005**
Fungi	Materials within week 1	0.473
	Materials within week 3	0.503
	Materials within week 6	0.452
	Weeks within LDPE	**0.003**
	Weeks within PLA	0.611
	Weeks within Glass	**0.003**

B.8 Methods and list of specific taxa of interest which were investigated

List of specific (pathogenic, plastic degrading or xenobiotic degrading) taxa of interest investigated and the literature which motivated their investigation.

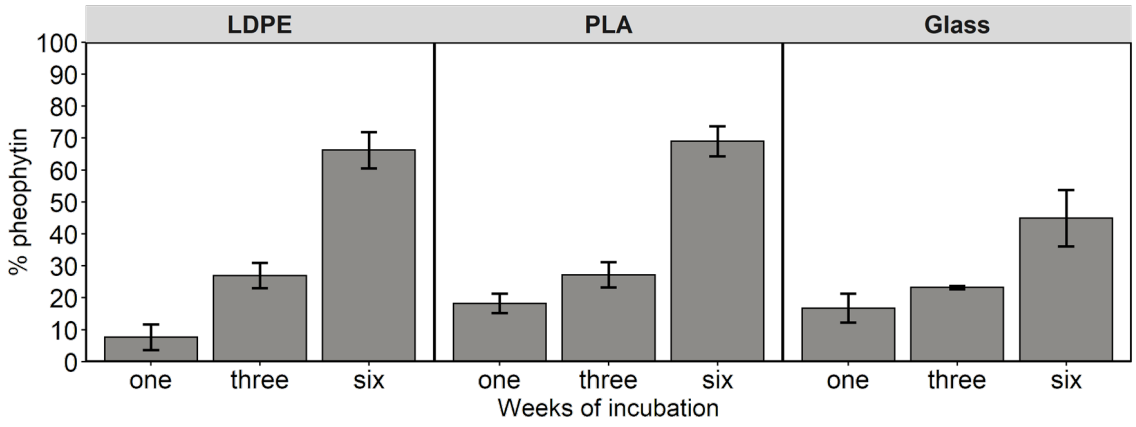
Taxa investigated	Literature motivating investigation	Present at >1% RA on at least two samples?	Significant difference?
Flavobacterium	(Gong et al., 2019)	**Yes**	**Yes**
Ideonella	(Morohoshi et al., 2018)	**Yes**	**Yes**
Acidovorax	(Morohoshi et al., 2018)	**Yes**	No
Undibacterium	(Morohoshi et al., 2018)	No	No
Chitinimonas	(Morohoshi et al., 2018)	No	No
Variovorax	(Eriksson et al., 2002; Posman et al., 2017)	**Yes**	**Yes**
Polaromonas	(Eriksson et al., 2002)	**Yes**	**Yes**
Sphingomonas	(Eriksson et al., 2002)	No	No
Alcaligenes	(Eriksson et al., 2002)	No	No
Caulobacter	(Eriksson et al., 2002)	No	No
Xenophilus	(Bai et al., 2020)	**Yes**	**Yes**
Rhodococcus	(Bai et al., 2020)	No	No
Niabella	(Bai et al., 2020)	No	No
Sphingopyxis	(Bai et al., 2020)	No	No
Achromobacter	(Bai et al., 2020)	No	No
Tahibacter	(Bai et al., 2020)	No	No
Pseudomonas	(Kelly et al., 2021)	No	No
Klebsiella	(Kelly et al., 2021)	No	No
Acinetobacter	(Kelly et al., 2021)	No	No
Bacillus	(Wu et al., 2020)	No	No
Aquabacterium	(Kelly et al., 2021)	**Yes**	**Yes**

Eukaryotic photosynthetic taxa identified to be DMS-producing in Bechard and Rayburn (1979) and Steinke et al. (2018) were also investigated. These included *Synechococcus* sp., *Oscillatoria* sp., *Nostoc* sp., *Spirogyra* sp., *Oedogonium* sp., *Chlorella* sp. and *Scenedesmus* sp. None of these taxa were found to be present at > 1 % relative abundance on more than one sample.

Bacterial taxa which have been identified for their potential to contain a gene functionally similar to the *mddA* gene, which facilitates DMSP-independent DMS production in terrestrial and freshwater environments, were also investigated. These were outlined by Carrión et al. (2015) and included the taxa: *Mycobacterium, Gordonia, Catelliglobosispora, Rhodococcus, Bradyrhizobium,* ***Hyphomicrobium****, Afipia, Dyella, Methylobacterium, Robiginitomaculum, Maricaulis, Mesorhizobium, Rhodopseudomonas, Hirschia, Nodosilinea, Cyanothece, Pseudanabaena, Opitutus, Thioalkalivibrio,* ***Pirellula****, Blastopirellula, Cycloclasticus, Nevskia, Trichodesmium, Leptolyngbya, Crocosphaera, Maribacter, Henriciella,* ***Novosphingobium****, Nocardioides, Terracoccus, Actinoplanes, Nocardia, Kangiella, Massilia, Leptospira, Congregibacter, Glaciecola, Hahella, Sulfuricurvum, Sulfurovum, Nitratifractor*. Those in bold were present at a > 1 % relative abundance on at least two samples and were tested for significant differences across time and materials.

The presence of the benthic diatom *Achnanthes biasolettiana* and the green algae *Ulothrix fimbriata* which are known to produce odorous compounds which attract grazing gastropods (Fink, 2007) was investigated but were not found to be present within biofilms.

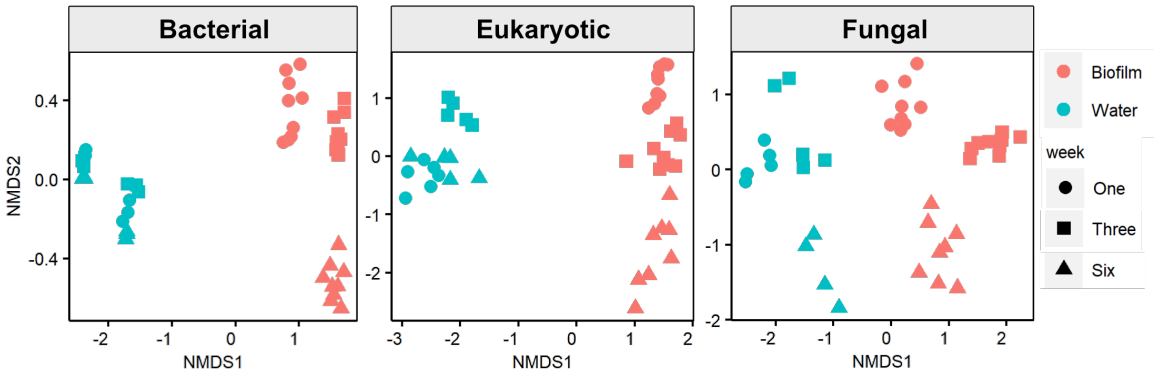
B.9 Proportion of pheophytin on samples



Percentage pheophytin pigment on glass, LDPE and PLA samples.

B.10 Comparison of free-living and biofilm-forming species composition

The free-living water community composition showed a clear separation from the biofilm community for bacterial, eukaryotic and fungal groups with a significant difference present for all taxa at all weeks ($p < 0.05$ for all). For bacteria and eukaryotes, sample type (biofilm *vs.* water) explained 51 – 77 % of the community variation observed; for fungi this was between 56 – 57 % in week one and week three and 38 % in week six. The Shannon-Wiener diversity of communities also differed between water and biofilms, with biofilms showing a significantly higher bacterial diversity ($p < 0.001$ for all), but a significantly lower eukaryotic diversity ($p < 0.001$ for all) than water in all weeks. The fungal community showed a significantly lower diversity in biofilms in week three and six ($p < 0.01$) but no significant differences in week one. Following the establishment of clear differences between water and biofilm communities, water samples were excluded from subsequent analyses to focus on biofilm community characteristics.



NMDS plots of free-living water community compositions compared to biofilm community compositions for bacterial, eukaryotic and fungal taxa.

B.11 Statistical details for Shannon-Wiener diversity analysis

Significance (p) values for all statistical contrast tests for Shannon-Wiener diversity tests.

