PLASTIC POLLUTION IN THE HYPORHEIC ZONE: OCCURRENCE AND ITS INTERACTION WITH MICROORGANISMS

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Doctoral Dissertation
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ONESNAŽENJE S PLASTIKO V HIPOREIKU: POJAVLJANJE IN INTERAKCIJA Z MIKROORGANIZMI

Doktorska disertacija

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Očetu

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Abstract

Plastic pollution is a global issue, yet our understanding of its impact on organisms and ecosystems remains limited. My PhD thesis explores the presence of microplastics (MP) in river ecosystems, their interaction with freshwater hyporheic biofilms and specifically, the response of hyporheic biofilms to polyethylene terephthalate (PET) pollution in the laboratory and natural settings.

The thesis begins with assessing MP pollution in Slovenian rivers and reviewing global data on bacterial biodegradation of plastic. In small-scale Slovenian rivers (500 km^2 < catchment <800km^2, river length < 45km), MP pollution increased in the direction of flow and land use intensification. The results showed a prevalence of fibres in the water column and fragments in the sediments. The most frequent polymer types reported were polyethylene (PE) and polypropylene (PP) in both river compartments. Variability in MP colouration, size and shapes indicated diverse sources of MP pollution.

As part of a systematic review of 145 scientific papers, a list was made of all known strains of bacteria capable of degrading plastic. The main findings indicate that research on plastic biodegradation predominantly centres around polyethylene (PE) and its variants. Moreover, regarding biodegradation experiments, studies suggest that plastics should undergo pre-treatment, such as UV irradiation, to simulate natural environmental conditions better. They also highlight the lack of standardization and reporting consistency and the need for long-term studies.

A preliminary study was conducted on biodegrading different plastic materials in a low-carbon environment using activated sludge from a municipal wastewater treatment plant (WWTP). After two months, scanning electron microscopy (SEM) confirmed the presence of different biofilms on different plastic materials. This study was then followed by an examination of the interactions between plastics as a substrate and freshwater hyporheic biofilms as colonisers in the natural environment. The study focused on the one-month colonization and one-year seasonal changes of biofilm responses exposed to PET fibres. Different methods, such as respiratory electron transport system activity (ETSA), total protein content (TPC) and community-level physiological profiling (CLPP), were employed to characterise microbial community activity, biomass and metabolism. The study was then repeated, using the same methods but extending the time to two years and broadening the geographical scope to include four geomorphologically distinct locations. This study confirmed the inhibitory effect of PET on microbial biofilms in the hyporheic zone.

Finally, the effects of three different water regimes–flow, stagnant and unsaturated– on hyporheic biofilms under simultaneous pollution with PET fibres were investigated. Significant inhibitory effects of water regimes and PET pollution were observed for bacterial abundance and microbial metabolism on a specific substrate (CLPP) but not on microbial biomass (TPC) or activity (ETSA).

The knowledge acquired during this work enhances our understanding of MP pollution at local and global scales and its significance for hyporheic biofilms, which play a pivotal role in global nutrient cycling within freshwater environments. The study also highlighted

the spatial variability within the hyporheic zone as an important factor in hyporheic biofilm research.

Povzetek

Onesnaževanje s plastiko je svetovni problem, vendar je naše razumevanje njenega vpliva na organizme in ekosisteme še vedno omejeno. V svoji doktorski disertaciji sem raziskovala prisotnost mikroplastike (MP) v rečnih ekosistemih, zlasti interakcijo med hiporeičnim biofilmom celinskih voda in MP, ter odziv hiporeičnih biofilmov na onesnaženje s polietilen teraftalatom (PET) v naravnih okoljih.

Raziskavo sem začela z oceno onesnaženosti slovenskih rek z MP in pregledom svetovnih podatkov o bakterijski biorazgradnji plastike. V slovenskih rekah se je onesnaženost z MP povečevala v smeri rečnega toka in z intenzifikacijo rabe tal. Rezultati so pokazali, da vlakna prevladujejo v vodnem stolpcu, fragmenti pa v sedimentih. Najpogosteje poročana tipa plastike tako v sedimentih kot tudi v vodnem stolpcu sta bila polietilen (PE) in polipropilen (PP). Raznolikost v obarvanosti, velikosti in oblikah MP je kazala na različne vire onesnaženja z MP.

V sistematičnem pregledu znanstvene literature, ki je vključeval 145 objavljenih znanstvenih člankov, je bila sestavljena tabela bakterijskih sevov, ki naj bi biološko razgrajevali različne plastične materiale. Glavne ugotovitve sistematičnega pregleda so bile, da se raziskave osredotočajo predvsem na PE in njegove različice, da bi bilo plastiko potrebno predpripraviti (npr. izpostaviti UV žarkom), da bi morale biti študije biorazgradnje bolj podobne razmeram v okolju, in da bi bilo potrebno izvesti več dolgoročnih študij. Poudarjeno je bilo tudi pomanjkanje standardizacije študij biorazgradnje in doslednosti poročanja.

Izveden je bil preliminaren poskus biorazgradnje različnih plastičnih materialov v okolju z nizko vsebnostjo ogljika z uporabo aktivnega blata iz čistilne naprave. Po dveh mesecih so mikrografije SEM potrdile različne tipe biofilma na različnih plastičnih materialih.

Nadalje so bile raziskane interakcije med plastiko kot substratom in hiporeičnimi biofilmi celinskih voda kot kolonizatorji v naravnem okolju. Prva študija se je osredotočila na enomesečno kolonizacijo in enoletne spremembe odzivov biofilmov v bližini PET vlaken. Za opredelitev mikrobne aktivnosti, biomase in metabolizma so bile uporabljene različne metode, kot so aktivnost respiratornega sistema za prenos elektronov (ETSA), skupna vsebnost proteinov (TPC) in fiziološko profiliranje na ravni skupnosti (CLPP). Druga študija je z uporabo istih metod, a v daljšem časovnem obdobju (2 leti) in z večjo prostorsko variabilnostjo (4 geomorfološko različne lokacije), potrdila inhibitorni učinek prisotnosti PET na mikrobne biofilme v hiporeičnem območju.

Nazadnje smo z enakimi metodami analizirali učinke treh različnih vodotočnih režimov (pretočnega, stoječega in nenasičenega) na hiporeične biofilme ob hkratni prisotnosti PET vlaken. Pomembni inhibitorni učinki vodotočnih režimov in onesnaženja s PET so bili opaženi pri številčnosti bakterij in mikrobni presnovi nekaterih substratov (CLPP), ne pa tudi pri mikrobni biomasi (TPC) ali aktivnosti (ETSA). Raziskava je poudarila tudi prostorsko heterogenost v hiporeiku kot pomemben dejavnik pri raziskavah hiporeičnih biofilmov.

Ugotovitve doktorata pomembno prispevajo k boljšemu razumevanju onesnaženja z MP na lokalni in globalni ravni ter razumevanju vpliva na hiporeične biofilme, ki so ključni promotorji globalnega kroženja hranil v celinskih vodah.

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Abbreviations

ANOSIM	... Analysis of similarities
ANOVA	... Analysis of variance
CLPP	... Community-level physiological profiling
DOM	... Dissolved organic matter
DW	... Dry weight
ETSA	... Respiratory electron transport system activity
HZ	... Hyporheic zone
IWRS	... Institute for Water of the Republic of Slovenia
JSI	... Jožef Stefan Institute
LOI	... Loss on ignition
MP	... microplastic
NIB	... National Institute of Biology
NMDS	... Non-metric multidimensional scaling
NP	... nanoplastic
PA	... polyamide
PE	... Polyethylene
PERMANOVA	... Permutational multivariate analysis of variance
PES	... Polyester
PET	... Polyethylene terephthalate
PICT	... Pollution-induced community tolerance
POM	... Particulate organic matter
PP	... Polypropylene
PVC	... Polyvinyl chloride
TN	... Total nitrogen
TPC	... Total protein content
UNEP	... United Nations Environment Programme
WWTP	... Wastewater treatment plant

Chapter 1

Introduction

Water is one of the most essential natural resources for all humanity and the planet's well-being. Moreover, water supports an array of freshwater and marine ecosystems where many organisms spend at least a part of their life cycle. Consequently, freshwater ecosystems are one of the most exploited worldwide. They provide potable water, water for irrigation, industrial processes (e.g., paper production), power generation (electricity), construction, as well as facilitating transportation (Mazor et al., 2018; van Rees et al., 2021).

When fully functional, freshwater ecosystems are critical to solving or mitigating the three interlinked planetary crises recognised by the United Nations Environment Programme (UNEP): climate change, nature and biodiversity loss, and pollution and waste (UNEP, 2022). Despite providing crucial services tied to human well-being, such as provisioning (drinking water, food, genetic resources), regulation (climate regulation, flood control, disease mitigation, water quality maintenance and wastewater treatment), and cultural services (recreation, aesthetic enjoyment, and spiritual fulfilment), and support services like soil formation and nutrient cycling (Daily et al., 1997; Reid et al., 2005), freshwater ecosystems are at risk due to anthropogenic activities including the modification, degradation and fragmentation of freshwater habitats, overexploitation of water and sediments, climate change and pollution (Vorosmarty et al., 2010; Reid et al., 2019).

Rivers and streams are highly dynamic and heterogenic freshwater bodies, supporting life in and around them by providing habitats for various organisms (bacteria, fungi, protozoans, flora, and fauna). They are tightly connected to all parts of a catchment by the bidirectional water movement in three dimensions (Ward, 1989; Closs et al., 2010). Longitudinal water flows down the channel, vertically between the open channel and underlying sediments and laterally to and from the riparian zone (Ward, 1989; Jones & Holmes, 1996). The fourth dimension is time (temporal, Figure 1), which depends on the organism and phenomenon being investigated and can range from the time required to observe a behavioural change to the time needed for evolutionary change (Ward, 1989).

Because rivers and streams are connected to their surroundings, they are highly susceptible to land and water-based anthropogenic activity (Clapcott et al., 2012). Structural changes in river ecosystems are induced by changes in land use, variation in

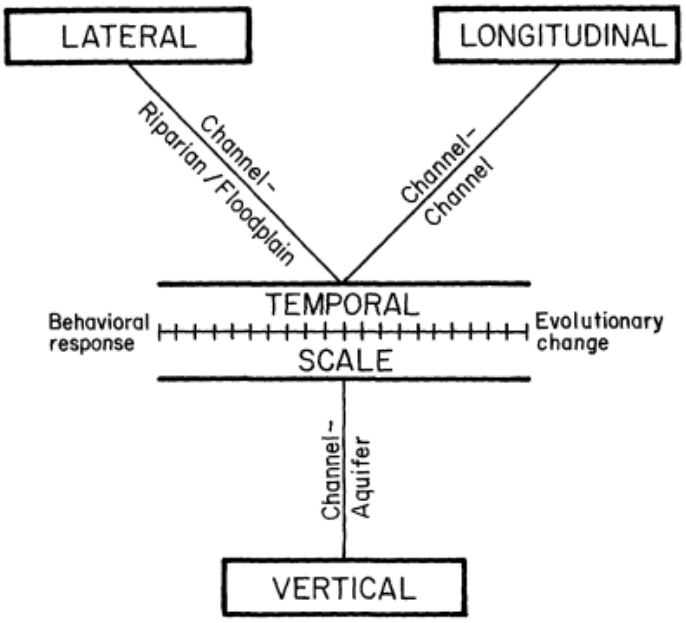

Figure 1: The four-dimensional concept of lotic ecosystems (Ward, 1989).

discharge, inputs of organic matter, nutrients and toxic pollutants (both point and non-point sources), changes in surface and subsurface hydrology and geomorphology (i.e., clogging) and changes in biological communities (Clapcott et al., 2012; Koch et al., 2018; Sagova-Mareckova et al., 2021). These changes (i.e. various abiotic and biotic components), in turn, determine ecosystem functioning, such as ecological processes such as metabolism, organic matter decomposition, and primary and secondary production (von Schiller et al., 2017).

A major issue regarding freshwater ecosystems is pollution by newly emerging pollutants such as micro- and nanoplastics (Eerkes-Medrano et al., 2015; Avio et al., 2017; Arias-Andres et al., 2018; Gigault et al., 2018; Wagner & Lambert, 2018; Caruso, 2019; Ahmad et al., 2020; Cera et al., 2020; Mammo et al., 2020; Wong et al., 2020; Yang et al., 2020; Miloloža et al., 2021; Yang et al., 2021; Bank & Hansson, 2022; Castro-Castellon et al., 2022; Ziani et al., 2023). Microplastics (MPs) are synthetic polymer particles of 5 mm or less in size. They are typically distinguished as primary and secondary MPs. Primary MPs are produced intentionally, usually as raw materials for plastic production or as abrasives in personal care products, and often enter the environment through improper disposal, littering or accidental releases. Alternatively, secondary MPs result from the degradation and fragmentation of large plastic items, such as bottles and packaging materials, textiles, construction materials and tyre abrasion (Hidalgo-Ruz et al., 2012; Waldschläger et al., 2020).

The majority of plastics and MPs degrade very slowly. Their degradation rate depends on physical factors such as UV light exposure, physical disturbance, oxygen and temperature (Matjašič et al., 2021b). It also depends on the microorganisms present in the surrounding environment. Consequently, degradation rates vary between landfills, terrestrial, freshwater and marine environments and can take several hundred years (Kyrikou & Briassoulis, 2007; Hopewell et al., 2009).

Freshwater ecosystems are known to receive a large amount of plastic particles (large plastic debris, microplastics, and nanoplastics) from the air and land, which they retain or transport to the ocean (Lebreton et al., 2017; Constant et al., 2020; Wang et al., 2021). Whenever a plastic particle enters the environment, it is exposed to not only physical factors (e.g. temperature, UV light, abrasion) but also to the biosphere (McCormick et al., 2014; McCormick et al., 2016; Rummel et al., 2017; Parrish & Fahrenfeld, 2019). The latter can be concerning since plastic particles can act as a carrier of toxic chemicals and pathogenic organisms (Shah et al., 2008; Thompson et al., 2012; Wu et al., 2019b). Moreover, MPs can impact ecosystem processes in freshwater ecosystems, such as

biogeochemical cycles taking place in the sediments that are driven by the microbial community (Battin et al., 2003; Seeley et al., 2020; You et al., 2020; Wang et al., 2021; Yang et al., 2021). However, MP pollution is only one among multiple stressors (e.g., land use changes, temperature increases, floods, droughts, and nutrient inputs) affecting structural and functional characteristics of impacted freshwater ecosystems (Mori et al., 2018; Lemm et al., 2021). Given the overarching threat of climate change, there is a need for further research to gain a better understanding of how freshwater ecosystems respond to MP pollution under various environmental conditions and multiple stressors.

1.1 The Hyporheic Zone and Freshwater Biofilms

1.1.1 The hyporheic zone

Several freshwater scientists, including Orghidan (1959, 2010), Hynes (1975), Danielopol (1976), Stanford and Ward (1988), Boulton et al. (1992), Mulholland et al. (1997), and Gibert et al. (1997), have recognised the existence of a transitional habitat type within river ecosystems that connects surface and groundwater habitats. This habitat, known as the hyporheic zone (HZ, Figure 2), is a dynamic ecotone where water, nutrients, biota, and other materials are exchanged between surface water and groundwater (Boulton et al., 2010). It extends vertically and laterally from the river's channel, serving as a zone of interaction. The HZ shares similarities and differences between groundwater and surface water while exhibiting some unique features (Table 1). The hyporheic infiltration zone is particularly significant. Here, sediments act as physical, chemical, and biological filters for compounds, particles (including MPs), and organisms from the surface water. This filtration process significantly influences the quality and quantity of adjacent groundwater (Orghidan, 1959, 2010; Williams et al., 2010; Battin et al., 2016).

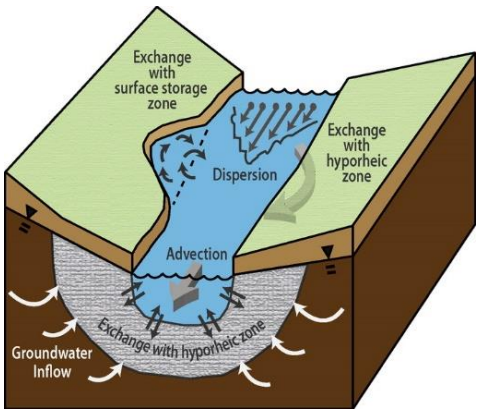

Figure 2: Hyporheic zone exchange (Harvey & Gooseff, 2015).

Table 1: Comparison of physical, chemical and biological characteristics between groundwater, the hyporheic zone and surface water (after (Krause et al., 2011; Zhou et al., 2014; Debeljak, 2018).

Characteristics	Groundwater	Hyporheic zone	Surface water
PHYSICAL CHARACTERISTICS:			
Light	Constant darkness	Constant darkness	Daylight fluctuations
Current velocity	Low	Intermediate	High
Annual and daily temperature range	Very low	Low	High
Substrate stability	High	Intermediate	Low

Gradient of physical and chemical parameters	Low	Steep	Steep
CHEMICAL CHARACTERISTICS:			
Amount of organic matter	Low	High	Intermediate
Amount of dissolved oxygen	Low	Intermediate	High
Amount of nutrients	Low	High	Intermediate
BIOLOGICAL CHARACTERISTICS:			
Habitat diversity	Low	Intermediate	High
Food webs	Simple and short	Intermediate	Complex and long
Productivity	Low	Intermediate	High

The size of the HZ is difficult to gauge due to the spatio-temporal variability of its boundaries. The interaction between surface streams and underlying groundwater, coupled with the geomorphology of the river, also contributes to this variability (Boulton et al., 1998). In certain instances, such as river channels confined by bedrock, the HZ may extend only a few centimetres. However, in large river systems with broad floodplains and sandy/alluvial substrates, it can extend vertically by several meters and longitudinally by several kilometres (Stanford & Ward, 1988; Marmonier et al., 1992; Hucks Sawyer et al., 2009; Chen, 2011; Lapworth et al., 2011; Stelzer et al., 2011).

The vertical dimension of the HZ is influenced by various factors, including fluctuations in surface water level, flow velocity, groundwater table level, and water temperature, which affect subsurface flow paths. However, the upper boundary is primarily determined by the riverbed's surface (Lewandowski et al., 2019). Understanding the temporal patterns of groundwater flow and its interaction with surface water is crucial, as these patterns control critical biogeochemical processes like nitrification and denitrification. Rapid groundwater recharge, for example, results in shorter residence times within the HZ, limiting the biodegradation of organic contaminants (Triska et al., 1993; Landmeyer et al., 2010).

The relationships between the volumes and residence times of water within different compartments of the hydrological cycle are significant because they have implications for the vulnerability of water to anthropogenic pollution. Water with extended residence times, such as glaciers or groundwater, can retain pollutants for long periods, even after the source of pollution has been removed. Furthermore, pollution spreads more slowly in systems with long residence times, resulting in substantial amounts of pollutants entering the system before the problem is detected (Closs et al., 2010).

Hyporheic processes can be best described as interactions among physical, chemical and biological systems (Figure 3). First, the physical system comprises the geological structure, which supports the processes occurring upon and within it while water, matter, and energy flow into and out of the HZ. Second are the chemical reactions and transformations occurring within the HZ, and lastly, the biological systems encompass all organisms, organic matter, and their interactions and relationships (Ward, 2016).

Both physical and chemical systems play a crucial role in establishing habitats for organisms, with the physical system providing the physical habitat and the chemical system determining the key chemical conditions. For instance, the physical and chemical systems interact by controlling the exchange flux of water between the stream and the HZ and the residence time of water within the HZ.

Alternatively, biota, whether permanently or temporarily residing in the HZ, can also alter its surface and subsurface structure through their movements and deposition.

Microbially mediated biogeochemical processes also involve interactions between chemical and biological systems, as primary and secondary biological production transforms major and minor nutrients within the HZ. An example of this natural interaction is salmonid spawning in the HZ (biological), which occurs at sites with upwelling cool (physical), well-oxygenated (chemical) water. Understanding these interactions is essential for comprehending hyporheic processes (Ward, 2016).

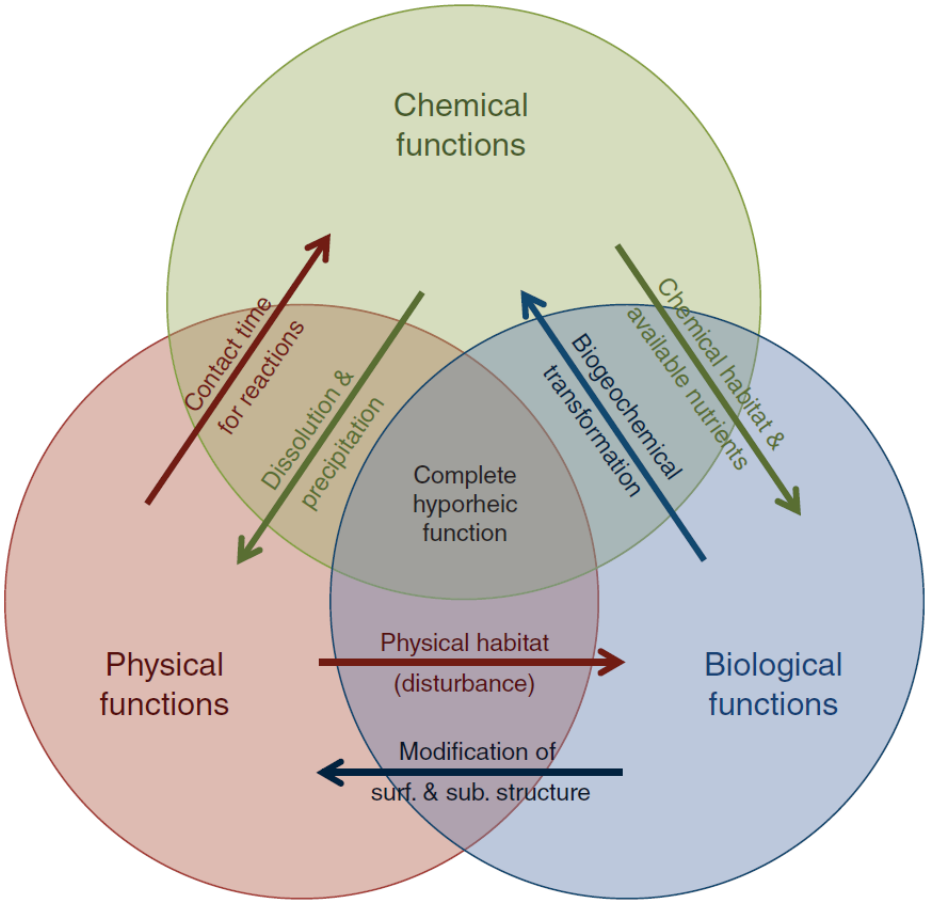

Figure 3: Chart showing the interactions between chemical, physical and biological functions. Adapted from Ward (2016).

The HZ is a habitat where diverse communities of organisms exist, from microorganisms to invertebrates (Orghidan, 1959, 2010; Robertson & Wood, 2010). The high surface area of the HZ's sedimentary matrices provides habitats for various microorganisms and offers protection to invertebrate and biofilm communities against extreme temperatures, drought and high discharge. Food for biota is provided through surface water infiltrating the HZ and carrying particles of organic material and nutrients into and out of the HZ.

Resident animals are either detritivores, predators or microbial heterotrophs. The photoautotrophic organisms are lacking since photosynthesis is impossible due to the absence of sunlight (Closs et al., 2010). Invertebrates in the HZ (hyporheos) are typically stygoxenes, stygophiles and stygobionts. Stygoxenes are organisms that accidentally find themselves in the HZ and have no affinities with groundwater systems. Stygophiles spend part of their life in underground water and are intermediate between stygobionts and stygoxenes. Stygobionts, in contrast, are specialized groundwater-dwelling organisms that exclusively inhabit groundwater environments and exhibit distinct characteristics such as

reduction of eyes, loss of body pigment, reduced size, and adaption to oligotrophic conditions (Gibert et al., 1994).

Microorganisms are usually attached to sediment grains, as opposed to protists, meiofauna and macroinvertebrates that live in the pore space or burrow into the sediment, occupying the interstitial spaces among sediment particles. This diverse community of organisms plays a critical role in the ecological functioning of the HZ (Lewandowski et al., 2019).

Because the HZ plays a crucial role in a river's self-cleaning process, it is often called the river's "liver". Additionally, it is one of the systems highly susceptible to and profoundly impacted by climate change (Orghidan, 1959, 2010; Closs et al., 2010; Williams et al., 2010; Lawrence et al., 2013; Battin et al., 2016; Mori et al., 2018). Regarding climate change, it is crucial to acknowledge the global significance of the HZ, given that it is found in cold (Edwardson et al., 2003), temperate and tropical streams (Covich et al., 2003). For example, the HZ's hydrological, chemical, and ecological buffering capacity becomes crucial in extreme climate conditions such as temperature extremes and floods. However, despite its significance, research on the presence of microplastics and their effect on biofilm function is lacking. Equally, knowledge about microbially driven processes in the HZ is essential to ensuring good ecological status of rivers and healthy drinking water. At the same time, a comprehensive understanding of these processes holds significant potential for application in bioremediation.

1.1.2 Freshwater biofilms

Freshwater biofilms are a diverse, complex group of bacteria, archaea, algae, protozoa, and fungi, in association with fine organic and inorganic particles embedded in extracellular matrix (Closs et al., 2010; Battin et al., 2016; Penesyan et al., 2021). They grow on any surface within a stream, usually making it slimy (Closs et al., 2010), and are often viewed as the initial biotic interactions with solid substrates in aquatic environments (Hoellein et al., 2014). They also represent an essential part of the food web (Akbari et al., 2017), serving as food sources for higher trophic-level organisms, such as invertebrates and fish (Hoellein et al., 2014). Furthermore, they include diverse microbial partners in obligate mutualism and can exhibit characteristics of multicellular organisms, such as division of work (Flemming & Wuertz, 2019; Penesyan et al., 2021). Biofilms can be beneficial, for example, when used to remove organic compounds during wastewater treatment (Ehlers & Turner, 2012). However, they can be problematic, when they form on surfaces within man-made systems, such as drinking water distribution systems (Akbari et al., 2017).

The life cycle of a biofilm comprises the following five stages: reversible attachment, irreversible attachment, maturation-I, maturation-II, and dispersion. During stage one, cells attach and re-attach to the newly available surface. These irreversibly attached cells, which have reduced mobility, produce biofilm matrix components, proliferate and recruit cells from the surrounding environment. Such cell assemblages are several cells thick at the beginning and embedded in the biofilm matrix (maturation I) and form microcolonies (maturation II). Once fully matured, they disperse motile cells and cell aggregates, which spread and serve as an inoculum for new biofilm formation. However, there is evidence that not all biofilms attach to surfaces (Penesyan et al., 2021; Sauer et al., 2022).

There are four main advantages of bacteria forming a biofilm instead of being in planktonic forms (Jefferson, 2004):

1. Defence: Biofilms can resist better physical forces, withstanding nutrient deprivation, pH changes, oxygen radicals, disinfectants, antibiotics, resistance to phagocytosis, and protection from protozoa grazing.

2. Biofilrms afford the ability to remain in a favourable habitat (understood as a mechanism to remain attached in a favourable niche), e.g., the human body as a nutrient-rich and stable environment provides a habitat for a large number of commensals, many of which exist as biofilms.

3. Living in an interactive community also means organisms can share the metabolic burden, transfer genes, and express altruistic behaviour.

4. In the default mode, i.e., bacteria grown planktonically in the laboratory have all the nutrients they need, but this may not be the case in the environment.

Freshwater biofilms usually have a high diversity of operational units, representing diverse assemblages of archaea and bacteria, fungi, and algae (in the top sediment layers), diverse metabolic capabilities, and high enzymatic activity (Romaní et al., 2008; Battin et al., 2016; Lewandowski et al., 2019). In benthic and hyporheic biofilms, the most numerous bacterial species are members of the Proteobacteria and Acidobacteria phyla, both of which include diverse gram-negative bacteria. Archaea thrive only in specialised areas, such as methanogenic archaea in anoxic pockets (Wagner et al., 2014; Battin et al., 2016; Hou et al., 2017; Sienkiewicz et al., 2020; Gao et al., 2022).

The highly diverse microbial community composition of rivers and streams changes with geographic distance and is susceptible to changes in environmental conditions such as temperature, pH, conductivity and nutrients (Beier et al., 2008; Zhang et al., 2022a), but also to anthropogenic disturbances (Liao et al., 2019; Wang et al., 2023). In turn, protists, meiofauna, and other hyporheos inhabiting the streambed sediments boost biofilm activity by grazing and bioturbating the hyporheic sediments (Lewandowski et al., 2019). Hyporheic biofilms, attached to sediment surfaces, typically include heterotrophs and chemolithotrophs embedded in a polysaccharide matrix (Claret et al., 1998; Romaní et al., 1998; Closs et al., 2010; Battin et al., 2016). They inhabit both nutrient-rich, reducing HZ sediments and nutrient-poor, oxidizing HZ sediments (Krause et al., 2017). They also efficiently uptake, immobilise and transform organic substances while providing energy for the stream community (Claret & Fontvieille, 1997).

The development and activity of heterotrophic biofilms are closely linked to nutrient quality, quantity, and other factors such as water exchange and sediment structure. Recently, Höhne et al. (2022) demonstrated their ability to attenuate trace organic compounds. Such processes, occurring in the HZ and driven by biofilms, are known to be influenced by anthropogenic pressures, allowing HZ biofilms to be used as a functional indicator of those pressures (Boulton et al., 1998; Battin et al., 2016; Estevez et al., 2017).

Overall, microbial organisms play a crucial role in primary production and the breakdown and recycling of materials and nutrients (Closs et al., 2010; Battin et al., 2016; Akbari et al., 2017). They are also vital components of the global biogeochemical exchange of carbon, nitrogen and phosphorous and for ecosystem functioning, degrading and transforming a broad range of nutrients, pollutants, and trace organic compounds (Akbari et al., 2017; Roberts & Cooper, 2018; Lewandowski et al., 2019).

1.2 Freshwater Plastic Pollution

Plastics, as a general term, encompasses a wide variety of synthetic polymer products primarily derived from non-renewable resources such as coal, oil, and natural gas and processed using a range of chemical additives. These polymers comprise repeating monomers linked by strong covalent bonds, forming large chains. The end products can vary from everyday items like plastic bags and food wraps to synthetic fibres for clothing

and construction materials. Plastic is durable and lightweight, with low- energy and production costs (Hopewell et al., 2009; North & Halden, 2013).

Global plastic production in 2020 reached 367 million tonnes, with the European Union (EU) accounting for 55 million tonnes. In the EU, the largest markets for plastics in 2020 were packaging and, building and construction, accounting for 40.5% and 20.4%, respectively (PlasticsEurope, 2021). The first plastic made from synthetic components was Bakelite, developed by Leo Baekeland in New York in 1907 and patented two years later (Roncone Zappas, 2007). Today, there are over 20 major types of plastics utilised worldwide, including Polypropylene (PP), Polyethylene (PE), Polyvinyl chloride (PVC), and Polyethylene Terephthalate (PET) (Hopewell et al., 2009; North & Halden, 2013; PlasticsEurope, 2021). A consequence of the widespread use of polymers is the release of large amounts of plastic waste and particles into the environment.

The major sources of plastic pollution are households (e.g., personal care products and fibres released during laundry), industry and construction, transport (e.g., tyre abrasion) and ports (shipping, fishing), littering (misplacement of plastic waste), and agriculture (mulching of plastic covers, application of WWTP sludge as fertiliser) (Waldschläger et al., 2020). Most plastic waste ends up in the marine environment through surface drains, rivers, coastal waste, lost cargo, and deliberate littering (Eerkes-Medrano et al., 2015). Once in the environment, due to weathering, larger plastic debris undergoes fragmentation, which does not equal degradation. Consequently, plastic debris and fragmented plastic pieces accumulate in landfills and can be transported across natural environments.

Primary MPs are intentionally produced, for example, as industrial plastic resin pellets (raw material for remelting and moulding into final products) and as abrasive particles (microbeads) in personal care products, while secondary MPs are the result of the fragmentation of displaced larger plastic (Science Advice for Policy by European, 2019; Waldschläger et al., 2020; Yang et al., 2021). Resin pellets are used because they are easier to transport, while microbeads improve stability and act as replacement natural abrasives such as walnut shells or activated carbon (Wagner & Lambert, 2018; Waldschläger et al., 2020). Sources of secondary MP in the environment are microfibers released from laundry, city dust, agricultural foils, MPs from tyre abrasion, coatings used in the marine industry, and paints (Wagner & Lambert, 2018; Science Advice for Policy by European, 2019; Waldschläger et al., 2020). Both primary and secondary MP threaten terrestrial and aquatic ecosystems (Dris et al., 2015b).

Microplastic particles are usually characterised by size, shape, type, and colour (Yang et al., 2021). The definition of MP is based on the maximum size of the particle, while the sampling mesh or mesh size of the filter used determines the minimum size. Some consider microplastic as particles between 1 and 5 mm and particles < 1 µm as nanoplastic (Gigault et al., 2018). As plastic is an umbrella term for synthetic polymers, the chemical composition is the basic criterion for characterizing a plastic particle and is typically determined using Fourier-transform infrared spectroscopy (Matjašič et al., 2021b).

Microplastic particles include pellets, spherules, fragments, foam, fibres and films (Hidalgo-Ruz et al., 2012). The shape of a particle can provide insights into its origin. For instance, fragments are typically produced through the fragmentation of household plastic products. At the same time, fibrous particles can be traced back to textile wastewater effluents from shredded cloth and synthetic fibres released during washing or due to textile wear and tear and fishing gear such as nets. Both fragments and fibres can be easily mistaken for food and ingested by various aquatic organisms (Egbeocha et al., 2018; Chang et al., 2022). Foams may be associated with products like packaging and insulation materials, while films represent thin plastic materials commonly used in packaging. Foams and films can accumulate on the water surface, disrupting light penetration and oxygen exchange. Spherical pellets are typically used in plastic manufacturing and are typically

misplaced during production or transportation (Fahrenfeld et al., 2019; Yang et al., 2021; Matjašič et al., 2023).

Freshwater (surface and sediments) MPs are primarily PP and PE and are most abundant in areas with dense human activities. The particles are mainly white and transparent fibres, smaller than 1 mm in size (Wang et al., 2021; Yang et al., 2021). Microplastics come in various colours, from transparent to white to red, yellow, green, blue and black. More colourful particles are more likely to be digested by aquatic animals (Berglund et al., 2019; Lopes et al., 2020). Additionally, based on colour, potential sources can be detected (Fahrenfeld et al., 2019). However, since colour perception can be subjective, MPs are typically sorted into transparent, black, white and coloured particles (Lu et al., 2021; Yang et al., 2021).

Because of their small size and often low density, MPs are exposed to wind erosion and are susceptible to entrainment and transport (Bullard et al., 2021). Particles can be transported over long distances, even to remote areas (Evangeliou et al., 2020), such as glaciers of the Tibetan Plateau (Zhang et al., 2021), The Arctic (Bergmann et al., 2019) and even above the planetary boundary layer (González-Pleiter et al., 2021). However, the existence of MPs in urban and rural areas has also been demonstrated (Dris et al., 2015a; Zhang et al., 2020b). Frequently detected shapes of atmospheric MPs are spherical particles (road dust; tyre abrasion and paint particles) and textile fibres (Dris et al., 2017; Zhang et al., 2020b), but vary significantly in chemical composition over different regions (Zhang et al., 2020b). Microplastics can also be deposited in remote areas by precipitation (Bergmann et al., 2019) since MPs can adhere to raindrops and enter inland waters (Xia et al., 2020). Ganguly and Ariya (2019) showed how MPs could be efficient cloud ice nuclei. Similarly, several studies confirmed freshwaters to be significantly affected by a rain event, an effect that can last for hours after the event (Dris et al., 2015a; Bondelind et al., 2019; Xia et al., 2020). Further, stormwater runoff has been recognised as a major source of MP pollution (Piñon-Colin et al., 2020; Treilles et al., 2021; Werbowski et al., 2021), most of which does not receive any treatment before reaching a water body (Piñon-Colin et al., 2020).

MP pollution from land-based sources accumulates in wastewater treatment plants (WWTP). These are considered a point source that links pollutants and aquatic environments (Ziajahromi et al., 2016; Kovač Viršek et al., 2017). Although wastewater treatment plants are highly efficient in removing MPs ($>$ 97 % in WWTP with tertiary treatment), the contamination of aquatic ecosystems is still considerable due to the high volume of effluents released daily. Conversely, the MPs retained in the WWTP end up in sewage sludge, which can then be applied to agricultural land, where rain can wash them into surface waters (Sun et al., 2019).

The most commonly detected plastic pollutants in the WWTPs are polyester (PES), PE, PET and polyamide (PA), with fibres as the most common shape. A primary source of MP fibres in WWTP influents and freshwaters is washing machine effluents (Prata, 2018; Sun et al., 2019). Also, McCormick et al. (2014) showed that the amount of MPs upstream of the WWTP inlets is lower than downstream from the WWTP discharge. Lentic environments can also be a source of MPs (Nel et al., 2018) and serve as a sink for MP contaminants. For example, Lake Hovsgol, Mongolia, exhibited a high MP load (mean = 20.264 particles km^{-1}).

If microplastic particles enter the water directly, their first interaction with the biosphere is with microbes (McCormick et al., 2014; Hoellein et al., 2017). Scientists have recently introduced a new term, “plastisphere”, which refers to a diverse community of heterotrophs, autotrophs, predators and symbionts collected on plastic marine debris at multiple locations in the North Atlantic (Zettler et al., 2013). However, the term only applies when there are distinct microbial communities on plastic particles compared to other substrates in aquatic environments (Oberbeckman et al., 2018). Biofouling, i.e. the

attachment of microorganisms, can have a significant effect on the hydrodynamics of plastic particles and lead to an increase in a particle's density and a decrease in their buoyancy, causing sedimentation (Rummel et al., 2017; Miao et al., 2021a). Settling particles can infiltrate deeper into sediments or become re-suspended under strong flow conditions, depending on river hydrology and geomorphology. Additionally, the microbiota overgrowth depends on the type of plastic (Parrish & Fahrenfeld, 2019).

Research on freshwater pollution with plastics and MPs and its significance for ecosystem processes is still in its infancy, and further investigations are needed. For example, few studies have investigated the spatiotemporal aspects of MP pollution across different scales (e.g., cathment scales, geomorphological). Developing standardised sampling and processing procedures is critical to ensure reliable study comparison (Talbot & Chang, 2022; Viaroli et al., 2022).

While MP pollution is a highly relevant topic, only a few studies currently provide field data about groundwater. Groundwater receives less attention than other water bodies because contamination may appear less evident. Also, effect of MP pollution on the HZ and HZ biofilms has yet to be studied, even though they play a crucial role in global biogeochemical cycles. Consequently, a significant knowledge gap remains regarding the impact of plastic presence on these cycles.

Chapter 2

Research Aims and Hypothesis

Despite the significance of freshwater ecosystems as sources of potable water and habitats for diverse organisms, our understanding of the impact that microplastics (MPs) have on river and riverbed sediment biota and ecosystem processes remains limited. Also, compared to marine environments, there is a lack of information regarding sources, concentrations, transport pathways, environmental fate, and biological effects of MPs in freshwater. Furthermore, most studies on MPs in freshwater have been conducted in China and the USA, leaving a significant knowledge gap for Europe.

My research addresses these gaps by providing insights into the patterns and types of MP pollution in selected Slovenian rivers, the biodegradability of different plastic materials and biodegradation potential of microorganisms, colonization processes on different plastics, and the influence of PET fibres on hyporheic biofilm structure and function, and on freshwater ecosystem processes. These findings are crucial for developing effective strategies to mitigate the environmental impact of plastic pollution on freshwater systems and their ecosystem services, such as water purification.

The primary objective of my PhD thesis was to obtain an in-depth understanding of the interactions between plastics and freshwater biofilms in the hyporheic zone. To achieve this, I first investigated MP pollution in the shallow hyporheic zone and explored colonization patterns of microorganisms on the selected plastic materials to better understand their biodegradation potential. This part of my studies also included a systematic literature review and preliminary experiments involving common plastics and microorganisms from heavily burdened environments (active sludge).

The second part of the research involved assessing the impact of plastic pollution on hyporheic biofilms under differing environmental conditions. This task involved a series of field and laboratory experiments. In the field experiments, PET fibres, one of the most common MP pollutants in freshwaters, were used to study the response of the hyporheic biofilm to different environmental conditions and across different temporal scales.

This study is among the first to address not only plastic pollution in freshwater environments but also to investigate the response of hyporheic microorganisms to the presence of plastic pollution. The findings should prove valuable for decision-makers responsible for protecting freshwater and their ecosystem services since maintaining a healthy environment is more cost-effective and efficient than remediation.

2.1 Specific Aims

The specific aims of my PhD Thesis are:

- Understanding the scale of MP pollution of the hyporheic zone of selected Slovenian rivers
- Exploring the relationships between freshwater microorganisms and plastic substrates
- Investigating the mechanisms of potential plastic biodegradation
- Analysing the colonization patterns and seasonal dynamics of the hyporheic biofilm functioning in the presence of PET fibres in a pre-alpine river gravel bed,
- Assessing the spatiotemporal diversity of a hyporheic biofilm's structural and functional response to the presence of PET fibres across distinct ecohydrological regions (e.g., karstic and pre-alpine)
- Investigating the impact of the presence of PET fibres on the hyporheic biofilm structure and function under simulation water regimes (flowing, stagnant, and unsaturated)

2.2 Hypothesis

1. MPs are present in larger quantities in the hyporheic zone of river reaches exposed to WWTP effluents than in un-impacted river reaches.
2. Biofilms formed on different plastic types will likely contain bacteria exhibiting biodegrading potential than biofilm not exposed to plastic.
3. The presence of PET fibres inhibits the function of hyporheic biofilms.
4. Seasonal dynamics in hyporheic biofilm structure and functioning over one year differ when comparing PET-polluted and PET-unpolluted hyporheic environments.
5. The presence of PET fibres in the hyporheic zone inhibits the variability of biofilm function across a larger spatiotemporal scale.
6. An increasing amount of PET fibres in combination with reduced flow (stagnant conditions) will have an inhibitory effect on hyporheic biofilm functioning.

Chapter 3

Publications

Publications included in this thesis consist of three published scientific articles, one published review paper and two scientific articles in preparation. The publications are presented in the following order:

MATJAŠIČ T., MORI N., HOSTNIK I., BAJT O., KOVAČ VIRŠEK M. 2023. Microplastic pollution in small rivers along rural–urban gradients: variations across catchments and between water column and sediments. Science of the total environment 858: 1-11. https://doi.org/10.1016/j.scitotenv.2022.160043.

MATJAŠIČ T., SIMČIČ T., MEDVEŠČEK N., BAJT O, DREO T, MORI, N. 2021. Critical evaluation of biodegradation studies on synthetic plastics through a systematic literature review. Science of the total environment 752: 141959, 1-16. DOI: https://doi.org/10.1016/j.scitotenv.2020.141959.

MATJAŠIČ T., DREO T., SAMARDŽIJA Z., BAJT O., KANDUČ T., SIMČIČ T., MORI, N. 2020. Preliminary experiments into colonization of microorganisms from activated sludge on different types of plastics = Preliminarni poskusi kolonizacije različnih tipov plastike z mikroorganizmi iz aktivnega blata. Acta biologica slovenica: ABS. 63(1): 45-61. http://www.dlib.si/details/URN:NBN:SI:doc-ILJ53CMY.

MATJAŠIČ T., SIMČIČ T., KANDUČ T., SAMARDŽIJA Z., MORI, N. 2021. Presence of polyethylene terephthalate (PET) fibers in hyporheic zone alters colonization patterns and seasonal dynamics of biofilm metabolic functioning. Water Research 203: 1-13. https://doi.org/10.1016/j.watres.2021.117455.

MATJAŠIČ, T., SIMČIČ, T., KANDUČ, T., SAMARDŽIJA, Z., MORI, N. Hyporheic microbial community structure and functioning during 3-years exposure to pollution with polyethylene terephthalate (PET) fibres.

MATJAŠIČ, T., SIMČIČ T., AKBARI, E., WEIGELHOFER, G., MORI, N. Resilience of hyporheic biofilms to non-flow events and PET pollution. Weigelhofer, G., and Mori, N. share last co-authorship.

3.1 MP Pollution of the Hyporheic Zones in Selected Slovenian Rivers

3.1.1 Published scientific article: "Microplastic pollution in small rivers along rural-urban gradients: Variation across catchments and between water column and sediments"

The study focused on types of MP pollution in smaller catchment rivers in both water column and riverbed sediments. The main objectives were to understand the influence of hydrogeomorphology, urbanization and a WWTP on the concentration, types, shapes, sizes and colours of MP pollution and the transport, retention and distribution of MP in streams.

The MP concentration increased with the distance from the river spring in the water column and sediments. The most frequent MPs found were PE and PP particles. Fragments dominated the sediment samples, while fibres dominated the water column. Also, particles in the water column were smaller than those in sediment samples. This variability hints at a wide array of pollution sources. These sources play a pivotal role in determining the types and quantities of MP in rivers. However, the distribution, retention and transport of MP particles depend on the hydrogeomorphological characteristics of the river.

The published article was prepared in collaboration with the Institute for Water of the Republic of Slovenia (IWRS) under the following authorship: Tjaša Matjašič (NIB), Nataša Mori (NIB), Irma Hostnik (IWRS), Oliver Bajt (NIB) and Manca Kovač Viršek (IWRS). The article was published in Science of the Total Environment in 2023. I designed the sampling strategy, developed the sampling methodology (hyporheic zone at a depth between 20 and 40 cm), conducted field sampling, performed the laboratory work, and analysed the data, all under the supervision of Nataša Mori. I also prepared the first draft of the manuscript and submitted the paper in a co-authorship with other authors. IWRS carried out the sampling of MPs from the river water under the leadership of Manca Kovač Viršek. The MP particles extracted from water and sediment samples were photographed, counted and characterised by Irma Hostnik under the supervision of Nataša Mori (NIB), Oliver Bajt (NIB-Marine Biological Station) and Manca Kovač Viršek (IWRS) using FTIR and micro-FTIR.

Link to the article: https://doi.org/10.1016/j.scitotenv.2022.160043

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Microplastic pollution in small rivers along rural–urban gradients: Variations across catchments and between water column and sediments

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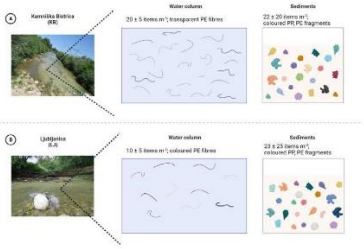
HIGHLIGHTS

- Microplastic pollution of a pre-alpine and karstic catchment was compared.
- Water and sediments of small sized catchment (< 800 km^2) were investigated.
- PE and PP were most common polymers with fibres in water and fragments in sediments.
- Sampling location, catchment characteristics and hydrogeomorphology are important.

GRAPHICAL ABSTRACT



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ABSTRACT

The aquatic ecosystems of the world are highly burdened with microplastics (MPs; particles <5 mm). There is a great need for better understanding of patterns of MP pollution across catchments and rivers of different sizes, anthropogenic pressures and hydrogeomorphological features. In this study, we investigated the MP concentrations including their characteristics (polymer type, shape, size and colour), and MP distribution in water and sediments of two hydrogeomorphologically different small-scale catchments (< 800 km^2), namely Kamniška Bistrica (KB) and Ljubljanica (LJ), Slovenia. The main objective of this study was to gain a better understanding of how WWTP effluents and catchment urbanisation together with the diversity of natural hydrogeomorphology, affect the quantity and quality of MP pollutants in the rivers with smaller catchments. Significantly different mean MP concentrations were found in the water columns (KB: 59 ± 16 items m^{-3}; LJ: 31 ± 14 items m^{-3}), but not in the sediments (KB: 22 ± 20 items kg^{-1}; LJ: 23 ± 25 items kg^{-1}). A longitudinal gradient with increasing particle concentration was observed in both water and sediment samples and in both catchments. Polyethylene (PE) and polypropylene (PP) particles dominated in all samples. Fibres were predominant in the water column samples, while fragments were more common in the sediment samples. MP particles were mostly coloured, and most of them were smaller than 2 mm in both water and sediment samples. The critical evaluation of the results and previous studies suggest that the characteristics of the catchment (anthropogenic pressures, size, climate, etc.), the hydrogeomorphology of the river (sediment type, discharge, flow velocity etc.), the sampling location along the river, the sampled compartment (water, sediment), the sampling method, and the hydrometeorological characteristics at the time of sampling, are important factors for observed MP concentrations and other characteristics.

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1. Introduction

Microplastic particles (MPs; particles <5 mm) pose an increasing threat to human health and the environment worldwide. Research on MPs and their sources, concentrations, transport pathways, environmental fates and impacts on biota, while rapidly increasing, is primarily focused on the marine environment; the origin, distribution patterns, transport pathways and impacts of MPs in the freshwater environment remain largely unexplored (Wang et al., 2021). As rivers are considered highways for plastic debris of various sizes, including MPs, an estimated 0.47–2.75 million tonnes of plastic are transported by rivers into the oceans each year (Lebreton et al., 2017; Schmidt et al., 2017). Therefore, research focusing on freshwater systems is crucial to better understand MP accumulation in the oceans and global distribution patterns.

Most studies dealing with freshwaters have investigated pollution patterns of MP in larger rivers (catchment area > 50,000 km^2 or main tributaries with catchment area > 5000 km^2), and provide information on the large-scale regional and/or global drivers of MP pollution either in the water columns or in the riverbed sediments (Klein et al., 2015; Constant et al., 2020; Eibes and Gabel, 2022). The most common polymers in freshwaters in general, and also in large rivers, are polypropylene (PP) and polyethylene (PE), with fibres and fragments being the most commonly detected shapes (Wang et al., 2021; Yang et al., 2021). Concentrations in water and sediment samples can vary considerably, from 0 to over 80 items in m^{-3} of water, depending on the sampling method (i.e. water pump, net), anthropogenic pressures and catchment size (Constant et al., 2020; Eibes and Gabel, 2022), and from 9 to over 15,000 items per kg^{-1} in river sediments, with the location of sampling playing an important role (Scherer et al., 2020).

MP are mostly produced on land and subsequently distributed throughout ecosystems, including freshwater bodies (Ziajahromi et al., 2016). Primary MPs are intentionally produced, such as industrial resins (e.g. pellets for easier transport) or personal care products (e.g. abrasive particles in toothpaste or skin care) (Waldschläger et al., 2020), while secondary MPs are products of larger displaced plastics. Under anthropogenic or environmental factors, large plastics are fragmented into numerous small pieces, becoming important sources of MP in the environment (Hidalgo-Ruz et al., 2012; Yang et al., 2021). MPs from households, industrial and other wastewater, and urban or road runoff are concentrated in wastewater treatment plants (WWTPs), which serve as a major point-source link between MP pollutants and the aquatic environment (Ziajahromi et al., 2016; Kovač Viršek et al., 2017). Although most MPs in sewage sludge are retained by WWTPs, millions of MP particles are still discharged into rivers per day due to the large amount of treated water (Prata, 2018). In addition, sewage sludge from WWTPs, when used as a fertiliser on agricultural land, can also be a source of MP pollution to freshwaters; it can be washed off by precipitation and enter surface waters (Sun et al., 2019) or be released into the atmosphere during surface treatment (Sommer et al., 2018). Atmospheric MP particles origin from a range of anthropogenic sources (WWTPs, transport, industry, agriculture, etc.) reach the land (forest, urban and agricultural) via precipitation, and flow into rivers and lakes via surface runoff (Waldschläger et al., 2020; Wang et al., 2021; Kallenbach et al., 2022).

Catchment characteristics and river hydrogeomorphology can influence the flux of MP pollution across a landscape and within water bodies. Climate, topography, hydrology and land use, for example, alter the mass balance of MP within a catchment by affecting the diversity and volume of MP emitted from different sources, the nature and extent of transport processes and the likelihood of temporary storage in ecosystems (Windsor et al., 2019). For example, heavy rainfall causes the flux of MP particles into river systems to increase due to atmospheric fallout (Dris et al., 2015; Bergmann et al., 2019), soil erosion (Bläsing and Amelung, 2018) and stormwater runoff (Piñon-Colin et al., 2020; Treilles et al., 2021; Werbowski et al., 2021). In slow-moving stretches of water, MP are likely to settle along with sinking sediment particles, with sediment deposition further contributing to MP particles being buried (Horton and Dixon, 2018). Particles that settle on the riverbed can infiltrate the sediments into a deeper layers or be resuspended during stronger flow conditions, such as floods (Hurley et al., 2018), or even under baseflow conditions (Drummond et al., 2020). Resuspension is strongly influenced by the geomorphology of the riverbed, MP particle concentration and diameter (Nizzetto et al., 2016; Waldschläger and Schüttrumpf, 2019). In some rare cases, MP can even return to land (e.g. during floods) (de Souza Machado et al., 2018). Although the oceans act as sinks for much of the MP, freshwater bodies and soil are also involved in the plastic cycle and retain much of the MP they receive (Klein et al., 2015; Horton and Dixon, 2018; Drummond et al., 2020; Scherer et al., 2020). A study by Drummond et al. (2022) found that MPs are largely retained in headwater systems at low-flow conditions, with residence times of up to 1.7 years km^{-1}. On the other hand, the faster a river flows, the more energy it has to entrain and transport a large amount of particles. High-energy flood flows, for example, lead to the resuspension of dense MP particles together with other sediment particles (Knighton, 2014; Hoellein et al., 2017).