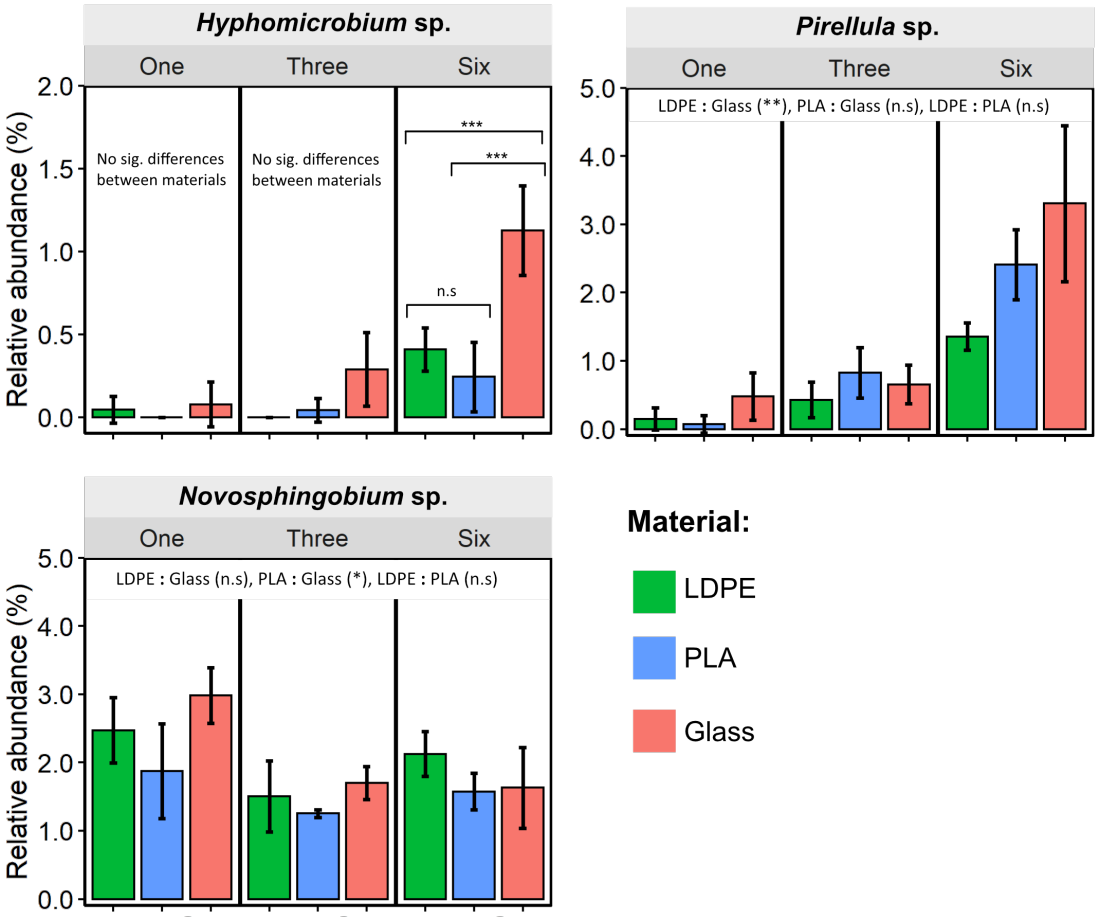
Contrast comparison	**Adjusted p-value**
Bacterial (significant main effect of week)	
Week One – Week Three	**0.009**
Week Three – Week Six	**0.049**
Eukaryotic (significant interaction effect)	
LDPE:One – Glass:One	0.072
PLA:One – Glass:One	0.339
PLA:One – LDPE:One	0.987
LDPE:Three – Glass:Three	0.999
PLA:Three – Glass:Three	0.733
PLA:Three – LDPE:Three	0.880
LDPE:Six – Glass:Six	0.539
PLA:Six – Glass:Six	0.082
PLA:Six – LDPE:Six	0.894
Glass:Three – Glass:One	0.999
LDPE:Three – LDPE:One	0.077
PLA:Three – PLA:One	**0.031**
Glass:Three – Glass:Six	$<$**0.001**
LDPE:Three – LDPE:Six	$<$**0.001**
PLA:Three – PLA:Six	$<$**0.001**
Fungal (significant main effect of week)	
Week One – Week Three	**0.009**
Week Three – Week Six	0.976

B.12 Linear discriminant effect size analysis (LEfSe) analysis details

Number of bacterial, eukaryotic and fungal ASVs that were identified to significantly discriminate (at the 5 % level) for material type or week with a Linear discriminant analysis Effect Size (LEfSe) analysis. First number shown it the total number of significant discriminatory ASVs, the number shown in brackets is the number of the discriminated ASVs which had > 1 % relative abundance on the discriminated feature.

		Number of discriminatory ASVs		
		Bacteria	**Eukaryote**	**Fungi**
Week one	LDPE	7 (6)	3 (0)	-
	PLA	17 (6)	0 (0)	-
	Glass	11 (2)	1 (0)	-
Week three	LDPE	5 (0)	10 (6)	1 (1)
	PLA	2 (1)	4 (0)	2 (1)
	Glass	4 (1)	4 (3)	1 (1)
Week six	LDPE	6 (3)	-	-
	PLA	10 (5)	-	-
	Glass	7 (0)	-	-
LDPE	One	17 (11)	24 (13)	4 (3)
	Three	17 (7)	27 (14)	3 (3)
	Six	21 (9)	5 (4)	4 (2)
PLA	One	14 (5)	18 (12)	7 (6)
	Three	15 (10)	26 (8)	7 (6)
	Six	26 (12)	3 (3)	1 (1)
Glass	One	18 (8)	4 (4)	1 (0)
	Three	21 (11)	8 (4)	2 (2)
	Six	20 (7)	0 (0)	1 (1)

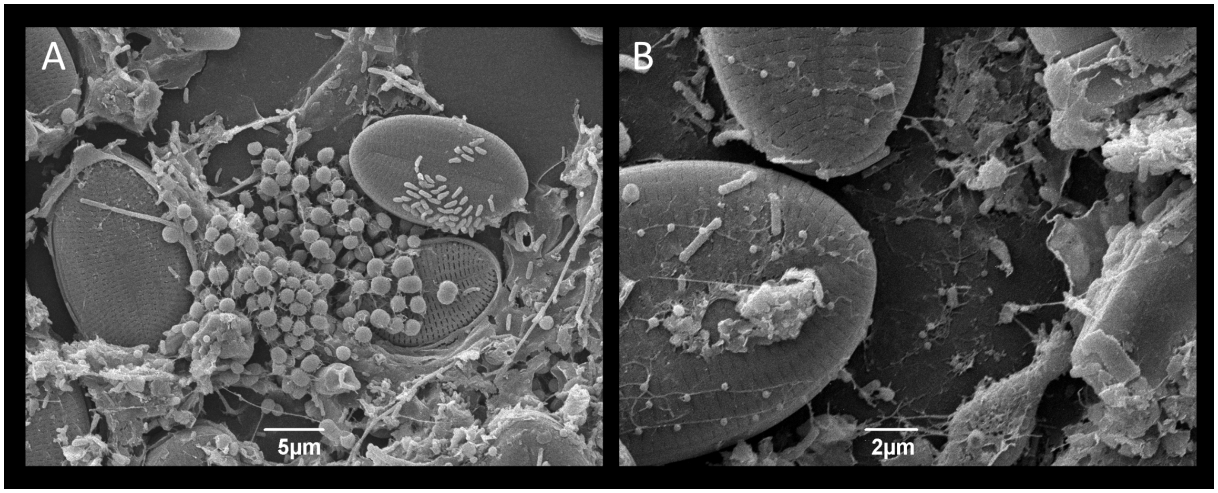
B.13 Presence of potential dimethyl sulfide producing species



Relative abundance of three bacteria genera which have been linked to their potential genetic ability to produce the odour compound dimethyl sulfide.

B.14 Examples of inter-domain interactions within plastic biofilms

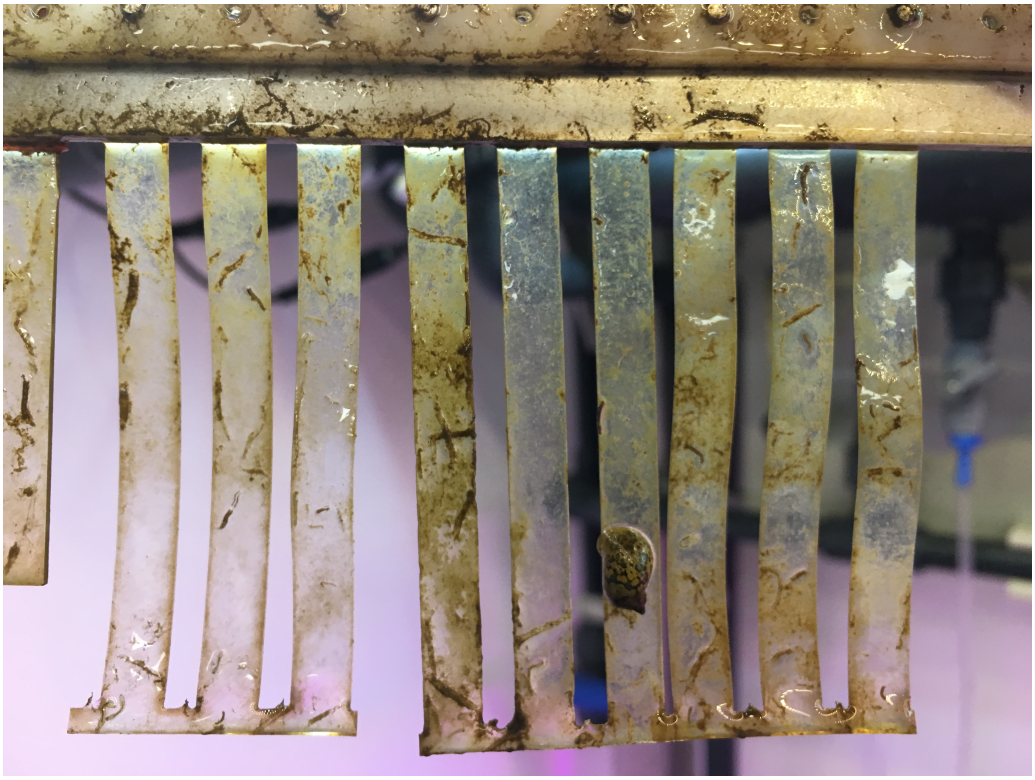
Scanning electron micrograph image of inter-domain interactions occurring between diatoms and bacteria within biofilms attached to low-density polyethylene (left) and polylactic acid (right) after three weeks of submersion in a UK river.



B.15 Images and videos of invertebrates qualitatively identified as being present within or in close association with plastic biofilms

Videos of chironomid larvae and black fly pupa within plastic biofilms can be seen at: https://figshare.com/s/6de9f6897858c6a91e33 or by scanning the QR code.

Adult fly, identified as a chironomid, which emerged from plastic surfaces.

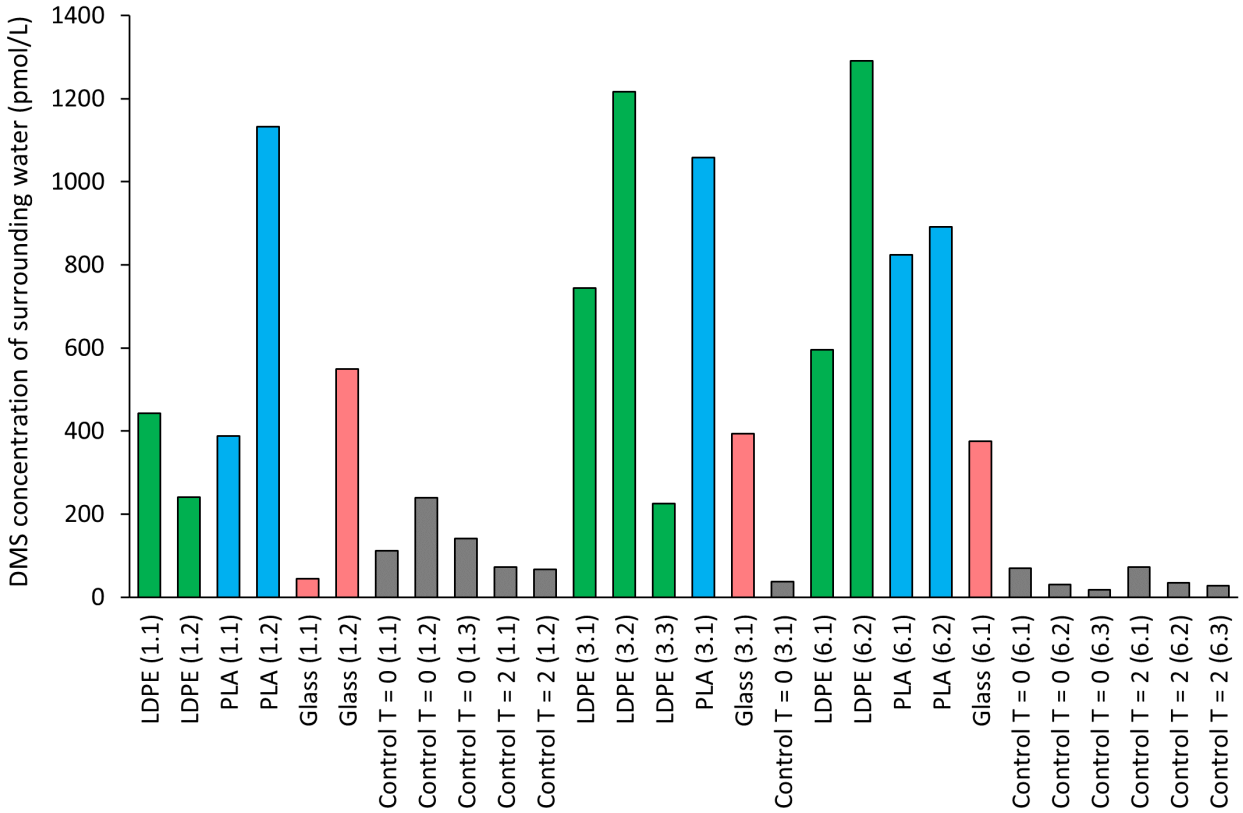


Tube structures found to be common on sample surfaces and Physidae snail found associated with samples.

Appendix C

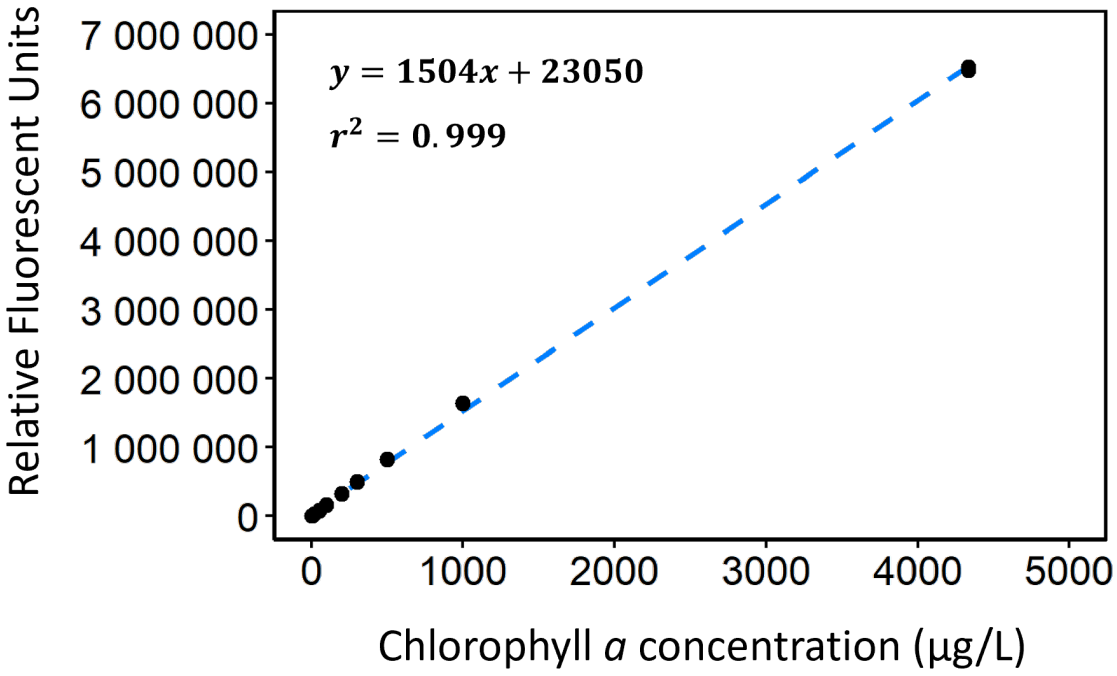
Appendix for Chapter 3

C.1 Preliminary detection of DMS



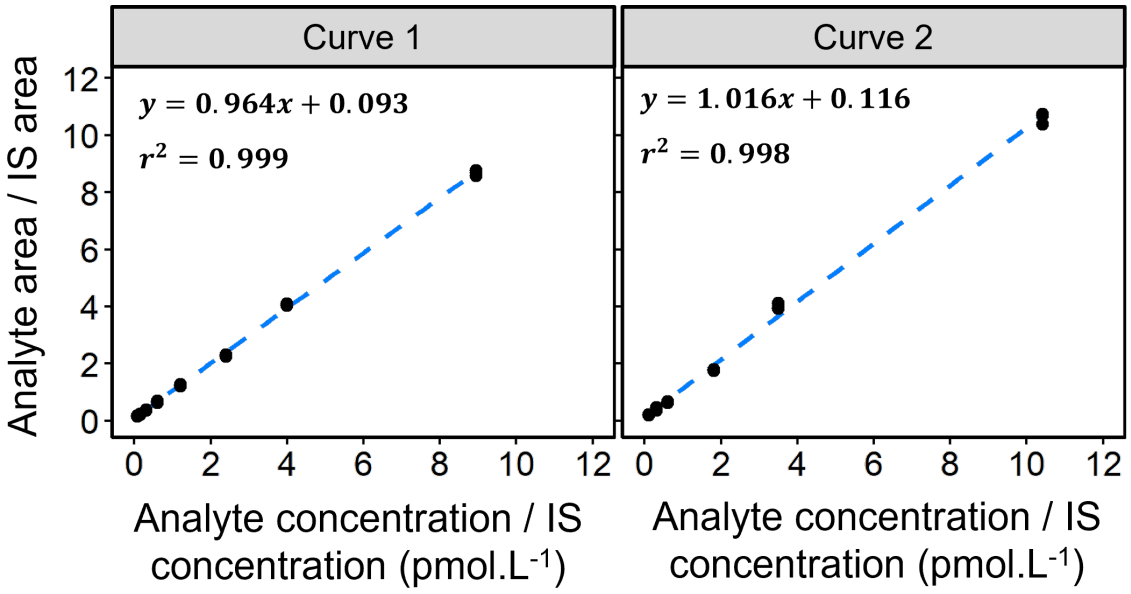
Preliminary detection of DMS in water which has been in close contact with microbially colonised low-density polyethylene (LDPE) and polylactic acid (PLA) films after either one, three or six weeks of submersion in a UK river. Plastic films were removed from the river and incubated in sterile river water for 48 hours and measured in the same way as described in the main text. Each bar shows one replicate, 1.1 denotes replicate one for one week of incubation. Control bars indicate water treated identically to treatment water but where no microbially colonised plastic films had been submerged. T = 0 controls shows water which was measured prior to the 48 hours incubation, T = 2 controls show water which was measured after the 48 hours incubation.