Flow conditions (flow velocity, flood events) and particle properties (shape, density, type of material, microbial overgrowth) are the most important factors controlling the transport of individual MP particles in rivers (Horton and Dixon, 2018; Waldschläger et al., 2020; Winkler et al., 2022). Depending on their density, particles can either float or sink when introduced into the water (Waldschläger and Schüttrumpf, 2019). Floating particles tend to be transported to the ocean by the river current unless retained by barriers (Scherer et al., 2020), while higher density particles, such as PET or nylon, sink to the river bed unless a high-energy current transports them downstream to a low-flow area (Nizzetto et al., 2016). Furthermore, Waldschläger and Schüttrumpf (2019) reported significant differences in the sinking behaviour of pellets, fibres and fragments, indicating the importance of MP shape. Additionally, researchers have found that most fibres are of natural origin (Constant et al., 2020), in some cases as much as 90 % (Stanton et al., 2019).

Due to biofouling, i.e. the attachment of microorganisms to particles, MPs become denser and have a higher settling velocity (Miao et al., 2021). In addition, the shape and size of particles affect the retention of MP. Irregular particles such as fragments and pellets tend to sink or rise more slowly than spheres because they are slowed down by secondary movements (e.g. rotation, lateral oscillation). Fibres appear to orient themselves horizontally in the water regardless of their initial position, and rise with the same velocity, regardless of their length. However, the speed of the fibres may increase as their diameter increases (Waldschläger and Schüttrumpf, 2019). When characterising MP particles, it is also important to consider the colours of MPs, because of the interaction with the microbiota. For example, Lopes et al. (2020) analysed fish stomachs and found a prevalence of blue and black MPs, while Berglund et al. (2019) found predominantly black and transparent fibres in mussels, suggesting that mussels show a colour preference for food.

The present study focused on determining the patterns and types of MP pollution at smaller scales (500 km^2 < catchment <800 km^2, river length < 45 km) in both abiotic compartments: water columns and riverbed sediments, and along the rural-urban gradient. Since understanding MP sources and sinks is important to effectively reduce the impact of plastic pollution on receiving ecosystems (Wang et al., 2021), the main objective of the study was to understand in-depth how environmental and anthropogenic factors, such as hydrogeomorphology, urbanisation and WWTPs, impact MP pollution (i.e., concentrations, types, shapes, sizes and colour of MPs), on smaller scales. In addition, these MP characteristics were studied in both water columns and riverbed sediments to better understand the transport, retention and distribution of MPs in streams. Notably, two geologically and hydrologically different catchments (turbulent pre-alpine river and meandering lowland karst river) with similar anthropogenic pressures were compared. The hypotheses addressed in this paper are: (1) MP pollution gradually increases along the river, both in the water column and in sediments, which is due to an increase in the anthropogenic impacts, (2) MP pollution is significantly higher downstream from WWTP effluents in both water columns and sediments, (3) MP pollution in the two catchments studied differs due to differences in catchment characteristics,

including anthropogenic pressures and hydrogeomorphology, and (4) MP pollution differs between water columns and sediments. Furthermore, this study calls for the standardisation of methods for estimating MP pollution in rivers by comparing and analysing the results of existing publications on European rivers MP pollution and contributes to the development of efficient mitigation methods against MP pollution in freshwater bodies by providing new field data.

2. Methods

2.1. Study catchments

The samples were obtained from two catchments in Slovenia: Kamniška Bistrica (KB) and Ljubljanica (LJ) (Fig. 1). The Ljubljanica is a typical karst river in the south-central part of Slovenia, sinking underground in several places and then reappearing back on the surface. At its last appearance on the surface, the Ljubljanica wells from numerous springs near Vrhnika, flows through the Ljubljana Moor and through the city of Ljubljana, merging with the Gradaščica and then flows as a right tributary into the Sava River. The length of the river in this section is 41 km and the catchment area of this part is about 787 km^2 (Table 1). The NW part of the Ljubljanica catchment is classified as a pre-alpine or isolated karst with steep dolomite slopes, while the background of its springs is a typical karst area. The Kamniška Bistrica, on the other hand, is a pre-alpine river with a gravel-bed in north-central Slovenia, rising in the southern Kamniško-Savinjske Alps (630 m elevation). It is 33 km long and has a catchment area of 534 km^2 (Table 1). The river flows through a narrow valley, continues through the town Kamnik, enters the Ljubljansko polje – the Ljubljana basin tectonic depression – and reaches the Sava River as its left tributary, near the outflow of the Ljubljanica. In the upper reaches, the catchment area consists of limestone and dolomite and is surrounded by forest. The catchment area around Kamnik is composed of clastic rocks (tuff, sandstone, conglomerate, clay, marl), with the lower part of the river containing alluvium.

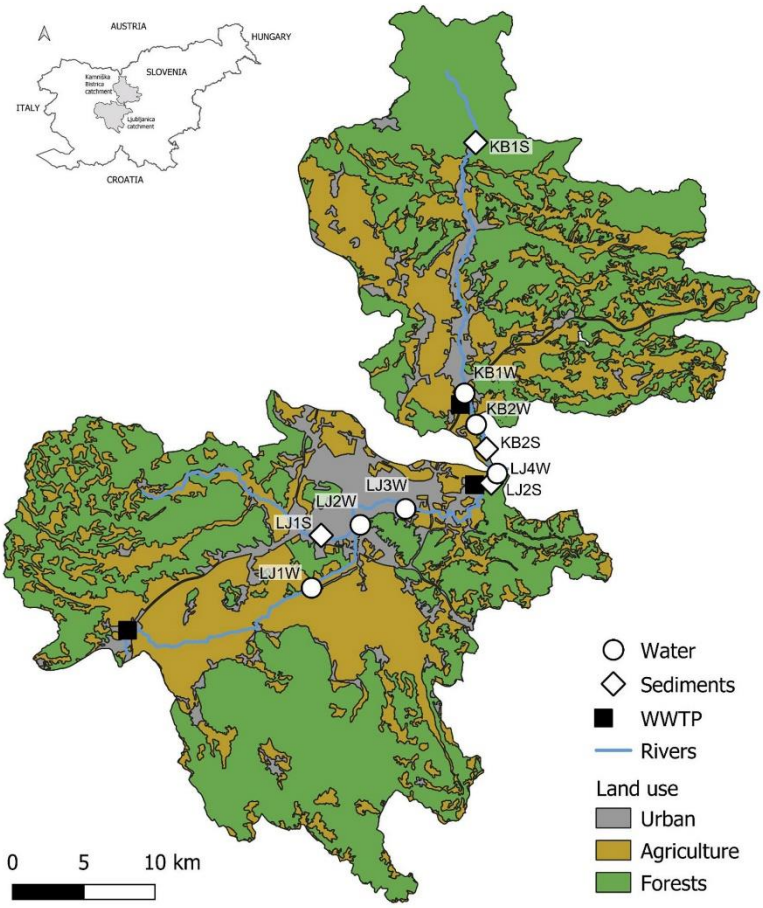

Fig. 1. The study area.

Table 1
Characteristics of study catchments. Source of data: Slovenian Environmental Agency; gauge stations: Moste I (LJ) and Vir (KB).

River	Catchment area (km^2)	River length (km)	Mean daily discharge (±SD) (2010–2020) (m^3/s)	Min daily discharge (2010–2020) (m^3/s)	Max daily discharge (2010–2020) (m^3/s)
Ljubljanica (LJ)	787	41	52.9 ± 53.6	4.4	343.8
Kamniška Bistrica (KB)	534	33	5 ± 8.7	0.1	156.8

2.2. Field sampling

2.2.1. Water column

Samples were obtained from two locations along the Kamniška Bistrica (upstream and downstream of the WWTP; 20 September 2019) and at four locations along the Ljubljanica (upstream, in, and downstream the city of Ljubljana; 1 October 2019) (Table 2 and Fig. 1). At each sampling location, 1 m^3 of water was pumped three times. Water samples were collected at a depth of 15–20 cm below the water level using a motor pump (Makita EW1060H) and a suction basket with a pore size of 1 × 1 cm. The water was filtered through a sieve with a pore size of 150 μm. The collected material was washed with 70 % ethanol and stored in glass containers until further analysis.

2.2.2. Sediments

Sediment samples were taken from two locations along the Kamniška Bistrica (at the pristine river reach and downstream of the WWTP; 26 August and 28 June 2019, respectively) and at two locations in the Ljubljanica catchment (in and downstream of the city of Ljubljana; 26 August 2019). Three sediment samples (500 mL) were randomly collected from the frequently flooded banks and three from the wetted channels in summer 2019 (base flow conditions). In the wetted channels, a tube (diameter = 30 cm; height = 60 cm) was inserted into the river bed. Large stones were removed and sediments (< 4 cm grain size) were carefully sieved and washed and collected in a 500 mL PP flask. 1 L of water with suspended material was also collected from the tube to obtain MPs that may have been released from the sediments during sampling.

2.3. Laboratory sample processing

2.3.1. Preprocessing of sediment samples

The sediments had to be preprocessed in order to extract MPs. In the laboratory, 300 g of sediment was thoroughly mixed with a saturated NaCl solution and left for 24 h. The supernatant was then removed and washed through a 0.063 mm steel sieve. The sediment was again mixed with NaCl solution and allowed to stand for 2 h; the supernatant was then washed through the 0.063 mm sieve and the sediment was allowed to stand in saturated NaCl solution for 2 h before being sieved again. The material was collected in glass containers and washed; $FeSO_4$ and H_2O_2 were added to break down any organic particles. The solution was heated at 75 °C for half an hour, until the chemical reaction ended. If any material remained, additional H_2O_2 was added and the solution was heated. Finally, the residues were washed again over the steel sieve and then the residues in the sieve were filtered through a glass filter (Whatman, GFF, No. 1825-047). The remains on the filter were stored in closed glass petri dishes at room temperature until they dried.

2.3.2. Characterisation of MPs from water and sediment samples

All glass containers used for sampling the water column and filters used for sediment preprocessing were thoroughly checked using a stereo microscope (20–120× magnification, StereoDiscovery V8, Zeiss, Germany). Each assumed MP particle was isolated from the samples with precise tweezers and sorted into one of five categories: fragments, fibres, foams, films and pellets/granules. The particles were then measured according to their longest length, their colours noted and their chemical composition determined using attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR; SpectrumTwo, Perkin Elmer), except for fibres in the water column (see Chapter 2.3.3). Colours were categorised into one of the four obvious groups – transparent, black, white and coloured – as colour perception is subjective and can be influenced by factors such as microscope illumination/background or personal factors (Lu et al., 2021; Yang et al., 2021). MP particles were measured and sorted into size categories determined as 0–0.99 mm, 1–1.99 mm, 2–2.99 mm, 3–3.99 mm, and 4–4.99 mm, with a difference of 0.99 mm between them.

2.3.3. Characterisation of fibres

Individual textile fibres from environmental samples may be too small to be analysed by micro-FTIR. Therefore only a representative sample of fibres from the Ljubljanica River were chemically analysed using this method (Spotlight 200i, PerkinElmer). The fibre particles obtained from the water columns of the LJ were classified according to their origin – natural or anthropogenic – with the latter additionally divided into group of either semi-natural or synthetic origin. No such distinction was made for the samples from KB and they were therefore not included in the chemical analysis. From the LJ samples, a representative sample (5 %) of the fibres was selected for micro-FTIR analysis and fixed on gold-coated polyester membranes (i3-TrackPor P Membrane; i3-Membrane) to avoid interference. In Fig. 2, all fibres of both origins are included. In Fig. 3, all fibres (semi-natural and synthetic) were included in the "Shape" section, while for the presentation of material analysis, colour and size ranges, fibres found from water column samples were not included. In Fig. 4, only fibres that were visually determined to be synthetic were included.

2.3.4. Quality control

During the sampling and analytical processes, plastic was avoided where possible to minimise interference with the samples. All researchers wore non-synthetic clothes and/or cotton lab coats. Glassware was washed thoroughly with Milli-Q water before use, and the workspace was cleaned

Table 2
The sampling sites with location coordinates and location IDs, used in graphics (IWRS – Institute for Water of the Republic of Slovenia; NIB – National Institute of Biology).

Sampling site (river)	Sampler	Sample ID	Coordinates	
Črna vas (LJ)	IWRS	LJ1W	45°59′59.9″N	14°28′06.0″E
Prule – Špica (LJ)	IWRS	LJ2W	46°2′23.9″N	14°30′44.0″E
Fužine (LJ)	IWRS	LJ3W	46°3′00.8″N	14°33′09.9″E
Zalog (LJ)	IWRS	LJ4W	46°4′22.5″N	14°38′8.9″E
Above WWTP Domžale-Kamnik (KB)	IWRS	KB1W	46°07′24.6″N	14°36′21.1″E
Under WWTP Domžale-Kamnik (KB)	IWRS	KB2W	46°06′13.5″N	14°36′59.3″E
Stahovica (KB)	NIB	KB1S	46°16′55.3″N	14°36′53.3″E
Bišče (KB)	NIB	KB2S	46°05′18.4″N	14°37′33.1″E
Mali Graben (GRA)	NIB	LJ1S	46°01′59.4″N	14°28′36.1″E
Cesta v Kresnice (LJ)	NIB	LJ2S	46°04′00.6″N	14°37′49.5″E

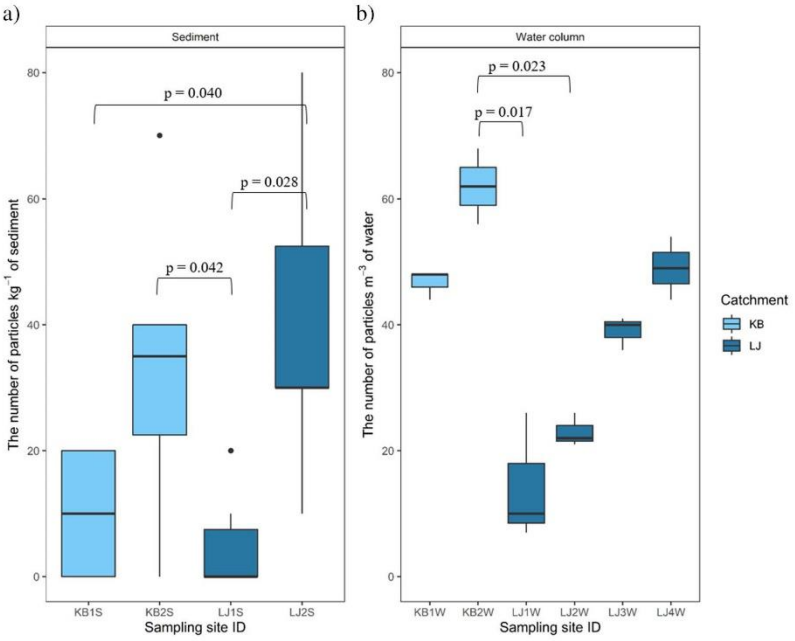

Fig. 2. Boxplots of the number of particles found in a) 1 kg of sediments and b) 1 m^3 of filtered water. Samples were taken as triplicates, and means and standard deviations were calculated as pictured in the graph. All fibres found (including of synthetic/seminatural origin) found were included in the graph. The added *p*-values are the results of the Dunn post-hoc test between the locations. In addition, the Kruskal-Wallis test revealed significant differences between the catchments ($H = 7.6575$, $df = 1$, $p = 0.006$) for the water column samples.

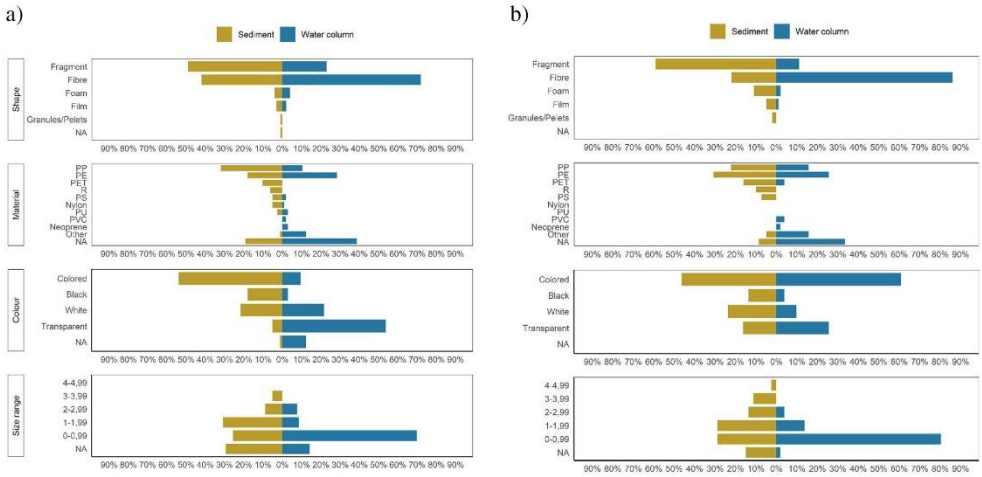

Fig. 3. Characteristics of MPs reported in the sediment and water samples as a percentage of the MPs found in the catchments a) Kamniška Bistrica and b) Ljubljanica. Shapes: fragment; fibre; foam; film; granule/pellet; NA (not defined). Materials: polypropylene (PP); polyethylene (PE); polyethylene terephthalate (PET); rubber (R); polystyrene (PS); nylon; polyurethane (PU); polyvinylchloride (PVC); neoprene; other (different single polymer materials or blends such as poly(2-hydroxyethyl acrylate); NA (not defined). Colours: coloured; black; white; transparent; NA (not defined). Size ranges (mm): 0–0.99; 1–1.99; 2–2.99; 3–3.99; 4–4.99; NA (not defined). Material, Colour and Size range do not take into account water column fibres.

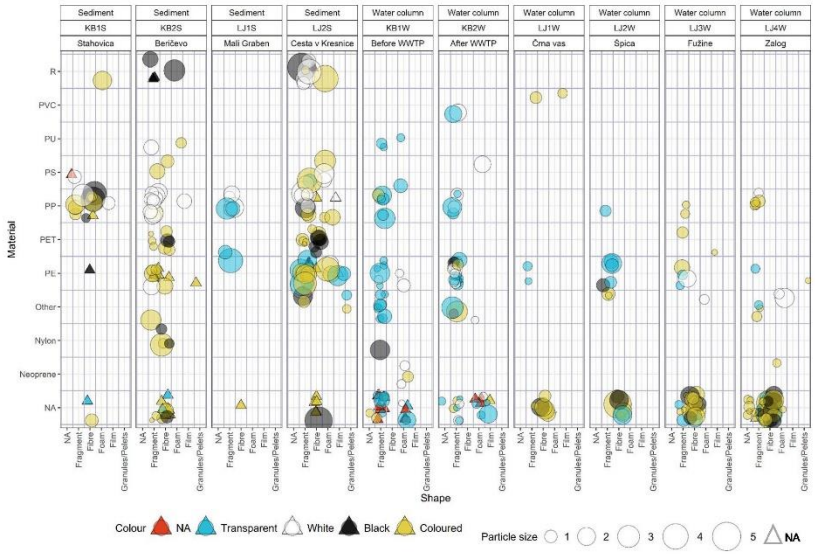

Fig. 4. Visualisation of the various materials, shapes, colours and size ranges of the MP particles collected in this study. Each circle represents one particle. The triangles represent particles whose size was not defined. For the water column samples, only fibres that were visually determined to be synthetic were included. Fibres found in KB were excluded as they were not differentiated according to their origin (semi-natural or synthetic).

with Milli-Q water before and during the research procedure. For the most part, the filters were handled in a laminar flow to prevent MP contamination from the air. When not in the laminar flow, the glass petri dishes containing the sample filters were kept closed. Microscopy was performed in a clean room with air purifier turned on. When extracting MPs from sediments, a spiked positive control was used to evaluate the MP return rate. Furthermore, distilled water was subjected to the same procedure as the environmental samples to determine blank values. The data were corrected according to the contamination levels found during the laboratory analysis.

2.4. Statistical analyses

Statistical analyses were performed using R version 4.1.2 (R Core Team, 2021) and the tidyverse package (Wickham et al., 2019) for visualisation. The level of significance for all analyses was $p < 0.05$. Due to the different methods used for sampling the water column and sediments, the data were analysed separately. As the samples were not normally distributed, the Kruskal–Wallis one-way analysis of variance was used to determine whether there was a significant difference between the mean abundance of particles in the water columns of both catchments and that in the sediments. In addition, Dunn's test was performed post hoc.

3. Results

3.1. Quantification of MPs

MPs were found in all water and sediment samples. A total of 378 particles were characterised from both the water column samples (LJ, $N = 12$; KB, $N = 6$) and the sediment samples (LJ, N = 6; KB, N = 6). These and the following numbers include synthetic fibres visually determined to be of synthetic origin. For the LJ water column samples, a representative number of fibres were analysed by micro-FTIR. An average of 41 ± 20 items m^{-3} (range: 7–89 items m^{-3}) were found in the water column samples ($N = 18$), and an average of 22 ± 22 items kg^{-1} (range: 5–40 items kg^{-1}) in the sediment samples ($N = 24$) (Fig. 2). A longitudinal gradient with increasing particle concentrations was observed in the water and sediment samples from both catchments. In general, higher average concentrations of MPs were observed in the water samples from the pre-alpine river KB (Table 3). The MP concentrations in the sediments highly varied within, between the sites and the two catchments. For water columns, the lowest amount of MP particles was found in the sample obtained above the Ljubljana city in the LJ catchment (LJ1W, 14 ± 8 items m^{-3} of water), and the highest amount was found in the sample downstream of the WWTP effluent in the KB river (KB2W, 71 ± 14 items m^{-3} of water). Among the sediments, the lowest number of MP particles was detected in the city, from the samples from the regulated tributary of the LJ (LJ1S, 5 ± 7 items kg^{-1} of sediment), and the highest number was detected in the samples below the WWTP of the LJ (LJ2S, 40 ± 23 items kg^{-1} of sediment). The Kruskal–Wallis test revealed significant differences between catchments ($H = 7.6575$, $df = 1$, $p = 0.006$) and between sampling locations ($H = 15.868$, $df = 5$, $p = 0.007$) for the water columns. The Dunn post-hoc test showed that this difference was specifically between KB2W and LJ1W ($p = 0.017$) and LJ2W ($p = 0.023$). The Kruskal–Wallis test was conducted to check for differences between the sediment samples of the two catchments (KB vs LJ), but no significant difference was found. However, when considering the different sediment sampling locations (KB1S, KB2S, LJ1S and LJ2S; Fig. 1), the results of the Kruskal–Wallis test were significant ($H = 11.8$, $df = 3$ with $p = 0.0081$). The post-hoc Dunn test revealed significant differences between KB2S and LJ1S ($p = 0.042$), KB1S and LJ2S ($p = 0.040$), and LJ1S and LJ2S ($p = 0.028$), which also differed in terms of catchment land use and hydrogeomorphological characteristics.

3.2. In-depth characterisation of MP particles found in water and sediment samples

3.2.1. Water column

In terms of shape, the predominant particles in both catchments were fibres of both natural or synthetic origin (LJ 86 %, KB 72 %), followed by fragments (LJ 10 %, KB 22 %) (Fig. 3a, b). In the polymer/material analysis,

Table 3
A summary of the results of field studies investigating presence of MPs in the water column and/or sediments carried out in Europe over the last 7 years. For data on shape, size, material and colour, only the most common are given, as written by the authors. Empty cells – no data reported. EVA – Ethylene Vinyl Acetate.

		Rhine	Elbe	Rhône	Po	Dalälven	Main	Ems	Ticino	Têt	Ljubljanica	Kamniška Bistrica	Antuã
Mean river characteristics	Country	Germany	Germany	France	Italy	Sweden	Germany	Germany	Italy	France	Slovenia	Slovenia	Portugal
	Catchment area (km^2)	185,000	148,268	96,364	71,000	28,927	27,208	17,800	7228	1373	787	534	149
	Length (km)	1230	1091	783	652	520	524	371	248	116	41	33	38
Water column	Sampling method		Apstein plankton net	Manta trawl & conical plankton net	Water pump	Water pump		Driftnet	Neuston trawl	Manta trawl	Water pump	Water pump	Water pump
	Samples		n = 10	n = 13 (trawl); n = 15 (net)	n = 5	n = 10		n = 36	n = 18	n = 35	n = 12	n = 6	n = 6
	Mean density (items m^{-3})		5.57 ± 4.33	12 ± 18 (trawl); 19 ± 28 (net)	20.3 ± 13.2	4.5 ± 3		1.54 ± 1.54	33.3 ± 20.4	42 ± 18	31 ± 14	59 ± 16	
	Min – max densities (items m^{-3})		0.88–13.24	trawl: 0.3–59; net: 2.4–88	9.6–43.2	0.4–10.2		0–5.28		0.8–618	7–49	44–89	58–1265
Sediments	Density separation	NaCl	$ZnCl_2$				NaCl			NaCl	NaCl	NaCl	$ZnCl_2$
	Samples	n = 8	n = 11				n = 2		n = 18	n = 32	n = 6	n = 6	n = 6
	Mean density (items kg^{-1})		2080 ± 4670						11 ± 7.7	258 ± 259	23 ± 25	22 ± 20	
	Min – max densities (items kg^{-1})	228–3763	9–15,962				786–1368			7.3–1029	5–40	10–33	18–629
	Shape	Fragments	Spheres, fragments				Fragments		Irregular particles	Fragments	Fragments	Fragments	Fragments
	Size (mm)	0.630–5	0.125–5				0.630–5				0.15–0.99, 1–1.99	0.15–0.99, 1–1.99	0.055–5
	Material	PE, PP, PS	PE, PP				PE, PP, PS		EVA	PE, PP	PE, PP	PP, PE	PE, PP
	Colour	Various	Transparent								Coloured	Coloured	Coloured
Reference		Klein et al., 2015	Scherer et al., 2020	Constant et al., 2020	van der Wal et al., 2015	van der Wal et al., 2015	Klein et al., 2015	Ribes and Gabel, 2022	Winkler et al., 2022	Constant et al., 2020	This study	This study	Rodrigues et al., 2018

due to methodological limitations, 63 % of the particles could be identified from the water column samples: 61 % from KB and 67 % from LJ; fibres not included. In both, most of the particles were PE (28 % and 25 % respectively; fibres not included). The chemical composition could not be determined for all particles due to their small sizes, which led to accidental losses during handling or the particles were too small to handle at all, or the instrument could not provide a signal. Of the LJ samples, a representative sample (5 %) of anthropogenic fibres was selected for chemical analysis, and 75 % of the fibres characterised were polyethylene terephthalate (PET).

Most particles in the LJ sample were coloured (60 %), while most particles in the KB sample were transparent (53 %). The majority of particles in the water samples (fibres not included) were small (0–0.99 mm), both in LJ (80 %) and KB (70 %), followed by particles of size 1–1.99 mm (LJ: 13 %; KB 9 %) and 2–2.99 mm (LJ: 4 %, KB 8 %). For some particles, the size range could not be determined (LJ 2 %; KB 14 %).

3.2.2. Sediments

Generally, the MP particles found in both the LJ (59 %) and KB (49 %) sediments were classified as fragments, followed by fibres (22 % and 42 %, respectively). The LJ sediment sample consisted primarily of PE particles (31 %), followed by PP (22 %), PET (16 %), rubber (R; 10 %) and polystyrene (PS; 7 %); the material composition of 9 % of the particles could not be determined. In contrast, the majority of the KB sediment sample consisted of PP particles (32 %), followed by PE (18 %), PET (10 %), R (6 %), PS and nylon (both 5 %) and PU (3 %); 19 % of the particles could not be determined. Materials classified as "other" in both LJ (5 %) and KB (1 %) included polymethyl methacrylate (PMMA), PE-PP copolymers and acrylonitrile butadiene styrene (ABS), among others. The sediment particles were mostly coloured (LJ 46 %; KB 54 %) or white particles (LJ 23 %; KB 22 %). The majority of MP particles in the sediment samples fell into the smallest size categories: 0–0.99 mm (LJ 28 %; KB 26 %), 1–1.99 mm (LJ 28 %; KB 31 %), and 2–2.99 (LJ 14 %; KB 9 %). The size ranges of some particles could not be determined (LJ 15 %; KB 30 %).

The particles in the sediments were generally larger than in the water column samples (Fig. 4). The largest particle from the sediment samples was a black, 4.73-mm fibre in LJ2S, while the largest particle from the water column samples was a pink, 4.69-mm fibre in LJ2W. All identified particles in the samples from KB were smaller than 4 mm. No rubber particles were found in either of the water column samples and no neoprene or PVC particles were found in the sediments. In addition, no nylon or PU was found in the LJ sediment or water column samples. The most common particles in the water column samples were transparent PE fragments with a size between 0 and 0.99 mm, while the most common particles in the sediments were white PP fragments with a size of 1–1.99 mm. Almost no white particles were found in the LJ water column sample; most were coloured. Most particles from the KB water column were transparent fragments.

4. Discussion

4.1. Potential drivers of MP concentrations in river water

In this study MP sampling in the water column was carried out using the water pump method, which proved to be a very suitable method, as also shown by the linear trend of increasing concentrations downstream of the Ljubljanica River. The average concentrations of MP particles in the water were significantly lower in the Ljubljanica catchment (31 ± 14 items m^{-3}) compared to Kamniška Bistrica (59 ± 16 items m^{-3}), although KB is shorter and has a smaller catchment area. The main reason for this is most likely the selection of sampling sites, which in the case of KB was in the immediate vicinity of the WWTP. The comparison between Po and Kamniška Bistrica Rivers (Table 3) is also interesting, as the sampling methods are the same, but the MP concentrations much lower in the Po River, although the Po catchment is many times more densely populated (17 million inhabitants). The study in the Po reports on increased discharge (floods) during the sampling which could cause the dilution of MP pollution. In general, comparison of the results of other studies does not show any straight forward linkages between MP concentration, the size of catchment area and sampling method. Most likely, the main factors affecting the MP concentration in the riverine water column is the selection of sampling site, where the local anthropogenic pressures and the hydrological features of the river at the time of sampling play the most important role. This is in accordance with the study on the Ems River, where higher amounts of MP items m^{-3} have been found in the Ems downstream of WWTP (Eibes and Gabel, 2022). Interestingly, Eibes and Gabel (2022) also reported a significantly lower concentration of MP downstream of cities, thus identifying cities as potential sinks of MPs. A possible explanation for this phenomenon was the presence of obstacles (e.g. weirs) that reduced flow velocities upstream to the weir, which in turn increased sedimentation of floating MPs. A similar observation regarding obstacles altering flow velocities was made in the Elbe study (Scherer et al., 2020), where the MP concentration tended to decrease along the river course (higher in Middle Elbe than in the Lower and Outer Elbe). In the case of the Antuã River (Portugal), there was no significant upstream-to-downstream gradient in MP concentration (Rodrigues et al., 2018), as observed in our study. However, some seasonal differences were observed with higher concentrations in autumn in comparison to spring, while the opposite was true for sediment samples (Rodrigues et al., 2018). This is most likely due to precipitation. It is known, that the MP concentration in rivers increases with the amount of precipitation and flow velocity (Gündoğdu et al., 2018; Winkler et al., 2022), which is due to the resuspension of plastic particles from river sediments and river banks. This is also supported by the observation that the sampling sites with the lowest MP concentrations in the surface water samples had the highest concentrations in the sediment samples (Winkler et al., 2022). The findings of our and previous studies suggest that the hydrogeomorphology of the river studied is important for the MP distribution, indicating the need for further studies that take into account both spatial and temporal variability and carefully designing the sampling campaign.

4.2. Potential drivers and main characteristics of MPs in river sediments

Compared to previous studies (Table 3), the amounts of the MPs in the sediments of the two catchments studied were much lower. It seems that in the case of sediments, the intensity of MP pollution is related to the size and length of the catchment, followed by the intensity of anthropogenic pressures. For example, similar MP concentrations were found in samples from the Antuã, Têt and Ticino rivers, which have a comparable length and catchment area, while the larger Elbe, Rhine and Main rivers had much higher concentrations of MP in the sediments (Table 3). Furthermore, a strong gradient of higher MP concentrations along the river was observed in both study catchments, which is consistent with the pattern in the water samples.

Most of the particles found in the samples of previous studies were PE and PP in the form of fragments, which is consistent with the present study (Table 3). These results are in agreement with the review paper by Yang et al. (2021): they reported that PE was the main polymer type identified, followed by PP and PS, but also emphasised the diversity of MPs found in different regions of the world. For example, ethylene-*co*-vinyl acetate (EVA) was the main polymer found in the Ticino River (Winkler et al., 2022). On the contrary, Yang et al. (2021) reported that fibres were the most commonly occurring particles in freshwater sediments; however, it is important to note that the results of many studies may have been erroneous, as fibres were usually of natural origin (semi-natural fibres, most likely cotton) (Stanton et al., 2019).

Most of the MP particles were coloured in the present study, similar to the Antuã River, while Klein et al. (2015) found different colours and Scherer et al. (2020) found mostly transparent particles (Table 3). The latter was probably due to the different consideration of coloured MPs: Scherer et al., 2020 considered each individual colour in their calculations, while coloured MPs were grouped under the category "coloured" in the present study, highlighting the importance of standardised methods and reporting procedures.

Similar to water samples, the sampling season and hydrological events prior to sampling can have an important influence on MP concentrations

in sediments. In the Têt River, samples taken just after a rain event in October 2015 had the highest concentration (13 mm, 1029 items kg^{-1}), while the lowest mean concentration was found in samples collected a few days after a major rain event in December 2016 (59 mm, 24 ± 19 items kg^{-1}), which caused peaks in the river flow rate (87 m^3/s); this suggests that precipitation and river flow rate influence the MP concentrations in sediments (Constant et al., 2020).

4.3. Differences in MPs' concentrations and characteristics between water and sediments

Due to differences in the field sampling, sample processing, characteristics of water and sediments and the units used in this study, a direct comparison of the number of MPs between water and sediments is not meaningful, and only a qualitative comparison could be made. The qualitative analysis of the particles shows the difference in the shape of the particles – water samples are dominated by fibres, while sediment samples are dominated by fragments. The shape is one of the crucial factors (besides the material structure – density and size) that influence the buoyancy of the particles. Hoellein et al. (2017) have documented that fibres can float longer and have a longer transport length compared to fragments. In addition to shape, the surface-to-volume ratio also influences the state of aggregation and sinking behaviour of the particles. Additionally, Hoellein et al. (2017) observed that of the differently shaped plastics, for instance, fragments have the shortest transport length in the streams due to the surface-to-volume ratio. The results showed that the majority of particles in both types of samples (water and sediments) fit in the size range 0–0.99 mm and are coloured PE or PP.

The particles found in the water column samples were on average smaller than those found in the sediments. In the case of the Ticino River, for example, the sediment samples contained a significantly larger proportion of smaller MP than the water column (Winkler et al., 2022). However, comparison between different studies in terms of MP concentration by size can be difficult, as noted in recent reviews (Hartmann et al., 2019; Lu et al., 2021; Yang et al., 2021). MP studies consider pieces smaller than 5 mm and are limited by the sampling mesh or filter used. In addition, studies use a different size binning between their size classes (Klein et al., 2015; Constant et al., 2020; Yang et al., 2021). Lu et al. (2021) found that 73 different size classes were reported in the reviewed studies, while some of the reviewed studies did not mention particle size at all. Studies should report on MP size with standardised range categories to avoid overestimation.

In order to confirm and better understand the sources of the identified MP items, the visual placements of these items were confirmed using FTIR analysis. In addition, due to the difficulties in handling smaller pieces with FTIR, visual inspection remains important. Fibres of natural origin are flat, twisted and of uneven diameter, whereas fibres of synthetic origin are of uniform diameter along their entire length. Facilitating the digestion of organic matter in MP samples, along with visual separation of MPs, is effective for MP separation and is used in most studies (Stanton et al., 2019). Sediment samples were found to contain mostly PP followed by PE particles, while conversely most water column samples contained mostly PE particles followed by PP (Fig. 3). In most rivers, the predominant polymer was PE (Table 3) and these results are also consistent with the report by Lu et al. (2021) that PE, PET and PP were the predominant MP types in the water samples of previous studies, while PE and PP dominated in the sediment samples, which is consistent with the present study. PE is in high demand in Europe (17.4 % and 12.9 % for low and high density, respectively) and is mainly used for creating reusable bags, food packaging, shampoo bottles and agricultural film (PlasticsEurope, 2021).

In the present study, half of the items found in the sediments were coloured, followed by white, black and transparent items. In the water column samples, coloured and transparent items were common, followed by white and black items, and some were non-defined (6 %) (Fig. 3). Previous studies have reported similar results, different rivers were polluted with particles of different colours (Rodrigues et al., 2018; Scherer et al., 2020). Coloured MPs are usually associated with products with long shelf life (Prata et al., 2019). Analysing the colour of MP can also identify possible sources of MP or contaminants during sample preparation (Prata, 2018; Fahrenfeld et al., 2019; Stanton et al., 2019), which in turn can also facilitate the identification of shapes (Lu et al., 2021). In this study, the most common shapes were fibres and fragments. Coloured fibres may be associated with effluent from a nearby WWTP, while transparent (or discoloured) particles could be due to long-term environmental exposure to UV light or chafing against sediments. In contrast, coloured particles could be indicate fragments of recently discarded (and brightly coloured) larger plastic particles, usually from products with a long shelf life.

4.4. Methodological constraints and recommendations for improving MP characterisation

Due to differences in sampling methods and laboratory analyses, it is difficult to compare the results of MP concentrations in riverine systems between studies. Table 3 compares studies on European rivers of different sizes and under high anthropogenic pressure, using different sampling methods.

Water sampling with a water pump is a rather rare method in MP research. Before commencing the present study, the epineuston net and pump-based sampling methods were tested and it was found that the MP concentrations obtained by sampling with the pump were 10 times higher at smaller volumes of individual samples. Sampling with a net was deemed unsuitable for rivers, because a lot of organic (leaves, branches, aquatic organisms) and inorganic (sand, fine sediment) material gets trapped in the net, making further separation of sample particles difficult. Techniques such as organic material degradation and density separation are not suitable because organic particles such as leaves, cannot be completely degraded by techniques that do not simultaneously affect plastic particles. Furthermore, density separation would not work because the organic particles float on the surface together with the MPs. MP particles are therefore hidden under the other materials. In contrast, the samples obtained with the pump sample were extremely clean because the suction basket did not allow particles larger than 1 cm to pass through. Therefore, the samples did not need to be processed further before MP separation. This method also allowed sampling at different depths. In rivers, MPs tend to be more evenly distributed in the water column due to the river flow, and their concentrations are not necessarily highest at the water surface, as is typical for seawater, so sampling with a water pump in the upper half of the water column made even more sense.

An important consideration when investigating MP concentrations is the choice of an appropriate sample size. Sufficiently large water and sediment samples should be taken to reduce the error in the results, as extrapolating results from, say, 100 g sample to one kilogramme is not realistic as the samples are not homogeneous, and can lead to an error.

In MP research, laboratory analysis of MP particles is often based on separating particles using tweezers. However, this is limited by particle size and may result in a small proportion of particles whose chemical composition can be determined. We therefore propose to filter the washed samples from the filter on an inorganic membrane in the laboratory and examine them with an FTIR microscope. This avoids manual separation of the particles, which saves time and reduces the risk of sample contamination. The use of a glass fibre filter should be avoided, as such filters consist of several layers between which MP particles can hide, affecting the final result.

A current debate among researchers revolves around which fibres can be counted as MP. In particular, studies have differed on the counting of cotton fibres, which are chemically cellulose but are often dyed (Stanton et al., 2019). In the present study, anthropogenic fibres of synthetic and natural origin were distinguished by visual recognition using a stereomicroscope. Anthropogenic fibres of natural origin were classified as microlitter – a term also used in the implementation of marine litter monitoring in the context of the Marine Strategy Framework Directive (Hanke et al., 2013). Therefore, the term microlitter was deemed prudent to describe microplastics as well as particles of other materials (e.g. rubber, paints). Accordingly, all particles from anthropogenic materials in the natural

samples were classified to be microlitter, and those particles originating from polymer were categorised as microplastics.

5. Conclusion

The present study offers one of the rare in-depth insights into the quantities, distribution and main features (polymer type, shape, size and colour) of MPs in rivers with small catchments (< 800 km^2) along rural-urban gradient, differing in the intensity of anthropogenic pressures and hydrogeomorphology and addressing MP pollution in both the water column and sediments.

In both the water and sediment samples and in the two rivers studied, the MP concentrations increased further downstream, i.e. with increasing distance from the river spring. PE and PP particles were most frequent and abundant in the water and sediment samples, with smaller particles dominating in the water columns. Fragments prevailed in the sediments, and fibres were predominant in the water columns; this can be attributed to differences in the horizontal movement of the particles in the water. The colouration of the particles was not consistent across catchments, indicating that the sources of MP pollution are highly variable. While pollution sources of MP and pollution intensity are key factors determining the types and quantities of MP present in rivers, patterns of downstream distribution, retention and transport depend on the hydrogeomorphological characteristics of the river.

In past studies on MP pollution, different field sampling and processing methods have been used, making a comparison of different field studies difficult. Therefore, standardisation of sampling methods, laboratory procedures and reporting on results is crucial. In addition, more data needs to be collected in order to make a comprehensive risk assessment of MP in the environment. In sum, further research, with standardised methodologies and reporting procedures, is needed for a better understanding of MP distribution and its influencing factors including catchments with different anthropogenic pressures and of different sizes and natural characteristics.

CRediT authorship contribution statement

TM participated in conceptualization and development of methodology, in field and laboratory work, analysed and organized the data and wrote the first draft of MS; NM lead the conceptualization, participated in field sampling, data analysis and writing of MS; IH participated in conceptualization and development of methodology, laboratory work and writing of MS; OB participated in development of methodology, as an expert in FTIR and writing; MKV lead the development of methodology, participated in field sampling, data analysis and writing of MS.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3.2 Relationship Between Microorganisms and Plastics as Substrate and Food Source

When introduced to aquatic ecosystems, it is well known that plastics and MPs are immediately overgrown by biofilms (Zettler et al., 2013; McCormick et al., 2014; Jemec Kokalj et al., 2019). Microbial assemblages gain advantages through surface colonization and biofilm formation, such as improved resource access (Dang & Lovell, 2016). While the structure and taxonomic composition of microbial communities may vary with plastic-type and surface characteristics (Oberbeckmann et al., 2014; Oberbeckmann et al., 2018), the interaction between microorganisms and plastics in freshwater environments is poorly understood despite its importance for environmental remediation (Wu et al., 2019a). In addition to serving as a substrate for biofilms, plastics also act as a potential food source for certain microorganisms that biodegrade them (Yoshida et al., 2016; Miloloža et al., 2022).

Since knowledge of such interactions and plastic-degrading microorganisms is scattered across different scientific fields, a systematic literature review with pre-defined criteria was conducted to critically evaluate the existing studies on the interactions between plastics and bacteria, propose directions for future studies, provide an updated list of all currently known bacteria capable of degrading plastics, and to identify the best methods to assess their biodegradation (Chapter 3.2.1). An important conclusion of the meta-analysis was that most of the bacteria thought to be capable of biodegradation were isolated from contaminated sites (Matjašič et al., 2021b). Furthermore, most MPs removed during WWT are in the final waste sludge basin (Sun et al., 2019). Based on these findings, an experiment was conducted to investigate whether the activated sludge bacteria can colonise common MPs. While previous studies used activated sludge as seed in their experiments, they used only artificially produced polypropylene (PP) spherical particles as the substrate (Khatoon et al., 2014). In my preliminary study, colonization processes and biofilm formation were observed on different substrates (i.e., PET fibres and bottles, HDPE thin plastic bags) commonly present in treated wastewater and in freshwater (Lv et al., 2019).

3.2.1 Published scientific review article: "Critical evaluation of biodegradation studies on synthetic plastics through a systematic literature review"

This section is a review article authored by Tjaša Matjašič (NIB), Tatjana Simčič (NIB), Neja Medvešček (NIB), Oliver Bajt (NIB), Tanja Dreo (NIB) and Nataša Mori (NIB) that was published in Science of Total Environment in 2021. I conceptualised and developed the methodology, including the overall questionnaire design, all under Nataša Mori's supervision. I systematically searched for papers in scientific databases such as Scopus and Web of Science. I then extracted the data from the relevant published papers according to pre-determined criteria, analysed the extracted data and wrote the first draft of the manuscript. Tatjana Simčič and Nataša Mori were actively involved in developing the methodology, designing the questionnaire, and systematically extracting data. They also provided valuable feedback on the manuscript. Additionally, Neja Medvedšček assisted with the systematic extraction of data. Oliver Bajt and Tanja Dreo provided insightful feedback on environmental chemistry and microbiology.

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Review

Critical evaluation of biodegradation studies on synthetic plastics through a systematic literature review

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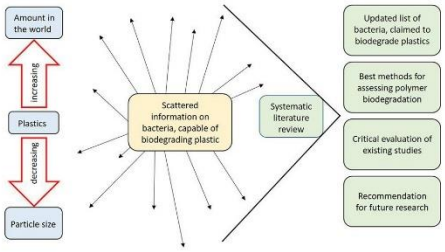
HIGHLIGHTS

- 145 papers on plastics bacterial biodegradation were systematically reviewed.
- 246 bacterial strains were identified; most frequent were *Pseudomonas* and *Bacillus*.
- 44% of cases tested polyethylene and derivates, 9.7% tested microplastics
- Research gaps and priorities were defined.

GRAPHICAL ABSTRACT



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ABSTRACT

Increasing amounts of plastic waste in the environment and their fragmentation into smaller particles known as microplastics (particles, <5 mm) have raised global concerns due to their persistency in the environment and their potential to act as vectors for harmful substances or pathogenic microorganisms. One possible solution to this problem is biodegradation of plastics by microorganisms. However, the scientific information on plastic-degrading microorganisms is scattered across different scientific publications. We conducted a systematic literature review (SLR) with predefined criteria using the online databases of Scopus and Web of Science to find papers on bacterial biodegradation of synthetic petroleum-based polymers. The aims of this SLR were to provide an updated list of all of the currently known bacteria claimed to biodegrade synthetic plastics, to determine and define the best methods to assess biodegradation, to critically evaluate the existing studies, and to propose directions for future research on polymer biodegradation in support of more rapid development of biodegradation technologies. Most of the bacteria identified here from the 145 reviewed papers belong to the phyla Proteobacteria, Firmicutes and Actinobacteria, and most were isolated from contaminated sites, such as landfill sites. Just under a half of the studies (44%) investigated the biodegradability of polyethylenes and derivates, particularly low-density polyethylenes. The methods used to monitor the biodegradation were mainly scanning electron microscopy and Fourier-transform infrared spectroscopy. We propose that: (1) future research should focus on biodegradation of microplastics arising from the most common pollutants (e.g. polyethylenes); (2) bacteria should be isolated from environments that are permanently contaminated with plastics; and (3) a combination of different observational methods should be used to confirm bacterial biodegradation of these plastics. Finally, when reporting, researchers need to follow standard protocols and include all essential information needed for repetition of the experiments by other research groups.

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Contents

1. Introduction

Plastics are long-chain manmade polymeric molecules (Scott, 1999) that have many favourable traits, such as strength, flexibility, extreme durability, low weight and easy and low-cost production. Their uses and applications have been steadily growing over the last 150 years (Sivan, 2011), resulting in an increase in worldwide production of plastics from 1.5 million tonnes in 1950 to 360 million tonnes in 2018 (APM, 2019). Until recently, plastics pollution in the environment was considered merely an aesthetic inconvenience; however, new discoveries have raised concerns about the potential deteriorative impacts of plastics on the biota and on human health (Sivan, 2011). The main environmental and health threats of plastics arise from their accumulation and persistence in the environment and their fragmentation into small particles over time (i.e. microplastics; <5 mm), as well as their potential "for serving as" vessels for toxic chemicals and pathogens (Mato et al., 2001; Shah et al., 2008; Chua et al., 2014; Caruso, 2019).

Plastic pollution of marine environments is now recognised as a global-scale threat with adverse effects that span from the molecular level to physiological performance and organism health and beyond to the loss of ecosystem services (Avio et al., 2017; Kanhai et al., 2020). Estuarine and freshwater environments most likely represent a major source of inland plastic pollution to marine environments (Eerkes-Medrano et al., 2015; Nel et al., 2018; Luo et al., 2019; Amrutha and Warrier, 2020; Zhang et al., 2020), and in the form of microplastics, plastics are also concentrated in wastewater treatment plants (WWTPs) during the primary and secondary treatment processes (Talvitie et al., 2017; Masiá et al., 2020), and they accumulate as plastic debris at landfill sites (Canopoli et al., 2020). Plastics also increasingly enter the soil through sewage sludge and wastewater used as fertilisers, and they are widely used in agriculture as protecting covers (e.g. greenhouses, low tunnels, mulching, and other uses), as coatings for fertilisers, pesticides, hormones and seeds, and as packaging materials (Riggi et al., 2011; Nizzetto et al., 2016). Currently, the problem of increasing and unknown quantities of microplastics in the environment is recognised as equally important as the plastic debris accumulating along the coasts and in the oceans around the world (Horton et al., 2017; Wu et al., 2020). New approaches are needed to cope with this extensive environmental issue, and microbial degradation is one promising strategy (Shah et al., 2008; Jacquin et al., 2019; Malachová et al., 2020).

The most commonly used polymers that end up as plastic debris in the environment are polyethylenes (PEs, including; linear low density [LLDPE], low density [LDPE], medium density [MDPE], high density [HDPE]), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS), nylon, polyethylene terephthalate (PET), polybutylene terephthalate (PBT), and polyurethane (PUR) (Alauddin et al., 1995). Most of these are thermoplastics (polymers built of long carbon chains), which makes them highly resistant to degradation or hydrolytic cleavage of chemical bonds. Some, such as PUR, are thermosetting plastic polymers with highly cross-linked structures that are potentially susceptible to degradation by hydrolytic cleavage of chemical bonds (Alauddin et al., 1995). When plastic particles are exposed to abiotic factors, such as UV irradiation from sunlight, they undergo photo oxidation, which causes them to deteriorate, lose their tensile strength, become brittle and crumble into small fragments and particles, known as microplastics (Sivan, 2011). These microplastics are characterised as water-insoluble solid polymer particles that are ≤5 mm in size; particles ≤1 μm in size are usually referred to as nanoplastics (Koelmans et al., 2019).

In general, the degradation of plastics involves conversion of the polymers into smaller subunits through breakage of their bonds by physical, chemical or biological processes with a resulting alteration in the physicochemical properties of the polymers (Chauhan et al., 2018). This degradation is therefore influenced by both abiotic (e.g. mechanical stress, light or temperature) and biotic (e.g. naturally occurring microorganisms like bacteria, fungi and algae) factors, as well as the time frame (Haider et al., 2019). Biodegradation is a complex process of physicochemical transformation of polymers into smaller units through the actions of microorganisms (Chiellini and Solaro, 1996). Therefore, biodegradable polymers are materials that are completely converted (over a limited period of time) by aerobic microorganisms into carbon dioxide, water, minerals and biomass, or in the case of anaerobic biodegradation, into carbon dioxide, methane and humic material. This biodegradation into their constituent parts does not leave any potentially harmful substances (Kyrikou and Briassoulis, 2007). The degree of biodegradability of plastics depends on their chemical and physical properties. The features with the most influence on biodegradation are the surface conditions (surface area and hydrophilic and hydrophobic properties), the first-order structures (chemical structure and molecular weight and its distribution), and the higher-order structures (glass transition temperature, melting temperature, modulus of elasticity, crystallinity and crystal structure) (Tokiwa et al., 2009).

The number of scientific publications on biodegradation of different types of plastics is gradually increasing, and several reviews have already summarised the emerging state of the art in this field. For example, Zheng et al. (2005) synthesised the knowledge at the time on the technological advances that had been made in the development of more easily biodegradable plastics. This included an evaluation of the biodegradation of conventional plastics by microorganisms, in order to adopt alternative methods for reducing the impact of plastic waste on the environment. Similarly, Kyrikou and Briassoulis (2007) delineated the degradability of polymers used in agriculture by reviewing the state of the art at the time. They listed 12 studies on LDPE biodegradation and one for PE, HDPE, PP and polyaromatic hydrocarbons (PAHs). They also summarised the existing standards for biodegradation and compostability of plastic materials. Gautam et al. (2007a) provided an overview of their current state of plastic foam biodegradation, including the biodegradation pathways of a few selected synthetic polymers. Shah et al. (2008) published an excellent review of 29 biodegradation studies on different types of plastics. They summarised various techniques that had been applied for the analysis of plastic degradation in vitro, provided a list of microorganisms reported to degrade different types of plastics, and recommended more uniform standards for assessment of the degradative abilities of these microorganisms. Two subsequent reviews on biodegradation of PEs by Sangale et al. (2012) and Restrepo-Flórez et al. (2014) examined 34 and 57 studies, respectively. In 2015, a similar review to that of Shah et al. (2008) was provided by Kale et al. (2015), where they presented a new list of 20 biodegradation studies carried out between 2004 and 2014 on different types of plastics. Raziyafathima et al. (2016) reviewed the plastic-degrading efficiency of microbes and their involvement in the degradation of plastic waste; the authors included different references to those provided by Kale et al. (2015). Ho et al. (2017) provided an extensive review of 20 studies on the biodegradation of PS and concluded that PS can be biodegraded, although at a slow rate. Wilkes and Aristilde (2017) carried out a review of 16 studies on the factors that control the processing of different plastic polymers and their by-products by *Pseudomonas* spp. In 2017, Pathak and Navneet (2017) derived a list of microorganisms associated with degradation of synthetic (i.e. PE, PVC and PUR) and bio-based polymers based on the data from seven studies, including some reviews. Urbanek et al. (2018) carried out a review of 16 studies to assess the knowledge of plastic degradation and plastic–microorganism interactions in cold marine habitats and indicated that some of the microorganisms isolated from these environments could degrade plastic waste from ecosystems. More recently, two comprehensive reviews that addressed biodegradation by marine microorganisms and the recent achievements in the area of biodegradation have provided extended and updated lists of microbes reported to degrade different types of synthetic polymers (Jacquin et al., 2019; Moharir and Kumar, 2019). Very recently, Ghatge et al. (2020) and Montazer et al. (2020) summarised the current state of the art in the field of PE biodegradation. In addition to these reviews, several shorter reviews have been published (e.g. Gnanavel et al., 2012; Muthukumar and Veerappapillai, 2015; Sharma et al., 2015; Kumar et al., 2017).