C.2 Calibration curve used to calculate the chlorophyll *a* and pheophytin concentration of biofilm samples



Calibration curve used to calculate the chlorophyll *a* and pheophytin concentration of biofilm samples. Detailed methods and equations used for these calculations are given in Chapter 2 and Appendix B.3

C.3 Calibration curves used to calculate the DMS concentration



Calibration curves used to calculate the DMS concentration of samples. Curve 1 was used for week three samples, curve 2 was used for week 6 samples and virgin control samples. Line of best fit is shown with the dashed line, equations of the line and the r^2 value are given on each plot.

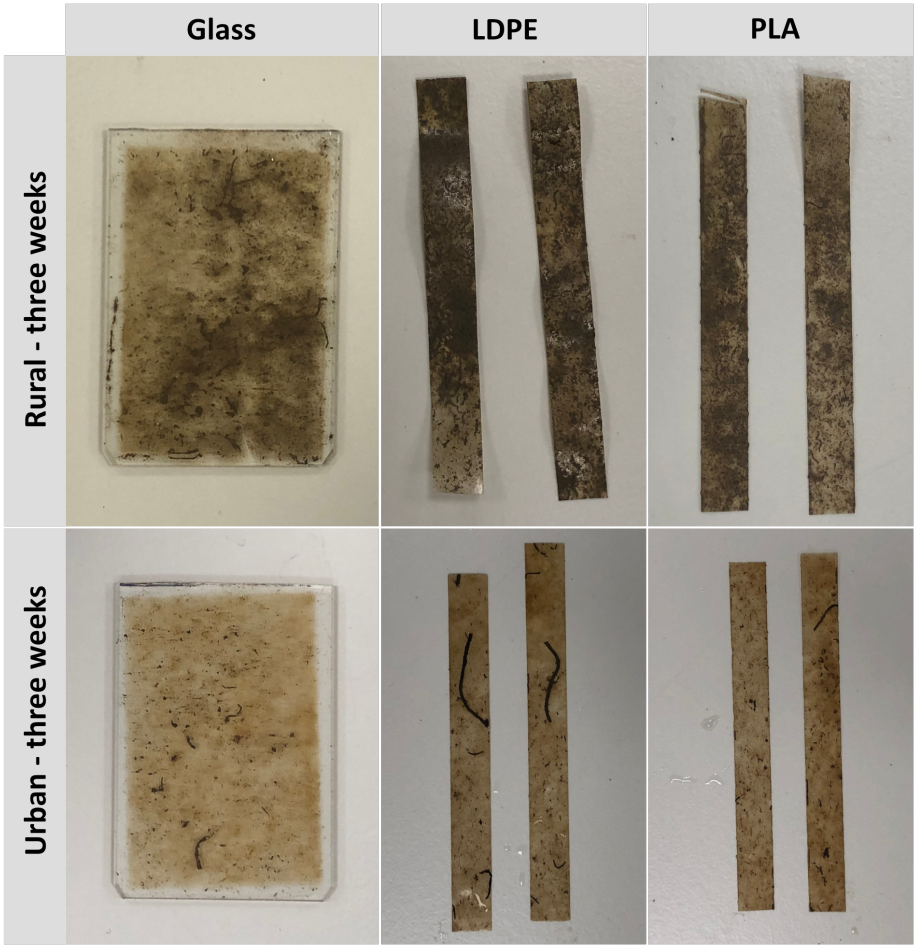
C.4 Details of statistical analyses carried out

Outline of the statistical tests and transformations carried out in the study. For tests where a non-parametric aligned ranks ANOVA was used, data did not meet either normality or equal variance after transformation.

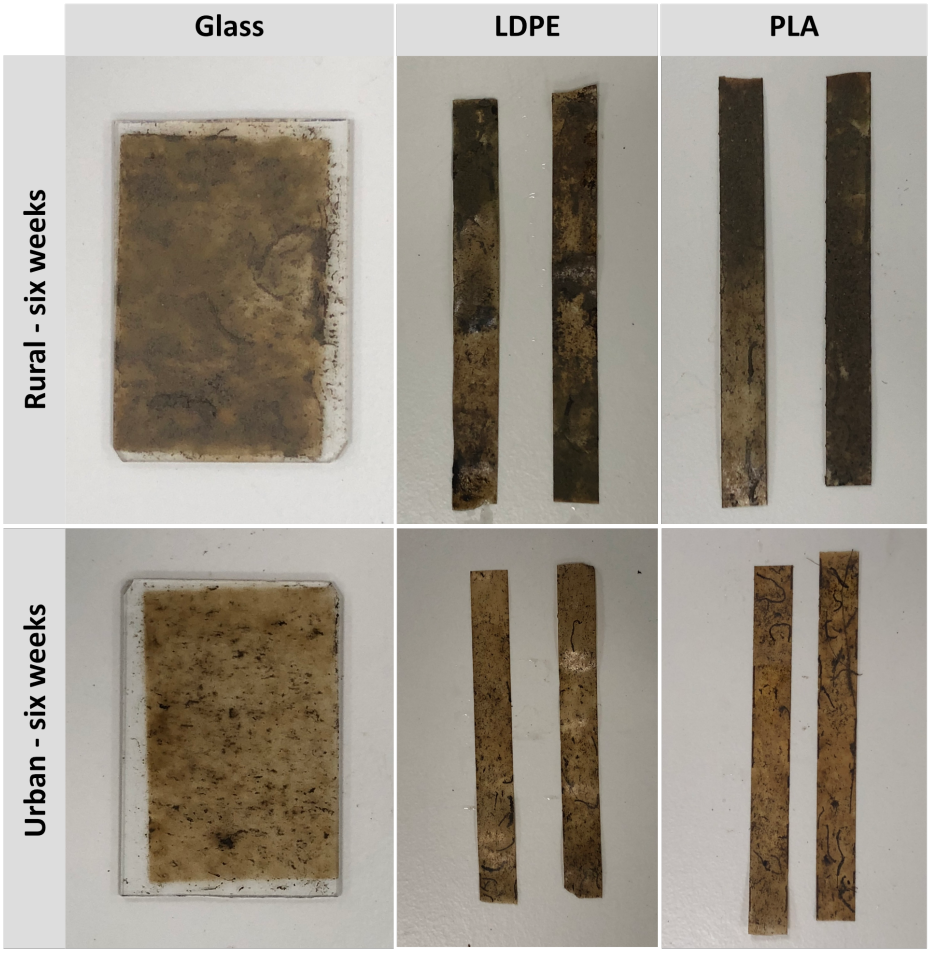
Endpoint comparison	Transformation	Test
DMS/cm^2: comparing material type and location week 3 + interaction effect	Square-root	Two-way ANOVA
DMS/cm^2: comparing material type and location week 6 + interaction effect	Square-root	Two-way ANOVA
Biofilm weight/cm^2: comparing material type and location + interaction effect for week 3	None	Non-parametric, aligned ranks ANOVA (ARTools package)
Biofilm weight/cm^2: comparing material type and location + interaction effect for week 6	None	Non-parametric, aligned ranks ANOVA (ARTools package)

DMS produced per mg of biofilm: comparing material type and locations + interaction effect for week 3	Square-root	Two-way ANOVA
DMS produced per mg of biofilm: comparing material type and locations + interaction effect for week 6	Square-root	Two-way ANOVA
Total pigment content per mg of biofilm: comparing material type and locations + interaction effect for week 3	None	Non-parametric, aligned ranks ANOVA (ARTools package)
Total pigment content per mg of biofilm: comparing material type and locations + interaction effect for week 6	None	Non-parametric, aligned ranks ANOVA (ARTools package)
% of total pigment which was pheophytin: comparing material type and locations + interaction effect for week 3	None	Non-parametric, aligned ranks ANOVA (ARTools package)
% of total pigment which was pheophytin: comparing material type and locations + interaction effect for week 6	None	Non-parametric, aligned ranks ANOVA (ARTools package)

C.5 Example images of materials after submersion in the river

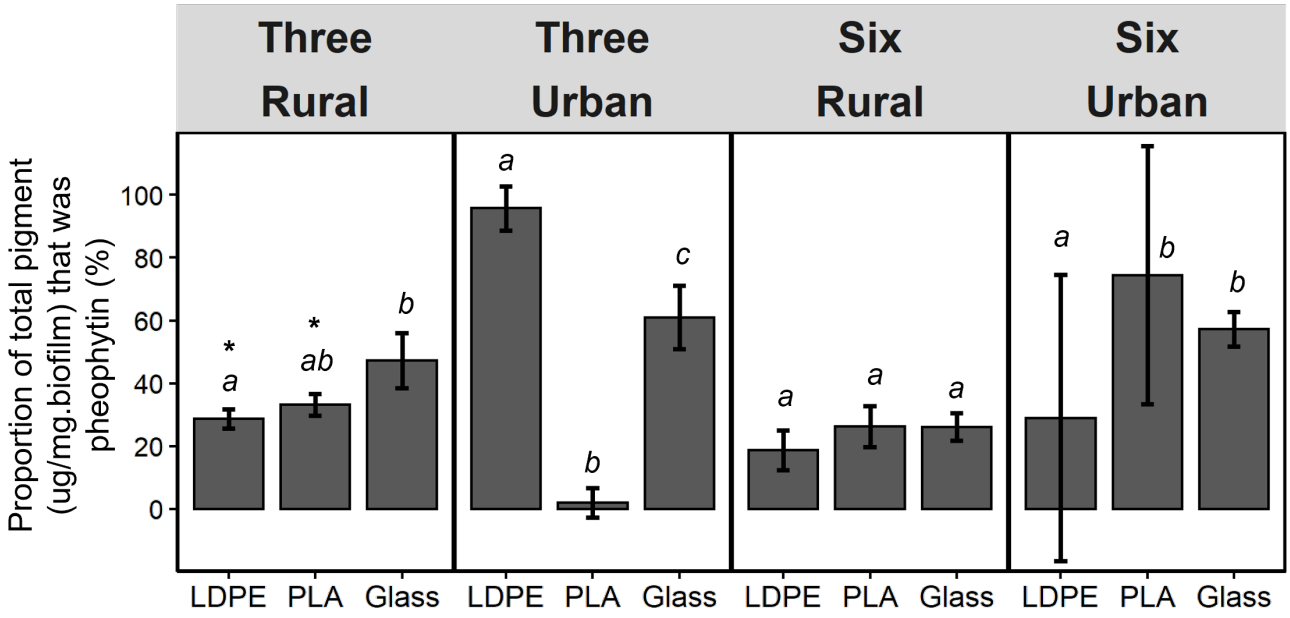


Examples of low-density polyethylene (LDPE), polylactic acid (PLA) and glass sample coupons after submersion in either a rural or urban river for three weeks.



Examples of low-density polyethylene (LDPE), polylactic acid (PLA) and glass sample coupons after submersion in either a rural or urban river for six weeks.

C.6 Proportion of pheophytin pigment in biofilms

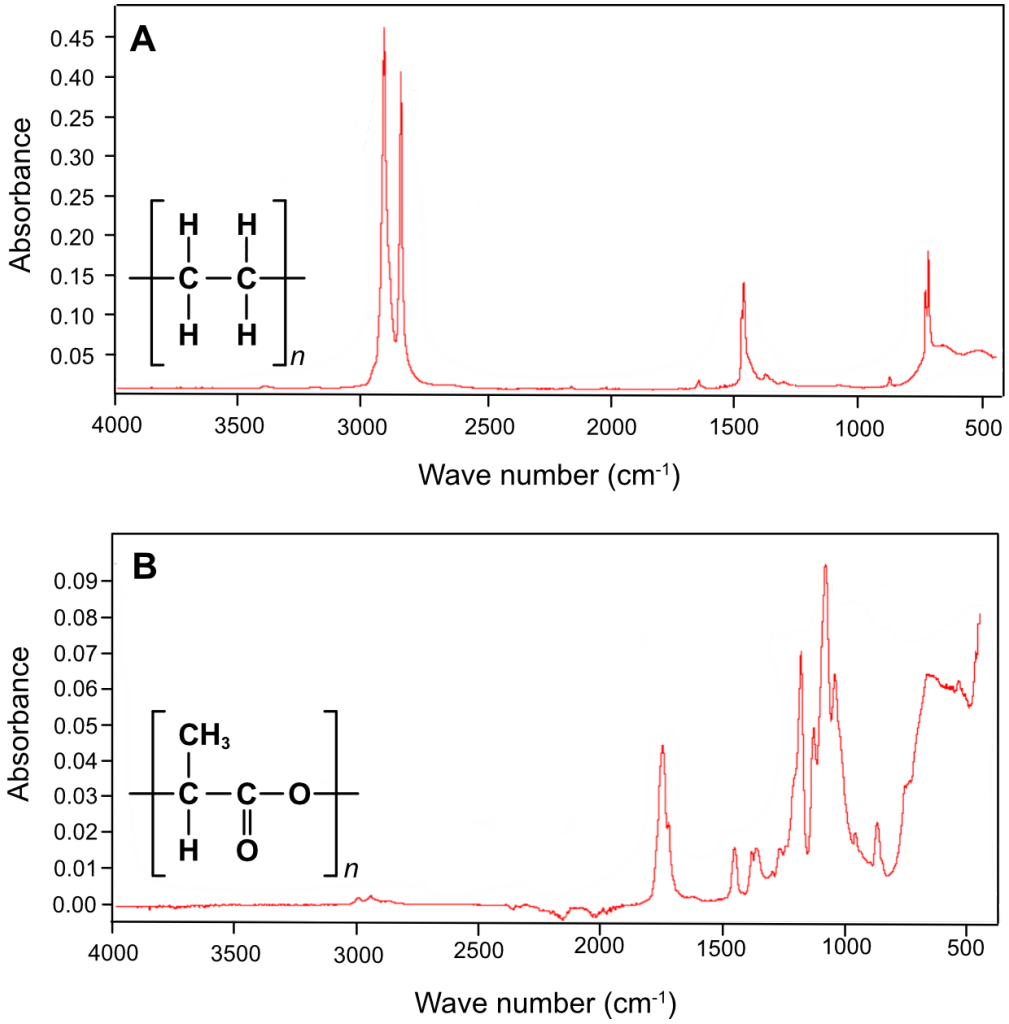


Proportion (%) of the total photosynthetic pigment content per mg.biofilm that was pheophytin on low-density polyethylene (LDPE), polylactic acid (PLA) and glass samples. Letters above bars indicate significant differences between materials within each individual subplot only, asterisk (*) above bars in rural plots indicates that the amount of DMS produced by these materials differed significantly from the amount produced by the same material in the urban location after the same incubation time.

Appendix D

Appendix for Chapter 4

D.1 Fourier transform infrared spectra for plastic films used in experimental work



Fourier transform infrared (FTIR) spectra of virgin LDPE (A) and PLA (B) film material used in this study. Spectra were collected at The Centre for Ecology and Hydrology, Wallingford, with an imaging μFTIR spectrometer (PerkinElmer Spotlight 400) set to collect spectra in the range between 4000 and 700 cm^{-1} wave numbers. LDPE film was found to closely match the spectra obtained from consumer LDPE material given in (Huppertsberg & Knepper, 2020). PLA film was found to closely match spectra obtained from pure PLA pellets given in Yuniarto et al. (2016) and material identity was therefore confirmed.

D.2 Weight of biofilms attached to plastic surfaces

Average weight change of dried LDPE and PLA coupons before and after surface brushing to remove attached biofilms. To ensure that weight changes in colonised samples that had been incubated in a river for three weeks was due to the biofilm weight, the same methods were also applied to control virgin samples which had been incubated in sterile Milli-Q water for the same time period and did not have a microbial biofilm on their surface. Coupons of colonised and virgin plastic were dried in an oven at 30°C for three hours before being weighed using an analytical balance. The biofilm was then removed from samples using a soft natural bristle brush and Milli-Q water; samples were checked under the microscope to ensure complete removal of the biofilm. The surface of control samples was brushed in the same way to replicate methods. Coupons were then re-dried and re-weighed and the change in weight per unit area was calculated. One virgin LDPE and one virgin PLA sample showed a weight decrease of 0.0001 grams and one virgin PLA sample showed an increase of 0.0001 grams with all other virgin samples maintaining the same weight. Weight changes in colonised samples are therefore determined to be due to the weight of the attached biofilm.

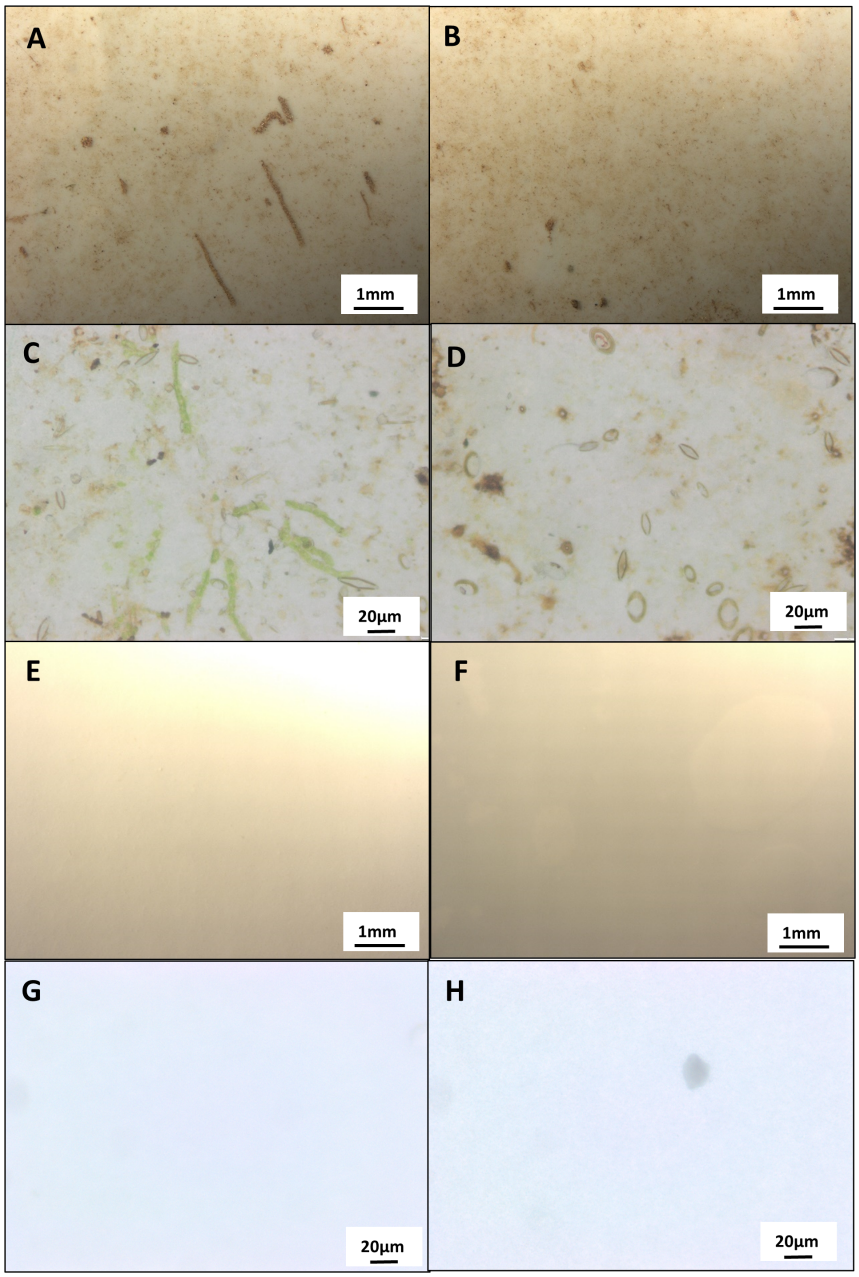
Sample	Average weight change (µg/cm2)
Colonised LDPE	120 ± 44.72
Virgin LDPE	2.69 ± 6.01
Colonised PLA	73.3 ± 22.36
Virgin PLA	-0.05 ± 9.64