Scientific reviews usually provide the expert with a synthesis of scientific evidence, and if a review is carefully constructed, it is termed systematic literature review (SLR) and can provide insights into the reproducibility of published data and the pooled estimates of 'common truth' through meta-analyses (Daldrup-Link, 2018). Our aim in the present paper was to achieve this type of insight into the current state of the art in the field of synthetic polymer biodegradation by carrying out a SLR with clearly defined selection criteria and using consistent methodologies for data extraction. In this SLR, we have synthesised the major findings of an extensive literature review on the biodegradation of synthetic (petroleum based) polymers that includes 145 studies published in peer-reviewed scientific journals, and we use meta-analysis (i.e. the quantitative synthesis, analysis and summary of a collection of studies; Hedges and Olkin, 1985) to summarize our findings. Further objectives of this SLR are to identify the missing gaps in the current state of the art in the field of biodegradation of synthetic polymers and to provide recommendations for future research. For this SLR, we were specifically interested in identifying: (a) all bacteria that have been clearly shown to break the chemical bonds in synthetic polymers; (b) the most practical and efficient observational methods for biodegradation studies; and (c) the research gaps and the most promising research directions in the field of polymer biodegradation that will help to remediate the problem of the increasing presence of both larger plastic debris and microplastics in the environment.

2. Methodology

The focus of this SLR is those studies that report bacterial degradation of synthetic plastics, rather than bioplastics (i.e. plastic materials produced from renewable biomass resources, such as vegetable fats and oils, corn starch, straw, woodchips, sawdust, recycled food waste and others). The search for relevant peer-reviewed papers on bacterial degradation (biodegradation) of synthetic plastics was conducted across two databases of peer-reviewed literature: Scopus and Web of Science (WoS). The following search strings were used:

- Scopus: (biodegradation OR "biological degradation") AND (microorganism* OR bacteria) AND (plastic OR plastics) NOT (bioplastic* OR "biodegradable plastic")
- WoS: TITLE-ABS-KEY ((biodegradation OR "biological degradation") AND (microorganism OR bacteria) AND (plastic) AND NOT (bioplastic OR "biodegradable plastic"))

These searches included papers published up to 17 June, 2020. Potentially, some papers might have been overlooked, especially those that are not included in the Scopus or WoS databases or did not use the term 'biodegradation'. The records obtained were first checked for duplication. If a publication was present in both databases, one record was removed. The abstracts were then read to check the suitability of the papers, as predefined. Papers were included if they reported an original study in English and were excluded if they reported experiments with only fungi or involving biodegradation of only bioplastics, or if they examined solely physical or chemical processes, and not biological degradation. Each abstract was checked by at least two authors to confirm suitability.

For improved clarity, we separately listed papers that were included in this SLR through systematic extraction of the required data using questionnaires (listed in Appendix A). The selected papers were then thoroughly read and the relevant information was extracted and gathered using an online predesigned questionnaire built using an open-source application (https://www.1ka.si/d/en). The questionnaire enabled us to predefine the information we wanted to obtain from the papers examined; thereby allowing extraction of structured and consistent data for to objective responses to the identified research questions. Again, two authors examined each paper and filled in the questionnaires.

In the questionnaire, general information about a paper, including unique ID, first author, publication year, and DOI number (where provided), was gathered and its suitability confirmed. Further information was then collected for those papers deemed suitable, including the origin of samples, bacteria tested and type of experiment conducted (e.g. degradation). Information on methodological approaches (i.e. study design, experimental conditions and observation methods) and results was then collected. When all relevant papers had been examined and the questionnaires completed, the data were extracted in the form of data worksheets for further analysis. Possible different outputs of questionnaires by different examiners were discussed to obtain mutual agreement on the answers.

The data obtained were consolidated in the form of Tables and Figures, and statistical analyses were performed when possible. Both

SigmaPlot (Systat Software, San Jose, CA) and the ggplot2 package (Wickham, 2016) in R software (R Core Team, 2019) were used to construct graphs, and R was to calculate Pearson's correlation coefficients between treatment temperatures and lengths of studies for the two most commonly reported bacterial genera, *Pseudomonas* and *Bacillus*, and their mixtures. Prior to analysis, the data were log(x + 1) transformed to meet linear assumptions. We also compared correlations against each other by testing for significant differences using an online test available on Psychometrica (Eid et al., 2011; Lenhard and Lenhard, 2014).

3. Biodegradation studies

The application of the above-mentioned search strings identified 1901 papers: 370 from WoS and 1531 from the Scopus database. The final number of studies obtained for this SLR was 145. The number of published articles reporting on bacterial degradation of plastics has steadily increased over the last 50 years (Appendix B, Fig. B.1). The affiliations of the first authors indicated, a varying number of SCI papers from 35 different countries (Appendix B, Table B.1) with highest number coming from India (33.8% of all 145 studies). India is also one of the countries with the highest rates of inadequately disposed waste (i.e. plastic waste that has the intention of being managed through waste collection or storage sites, but is not sufficiently managed and can be lost to the surrounding environment) (Jambeck et al., 2015). An interesting idea for future analyses would be to compare these results to those of national statistics in terms of waste management or demographics, for example.

3.1. Types of polymers

Over 20% of the studies included reports on investigations into LDPE biodegradation, while 14.5% of them reported on PE biodegradation (Fig. 1). PS, PP, HDPE, PET and the other plastics have been tested less often for their biodegradability (<10% of the studies). About a quarter used different combinations of plastic materials (i.e., mixtures) of, for example, LDPE and HDPE or of LDPE and PP to investigate biodegradation; therefore, a clear understanding of the individual biodegradation mechanisms could not be obtained. Some studies (3.4%) also did not report the specific type of plastics used in the experiments but instead used ambiguous names (e.g. plastics) or plastic products (e.g. e-waste, keyboards). Again, this precluded drawing any general conclusions regarding on the interactions between certain polymers and the bacteria being tested.

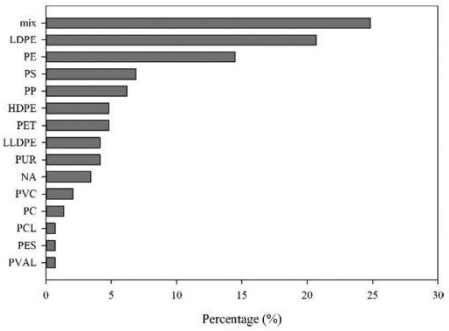

Fig. 1. The frequency of application of different plastic materials in biodegradation studies ($N = 145$). Mix – two or more different materials jointly treated, NA – not defined.

Among these 145 studies, only 9.7% used a material that can be classified as microplastic (i.e. particles <5 mm), whereas 52.4% of the studies were related to larger plastic pieces (>5 mm). In total and 37.9% of the studies did not define the size of the plastics. Most studies reported the use of plastics in the form of films, foils or sheets (49.0%) or strips (12.4%) (Table 1). Overall 9.0% of the studies, investigated plastic dust or nanoparticles, whereas 4.1%, of the studies did not report the physical form of the plastics used. Almost half studies (46.9%) provided data on the material surface area used in experiments, and another 13.8% reported the material volume (Table 1). However, 37.2% of the studies did not report on the amount of material used in biodegradation experiments (e.g. surface area or volume). A study by Chinaglia et al. (2018) revealed that the form of material is highly relevant, as the rates of biodegradation of the biodegradable aliphatic polyester polybutylene sebacate is affected by its particle size and surface area. The biodegradation rate was significantly slower for pellets (1 g, surface area, 33 cm^2) than for other smaller samples (double dose 2 g; with surface areas of 193 cm^2, 825 cm^2), which had a biodegradation rate similar to that of cellulose.

Globally, approximately 42% of all non-fibre plastics are used for packaging (mostly PE, PP and PET) and 19% in the building and construction sectors (mostly PVC) (Geyer et al., 2017). Similarly, in Europe, the biggest demand for plastics is for packaging (40%), followed by building and construction (20%), and then other uses (e.g. in appliances, mechanical engineering, furniture and medicine; 17%). The most widely used plastic materials are PP (19.3%), LDPE/LLDPE (17.5%) and HDPE/MDPE (12.2%) (APM, 2019). The compositions of the microplastics that contaminate different environmental compartments therefore mainly correspond to those contributing to the global demand and production. For example, in freshwaters and drinking water, microplastic particles occur in amounts of PE ≈ PP > PS > PVC > PET (Koelmans et al., 2019), whereas PP prevails in freshwater sediments (Peng et al., 2018), PE in floodplain soils (Scheurer and Bigalke, 2018), PAHs and PET in marine sediments (Martin et al., 2017), and PAHs in activated sludge (Liu et al., 2019). The findings of this SLR indicate that almost half of the biodegradation studies have focused on LDPE or PE biodegradation, in agreement with global use and waste production, as well as with the presence of microplastics in freshwaters. By contrast, studies on the biodegradation of PP, PS, PAHs and PET are relatively rare, although these are certainly also important pollutants. Hence, more studies should investigate the biodegradation of these other materials to develop techniques for their efficient removal them from the environment, especially when they occur in the form of dispersedly distributed microplastics.

Table 1
Summary of polymer characteristic reported in biodegradation studies (N = 145). Other = variety of different types i.e., foam, balls, tiles, pellets, plastic cups, bags, pieces, etc. NA = data not available.

Reported polymer characteristics	Proportion of studies (%)
Amount	
Surface area	46.9
Volume	13.8
NA	37.2
Form	
Film/foil/sheet	49.0
Other	20.0
Strips	12.4
Dust/nanoparticles	9.0
Film/foil/sheet; dust/nanoparticles	3.4
Pellets; strips	2.1
NA	4.1
Size	
Larger particles (>5 mm)	52.4
Microplastics (<5 mm)	9.7
NA	37.9

3.2. Experimental conditions

The environmental conditions are key for microbial metabolism. Accurate information on the autecological requirements of plastic-degrading bacteria are needed when they are tested in biodegradation studies. Hence, reports on biodegradation should include all essential information needed to repeat the studies, or to improve and upgrade the experiments. At a minimum, this information should include the information on pretreatment, growth medium, temperature and length of experiments.

3.2.1. Pretreatment

Although physical and chemical pre-treatments can greatly increase the bioavailability of polymers, around 11.7% of the 145 studies did not perform any pre-treatments, and 15.9% did not report on this. Most of the studies using pre-treatments only used one form (61%). Most of them reported on washing the original materials using distilled water or ethanol (26%), or by exposure to UV (4.8%). Around 18.6% of these pre-treatment studies reported on the use of two different forms of pre-treatment, most of which were thermal pre-treatments in combination with washing with water or ethanol (4.1%). Pretreatment with UV light was the second most commonly used pretreatment, although this was applied in only eight studies. Several plastic materials are indeed photosensitive, and according to Lucas et al. (2008), one of the most important parameters in abiotic degradation is exposure to light radiation. Photo-degradation is a process of decomposition that is generally initiated by UV and visible light and structural and morphological changes in plastics caused by UV irradiation have been reported in several studies (e.g. Fechine et al., 2002; Zhang et al., 2016). Pretreatment of plastics using abiotic factors like UV light or high temperatures can substantially improve their biodegradation (Lucas et al., 2008; Arkatkar et al., 2009; Carol et al., 2012). These abiotic factors can weaken of the polymeric structure, initiate the biodegradation process, and can even be considered as synergistic factors (Lucas et al., 2008).

Thermal and photo-degradation are similar under normal conditions (Fotopoulou and Karapanagioti, 2019). Thermal degradation is basically a 'molecular deterioration as a result of overheating', where the splitting of the chemical bonds is initiated by high temperatures. The components of the long-chain backbone of the polymer begin to separate and react with one another, thereby changing the polymer properties (Shah et al., 2008). Thermal degradation of thermoplastic polymers occurs when the polymer is transformed from a solid to a liquid. At melting temperatures, the thermosetting plastics do not have a fluid state; therefore the original material does not change state at temperatures below the temperature at which thermal decomposition occurs. Consequently, the material does not undergo any notable physical transformation before decomposition (Beyler and Hirschler, 2002). The papers reviewed in this SLR have included thermal pretreatments consisting mainly of autoclaving (Gautam et al., 2007b) and drying at 70 °C to 90 °C for 10 days in a hot air oven (Sudhakar et al., 2008; Arkatkar et al., 2009; Awasthi et al., 2017).

Most polymeric materials that end up in landfill sites or disperse in the environment are exposed to weathering, ageing and burying, which can induce mechanical, light, thermal and/or chemical transformations that can modify the biodegradation of these polymeric materials. Hence, biodegradation experiments should include pretreatment as an essential component to imitate natural in-situ conditions and to facilitate efficient biodeterioration of plastic debris.

3.2.2. Study length

There is no conclusive data on how long it takes for plastics to be biodegraded in the environment (but see Chamas et al., 2020). Only a few studies on degradation of plastics have been performed in the natural environment; therefore, many questions remain unanswered (Fotopoulou and Karapanagioti, 2019). The chemical bonds in polymers are difficult to break, so they are not easily accessible as nutrients for bacteria growing on plastics. Generally, long incubations should be used when bacteria have access to other nutrients during biodegradation experiments.

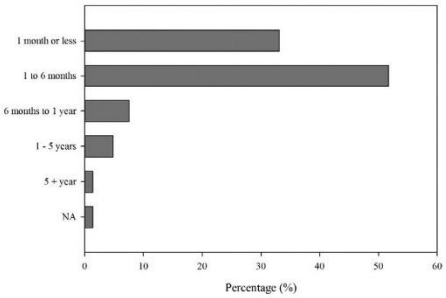

Fig. 2. The frequency of application of different treatment lengths in biodegradation studies (N = 145). Length of studies ranged from as short as 4 days to as long as 10 years. NA – not known.

Just over half the studies carried out degradation experiments extending from 1 to 6 months (51.7%), while one third exposed plastics to bacteria for one month or less (33.1%) (Fig. 2). Longer studies of 6 months to 1 year or 1 to 5 years were rare as 7.6% and 4.8%, respectively. Two studies were conducted for 10 years or more, and two studies did not indicate the duration of the degradation experiments.

Davis et al. (2005) proposed incubation times for soil bacteria ranging from one week to one month, as has been used in biodegradation studies in many cases, but many studies treated polymers for periods longer than one month. These longer durations have resulted in many successful experiments with isolated biodegrading bacteria and in changes in the polymer materials. Hence, giving the microorganisms enough time to grow and actually use the carbon from the plastics is an important step in their degrading of these synthetic materials.

3.2.3. Growth condition

Temperature is considered as probably the most important environmental factor that affects the growth and survival of microorganisms (Madigan et al., 2012). Most of the 145 studies involved experiments conducted at temperatures from 26 °C to 30 °C (30.3%) and from 36 °C to 40 °C (13.1%) (Fig. 3). Overall, 22.1% of the studies did not report

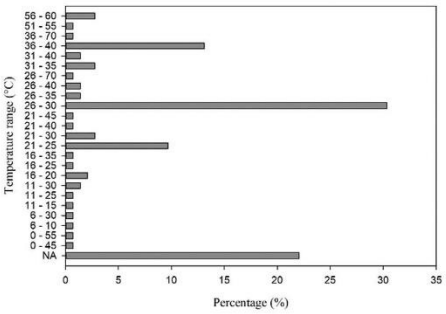

Fig. 3. The frequencies of temperature ranges used in biodegradation experiments (N = 145); number on the left side shows temperature range used in studies. Cases with wide ranges include studies that carried out the same experiments at different temperatures. The ranges starting at 0 °C include studies where experiments were carried out at 4 °C.

the temperature for the degradation Skariyachan et al. (2017) reported an experimental temperature range from 4 °C to 55 °C, and they observed bacterial growth in media with a mixture of LDPE and HPDE at five different temperatures (4, 25, 37, 45 and 55 °C). Generally, as temperature increases, the rate of metabolism increases, until it rapidly declines at higher temperatures where irreversible changes in cells occur (Schulte, 2015). It is likely that temperature has a direct impact also on plastic degradation rates. Pischedda et al. (2019) addressed the effects of temperature on the speed of plastics biodegradation in soil media, and demonstrated that the biodegradation rate is perfectly described by the Arrhenius equation in the tested temperature range (15–28 °C). While different microorganisms can grow also at temperatures below freezing or even above the boiling point of water, no known organisms can grow across this whole temperature range, as the range for any given organism is typically 25 °C to 40 °C (Madigan et al., 2012). Generally, four classes of microorganisms can be defined based on their growth temperature optima: psychrophiles (0–20 °C, optimal temperature, ≤15 °C); mesophiles (20–45 °C); thermophiles (45–80 °C, optimum temperature, >45 °C); and hyperthermophiles (optimum temperature, >80 °C) (Madigan et al., 2012). The microorganisms investigated in the reviewed studies here were mostly isolated from environmental samples that were collected from temperate or tropical latitudes, and they are hence most probably mesophiles. While about half of the studies reported on degradation temperatures from 26 °C to 40 °C, 22.1% of the studies did not report the temperature for the degradation experiments, or they were not precise enough (e.g., 'ambient temperature'). The temperature is one of the most important parameters in these experiments, and it should be chosen and reported fully and accurately. As such, it is our suggestion that the temperature should always be measured, even during outdoor experiments, and then included in scientific reports. Within the reviewed studies here, the greatest temperature range covered was from 4 °C to 55 °C, where Skariyachan et al. (2017) compared the degradation potential of formulated microbial consortia (IS1-IS4) from cow dung under thermophilic conditions (55 °C) in comparison to other temperatures (4, 25, 37, 45 °C). It would be interesting to check for biodegraders among the microorganisms that live in extreme environments (i.e., very hot or very cold).

Most studies (65.5%) did not report the pH of the medium, but most cases that did report pH (9.0%) indicated pH 7. Some studies tested several different pH values (Peng et al., 2014; Sarkhel et al., 2019), or samples with different pH readings (Lim et al., 2005; Tourova et al., 2020). Bacteria were often grown in vivo (28.3%), and only two studies did not disclose the media used. A popular medium choice was mineral salt medium (in 15.2%), as well as, Bushnell-Haas or the liquid carbon-free basal medium (both in 3.4% of the studies). Several studies have used media without the addition of a carbon; like mineral medium (Pandey, 2014; Satyalakshmi, 2016; Farzi et al., 2019), thereby forcing the bacteria to use the carbon from the plastics and consequently degrade them. By contrast, Shabbir et al. (2020) added different carbon sources when assessing changes in PE. The polydispersity index (M_w/M_n) was 7.82 for the control, 7.68 without a carbon source, and 6.82 with glucose. Similarly, M_w/M_n was lower for PP (4.47 for the control, 4.35 without a carbon source, and 3.90 with glucose) and for PET (2.44 for the control, 2.32 without a carbon source, and 2.28 with glucose). They reported higher relative abundances of the phyla in the glucose-treated samples, and this could explain the greater biodegradation of microplastics after glucose addition. This finding will be particularly interesting to consider in planning future studies.

3.3. Observational methods

Many studies (15.9%) reported either on the changes in surface texture of the treated plastics, weight loss, tensile strength, or chemical attributes during the biodegradation experiments. However, in many studies, a combination of two (27.6%) or three (25.5%) of these methods was used to observe the changes to the treated plastics. The most common techniques used to evaluate polymer degradation were a combination of visual surface changes, determined mainly using scanning electron microscopy (SEM), and measurements of weight loss and chemical changes, determined mainly using Fourier-transform infrared (FTIR) spectroscopy. Alongside FTIR spectroscopy, gel permeation chromatography (GPC), high temperature GPC, UV–visible spectroscopy, thin layer chromatography (TLC), gas chromatography–mass spectrometry (GC–MS), high performance liquid chromatography (HPLC), X-ray photoelectron spectroscopy (XPS), and nuclear magnetic resonance (NMR) when applied. Analysis of thermal profiles by thermal gravimetric analysis (TGA), thermogravimetry/derivative thermogravimetry/differential thermal analysis (TG-DTG-DTA), and differential scanning calorimetry (DSC), also provided information on both the physical and chemical characteristics, in 8, 4 and 10 studies, respectively. 84.1% of the studies reported using methods for observing the growth of bacteria exposed to polymer materials. The most widely used methods were genome/metagenome analyses (10.3%), measurements of respiration rates (6.9%), viable colony counts (6.2%) and direct visual observations (5.5%). Respiration rates in most studies were assessed by measurements of carbon dioxide production.

Degradation is any change in the physical or chemical properties due to chemical, physicochemical (e.g. photo-degradation, thermal and mechanical degradation) or biological processes (Shah et al., 2008). Biodeterioration, which includes the combined actions of members of the microbial community, is considered the first step in biodegradation and involves a superficial degradation that modifies the mechanical, physical and chemical properties of a given material. The second step is the cleavage of the polymeric molecules into oligomers, dimers or monomers through catalytic agents secreted by microorganisms; this process is referred to as depolymerisation. The next step is assimilation, which refers to the integration of the molecules to produce energy and new biomass and for other metabolic processes. The last step is mineralisation, which is the excretion of simple and different salts and of complex metabolites into the extracellular surroundings (Fotopoulou and Karapanagioti, 2019). The complete breakdown of large polymers to carbon dioxide usually requires several different organisms, each of which can attack different chemical bonds, first within the polymers and then within the monomers (Gu et al., 2011).

Polymer degradation, whether occurring by photochemical, thermal, chemical or biological processes, has to result in bond scission and the subsequent chemical transformations of the plastics, with the formation of new functional groups. This is reflected in the mechanical, optical and electrical changes observed in the material characteristics (Shah et al., 2008). Hence, studies of degradation processes can include a variety of approaches that monitor changes in different polymer characteristics, although careful design is needed to discriminate between biologically driven degradation and other degradation processes. Light (i.e. UV-A, UV-B), high temperatures and pressures, chemical conditions and biological activities are all possible sources for changes in polymer characteristics. All studies should therefore provide full and accurate reports of all parameters important for the biodegradation of plastics.

A clear understanding of microbial–plastic interactions requires, that all studies reporting on plastic biodegradation by microbes are able to link this biodegradation to a biotic factor (e.g. a bacterial species or consortium), while excluding abiotic factors. One method (e.g. weight loss) can be a beneficial tool for preliminary studies, but different methods need to be used in combination for a reliable establishment of the link between microbes and biodegradation. The methods used should be limited to those that have been shown to produce the most relevant data. This limitation should be followed to minimise non-standardised reporting on methodological approaches and the data obtained, as this is one of the main obstacles that currently prevents a clear analysis of the present state of the art, and that in turn inhibits further technological developments.

A typical case is seen with the use of FTIR spectroscopy analysis. Although this is a powerful tool for observing changes in polymers during biodegradation, carefully designed studies and monitoring schemes need to be set up, and standardised reporting is desperately needed. For example, Tribedi and Dey (2017), Ullah Jamil et al. (2017) and Kyaw et al. (2012), all studied biodegradation of LDPE. In the first study, the researchers scanned each film at room temperature 20 times from 400 cm^{-1} to 4000 cm^{-1} and repeated this three times. In the second study, the spectrum was recorded at 500 cm^{-1} to 4000 cm^{-1} without disclosing the temperature. In the third study, the scanning area was not disclosed. In this third study by Kyaw et al. (2012), the carbonyl index was used to measure the degree of biodegradation, which was obtained through a formula that used absorption at 1740 cm^{-1} (the maximum of the carbonyl peak) divided by the absorption at 1460 cm^{-1} (the maximum of the carbonyl peak). By contrast, Tribedi and Dey (2017) used the carbonyl bond index, which represents the ratio of absorbance peaks of carbonyl (1712 cm^{-1}) and CH_2 (1462 cm^{-1}), as well as the terminal double-bond index, which represents the ratio between the absorbance of the terminal double-bond peak (908 cm^{-1}) and the mean of the two CH_2 peaks (1461, 1471 cm^{-1}). Conversely, Ullah Jamil et al. (2017) simply reported the different absorbance peaks that were seen. Thus, although all three of these studies reported on microbial degradation of LDPE using FTIR spectroscopy to assess the chemical changes, the results cannot be compared with any degree of certainty.

4. Plastic degrading bacteria

Of the 145 studies included here, 138 clearly reported a demonstration of some kind of polymer degradation. Among these 138, 103 reported 246 bacteria strains that biodegraded plastics (Table 2). An important part of the studies investigated the efficiency of degradation of microbial consortia from different environments. Most of the identified bacteria belong to the phyla Proteobacteria (48%), Firmicutes (37.4%) and Actinobacteria (9.8%). Bacteria from different phyla are already widely used for biodegradation of human wastewater (Wu et al., 2019) and contaminants at oil spill sites (Das and Chandran, 2010), and they have been applied in many other biotechnological processes (e.g. food production and pharmaceuticals). Proteobacteria are by far the largest and most metabolically diverse phylum of all bacteria, and they have great importance medically, industrially and agriculturally (Madigan et al., 2012). They have been reported as the most abundant phylum in activated sludge in wastewater treatment plants (WWTPs) around the globe (82% of all operational taxonomic units) (Wu et al., 2019), as well as in contaminated riverbed sediments of the Maozhou river in China (59%) (Liao et al., 2019), in marine sediments (38.6%) (Borin et al., 2009), and in different types of soils (Buckley and Schmidt, 2001). All are Gram-negative bacteria, and they show an exceptionally wide range of energy-generating mechanisms (e.g. chemolithotrophic, chemoorganotrophic and phototrophic species), relationships to oxygen (e.g. anaerobic and facultatively aerobic species) and cell shapes (e.g. straight and curved rods, cocci and filaments) (Madigan et al., 2012).

The most common genus mentioned in the reviewed papers was *Pseudomonas*, a genus that is ecologically important in soil and water environments and that currently has 216 known valid species (Parte, 2018; accessed on 19 March, 2020). Similarly, Jacquin et al. (2019) listed several *Pseudomonas* strains that degrade PE. Other commonly listed strains were from the genera *Bacillus* and *Brevibacillus*, and several strains of fungi were also mentioned but are not considered in the present SLR. One characteristic property of these plastic-degrading bacteria is their use of many different organic compounds as carbon and energy sources (Seo et al., 2009; Shourian et al., 2009; Wilkes and Aristilde, 2017). Some species can use over 100 different compounds, even materials of xenobiotic origin, making them important agents of bioremediation in the environment (Madigan et al., 2012).

Most members of the phylum Firmicutes are Gram-positive bacteria that can form endospores and are found primarily in the soil (Madigan et al., 2012). The most commonly mentioned organisms were members of the *Bacillus* genus, which currently has 282 known valid species (Parte, 2018; accessed on 19 March, 2020). The bacteria of this genus can form oval or cylindrical endospores, and they can function as aerobes or facultative aerobes. Several strains can produce extracellular hydrolytic enzymes that can break down complex polymers (e.g. lipids), for use as carbon sources and electron donors. Many of these bacteria also produce antibiotics and insecticides (Madigan et al., 2012).

Actinobacteria are a Gram-positive group that includes rod-shaped to filamentous, primarily aerobic bacteria commonly found in the soil and in plant materials. Commercially, they are used for production of antibiotics and certain fermented dairy products (Madigan et al., 2012).

Among the bacteria identified as effectively initiating the biodegradation of the synthetic polymers, 21.0% of the studies indicated *Pseudomonas*, 15% indicated *Bacillus* and 17% indicated mixtures of these two genera. A comparison of temperature and exposure time (i.e. study length) effects across the studies indicated weak negative correlations and no significant relationships, most probably due to the scarce data and the prevalence of studies carried out in a temperature range of 26 °C to 30 °C (Fig. 4). In case of *Pseudomonas* ($n = 32$) the correlation was weak (−0.34), although close to the significance level of $p < 0.05$ ($p = 0.05$), suggesting that exposure to higher temperatures with, shorter incubation times will be needed for this genus to achieve polymer biodegradation. In the example with *Bacillus* the Pearson's coefficient of the linear relationship between temperature and study length was also weak ($r = -0.29$, $n = 12$, $p = 0.36$), and a very weak coefficient for their mixtures ($r = 0.001$, $n = 17$, $p = 1.00$). Comparisons of correlations between datasets revealed no significant differences ($p > 0.05$) for *Bacillus* and *Pseudomonas* ($z = 0.23$, $p = 0.41$), *Bacillus* and mixtures ($z = -0.70$, $p = 0.24$), or *Pseudomonas* and mixtures ($z = -1.11$, $p = 0.13$), indicating similar behaviours of all three tested correlations. Hence, the SLR showed that shorter treatments are needed during biodegradation when experimental temperatures are above room temperature.

The studies identified bacteria that were isolated from contaminated sites, such as landfill sites or plastic waste dumps (Kowalczyk et al., 2016; Mehmood et al., 2016; Awasthi et al., 2017) from oil contaminated sites (Das et al., 2012) and from activated sludge from WWTPs (Khatoon et al., 2014) (45.9%) (Table 3). Some studies used, bacteria from banks or synthetic mixtures, while the rest used bacteria obtained from mixed environmental samples (polluted/unpolluted) or from natural environments, such as mangrove swamps, soils, sediments, freshwater, marine environments, animal guts or plant nodes. Contaminated sites are extreme environments due to the presence of biologically toxic compounds and different types of synthetic substrates that microorganisms can alternatively use. Similarly, the soil is a highly variable environment in terms of nutrient levels, temperature and water activity (Madigan et al., 2012). These differences promote a high adaptation rate of microbes.

Increasingly more studies have examined the polymer degradation potential of consortia of different bacteria (Skariyachan et al., 2015; Skariyachan et al., 2017; Tribedi and Dey, 2017; Skariyachan et al., 2018; Tourova et al., 2020). Some future studies that might be beneficial will be investigations into biodegradation processes occurring via 'syntrophy metabolism', in which the degradation of compounds follows a metabolic chain where the catabolic product of one species is a source of carbon for another (Santisi et al., 2015).

5. The most common changes in the polymers

5.1. Weight loss

Weight losses of the plastics during biodegradation were mainly reported as percentage weight reductions calculated from difference

Table 2
List of bacteria proven to biodegrade polymers as stated in the analysed papers. Banks/SM – bacteria from strain banks and/or their synthetic mixtures (SM); contaminated sites – samples from contaminated sites (e.g., landfill/dump sites, activated sludge, contaminated soils, etc.); mix – mixture of bacteria from different environments (polluted/unpolluted); other – samples from different natural environments (i.e., mangroves, soils, sediments, freshwater, marine environments, animal gut, plant nodes). *not clearly defined which genus.

Polymer	Bacteria/sample origin	Phylum	Identified bacteria capable of plastic biodegradation	References
LDPE	Banks/SM	Acidobacteria, Firmicutes & Proteobacteria	*Consortium of: Pseudomonas otitidis SPT1, Bacillus aerius SPT2, Acanthopleuribacter pedis SPT3, Bacillus cereus SPK1*	Anwar et al., 2013
LDPE	NA	Acinetobacteria, Gemmatimonadetes & unknowns	Consortium of: *Actinobacteria, Gemmatimonadaceae and some unknowns*	Zhang et al., 2020
LDPE	Banks/SM	Actinobacteria	*Arthrobacter oxydans, A. globiformis, Microbacterium paraoxydans* (GenBank ID: HQ185284)	Carol et al., 2012; Rajandas et al., 2012
LDPE	Contaminated site	Actinobacteria	*Cellulosimicrobium funkei, Micrococcus luteus*	Montazer et al., 2018; Muhonja et al., 2018
LDPE	NA	Actinobacteria	*Arthobacter paraffineus*	Albertsson et al., 1998
LDPE	Contaminated site	Bacteriodetes	*Spingobacterium multivorum*	Montazer et al., 2018
LDPE	Contaminated site	Cyanobacteria	*Oscillatoria subbrevis, Phormidium lucidum*	Sarmah and Rout, 2018
LDPE	Banks/SM	Firmicutes	BP/SU1 of *Staphylococcal epidermis*	Chatterjee et al., 2010
LDPE	Contaminated site	Firmicutes	*Bacillus cereus, B. niacini, B. pseudomycoides, B. safensis, Bacillus* sp., *Bacillus* sp. *ISJ55, B. subtilis, B. thuringiensis, B. toyonensis, Brevibacillus borstelensis, B. parabrevis, Lysinibacillus macroides, Paenibacillus sp. (GenBank MK053775), Staphylococcus* sp., *Streptococcus, Streptococcus/Staphylococcus**	Anbuselvi Vatsaldutt Pandey, 2015; Bardají et al., 2019; Kumar Gupta and Debvi, 2019; Muhonja et al., 2018; Muthumani and Anbuselvi, 2014-2015; Pandey & Anbuselvi, 2014
LDPE	Mix	Firmicutes	*Bacillus subtilis* MTCC 9447	Skariyachan et al., 2016
LDPE	Other	Firmicutes	*Bacillus* sp. YP2, *Bacillus* sp. *B. sphericus, B. cereus, Bacillus* spp., *Paenibacillus* spp.	Kumari et al., 2019; Skariyachan et al., 2017; Sudhakar et al., 2008; Yang et al., 2014
LDPE	Banks/SM	Proteobacteria	*Pseudomonas aeruginosa* PAO1 ATCC 15729, *P. aeruginosa* ATCC 15692, *P. aeruginosa* (GenBank ID: HQ185285), *P. putida* KT2440 ATCC 47054, *P. syringae* DC 3000 ATCC 10862	Kyaw et al., 2012; Rajandas et al., 2012
LDPE	Contaminated site	Proteobacteria	*Acinetobacter pitti, Alcanivorax borkumensis, Citrobacter amalonaticus, Delftia tsuruhatensis, Escherichia coli, Klebsiella* sp., *Ochrobactrum intermedium, O. oryzae, O. pseudintermedium, Pseudomonas aeruginosa, P. aeruginosa SKN1 (ID: 9702593), P. citronellolis* EMBS027 KF361478, *P. putida, Pseudomonas* spp., *Stenotrophomonas humi, S. maltophilia, S. pavanii* CC18, *P. stutzeri*	Anbuselvi Vatsaldutt Pandey, 2015; Anbuselvi and Pandey, 2015; Bhatia et al., 2014; Deepika & Jaya Madhuri, 2015; Delacuvellerie et al., 2019; Mehmood et al., 2016; Montazer et al., 2018; Muhonja et al., 2018; Muthumani and Anbuselvi, 2014-2015; Nourollahi et al., 2019; Pandey & Anbuselvi, 2014; Sharma & Sharma, 2004; Skariyachan et al., 2015
LDPE	Mix	Proteobacteria	*Enterobacter* spp., *Pantoea* spp., *Proteus* spp., *Pseudomonas putida* MTCC 2445, *Pseudomonas* spp., *P. stutzeri* MTCC 2643	Skariyachan et al., 2016
LDPE	Other	Proteobacteria	*Enterobacter asburiae, Enterobacter cloacae AKS7,Pseudomonas* spp., *Stenotrophomonas* spp.	Sarker et al., 2020; Skariyachan et al., 2017; Yang et al., 2014
LDPE	Other	Firmicutes & Proteobacteria	Consortium of: *Serratia* sp. KC1-MRL, *Bacillus licheniformis* KC2-MRL, *B.* sp. KC3-MRL and *Stenotrophomonas* sp. KCMRL	Ullah Jamil et al., 2017
LLDPE	Bank/SM	Actinobacteria & Proteobacteria	Consortium of: *Pseudomonas aeruginosa & Brevibacterium sp.*	Fachrul et al., 2020
LLDPE	Bank/SM	Proteobacteria	*Microbulbifer hydrolytic IRE-31 (ATCC 700072)*	Li et al., 2020
LLDPE	Other	Firmicutes	*Bacillus amyloliquefaciens* (GenBank accession no. KT185076)	Novotný et al., 2018
LLDPE	Other	Proteobacteria	*Serratia marcescens marcescens*	Azeko et al., 2015
LMWPE	Contaminated site	Proteobacteria	*Stenotrophomonas panacihumi* PA3-2.	Jeon & Kim, 2016
PE	Banks/SM	Actinobacteria	*Rhodococcus ruber* strain C208	Santo et al., 2013
PE	Contaminated site	Actinobacteria	Arthrobacter spp., consortium of: *Arthrobacter, Curtobacterium, Gordonia* and *Rhodococcus*	Jin & Kim, 2017; Puglisi et al., 2019
PE	Other	Actinobacteria	*Micrococcus* sp., *Brevibacterium* sp., *Streptomyces albogriseolus* LBX-2	Brandon et al., 2018; Kathiresan, 2003
PE	Banks/SM	Firmicutes	*Lysinibacillus fusiformis*	Mukherjee et al., 2017; Shao et al., 2019
PE	Contaminated site	Firmicutes	*Bacillus aquimaris, B. boroniphilus, B. drentensis, B. firmus, B. idriensis, B. luciferensis, B. marisflavi, B. megaterium, B. muralis, B. mycoides, B. pumilus, B. simplex, B. subtilis, B.* sp., *Paenibacillus woosongensis*	Puglisi et al., 2019
PE	Mix	Firmicutes	*Bacillus cereus* VASB1/TS, *Lysinibacillus fusiformis* VASB-14/WL, *Staphylococci*	Rani and Rao, 2012; Shahnawaz et al., 2016
PE	Other	Firmicutes	*Bacillus* sp., *B. gottheilii, B. cereus, Staphylococcus* sp., *Streptococcus* sp.	Kathiresan, 2003; Auta et al., 2017
PE	Other	Fusobacteria	*Sebaldella termitidis*	Brandon et al., 2018
PE	Contaminated site	Proteobacteria	16 isolates from three genera: *Comamonas, Delftia,* and *Stenotrophomonas,* recombinant strains by *Escherichia coli* BL21 and *P. aeruginosa* E7, *Acinetobacter johnsonii, Comamonas testosteroni, Pseudomonas* sp., *P. aeruginosa, P. alcaligenes, P. plecoglossicida, P. thivervalensis, Stenotrophomonas maltophilia*	Jeon & Kim, 2016; Peixoto et al., 2017; Puglisi et al., 2019; Satyalakshmi, 2016; Shahreza et al., 2019; Skariyachan et al., 2015
PE	Mix	Proteobacteria	*Pseudomonas putida*	Rani and Rao, 2012
PE	Other	Proteobacteria	*Citrobacter* sp., *Diplococcus* sp., *Enterobacter* sp., *Kosakonia*	Brandon et al., 2018; Li et al., 2020; Kathiresan, 2003;

Table 2 (*continued*)

Polymer	Bacteria/sample origin	Phylum	Identified bacteria capable of plastic biodegradation	References
			sp., *Moraxella* sp., *Pseudomonas aeruginosa*, *Pseudomonas* sp.	Nanda et al., 2009; Ren et al., 2019
HDPE	Contaminated site	Actinobacteria	*Arthrobacter* sp. GMB5, *Leucobacter* sp., *Micrococcus* sp.	Balasubramanian et al., 2010; Kunlere et al., 2019; Sangeetha Devi et al., 2019
HDPE	Contaminated site	Firmicutes	*Bacillus spp.*, *B. amyloliquefaciens*, *B. aryabhattai*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. subtilis*, *Staphylococcus sp.*	Kunlere et al., 2019; Sangeetha Devi et al., 2019
HDPE	Mix	Firmicutes	*Brevibacillus borsteiensis* KY49486	Mohanrasu et al., 2018
HDPE	Other	Firmicutes	*Bacillus* spp., *B. cereus*, *B. sphericus*, *Paenibacillus* spp.	Kumari et al., 2019; Sudhakar et al., 2008; Skariyachan et al., 2017
HDPE	Contaminated site	Proteobacteria	*Achromobacter xylosoxidans* PE-1, *Acinetobacter sp.*, *Klebsiella pneumoniae* CH001, *Pseudomonas* sp. GMB7, *P. aeruginosa*	Awasthi et al., 2017; Balasubramanian et al., 2010; Kowalczyk et al., 2016; Kunlere et al., 2019; Sangeetha Devi et al., 2019
HDPE	Other	Proteobacteria	*Pseudomonas* spp., *P. fluorescens*, *Serratia marcescens*, *Stenotrophomonas* spp.	Baculi et al., 2017; Skariyachan et al., 2017
PS	Contaminated site	Actinobacteria	*Mycobacterium* sp.	Chen et al., 2020
PS	Other	Actinobacteria	*Aquihabitans* sp., *Paenibacillus urinalis*, unclassified Xanthomonadaceae	Atiq et al., 2010; Brandon et al., 2018
PS	Contaminated site	Deinococcus-Thermus	*Thermus* sp.	Chen et al., 2020
PS	Other	Bacteriodetes	unclassified Saprospiraceae	Brandon et al., 2018
PS	Other	Firmicutes, Proteobacteria & Tenericutes	Spiroplasmataceae, Enterococcaceae, Enterobacteriaceae	Peng et al., 2019
PS	Contaminated site	Firmicutes	*Aneurinibacillus* sp., *Bacillus* spp., *Brevibacillus* sp., *Desulfotomaculum* sp., *Geobacillus* sp., *Proteiniclasticum* sp.	Chen et al., 2020; Mohan et al., 2016
PS	Contaminated site	Proteobacteria	*Alcaligenes* sp., *Azospirilum* sp., *Cupriavidus* sp.	Chen et al., 2020
PS	Other	Firmicutes	*Exiguobacterium sibiricum* DR11, *E. undae* DR14, *E.* sp. strain YT2, *Bacillus* sp., *B. gottheilii*, *B. cereus*, *Listeria* sp.	Auta et al., 2017; Atiq et al., 2010; Brandon et al., 2018; Chauhan et al., 2018; Yang et al., 2015
PS	Other	Nitrospirae	*Nitrospira defluvii*	Brandon et al., 2018
PS	Contaminated site	Proteobacteria	*Alcaligenes* sp., *Citrobacter sedlakii*, *Enterobacter* sp., *Pseudomonas* spp.	Mohan et al., 2016; Sekhar et al., 2016
PS	Other	Proteobacteria	*Acinetobacter* sp., *Pseudomonas aeruginosa*, *Citrobacter* sp., *Kosakonia* sp., *Pedomicrobium* sp., unclassified *Burkholderiales*	Atiq et al., 2010; Brandon et al., 2018; Wang et al., 2020
PUR	Contaminated site	Actinobacteria	*Corynebacterium* sp. AF14	Shah et al., 2008b
PUR	Mix	Actinobacteria	*Arthrobacter globiformis* SBI-5	El-Sayed et al., 1996
PUR	Banks/SM	Firmicutes	*Bacillus subtilis*	Stepien et al., 2017
PUR	Contaminated site	Firmicutes	*Bacillus* sp. AF8	Shah et al., 2008
PUR	Banks/SM	Proteobacteria	*Pseudomonas chlororaphis*, *P. denitrificans* ATCC 19244	Gautam et al., 2007b; Stepien et al., 2017
PUR	Contaminated site	Proteobacteria	*Arthrobacter* sp. AF11, *Micrococcus* sp. AF10, *Pseudomonas* sp. AF9	Shah et al., 2008
PUR	Mix	Proteobacteria	*Burkholderia gladioli*, *Pseudomonas otitidis*, *P. putida*	Peng et al., 2014
PP	Other	Actinobacteria	*Rhodococcus* sp. strain 37	Auta et al., 2018
PP	Contaminated site	Firmicutes	*Bacillus flexus* AB021185, *B. flexus* FJ948078, *B. subtilis* GQ241354	Arkatkar et al., 2009; Arkatkar et al., 2010
PP	Other	Firmicutes	*Bacillus* sp. strain 27, *Bacillus* sp., *B. gottheilii*, *B. cereus*	Auta et al., 2017, 2018; Cacciari et al., 1993
PP	Contaminated site	Proteobacteria	*Pseudomonas azotoformans* MTCC 7616, *Pseudomonas stutzeri*	Arkatkar et al., 2010; Sharma & Sharma, 2004
PP	Other	Proteobacteria	*Pseudomonas chlororaphis*, *P. stutzeri*, *Vibrio* sp.	Cacciari et al., 1993
LMWPP	Contaminated site	Proteobacteria	*Stenotrophomonas panacihumi* PA3-2.	Jeon & Kim, 2016
PET	Banks/SM	Actinobacteria	*Streptomyces* sp.	Farzi et al., 2019
PET	Other	Actinobacteria	*Streptomyces* sp., *Brevibacterium* sp.	Farzi et al., 2019; Narciso-Ortiz et al., 2020
PET	Other	Firmicutes	*Bacillus gottheilii*, *B. cereus*, *B. muralis*	Auta et al., 2017; Narciso-Ortiz et al., 2020
PET	Mix	Proteobacteria	*Ideonella sakaiensis* 201-F6	Yoshida et al., 2016; response
PET	Other	Proteobacteria	*Serratia proteamaculans*, *Vibrio* sp. GenBank: KY941137.1	Narciso-Ortiz et al., 2020; Sarkhel et al., 2019
PC	Contaminated site	Actinobacteria	*Arthrobacter* sp. DQ205429	Goel et al., 2008
PC	Contaminated site	Firmicutes	*Bacillus megaterium*, *B. cereus*	Arefian et al., 2020
PC	Contaminated site	Proteobacteria	*Enterobacter* sp. DQ205431	Goel et al., 2008
PCL	Other	Proteobacteria	*Pseudomonas sp.*	Wang et al., 2019
PES	Contaminated site	Proteobacteria	*Pseudomonas* sp. AKS2 added to microbial natural consortia	Tribedi & Sil, 2013
PVAL	Contaminated site	Actinobacteria & Firmicutes	Recombinant strain N-2 (*Micrococcus* sp. PVC-4 and *Bacillus* sp. PVA-7)	Patil & Bagde, 2016
PVAL	Contaminated site	Firmicutes	*Bacillus* sp.PVA-7	Patil & Bagde, 2016
PVAL	Contaminated site	Proteobacteria	similar to *Pseudomonas putida*, *P. aeruginosa*, *Pseudomonas* sp. PVA-2	Hoffmann et al., 2003; Patil & Bagde, 2016

(*continued on next page*)

Table 2 (*continued*)

Polymer	Bacteria/sample origin	Phylum	Identified bacteria capable of plastic biodegradation	References
PVC	Contaminated site	Actinobacteria	*Micrococcus* sp. PVC-4	Patil & Bagde, 2016
PVC	Contaminated site	Actinobacteria & Firmicutes	Recombinant strain N-2 (*Micrococcus* sp. PVC-4 and *Bacillus* sp. PVA-7)	Patil & Bagde, 2016
PVC	Other	Firmicutes	*Bacillus* sp.	Kumari et al., 2019
PVC	Banks/SM	Firmicutes	*Bacillus flexus*	Giacomucci et al., 2019
PVC	Contaminated site	Proteobacteria	*Achromobacter* sp., *Pseudomonas aeruginosa*	Das et al., 2012
PVC	Banks/SM	Proteobacteria	*Pseudomonas citronellolis*	Giacomucci et al., 2019
PVC	NA	Proteobacteria	*Pseudomonas aeruginosa*	Wolkober et al., 1978
PVC	Banks/SM	Acidobacteria, Firmicutes & Proteobacteria	consortium of: *Pseudomonas otitidis SPT1*, *Bacillus aerius SPT2*, *Acanthopleuribacter pedis SPT3*, *Bacillus cereus SPK1*	Anwar et al., 2013

between the initial polymer weight and the weight after the exposure to bacteria that achieved biodegradation. These observations of weight loss were typically combined with another method, such as surface changes and/or FTIR spectroscopy. Some studies reported no changes, usually when an untreated polymer was used as the control to compare polymer blends (Niño et al., 1999; El-Wakil et al., 2020). Among the studies that reported an observed degradation ($n = 138$), 65.2% reported weight losses, of up to 10% (Yang et al., 2014; Mukherjee et al., 2017; Auta et al., 2018; Kumari et al., 2019; Shahreza et al., 2019) or 10% to 20% (Kyaw et al., 2012; Bhatia et al., 2014; Awasthi et al., 2017; Wang et al., 2020). The extent of the weight loss depended on the polymer type and its characteristics, the type of bacteria or consortium, the pretreatment of the plastic, and the conditions during the experiments (e.g. study length, temperature and pH). The greatest weight losses were reported for LDPE and HDPE in the form of foils, films or strips and for *Klebsiella pneumonia* CH001 (Awasthi et al., 2017) and different *Pseudomonas* strains (Kyaw et al., 2012; Bhatia et al., 2014). In many studies, the exact extent of the weight loss could not be extracted from the publications due to insufficient reporting.

5.2. Tensile strength

The changes in the tensile strength of the plastics were usually determined using a tensile testing machine (e.g. INSTRON 5566, as used by Kyaw et al., 2012) and were measured as the percentage losses in tensile strength (Kyaw et al., 2012), elongation at the break point (%), or ultimate tensile strength (i.e. the stress that the material withstood while being stretched to breaking point) measured in units of megapascals (MPa) (Kyaw et al., 2012; Sarmah and Rout, 2018) or newtons per square metre (N m^{-2}) (Tribedi and Dey, 2017). Some international standards, such as ASTM D 882-97, are already in use. About 17.4% of the studies reported using tensile strength changes as an indication of biodegradation. The tensile strength reductions were usually characterised as lower for virgin polymers than for blends (Leonas and Gorden, 1996; Francis et al., 2011); the reductions could even be zero (Leonas and Gorden, 1996). However, some studies reported substantial reductions in tensile strength during the biodegradation experiments. For example, a study of biodegradation of thermally pretreated HDPE by *Klebsiella pneumoniae* CH001 reported a significant reduction

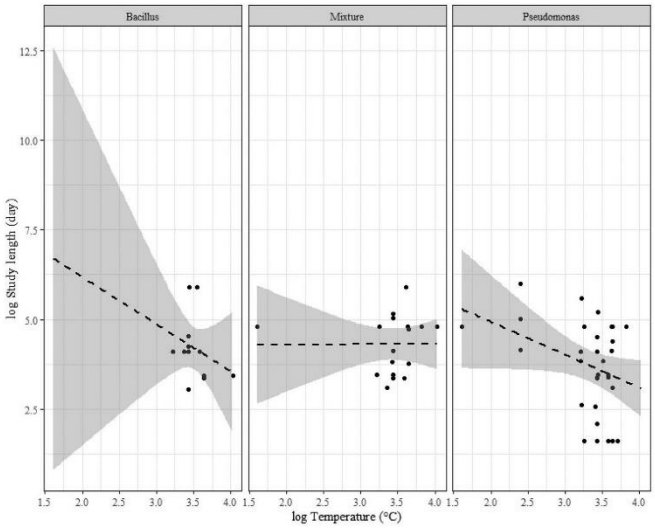

Fig. 4. Degradation capacity over temperature ranges and different lengths of exposure for *Bacillus*, *Pseudomonas* or their mixtures. Range of study lengths is from 4 to 400 days and temperatures from 4 to 55 °C. Grey area indicates the confidence level (0.95) of linear model regression.

Table 3
Origin of bacteria used in biodegradation studies (N = 138). Mix = when samples from different sites were used in the experiments (e.g., freshwater and soil), NA = information unavailable.

Origin of tested bacteria	Proportion of studies (%) (N = 138)	Proportion of bacteria strains (%) (N = 246)
Contaminated site	36.2	54.9
Landfill and dump sites	29.0	
WWTPs	3.6	
Oil spillage sites	2.2	
Marine plastic waste	1.4	
Other	37.0	30.1
Soil	12.3	
Animal gut	5.8	
Marine	5.1	
Compost	4.3	
Mangrove	3.6	
Freshwater	3.6	
Root nodules	0.7	
Cow dung	0.7	
Sludge samples from denitrification reactor in lab	0.7	
Mix	12.3	6.9
Banks/SM	13.0	7.3
Bank	12.3	
Synthetic enzymes/strains	0.7	
NA	1.4	

(~50%) after a 60-day incubation (Awasthi et al., 2017). Similarly, an investigation of PE biodegradation by bacterial strains from the guts of plastic-eating waxworms indicated a >50% decrease in tensile strength in PE exposed to *Bacillus* sp. YP1 or *Enterobacter asburiae* YT1 (Yang et al., 2014). Moderate decreases were observed by Agamuthu and Faizura (2005), where 45-day composting resulted in a 20% decrease in tensile strength for both HDPE with 3% additive and LLDPE with 7% additive.

5.3. Surface changes

Surface changes of the plastics were evaluated through visible changes in the 'before' and 'after' images from SEM in 60% of the studies, and these were mainly described as the formation of cracks, pores and holes. Awasthi et al. (2017) performed SEM image analysis to determine the degradation of HDPE films by the bacterium *Klebsiella pneumoniae* and claimed that formation of biofilms occurred within 15 days and that the HDPE films were cracked and developed holes when incubated with *K. pneumoniae*. Similarly, Kyaw et al. (2012) provided SEM images of LDPE that revealed surface changes after 3 months of incubation with selected *Pseudomonas* strains. Surface changes have also been observed after 12 months of exposure of PP to different *Bacillus* strains (Arkatkar et al., 2009). Similarly, SEM experiments have revealed deterioration of PET (Austin et al., 2018; Narciso-Ortiz et al., 2020), PS (Chauhan et al., 2018; Wang et al., 2020) and PE (Deshpande et al., 2015; Mukherjee et al., 2017) by different strains at various incubation times (i.e. 4 days to a few years).