D.3 Details of statistical analyses

Details of all statistical tests carried out for the three experiments in the study.

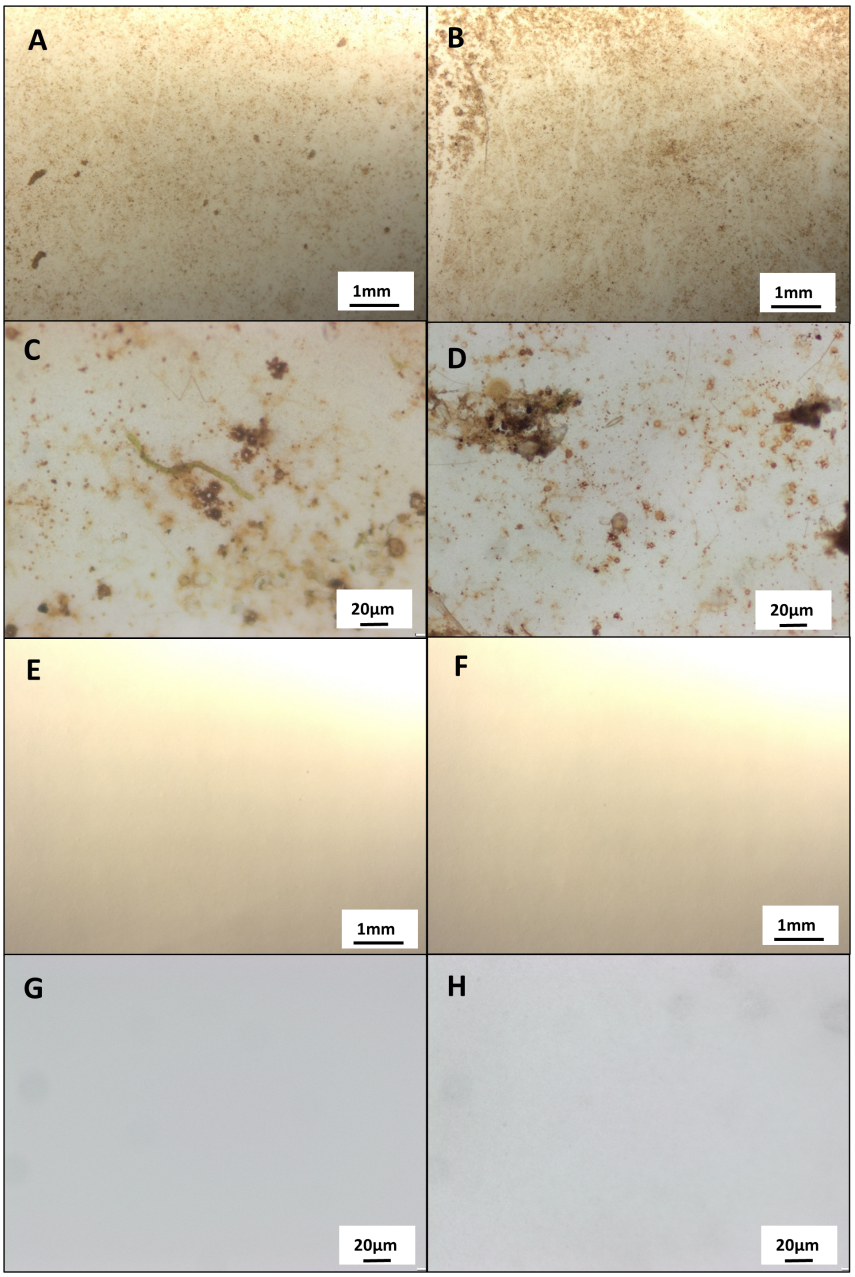
Comparison	Details	Test
	Plastic disc area	
Virgin LDPE: Gammarus *vs.* no Gammarus	Data passed Shapiro-wilk normality but not equal variance even after transformation	Welch's t-test
Colonised LDPE: Gammarus *vs.* no Gammarus	Data passed Shapiro-wilk normality and equal variance. Performed Welch's t-test to maintain consistency and comparable results	Welch's t-test
Virgin PLA: Gammarus *vs.* no Gammarus	Data passed Shapiro-wilk normality and equal variance. Performed Welch's t-test to maintain consistency and comparable results	Welch's t-test
Colonised PLA: Gammarus *vs.* no Gammarus	Data passed Shapiro-wilk normality and equal variance. Performed Welch's t-test to maintain consistency and comparable results	Welch's t-test
	Leaf disc area	
Area of leaf dics between different treatments after three-day exposure period	Data did not pass Shapiro-wilk normality test even after transforming data using square root and cube root functions.	Kruskal Wallis test. Followed by a post hoc pairwise Wilcoxon test with a Benjamini-Hochberg (BH) adjustment for multiple comparisons
	Behaviour experiment	
Time spent on leaf between all three treatments	Normality of residuals could not be achieved so non-parametric test conducted	Kruskal Wallis test
Number of visits to leaf between all three treatments	Normality was achieved though a cube root transformation. However, normality could not be achieved for the number of plastic visits data. To maintain consistency and allow better comparisons a non-parametric test has been carried out for both comparisons.	Kruskal Wallis test
Time spent swimming between all three treatments	Transformation achieved normality but non-parametric test carried out to maintain consistency between statistical comparisons within the same experimental comparisons	Kruskal Wallis test
Time spent on plastic between LDPE choice and PLA choice treatments	Transformation achieved normality but non-parametric test carried out to maintain consistency between statistical comparisons within the same experimental comparisons	Kruskal Wallis test
Number of visits to plastic between LDPE choice and PLA choice treatments	Normality of residuals could not be achieved	Wilcoxon signed rank test

D.4 Images of virgin and microbially colonised low-density polyethylene films



Images of the surface of LDPE coupons used in *Gammarus* feeding and behaviour experiments. Colonised plastic which had been incubated in a river for three weeks is shown in images A-D and virgin control plastic soaked in sterile Milli-Q water for three weeks is shown in images E-H.

D.5 Images of virgin and microbially colonised polylactic acid films



Images of the surface of PLA coupons used in *Gammarus* feeding and behaviour experiments. Colonised plastic which had been incubated in a river for three weeks is shown in images A-D and virgin control plastic soaked in sterile Milli-Q water for three weeks is shown in images E-H

D.6 Area of plastic film discs in second feeding experiment

Average disc area ± standard deviation of virgin and colonised LDPE and PLA discs after five days exposure to either control conditions (with no *Gammarus pulex* present) or under treatment conditions with one *Gammarus pulex* individual present.

Treatment	Average area of disc after exposure ± standard deviation
Virgin LDPE – Control (No Gammarus)	751.1 ± 3.2
Virgin LDPE – Exposed to Gammarus	746.3 ± 8.7
Colonised LDPE – Control (No Gammarus)	740.2 ± 7.3
Colonised LDPE – Exposed to Gammarus	748.3 ± 11.9
Virgin PLA – Control (No Gammarus)	730.7 ± 7.5
Virgin PLA – Exposed to Gammarus	734.7 ± 5.6
Colonised PLA – Control (No Gammarus)	744.9 ± 12.6
Colonised PLA – Exposed to Gammarus	745.2 ± 10.1

D.7 Water quality parameter of river water

Water quality parameters of collected river water used throughout the study.

Water parameter	Value
Temperature	15°C
pH	8.04
Alkalinity	101 ppm
Conductivity	439 (µs/cm)

Appendix E

Appendix for Chapter 5

E.1 Images of original caddisfly cases

Examples of the original cases that *Agrypnia* sp. were removed from at the beginning of the experiment - built from fragmented flexible organic material such as leaves and other plant debris.

E.2 Modified algal pellet recipe

Algal pellets were made by boiling 12 g of nutrient agar with 500 ml deionized water before adding 15 g of cellulose and 15 g of food-grade Chlorella powder and 5 mg ascorbic acid. The pellet mixture was homogenised, poured into a glass dish, covered and then cooled for 30 minutes before being cut into uniform 0.95 g cylindrical pellets.

E.3 FTIR methods used to confirm visual identification of PLA fragments

To confirm that visual counting correctly identified PLA fragments, micro-Fourier transform infrared spectroscopy (μFTIR) was used to confirm the polymer identity of the white fragments. Both control samples (containing no added PLA) and samples from the exposure waters were analysed. Samples were thoroughly mixed by vigorously shaking for 10 seconds, then immediately deposited onto a 25 mm diameter 3 μm pore size silver membrane filter (Sterlitech, Washington USA) using a glass pipette. Where the entire sample could not be deposited, a sub-sample was determined by weight to 0.1 mg accuracy. All particles within the deposition area (about 11 x 11 mm) were identified and quantified with an imaging μFTIR spectrometer (PerkinElmer Spotlight 400) set to collect spectra in the range between 4000 and 700 cm^{-1} wave numbers. A background spectrum of the silver filter was collected prior to each sample and removed from resulting data. A first analysis scanned the whole area at a pixel size of 25 μm to give a reasonable compromise between resolution, processing time and resulting file size. The minimum particle size that can be enumerated in these scans is therefore 25 μm. A higher resolution scan on selected areas of the filter was then also performed at 6.25 μm pixel size (the lowest resolution pixel size on the Spotlight FTIR system, and near the theoretical spatial limits of conventional FTIR imaging), enabling the imaging and enumeration of particles smaller than 25 μm. All mapping was carried out at a resolution of 8 cm^{-1}, with two scans per pixel, and an interferometer speed of 2.2 cm/s. The generated spectra were analysed using the siMPle software (http://simple-plastics.eu). Spectra were matched against an expanded polymer database, as developed and described by (Primpke et al., 2018). Spectra were matched against the database at an initial Pearson's correlation coefficient value of 0.65 (where 1.0 is a perfect match and 0 is a complete mismatch) against the raw and first derivative spectra, with subsequent particle building thresholds of 0.4 and 0.3. This means that a lower level of certainty is accepted for a pixel that is adjacent to an already positively identified pixel of the same polymer.

E.4 Details of statistical analyses

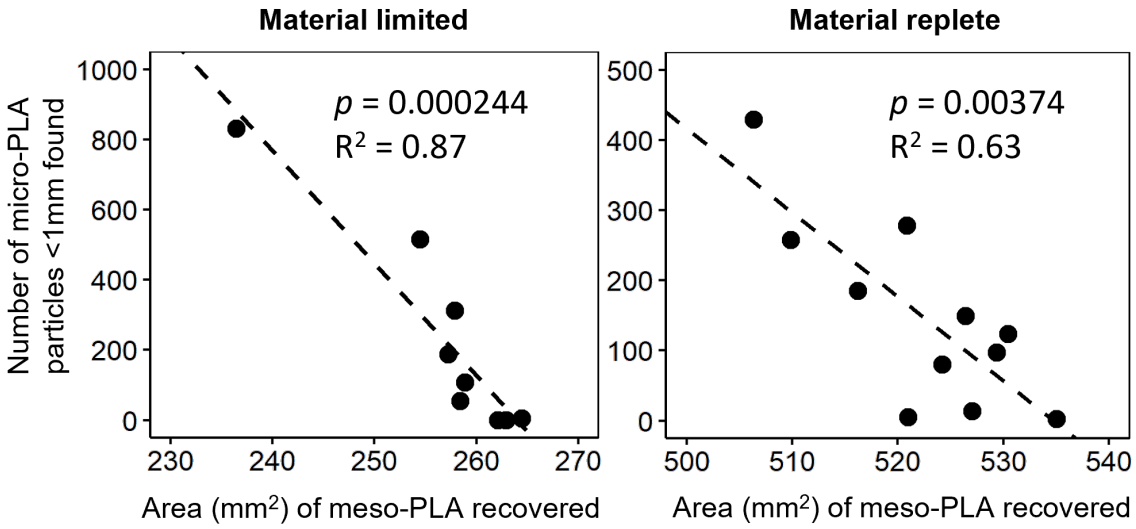
Details of all statistical tests carried out in the study and any transformation that were made to meet assumption of normality and equal variance.

Comparison	Transformation	Test
Old case length	None	Unpaired, two-tailed t-test (equal variance)
New case length	None (normality not achieved through transformation)	Mann-Whitney U
Surface area of total material used to build new case	None	Unpaired, two-tailed t-test (equal variance)
Wet weight of larvae at end	None	Unpaired, two-tailed t-test (equal variance)
Amount of plastic used compared to leaf within each treatment	None	Paired, two-tailed t-test (equal variance)
Amount of PLA used ML vs MR	None	Unpaired, two-tailed t-test (equal variance)
Amount of leaf used ML vs MR	None	Unpaired, two-tailed t-test (unequal variance)
Proportion of meso-PLA pieces that were intact between ML and MR	None	Unpaired, two-tailed t-test (equal variance)
Size of intact, ML chewed and MR chewed pieces	None (normality not achieved through transformation)	Kruskall Wallis, followed by a Dunn's post hoc test
Number of microplastics found between ML and MR	Square-root	Unpaired, two-tailed t-test (equal variance)
Regression analysis between number of PLA microparticles and surface area of meso PLA recovered ML treatment	None	Linear regression
Regression analysis between number of PLA microparticles and surface area of meso PLA recovered MR treatment	None	Linear regression
Regression analysis between number of PLA microparticles and larvae weight ML treatment	None	Linear regression
Regression analysis between number of PLA microparticles and larvae weight ML treatment	None	Linear regression
Difference between micro-particle size between treatments	None (normality not achieved through transformation)	Mann-Whitney U

E.5 Video of a *Agrypnia* sp. larva chewing and fragmenting polylactic acid film, before incorporating it into its protective case. Video was recorded for a larva outside of the main study, during preliminary trials. Video is edited together clips from a longer video which records this behaviour over a roughly 45 minute period and is shown at a playback speed of times three.

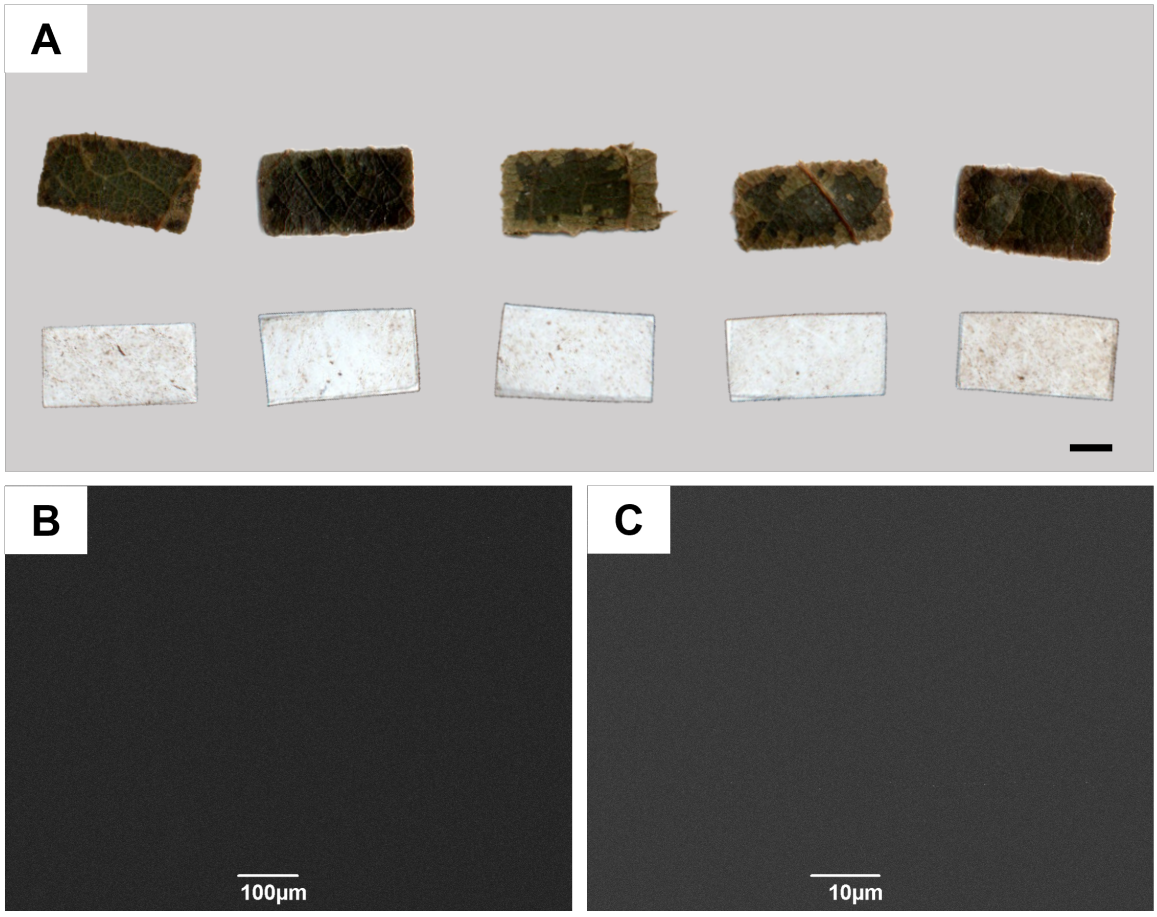
Video can be viewed at https://figshare.com/s/6de9f6897858c6a91e33 or by scanning the QR code.