5.4. Chemical changes

Fourier-transform infrared spectroscopy has been the most widely used method (60.9% of the studies) to evaluate chemical changes in treated polymers. Changes have usually been depicted as FTIR spectra and explained as shifts and changes in intensity of the carbonyl bands. For example, Albertsson et al. (1998) reported changes in the carbonyl index from 0.196 to 0.143 for initially pretreated LDPE after treatment with *Arthrobacter paraffineus*, and changes in the double-bond index from 0.275 to 0.250. Arkatkar et al. (2009) measured the FTIR spectroscopy absorbance of treated PP at 1377 cm^{-1} and observed decreases over the time when PP was exposed to bacteria. The methyl group index showed a gradual decrease from an initial value of 1.0 to 0.85 after 6 months, to 0.75 after 9 months, and to 0.70 after 1 year, whereas the carbonyl index increased from 0.0125 to 0.016. The methyl group index for the untreated PP decreased to 0.76 after 1 year. Novotný et al. (2018) measured the FTIR spectra of LLDPE films before and after 60-day treatments with a *Bacillus amyloliquefaciens* strain and reported a decreased carbonyl band and flattening of the 1300–1100 cm^{-1} zone due to the bacterial action.

Changes in thermal profiles have also been assessed using TGA to follow the degradation of plastics. For example, Bhatia et al. (2014) showed that the thermal profile of virgin LDPE had a steep degradation curve between 450 °C and 500 °C, whereas degraded LDPE showed a three-step weight loss of 22%, 33% and 46% at 50 °C, 100 °C and 175 °C, respectively. They concluded that bacterial cultures can accelerate the degradation rate because of direct enzymatic scission and assimilation of low-molecular-weight chains. The three-step degradation curves were a result of digestion of the hydrocarbon backbone by the isolated bacteria, *Pseudomonas citronellolis* EMBS027 (Bhatia et al., 2014).

In addition to FTIR spectroscopy and TGA, Mohan et al. (2016) used NMR and HPLC analysis to assess microbial-assisted high-impact PS degradation. They used deuterated chloroform as the internal reference before and after analysis of high-impact PS samples by NMR. One of the major peaks reported was between 3 ppm and 4 ppm, which generally corresponds to $-CH_2-Br$; the control showed, no peak in that region. They concluded that, because of the bacteria, bromine was released from the high-impact PS in the form of methyl bromine. They also conducted HPLC analysis of the culture medium following treatment with *Bacillus* spp. and *Pseudomonas* spp. detected the presence of phenyl ethanol, an intermediate product in the microbial degradation of PE.

GPC has also been used to determine the number-averaged molecular mass (M_n) and the weight-averaged molecular weight (M_w) of biodegraded plastics, as well as the Polydispersity Index (M_w/M_n). Novotný et al. (2018) used GPC combined with other methods (e.g. FTIR spectroscopy) to assess the deterioration of pretreated LLDPE by *Bacillus amyloliquefaciens*. After 60 days of exposure to the bacteria at 28 °C, the properties of virgin LLDPE did not change, nor did those of an abiotic control. Exposure of the pretreated LLDPE to the bacteria resulted in a steady increase in M_n, and increase in M_w and a decrease in the polydispersity index from 16.9 to 9.4. The authors concluded that the oligomers in the pretreated LLDPE were removed through the microbial action, and this conclusion was confirmed by other measurements (Novotný et al., 2018).

6. Recommendations for future research

The increasing accumulation of plastics in the environment has created an urgent need to develop technologies for the degradation of the plastic wastes that currently cannot be recycled and especially for degradation of plastics that pose serious threats to the environment. Especially critical attention is needed for the microplastics that accumulate in activated sludge during wastewater treatment processes, as this water is then further used in agriculture or discharged into streams. Alongside photo, thermal and mechanical deterioration, biodegradation is a promising approach, and it can provide energy-efficient and cost-efficient technologies that are also environmentally friendly, although only if this is carried out under controlled conditions. Hence, understanding what conditions are needed for biodegradation processes is critical, as is understanding the mechanisms involved.

As a first step, microorganisms with high potential for catalysing biochemical changes of these inert materials need to be identified, and their interactions with these plastic materials need to be understood more fully. The second step is to obtain a complete understanding of the autecology (i.e. the ecological requirements) and metabolic pathways of the relevant organisms, followed by establishment of the changes caused by the biochemical activity of microbes upon exposure to plastic materials. Our SLR indicates that the identification of potential

microorganisms is undergoing rapid progress, with 246 bacteria currently identified as capable of causing some kind of synthetic polymer change.

The important pools of potential bacteria that can biodegrade plastics are generally located in contaminated sites, such as landfill sites, where bacteria are in close contact with plastic waste. Shah et al. (2008) suggested that standardised screening of organisms that can degrade polymers, or that produce enzymes or enzyme systems that can degrade polymers, might well be environmentally profitable in the 21st century, in analogy to the screening programmes used for antibiotics in the 1950s and 1960s. Moreover, rapid technical developments in our analytical tools for observing biochemical changes in plastics and in the development of molecular techniques are now providing a profound understanding of the biodegradation processes. These advances can be accelerated if future experiments are carefully designed and are clearly, fully and accurately described when they are published.

Very recently, an excellent comprehensive review concluded that 'current international standards and regional test methods are insufficient in their ability to realistically predict the biodegradability of carrier bags in marine environment, due to several shortcomings in experimental procedures and a paucity of information in the scientific literature' (Harrison et al., 2018). Hence, the careful design of biodegradation experiments and the provision of clear and standardised descriptions of these studies when published will accelerate scientific progress in the field of plastic biodegradation. Another critical consideration is the need for careful definition of the terminology used in scientific publications, such as references to dilution, enrichment, isolation and screening, to ensure reproducibility of the studies.

When planning, carrying out and reporting biodegradation studies, the following should be considered:

- Biodegradation studies should focus on the materials that are most commonly present as environmental pollutants. Special concern should be focussed on the polymers that occur as microplastic pollutants (e.g. PEs, PP, PS, PAHs and PET).
- Contaminated sites (e.g. landfill sites, activated sludge from WWTPs, contaminated soils or sediments), and gut bacteria from invertebrates that feed on detritus that contains synthetic polymers should be investigated as promising pools of biodegrading bacteria.
- Pretreatment of plastics with UV light or imitation sunlight and moderate temperatures (in accordance with environmental conditions), and/or by mechanical deterioration (all of which imitate natural conditions) should be included in all biodegradation studies, as no plastic pollutant or waste is exposed to microorganisms in its virgin form. Moreover, the synergistic effects should be studied.
- Descriptions of experiments should include at least the information on the bacteria tested (name, origin) and the plastic material (type, form, additives), and should report the essential experimental conditions (temperature, pH, growth media, length of study, amount of plastics, cell density, particle size and surface area, condition of polymer material used – new or aged).
- FTIR spectroscopy has been identified as the most frequently used method for observation of biodegradation, as this enables an understanding of the biochemical changes occurring on the polymer surface; however, a standardised approach for reporting should be developed that includes the identification of the areas that change due to the biological degradation, the definition of the parameters that should be observed for each plastic, and the application of standardised indices that can sufficiently detect changes in the polymer structure due to biological activities. Ideally, FTIR spectroscopy should be used in combination with other methods, such as HPLC, which enables the identification of intermediate products of biodegradation, or with methods that enable more detailed interpretation of FTIR spectroscopy measurements, such as DSC, NMR, XPS or GPC.

7. Conclusions

In this SLR, we have provided a systematic and objective presentation of the data extracted from 145 scientific papers reporting synthetic polymer degradation by bacteria. We have shown that, when exposed to suitable bacterial strain(s), all the tested synthetic polymers show some kind of surface deterioration or the initiation of chemical changes due to bacterial activity. However, these changes are minimal and slow, and reports on them for various tested materials in terms of the biological processes involved have been highly inconsistent between publications. As already highlighted by Shah et al. (2008), a need remains for international standardisation of biodegradation tests. Even more importantly, researchers should report all of the relevant details of their biodegradation studies to enable replication by others.

We have identified several gaps in the existing research. A first deficiency is that the biodegradation of global pollutant–environmental microplastics has rarely been studied. A second is that, apart from the biodegradation of PEs, biodegradation of other common plastic pollutants, such as for PP, PS, PVC, PET and PAHs, has been much less intensively investigated. Long-term experiments with incubation times exceeding 6 months are also lacking; hence, our understanding is limited only to superficial degradation (biodeterioration) under laboratory conditions and the resultant modification of mechanical, physical and chemical properties. The processes of polymer molecules cleavage by bacteria (depolymerisation) remain essentially unknown. Studies are also lacking that mimic 'outdoor' biodegradation conditions, where the bacteria are exposed to natural temperature ranges (4–20 °C; i.e. most studies are conducted at room temperature of: 26–30 °C). Another serius gap is that few studies have investigated biodegradation by microbial consortia. Only *in- situ* studies can provide relevant information on natural biodegradation processes that can be further transferred to bioremediation technologies.

Future studies should be oriented towards the use of advanced chemical analysis to generate a better understanding of the metabolic pathways at the interface between the exposed polymers and the bacteria, with the aim of including the identification of relevant enzyme complexes and the chemical changes they incur. An understanding of the autecological requirements of potential biodegraders is also important, along with their optimal niches where their biodegradation is at its highest. Finally, plastic-contaminated environments are not free of other more easily accessible carbon resources, so the microbial activities in such environments should be more intensively studied.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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3.2.2 Published scientific article: "Preliminary experiments into colonization of microorganisms from activated sludge on different types of plastics"

This section is a scientific article authored by Tjaša Matjašič (NIB), Tanja Dreo (NIB), Tatjana Simčič (NIB), Tjaša Kanduč (JSI), Oliver Bajt (NIB) and Nataša Mori (NIB) published in Acta Biologica Slovenica in 2020. The article was prepared in collaboration with colleagues from the Jožef Stefan Institute (JSI). I conceptualised and developed the methodology, sampled the bacterial inoculates, conducted laboratory work, organised and analysed the data, and wrote the initial manuscript. Zoran Samadržija (JSI) performed SEM and assisted with sample preparation. Tjaša Kanduč (JSI) performed the isotopic analyses of the prepared plastic samples and assisted with interpretation. Oliver Bajt (NIB) offered expert insight on FTIR technology and provided the FTIR spectrograms. Tanja Dreo, Tatjana Simčič and Nataša Mori (NIB) helped conceptualise the methodology and provided valuable feedback on the manuscript.

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Preliminary experiments into colonization of microorganisms from activated sludge on different types of plastics

Preliminarni poskusi kolonizacije različnih tipov plastike z mikroorganizmi iz aktivnega blata

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Abstract: The presence of plastics in the environment is currently one of the most pressing global environmental problems. Microorganisms start to form biofilms on plastic surfaces when they first come in contact with the biosphere; however, these interactions and processes are little understood, especially in freshwaters. This study aimed to better understand the colonization process of microorganisms from activated sludge on plastic materials exhibiting different surface characteristics. We inoculated synthetic fabric (PET), water bottles (PET), and plastic bags for packing vegetables and fruits (HDPE) with microorganisms from activated sludge. Mixtures of plastics and activated sludge, as well as the control, were incubated at 22-24°C in Bushnell Haas (BH) liquid medium and shaken at 120 rpm for two months. The mixtures were sub-sampled weekly and seeded into fresh BH medium with test plastic materials to avoid feeding microorganisms on dead biomass. The colonization was followed by measuring optical density (OD_{600}) of liquid medium, by measurements of isotopic composition of carbon ($\delta^{13}C$) in untreated and treated plastic materials and, with inspecting the plastics surface with scanning electron microscopy (SEM). Overall, the study confirmed differences between colonizing microorganisms on different plastic material when comparing SEM micrographs of materials from the flasks inoculated with activated sludge. The texture of the HDPE bag changed during the experiment in both, control and inoculated flasks, but it is not clear whether the observed changes were due to abiotic or biotic factors. We concluded that microorganisms from activated sludge are capable of colonizing both PET and HDPE materials, and biofilm formation is most probably influenced by the chemical composition of plastics and their surface characteristics.

Keywords: biofilm, plastics, SEM, isotopic composition of carbon, co-cultivation, UV sterilization

Izvleček: Prisotnost plastike v okolju postaja eden izmed največjih globalnih problemov. Prvi stik plastike z biosfero je običajno s kolonizirajočimi mikroorganizmi, ki tvorijo biofilm, vendar je ta interakcija dokaj neznana, še posebej v celinskih vodah. Cilj študije je bil bolje razumeti proces mikrobne kolonizacije različnih plastičnih materialov z različnimi površinskimi lastnosti. Uporabili smo sintetična vlakna blaga (PET), plastenke za vodo (PET) in plastične vrečke za pakiranje zelenjave in sadja (HDPE) ter jih zmešali z okoljskim vzorcem aktivnega blata. Erlenmajerica z mešanico različnih plastik, inokulirana z vzorcem aktivnega blata v Bushnell Haas (BH) tekočem gojišču, ter negativna kontrola (mešanica plastik v sterilnem BH gojišču) so bile 2 meseca inkubirane na 22-24°C in stresane s 120 rpm. Vzorce smo tedensko prestavljali v sveža BH gojišča s testnimi plastičnimi materiali, da smo izključili rast na odmrli biomasi. Proces smo spremljali z merjenjem OD_{600} v tekočem mediju, z meritvami izotopske sestave ogljika ($\delta^{13}C$) v plastiki, in z opazovanjem površine plastike z vrstičnim elektronskim mikroskopom (SEM). S študijo smo potrdili različno rast mikroorganizmov na različnih materialih. V primeru HDPE vrečke se je spremenila tekstura tako v sterilnem kontrolnem gojišču kot v gojišču z mikroorganizmi iz aktivnega blata, vendar ni jasno, če zaradi abiotskih ali biotskih faktorjev. Zaključili smo, da so bakterije iz aktivnega blata zmožne kolonizacije plastike, ki je v vsakdanji uporabi, in da je pestrost in struktura biofilma odvisna od kemične sestave in površinskih lastnosti plastičnih materialov.

Ključne besede: biofilm, plastika, SEM, izotopska sestava ogljika, ko-kultivacija, UV sterilizacija

Introduction

Continuous increase in the production of synthetic or semi-synthetic organic compounds (plastics) and its wide use in every aspect of human life has led to increasing occurrence of plastic waste in freshwater environments (Li et al. 2018). During its time in the environment, larger plastic debris undergoes fragmentation due to weathering processes generating secondary microplastics (MP) (i.e. particles <5 mm). MP can also be manufactured as such and used as resin pellets to produce larger items or used directly (primary MP), as used in cosmetic products (Wagner and Lambert 2018). In concordance with global production rates most commonly found polymers in the environment are high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC). They occur mostly as fragments (rounded, angular), pellets (cylinders, disks, spherules), filaments (fibres), and granules (Wagner et al. 2014).

The presence of MP in the environment, as a new type of emerging contaminant, has become an issue of great concern and has drawn the attention of public and government authorities (Li et al. 2018). The main concerns are that MP particles can be easily ingested throughout the food chain; MP is a vector for toxic contaminants including metals and persistent, bioaccumulative and toxic compounds such as pharmaceuticals and endocrine-disrupting compounds; and MP can act as a vector for waterborne (human) pathogens influencing the hygienic water quality (Wagner et al. 2014, Eckert et al. 2018). There are many sources of MP for freshwater systems, with the largest portion from wastewater treatment plants (WWTP) (Li et al. 2018). When entering the environment, the plastic is exposed to physical factors (temperature, UV light, abrasion, etc.) and adsorbs organic and inorganic substances due to its high adsorptive properties (Rummel et al. 2017). After that, further colonization by bacteria, algae, fungi, and protozoa occur, which results in biofilm formation (McCormick et al. 2014, Rummel et al. 2017, Jemec Kokalj et al. 2019, Parrish and Fahrenfeld 2019).

The interaction between microorganisms and plastics occurring in freshwaters is not well known, even though these interactions and potential for biodegradation is a highly relevant topic in the field of environmental remediation (Wu et al. 2016). MP-associated microbial assemblages in forms of biofilms are likely to influence the distribution, impacts and fate of these pollutants, but most research has focused on marine environments (Harrison et al. 2018). Several researchers pointed out, that plastic particles are rapidly colonized once submerged in marine waters (Lobelle and Cunliffe 2011, Dang and Lovell 2016, Jacquin et al. 2019). In streams, biofilms are primary sites for carbon and nutrient transformations; thus they are also essential for pollutant biodegradation (Battin et al. 2016, Harrison et al. 2018). A recent study of Parrish and Fahrenfeld (2019) demonstrated that biofilm community structures varied as a function of source water and that PS spheres had different microbial community structures from PE microparticles indicating that the characteristics of plastics that is being colonized affects the biofilm structure.

Biofilm formation is composed of four distinct phases: (i) adsorption of dissolved organic molecules, (ii) attachment of bacterial cells, (iii) attachment of unicellular eukaryotes, and (iv) attachment of larvae and spores (Dobretsov 2010). Bacterial adhesion is divided into two stages, primary (docking) and secondary (locking) bacterial adhesion (Dunne 2002). Generally, plastics are first covered by inorganic and organic matter, referred to as "conditioning film" and shortly after by bacteria (mainly *Gammaproteobacteria* and *Alphaproteobacteria*) (Oberbeckmann et al. 2015). Microorganisms attach more firmly to hydrophobic materials, which is opposite to hydrophilic materials such as glass (Dobretsov 2010). The factors that affect the composition of microbial communities attached to artificial substrates are not well known (Caruso 2020), but they certainly gain advantages through surface colonization and biofilm formation, the most critical being better access to resources (Dang and Lovell 2016). Certain bacterial groups belonging to the phyla Proteobacteria, Bacteriodetes, Firmicutes, and Cyanobacteria are associated with plastic as colonizers more than others, suggesting an ecological niche for some taxonomic groups and indicating metabolic adaptation (Roager and Sonnenschein 2019). However, more information is needed to better understand how bacteria are associating with different type of plastics and under what conditions. A study conducted by Khatoon et al. (2014) used activated sludge as seed in their experiments. Similarly, they used SEM to characterize biofilm and surface morphology, but of polypropylene (PP) balls. A study by Huang and Cui (2012) also used activated sludge but investigated poly(lactic acid) (PLA), poly(butylene succinate) (PBS) and poly(caprolactone) (PCL). Using SEM, they found that biodegradation of PCL is best, PLA follows, and lastly PBS. Although some papers study HDPE degradation using SEM, the bacterial seeds come mostly from plastic waste dumpsites' soil or plastic samples and to lesser extent from activated sludge (Kowalczyk et al. 2016, Awasthi et al. 2017).

This study aimed to better understand the colonization process of microorganisms from activated sludge on plastics differing in chemical (PET and HDPE) and surface characteristics (textile fabric, thin plastic bags, thick plastic bottles). The colonization process and biofilm formation were observed on PET and HDPE materials, which are commonly present in treated wastewaters (Lv et al. 2019) and also occur as pollutants in freshwater environments (Koelmans et al. 2019) and, to our knowledge, have never been studied in a similar experiment. We hypothesized that the microorganisms from the activated sludge would be able to colonize and utilize carbon from experimental plastics in order to survive, that different biofilms would form on the PET textile, PET bottle, and HDPE bag, and that the thin plastic bag would reveal the greatest structural surface changes due to its thinness. Since scanning electron microscopy (SEM) enables visualization of bacterial colonization and biofilm architecture, including extracellular polymeric substance (EPS) deposits, we used it to investigate the colonization process by microorganisms from activated sludge, which are investigated less often than microorganisms from landfill and dump sites (Matjašič et al. in prep.). Moreover, we tested whether the measurements of the isotopic composition of carbon ($\delta^{13}C$), as proposed by Lucas et al. (2008), can provide reliable information on microbial degradation of experimental plastics. This preliminary study

also identified methodological improvements necessary for further experiments where we will try to better understand the survival and colonization processes on plastic materials by microorganisms from different polluted sites. Our final, long term aim is to isolate environmental microorganisms capable of efficient biodegradation.

Material and methods

Preparation of plastic materials

We used three types of plastics in this study. The store-bought new materials consisted of synthetic fabric (textile for clothes), plastic water bottles (bottle), and plastic bags used for packing vegetables and fruits in grocery stores (bag). The determination of the chemical composition of the materials was carried out using Fourier-transform infrared spectroscopy (FTIR) (Perkin Elmer Spectrum Two) in ATR mode. FTIR identifies the functional groups present in organic or inorganic compounds and characterizes covalent bonding information by measuring their absorption of infrared radiation over a range of wavelengths (Smith 2011). By FTIR, we can identify the analysed polymer, but cannot see the detailed chemical composition, including additives. The textile and water bottle were identified as polyethylene terephthalate (PET) and the bag as high-density polyethylene (HDPE), all with 98% similarity (Fig. 1).

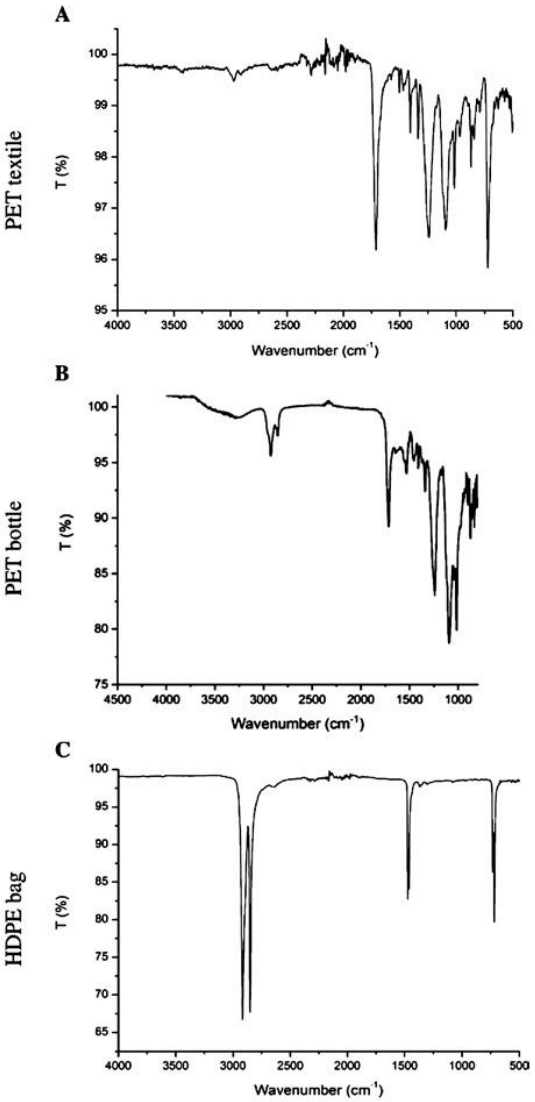

Figure 1: FTIR spectra of PET textile (A), PET bottle (B) and HDPE bag (C).

Slika 1: FTIR spekter PET blaga (A), PET plastenke (B) in HDPE vrečke (C).

Usually, in colonization studies, sterilization with 70% of ethanol is applied (Arkatkar et al. 2009, Arkatkar et al. 2010, Francis et al. 2011, Mohanrasu et al. 2018) but, because treatment with ethanol was indicated not to be sporicidal (McDonnell and Russell 1999, Yoo 2018), we decided to expose plastic materials to UV light. The materials were first exposed to UV-A light (365 nm) for seven days (lamp Osram Ultra-Vitalux, 300 W) to partly mimic the exposure of materials to natural light present in the environment. Next, the plastics were cut into 2 x 2 cm squares and exposed to UV light (ozone free UV-C (λ = 253.7 nm), with a UV radiation level of 15 mW/cm^2/sec; (UVC/T-M-AR Cleaner Box, BioSan) for 30 minutes, turning the materials half-way through to sterilize them.

Co-cultivation experiment

Erlenmeyer flasks (100 mL) were sterilized (dry sterilization, 5h, 180 °C) twice within a two-day window. To each flask, 27 mL Bushnell Haas (BH; 1.0 g K_2HPO_4, 1.0 g KH_2PO_4, 1.0 g NH_4NO_3, 0.2 g $MgSO_4$, 0.05 g $FeCl_3$, 0.02 g $CaCl_2$, in 1000 mL of deionized water) liquid medium (Bushnell and Haas 1941) was added, together with two pieces (2 x 2 cm) of each type of plastic material. We used BH medium because it was designed as a medium for bacteria that degrade hydrocarbons (Bushnell and Haas 1941), and recent studies dealing with microbial plastic degradation used it as a suitable medium without added carbon (Mohan et al. 2016, Auta et al. 2017, Auta et al. 2018, Mohanrasu et al. 2018). We inoculated one flask with microorganisms from an activated sludge sample, and one flask was used as a negative control. We collected the environmental sample of activated sludge on June 17th, 2019, at the wastewater treatment plant (WWTP) Domžale-Kamnik, located near Ljubljana, Slovenia. The volume of inocula was 3 mL. The flasks were incubated for two months at room temperature (22-24 °C) with continuous shaking (120 rpm, HS 501 digital, IKA Labortechnik). The samples were shaken because the medium and the plastic were settling, and because we wanted to maximize the available surface for bacteria. Shaking also increased the probability of bacteria encountering the plastic surface used in the experiments. During incubation, we carried out weekly subculturing to exclude growth on dead biomass, so that bacteria could use plastics as the sole carbon source. The first inoculation was conducted on June 18th, 2019. Then, regular weekly subculturing was carried out three times by transferring 3 mL of mixture from the inoculated flask and the negative control into flasks with fresh BH medium containing plastics. One and a half month after the last subculturing, OD_{600} of liquid media was measured (Lambda UV/Vis spectrophotometer, PerkinElmer, Waltham, MA, USA) and samples for the SEM and isotopic analysis were taken.

Characterization of biofilms on plastics

We took pieces of plastics from the final subculturing where plastic was exposed to microorganisms for month and a half (treated plastics) and corresponding negative controls (control plastics) for further investigation of colonization by SEM. The biofilm growth was characterized by comparing the SEM micrographs of plastics from the mixture with activated sludge with those of negative control and plastics, both not exposed (untreated) and exposed to UV-C (UV exposed), in order to obtain information on their initial texture. The potential biofilms on plastics were fixed with 2.5 % glutaraldehyde and 0.4 % paraformaldehyde in a 0.1 M Phosphate Buffer, pH 7.4, for 2 hours and subsequently subjected to desiccation in sorted series of ethanol concentrations (10%, 20%, 30%, 40%, 50%, 75%, 85%, 95%, 100%). The samples were coated using an ion-beam precision etching coating system (PECS 682, Gatan Inc. USA) with 5 nm thick conductive Au-Pd layer and observed at various magnifications under scanning electron microscope (SEM, JSM 7600F, JEOL, Japan) at SEM accelerating voltage of 10 or 5 kV. The whole surface of colonized plastic materials was systematically examined by using 5000 x magnification, and, in cases of indication on biofilm formation or microbial occurrence the selected area was inspected under 6000, 10 000 and 15 000 x magnifications. Highly indicative micrographs were extracted for this study. Due to space limitation, the presented micrographs (Fig. 2 – Fig. 6) are the most representative images.

Isotopic analysis

Following the co-cultivation experiment, plastics were pre-prepared for isotope analysis. Materials were taken from the final subculturing, same as for OD_{600}, submerged in deionized water separately, and rinsed two more times with fresh deionized water to remove the potential biofilm (treated material). Similarly treated were plastics from control flasks (control material) and material that was not incubated in the flask (untreated material). The isotopic composition of carbon in plastics was determined using a Europa 20-20 continuous flow IRMS ANCA-SL preparation module. Approximately 0.5 mg of plastics (textile - PET, bottle - PET, bag - HDPE) were weighed in a tin capsule for carbon analysis. The isotopic composition of carbon was determined after combustion of the capsules in a hot furnace (temperature 1000°C). Generated products were reduced in a Cu tube (600°C), where excess O_2 was absorbed. H_2O was trapped on a drying column composed of $MgClO_4$. Gases were separated on a chromatographic column and ionized. IAEA CH-3 (-24.724±0.041), CH-7 (-32.151±0.050), CH-6 (-10.449±0.033) reference materials were used to relate the analytical results to the VPDB standards. The sample reproducibility for carbon was ±0.2‰. The isotopic composition of carbon ($\delta^{13}C_{sample}$) is expressed in ‰ and was reported in the δ notation:

$$\delta^{13}C_{sample}\ (‰) = ((^{13}C/^{12}C_{sample})-(^{13}C/^{12}C_{RM}))/(^{13}C/^{12}C_{RM}) * 1000\ [‰]$$

with VPDB – Vienna Pee Dee Belemnite as the reference material (RM).

During the two months incubation in sterilized liquid BH (negative controls), plastic pieces of PET textile (Fig. 3) and PET bottles (Fig. 4) did not show substantial textural changes. However, individual rod-shaped cells or coccoid microorganisms occurred on the plastics, mostly on PET bottles, indicating microbial contamination. On the contrary, changes in surface texture were obvious for HDPE bags in control flasks (Fig. 5), which at the end of experiments showed rougher texture with cracks. PET bottles, PET textile and HDPE bag materials from flask inoculated with activated sludge, differed in both density of microorganisms and biofilm development. The highest microbial diversity and the most developed biofilm was observed on material from the PET bottle, and the least or almost none was found on PET textile. Colonization on PET bottles included the formation of deposits of extracellular polymeric substance (EPS) and the presence of different types of cells (coccoid, rods, spirals, corkscrews) in the form of single cells, pairs, chains or clusters (Fig. 6). The low colonization rate on textiles may be due to impregnation with antimicrobial additives, physical characteristics of the material, or other factors, such as not suitable growth temperature. FTIR analysis did not indicate substantial differences in PET structure and composition between the PET bottle and PET textile. However, some antimicrobial additives could be present in the PET textile that were not detected by FTIR.

Results and discussion

Visual analysis of the surface of untreated and treated plastics

The surface of the three types of untreated plastics, as seen by SEM micrographs, was smooth and without any visible surface overgrowth (Fig. 2). Treatment of HDPE bag with UV-C resulted in a seemingly smoother surface (Fig. 2D). Antimicrobial additives are widely used in the production of polyester (PET) and polyamide (PA) fibres in order to avoid pathogenic microorganism infection, control microbe infestation, limit the deterioration of textiles, control the spread of disease, reduce the formation of odour by microbial metabolism, and protect the textile products from staining, discolouration, and quality deterioration (Al-Balakocy and Shalaby 2017). The plastics of PET bottles as substrate lead to the highest perceived variety of cell morphology and the most structured and mature biofilm (Fig. 6). Despite this high growth, no obvious textural changes in the plastics itself could be

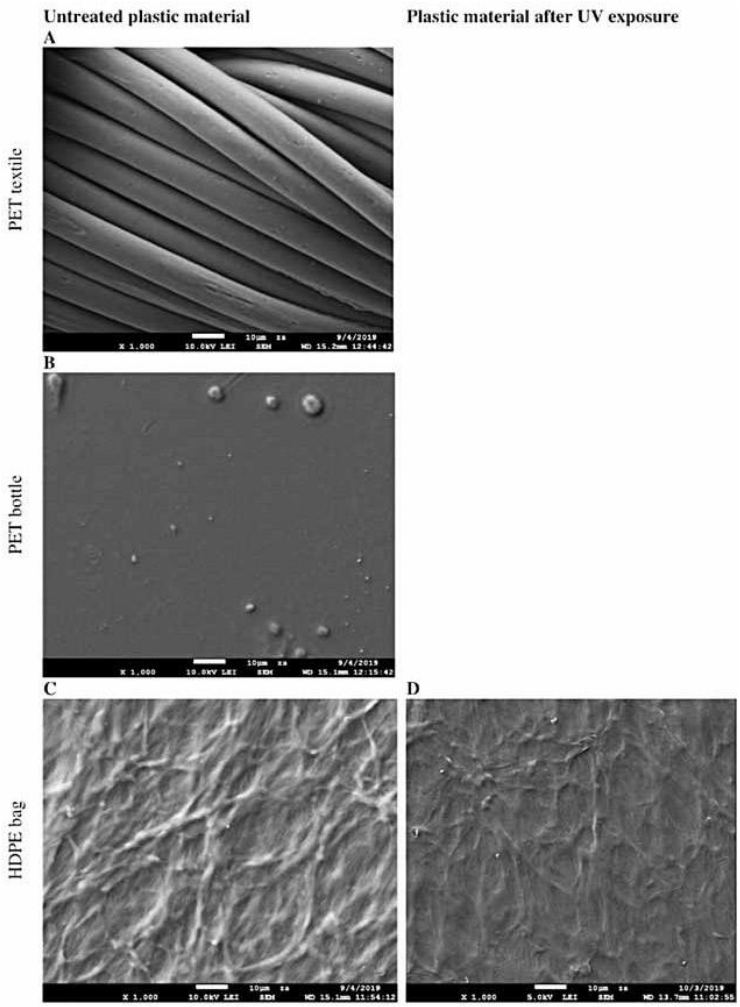

Figure 2: SEM micrographs of untreated PET textile (A), PET bottle (B) and HDPE bag (C) at 1000 x magnification and of HDPE bag (D) exposed for 7 days to UV light at 1000 x magnification.

Slika 2: SEM mikrograf neobdelanega PET blaga (A), PET plastenke (B), HDPE vrečke (C) in HDPE vrečke (D), 7 dni izpostavljene UV svetlobi na 1000 x povečavi.

observed. The HDPE bag, a material successfully sterilized with UV-C, supported the growth of microorganisms from activated sludge (Fig. 5). As with the PET bottles, the microorganisms seem to form a biofilm with cells embedded in the matrix. Contrary to PET plastics, the HDPE texture changed during the experiment; however, these changes were observed with and without activated sludge. Therefore, it is not clear whether the changes are due to abiotic or biotic factors.

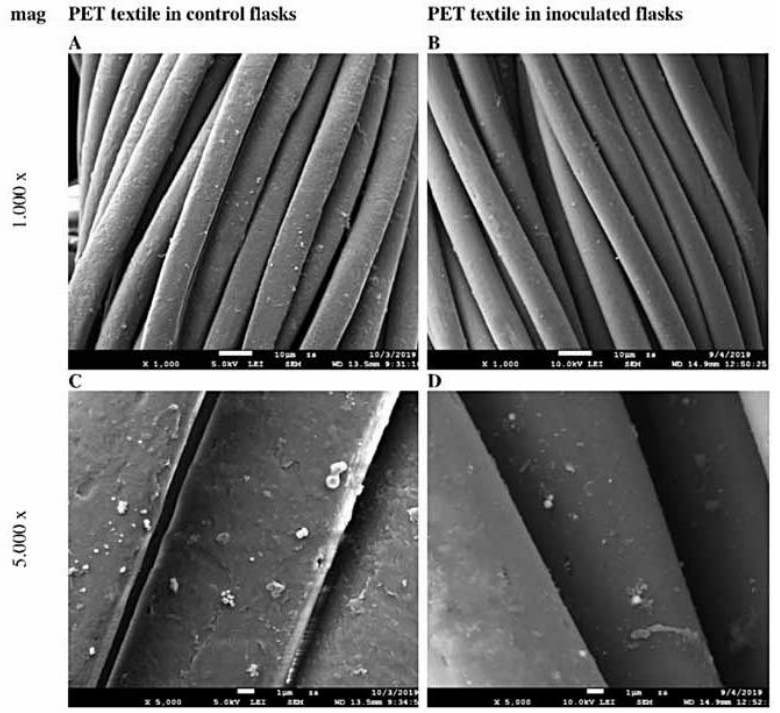

Figure 3: SEM micrographs of PET textile incubated for two months in sterilized media (A, C), and PET textile incubated in media with added microorganisms from activated sludge (B, D). Magnification (mag) showed left.

Slika 3: SEM mikrograf PET blaga, inkubiranega 2 meseca v steriliziranem gojišču (A, C) in PET blaga, inkubiranega v gojišču z dodanimi mikroorganizmi iz aktivnega blata (B, D). Povečava (mag) prikazana levo.

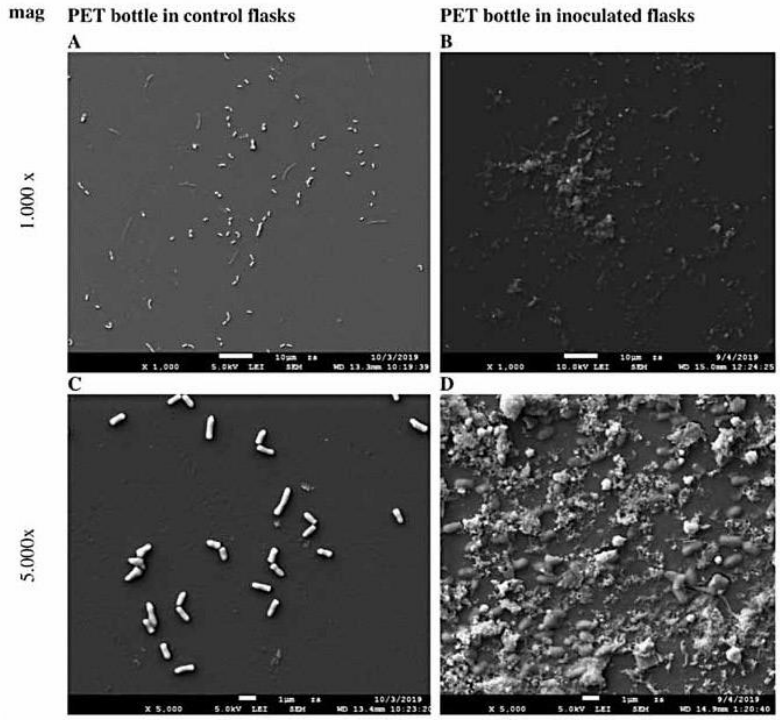

Figure 4: SEM micrographs of PET bottles incubated for two months in sterilized media (A, C), and PET bottles incubated in media with added microorganisms from activated sludge (B, D). Magnification (mag) showed left.

Slika 4: SEM mikrograf PET plastenk, inkubiranih 2 meseca v steriliziranem gojišču (A, C) in PET plastenk, inkubiranih v gojišču z dodanimi mikroorganizmi iz aktivnega blata (B, D). Povečava (mag) prikazana levo.

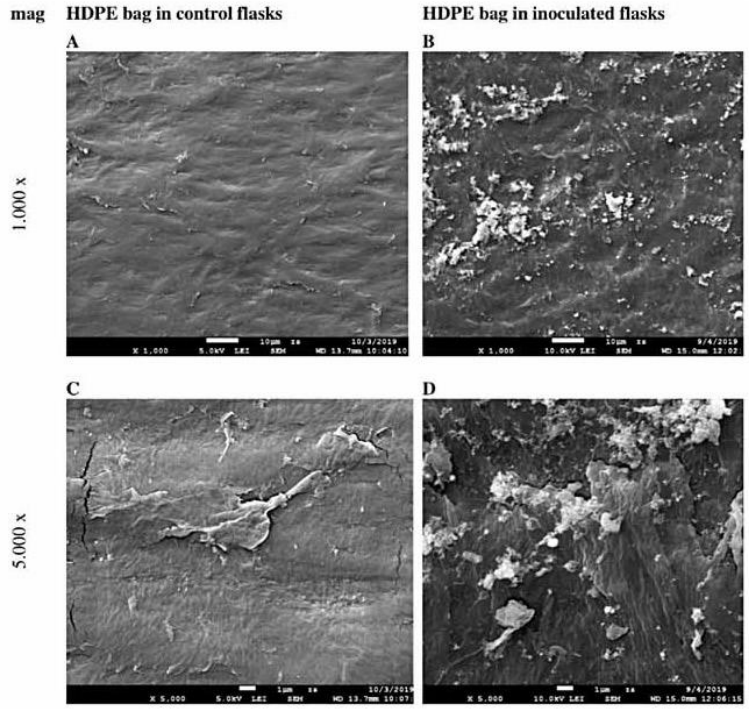

Figure 5: SEM micrographs of HDPE bag incubated for two months in sterilized media (A, C), and HDPE bag incubated in media with added microorganisms from activated sludge (B, D). Magnification (mag) showed left.

Slika 5: SEM mikrograf HDPE vrečk, inkubiranih 2 meseca v steriliziranem gojišču (A, C) in HDPE vrečk, inkubiranih v gojišču z dodanimi mikroorganizmi iz aktivnega blata (B, D). Povečava (mag) prikazana levo.

Overall, this study confirmed the capabilities of plastics to support growth of microorganisms and formation of biofilms from samples of activated sludge. An ecotoxicity study of primary MP by Jemec Kokalj et al. (2019) demonstrated that primary MP from cosmetic products became coated by organic/inorganic material and possibly microorganisms when incubated for three weeks in different environmental samples (spring water, river water, landfill leachate, WWTP effluent), as visualized by light microscopy. They observed the most pronounced overgrowth on MP incubated in the leachate collected from a landfill collection basin and the least in those from WWTP effluent (Jemec Kokalj et al. 2019). Since they did not use samples from activated sludge, a direct comparison with our study is not possible. Nevertheless, our observation of biofilm formation on plastics after exposure to activated sludge is in general agreement with their results. A study by Khatoon et al. (2014) revealed biofilm succession associated with degradative effects on plastic (PP) and contaminants in the sludge. Their surface analysis of plastics by SEM revealed the emergence of profound bacterial growth on the surface of PP beads. Biofilm development started after the third week of incubation. Six-week-old biofilm showed maximum growth and long chains of bacilli, which were succeeded by bacilli of larger sizes, followed after nine weeks by the predominance of mostly rod-shaped bacteria embedded in thick EPS. During biofilm formation they identified

13 microbial strains (*Escherichia coli, Pseudomonas aeruginosa, Enterobacter aerogenes, Salmonella typhimurium, Proteus vulgaris, Alcaligenes faecalis, Shigella dysenteriae, Klebsiella pneumonia, Bacillus subtis, Staphylococcus aureus, Micrococcus luteus, Streptococcus lactis,* and *Cornyebacterium xerosis*) by biochemical characterization. Since a global study by Wu et al. (2019) demonstrated that activated sludge over the globe has a small, core bacterial community (28 operational taxonomic units), we expect that strains from our samples, at least to some extent, coincide with the strains identified by Khatoon et al. (2014).

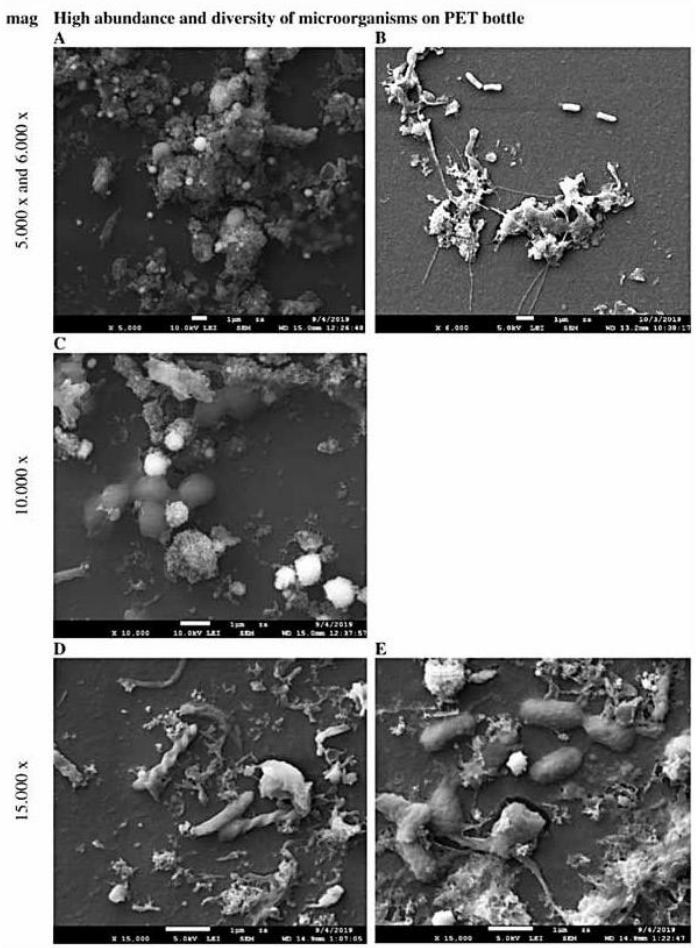

Figure 6: SEM micrographs indicating high abundance and diversity of microorganisms growing on PET bottles. The magnification was 5.000 x (A), 6.000 x (B), 10.000 x (C), 15.000 x (D, E). Magnification (mag) showed left.

Slika 6: SEM mikrografi nakazujejo visoko abundanco in diverziteto mikroorganizmov, rastočih na PET plastenkah. Povečava je 5.000 x (A), 6.000 x (B), 10.000 x (C), 15.000 x (D, E). Povečava (mag) prikazana levo.

Microbial diversity, based on observation of their cell morphology, was different on different types of plastics. We found the highest diversity of cell morphological diversity on PET water bottle and the lowest on PET textile fibres. PET water bottles are more closely regulated for any toxic compounds because they are used for storing drinking water consumed by humans, which may explain the highest cell morphological diversity found in them. Textile fibres, however, may be treated with different antimicrobial chemicals widely used in textile production (Al-Balakocy and Shalaby 2017). Parrish and Fahrenfeld (2019) found differences in biofilm composition between polystyrene (PS) and polyethylene (PE) microparticles that were exposed to environmental samples for only 48 hours. In their study, Deltaproteobacteria and Acidomicrobia (two classes common to wastewater), and Saprospirae of Bacteroidetes prevailed in wastewater PS and PE biofilms, and they observed elevated Gammaproteobacteria in PE microparticle biofilm and Betaproteobacteria in the PS biofilm in river water. They concluded that differences were due to the morphology/surface texture rather than polymer composition. They did not observe differences in biofilm due to particle size (particles smaller or larger than 250 µm), and they proved that biofilm microbial communities differed from communities in the surrounding water. Ogonowski et al. (2018) exposed ambient Baltic bacterioplankton to plastic substrates commonly found in marine environments (PE, PP, and PS) as well as native (cellulose) and inert (glass beads) particles for two weeks under controlled conditions. They found significant differences between plastics and non-plastic substrates in their community composition and diversity. Through operational taxonomic units (OTUs) data, the PE and PP communities were quite similar, whereas that on PS was more distinct. They determined that the observed differences were most probably due to surface hydrophobicity of the materials. Similarly, significantly different communities were observed on low density polyethylene (LDPE), polyethylene terephthalate (PET), and polypropylene (PP) materials from marine environments using denaturing gradient gel electrophoresis (DDGE) profiles (Oberbeckmann et al. 2014).

Microorganisms growth in liquid BH media

After one and a half months, measurements of optical density (OD_{600}) did not indicate microbial growth in the liquid medium. The OD_{600} of BH media was low (0.0603) and similar to the one in the control flask (0.0678). Optical density (OD) measurements of microbial growth are one of the most common techniques used in microbiology, including investigations of growth under different nutritional or stress environments, where the OD value obtained is assumed to be proportional to the cell number (Stevenson et al. 2016). For example, for *E. coli* cell cultures, OD_{600} of 1 corresponds to 8×10^8 cells mL^{-1}. However, to be accurate, the OD method needs thorough calibration and depends on the type of instrument, size and shape of the cells, and changes in the refraction of medium or cells (Stevenson et al. 2016). Our results indicate no microbial growth in liquid media in comparison with the growth on plastics itself as seen by SEM. We speculate that the microbial growth was not present in liquid BH medium containing only minerals, because of nutrient limitations. The food was present only in the form of carbon bonded within synthetic polymers (i.e. particles of PET textile and bottle and HDPE bag) localizing at the bottom of the 250 mL flasks. In contrast, when comparing surrounding media and biofilm, the study of Eckert et al. (2018) demonstrated that after 15 days, the bacterial community composition was not different between biofilm and free-living communities in the 750 mL vessels with different concentrations of PS microparticles (sizes 4 mm x 4 mm x 0.1 mm). Nevertheless, their experiment aimed to demonstrate that microplastics can be a vector for microorganisms from the WWTPs, not a source of carbon. Consequently, they used liquid media with a carbon source in the form of chitin. Moreover, the plastic particles added to the experiment were continually floating in the water column enabling uniform distribution of microorganisms within the experimental vessels.

Isotopic analysis

Values for $\delta^{13}C$ in untreated and treated materials, incubated in liquid BH medium in this study are presented in Table 1. The comparison

of the $\delta^{13}C$ values in treated and untreated materials, indicated that the $\delta^{13}C$ value increased (enriched with heavy carbon isotope $\delta^{13}C$) in both the control and inoculated flasks (Table 1). Berto et al. (2017) preliminary study reported on $\delta^{13}C$ values for various plastic materials, including PET bottles for drinking water and HDPE bags. They characterized plastic polymers using EA/IRMS where mean $\delta^{13}C$ for PET was -27.84 ± 1.71 ‰ as opposed to this study where $\delta^{13}C$ for untreated PET was -29.0 ±0.2 ‰. Mean $\delta^{13}C$ value for HDPE bag was -33.97 ± 0.70 ‰ in the study of Berto et al. (2017) and we measured -30.2 ± 0.1 ‰ for untreated HDPE in this study. Plastics degraded in the marine environment showed an increase of $\delta^{13}C$ values (Berto et al. 2017), which was also the case in our study. The shift of $\delta^{13}C$ could be related to physical/chemical or biological degradation although it was not possible to evaluate the degradation rate. The results indicated that this method has the potential to be used in biodegradation studies but careful experimental design is needed.

Critical evaluation of methodology

The chosen sterilization approach for plastics, namely UV-C, was found not to be totally efficient since microorganisms were detected on the plastics from control flasks. The UV-C sterilization was more suitable for thinner plastic bags of HDPE and less for PET textile and thicker plastic of PET water bottles. The morphology of microorganisms observed on the two materials from control flasks was different. Coccoid microorganisms were present in PET textile (Fig. 3), and rod-shaped cells were dominant in PET bottles (Fig. 4). This indicates that the contamination is not a consequence of an experimental error but instead is linked to the materials themselves. Most likely, the contamination is due to the limited penetration of UV-C through these materials. The results indicate that a combination of sterilization approaches may be required and will be used in further studies. The observations are in line with a previous study of Meechan and Wilson (2006) which shows that while UV-C is germicidal and virucidal, it does not penetrate well and will only disinfect the outer

Table 1: Isotopic composition of carbon ($\delta^{13}C$) measured in plastic materials prior to the experiment (untreated material) and these incubated in control flask and flask inoculated with activated sludge (treated material). The average values and SD of the replicate measurements on the same sample are shown. Sign ε (enrichment factor) represents the difference to respective untreated material.

Tabela 1: Izotopska sestava ogljika ($\delta^{13}C$) merjena v plastičnih materialih pred poskusom (netretiran material) in po poskusu v kontrolni Erlenmajerici in Erlenmajerici z dodanim inoculum iz aktivnega blata (tertian material). Prikazane so srednje vrednosti in SD zaporednih meritev istega vzorca. Znak ε (obogatitveni faktor) predstavlja razliko do neobdelanega materiala.

	Untreated material $\delta^{13}C$	**Treated material $\delta^{13}C$**	
	[‰ ± s‰]	Control flask [‰ ± s‰]	Flask with activated sludge [‰ ± s‰]
Textile (PET)	-28.9 ± 0.1	-28.2 ± 0.1	-28.1 ± 0.1
		ε = 0.7	ε = 0.8
Bottle (PET)	-29.0 ± 0.2	-28.6 ± 0.1	-28.6 ± 0.2
		ε = 0.4	ε = 0.4
Bag (HDPE)	-30.2 ± 0.1	-29.7 ± 0.1	-29.8 ± 0.2
		ε = 0.5	ε = 0.4

surface of a material. Plastics of PET bottles were also not efficiently sterilized with UV-C before to the experiment and showed contamination (Fig. 4). However, the final abundance of microbial growth in control was far below that observed on plastics exposed to activated sludge.

Conclusions

This study represents one of the rare insights into the differences in interactions between PET textile, PET bottle, and HDPE bag and microorganisms outside of the marine environments, namely with microorganisms from activated sludge. Through a combination of SEM and stable isotopic analyses, we demonstrated that the chemical composition of plastics and its surface characteristics (morphology, texture) play a significant role in biofilm development regarding both its diversity and complexity, presumably because of easier adhesion. The value of $\delta^{13}C$ slightly increased in all tested materials compared to the source material suggesting certain level of degradation. The study results show that microorganisms are capable of colonizing plastics in environments without other carbon sources. Among the PET textile, PET bottles, and HDPE bags, the last two promoted the most abundant growth and seemingly more structured and mature biofilm as evidenced by SEM micrographs. Further studies with improved experimental design, including metagenomic approaches, which may be useful in identifying the microorganisms present in activated sludge that are more successful in the colonization of plastics. As potential bio-degraders, these microorganisms are of interest to isolate in pure cultures as well.

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Povzetek

Posledica povečanja produkcije sintetičnih ali pol-sintetičnih organskih spojin (plastike) in njihove vse bolj razširjene uporabe je tudi povečano pojavljanje plastike v celinskih vodah. V okolju najdemo različne vrste plastičnih materialov (npr. polietilen visoke oz. nizke gostote (HD/LD-PE), ali polietilen tereftalat (PET)), ki se pojavljajo v različnih oblikah (npr. fragmenti, peleti, filamenti). Prvi stik plastike z biosfero so običajno kolonizacijski mikroorganizmi, vendar je ta interakcija v celinskih vodah slabo raziskana. V študiji smo poskušali bolje razumeti, kako poteka kolonizacijski proces mikroorganizmov iz aktivnega blata na različnih plastičnih materialih, prepoznali smo tudi nekatere metodološke izboljšave, ki jih bomo upoštevali v nadaljnjih eksperimentih.

V preliminarni raziskavi smo uporabili plastične materiale, ki so se razlikovali tako v kemični sestavi (PET in HDPE) kot tudi v površinskih lastnostih (blago, debelejše plastenke in tanjše plastične vrečke). Materiale smo najprej teden dni izpostavili UV-A, s čimer smo simulirali sončno svetlobo, nato smo ga pred vnosom v gojišče sterilizirali z UV-C. V erlenmajerice z BH gojiščem smo zraven različnih kosov plastike (2 x 2 cm) dodali še mikrobe iz vzorca aktivnega blata iz centralne čistilne naprave (CČN) Domžale-Kamnik, eno smo pustili ne-inokulirano kot negativno kontrolo. Erlenmajerice smo 2 meseca stresali na sobni temperaturi (22-24°C) in 120 rpm ter jih redno prestavljali v sveže gojišče s testnimi plastičnimi materiali, da bi bakterijam preprečili rabo ogljika, sproščenega iz odmrlih bakterij. Ob koncu eksperimenta smo v tekočih gojiščih izmerili OD_{600} ter izvedli analizo izotopske sestave ogljika ($\delta^{13}C$) in vizualno analizo z uporabo vrstičnega elektronskega mikroskopa (SEM) vseh treh plastičnih materialov.

Ugotovili smo, da so mikroorganizmi iz aktivnega blata sposobni kolonizirati plastiko brez dodatnih virov ogljika in da kemijska sestava plastike najverjetneje vpliva na razvoj biofilma. Biofilm, ki se je tvoril na PET plastenkah in HDPE vrečkah, je bil glede na SEM mikrografe na videz bolj strukturiran in zrel, na kar najverjetneje vpliva kemična sestava plastike, aditivi in njene površinske lastnosti. Ugotovili smo tudi, da sterilizacija plastike z UV ni zadostna. Vrednost $\delta^{13}C$ je bila

višja v materialih izpostavljenih mikroorganizmom v primerjavi z ne-tretiranim materialom, kar najverjetneje nakazuje degradacijo. V nadaljnjih eksperimentih bomo uporabili več različnih okoljskih virov mikroorganizmov, izboljšan način sterilizacije in metagenomske pristope, s končnim ciljem izolirati seve, ki so sposobni razgrajevati težko razgradljive plastične materiale.

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3.3 Structure and Function of the Hyporheic Biofilms in the Presence of Plastic Pollution

Freshwater sediments in the HZ are assumed to be sinks for MPs (Frei et al., 2019; Drummond et al., 2022). The HZ is also important in shaping MP particle transport and fate, i.e., deposition, retention and long-term accumulation (Frei et al., 2019; Drummond et al., 2020). The most common types of MPs in freshwater sediments globally are PE, PP and PS, but PET is also frequently found in river sediments (Yang et al., 2021). Similarly, the investigation performed as part of my studies showed that PE and PP are the most common MPs in the sediments of central Slovenian rivers (Matjašič et al., 2023). In WWTP, effluents represent major sources of MP pollution and PET fibres and irregularly shaped PE particles are the most frequently MP types (Ziajahromi et al., 2017).

In rivers, the principal metabolic pathways occur in the HZ (Orghidan, 1959, 2010; Battin et al., 2016), and the effects of MPs could potentially impair ecosystem functioning (Arias-Andres et al., 2019; Ahmad et al., 2020). A study by Scherer et al. (2020) found that the concentration of MP in sediments is higher than that in water columns for rivers and lakes. Several studies have indicated that pollution with MPs affects aquatic biofilms by altering the composition of the sediment microbial community (Li et al., 2020; Seeley et al., 2020; Miao et al., 2021b).

In this part of my work, I performed a field study to test whether PET fibres in riverbed sediments affect colonization, the seasonal dynamics of HZ microbial structure and metabolic activities (Chapter 3.3.1). Following this, I extended the field study to include three additional locations and prolonged it for 2-years to encompass both temporal and spatial variability in HZ biofilm response to pollution with PET fibres (Chapter 3.3.2).

3.3.1 Published scientific article: "Presence of polyethylene terephthalate (PET) fibres in hyporheic zone alters colonization patterns and seasonal dynamics of biofilm metabolic functioning"

This section is a scientific article authored by Tjaša Matjašič (NIB), Tatjana Simčič (NIB), Tjaša Kanduč (JSI), Zoran Samardžija (JSI) and Nataša Mori (NIB) that was published in Water Research in 2021. As part of this work, I collaborated with the Jožef Stefan Institute (JSI) in this paper. I conceptualised the experiment, developed the methodology, collected and processed the field samples, conducted laboratory work, analysed and organised the data, and wrote the initial draft of the manuscript under Nataša Mori's (NIB) supervision. Tatjana Simčič (NIB) contributed to the methodology's conceptualisation and development, provided laboratory assistance, and offered feedback on the manuscript. Nataša Mori (NIB) participated in field sampling, data analysis and manuscript writing. Tjaša Kanduč (JSI) performed the isotopic analysis of PET. Zoran Samadržija (JSI) provided SEM images of PET fibres. Both Tjaša Kanduč and Zoran Samardžija contributed to the manuscript's writing.

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Presence of polyethylene terephthalate (PET) fibers in hyporheic zone alters colonization patterns and seasonal dynamics of biofilm metabolic functioning

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ABSTRACT

Worldwide, the production of plastics is increasing, and plastic pollution in aquatic environments is a major global concern. Under natural conditions, plastic weathers to smaller pieces called microplastics (MP), which come in various shapes, with fibers often being the most common in freshwater sediments. The hyporheic zone, an ecotone between surface and groundwater, is important for the transport and fate of all MP particles. The main metabolic pathways in rivers take place in the hyporheic zone and are driven by a diverse microbial community. The objective of this study was to investigate *in situ* whether the presence of PET fibers in riverbed sediments affects patterns of colonization and the seasonal dynamics of microbial metabolic activities in the hyporheic zone. The effects of the presence of PET on microbial metabolism were evaluated *in situ* over a month (colonization study) and over a year (seasonal study) by measuring total protein content (TPC), and microbial respiration as respiratory electron transport system activity (ETSA) and by community-level physiological profiling (CLPP). Additionally, PET fibers were examined under a scanning electron microscope (SEM), and isotopic analysis ($\delta^{13}C$) of PET was performed after one year of exposure to field conditions. The findings demonstrated that during colonization and biofilm formation, and also over the seasons, the date had a large and significant impact on biofilm growth and activity, while PET presence slightly suppressed microbial biomass (TPC) and respiratory activity (ETSA). Overall microbial activity was repressed in the presence of PET fibers but there was a higher capacity for the utilization of complex synthetic polymer substrates (i.e., Tween 40) which have previously been linked to polluted environments. SEM micrographs showed diverse microbial communities adhering to PET fibers but little surface deterioration. Similarly, isotopic analysis suggested little deterioration of PET fibers after one year of *in situ* conditions. The study indicated that PET fibers present in riverbed sediments could have impacts on the metabolic functioning in rivers and thus affect their self-cleaning ability.

1. Introduction

Plastic pollution of aquatic environments is a global health and environmental concern. Worldwide, the production of plastics had increased from 1.5 million tonnes in 1950 to 360 million tonnes in 2018 (PlasticsEurope, 2019). Due to their durability and the low recycling rate, plastics have now been accumulating in landfills and the natural environment for almost a century. Under natural conditions, plastic waste weathers to smaller pieces called microplastics (particles < 5 mm; MP), which thus more easily enter food webs and serve as vectors for toxic chemical compounds (Thompson et al., 2004) or pathogens (Wu et al., 2019). In concordance with global production rates, high- and low-density polyethylene (HD/LD-PE), polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC) are the most common polymers found in the environment (Wagner et al., 2014). In freshwater sediments, the main types of MP, according to a review by Yang et al. (2021), are PE, PP, and PS, but PET is also frequently found in river and lake sediments across the globe. Various shapes of microplastics, including fragments, foams, fibers, and films, have been detected in freshwater sediments, but the fibers, probably closely connected to the washing of textiles, are those most commonly occurring in freshwater sediments (Yang et al., 2021). In

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waste waters, which are among the most significant sources of freshwater pollution, the most frequently found MPs are PET fibers and irregularly shaped PE particles (Ziajahromi et al., 2017). The major sources of MP particles in freshwater are secondary MP, generated by the breakdown of larger items (such as single-use packaging, tires, and fibers). These enter freshwaters due to poor waste management through surface and agricultural runoff or as wastewater treatment plant (WWTP) effluents (Wong et al., 2020; Matjašič et al., 2021). Despite the fact that WWTPs are highly efficient in retaining MP, with 78–98% of MP removed in the primary treatment, due to the high volume of treated effluents released to recipient water bodies every day, the MP contamination received into aquatic ecosystems is still considerable (Prata, 2018; Wong et al., 2020).

The main metabolic pathways in rivers take place within the riverbeds, in the so-called hyporheic zone (Orghidan, 2010). Here, surface water, organic matter particles, organisms, and pollutants enter the subsurface sediments, mix with the groundwater, and are in close contact with the biofilms that overgrow sediment particles (Boulton et al., 1998; Hancock et al., 2005). In the hyporheic zone, biofilms generally include heterotrophs and chemolithotrophs embedded in a polysaccharide matrix (Battin et al., 2016). Freshwater sediments are considered sinks for MP. For example, a study by Scherer et al. (2020) found that the concentration of MP in sediments is higher than that in water columns, for both rivers and lakes. Moreover, Frei et al. (2019) demonstrated that riverbed sediments of up to 0.6 m (i.e., the hyporheic zone) can be an accumulation area for MP of smaller size (< 50 μm) and pointed out that hyporheic exchange is a potential mechanism for smaller MP particles to enter an underlying aquifer and thus contaminate the groundwater. Similarly, a field stream study by Drummond et al. (2020) indicates that, in streambed sediments, hyporheic exchange is important for the transport and fate (i.e., deposition, retention, and long-term accumulation) of all MP particles < 100 μm, irrespective of polymer type.

Several studies have indicated that pollution with MP affects aquatic biofilms. Miao et al. (2021) demonstrated substrate-specific functional diversity for biofilms growing on PET or PVC substrates taken from three rivers. They used Ecoplates™ (BIOLOG, USA) as detectors for the metabolic functioning of microbial communities and observed the differences in metabolic profiles between biofilms growing on either PET or PVC substrates in all three rivers by measuring the intensity of utilization of 31 substrates from the Ecoplates. An interesting laboratory study by Seeley et al. (2020) clearly indicates that the amendment of sediments with either PE, PVC, PUF, or PLA microplastics alters the composition of the sediment microbial community and the nitrogen cycling processes. Li et al. (2020) found a decrease in the α-diversity of microorganisms from river sediments under the impacts of MP and significant effects on the structure and functioning of the microbial community. It is well known that loss of bacterial species richness can have a strongly negative impact on ecosystem functioning and services (Delgado-Baquerizo et al., 2016). For example, for heterotrophic bacteria to degrade complex compounds, the bacteria need to create a syntrophic metabolic chain. This means that one bacteria uses the products of catabolism from another as a source of carbon (Santisi et al., 2015). Losses in microbial diversity may result in the disruption of these metabolic "chains," because, for some species, resources become available only if another species degrades and consumes a part of that resource (Delgado-Baquerizo et al., 2016). Further studies are needed to fully understand how loss and changes in microbial structural and functional diversity due to the presence of plastic pollution impact the ecosystem processes in riverbed sediments.

After disturbances such as drought or high water levels, the colonization and re-colonization of natural sediments or anthropogenic debris by biofilm takes place within a few days, and the rate increases with warmer temperatures (Villanueva et al., 2011; Hoellein et al., 2014). The development of freshwater biofilms and their composition and functional diversity also depends on hydrological shear stress (Rickard et al., 2004), hyporheic exchange rates (Simčič and Mori, 2007), substrate surface and type of substrate (Wang et al., 2020), available nutrients in the surrounding water (Griebler et al., 2002), and pollution with MP (Li et al., 2020; Niu et al., 2021). The complex microbial communities (i.e., biofilms) living here are the driving force behind the ecosystem processes and biogeochemical cycles (Arias-Real et al., 2020), and are therefore an essential element of the self-purification capacity of rivers (Fischer et al., 2005; Battin et al., 2016; Harrison et al., 2018). Alterations in microbial communities can affect carbon cycling (Arias-Andres et al., 2019). To obtain information on the structure of the biofilms related to plastic or MP pollution, studies of environmental genetic material (metagenomics) are increasingly popular, but they are expensive and require extensive knowledge of bioinformatics. Alternatively, quick and simple methods, such as community-level physiological profiling (CLPP), can broaden the understanding of *in situ* ecological and ecosystem functions as based on community metabolic activity, and thus provide a quick way to assess the ecological health of a freshwater system. In addition, they can provide simple indicators of the impacts of MP on biofilm functioning (Weber and Legge, 2010).

The objective of this study was to investigate, *in situ*, whether the presence of PET fibers in riverbed sediments affects patterns of colonization and the seasonal dynamics of microbial metabolic activities in the hyporheic zone. This can be achieved by using either simple (respiratory electron transport system activity—ETSA) or more complex (community level physiological profiling—CLPP) measures of microbial functioning. The specific aims of this research were, (1) to investigate the colonization patterns of natural biofilms in plastic-free sediments and to compare them with colonization in PET-contaminated natural sediments, (2) to observe seasonal dynamics in biofilm functioning in both PET-polluted and unpolluted sediments over a one-year period, and (3) to observe whether PET fibers exposed to natural conditions in the hyporheic zone for a year showed any indications of deterioration or biodegradation. New biofilm development is usually driven by extreme events such as flooding or droughts over the different seasons. This study provides initial insights into the impacts of PET pollution on hyporheic biofilm metabolic activity and functioning following such events.

2. Methods

2.1. *In situ* experimental design

The study was carried out in the pre-Alpine gravel-bed river Kamniška Bistrica, a left tributary of the Sava River in north-central Slovenia (SE Europe). The study river emerges at a 630 m elevation in the southern Kamniško-Savinjske Alps and is 33 km long with a catchment area of 539 km^2. In the upper part of the catchment, Kamniška Bistrica is a typical, undisturbed pre-Alpine river, while in the lower reaches, it is strongly impacted by urbanization, intensive agriculture, and wastewater treatment effluents.

A 100 m river stretch between the two settlements, Beričevo and Videm, (46°05′18.4″N, 14°37′33.1″E, 268 m altitude) was selected as a study site. Within the study stretch, six sites, which included pools (P1–3; deeper areas with slower water) and runs (R1–3; areas with faster water), were selected to encompass the spatial heterogeneity of the environmental parameters within the riverbed, such as hydraulic conductivity, temperature, redox conditions, and water chemistry (Table 1). At each site, three types of artificial substrates (control, low PET, high PET), pre-prepared in the laboratory as described below, were inserted into the riverbed. The substrates were inserted at depths of 20 to 40 cm and then covered with natural sediments on 28th of June 2019 (D0). The natural riverbed sediments are composed of cobbles, pebbles, gravel, sand, and silt, with cobbles and gravel as the dominant particles. A temperature logger (Vemco, InnovaSea, USA) was inserted together with the artificial substrates. The artificial substrates in pockets and bags were inserted perpendicular to the subsurface stream flow. During the colonization, the first four samplings (i.e., collections of the pre-

Table 1
Environmental parameters during the seasonal samplings. T – water temperature; O_2 – oxygen concentration; cond – water conductivity; DOC – dissolved organic carbon; TN – total nitrogen; POM – particle organic matter in sampled sediments.

season	Surface water water level [cm]	T [°C]	cond [µS cm^{-1}]	O_2 [mg l^{-1}]	DOC [mg l^{-1}]	TN [mg l^{-1}]	Riverbed sediments T [°C]	POM [g kgDW^{-1}]
autumn	5 ± 4.1	12.9 ± 0.1	462 ± 2	9.5 ± 0.5	2.1 ± 0.1	3.3 ± 0.0	13.7 ± 0.2	10.2 ± 1.6
winter	13±4.7	8.3 ± 0.4	497 ± 1	11.1 ± 0.3	2.4 ± 0.0	3.3 ± 0.0	5.4 ± 0.5	9.8 ± 1.2
spring	4 ± 0.0	11.6 ± 0.1	351 ± 0	10.2 ± 0.0	1.5 ± 0.1	1.4 ± 0.1	13.1 ± 0.1	10.3 ± 1.2
summer	8.3 ± 2.4	15.3 ± 0.0	430 ± 0	9.1 ± 0.0	3.1 ± 0.2	1.8 ± 0.0	17.0 ± 1.2	10.7 ± 1.4

prepared substrates) were carried out in weekly to monthly intervals (D1: one week, D2: two weeks, D3: a month, D4: two months). After that, the samplings were carried out seasonally (D5: autumn 2019, D6: winter 2019, D7: spring 2020, D8: summer 2020) (Fig. 1). Concurrently, river water levels, surface water temperatures, conductivity, and oxygen concentrations were measured using field probes (Multi 3430, WTW, Germany). In addition, surface water samples were collected for laboratory analysis of dissolved organic carbon (DOC) and total nitrogen (N_{tot}) (analyzed at the Slovenian Forestry Institute).

2.2. Artificial substrate preparation

Sediments for the preparation of the artificial substrates were taken from the same site where the *in situ* experiment was later to be carried out. About 15 kg of sediments were washed and sieved in the laboratory to obtain sediments with particle sizes between 2 and 4 mm and between 1 and 2 mm. These two size classes were mixed in a ratio of 2:1 before filling the pockets with them so as to standardize the sediment surface, which is one of the most important factors when estimating biofilm activity (i.e., Simčič and Mori 2007). Since the pockets were buried and thus fixed in the riverbed sediments, they were not exposed to being continuously washed out by the surface water, and the loss of smaller fraction (<2 mm) in the sediments was minor.

Store-bought fabric was used for the colonization study and characterized by Fourier-transform infrared spectroscopy (FTIR) as polyethylene terephthalate (PET), as described in Matjašič et al. (2020). The fabric was washed in a washing machine at 40 °C and exposed to sunlight for about a month. After exposure, the fabric was cut into 120 × 120 mm pieces for use in the experiment.

Galvanized net (2 × 2 mm mesh size) was cut into bigger (i.e., 350 × 400 mm, for the bags) and smaller pieces (i.e., 160 × 120 and 160 × 180 mm, for the pockets). The pieces were folded in half and stapled together with galvanized staples to construct the "pockets," later to be filled with the artificial substrate, or the larger "bags" to be filled with the pockets. Placing the pockets into the bags was to prevent the loss of the artificial substrates during the one-year study in the event of floods or increased discharge.

For each sampling site (P1–3; R1–3), three bags of size 35 × 40 cm, each containing 13 smaller pockets, were prepared. These three bags contained the control (C), the low PET (1P), and the high PET (5P) pockets, separately. The control pockets (C) were filled with 50 g of washed and sieved sediment. The "low PET" pockets (1P) were filled with a mixture of the washed and sieved sediment and PET fabric in the ratio 10 versus 0.2 (50 g of sediment, 1 g PET) and the "high PET" (5P) in

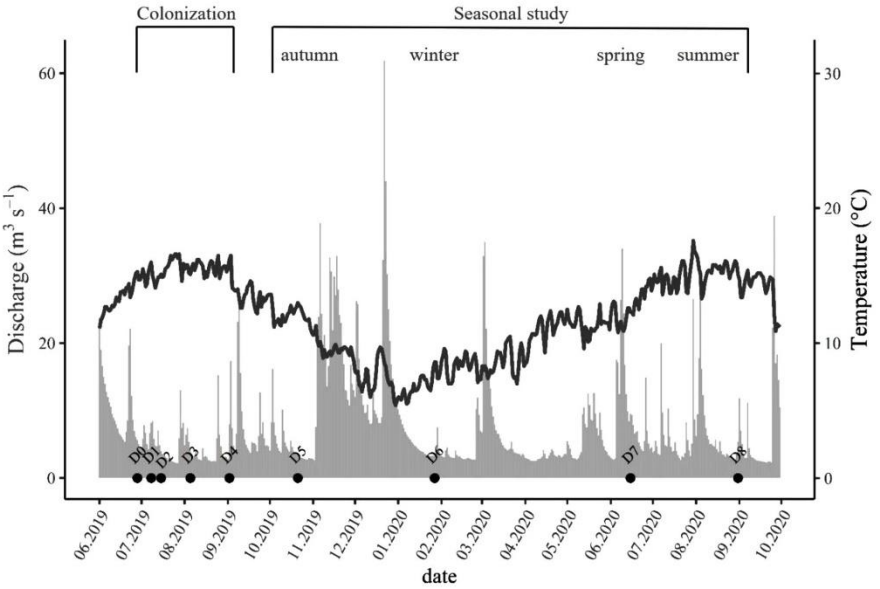

Fig. 1. Surface water temperature and river discharge during the field colonization and seasonal experiment. The sampling dates are indicated by circles (date of insertion: D0; colonization: D1-D4; seasonal study: D5: autumn 2019, D6: winter 2019, D7: spring 2020, D8: summer 2020).

the ratio 10 versus 0.7 (70 g of sediment, 5 g PET). Hence, we had an experimental set up with low (1P) and high (5P) amounts of PET fiber present. We used more sediment for the high PET treatment to maintain the conditions at the microscale similar to natural conditions in the hyporheic zone (i.e., a complex network of interstitial pores between sediment grains of different sizes). The artificial substrates were then autoclaved twice within a two-day window at 121.5 °C for 15 min, prior to application in the field.

2.3. Laboratory work

On each sampling date, one pocket was extracted from each bag (C, 1P, 5P) and from each site (P:1–3, R:1–3), altogether obtaining 18 pockets. In the laboratory, the pockets were cut with scissors, and the sediments were mixed well with a spoon before analysis. Part of a sediment (2 g) was used immediately for community-level physiological profiling (CLPP), while the remaining sediment was frozen at −80 °C for later use with other analyzes (POM, TPC, ETSA; described in Sections 2.4, 2.5 and 2.6, respectfully). All samples were frozen for at least a week prior to analysis. Once thawed, part of the sediment (8 g) was put into a centrifuge tube, and 2 ml of homogenizing buffer (0.1 M phosphate buffer pH = 8.4; 75 μM MgSO4; 0.15% (w/v) polyvinyl pyrrolidone; 0.2% (w/v) Triton-X-100) was added. The samples were processed with ultrasonic homogenizer 4710 (Cole-Parmer, Vernon, IL, USA) for 2 min and then put into a centrifuge (4 min, 10.000 rpm, at 0 °C) (refrigerated centrifuge Sigma 2K15, St. Louis, MO, USA) to collect the microorganisms from biofilms overgrowing the sediment particles. The supernatant containing the microorganisms was further used for TPC and ETSA analyzes. The rest of the thawed sediment (10 g) was used to determine particulate organic matter (POM) and dry weight of sediments (DW). DW was used for the calculations of TPC and ETSA. Samples of PET fibers were taken on the last date, after one year of *in situ* exposure (D8: summer), for isotopic analysis and SEM micrographs.

2.4. Particulate organic matter (POM)

The amount of particulate organic matter (POM) in the sediments was determined by loss-on-ignition (LOI). For the POM measurements, approximately 10 g of thawed sediment was placed in a ceramic bowl and weighed to obtain wet weight (WW). Next, the sediments were incubated at 105 °C until constant in weight and then weighed again to obtain dry weight (DW). The sediments were then put into an oven at 520 °C for 2 h to ignite the organic substance. When cooled, the bowls with sediments were weighed again to obtain loss on ignition. POM was expressed as gPOM $kgDW^{-1}$.

2.5. Total protein content (TPC)

Total protein content (TPC) was used as a proxy for microbial biomass. It was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). The assay is a spectrophotometric assay based on the alkaline reduction of the cupric to the cuprous ion by the protein, followed by chelation and color development by the bicinchoninic acid (BCA) reagent (Lovrien and Matulis, 2005). The BCA/copper complex is water soluble and exhibits a strong linear relationship between absorbance at 562 nm and increasing protein concentrations. Standards and reagents were prepared in accordance with manufacturers' instructions. The sample or standard (25 μl) was thoroughly mixed with the working reagent (200 μl) on the microplate and was then incubated for 30 min at 37 °C. The microplate was cooled to room temperature, and then the absorbance was immediately measured at 562 nm with spectrofluorometer SynergyMX (BioTek Instruments, USA). The results were expressed as μg protein per g of dry sediment (μg prot g sed^{-1}).

2.6. Respiratory electron transport system activity (ETSA)

The respiratory electron transport system activity (ETSA) estimates overall microbial respiratory activity by detecting the activities of dehydrogenases and cytochromes within the electron transport system that biochemically controls cell respiration (Debeljak et al., 2017). ETSA was measured by applying a modified assay adapted from Packard (1971). The supernatant obtained from thawed samples exposed to the homogenizer and centrifuged, as described above, was poured into a 2 ml microcentrifuge tube on ice, and later, microtiter plates were used. For every sample, 30 μl was put into each of two adjacent wells, while a third well was left empty (the blank). Next, 150 μl of substrate solution (NADH, NADPH) was carefully added to each well, followed by 50 μl of INT (iodonitrotetrazolium chloride) reagent. The plate was incubated at 20 °C in the dark for 30 min. After incubation, 50 μl of stopper solution was added to all wells. Finally, 30 μl of the corresponding sample was added to all the blanks. The absorbance was measured immediately with a microplate reader SynergyMX (BioTek Instruments, USA) at 490 nm. The measurements were converted to the concentration of oxygen used per dry weight of sediment during a given time interval. The blank was deducted from the average of the duplicates, and the result was used in the formula:

$$\text{ETS-activity } (\mu l\ O_2\ S^{-1}\ h^{-1}) = Abs^{490nm} \times V_r \times V_h \times 60 \times 1.30/(V_a \times S \times t \times 1.42)$$

Abs^{490nm} = sample absorption (duplicate average minus the blank)
V_r = final volume of reaction mixture (0.28 ml)
V_h = homogenate volume (2 ml)
V_a = volume of homogenate share in the sample (0.03 ml)
S = size of the sample (mass, g DW)
t = incubation time (30 min)
1.30 = length correction factor (Lampinen et al., 2012)
1.42 = factor for conversion of concentration of formazan to oxygen volume (Kenner and Ahmed, 1975).

2.7. Community-level physiological profiling (CLPP)

Community-level physiological profiling (CLPP) was carried out using the Biolog Ecoplates™ Assay (Biolog, California, USA), which provides information on community functioning. The assay was developed by Garland and Mills (1991) for soil samples and includes direct incubation of environmental samples in microtiter plates containing 31 different carbon sources, nutrients, and a redox dye. The assay enables fast characterization of microbial community metabolism by spectrophotometric measurement of the intensity of pre-prepared substrate utilization, which includes carbohydrates, polymers, carboxylic and ketonic acids, phenolic compounds, amino acids and amines/amides (Table 2). Among the polymer substrates on the plate are also synthetic polymers, such as Tween 40 and Tween 80. Increased utilization of these was previously shown to be typical for polluted environments (e.g., Sala, 2006), thus making them suitable indicators of pollution. After each sampling, 2 g of fresh sediment collected from the pockets exposed to different treatments (C, 1P, 5P) and from the six sites (P1–3; R1–3) (N = 18) was put into a marked sterilized glass beaker (100 ml) on an electro-balance (Sartorius BP 210 S, Germany) and 20 ml of chilled Ringer solution (Ringer solution ¼ strength tablets, Sigma-Aldrich; 1 tablet in 500 ml deionized water) was added. The beakers were transferred to an ultrasonic bath (Elmasonic *P*, Elma, Singen, Germany) for 1 min (37 kHz, 30%). Each beaker was gently shaken while the solution was poured into two tubes and centrifuged for 5 min at 800 rpm at 4 °C. Next, 150 μl of supernatant was transferred using multichannel pipettes to Ecoplates, and the absorbance of each well was measured using a spectrofluorometer SynergyMX (BioTek Instruments, USA) at 590 nm on the same day, and again, after 24, 48, and 72 h. Between the measurements, the plates were incubated in the dark at 20 °C. The Ecoplate consists of 96 wells divided into three groups, each containing one blank

Table 2
All 32 sources of carbon used in Ecoplates. Abbreviations indicate the labels used in Fig. 5 presenting heatmaps and Table 1 in Appendix A, displaying the results of SIMPER analysis.

Group of utilized substrates	Ecoplates label	Substrate	Abbreviation
Control	A1	water	wat
Carbohydrates	A2	beta-Methyl-D-Glucoside	BMDG
	B1	Pyruvic Acid Methly Ester	PAME
	B2	D-Xylose	Dxyl
	C2	i-Erythritol	iEry
	D2	D-Mannitol	DMan
	E2	N-Acetyl-D-Glucosamine	NADG
	G1	D-—Cellobiose	DCel
	G2	Glucose-1-Phosphate	Glu1P
	H1	alfa-D-Lactose	ADLa
	H2	D,L-alfa.Glycerol Phosphate	DLaGP
Polymers	C1	Tween 40	T40
	D1	Tween 80	T80
	E1	Alfa-Cyclodextrin	aCyc
	F1	Glycogen	Gly
Carboxylic and Ketonic Acids	A3	gama-Lactone	gLAC
	B3	D-Galacturonic Acid	DGalA
	E3	Gama- Hydroxy Butyric Acid	gHBA
	F2	D-Glucosaminic Acid	DGluA
	F3	Itaconic Acid	Ita
	G3	Alfa-Keto Butyric Acid	aKBA
	H3	D-Malic Acid	dMa
Phenolic compound	C3	2-Hydroxy Benzoic Acid	2HBA
	D3	4-Hydroxy Benzoic Acid	4HBA
Amino Acids	A4	L-Arginine	LArg
	B4	L-Asparagine	LAsp
	C4	L-Phenylalanine	LPhe
	D4	L-Serine	LSer
	E4	L-Threonine	LThe
	F4	Glycyl-L-glutamic Acid	GLGA
Amines/Amides	G4	Phenylethyl-amine	PheA
	H4	Putrescine	Put

well and 31 different carbon sources (Table 2), meaning the experiment is conducted in triplicate on one plate. Raw absorbance values measured for the substrate wells on each plate were corrected by the mean absorbance of the control wells (the three wells with no substrate). For CLPP, the substrate utilization metric measured after 72 h was used because most absorbance readings were below 2. According to Weber and Legge (2010), absorbance values taken later during the incubation can provide more information, but the values must not exceed 2, as higher values contribute to measurement error.

2.8. Isotopic analysis

The untreated (reference) PET fibers were compared to the PET fibers exposed to *in situ* conditions for a year. The PET fibers from the field were rinsed with deionized water to remove the majority of the organic matter, including the biofilm, so as not to interfere with the results.

About 0.5 mg of material was weighed in tin capsules. The isotopic composition of carbon ($\delta^{13}C$) was determined using a Europa 20–20 continuous flow IRMS ANCA-SL preparation module. The determination of isotopic composition followed combustion of the capsules in a hot furnace at 1000 °C. The products generated were reduced in a Cu tube (600 °C) where excess O_2 was absorbed. H_2O was trapped on a drying column composed of $MgClO_4$. gasses were separated on a chromatographic column and ionized. IAEA CH-3 (−24.724 ± 0.041), CH-7 (−32.151 ± 0.050) reference materials, and sugar with a value of −25.2‰ ± 0.2 as a working standard, were used to relate the analytical results to the VPDB standard.

2.9. Scanning electron microscopy (SEM)

The PET samples for scanning electron microscopy were coated with a 7 nm thick conductive Au-Pd layer using an ion-beam precision etching coating system (PECS 682, Gatan Inc. USA) and then observed at various magnifications in a field-emission gun scanning electron microscope (FEGSEM Verios G4, Thermo Fisher Scientific, USA) at SEM accelerating voltage of 5 kV. Multiple micrographs were taken. Due to limited publishing space, only the most relevant micrographs are shown.

2.10. Statistical analysis

The data from the first four dates (D1–D4) were used to investigate the biofilm colonization process (colonization) under the three different treatments (control (C), presence of low (1P), and high amounts (5P) of PET fibers in sediments). The data from the next four dates (D5–D8) were used to investigate whether there were any differences between the treatments over the four seasons (D5: autumn, D6: winter, D7: spring, D8: summer).

Two-way ANOVA with treatment (C, 1P, 5P) and date as factors was conducted on data for POM, TPC, and ETSA. Tukey's HSD (Honest Significant Difference) post hoc method was used to determine the significance of the factors and interactions. All data were transformed, using $\log_{10}(x+1)$ in order to achieve the ANOVA assumptions, and were analyzed separately for colonization and seasonal difference. All significance was $p < 0.05$ unless specified otherwise.

The CLPP absorbance data were transformed using $\log_{10}(x+1)$ and investigated using multivariate non-metric multidimensional scaling (NMDS) separately for colonization and the seasonal study. Bray-Curtis dissimilarity was used to calculate distance and visualize distribution patterns. The input data for the NMDS included the data on absorbance from all three treatments (C, 1P, 5P), separately for colonization and the seasonal study. However, due to the better visibility of variability between samples in metabolic functioning, the control, low, and high PET treatments are presented in three separate ordination diagrams for the colonization (Fig. 4a) and seasonal study (Fig. 4b), respectively. To test for significant differences between the dates and the differences between treatments, analysis of similarity (ANOSIM) was conducted. To search for the substrate utilization that contributed the most to the differences between groups (i.e., dates) for individual treatments, similarity percentage analysis (SIMPER) was used.

Heatmaps were constructed to demonstrate the intensity of individual substrate utilization under different treatments and dates. Heatmaps are graphical representations of data where the intensity of substrate utilization is depicted by color. A two-way ANOVA (treatment, date) and Tukey's HSD post hoc were performed on data for individual substrate utilization and groups of substrates (e.g., polymers, carbohydrates, etc.; Table 2). The same transformed data as for the CLPP analysis were used.

The analyzes were carried out using R studio software, R Core Team (2019), using packages such as tidyverse (Wickham et al., 2019), vegan (Jari Oksanen et al., 2020), and heatmaply (Galili et al., 2017). SIMPER was performed using PAST 3.22 (Hammer et al., 2001).

3. Results and discussion

3.1. Colonization patterns of river biofilms under different treatments (presence or absence of PET fibers)

The biofilm presence on artificial substrates (i.e., treated (1P, 5P) and untreated control (C) sediments), measured as total protein content (TPC) and microbial respiratory electron transport system activity (ETSA) over the first two weeks and until two months (D1–D4), could already be detected after one week (D1) of exposure to natural

Table 3
Significant results ($p < 0.05$), shown as asterisk (*), of two-way ANOVA for colonization (D0-D4) and seasonal (D5-D8) study with treatment (C, 1P, 5P) and date as factors and interaction between them (Interaction) for individual substrates and groups of substrates (italic).

Group of substrates/ individual substrate	D0-D4 Date	Treatment	Interaction	D5-D8 Date	Treatment	Interaction
water						
Carbohydrates	*	*		*		
beta-Methyl-D-Glucoside	*			*		
Pyruvic Acid Methly Ester	*		*	*		
D-Xylose	*	*	*	*		
i-Erythritol	*			*	*	
D-Mannitol	*					
N-Acetyl-D-Glucosamine				*		
D—Cellobiose	*			*	*	
Glucose-1-Phosphate	*			*		
alfa-D-Lactose	*					
D,L-alfa.Glycerol Phosphate	*			*		
Polymers	*				*	
Tween 40	*	*		*		
Tween 80	*			*	*	
Alfa-Cyclodextrin	*			*	*	
Glycogen	*					
Carboxylic and Ketonic acids	*	*	*			
gama-Lactone						
D-Galacturonic Acid	*			*	*	
Gama- Hydroxy Butyric Acid	*	*	*	*	*	
D-Glucosaminic Acid	*		*			
Itaconic Acid	*	*	*	*		
Alfa-Keto Butyric Acid	*			*		
D-Malic Acid	*			*		
Phenolic compound	*					
2-Hydroxy Benzoic Acid	*					
4-Hydroxy Benzoic Acid	*			*		
Amino Acids	*			*		
L-Arginine	*	*	*		*	
L-Asparagine	*	*	*		*	
L-Phenylalanine	*			*		
L-Serine	*	*	*	*		
L-Threonine	*			*		
Glycyl-L-glutamic Acid	*		*	*	*	
Amines/Amides	*			*	*	
Phenylethyl-amine	*				*	
Putrescine	*			*		

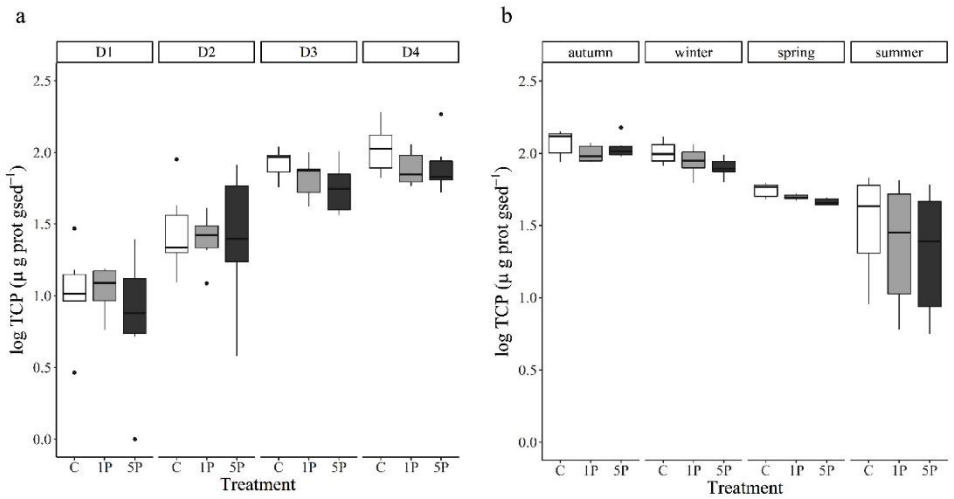

Fig. 2. Boxplots of TPC (total protein content) in a) colonization (D1: one week, D2: two weeks, D3: a month, D4: two months) and b) seasonal (autumn 2019, winter 2019, spring 2020, summer 2020) study for different treatments (C: control, 1P: low PET, 5P: high PET).

conditions (Figs. 2a and 3a), hinting that microbial colonization is fast in riverbed sediments. No significant differences were observed between treatments (C, 1P, 5P), while the date (D1–D4) was a statistically significant factor (TPC; two-way ANOVA, $F = 48.381$, $p < 0.001$; ETSA; two-way ANOVA, $F = 41.915$, $p < 0.001$). Differences were significant between all date combinations for both TPC and ETSA except for the last two dates (D3, D4; Tukey's post hoc test). However, despite the insignificant differences between treatments, generally lower mean TPC and ETSA were observed on the sediments impacted by both low (1P) and high amounts (5P) of PET fibers on all sampling dates (Figs. 2a and 3a).

The lower total protein content (TPC) and respiratory electron transport system activity (ETSA) observed on all dates in the presence of PET fibers indicated that PET possibly suppresses both the process of biofilm colonization and the development of mature biofilms. A measurement of respiratory electron transport system activity, ETSA is an ecological factor that is well studied in aquatic ecosystems. It is relatively simple to measure and is sensitive to ecosystem stress (Simčič et al., 2015). It has been used to highlight the importance of stressors, such as temperature and increased nutrient loads, on microbial respiration in the hyporheic zone (Debeljak et al., 2017; Mori et al., 2018), and it would seem that it also responds to stress due to PET pollution. During the presence of plastic pollution, substances can be released that have toxic effects on microorganisms, enabling only a few adapted bacteria to grow in their presence (Li et al., 2020), or such substances can have a general inhibitory effect on the respiratory activity of microorganisms. Since antimicrobial additives are often used in the production of polyester (PET) fibers (Al-Balakocy and Shalaby, 2017), this may have influenced the microbial community in this study.

The comparison of microbial community functioning (i.e., CLPP) between different treatments (C, 1P, 5P) during colonization (D1–D4) using NMDS (Fig. 4a, stress = 0.1159) indicated that the presence of PET suppressed shifts in microbial community functioning during colonization. The differences between samples from different dates were smaller under the presence of PET in comparison to the controls, especially in the case of high PET presence. However, the analysis of similarity (ANOSIM), which tested for significant differences in microbial functioning between dates and treatments, revealed the importance of the dates (D1–D4) (R: 0.3905; $p < 0.05$), but not of the treatments (R: 0.0203; $p = 0.102$) for the variability in microbial functioning. Still, differences between dates for the individual treatments were bigger for the control (ANOSIM; R: 0.7233, $p < 0.001$) relative to the treatments (ANOSIM 1P, R:0.5579, $p < 0.001$ and 5P R: 0.2523, $p < 0.001$), suggesting the suppression of functional shifts in microbial communities during colonization in the presence of PET fibers. The SIMPER analysis indicated that the substrates that contributed the most to the differences between the sample date were similar in the control and treatment 1P and included itaconic acid, gamma-hydroxybutyric acid, and L-phenylalanine while under treatment 5P, the utilization of L-phenylalanine, 2-hydroxybenzoic acid, and alfa-cyclodextrin contributed the most to the variability between the sample date. The former two substrates utilization was substantially lower in treatment 5P, the utilization of L-phenylalanine was slightly higher under treatment 5P, while the last two substrates were substantially more intensively utilized under treatment 5P (Appendix A). Itaconic acid is an ingredient known in medicine as salicylic acid (NCBI, 2021a). Phenylalanine is an essential aromatic amino acid for humans and is important in the structure and function of many proteins and enzymes. Its L-form is incorporated into proteins (NCBI, 2021b). Alfa-cyclodextrin is one of the most common natural cyclodextrins (Gidwani and Vyas, 2015), with high water solubility, the ability to form complexes, and relatively resistant to enzymatic hydrolysis. Its potential applications are increasing, especially in the food industry (Li et al., 2014).

The community composition and functioning of stream biofilms are driven by an array of environmental factors that are usually highly interconnected. In response to the physical and chemical structure of the streambed environment with its variability, biofilms adapt and evolve, which may lead to changes in biofilm carbon metabolism (Battin et al., 2016), but only to a certain extent. Certain previous studies suggest that plastics have an inhibitory effect on stream biofilms. Miao et al. (2019) compared natural and MP (PE and PP) substrates in terms of community structure and microbial function. Their results suggest that although MP serves as a new substrate for microbial colonization, it decreases the richness and diversity of biofilms compared to those inhabiting natural substrates. MP probably alters microbial survival strategies and negatively affects their ecological functions and other biogeochemical processes. Further, Miao et al. (2021) show that biofilms on PET have lower

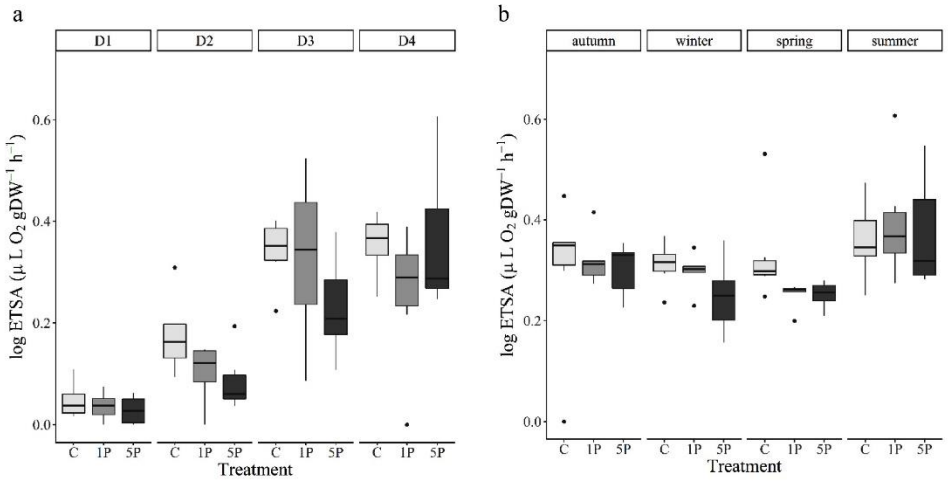

Fig. 3. Boxplots of ETSA (respiratory electron transport system activity) in a) colonization (D1: one week, D2: two weeks, D3: a month, D4: two months) and b) seasonal (autumn 2019, winter 2019, spring 2020, summer 2020) study for different treatments (C: control, 1P: low PET, 5P: high PET).

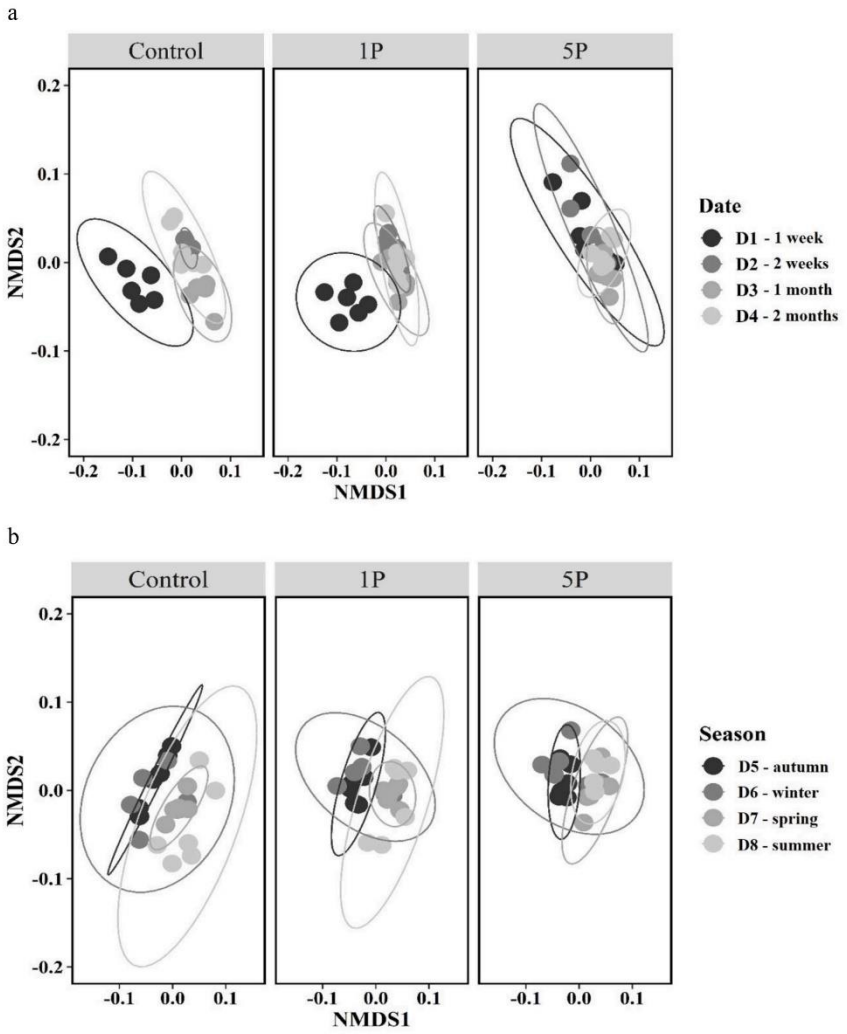

Fig. 4. Ordination diagrams of NMDS based on data on substrate utilization (Biolog Ecoplates) during colonization (a; stress = 0.1195) and seasonal (b; stress = 0.1945) studies and during different treatments: control (C), low plastic amount (1P) and high plastic amount (5P). The distances were determined using Bray-Curtis dissimilarity calculation.

capacities and carbon metabolism rates and the lowest diversity compared to those on glass or PVC. Li et al. (2020) demonstrated that MP reduced the α-diversity of microorganisms and had a significant effect on the structure of microbial communities. Additionally, Li et al. (2020) demonstrated differences in microbial diversity, community structure, and functioning when comparing sediments with low or high amounts of MP. Seeley et al. (2020) assessed bacterial community diversity based on MiSeq sequencing of 16S rRNA genes. Communities exposed to PE and PUF expressed the most variation over time, as opposed to the control and PLA communities, which exhibited minimal changes over time. A study by Niu et al. (2021) showed a significant difference between bacterial communities from MPs (sieved from river sediments) and sediments.

Using absorbance readings from Ecoplates, a comparison of the individual substrate utilization during colonization and between treatments and dates was visualized using heatmaps (Fig. 5a). Under PET stress, some substrates were poorly utilized in comparison to the control. One such case was carbohydrate D-xylose, indicating that PET presence

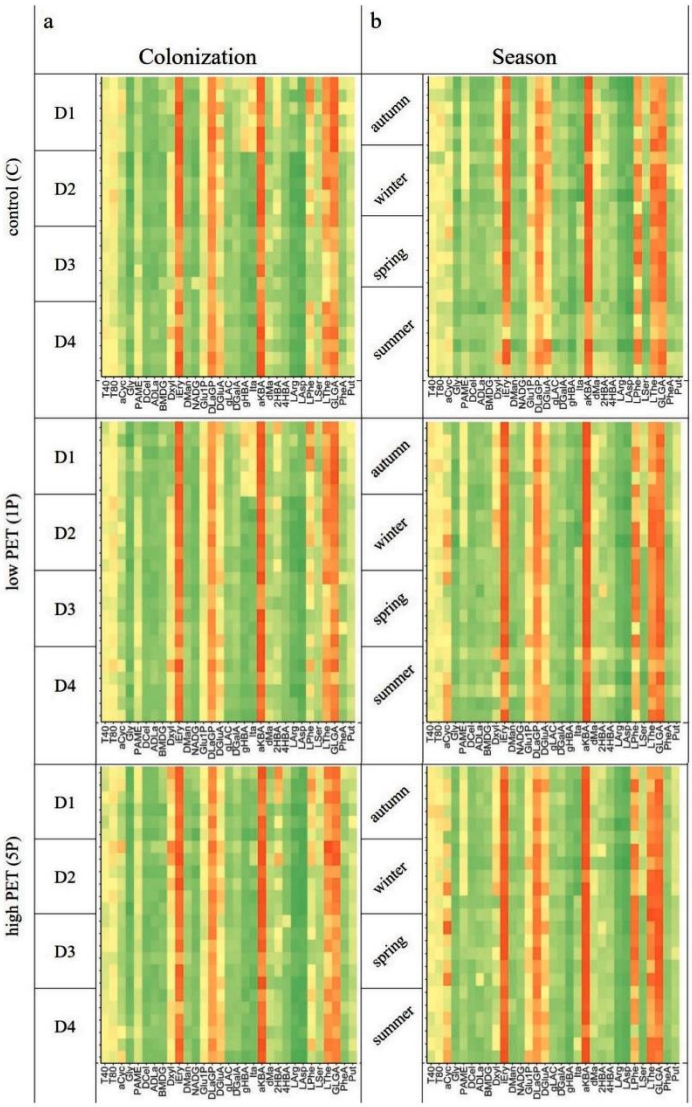

Fig. 5. Heatmaps indicating the intensity of substrate utilization of samples collected during colonization and seasonal studies and during different treatments: control (C), low PET amount (1P) and high PET amount (5P). The x-axis list 31 substrates (A2 – H4; abbreviations explained in Table 2) and the y-axis the samples, six replicates (P1–3, R1–3) for each date (colonization study: D1: one week, D2: two weeks, D3: a month, D4: two months; seasonal study: autumn 2019, winter 2019, spring 2020, summer 2020). The darkest green color indicates the log maximal substrate utilization (abs_{max} = 0.5) and red the minimal substrate utilization (abs_{min} = 0), both expressed as absorbance (abs), measured spectrofotometrically. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

could suppress the capacity of the microbial community for the degradation of allochthonous wood or plant debris containing xylose. In contrast, other substrates such as Tween 40, Tween 80 and alpha-cyclodextrin were utilized more intensely under PET stress.

Utilization of the substrates pyruvic acid methly ester, D-glucosaminic acid, and glycyl-L-glutamic acid changed significantly over the dates, and the interaction between date and treatment was significant (two-way ANOVA). The intensity of utilization of substrate D-xylose, gamma- hydroxybutyric acid, itaconic acid, L-arginine, L-asparagine and L-serine changed significantly with date and treatment and when in interaction, while utilization of Tween 40 significantly depended on the type of treatment and was more intense in the presence of higher concentrations of PET (Tukey's post hoc, $p < 0.001$). The polymers included in Ecoplates, Tween 40 (C1), Tween 80 (D1), alfa-cyclodextrin (E1), and glycogen (F1), are complex carbon substrates. Utilization of complex polymers (Tween 40) has previously been linked to polluted

environments (Sala et al., 2006; Oest et al., 2018).

Generally, significant differences between treatments were observed for carbohydrates (two-way ANOVA, $F = 3.602$, $p < 0.05$) and carboxylic and ketonic acids (two-way ANOVA, $F = 5.132$, $p < 0.01$), while carbohydrates and the other four substrate groups (polymers, phenolic compounds, amino acids, amines/amides) showed significant differences between dates (Table 3). In addition, for carboxylic and ketonic acids, date (two-way ANOVA, $F = 27.975$, $p < 0.001$) and interaction between date and treatment (two-way ANOVA, $F = 12.756$, $p < 0.001$) proved significant. The most significant difference in the degradation of carboxylic and ketonic acids was between the control and treatment 5P ($p < 0.01$). A large quantity of organic matter and nutrients is delivered to the hyporheic zone by river surface water penetrating the streambed where it becomes trapped and is further decomposed by microbial communities (Krause et al., 2011; Zhou et al., 2014). In general, a high consumption of carbohydrates and carboxylic acids in freshwater sediments has been reported by multiple studies (Oest et al., 2018; Melita et al., 2019; Miao et al., 2019). Carbohydrates and carboxylic and ketonic acids are important carbon sources for aquatic microorganisms (Sala et al., 2006; Arnosti et al., 2014). Studies consistently report that the presence of plastic lowers the biodiversity of microbial communities (Miao et al., 2019; Li et al., 2020; Seeley et al., 2020; Miao et al., 2021), and this was also indicated in our colonization study.

3.2. Seasonal dynamics of biofilms under different treatments (the presence or absence of pet fibers)

River systems are highly dynamic environments, driven by climate and hydrogeological characteristics and impacted by an array of anthropogenic pressures. The river studied has a strong seasonal pattern for discharge rates and water temperatures (Fig. 1). This is also reflected in the water physico-chemistry (Table 1) and consequently also in the biofilm structure and function (Figs. 2b and 3b). The highest mean value for TPC was in the autumn, and then it gradually decreased, exhibiting the lowest values but highest spatial variability in the summer. Microbial activity, measured as ETSA, was highest in the summer when the variability within the streambed was also the highest.