E.6 Regression of microplastic number against remaining PLA surface area



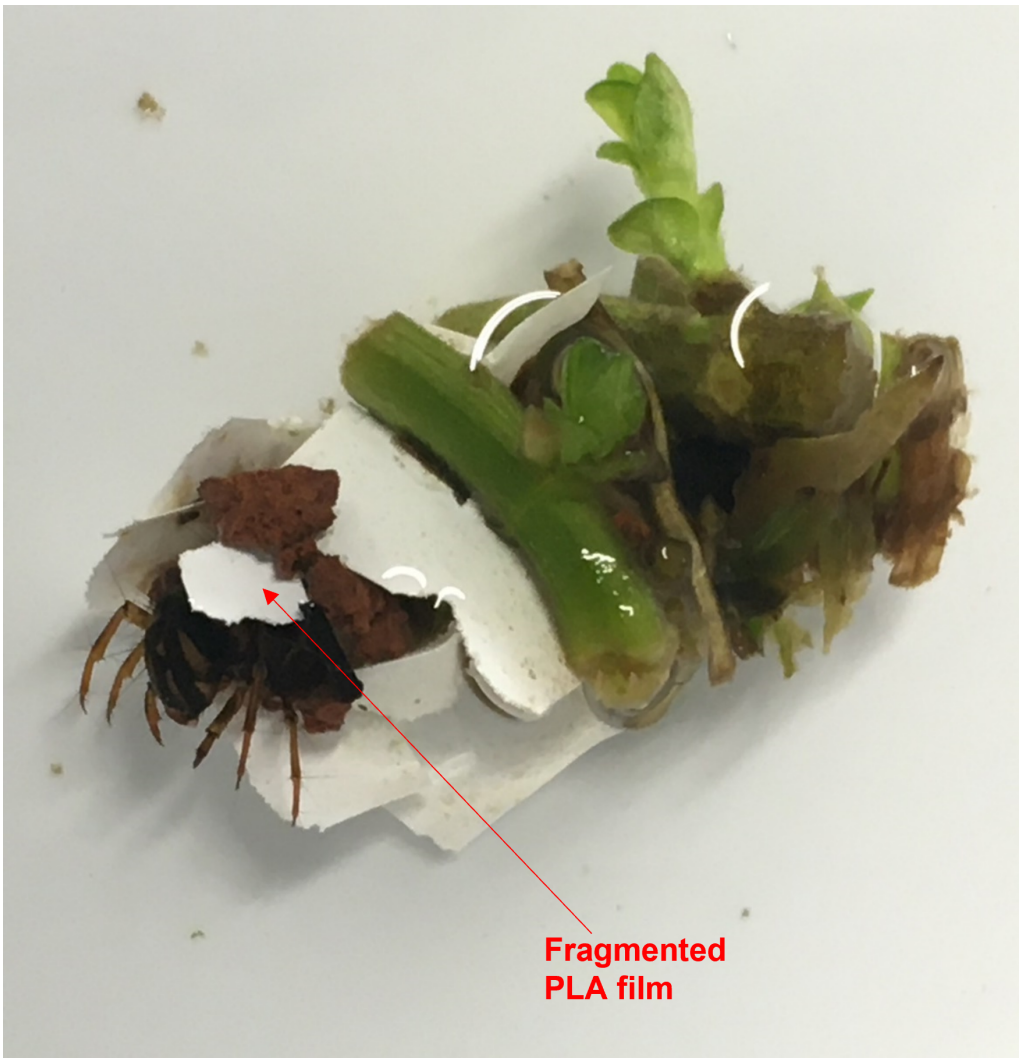
Number of micro-PLA particles found in the exposure water plotted against the total surface area of meso-PLA pieces recovered from the case and exposure jar for each replicate of material limited ($n = 9$) and material replete ($n = 11$) treatments. Significance (p) value of the regression test and R^2 values are given on each plot.

E.7 Images of polylactic acid film and leaf surfaces



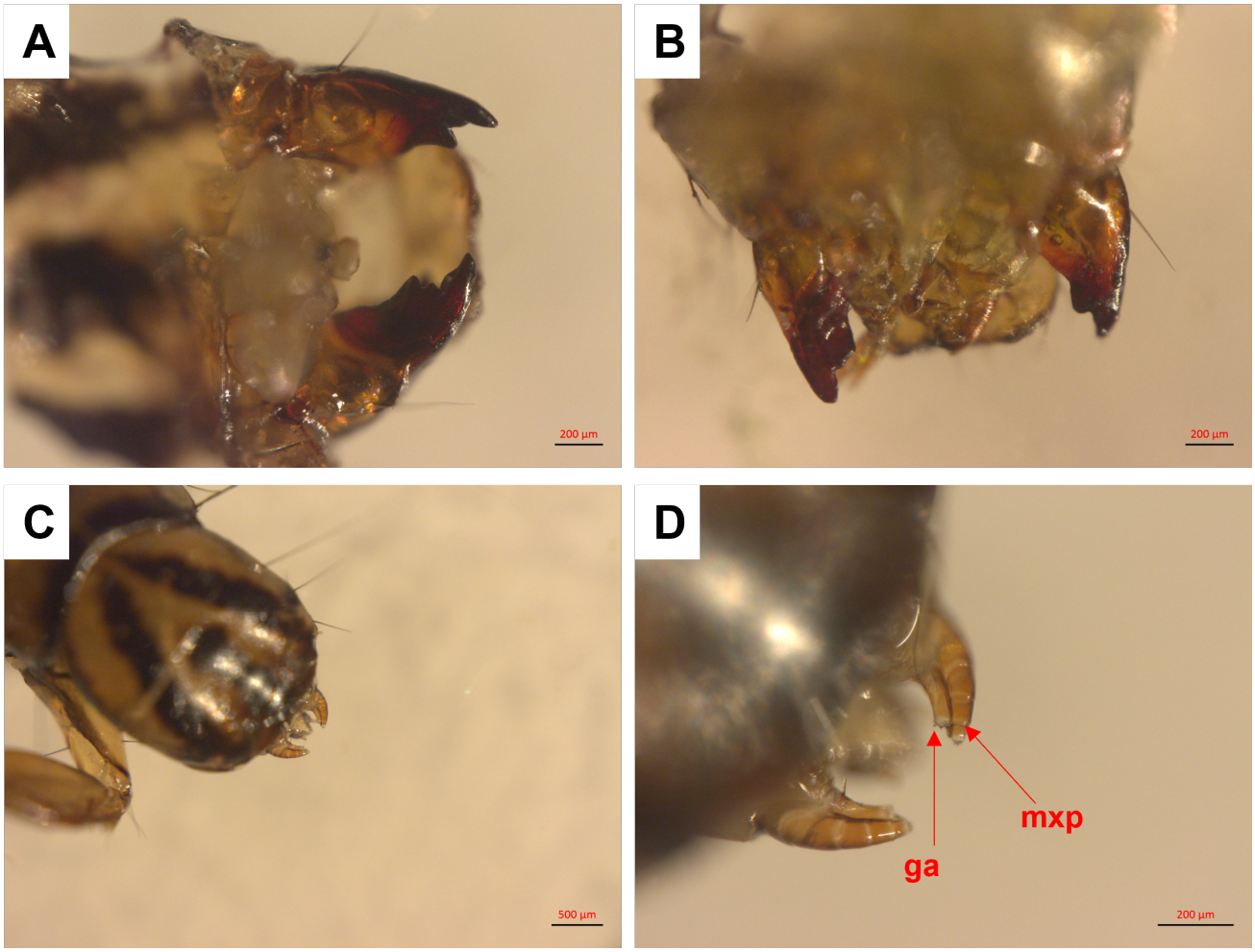
Images of polylactic acid film (PLA) and *Quercus robur* surfaces. (A) Coupons of PLA and *Q. robus* provided to caddisfly – note the textured surface of the *Q. robus* leaf material, scale bar = 10 mm. (B) & (C) Scanning electron microscope (SEM) images of the surface of virgin PLA. To be directly compared with the SEM image of the surface of mature *Q. robus* leaves presented in (Gülz & Boor, 1992) - Figure 5

E.8 Evidence of plastic use and fragmentation by other caddisfly species



Preliminary evidence of another caddisfly species, provisionally identified as *Limnephilus* sp., fragmenting and using PLA film in their case.

E.9 Feeding appendages of caddisfly larvae



Feeding appendages of *Agrypnia* sp. (A) & (B) show the serrated mandibles used to cut material. (C) & (D) show the maxillary palps (mxp) and galea (ga) which work in conjunction with the mandibles to sense and guide material.

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