For both the TPC and ETSA, the season was a significant factor (TPC; two-way ANOVA, $F = 31.886$, $p < 0.001$; ETSA; two-way ANOVA, $F = 5.178$, $p < 0.01$). While both ETSA ($p < 0.01$) and TPC ($p < 0.001$) were significantly different between summer and winter or spring, the TPC in summer was also significantly different from that in autumn, while there were also significant differences between autumn and spring and between spring and winter. The key driver of microbial activity in riverbed sediments is temperature, but nutrients and organic carbon are also important for the general functional response of the hyporheic biofilm (Debeljak et al., 2017; Mori et al., 2018). Seasonal changes in temperature, discharge, and water chemistry were most probably driving the seasonal patterns observed in TPC and ETSA in the control sediments in this study. The differences in TPC and ETSA between the control sediments and the sediments under PET pollution were insignificant, but generally, lower means for TPC and ETSA were observed in the sediments impacted by the PET fibers (1P, 5P) during all four seasons, suggesting, as in the colonization study, that the presence of PET fibers suppresses the biofilm biomass and respiratory potential throughout the year, the least in autumn and the most in summer.

Using CLPP for a comparison of microbial functioning between seasons and treatments also revealed that the presence of PET suppressed the seasonal dynamics. NMDS ordination plots (Fig. 4b; stress = 0.1944) indicate differences in microbial functioning between the control and treatments (1P and 5P). However, the microbial utilization of substrates differed significantly between seasons (ANOSIM; R: 0.4214; $p < 0.001$), but not between treatments (ANOSIM; R: 0.0181; $p = 0.1697$). These findings suggest that biofilms growing in the presence of a large quantity of PET fibers are less susceptible to seasonal changes than those in a control treatment. The three substrates that contributed the most to the differences between date samples in the control were D-xylose, L-phenylalanine, and itaconic acid, while in the samples under the PET fiber treatments (1P and 5P), alfa-cyclodextrin was the most intensively used substrate rather than itaconic acid. In addition, alfa-cyclodextrin was among those substrates contributing most to the differences between the control and the treatments (both 1P and 5P) (Appendix A, Table 1), and thus one of the four polymers contributing the most to the differences between seasons. This contribution was highest in the presence of the larger quantity of PET fibers (8.9% versus 4.2% (C) and 7.6% (1P)). Additionally, glucose-1-phosphate was present in the SIMPER results for the first 50% of the substrates, thus contributing to differences between the samples for both treatments (1P, 5P), but not for the control. This last substrate is a component in glycogen metabolism, explaining the lower contribution of glycogen in both instances (1.9% (1P) and 1.4% (5P), versus 2.4% in the control) as this was probably used as a carbon source and suggests a shift in the metabolic profile of the microbial community. Glycogen is a branched polymer of glucose, acting as its readily mobilized storage form for numerous organisms (Berg et al., 2002).

Investigation of the heatmaps presenting substrate utilization using Ecoplates absorbance readings (Fig. 5b), indicated either more intensive utilization of carbon sources (dark green) or none at all (dark red) in the treatment with the higher quantity of PET. This could be an indication that the community is shifting toward more specialized metabolism and therefore exhibits lower adaptability to newly available substrates (Melita et al., 2019).

When observing the separate utilization of substrates during the seasonal study, L-arginine, L-asparagine and phenylethyl-amine were significantly dependent on the type of treatment and were utilized more intensely in the presence of the higher amount of PET fibers (Tukey's post hoc, $p < 0.05$). Utilization of substrates D-cellobiose, i-erythritol, D-galacturonic acid, gamma-hydroxybutyric acids, glycyl-L-glutamic acids, Tween 80, and alfa-cyclodextrin were all affected by both date and treatment. Both polymers, Tween 80 (Tukey post hoc, $p < 0.05$) and alfa-cyclodextrin (Tukey's post hoc, $p < 0.01$), were utilized more intensely in the presence of higher amounts of PET fibers.

Significant differences between treatments were observed for the polymer group (two-way ANOVA, $F = 3.642$, $p < 0.05$) between the control and the highest presence of PET fibers (Tukey's post hoc, $p < 0.05$). Additionally, the amines/amides group showed significant differences between dates and treatments. Carbohydrates and amino acids showed significant difference between seasons, while the other two, carboxylic and ketonic acids and phenolic compound, showed no significance whatsoever (Table 3). After a year of exposure to PET fibers, bacterial communities had started to utilize the synthetic polymer (Tween 80). This indicates a shift in metabolic functioning and suggests that the community could adapt to utilizing synthetic polymers. The significance of the amines/amides group could indicate low nitrogen conditions and the presence of opportunistic classes of bacteria (Oest et al., 2018; Seeley et al., 2020).

3.3. Observed changes in PET fibers and $\delta^{13}C$ after one year of in situ exposure

The surface of untreated, reference PET fibers is smooth with occasional small bumps or irregularities (presumably arising during manufacture), without visible surface overgrowth or deterioration (Fig. 6). Despite washing with deionized water, diverse microorganisms, probably fungal hyphae and bacilli, were observed on the PET fibers exposed to *in situ* conditions for one year. This confirms that microorganisms in hyporheic sediments overgrow not only sediments but also PET fibers.

While the SEM micrographs indisputably indicate a diverse biofilm, they do not provide clear evidence about possible surface deterioration due to biodegradation or utilization of the plastic. Lucas et al. (2008) suggests that the isotopic composition of carbon can provide reliable information regarding microbial degradation of experimental plastics.

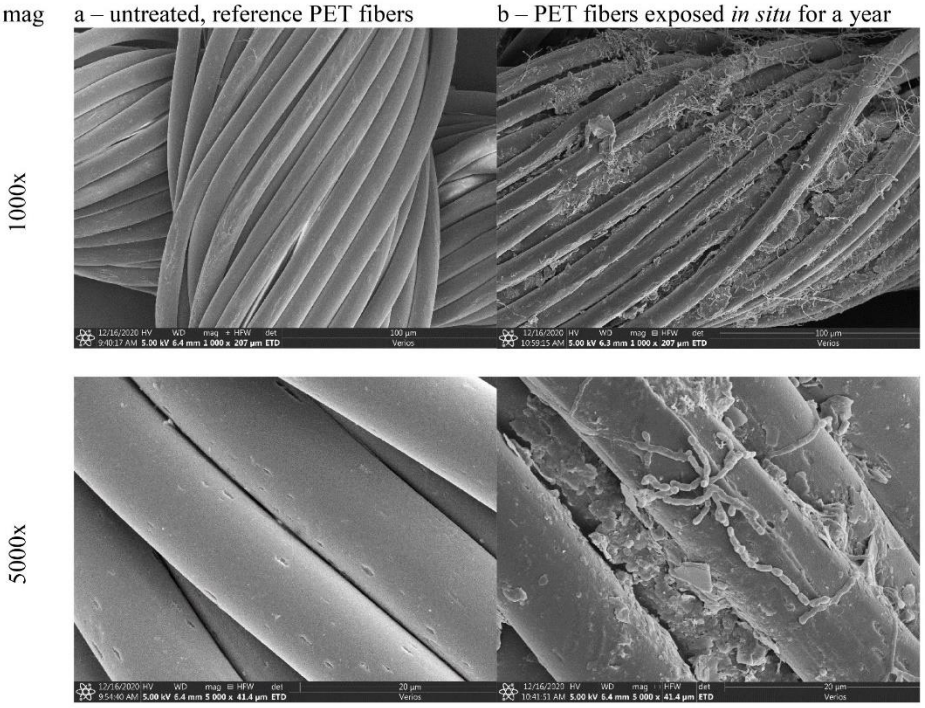

Fig. 6. SEM micrographs of untreated, reference PET fibres (a) and PET fibres exposed to natural weathering and microbes in riverbed sediments for a year (b).

IRMS (isotope ratio mass spectrometry) results show a difference between $\delta^{13}C$ values for plant-derived ("BIO" bag; $-25.3 \pm 0.7‰$; $n = 13$) and petroleum-based polymers (PET; $-27.8 \pm 1.7‰$; $n = 7$) (Berto et al., 2017). The study by Lucas et al. (2008) also revealed differences between plastic items composed of the same polymer but from different countries, and between some recycled and nano-recycled plastics. Furthermore, increased $\delta^{13}C$ were observed after exposure to UV light (Birch et al., 2021). In an earlier study by Matjašič et al. (2020), the isotopic composition of carbon ($\delta^{13}C$) was determined for different materials (PET textile, PET bottle, and HDPE bag). The study confirmed the ability of microorganisms from activated sludge to colonize plastics in an environment without other carbon sources. The value of $\delta^{13}C$ was slightly increased in all tested materials compared to the source material, suggesting a certain level of degradation. In this study, the value of untreated PET fibers was determined to be $-28.9 \pm 0.1‰$ and the average value for rinsed samples was $-28.6 \pm 0.2‰$ ($\varepsilon = 0.3$).

On performing an isotopic analysis, we found, that while not significant, the average values of treated samples were higher than those of untreated fabric. The shift in $\delta^{13}C$ could be due to either physical/chemical or biological degradation, although determination of the degradation rate was not possible. Biological degradation could occur due to the adhered biofilm, as seen in the SEM micrographs (Fig. 6).

4. Conclusion

The present study is one of the few studies investigating colonization patterns and seasonal dynamics of biofilm activity *in situ* riverbed sediments in the presence of PET fibers. We found that:

- During colonization and biofilm formation, the biofilm biomass (measured as TPC) and microbial activity (measured as ETSA) were slightly suppressed by the presence of PET, while metabolic functioning, measured as CLPP, differed between treatments due to different intensities in their use of 2-hydroxybenzoic acid (i.e., phenolic compound) and alfa-cyclodextrin (i.e., polymer).
- The presence of PET fibers suppressed biomass and microbial activity over the four seasons and suppressed the seasonal dynamics in microbial functioning.
- In both colonization and seasonal studies, utilization of synthetic polymer substrates Tween 40 (colonization) and Tween 80 (seasonal) was significantly higher in sediments polluted with PET fibers.
- Little surface deterioration of PET fibers was observed after one year of exposure to *in situ* conditions in riverbed sediments, suggesting PET biodegradation in the hyporheic zone takes a considerably longer time.

Further work should be conducted to understand better how the presence of PET and other plastic micropollutants affects the biogeochemical cycle and self-cleaning ability of rivers—a function of the hyporheic microbial community. To deepen the understanding of metabolic processes, wider studies with more rivers of varying hydrology and geomorphology should be carried out using a combination of controlled laboratory and mesocosm studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117455.

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3.3.2 Scientific article in preparation: "Hyporheic microbial community structure and functioning during 2-year exposure to pollution with polyethylene terephthalate (PET) fibres"

Introduction

The accumulation of plastic debris and smaller particles (i.e., MPs and NPs) in freshwater environments has become a pressing global concern (Zhang et al., 2022a; Zhang et al., 2022c; Ziani et al., 2023). One of the frequent MP pollutants in rivers and streams is polyethylene terephthalate (PET), predominately as fibres (Li et al., 2018; Matjašič et al., 2021b). These synthetic fibres, derived from various sources such as textiles and packaging materials, have the potential to persist in freshwater ecosystems and pose an unknown ecological risk.

The known toxic effects of microplastics (MPs) on aquatic organisms are greatly influenced by their size, shape, and chemical composition. These factors contribute to a range of documented impacts, including neurotoxicity, reproductive toxicity, oxidative stress, immunotoxicity, and reduced photosynthetic efficiency of aquatic organisms (Miloloža et al., 2021). Both MPs and NPs have been shown to affect freshwater macrophytes, leading to growth inhibition and root shortening. These particles also impact phytoplankton by affecting growth, photosynthetic efficiency, metabolic processes, and weakening membrane permeability, as summarised in a comprehensive review by Castro-Castellon et al. (2022). Moreover, this review provides an inclusive list of studies evaluating MP toxicity in planktonic and benthic consumers.

The list offers detailed insights into various physiological and behavioural changes from MP exposure. These changes can have far-reaching effects on ecosystem dynamics, as invertebrates and microorganisms play crucial roles in driving primary and secondary production within streams. However, the results were obtained from studies conducted in artificial streams such as mesocosms or laboratory settings. Furthermore, Schell et al. (2022) discovered a significant negative impact on the reproduction and survival of *Daphnia magna* caused by MP fibres due to entanglement and limited mobility.

Detritivorous shredders, such as amphipods, adapted to feed on non-digestible material, might exhibit reduced susceptibility to MP exposure, as suggested by a study on amphipod *Gammarus pulex* (Weber et al., 2018). PET particles could also pose a potential silent threat to benthic invertebrates through oxidative stress, despite a study on amphipod *Hyalella azteca*, where PET did not significantly affect their mortality rate (Queiroz et al., 2023).

Moreover, PET MPs have been shown to affect the digestive system of Gastropoda and cause villi damage in gastrointestinal walls (Song et al., 2019). Additionally, MP pollution has been observed in insects. For example, PET microfibers have been linked to decreased growth rates in the cricket species (Fudlosid et al., 2022), and a mixture containing mainly PET was linked to deformities in Diptera species (Stanković et al., 2020; Zhu et al., 2023).

Furthermore, it has been determined that MP PET particles induce increased CO_2 emissions from 7 to 30 days after entering freshwater sediment (Zhang et al., 2022b), thus directly affecting ecosystem processes. However, there is still a need for a deeper understanding of the impacts of PET MPs in complex and dynamic natural environments, such as hyporheic zones, where microplastic pollution is just one of several stressors shaping the biota, mostly microorganisms and invertebrates and ecosystem processes.

Flowing waters and their HZs are subjected to spatial (e.g., hydrogeomorphology, temperature, organic matter and nutrient inputs) and seasonal variability in natural factors (e.g., discharge, temperature, organic matter and nutrient inputs) and exposed to an array

of stressors, from climate change and both organic and inorganic point and non-point pollution to modifications in hydrogeomorphology and shifts in catchment land use (Ponsatí et al., 2016; Mori et al., 2018; Romero et al., 2018; Romero et al., 2019). Climate change affects the water bodies' hydrological and temperature patterns, brought about by alterations in precipitation distribution and rise in global air temperatures (Pörtner, 2022). With decreasing precipitation due to climate change (Pörtner, 2022), occurrences of non-flow episodes are becoming more frequent and enduring (Papadaki et al., 2016; Pumo et al., 2016; Skoulikidis et al., 2017), which in turn holds substantial implications, even for perennial water bodies (Döll & Schmied, 2012). Consequently, biofilms in perennial streams are growing increasingly vulnerable to shifts in environmental conditions caused by climate-induced weather changes and precipitation patterns (Colls et al., 2019). Furthermore, these biofilms display diminished resistance to non-flow circumstances, resulting in decreased structural resilience and reduced variability.

This impact does not appear to be mirrored at a functional level. It seems that biofilms adapted to drought conditions exhibit more robust responses to such circumstances (Timoner et al., 2020). Another consequence of shifting precipitation patterns and escalating temperatures is the connectivity disruption among water bodies. Stream networks constitute a vital element of the hydrological system, serving as the physical underpinning for hydrological processes, water storage and conveyance, aquatic habitats and human water requirements. Additionally, they play a pivotal role in maintaining water quality, water balance, water utilization and the integrity of aquatic ecosystems (Cabezas et al., 2011). Urbanization and land use changes frequently lead to the alteration or complete removal of stream networks in urbanised regions (Gao et al., 2020). In addition to these hydromorphological pressures, urbanization leads to increased input of nutrients, organic matter and MPs to the streams, primarily through surface runoff and WWTP effluents.

Besides anthropogenic pressures and natural variability in hydrogeomorphology and temperature regimes, streams undergo seasonal changes in the quantity and quality of organic substrates originating from various sources, such as leaf litter and surface runoff (McDowell & Fisher, 1976), thus affecting biofilm structure and functioning that can significantly influence ecosystem functioning (Sabater et al., 2002; Battin et al., 2003; Docherty et al., 2006; Martínez et al., 2017). The quality and amount of organic matter also affect microbial activity and strongly correlate to bacterial abundance and production (Fischer et al., 2002b).

In aquatic environments, organic matter can be categorised into two main types based on its size: dissolved organic matter (DOM) and particulate organic matter (POM). POM found in water systems can undergo different processes to become DOM, depending on internal (source, size) factors of POM and external (photochemistry, biology) effects on the POM (Lee et al., 2023). Further classification of POM is based on its source, with allochthonous POM originating from external sources outside the aquatic system and autochthonous POM produced within the aquatic system by primary producers (Langhans et al., 2013).

Considering natural variability and stressor interactions is important to understanding the ecological effects. For example, nutrient enrichment exhibits a higher occurrence of synergistic interactions, influencing biofilms when the flow velocity is reduced, or sediment is added (Juvigny-Khenafou et al., 2021). Studies have estimated that nutrient stress was involved in 71% to 98 % of multi-stress situations and 42 % of those in groundwaters (Nõges et al., 2016). Moreover, a short-term (4 h) drought increased the susceptibility of biofilms to herbicide, while the effects of herbicide were less pronounced in biofilms exposed to higher temperatures (Romero et al., 2018). Additionally, the positive effect of higher temperatures on dried leaf biomass loss was weaker when buried under sediment (Piggott

et al., 2015). These findings highlight the complex relationships between changing water regimes, nutrient enrichment, microbial responses, and ecosystem functioning in freshwaters (Greenwood et al., 2007; Segner et al., 2014). Understanding these connections is crucial for effectively managing and conserving freshwater ecosystems.

Long-term field studies and studies using artificial substrates offer a valuable approach to partially control the environmental drivers, such as MP pollution, and simultaneously observe spatial and seasonal patterns (Bonzini et al., 2008; Burrows et al., 2017). These studies are scarce but vital as they consider the time component, a fundamental aspect of the four-dimensional concept of lotic ecosystems (Ward, 1989).

Several long-term field studies have investigated the effects of different variables on freshwater sediment biofilms. For instance, Ackermann et al. (2011) conducted a 15-month study examining the dynamics of benthic biofilms in River Rhine, Germany. They observed a successional pattern of the total biovolume, including a summer depression in June, a plateau in autumn, a decrease in winter, and a significant increase in the following spring warming. Yan et al. (2021) collected groundwater samples over 75 months at 4-week intervals to explore the temporal dynamics of the bacterial communities using DNA extractions and hydrochemistry. They reported on community succession, noting that the observed patterns differed between groundwater, surface freshwaters, and oceans. Gautam et al. (2021) collected biofilm samples from submerged rocks in six streams over 30 months. They used PCR amplification and sequencing to determine the relative importance of spatial versus temporal factors for microbial community composition. Their findings indicated that temporal changes have a greater impact than spatial variability.

In another study conducted in France, a shorter 9-month investigation validated a pollution-induced community tolerance (PICT) approach to assess spatiotemporal variation in herbicide (diuron) contamination and its ecological impact in a polluted river. The study found that the mean *in situ* diuron exposure level during biofilm colonization periods was the main factor explaining the variation in diuron sensitivity. In Hill et al. (2012) study, the authors compared water and sediment chemistry and land use of more than 2100 streams in the USA. They found that catchment land cover impacted stream chemistry, microbial enzyme activities and assemblages. Moreover, they found that P and N seem to be limiting factors in streams and that elemental ratios govern nutrient retention.

Although the studies above examined spatiotemporal patterns, they mainly utilised DNA-based methodologies, not functional measures for biofilm characterization or focused on benthic biofilms, including photoautotrophs. It is important to note that photoautotrophs are typically absent in hyporheic biofilms. Although becoming more affordable, DNA methods still require significant time and expertise for data interpretation. Alternatively, analysing microbial activities, including community-level physiological profiling (CLPP), can provide a quicker and easier method of assessing the ecological health of a freshwater system (Weber & Legge, 2010). In this particular study, our main objective was to investigate spatiotemporal patterns in hyporheic biofilm functioning, specifically in the presence or absence of PET pollution, across a range of ecohydrologically diverse rivers (i.e., pre-alpine, karstic rivers), with a focus on community metabolic activity.

Aims and objectives

The overall aim of this field experiment using artificial substrates was to investigate the impact of PET fibres on hyporheic biofilm structure and functioning across eco-geomorphologically distinct river systems over two years. The biofilm growth and overall fitness were assessed in multiple ways, such as changes in total microbial biomass (TPC) and their metabolic functioning (ETSA, CLPP). In the study, I explored the adaptability of biofilms, compared the composition, functionality and overall fitness, and explored the

influence of seasonal variation, considering the effects of temperature, water availability and nutrient fluctuations in the different river systems. Finally, the structural changes and damages on PET surfaces during the two years were examined using scanning electron microscopy (SEM) and stable isotope analyses of 13C were conducted.

Hypothesis

1. PET fibres in river ecosystems' HZ inhibit biofilm growth and alter metabolism. Adaptable biofilms will thrive, reducing biodiversity. This results in decreased microbial biomass (TPC), metabolic activity (ETSA), and altered profiles (CLPP). Adaptable biofilm will dominate over less adaptable.
2. The pollution of sediments with PET fibres will significantly impact biofilm structure and function across eco-geomorphologically distinct study river sites exposed to different anthropogenic pressures.
3. PET fibres will inhibit biofilm biomass and metabolic pathways among these rivers. The inhibition rate (higher or lower) will depend on the difference in pollution and the plastic burden of the rivers.
4. Seasonal variations will influence the response of river biofilms in the presence of PET fibres. The effects of PET on biofilm growth, composition, and metabolic activity will differ between seasons due to varying environmental conditions. Specifically, greater inhibition of biofilm growth and changes in metabolic pathways during seasons characterised by higher temperatures, reduced water availability (drought), and increased nutrient availability are expected (i.e., in summer).
5. The isotopic composition of the PET sheets will remain relatively stable, reflecting limited nutrient exchange between the biofilms and PET surfaces. However, scanning electron microscopy (SEM) of the PET sheets will reveal visible damages and alteration resulting from biofilm colonization. Physical evidence of biofilm attachment and potential biofilm-mediated degradation mechanisms are expected, leading to visible structural changes and damage on the PET surfaces after 2-years.

Methodology

Study area

The field study was conducted within the catchments of the Kamniška Bistrica and Ljubljanica Rivers, nearby and within the city of Ljubljana, in central Slovenia (Figure 7). The Kamniška Bistrica originates in the southern Kamniško-Savinjske Alps at an elevation of 630 m. It flows for 33 km before joining the Sava River as a left tributary. This pre-alpine river's catchment area spans 534 km^2 and is in the north-central Slovenia. The upper reaches of the river, located in the alpine region, are surrounded by forests and characterised by limestone and dolomite formations.

In contrast, the lower part of the river contains alluvial deposits and is influenced by various clastic rocks such as tuff, sandstone, conglomerate, and more. The Ljubljanica River is a typical karstic river renowned for its karst formations and features. It undergoes sinking and re-emergence in several places, which has led to its seven different names. After the last emergence, the river stretches for 41 km in the south-central part of Slovenia, with an estimated catchment of approximately 787 km^2. One of the left tributaries of the Ljubljanica River, the Gradaščica River, joins the Ljubljanica River within the city of Ljubljana and is 34 km in length. The catchment area of the Ljubljanica and Gradaščica Rivers encompasses both urban and rural landscapes, and both have undergone extensive regulation and channelization to manage flood risks and accommodate urban infrastructure within the city of Ljubljana and its surroundings.

In situ experimental design

For the study, four study sites were selected on three rivers: a reference site, Stahovica (KBR); an impacted site, Beričevo (KBB) on the Kamniška Bistrica River; a site on the Ljubljanica River below the city of the Ljubljana (LJU), and a site on the Gradaščica river within the city of Ljubljana (GRA) (Table 3, Figure 5). Three site replicates were selected to cover reach scale spatial heterogeneity at each study site. At each site, artificial substrates were inserted into the riverbed sediments, containing a bag with control substrates (13 pockets containing only sterilized sediments of size > 4 mm) and a bag with treated substrates (13 pockets containing sterilised sediments of size > 4 mm and five cloths of pre-treated PET fabric of the size 12 x 12 cm; the mass ratio: 70 g sediments vs. 5 g PET fibres) (Figure 4).

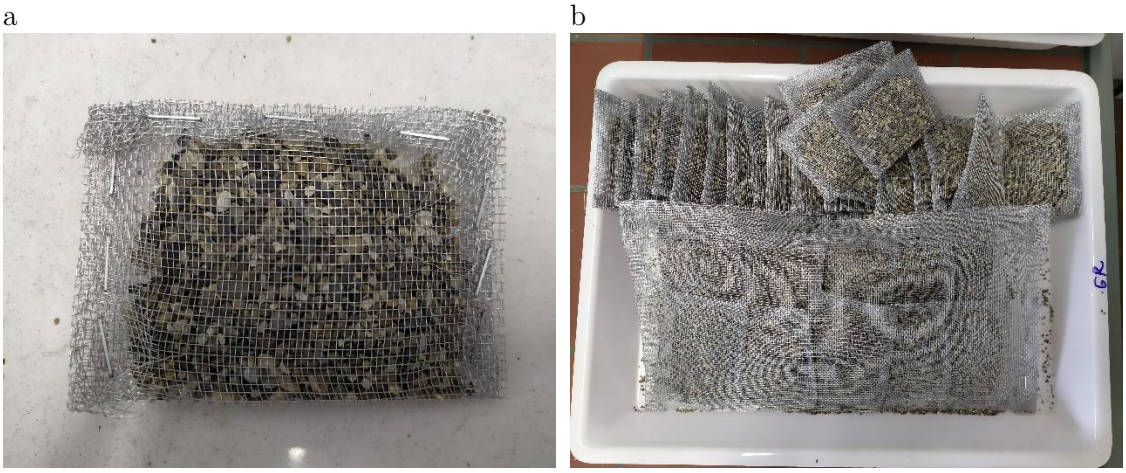


Figure 4: Photos showing control pockets (a) and control pockets in bags (b).

At site KBB, six spatial replicates were set to observe the detailed spatial heterogeneity in the biofilm response to PET pollution. Temperature data loggers (Vemco, Canada) were buried under the artificial substrates. The artificial substrates were buried in the sediments at a depth of 20 to 40 cm on the 26th of August 2019. The samples for the laboratory analyses were first collected after two months. Subsequently, the samples were collected regularly during each season until the summer of 2021 (Table 2). The hydrological data (discharge) and temperature data (river water) were obtained for the study period from the Environmental Agency of the Republic of Slovenia (Figure 6).

Table 2: Sampling dates with associated code and season.

Season	**Sampling date**
Summer	2.9.2019
Autumn	21 – 22.10.2019
Winter	27-28.01.2020
Spring	15-29.6.2020
Summer	31.8-1.9.2020
Autumn	9-10.11.2020
Winter	22-23.2.2021
Summer	23-24.8.2021

Table 3: Sample locations with the name and abbreviations of the river and coordinates.

River	**Location name**	**River abbreviation**	**Coordinates**	
Kamniška Bistrica	Stahovica	KBR	46°16’55.3''N	14°36’53.3''E
Kamniška Bistrica	Beričevo	KBB	46°05’18.4''N	14°37’33.1''E
Ljubljanica	Cesta v Kresnice	LJU	46°04’00.6''N	14°37’49.5''E
Gradaščica	Mali Graben – Cesta v mestni log	GRA	46°01’59.4''N	14°28’36.1''E

KBR

KBB

GRA

LJU

Figure 5: Photographs of the four study sites. KBR – Kamniška Bistrica Stahovica, KBB – Kamniška Bistrica Beričevo, GRA – Gradaščica, LJU – Ljubljanica.

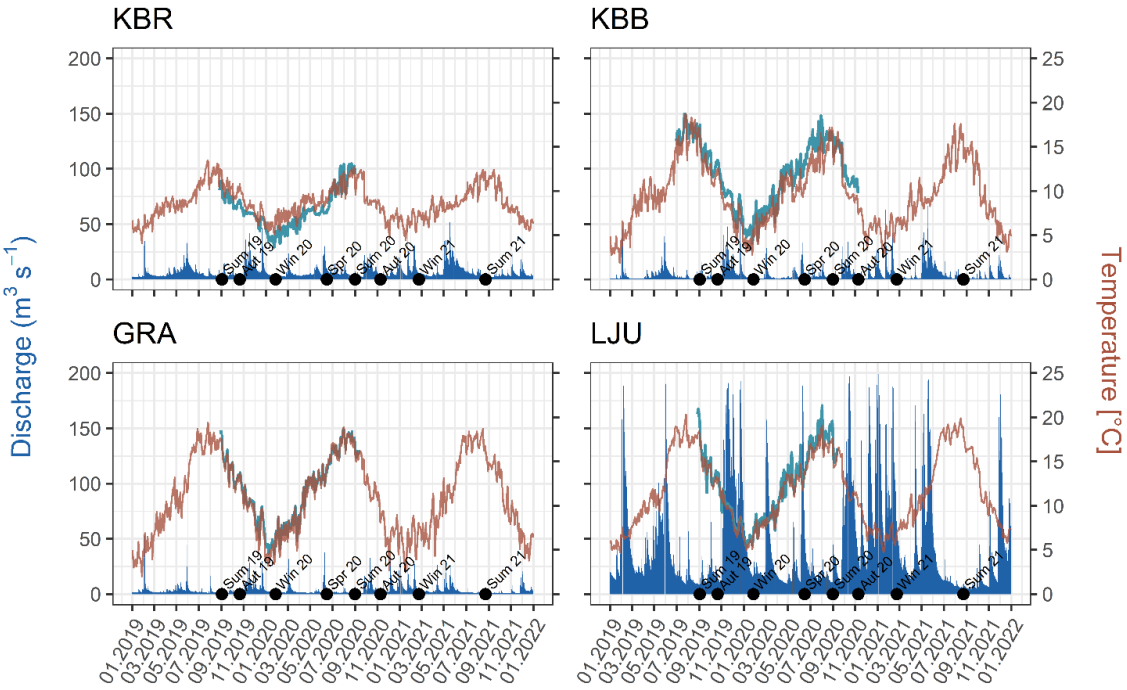


Figure 6: Plots of surface water temperature and river discharge during the experiment. The data are obtained from the Environmental Agency of the Republic of Slovenia. The sampling times are indicated by the black dots.

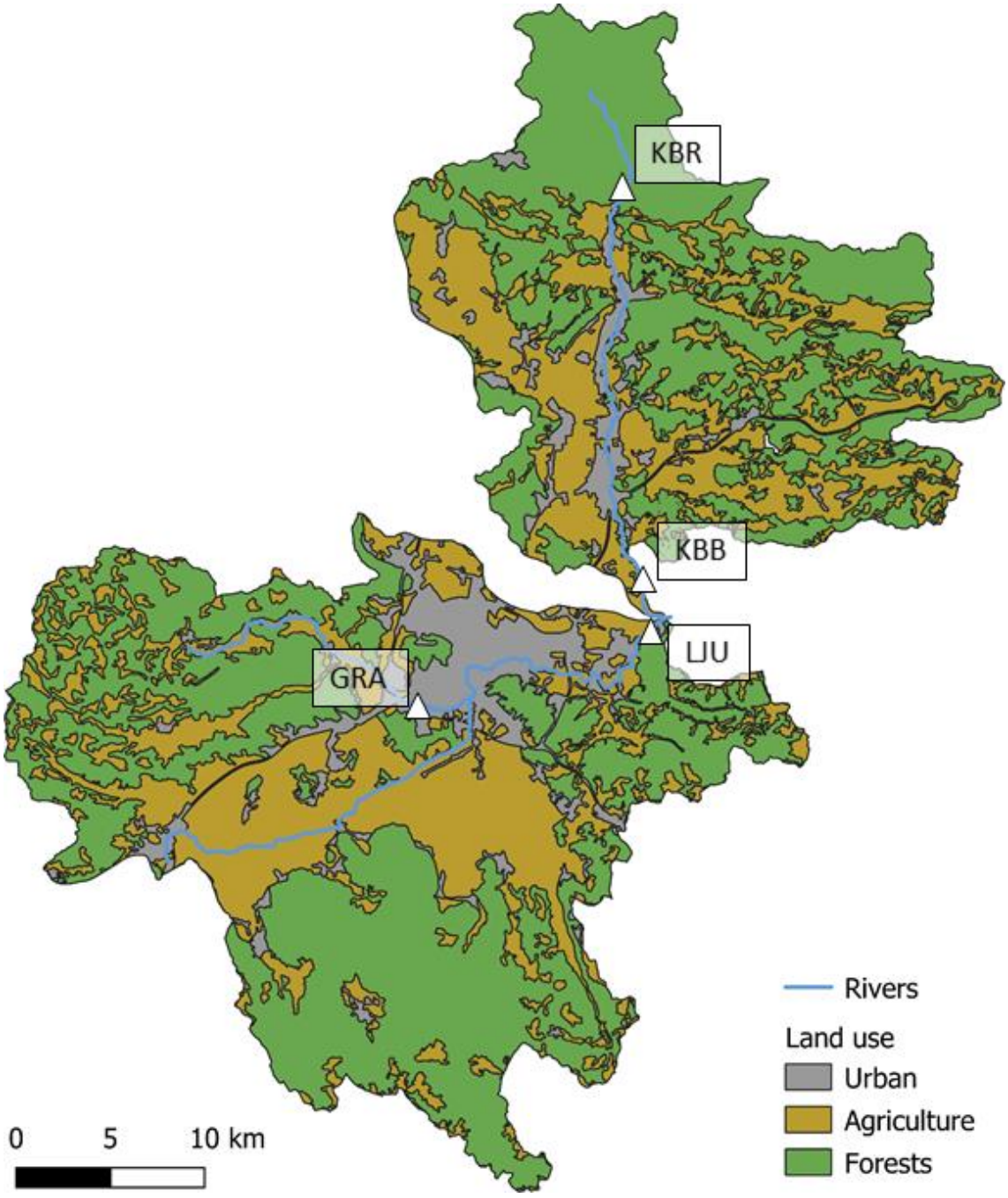


Figure 7: Map of the Ljubljanica and Kamniška Bistrica catchments indicating the four study sites where artificial substrates were inserted into the hyporheic zone. Source of data: Environmental Agency and Ministry for Agriculture, Forestry and Food Production (https://www.arso.gov.si/vode/podatki/).

Field sampling

Due to the large number of samples and the need to process them immediately, field sampling was conducted over two days. On the first day, samples were collected from KBB (n = 18), and on the second day, samples were collected from KBR, LJU and GRA, respectively (n = 6 for each site, n = 18 in total). The samples (control and with PET fibres contaminated artificial sediments) were carefully placed in a cooling box and promptly transported to the laboratory. Concurrently, on each sampling occasion, river water level, water temperature, oxygen concentrations, oxygen saturation, and

conductivity were measured on-site. Water samples were collected in HDPE bottles to analyse dissolved organic matter (DOC) and total nitrogen (TN).

Samples of PET fibres for isotopic analysis of carbon $\delta^{13}C$ and SEM micrographs were taken twice after one and two years of exposure to field conditions (Summer 2020 and 2021).

Laboratory work

The hyporheic biofilm characteristics from the control and with PET fibres-treated sediment pockets were assessed using total protein content (TPC), biofilm activity (ETSA) and metabolic profiling (CLPP) measurements. Additionally, particulate organic matter (POM) was measured.

The sediment pockets were opened in the laboratory, and their contents were thoroughly mixed. For CLPP analysis, 2 g of sediment and one sheet of fabric were used, respectively, while the remaining samples were frozen at -80 °C for at least one week and were analysed for POM, TPC, and ETSA.

Samples for analysis were first thawed, and 8 g were transferred to a centrifuge tube, to which 2 ml of homogenizing buffer was added. The cell walls were destroyed using an ultrasonic homogeniser 4710 (Cole-Parmer, Vernon, IL, USA) for 2 minutes, and then the lysate was centrifuged at 0°C for 4 minutes at 10,000 rpm. The supernatant was used for TPC and ETSA analysis, while the remaining thawed sediment was used for determining particulate organic matter (POM) and sediment dry weight (DW).

Particulate organic matter (POM)

The determination of particulate organic matter (POM) in the sediments was carried out by the loss-on-ignition (LOI) method. Approximately 10 g of thawed sediment was placed in a ceramic bowl and weighed to obtain the wet weight (WW). The sediments were then incubated at 105°C until a constant weight was achieved, after which they were weighed again to obtain the dry weight (DW). The sediments were then subjected to an oven temperature of 520°C for 2 hours to ignite the organic substances. After cooling, the bowls containing the sediments were weighed to calculate the loss on ignition. The POM content is expressed in gPOM $kgDW^{-1}$.

Total protein content (TPC)

The total protein content (TPC) is commonly utilised as an indirect indicator of microbial biomass. It involves measuring the colour development of the BCA reagent following the protein's alkaline reduction of the cupric ion (Lovrien & Matulis, 2006). The resulting BCA/copper complex is water soluble and shows a robust linear correlation between absorbance at 562 nm and protein concentration. The Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) was used, and all the standards and reagents were prepared following the manufacturer's instructions.

For the assay, a sample or standard (25 µL) was mixed with the working reagent (200 µL) in a microplate and incubated at 37°C for 30 minutes. After cooling the microplate to room temperature, the absorbance was measured at 562 nm using a spectrofluorometer (SynergyMX, BioTek Instrumentals, USA). The results were expressed as micrograms of protein per gram of dry sediment (µg prot g sed^{-1}).

Respiratory electron transport system activity (ETSA)

Respiratory electron transport system activity (ETSA) was used to investigate the functional capacity of microbial communities and their response to different environmental conditions. ETSA measures the electron transfer rate along the electron transport chain

during cellular respiration, providing insights into the microbial community's overall metabolic activity and vitality (Debeljak et al., 2017).

This study used a modified assay adapted from Packard (1971), as in chapter 3.3.1. Briefly, the supernatant obtained from the previously mentioned thawed samples was utilised. Each sample (30 µL) was added to two adjacent cells on a microplate, while a third cell was left empty as a blank. Substrate solution (150 µL) and reagent solution (50 µL) were added to each well. The microplate was then incubated in the dark at 20 °C for 30 minutes. Following the incubation, a stopper solution (50 µL) was added. Finally, 30 µL of the corresponding sample was added to the blanks. The absorbance was immediately measured at 490 nm using a microplate reader SynergyMX (BioTek Instruments, USA). The measurements were converted to the concentration of oxygen used per dry sediment weight during a specific time interval. The average of the duplicates was adjusted by subtracting the blank value, and the resulting value was utilised in the formula described in detail in Chapter 3.3.1 (Matjašič et al., 2021a).

Community-level physiological profiling (CLPP)

The functional diversity of the microbial community was assessed using the Biolog Ecoplates™ Assay (Biolog, California, USA). The assay consists of multi-well plates containing 31 different carbon sources and water in the blank. By adding the microbial sample to the wells, different organisms can utilise specific carbon sources, resulting in the development of distinct patterns, often called fingerprints, on the plate due to redox dye. These colony patterns can be analysed to determine the metabolic capabilities of the microbial community. The CLPP is a quick and cost-effective approach for evaluating microbial functional diversity (Garland & Mills, 1991). The intensity of substrate utilization was measured using spectrophotometry.

The sediment used for this analysis was immediately utilised upon transportation from the field. For each sample, 2 g of sediment (n = 24) and one fabric sheet (n = 12) were placed in labelled beakers, and 20 ml of chilled Ringer solution was added. The samples in the beakers were subjected to ultrasonic (Elmasonic P, Elma, Singen, Germany) treatment for 1 minute. The contents were then shaken, and the solution was centrifuged at 4°C for 5 minutes at 800 rpm. Subsequently, 150 µL of the supernatant was transferred to each 96 wells (administered in triplicates; 3x32) and measured at 590 nm using SyneryMX (BioTek Instruments, USA) at 0, 24, 48 and 72 hours. The plates were incubated in the dark at 20°C during the measurement intervals. The raw absorbance measurements were corrected by subtracting the blank. The substrate utilization measurements after 72 hours were used to compare the results with previous studies and minimise measurement errors (majority of values not exceeding 2) (Weber & Legge, 2010).

Isotopic analysis

The reference material used in this study was an untreated commercial PET cloth, previously characterised in a study by Matjašič et al. (2020) and used in the field study. The reference PET cloth was compared to PET cloths exposed to field conditions for one and two years (Summer 2020 and 2021, respectively). The isotopic composition of carbon ($\delta^{13}C$) was also determined.

Before conducting the $\delta^{13}C$ analysis, the field samples were treated in two ways to optimise the method and remove as much organic matter (including the attached biofilms) as possible. One set of samples underwent thorough rinsing with deionised water, while the other was subjected to ultrasonic treatment. The latter included placing the samples in an Elmasonic P ultrasonic bath (Elma, Singen, Germany) for 1 minute at 37 kHz and 30% power, followed by an additional 1-minute treatment using an ultrasonic homogeniser 4710 (Cole-Parmer, Vernon, IL, USA). This process was repeated after replacing the water with

fresh deionised water. Finally, the deionised water was replaced with fresh deionised water, and the samples were further processed as described in Matjašič et al. (2021a).

For isotopic analysis, all samples were treated in the same manner. Approximately 0.5 mg of material was weighed into tin capsules, and the $\delta^{13}C$ isotopic composition was determined using a Europa 20-20 continuous flow isotope ratio mass spectrometer (IRMS) with an ANCA-SL preparation module. The tin capsules were combusted at 1000 °C, and the resulting gases were reduced in a copper tube at 600°C to remove excess oxygen. Water vapour was trapped on a drying column composed of $MgClO_4$. The gases were then separated on a chromatographic column and ionised for analysis.

The following reference materials were used: IAEA CH-3 (-24.724 ± 0.041 ‰), IAEA CH-7 (-32.151 ± 0.05 ‰), and sugar with a value of -25.2 ± 0.2 ‰, to calibrate the analytical results to the VPDB standard.

Scanning electron microscopy (SEM)

The PET fibres were prepared for scanning electron microscopy (SEM) conducted by Zoran Samardžija from JSI by applying a 7 nm thick conductive Au-Pd layer using an ion-beam precision etching coating system (PECS 682, Gatan Inc. USA). Subsequently, the samples were examined using a field-emission gun scanning electron microscope (FEGSEM Verios G4, Thermo Fisher Scientific, USA) at an accelerating voltage of 5 kV and various magnifications. While multiple micrographs were captured during the observation, only a limited selection is included in the thesis. Additional micrographs can be made available upon request. The procedure used was described in full by Matjašič et al. (2021a).

Statistical analysis

The primary objective of this study was to investigate changes in biofilm structure and function over time (during the two years) and across different seasons and to explore variation between the study sites from different rivers. The normality of the data was assessed using the Shapiro-Wilk test. If the data followed a normal distribution, and other ANOVA assumptions were achieved, ANOVA and Tukey's Honest Significant Difference (HSD) test were applied. If the data did not meet the normality assumption, even after log transformation ($\log_{10}(x+1)$), the Kruskal-Wallis test and Dunn's post hoc test were used. The significance level was $p < 0.05$ unless specified otherwise.

To examine the changes in biofilm, the data on POM, TPC, ETSA and CLPP, from summer 2019 to summer 2021 from the study site Beričevo, were used (KBB). Eight temporal data points (Table 2) and six spatial replications were established, with sampling conducted once per season for two consecutive years. The data for TPC were transformed using $\log_{10}(x+1)$. The analysis done on this (temporal) data set was 2-way ANOVA for POM and logTPC (factors: year, treatment) together with Tukey's HSD post hoc, while for the ETSA, Kruskall-Wallis test and Dunn's post hoc were used for data interpretation. For all the substrates of the CLPP, 2-way ANOVA was used. Further, a Non-metric multidimensional scaling (NMDS) and analysis of variance (ANOSIM) was conducted to test for significant differences. At the same time, Permutational Multivariate Analysis of Variance (PERMANOVA) was used to test for group differences, and Canberra dissimilarity was used to calculate distances as it is a robust distance metric, particularly for outliers. The sampling in spring 2021 was not performed due to hazardous circumstances caused by high water levels at all sampling locations.

When looking at seasonal variations, we pooled the data from all four locations (KBB, KBR, GRA, and LJU). Next, the data for POM, TPC, and ETSA data were tested for normality using the Shapiro-Wilk test, and none were distributed normally; therefore, we carried out a Kruskall-Wallis test for POM, TPC, and ETSA (factors: treatment, season), together with Dunn's post hoc. For all the substrates of the CLPP, 2-way ANOVA (factors:

treatment, season) was used. For data on PET sheets, for TPC and ETSA, a Kruskall-Wallis test (factor: season) was used. The POM was not calculated due to the technical constraints of using a PET sheet (unable to burn it in the muffle oven). NMDS, ANOSIM, PERMANOVA (Canberra dissimilarity) and heatmaps were also used for data interpretation.

The data from all seasons (autumn, winter, spring, and summer) were pooled to assess spatial variation in biofilm function. Next, data on POM, TPC, and ETSA were tested for normality using the Shapiro-Wilk test. The data were not normally distributed; therefore, a Kruskall-Wallis test for POM, TPC, and ETSA (factors: treatment, river) and Dunn's post hoc test were performed. For all the substrates of the CLPP, a 2-way ANOVA (factors: treatment, river) was used. Also, NMDS, ANOSIM, and PERMANOVA were calculated, and heatmaps were used for data interpretation. For data on PET sheets, for TPC and ETSA, the Kruskall-Wallis test (factor: river) was used. The POM was not calculated due to the technical constraints of using a PET sheet, which could not be burned in the muffle furnace. For all CLPP substrates, a 2-way ANOVA was used. NMDS, ANOSIM, PERMANOVA (Canberra dissimilarity) and heatmaps were also used for data interpretation.

The CLPP data was log-transformed and then investigated using multivariate non-metric multidimensional scaling (NMDS), similar to the process described in Matjašič et al. (2021a). For NMDS, the input data set was separated based on treatment (data from sediments and data from PET sheets). Further, both data sets included absorbance and were presented separately, based on the factor being investigated: year (sediments: Figure 11, PET sheets: Figure 12), season (sediments: Figure 20, PET sheets: Figure 20) and river (sediments: Figure 27, PET sheets: Figure 28). The data were then further separated based on treatment (CT, Fig. 11, 20, 29 and P, Fig. 12, 21, 30). NMDS, ANOSIM, PERMANOVA (Canberra dissimilarity) and were used for data interpretation.

Additionally, heatmaps were constructed to visualise the intensity of substrate utilization under different treatments, with violet as the least intensive, blue-green in the middle and yellow as the most intensive. Statistical analysis was conducted using R studio to analyse the data. The statistical packages used for analysis included tidyverse (Wickham H et al., 2019), vegan (Oksanen et al., 2020), openxlsx (Schauberger & Walker, 2022), reshape2 (Wickham, 2007), viridis (Garnier et al., 2021), dunn.test (Dinno, 2017) and gridExtra (Auguie, 2017).

Results

Environmental characteristics of the study sites

The measured environmental variables of surface water showed a high between-site variability under ecological conditions important for biofilm structure and function. The mean discharge of the study rivers between January 1, 2019, and December 31, 2021, ranged from 3.73 ± 5.20 (GRA) to 46.41 ± 48.55 (LJU) $m^3 s^{-1}$ with a mean temperature of 8.6 ± 1.9 °C (KBR) to 11.6 ± 4.2 °C (LJU). Similarly, the mean temperature, measured in the hyporheic zone, ranged from 7.6 ± 2.1 °C in KBR to 12.4 ± 4.1 °C in LJU. However, the temperature in the hyporheic zone was measured from August 27, 2019, to September 13, 2020, when the temperature data loggers were active (Figure 6).

On the sampling occasions (N=8), the mean water levels ranged from 10 ± 7 cm in KBR to 29 ± 16 cm in LJU. Similarly, the mean temperature, measured in the hyporheic zone, ranged from 12.7 ± 1.3 °C in KBR to 14 ± 0.4 °C in the LJU (Table 4). However, the date range was limited to August 27, 2019, and September 13, 2020, when the temperature data loggers were active.

The highest oxygen (O_2) content was measured in the water from the KBR site, whereas the lowest was found in the water from the LJU site (11.7 ± 0.1 mg/L and 7.9 ± 2.0 mg/L, respectively). This trend also held for oxygen saturation, with KBR exhibiting the highest saturation (101 ± 2 %) and LJU showing the lowest (79 ± 16 %). The measurements for KBB and GRA fell within the range of those in KBR and LJU.

The water conductivity results indicated a noticeable variation, with the lowest conductivity recorded at the KBR site (211 ± 17 µS/cm) and relatively high values measured at KBB and LJU sites (446 ± 48 µS/cm and 447 ± 38 µS/cm, respectively). Similarly, the mean dissolved organic carbon (DOC) and total nitrogen (N_{tot}) measurements were the lowest at the KBR site (0.83 ± 0.16 mg/L and 0.57 ± 0.12 mg/L, respectively), while the highest DOC content was measured at LJU site (3.07 ± 0.40 mg/L), and the highest N_{tot} content was observed at KBB site (2.36 ± 0.84 mg/L). KBB's proximity to the WWTP Domžale – Kamnik effluents may account for higher N_{to}t levels.

Table 4: The mean values (±) standard deviation of the environmental data for all sampling sites throughout the sampling ($N_{sampling\ dates}$=8). Given that data from 10.11.2020 for $T_{hyporheic}$ for KBR, GRA, and LJU is missing $N_{sampling\ dates} = 7$.

Sampling site	**Abbr**	**Water level [cm]**	**T_{water} [°C]**	**$T_{hyporheic}$ [°C]**	**O_2 [mg l^{-1}]**	**Saturation [%]**	**Conductivity [µS/cm]**	**DOC [mg l^{-1}]**	**N_{tot} [mg l^{-1}]**
Kamniška Bistrica – Stahovica	KBR	10 ± 7	9.5 ± 2.1	13.0 ± 3.6	11.7 ± 0.2	101 ± 2	211 ± 17	0.83 ± 0.16	0.57 ± 0.12
Kamniška Bistrica – Beričevo	KBB	15 ± 12	11.3 ± 4.5	12.7 ± 1.3	10.2 ± 1.0	95 ± 4	446 ± 48	1.97 ± 0.61	2.36 ± 0.84
Gradaščica	GRA	18 ± 8	12.6 ± 4.3	13.4 ± 4.0	10.5 ± 1.0	98 ± 2	384 ± 33	1.78 ± 0.84	0.99 ± 0.23
Ljubljanica	LJU	29 ± 16	13.0 ± 4.8	14.0 ± 0.4	7.9 ± 2.0	79 ± 16	447 ± 38	3.07 ± 0.40	1.85 ± 0.71

A detailed spatiotemporal patterns in POM and hyporheic biofilm response to PET pollution over two years at the Kamniška Bistrica study river site Beričevo (KBB)

The mean POM contents in the control sediment pockets ranged from 8.88 ± 1.48 gPOM $kgDW^{-1}$ (winter 2021) to 11.38 ± 1.30 gPOM $kgDW^{-1}$ (summer 2020). In contrast, the mean POM contents in sediment pockets treated with PET fibres varied from 8.38 ± 1.48 gPOM $kgDW^{-1}$ (summer 2019) to 11.63 ± 1.63 gPOM $kgDW^{-1}$ (summer 2020) (Figure 8). Spatial variability within the study site (N=6) was lowest during the winter seasons for both cases.

Statistical analysis revealed significant differences in POM contents between years and between the control and treated sediment pockets (two-way ANOVA, factors: years x treatment, N=8 x 2 x 6, $F_{years} = 3.909$, p_{season}=0.001, $F_{treatment} = 6.829$, $p_{treatment}$= 0.012). The presence of PET fibres reduced POM contents in the sediment pockets. Tukey's HSD post hoc test indicated significant differences between Summer 2020 and Summer 2019 ($p = 0.0029$) and between Summer 2020 and Winter 2021 ($p = 0.0011$). Sampling in the summer of 2019 occurred shortly after the insertion of the sample in the river bed sediments, which likely resulted in lower POM levels.

Although the two-way ANOVA did not reveal any significant interaction between the factors (F= 1.241, p= 0.291), Tukey's post hoc did show significance between control pockets in Summer 2020 and treatment pockets in Summer 2019 (p = 0.034), as well as between treatment pockets in Summer 2019 and Summer 2020 (p = 0.008).

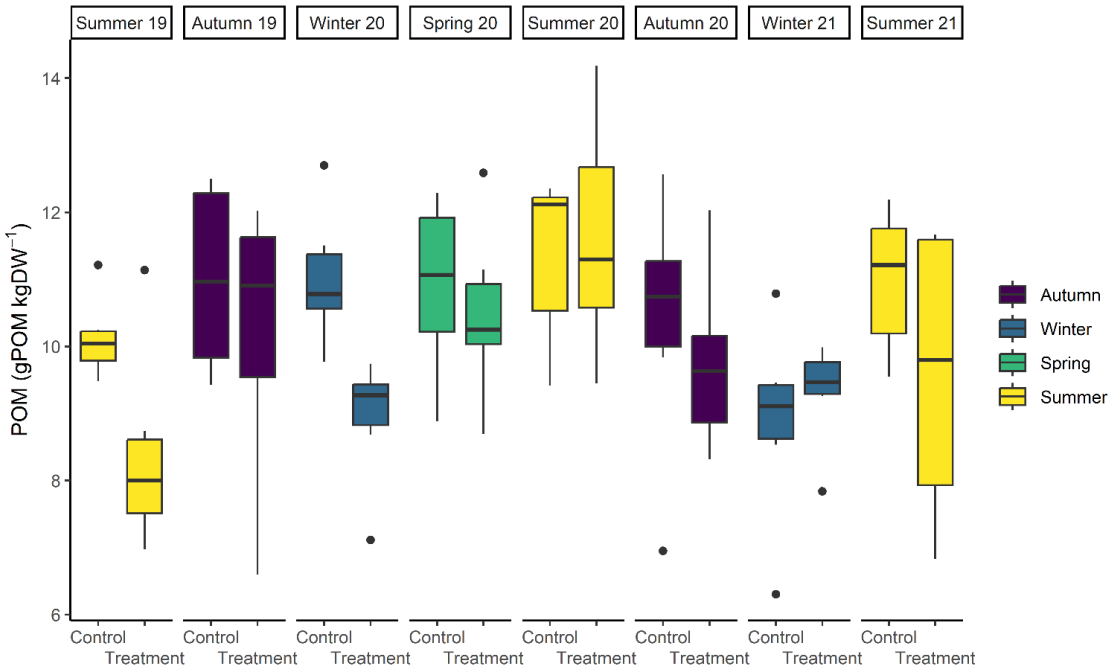


Figure 8: Boxplots indicating variation in POM contents in the control (N=3) and treatment (N=3) sediment pockets over two years (N=8) at the Kamniška Bistrica study site Beričevo (KBB).

The mean TPC in the control sediment pockets ranged from 38.58 ± 5.41 µg prot gDWsed^{-1} (Summer 2020) to 120.93 ± 72.12 µg prot gDWsed^{-1} (Summer 2021). Conversely, the mean TPC in sediment pockets treated with PET fibres (Figure 9) varied from 32.27 ± 3.49 µg prot gDWsed^{-1} (summer 2020) to 99.88 ± 73.12 µg prot gDWsed^{-1} (Autumn 2019). Significant differences were observed between the seasons over the two years, except for autumn.

A two-factor ANOVA analysis revealed a significant difference in logTPC contents only between years (two-way ANOVA, factors: years x treatment, N=8 x 2 x 6, F_{season}=9.772, $p_{season} < 0.0001$, $F_{treatment} = 0.795$ $p_{treatment}$= 0.380). Tukey's post hoc indicated significantly lower protein content in the spring compared to summer seasons ($p_{2019} = 0.0026$, $p_{2021} = 0.0039$), autumn seasons ($p_{2019} = 0.0007$, $p_{2020} = 0.0074$) and winter ($p_{2021} = 0.026$). Similarly, Tukey's test revealed significantly lower protein content in summer 2020 when compared to the summer seasons ($p_{2019} = 0.0001$, and $p_{2021} = 0.0002$), as well as between autumn ($p_{2019} < 0.0001$, $p_{2020} = 0.0004$) and winter ($p_{2020} = 0.041$, $p_{2021} = 0.0016$) seasons.

Although the "treatment" factor was not significant, Tukey's test revealed significant differences when the "years" factor was included. The protein content in the Summer 2020 control was significantly lower than in the Autumn 2019 control (p = 0.017) and Summer 2021 control (p = 0.0096). Similarly, the protein content in the summer 2020 treatment was significantly lower than other summer controls (p19 = 0.009, p21 = 0.001) and treatments (p19 = 0.021), autumn controls (p19 = 0.002, p20 = 0.036) and treatments (p19 = 0.015, p20 = 0.022), and Winter 21 treatment (p = 0.028). The control of Summer 2021 was significantly higher than the Spring 2020 control (p = 0.017) and treatment (p

= 0.037). The Spring 2020 control protein content was significantly lower than the Autumn 2019 control (p = 0.0296).

Lower TPC values were also measured in the treated sediment pocket during summer (2019, 2020, 2021) and autumn (2019). This observation can be attributed to high temperatures or nutrient inputs and consequently increased biofilm growth and activity.

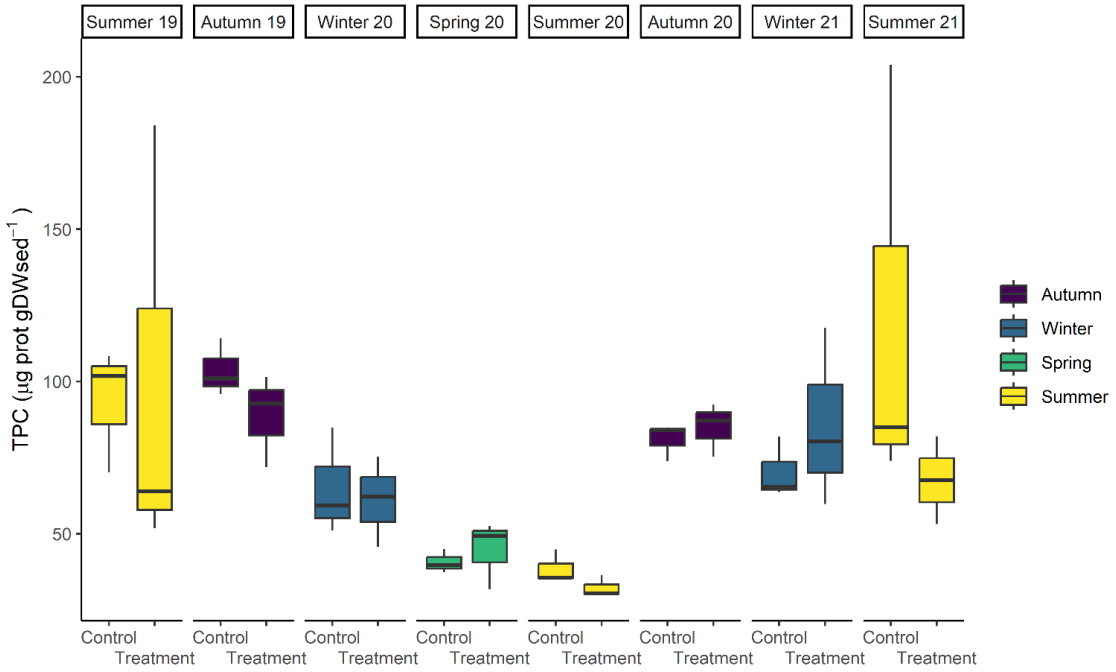


Figure 9: Box-plots indicating variation in TPC in the control (N=3) and treatment (N=3) sediment pockets over two years (N=8) at the Kamniška Bistrica study site Beričevo (KBB).

The mean ETSA in the control sediment pockets ranged from 1.28 ± 0.09 µL O_2 $gDWsed^{-1}$ h^{-1} (Winter 2021) to 2.19 ± 0.31 µL O_2 $gDWsed^{-1}$ h^{-1} (Autumn 2019), while the mean ETSA in the treatment sediment pockets ranged from 1.10 ± 0.15 µL O_2 $gDWsed^{-1}$ h^{-1} (Summer 2021) to 2.61 ± 1.12 µL O_2 $gDWsed^{-1}$ h^{-1} (Summer 2019) (Figure 10). The ETSA was lower in winter and spring while higher in the control sediment pockets during the same seasons.

The data were not normally distributed, as indicated by the Shapiro-Wilk normality test (W = 0.868, p < 0.0001). The Kruskall-Wallis test was used for data interpretation. The factor "treatment" was not significant (p = 0.54), but there were significant differences between years (p < 0.001). Specifically, the ETSA was significantly higher in Autumn 2019 than in Spring 2020 (p = 0.0106) or Winter 2021 (p = 0.0061), as Dunn's post hoc test revealed. Additionally, ETSA in Summer 2019 was significantly higher than in Winter 2021 (p = 0.0166). Although the "treatment" was not significant, a trend (with over half of the sampling points suggests that sediment pockets with PET present have lower ETSA in seasons with overall lower activity (winter, spring).

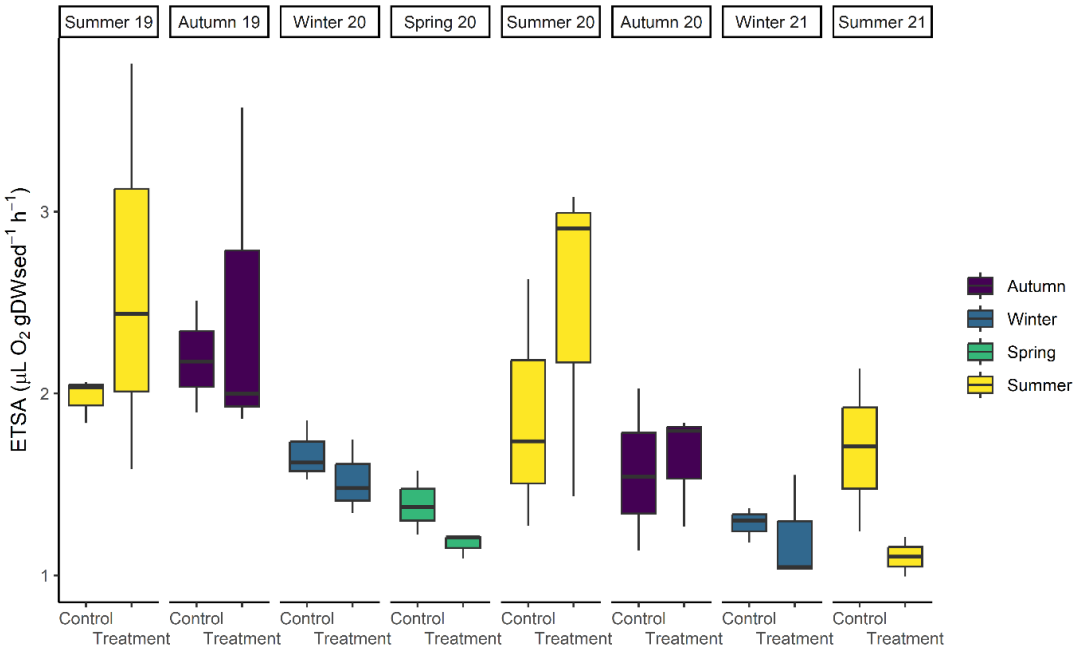


Figure 10: Box-plots indicating variation in ETSA in the control (N=3) and treatment (N=3) sediment pockets over two years (N=8) at the Kamniška Bistrica study site Beričevo (KBB).

The Community Level Physiological Profiling (CLPP) results revealed that, for most cases, the presence of PET had a suppressive effect on variability in microbial functioning throughout the 2-year monitoring period. However, a noticeable disparity was observed between the control and treatment groups (NMDS, stress = 0.2) when analysing the metabolic function of the microbial communities in sediment pockets (Figure 11). Specifically, during the Summer 2019 and Autumn 2020, the presence of PET was found to suppress the variability of the metabolic function of bacteria inhabiting the sediments, in contrast to the summer of 2020 and winter and spring, where bacteria exhibited higher variability in metabolic function compared to biofilms growing on sediments without PET. Caution, however, is needed in interpreting these results due to the relatively high-stress level obtained from the NMDS analysis. The metabolic functioning during autumn and winter appeared similar across different years for bacteria growing on PET (Figure 12, stress = 0.14). At the same time, there was notable dissimilarity between summer seasons, probably due to high discharge before the last sampling in Summer 2021.

The ANOSIM, which tested for significant differences in microbial functioning between treatments (sediment pockets) and years (Table 2), revealed the importance of both factors: "treatment" (R: 0.0228, p = 0.004) and "years" (R: 0.2853, p = 0.001). The R-value provides information about the strength of dissimilarity between groups, with higher values indicating greater dissimilarity. Therefore, the results suggest that the year influences microbial functioning the most compared to treatment or season. This observation was confirmed using PERMANOVA. The factor "treatment" explained only 1.7% (p = 0.006) of the total variation in the data, and years explained 24 % (p = 0.001). The data obtained from PET were analysed separately. The ANOSIM analysis revealed years as significant factors (R: 0.4011, p = 0.001), with years explaining 48 % (p = 0.001) of the total variation.

Based on the results of CLPP from both sediment pockets and PET samples, the trend indicates that time is the most influential factor affecting variation in microbial functioning, with an even more pronounced effect observed in the samples containing PET.

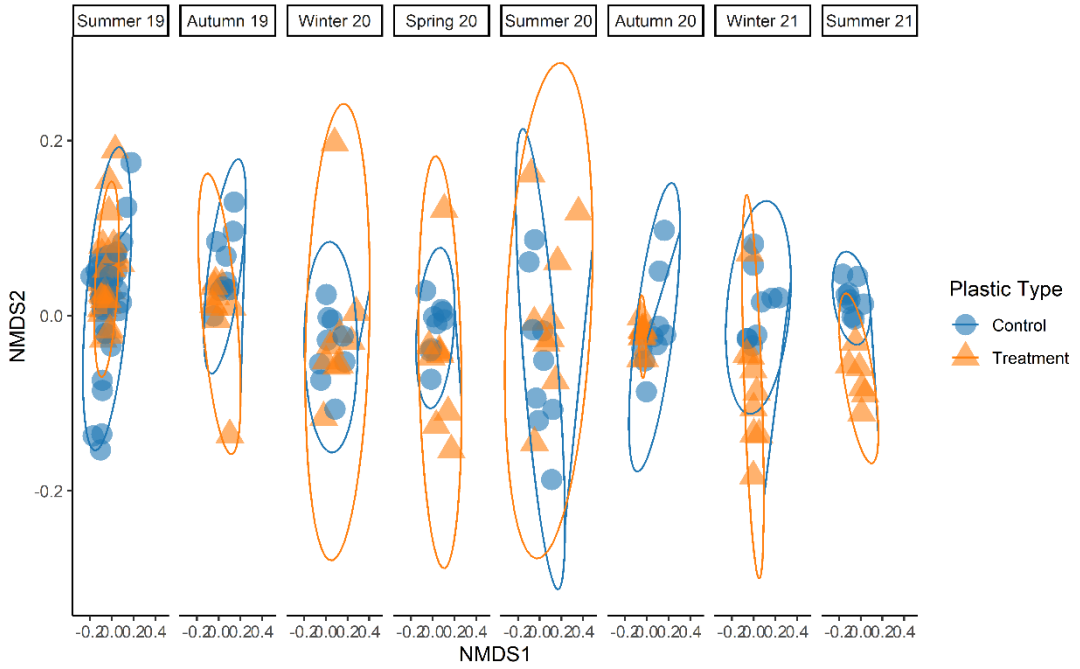


Figure 11: The NMDS carried out on CLPP data from KBB site, depicting the comparison between control and treated sediments over 2 years (Summer 2019 to Summer 2021). Stress: 0.2, distance = “canberra”.

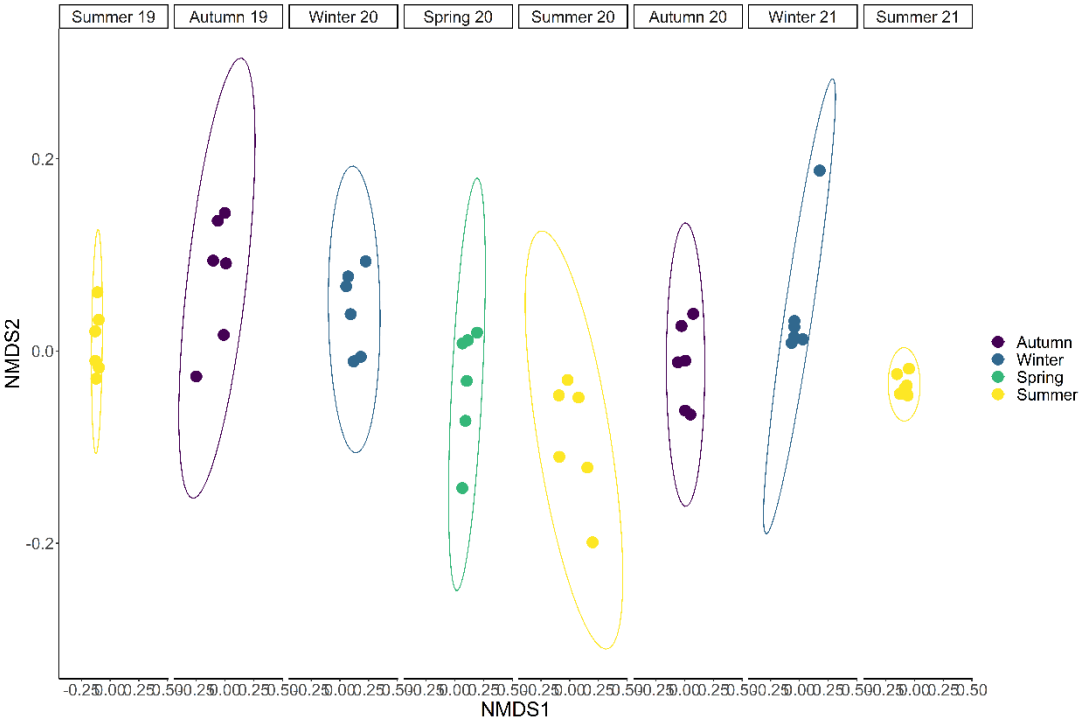


Figure 12: The NMDS carried out on CLPP data from KBB site, depicting the variations in functioning of biofilms grown on the PET sheets for 2 years. Stress: 0.14, distance = "canberra".

The heatmaps visually illustrate a progressive acquisition of substrate utilization capabilities over time, with a noticeable trend indicating an expanding range of utilised

substrates. During the summer of 2020, the lowest substrate utilization was observed across all treatments, while the sample from the summer of 2021 exhibited the highest utilization. This consistent increase in substrate utilization reflects an expanding microbial functional diversity throughout the study period. Substrate utilisation declined from Winter 2020 to Summer 2020 for bacteria growing on PET.

A comparison of absorbance readings between the control and treatment groups over the years also revealed distinct patterns. During the summer of 2021, substrate utilization by bacteria growing on PET (Figure 13, b) was initially lower compared to the control or treatment groups (Figure 13, a). However, it gradually improved, indicating the bacteria's adaptation to the PET substrate. The heatmap showed that bacteria growing on PET are exposed to higher stress and are less successful in utilizing the substrates than bacteria in unburdened environments or bacteria living only in the presence of PET.

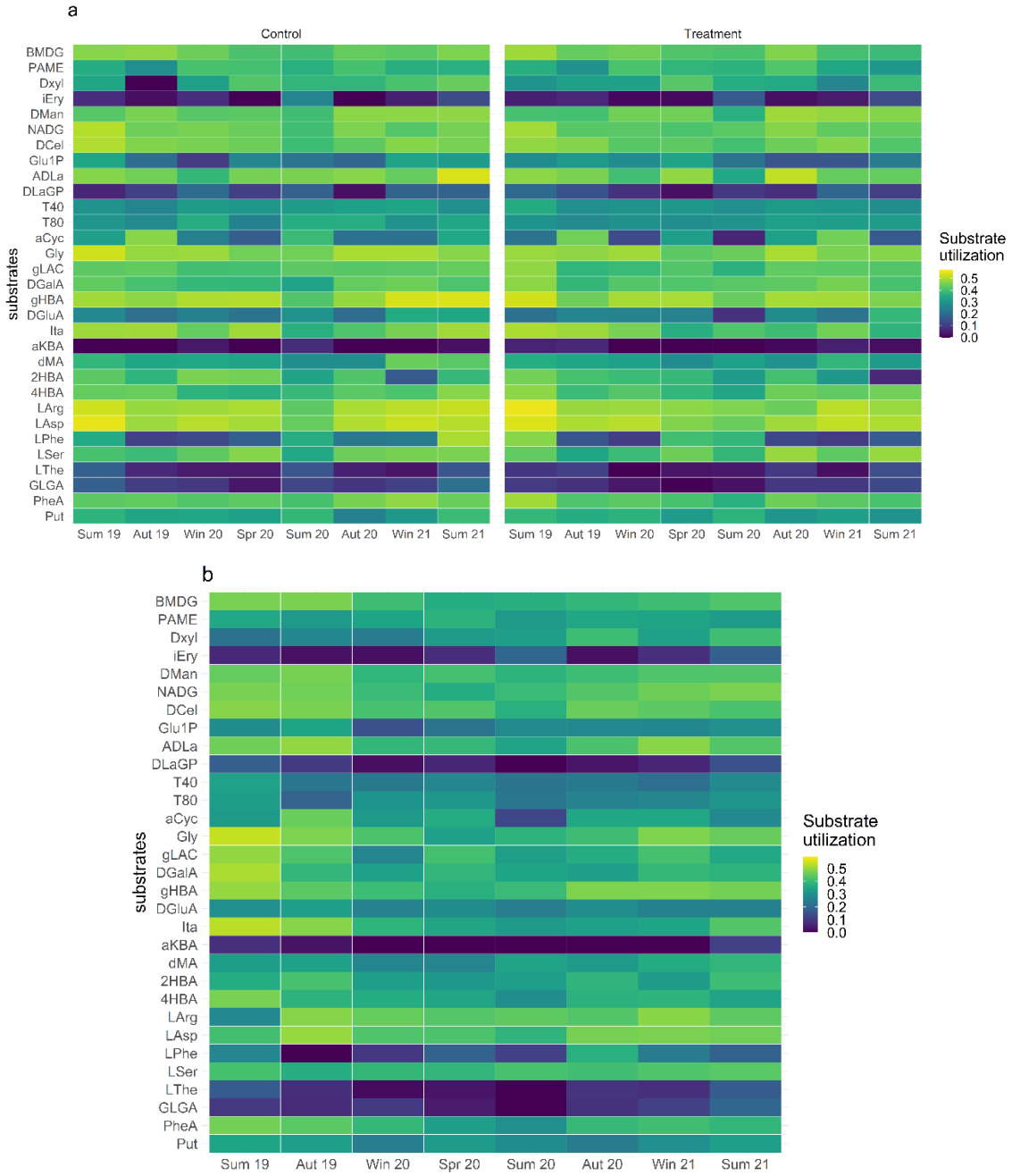


Figure 13: Heatmaps illustrating substrate utilization intensity during a two-year field study of bacteria from control sediments, treated sediments (a), and PET sheets (b). The y-axis displays the 31 substrates from the Biolog Ecoplates, measured spectrophotometrically.

Overall, the most significant differences in the utilization of substrates were found for factors "years" (n = 25 + 1 indication of trend ($p < 0.1$)), most of which were contributed by Carbohydrates (n = 7). All Polymer and Amino Acids group members were significant for this factor (Table 5). Two members of the polymer group, Tween 40 (C1) and alfa-cyclodextrin (E1) were significant for all factors and interactions tested. For the factor "treatment", 11 substrates were statistically significant ($p \leq 0.05$). Additionally, three substrates approached significance, suggesting that prolonged exposure to the treatment may reveal more significant substrates.

Table 5: Results of ANOVA comparing variability in the intensity of utilization of substrates from Biolog Ecoplates over 2 years for control and treated sediment pockets for the KBB location (temporal data). Treatment – Control, treatment. Years: Summer 2019 to Summer 2021. The sign ":" indicates the combination of effects. Significance codes: '***' ≤ 0.001, '**' ≤ 0.01, '*' ≤ 0.05, '.' ≤ 0.1.

Group of utilised substrates	**substrate**	**abbr**	**Ecoplates label**	**Years**	**Treatment**	**Years: Treatment**
Carbohydrates	beta-Methyl-Dglucoside	BMDG	A2	***	.	**
	Pyruvic Acid Methyl Ester	PAME	B1	***		
	D-Xylose	Dxyl	B2	***	*	**
	i-Erythritol	iEry	C2	***	.	*
	D-Mannitol	Dman	D2			*
	N-Acetyl-Dglucosamine	NADG	E2	***		
	D–Cellobiose	Dcel	G1	***		
	Glucose-1-Phosphate	Glu1P	G2	***		**
	alfa-D-Lactose	ADLa	H1	***		
	D,L-alfa Glycerol Phosphate	DlaGP	H2			.
Polymers	Tween 40	T40	C1	***	*	*
	Tween 80	T80	D1	***		*
	Alfa-Cyclodextrin	aCyc	E1	***	***	***
	Glycogen	Gly	F1	***		
Carboxylic and Ketonic Acids	Gamma-Lactone	gLAC	A3	***		.
	D-Galacturonic Acid	DgalA	B3		*	
	Gamma- Hydroxy Butyric Acid	gHBA	E3	.	***	.
	D-Glucosaminic Acid	DgluA	F2	***		
	Itaconic Acid	Ita	F3	***	.	.
	Alfa-Keto Butyric Acid	aKBA	G3	***		
	D-Malic Acid	dMa	H3	***		.
Phenolic compound	2-Hydroxy Benzoic Acid	2HBA	C3			
	4-Hydroxy Benzoic Acid	4HBA	D3	***	**	
Amino Acids	L-Arginine	Larg	A4	**	***	.

	L-Asparagine	Lasp	B4	***	***	
	L-Phenylalanine	Lphe	C4	***		*
	L-Serine	Lser	D4	***		
	L-Threonine	Lthe	E4	***	**	
	Glycyl-L-glutamic Acid	GLGA	F4	***		.
Amines/Amides	Phenylethylamine	PheA	G4		**	
	Putrescine	Put	H4	**	*	

A spatiotemporal patterns in POM and hyporheic biofilm response to PET pollution over two years at four study river sites (KBB, KBR, GRA, and LJU)

Seasonal patterns in biofilm response to pollution with PET

In this subchapter, we investigated the seasonal differences in biofilm structure and functioning across a large, regional scale, merging the data from all four study sites (KBR, KBB, GRA, LJU) on eight sampling occasions.

The mean POM contents (Figure 14) in the control sediment pockets ranged from 9.64 ± 2.49 gPOM kgDW^{-1} (winter) to 10.75 ± 0.79 gPOM kgDW^{-1} (spring). Similarly, the mean POM contents in the treatment sediment pockets were lowest in the winter (9.20 ± 2.35 gPOM kgDW^{-1}) and highest in autumn (10.22 ± 2.81 gPOM kgDW^{-1}).

Statistical analysis did not reveal any significance in POM contents between control and treatment pockets nor seasons (KW, p = 0.4594 and p = 0.5478, respectively). Despite no statistical difference, the data still reveals that in the autumn and summer, the POM content is higher in the treatment pockets compared to spring and winter.

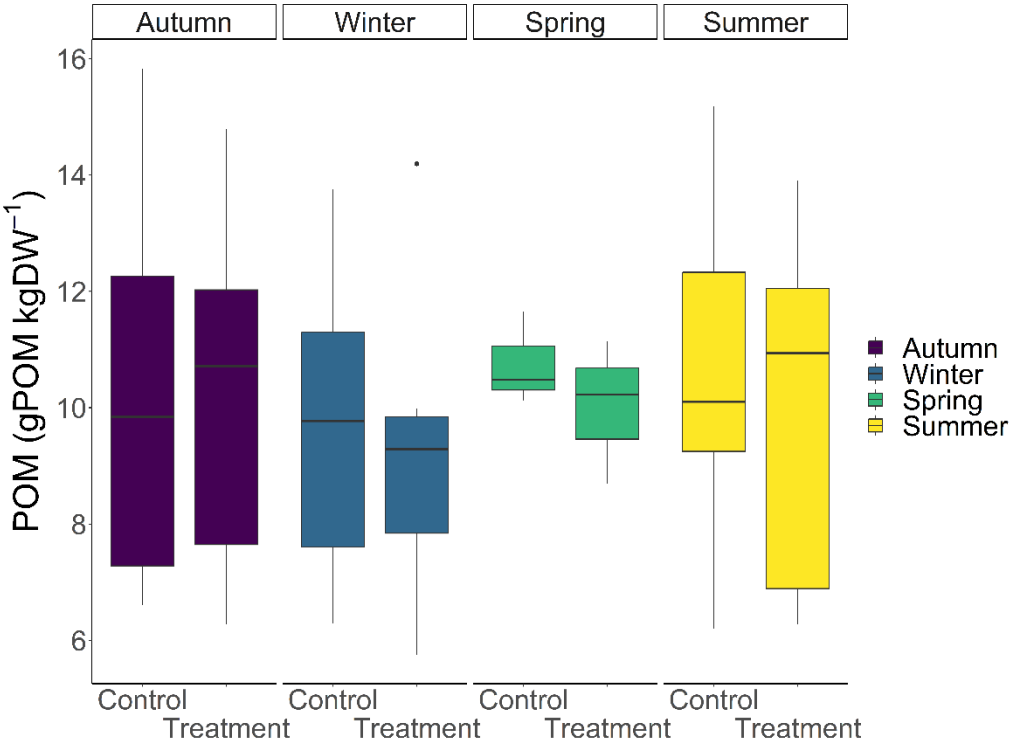


Figure 14: Box-plots indicating variation in POM contents in the control (N=4 sites x 3 replicates=12) and treatment (N=4 sites x 3 replicates = 12) sediment pockets over four seasons from all four locations (KBR, KBB, GRA, LJU).

The mean TPC content in the control sediment pockets ranged from 31.38 ± 25.56 µg prot g DW^{-1} (Spring) to 65.59 ± 45.47 µg prot g DW^{-1} (Summer). For the treatment sediment pockets, the mean TPC content ranged from 34.35 ± 30.37 µg prot g DW^{-1} (Spring) to 51.70 ± 33.03 µg prot g DW^{-1} (Autumn) (Figure 15).

Statistical analysis indicated no significant differences for TPC between the treatments (KW; $chi^2 = 2.0454$, $p = 0.15$) or seasons (KW; $chi^2 = 6.96$, $p = 0.073$). However, it is worth noting that the p-value approached significance, suggesting a potential trend if the sampling was expanded over a longer duration or a larger number of samples would be subjected to analyses. It is also evident that the mean TPC in treatment sediment pockets was generally lower than in control sediment pockets.

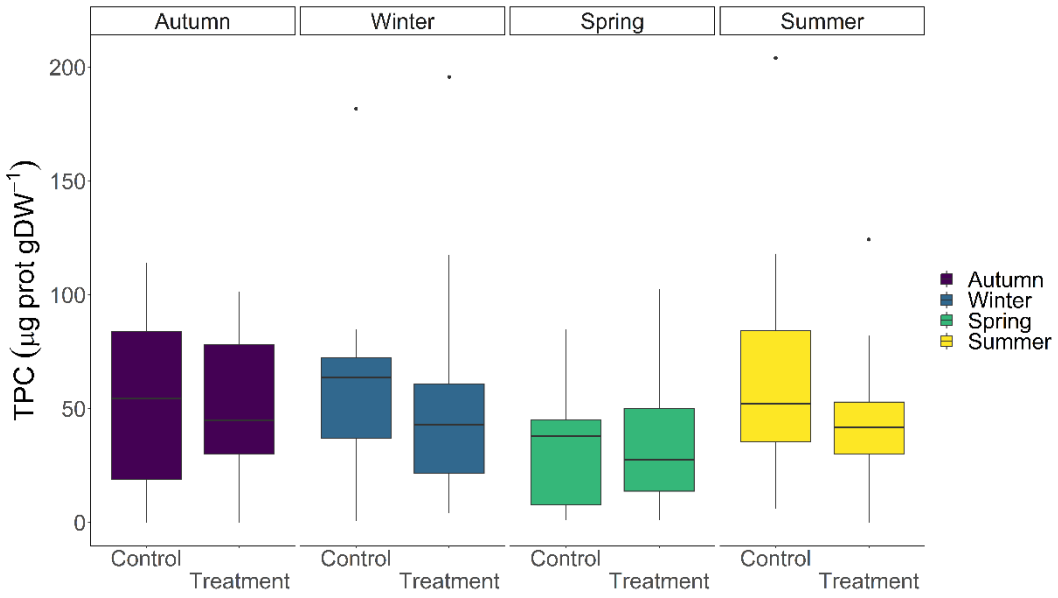


Figure 15: Box-plots indicating variation in TPC contents in the control (N=12) and treatment (N=12) sediment pockets over seasons from all four locations (KBR, KBB, GRA, and LJU).

The TPC content for microbiome on PET sheets varied from 184.21 ± 145.84 µg prot g DW^{-1} (Winter) to 408.72 ± 328.79 µg prot g DW^{-1} (Summer). Statistical analysis revealed significance (KW; $chi^2 = 8.5617$, $p = 0.036$), and subsequent Dunn's test, significantly lower TPC in winter compared to summer ($p = 0.0242$) and a near significance for a lower TPC in Autumn compared to summer ($p = 0.0569$). The microbiome on PET sheets was influenced by seasonal variations (Figure 16).

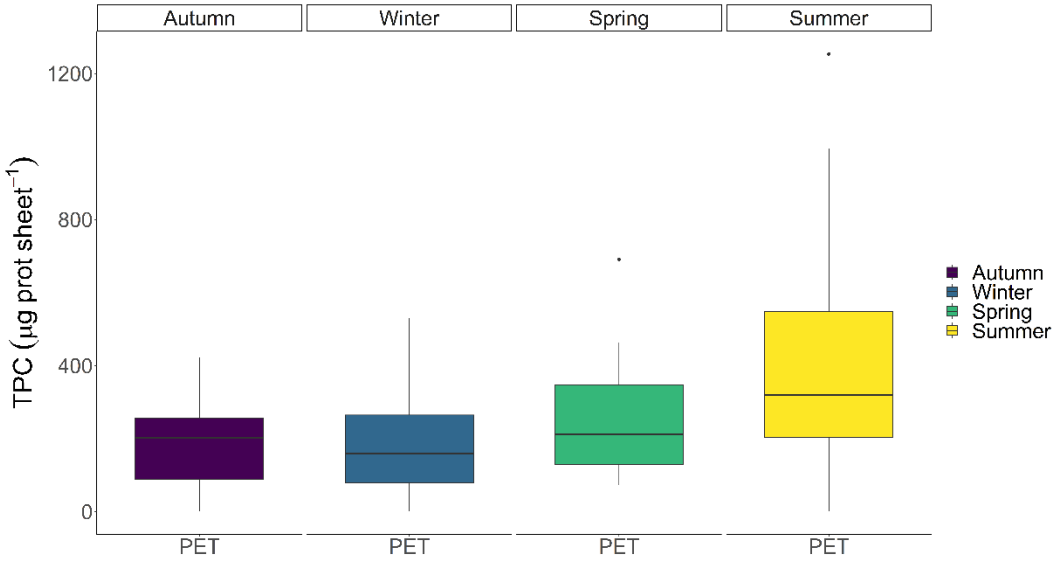


Figure 16: Box-plots indicating variation in TPC contents from PET sheets (N = 12) over seasons from all four locations (KBR, KBB, GRA, and LJU).

The mean ETSA in the control sediment pockets ranged between 0.59 ± 0.59 µL O_2 gDW^{-1} h^{-1} (Spring) and 0.81 ± 0.61 µL O_2 gDW^{-1} h^{-1} (Summer). The mean ETSA in the treatment pockets ranged between 0.43 ± 0.43 µL O_2 gDW^{-1} h^{-1} (Autumn) and 0.77 ± 0.93 µL O_2 gDW^{-1} h^{-1} (Summer). The ETSA was generally lower for the treatment pockets, indicating that PET fibres inhibit microbial activity (Figure 17).

Significant differences were confirmed between control and treatment (KW, chi^2 = 5.1205, p = 0.024). No significant difference was found for the factor "season" (KW, chi2 = 1.8944, p = 0.5946), although the activity is higher in summer.

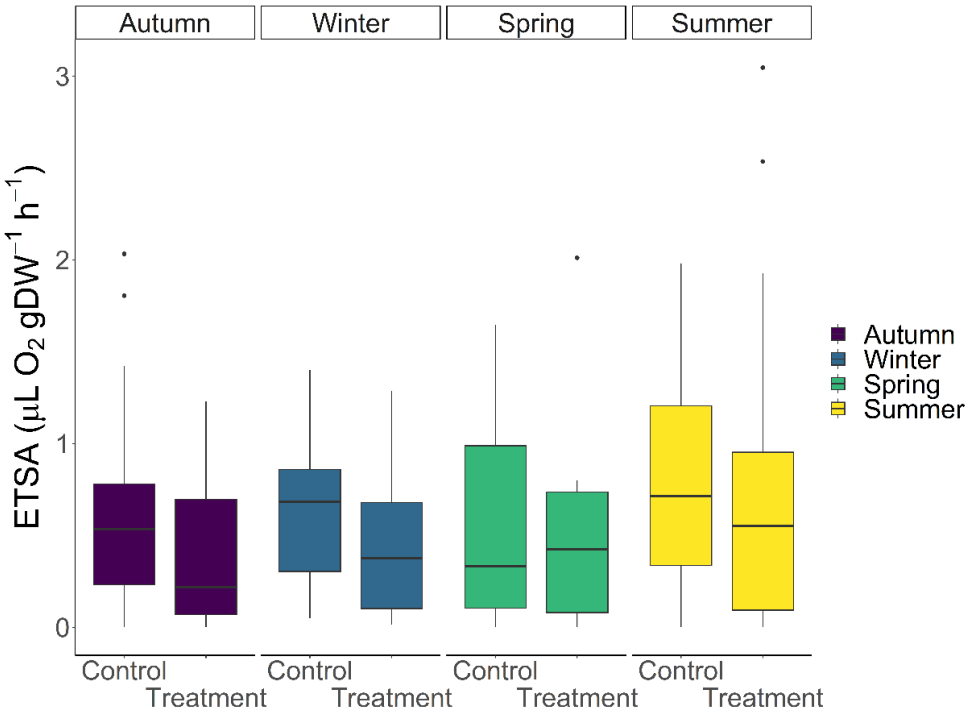


Figure 17: Box-plots indicating variation in ETSA in the control (N=12) and with PET fibres treated (N=12) sediment pockets over seasons from all four locations (KBR, KBB, GRA, and LJU).

The mean ETSA for biofilms on PET sheets ranged from 2.93 ± 2.69 µL O_2 gDW^{-1} h^{-1} (Autumn) to 8.73 ± 6.09 µL O_2 gDW^{-1} h^{-1} (Summer) (Figure 18). The factor "season" was significant for ETSA for biofilm on PET (chi^2 = 13.527, p = 0.0036). Significant lower activity was measured in Autumn compared to summer (Dunn's test: 0.0016).

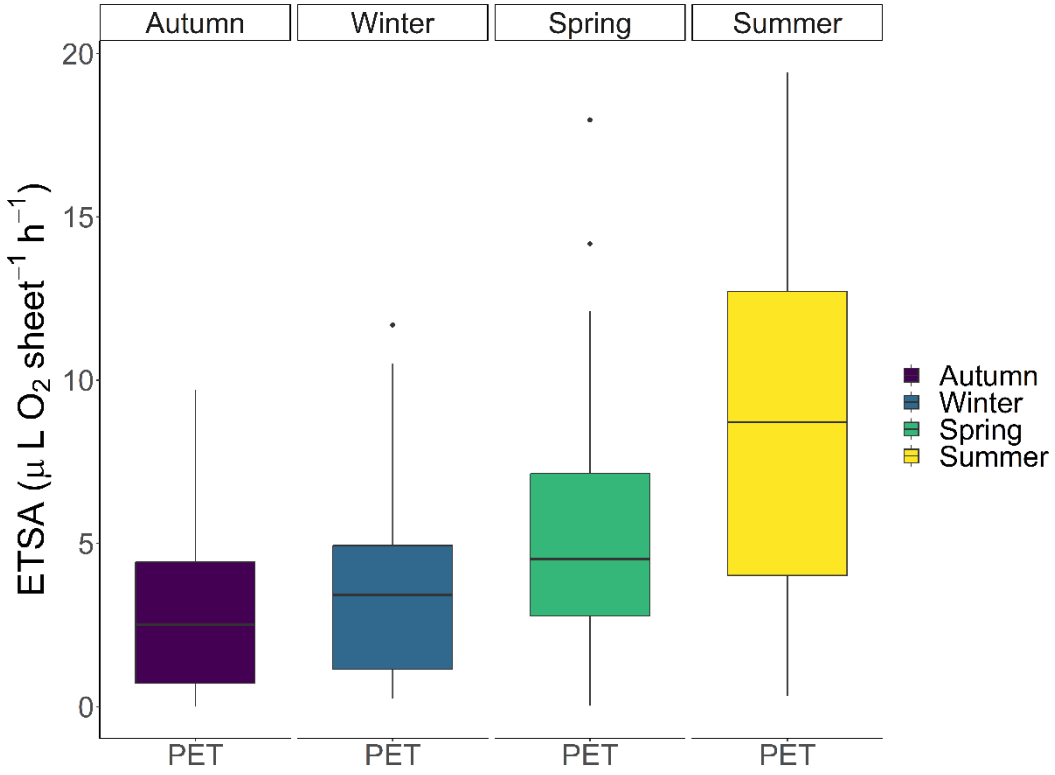


Figure 18: Box-plots indicating variation in ETSA from PET sheets (N = 12) over seasons from all four locations (KBR, KBB, GRA, and LJU).

Overall, the CLPP results revealed that the factor "season" had the most effect on the utilization of substrates (revealing 23 significant substrates) than "treatment", revealing nine significant substrates and four that are almost significant (Table 6). Further, a trend is noticeable for samplings from the autumn and spring, whereas in the sediment pocket containing PET, the variability in the metabolic function was suppressed, while for the winter and summer months, the opposite was true (Figure 19, NMDS stress = 0.1871). For the bacteria growing on PET, metabolic function variability was suppressed for all seasons. The calculated NMDS stress = 0.0436 is close to zero, indicating a good representation of the original data (Figure 20).

The ANOSIM showed significant differences in microbial functioning for both factors: "seasons" (R: 0.06311, p = 0.001) and "treatment" (R: 0.02, p = 0.001). Due to higher R statistics for factor "season", it can be concluded that they have a higher impact on microbial functioning than treatment. The PERMANOVA analysis for individual factors showed a similar trend, where the factor "season" (R^2 = 0.04535, p = 0.001) explains about 4.5 % of the total variation, while factor "treatment" explains about 0.6 % of the total variation (R^2 = 0.00597, p = 0.001). The PERMANOVA results of the combined effects of both factors are close to significant (R^2 = 0.00891, p = 0.068).

Similarly, "season" was also significant (p = 0.001), with a similar R statistic (R_{season} = 0.07799) for PET sheets. The season has a similar impact on the metabolic functioning of the biofilm growing on PET, with R statistics indicating a dissimilarity between tested groups. The PERMANOVA results were also significant (p = 0.001) and indicated that season attributed a total variation of 5.9 % (R^2 = 0.05887).

The analysis revealed a noticeable trend, with "season" appearing as a more influential factor in microbial functioning than the proximity of PET. However, it is essential to highlight that for biofilms on PET, microbial functioning was consistently suppressed across all seasons, while for bacteria from sediment pockets, some variability in metabolic function was observed.

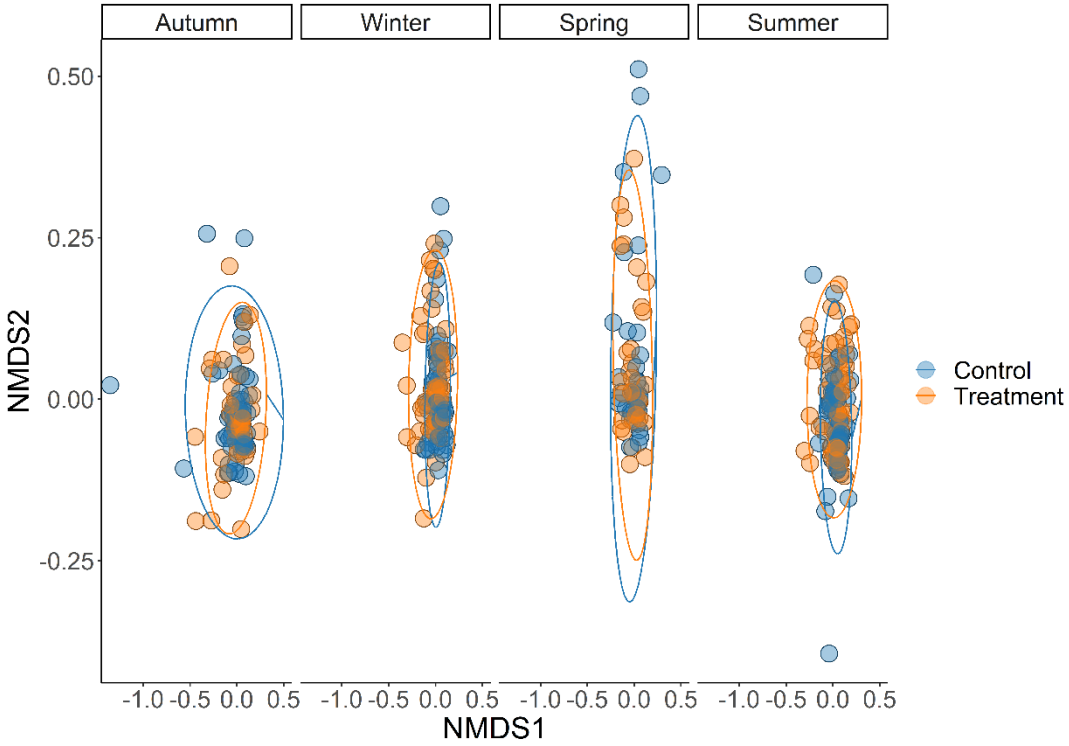


Figure 19: The NMDS done on CLPP seasonal data (N=4 sites x 3 replicates) from seven sampling occasions (autumn=2, winter = 2, spring = 1, and summer=2), depicting the comparison between control and treatment sediment pockets between different seasons. Stress: 0.1871, distance = "canberra".

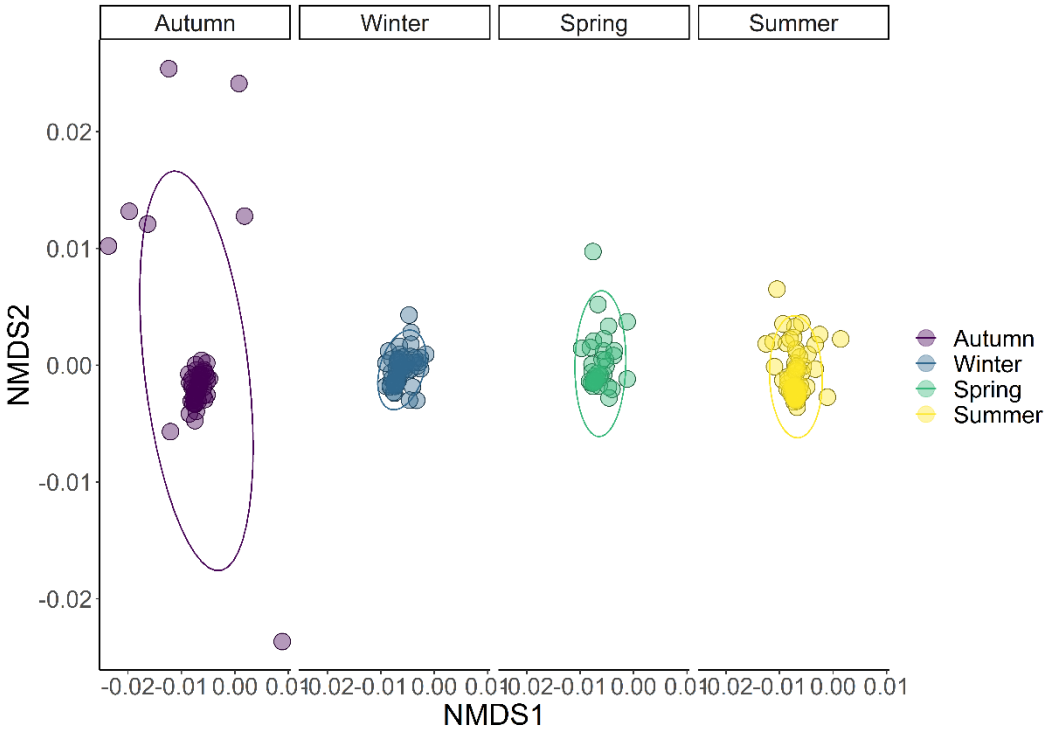


Figure 20: The NMDS using CLPP data, depicting the variations in microbial functioning on PET sheets across different seasons (N=4 sites x 3 replicates) from seven sampling occasions (autumn=2, winter = 2, spring = 1, and summer=2). Stress: 0.0436, distance = "canberra".

The heatmaps are presented for the factor “season” (Figure 21). An overall observation reveals that the control pockets exhibited a higher utilization of substrates in various seasons than the treatment pockets (Figure 21, a). All substrates were utilized efficiently in the control pockets during the summer. In winter, however, the treatment pockets showed a curious pattern, with some substrates utilised efficiently and others not. Conversely, in the case of samples from PET, the metabolic function remained suppressed across all seasons, but its utilization was highest in summer (Figure 21, b).

Both sediment pockets and PET show a trend of the most efficient substrate utilization during the summer.

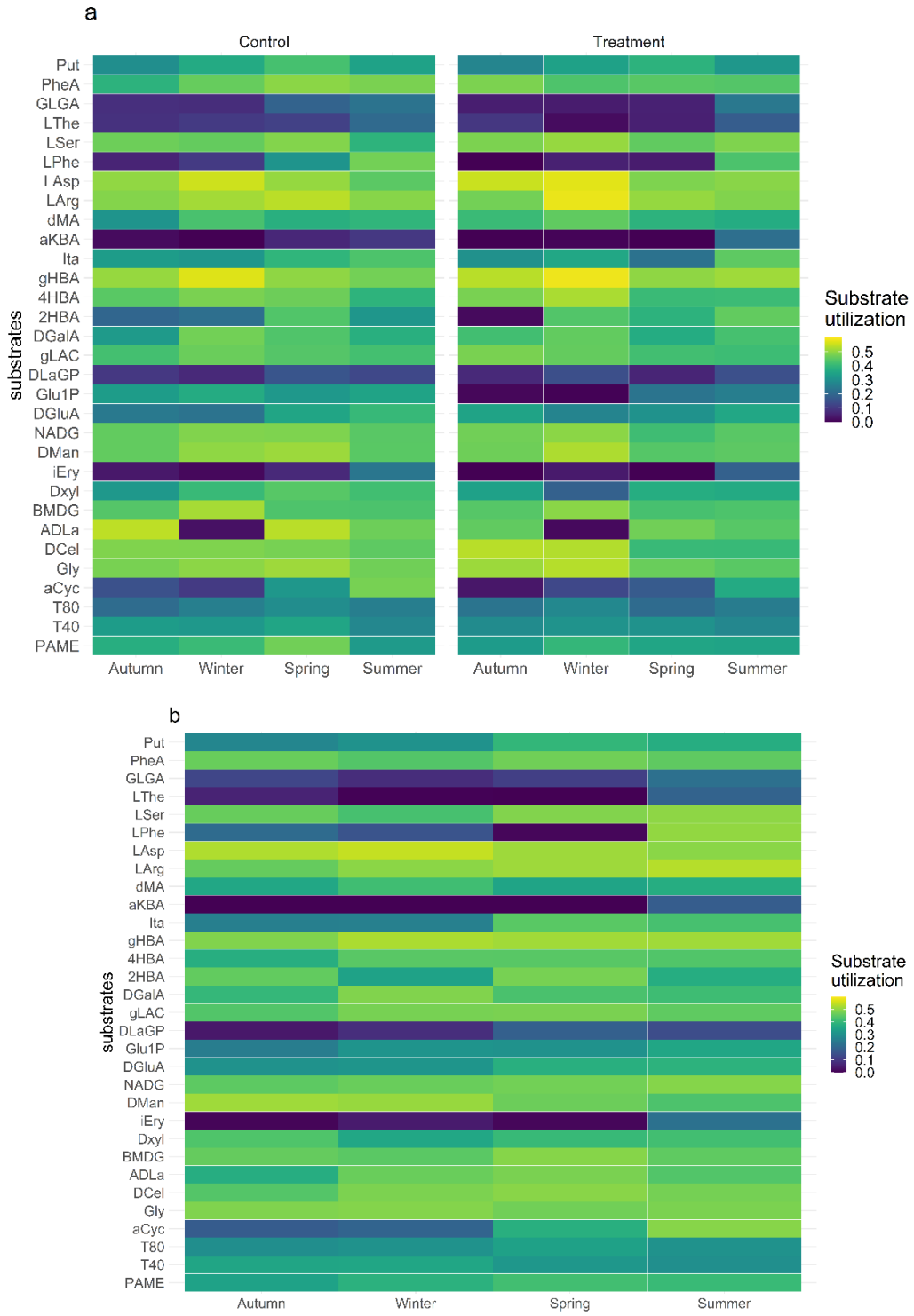


Figure 21: Heatmaps illustrating the intensity of substrate utilization during the seasons for bacteria from control and treatment sediment pockets (a), and PET sheets (b). The y-axis displays the 31 substrates from the Biolog Ecoplates, which were measured spectrophotometrically.

Overall, the factor "season" exhibited the highest number of significant substrates (n = 23), while treatment and a combination of treatment and season showed nine and three significant substrates, respectively (Table 6). Among the substrates, all polymers revealed

that "season" was a significant factor. Substrates Alfa-Cyclodextrin (polymers) and D-Xylose showed significance for both factors and their combination.

Table 6: Comparison of ANOVA variability in the intensity of utilization of substrates from Biolog Ecoplates over seasons for control and treated sediment pockets and PET sheets. Treatment – Control, treatment. Season – Autumn, Winter, Spring, Summer. The sign ":" indicates the combination of effects. Significance codes: '***' ≤ 0.001, '**' ≤ 0.01, '*' ≤ 0.05, '.' ≤ 0.1.

Group of utilised substrates	**substrate**	**abbr**	**Ecoplates label**	**season**	**Treatment**	**Season: Treatment**
Carbohydrates	beta-Methyl-Dglucoside	BMDG	A2	**		
	Pyruvic Acid Methyl Ester	PAME	B1	***		
	D-Xylose	Dxyl	B2	***	***	*
	i-Erythritol	iEry	C2	***	***	
	D-Mannitol	Dman	D2		*	
	N-Acetyl-Dglucosamine	NADG	E2	***		
	D–Cellobiose	Dcel	G1	***		
	Glucose-1-Phosphate	Glu1P	G2	***	.	*
	alfa-D-Lactose	ADLa	H1			
	D, L-alfa Glycerol Phosphate	DlaGP	H2	***	.	
Polymers	Tween 40	T40	C1	***	*	
	Tween 80	T80	D1	***	***	
	Alfa-Cyclodextrin	aCyc	E1	***	***	*
	Glycogen	Gly	F1	***		
Carboxylic and Ketonic Acids	gamma-Lactone	gLAC	A3		.	
	D-Galacturonic Acid	DgalA	B3			
	Gamma- Hydroxy Butyric Acid	gHBA	E3			
	D-Glucosaminic Acid	DgluA	F2	**		
	Itaconic Acid	Ita	F3	***		
	Alfa-Keto Butyric Acid	aKBA	G3	***	.	
	D-Malic Acid	dMa	H3			.
Phenolic compound	2-Hydroxy Benzoic Acid	2HBA	C3			
	4-Hydroxy Benzoic Acid	4HBA	D3	***		
Amino Acids	L-Arginine	Larg	A4			
	L-Asparagine	Lasp	B4	**	*	
	L-Phenylalanine	Lphe	C4	***		
	L-Serine	Lser	D4	***		
	L-Threonine	Lthe	E4	***	*	
	Glycyl-L-glutamic Acid	GLGA	F4	***	***	
Amines/Amides	Phenylethyl-amine	PheA	G4	**		
	Putrescine	Put	H4	***		

Spatial patterns in biofilm response to pollution with PET

The study investigated whether the biofilms from different study sites with different natural characteristics and different intensities of anthropogenic pressures respond differently to pollution with PET.

The mean POM content in the control sediment pockets varied from 7.56 ± 1.48 gPOM $kgDW^{-1}$ (KBR) to 13.58 ± 1.30 gPOM $kgDW^{-1}$ (GRA). Similarly, the mean POM content in the treatment sediment pockets ranged from 6.70 ± 0.51 gPOM $kgDW^{-1}$ (KBR) to 13.68 ± 0.98 gPOM $kgDW^{-1}$ (GRA) (Figure 22).

The statistical analysis revealed significant differences between river sites (KW; chi^2 = 57.233, $p < 0.0001$) but not between treatments (KW; $chi^2 = 0.5473$, $p = 0.46$). Dunn's test further indicated significant differences between KBR and all the other rivers (p_{KBB} = 0.0000, $p_{GRA} = 0.0001$, $p_{LJU} = 0.0001$), as well as between KBB and GRA ($p = 0.0001$). These findings suggest substantial variations in POM content among different river sites, especially highlighting the differences between KBR and the other rivers.

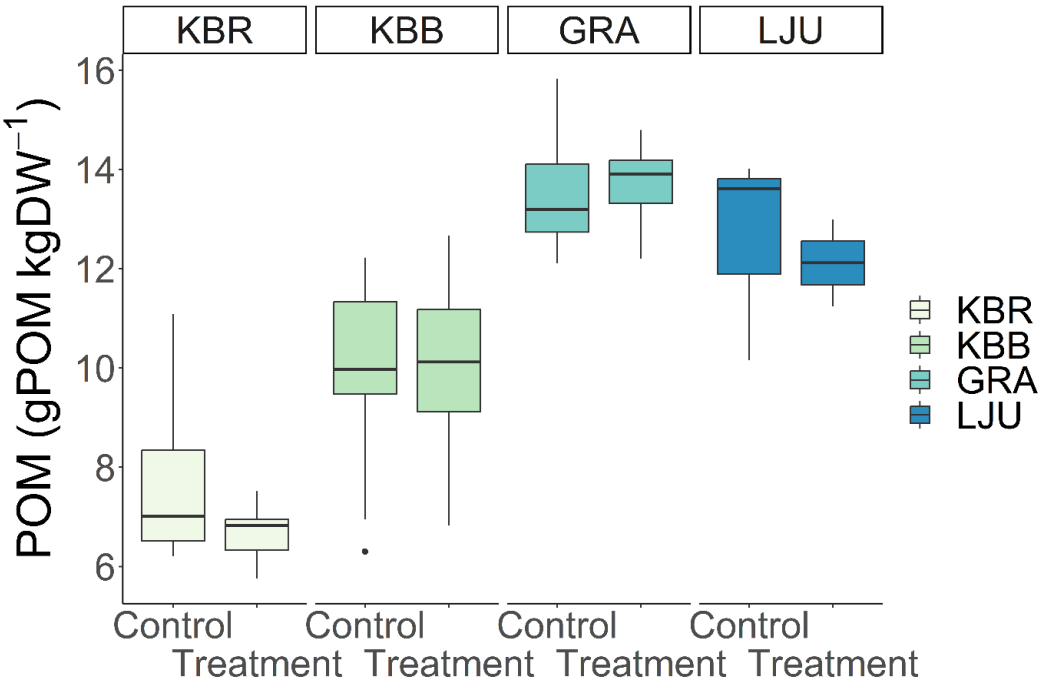


Figure 22: Box-plots indicating variation in POM contents in the control (N=4 sites x 3 replicates) and treatment (N=4 sites x 3 replicates) sediment pockets, comparing all four locations (KBR, KBB, GRA, and LJU).

The mean TPC content in control sediment pockets ranged from 32.16 ± 40.76 µg prot g DW^{-1} (KBR) to 74.30 ± 37.64 µg prot g DW^{-1} (KBB). The mean TPC content in treatment sediment pockets ranged from 18.79 ± 19.04 µg prot g DW^{-1} (KBR) to 80.11 ± 48.16 µg prot g DW^{-1} (LJU) (Figure 23). The statistical analysis indicated a significant difference between rivers (KW; $chi^2 = 51.886$, $p < 0.001$) but not between treatments (chi^2 = 2.0454, $p = 0.15$). Post-hoc Dunn's test revealed significantly lower TPC in KBR than in other rivers ($p_{KBB} = 0.000$, $p_{GRA} = 0.0202$, $p_{LJU} = 0.000$). Additionally, there were significant differences in TPC between KBB and GRA ($p = 0.002$) and between GRA and LJU ($p = 0.0111$). The findings highlight notable variation in TPC among different river sites, with KBR showing lower TPC than other rivers.

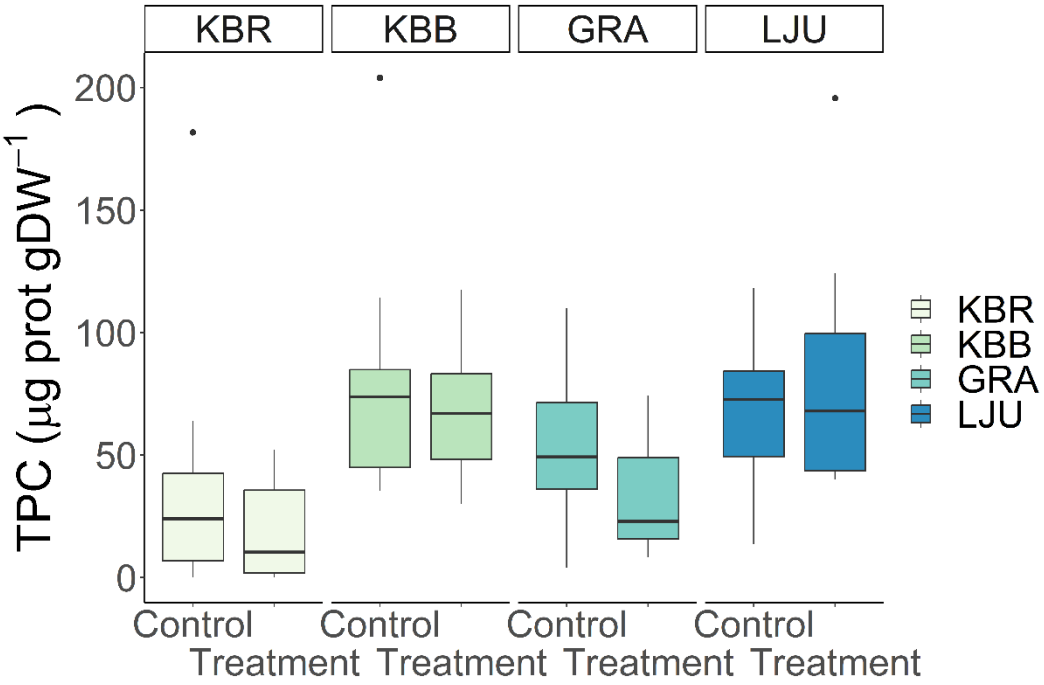


Figure 23: Box-plots indicating variation in TPC contents in the control (N=4 sites x 3 replicates) and treatment (N=4 sites x 3 replicates) sediment pockets, comparing all four locations (KBR, KBB, GRA, and LJU).

The mean TPC ranged between 269.11 ± 354.30 µg prot sheet^{-1} (LJU) to 279.02 ± 158.91 µg prot sheet^{-1} (GRA) (Figure 24). The statistical analysis did not reveal significant differences between rivers (KW; chi^2 = 1.9959, p = 0.5733).

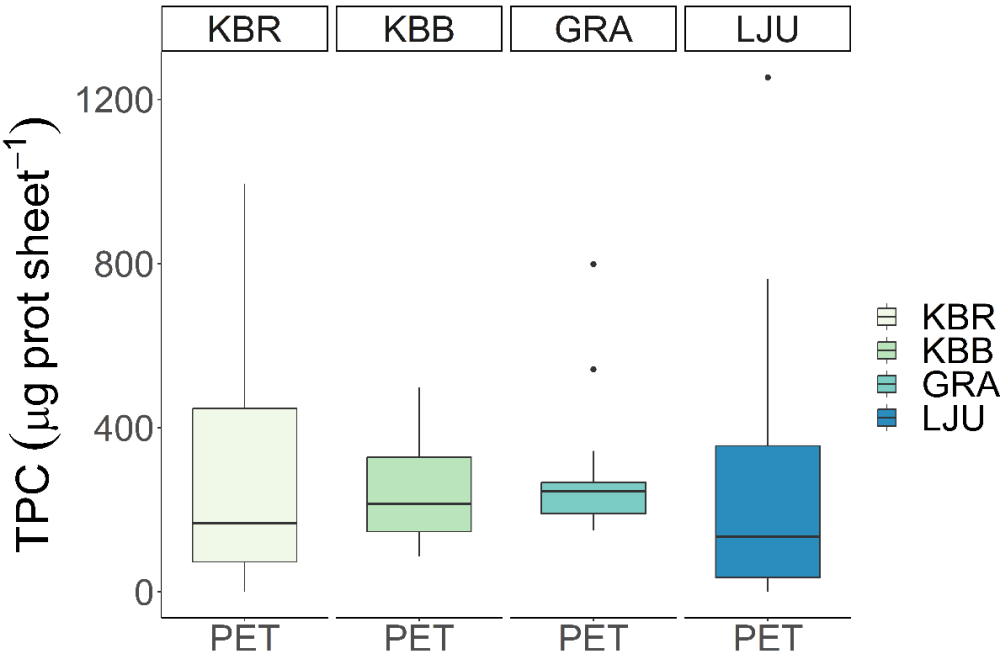


Figure 24: Box-plots indicating variation in TPC contents from PET sheets (N = 4 sites x 3 replicates) comparing all four locations (KBR, KBB, GRA, and LJU).

The mean ETSA ranged from 0.30 ± 0.46 µL O_2 gDW^{-1} h^{-1} (KBR) to 1.04 ± 0.07 µL O_2 gDW^{-1} h^{-1} (KBB) in control sediment pockets and between 0.07 ± 0.08 µL O_2 gDW^{-1} h^{-1} (KBR) and 0.98 ± 0.70 µL O_2 gDW^{-1} h^{-1} (KBB) in treatment sediment pockets (Figure 25). Statistical analysis revealed significant differences between rivers (KW; $chi^2 = 74.211$, $p < 0.001$) and between treatments (KW; $chi^2 = 5.1205$, $p = 0.024$). A post-hoc Dunn's test further indicated that ETSA was significantly lower in treatment than in controls ($p = 0.0118$). Additionally, Dunn's test revealed that ETSA in KBR was significantly lower than KBB and LJU ($p_{KBB} = 0.000$, $p_{LJU} = 0.000$, respectively) but not GRA ($p = 0.1573$). ETSA was also significantly lower in GRA than KBB ($p = 0.0026$) and LJU ($p = 0.0003$).

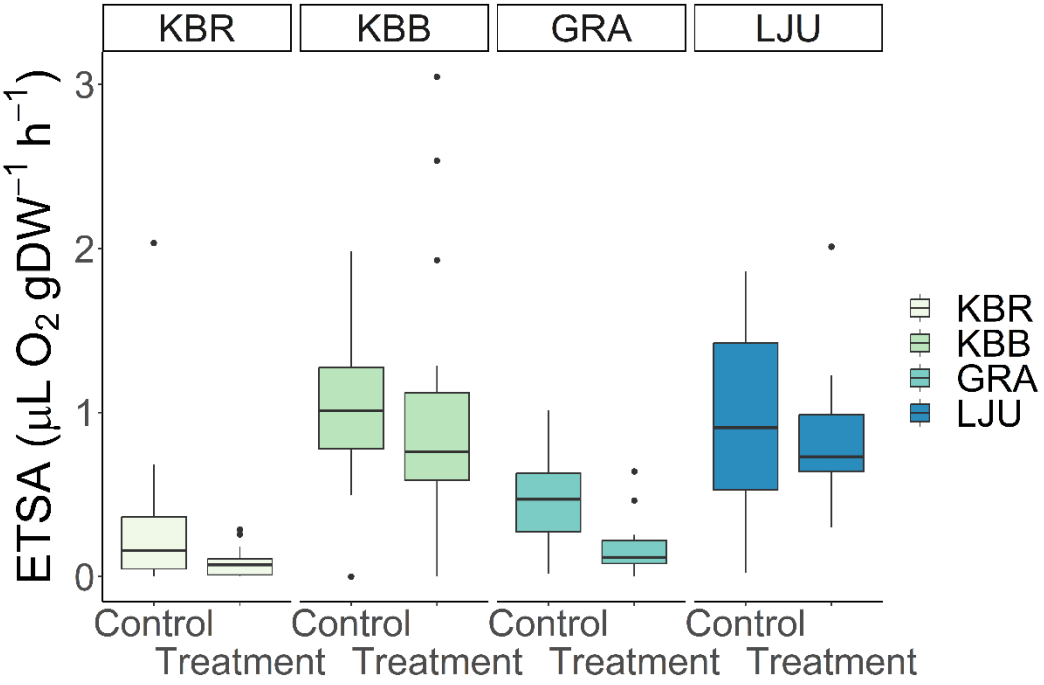


Figure 25: Box-plots indicating variation in ETSA in the control (N=4 sites x 3 replicates) and treatment (N=4 sites x 3 replicates) sediment pockets, comparing all 4 locations (KBR, KBB, GRA, LJU).

The mean ETSA from PET sheets ranged between 4.62 ± 3.89 µL O_2 $sheet^{-1}$ h^{-1} (KBB) to 6.23 ± 6.59 µL O_2 $sheet^{-1}$ h^{-1} (KBR) (Figure 26). Statistical analysis indicated no significant differences in ETSA among biofilms on PET sheets (KW, $chi^2 = 1.1651$, $p = 0.7614$).

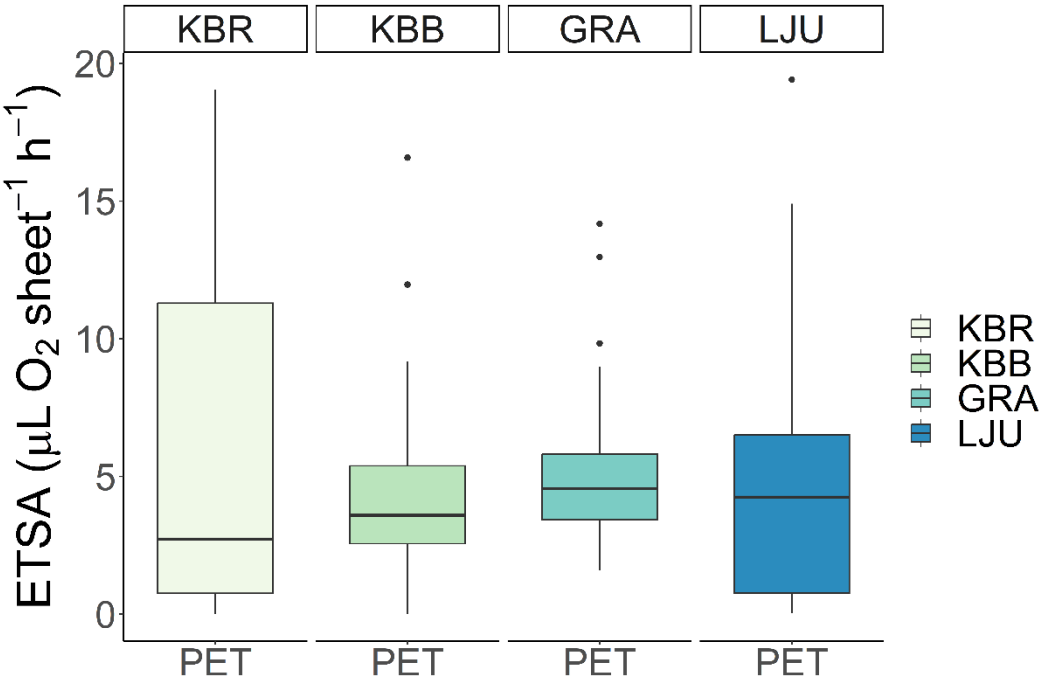


Figure 26: Box-plots indicating variation in ETSA from PET sheets (N = 4 sites x 3 replicates) comparing all four locations (KBR, KBB, GRA, LJU).

The results of the CLPP provide clear evidence of the impact of PET presence in different river locations. Among all the rivers sampled, KBR stands out as the most pristine, exhibiting the highest functional microbial diversity. Conversely, the functional diversity is the lowest in KBB, where sampling occurred a short distance downstream of the WWTP effluent outflow. Microbial metabolic functional diversity in GRA and LJU was also suppressed (Figure 27, NMDS stress = 0.1871). A similar trend is observed for bacteria on PET sheets (Figure 28, NMDS stress = 0.0436), with KBR displaying the highest microbial functional diversity, while the microbial functional diversity is notably lower in the other locations.

The outcomes of both ANOSIM and PERMANOVA analyses reinforce the influence of the "river" factor in significantly shaping the dissimilarity of microbial functioning among the various rivers. ANOSIM's R statistic of 0.2551 indicates substantial dissimilarity between the microbial communities of different rivers ($p = 0.001$). Furthermore, the PERMANOVA result confirms this finding with a high R^2 value of 0.11164 ($p = 0.001$), indicating significant differences in microbial functioning among the rivers, with approximately 11 % of the variation attributable to the differences between the rivers. Additionally, when considering both factors in the PERMANOVA analysis, the factor "treatment" contributes approximately 0.6% ($R^2 = 0.0058$, $p = 0.001$) to the total variation, while the factor "river" explains about 11 % of the total variation ($R^2 = 0.1116$, $p = 0.001$).

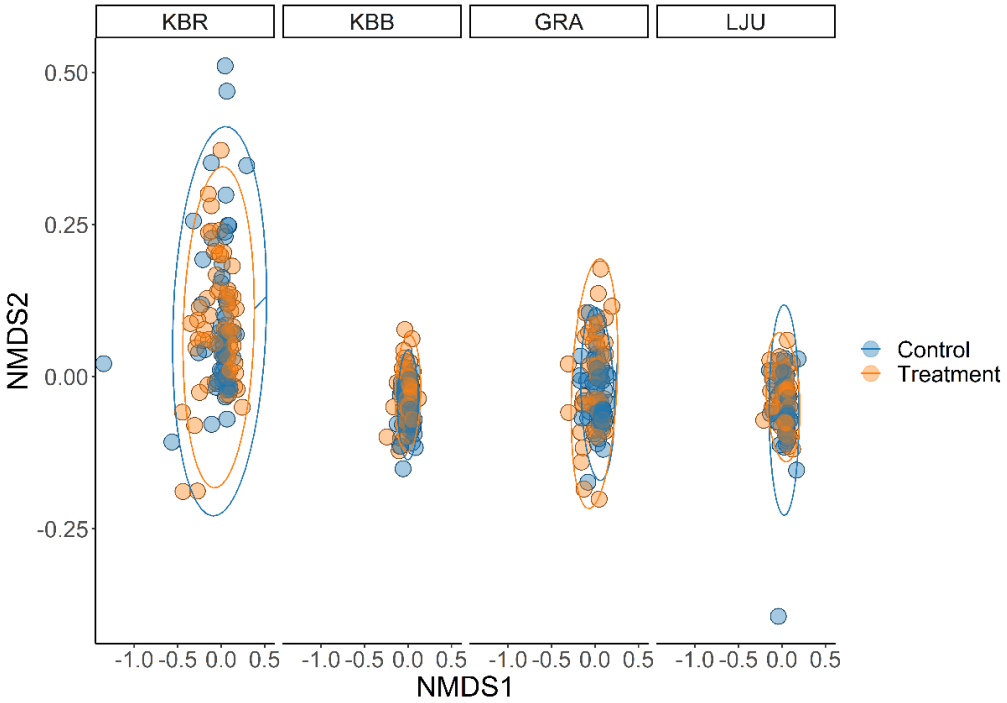


Figure 27: The NMDS done on spatial data set (N= 4 sites x 3 replicates) from 7 sampling occasions (autumn = 2, winter = 2, spring = 1, and summer = 2), depicting the variation in microbial functioning between control and treatment sediment pockets between different rivers. Stress: 0.1871, distance = “canberra”.

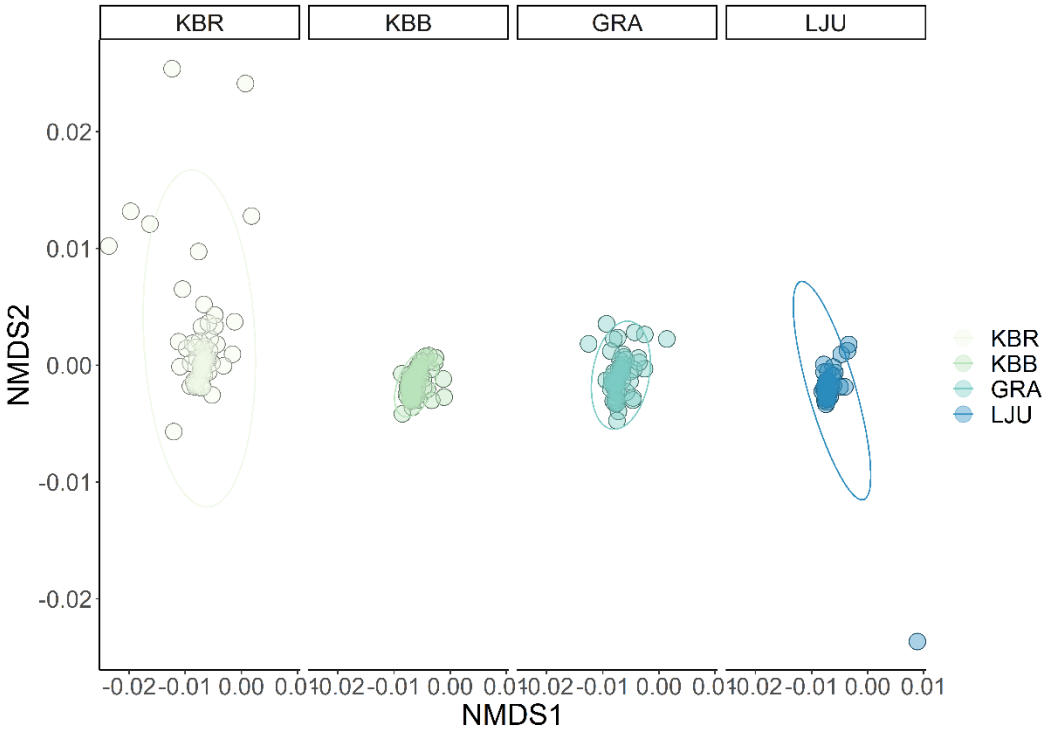
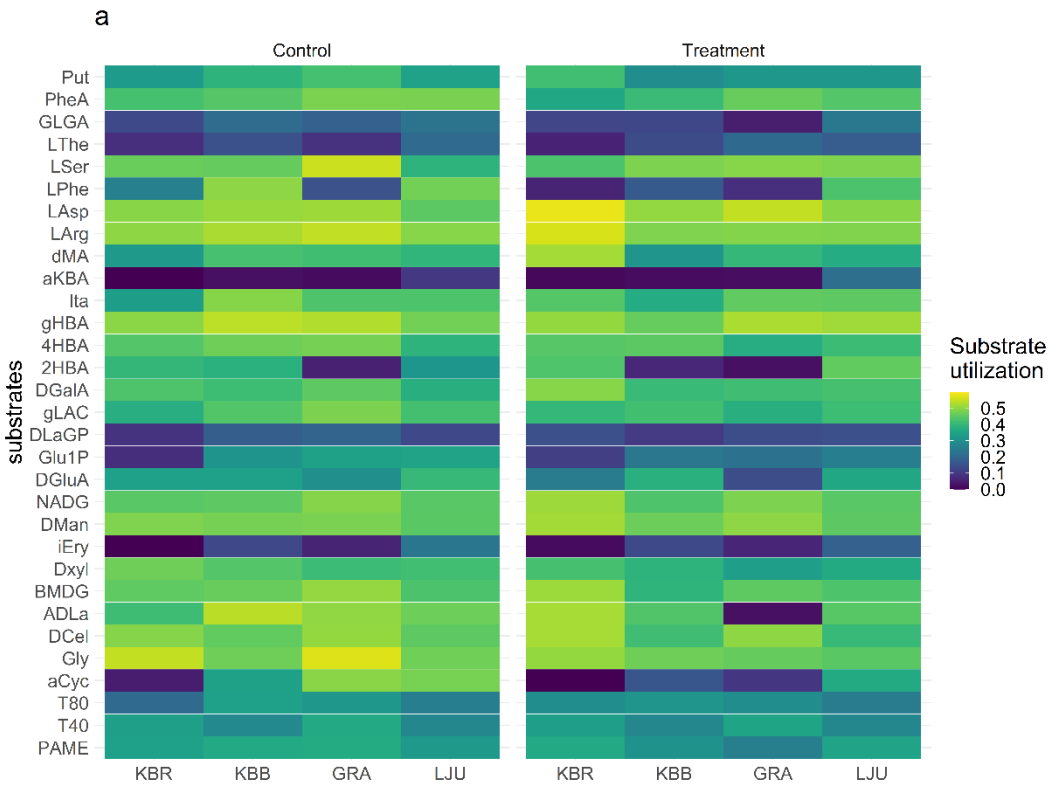


Figure 28: The NMDS using spatial data set, depicting the variations of microbial functioning on PET sheets across different rivers (N sites x 3 replicates) from 7 sampling occasions (autumn=2, winter = 2, spring = 1, and summer=2). Stress: 0.0436, distance = "canberra".

The heatmaps depicting substrate utilization among different rivers (Figure 29) highlight significant differences between bacteria from control sediment pockets and treatment sediment pockets and bacteria from PET sheets. The bacteria from LJU demonstrated excellent substrate utilization capabilities, irrespective of being sampled from control or treatment pockets. Bacteria from the KBB and GRA sites appeared to exhibit proficient utilization when sampled from control pockets but poor utilization from treatment pockets. In contrast, bacteria from KBR seem more proficient when sampled from the treatment pockets than the control pockets.

Regarding bacteria from PET sheets, substrate utilisation was overall weaker in KBR, with only a few substrates poorly utilised while others were utilised more efficiently. KBB and LJU were sites where bacteria could utilise all the substrates effectively. Bacteria from GRA showed similarities with KBR in terms of substrate utilisation.



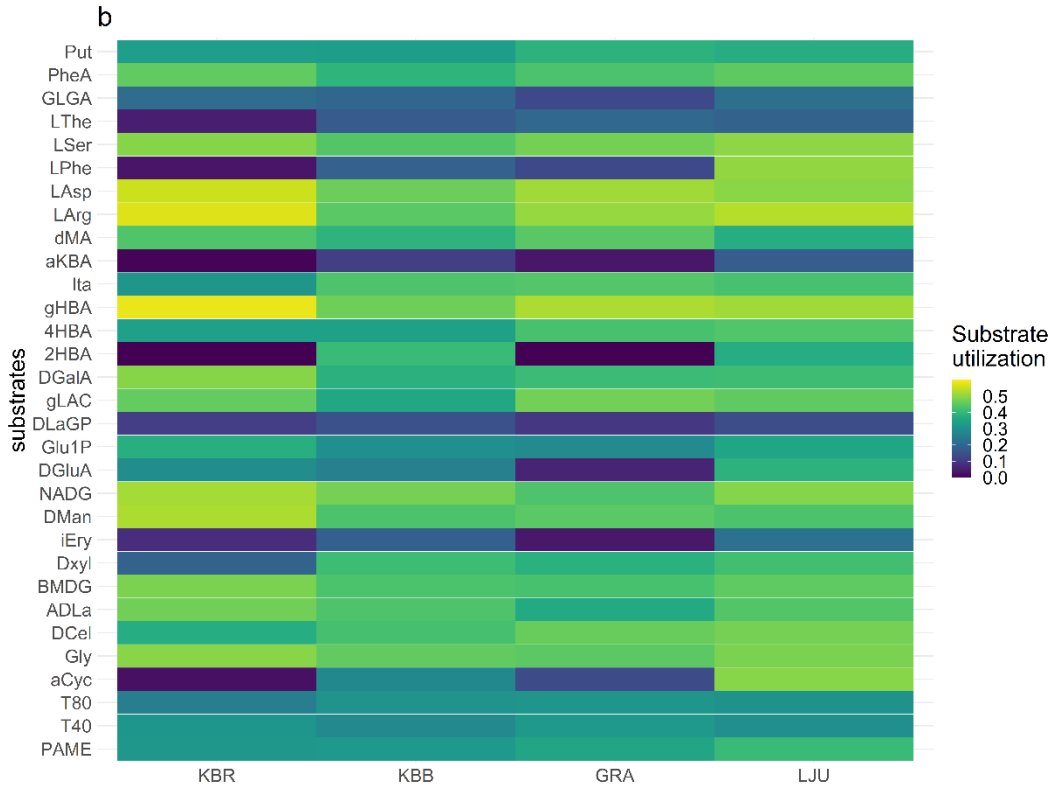


Figure 29: The heatmaps illustrate the intensity of substrate utilization across the rivers for bacteria derived both from control and treatment sediment pockets (a), as well as PET sheets (b). The y-axis displays the 31 substrates from the Biolog Ecoplates, which were measured spectrophotometrically.

Overall, the factor "river" showed the most substantial influence, significantly affecting 26 substrates at a high significance level (Table 7). The factor "treatment" displayed significance for nine substrates. Similarly, the combined effect of both factors was significant for nine substrates. Notably, four substrates did not show significance for either factor, whether it was treatment, river, or their combined effects: beta-methyl-dglucoside, gamma-lactone, D-glucosaminic acid, and L-arginine, although the last two ones were close to significance. Interestingly, glycogen (polymers) exhibited a significant effect when both factors were combined but not when considering individual factors.

Table 7: Results of ANOVA for spectrophotometric measurements based on Biolog Ecoplates. Treatment – presence or absence of PET; river – KBB, KBR, GRA, LJU. The sign ":" indicates the combination of effects. Significance codes: '***' ≤ 0.001, '**' ≤ 0.01, '*' ≤ 0.05, '.' ≤ 0.1.

Group of utilised substrates	**substrate**	**abbreviation**	**Ecoplates label**	**River**	**Treatment**	**River: Treatment**
Carbohydrates	beta-Methyl-Dglucoside	BMDG	A2			
	Pyruvic Acid Methyl Ester	PAME	B1	***		
	D-Xylose	Dxyl	B2	***	***	.
	i-Erythritol	iEry	C2	**	**	
	D-Mannitol	Dman	D2	***	*	*
	N-Acetyl-Dglucosamine	NADG	E2	**		

	D–Cellobiose	Dcel	G1	***		
	Glucose-1-Phosphate	Glu1P	G2	***		
	alfa-D-Lactose	ADLa	H1	***		.
	D, L-alfa Glycerol Phosphate	DlaGP	H2	***		*
Polymers	Tween 40	T40	C1	***		*
	Tween 80	T80	D1	***	***	
	Alfa-Cyclodextrin	aCyc	E1	***	***	
	Glycogen	Gly	F1			*
Carboxylic and Ketonic Acids	Gamma-Lactone	gLAC	A3			
	D-Galacturonic Acid	DgalA	B3	***		.
	Gamma- Hydroxy Butyric Acid	gHBA	E3	***	*	**
	D-Glucosaminic Acid	DgluA	F2	.	.	
	Itaconic Acid	Ita	F3	***		
	Alfa-Keto Butyric Acid	aKBA	G3	***		
	D-Malic Acid	dMa	H3	***		
Phenolic compound	2-Hydroxy Benzoic Acid	2HBA	C3	***		*
	4-Hydroxy Benzoic Acid	4HBA	D3	*		
Amino Acids	L-Arginine	Larg	A4		.	.
	L-Asparagine	Lasp	B4	***	**	
	L-Phenylalanine	Lphe	C4	***		
	L-Serine	Lser	D4	***		
	L-Threonine	Lthe	E4	***	**	
	Glycyl-L-glutamic Acid	GLGA	F4	***	***	*
Amines/Amides	Phenylethylamine	PheA	G4	***		*
	Putrescine	Put	H4	***		**

Temporal variation in carbon isotope composition ($\delta^{13}C$) among rivers

Isotope ratio mass spectrometry (IRMS) was used to determine the isotopic composition of carbon ($\delta^{13}C$), providing reliable information on potential surface deterioration (Lucas et al., 2008).

The reference PET material exhibited a value of -28.9 ‰ (Control). The measured PET mean $\delta^{13}C$ values in Summer 2020 ranged from -28.58 ± 0.11 ‰ (KBR) to -28.46 ± 0.19 ‰ (LJU). In Summer 2021, the PET mean $\delta^{13}C$ ranged from -28.65 ± 0.07 ‰ (GRA) to -28.43 ± 0.12 ‰ (KBR). Overall, all of the measured samples have higher values than the control, indicating some level of degradation (Figure 30). Notably, $\delta^{13}C$ values increased in KBR and KBB with time, while in GRA, they decreased in Summer 2021 compared to Summer 2020. For samples from LJU, the $\delta^{13}C$ value remained similar in both years.

Given the non-normal distribution of the data (Shapiro's test < 0.05), the potential difference between different pre-procedures was initially assessed but found to be non-significant (KW; $p = 0.8948$). Consequently, the data were pooled to enhance reliability due to the increased sample size. Even after pooling, no significant differences were observed between the two sampling seasons (Summer 2020 and 2021) or between rivers.

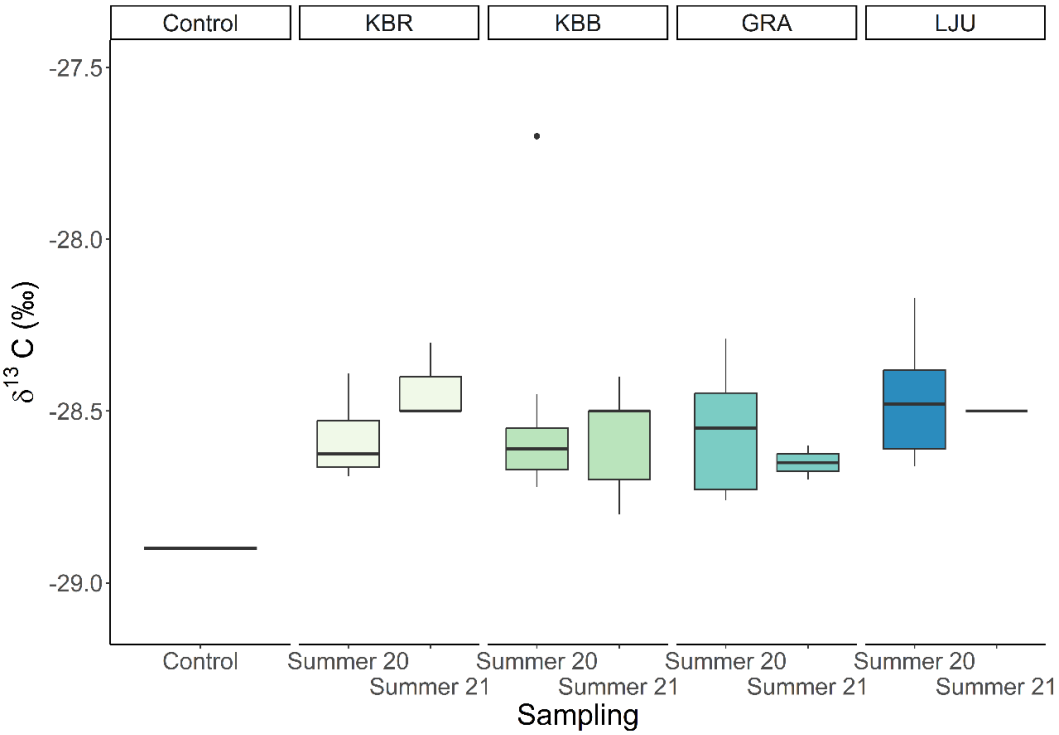
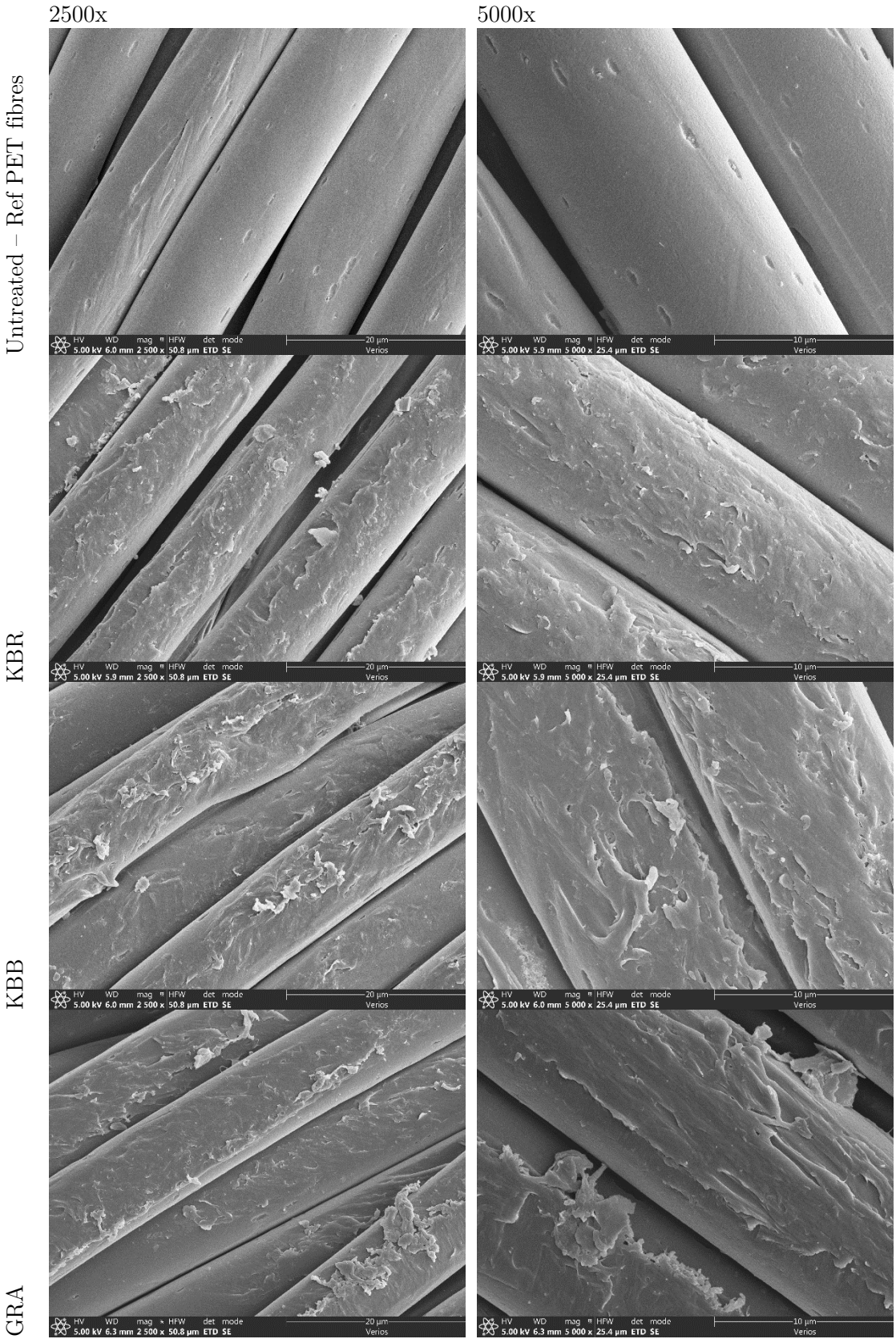


Figure 30: Boxplot of isotopic analysis on Summer 2020 and 2021 for all rivers (N=2 years x 4 rivers x 2 processing methods x 3 replicates) and a separate control.

Surface deterioration of PET during exposure to natural conditions

The untreated reference PET fibres appear smooth with minor holes and some straight lines visible under 2500x and 5000x magnification (Figure 31). In contrast, PET fibres from all four locations (KBR, KBB, GRA, and LJU), exposed to environmental conditions for two years, show obvious signs of damage compared to untreated material. The treated fibres also show surface damage with shallow indentations, with some sections appearing on the verge of breaking off (KBR). When observed at 2500x magnification, damage is already apparent but becomes even more pronounced at 5000x magnification. The microbiome is not visible since the samples were washed before fixing them for SEM. Nevertheless, it is reasonable to assume that the biofilm caused the damage.


2500x
5000x
Untreated – Ref PET fibres
KBR
KBB
GRA

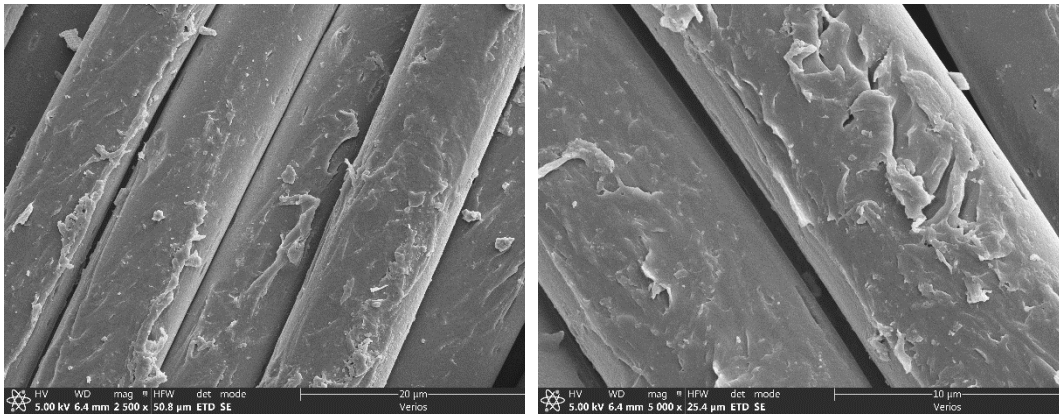


Figure 31: SEM photgraphs showing the untreated PET fibres and treated PET fibres from all different locations (KBR, KBB, GRA, and LJU), after two years under environmental conditions. Photo credits: Zoran Samardžija, IJS.

Discussion

In this comprehensive two-year field experiment using artificial substrates, the impact of PET fibres on hyporheic biofilm structure and function across diverse river systems was investigated. Through assessments of total microbial biomass (TPC), microbial activity (ETSA) and community-level physiological profiling (CLPP), the adaptability of hyporheic biofilms, as well as their overall fitness, while exposed to pollution with PET fibres over a prolonged period was explored. My hypotheses focused on the general prediction that PET fibres inhibit biofilm growth and shift the community metabolism due to changes in substrate and leaching of toxic substances, leading to reduced TPC, decreased ETSA, and altered CLPP. I expected to see variations in biofilm fitness and metabolic activity among different river systems, different effects of PET pollution during different seasons and the signs of degradation on exposed PET fibres.

Environmental characteristics of the study sites

The study river sites exhibited distinct characteristics shaped by their locations and surrounding environments (Figure 5 and 7). The KBR study site, situated near the river spring and surrounded by forests, featured a limestone and dolomite gravel-dominated riverbed with relatively shallow water depth. However, its proximity to hiking trails and mountain huts raised concerns about potential microplastic (MP) contamination (Matjašič et al., 2023). In contrast, the KBB study site, 25 km downstream from the KBR study site and next to the WWTP effluent and the confluence with the Sava River, presented a contrasting picture with its gravel-dominated riverbed composed of various clastic rocks and a larger catchment area with high population density and industrial activity. The LJU study site exhibited the highest mean and recorded water levels, necessitating rescheduling or being unable to sample due to dangerous conditions. Ljubljanica, a typical karstic river, flowed through the country's capital before merging with Sava, with its upstream catchment consisting primarily of agricultural land. Similarly, the Gradaščica, a left tributary to the Ljubljanica, is heavily influenced by its regulated streambed and flow through the capital.

Discharge data reveals significant hydrological variability between the sites and the two study years (Figure 6). KBR, GRA, and KBB all exhibited a moderate discharge rate (3-5 m^3 s^{-1}), while LJU displayed a variable discharge rate exceeding 45 m^3 s^{-1}, signifying its dynamic and robust water flow. High water flow was present throughout the year 2021. Similarly, the mean surface water temperature assessment highlighted the differences in

thermal characteristics among the sites (Figure 6, Table 4). The KBR site had the lowest mean surface water temperature, and the LJU site had the highest during the entire period when the temperature data loggers were active.

Similarly, the lowest mean hyporheic temperature was recorded at the KBB site, while the warmest was recorded at the LJU site (Table 4). Conductivity measurements revealed distinct dissolved ion concentrations, with KBB and LJU showing similarly high values, followed by GRA and KBR with the lowest values. Conductivity is linked with hydrological basis and pollutants (Chusov et al., 2014).

The oxygen (O_2) content and saturation levels provided valuable information regarding water quality and ecological conditions. The KBR site had the highest oxygen content and saturation levels, indicating well-oxygenated conditions, likely due to the pristine nature of the site and efficient oxygen exchange processes. In contrast, LJU displayed the lowest oxygen content and saturation levels, suggesting possible oxygen depletion and impaired water quality. The solubility of oxygen also decreases with increasing temperatures. The DOC and N_{tot} levels were lowest in KBR. The highest level of DOC was recorded in LJU, while the highest levels of N_{tot} were recorded at the KBB site.

In conclusion, variations in hydrological parameters (surface water and hyporheic temperatures, conductivity, oxygen saturation, DOC and TN content) were observed among the experimental sites, reflecting their distinct characteristics and environmental influences and likely hosting different hyporheic biofilm communities. These findings suggest that the KBR site has the lowest chemical burden, while the KBB and LJU sites are the most chemically impacted.

Variations in hyporheic biofilm response to PET pollution at Kamniška Bistrica Beričevo study site (KBB) over two years

The influence of PET fibres on microbial functioning and substrate utilization in the sediments from the experimental pockets was evident. Variations in environmental factors over seasons during two years and long-term PET fibre exposure affected microbial biomass and microbial activity, generally diminishing metabolic capabilities. Notably, the mean particulate organic matter (POM) content in the control pockets varied significantly with year, with the lowest in winter 2021 and the highest in summer 2020. Further, the proximity of PET fibres consistently showed a reduced POM content in the treatment pockets. The TPC in the control pockets also varied, reaching a minimum in Summer 2020 and a maximum in Summer 2021. While the "treatment" factor lacked statistical significance, TPC values varied in treatment pockets. Mean ETSA within control and treatment sediment pockets exhibited non-significant variations over time. Nevertheless, ETSA levels in treatment pockets were generally lower than in control pockets.

Analysis of similarity (ANOSIM) and PERMANOVA underscored the dominant influence of years on microbial functioning, surpassing the impact of treatment. Heatmaps depict a steady increase in the ability of bacteria to utilise the substrates. For example, in Autumn 19 and Summer 20, all substrates are utilized in the control pockets, adding Spring 2020 for the treatment pockets. Bacteria under PET stress exhibited heightened substrate utilization, implying enhanced metabolic capabilities within the microbial community exposed to PET stress. Temporal factors also had a noticeable effect on substrate utilization and levels of carbohydrates and amino acids.

The presence of PET fibres consistently inhibited the variability of microbial function, confirming a clear trend over the two-year monitoring period, suggesting that the introduction of PET into the environment may negatively affect microbial processes. Significant differences in microbial functioning were also observed between treatments and years, highlighting the pivotal roles of temporal changes in general in shaping microbial communities and the important role of pollution with PET. R-values show the differences

between groups, indicating distinct microbial functional patterns associated with different treatments and years. The differences between years emerged as the primary factor influencing microbial functioning, outweighing treatment effects. This observation underscores the substantial impact of temporal changes, potentially driven by long-term environmental conditions or microbial succession, on shaping microbial communities.

Interestingly, a similar trend was observed in both sediment and treatment pockets, further highlighting the changing nature of hyporheic biofilms over time, most probably driven by changes in environmental factors, such as discharge, temperature and resource availability. Prolonged exposure to PET sheets induces notable changes in microbial communities, leading to increased substrate utilization and potential adaptation to PET-associated compounds.

The results also indicate a capacity of bacteria to expand their utilization capabilities in response to limited food resources. This adaptive response reflects the dynamic nature of the microbial communities and their ability to optimise substrate utilisation for survival and growth. These findings underscore the critical influence of seasonal and annual variation on microbial community dynamics, potentially amplified by interacting stressors.

Existing studies agree with the importance of temporal scale on biofilms in freshwaters. A year-long stream and river water sampling campaign near Athens (GA, USA) unveiled temporal changes driving changes in downstream microbial communities, impacting diversity, composition, and nutrient cycling. Environmental disruptions also influence these trends, emphasizing the need for further research on ecosystem dynamics (Hassell et al., 2018). In a French Natural Regional Park, planktonic microorganisms were sampled monthly over two years, revealing distinct seasonal patterns and community shifts through DNA analysis (David et al., 2021).

Similarly, a study involving a 36-month sampling campaign of six streams, draining three catchment types, showed consistent seasonal patterns, with physicochemical factors like light, temperature, pH, dissolved oxygen, and nutrients linked to temporal community changes (Gautam et al., 2022). A three-year field study in the Little Miami River drainage basin (USA) observed seasonal changes in microbial community structure, correlating with stream discharge, temperature, and land development (Sutton & Findlay, 2003). Lauber et al. (2013) demonstrated that soil bacterial community composition evolves and is influenced by land-use types.

Additionally, higher ETSA levels were previously associated with shallow hyporheic zones adjacent to agricultural or urban land (Debeljak et al., 2017), and nutrient influx contributed to higher respiration rates, microbial biomass, and activity (Gulis et al., 2004). Even under similar environmental conditions and species pools, community variations occur due to the order of species colonization. Local- and regional-scale heterogeneity, characterised by redox gradients and nutrient availability, also emerged as important drivers of biodiversity (Nemergut et al., 2013).

It is worth considering the potential influence of the COVID-19 pandemic and associated regulations on factors such as POM, TPC, and ETSA in the study sites. The sampling location beneath the wastewater treatment plant's effluent and remote work arrangements during specific sampling periods may have impacted the variables and should be considered when discussing the results.

Seasonal and spatial variation in hyporheic biofilm response to PET pollution across 4 study river sites

Overall, the results indicated variation in microbial functioning among seasons, with certain seasons showing higher biomass, microbial activity and substrate utilization. However, the presence of PET fibres did not lead to significant differences in POM content and microbial biomass between control and treated sediments, although generally, the

measured variables were consistently lower in treated sediments compared to controls. However, microbial activity was significantly higher in control and treatment pockets. Additionally, there were variations in POM content, microbial biomass, microbial activity, and substrate utilization patterns among different river sites. The KBR site was consistently distinct, with lower POM content, microbial biomass, and enzyme activity than LJU, KBB and GRA sites. Similarly to seasons, the study sites were a significant factor affecting biofilm biomass and activity rather than pollution of sediments with PET fibres.

Interestingly, when looking at the characteristics of biofilms growing on PET fibres, variation was observed over the seasons but not between the sites. It seems that PET fibres are substrates supporting similar biofilm communities regardless of the surrounding environment and surrounding biofilm communities that change with the season (temperature, POM content), but this has to be investigated further by molecular approaches to be confirmed.

Seasonal variations across larger spatial scales

Despite observed seasonal differences, seasons had little impact on POM and microbial biomass but significantly affected microbial activity. A slight inhibition was observed for variables between biofilms in control and treated sediments, while for PET sheets, all measured variables showed some seasonal variation.

A study by Staley et al. (2015) confirmed significant bacterial seasonal variation, with minimal influence of spatial distance, but did so based on water samples. However, they found that microbial sediment communities significantly influenced the microbial community structure in the water column (Staley et al., 2015). Similarly, Brugger et al. (2001) revealed seasonal patterns. They found the highest abundance, production and ectoenzymatic activity in autumn and summer and the lowest in winter for Austria's oligotrophic alpine river Enns. However, in both studies, spatial variation was analysed longitudinally in the same river, while in the present study, geomorphologically diverse rivers were sampled.

Biofilm development and characteristics were found to depend on temperature, substrate characteristics and available food resources (Rao et al., 1997; Gulis et al., 2004; Sauer et al., 2022), which change seasonally. Furthermore, hydrological disturbances in streams, such as floods or high discharge events, can indirectly affect nutrient uptake by scourging stream biofilm communities (Mueller Price et al., 2015; Balik et al., 2021). For example, the content of POM in the hyporheic zone was shown to be higher in summer and autumn but lower in spring (Storey & Williams, 2004). These organic substrates can come from catchment vegetation like leaves, flowers, and fruit, and they can enter the stream through direct fall or lateral input from flooding or winds. Particulate organic matter typically peaks during autumn in temperate regions due to the input of leaves shed during that season (Pusch et al., 1998). Although significant differences between POM levels were not identified in the present study, a trend is visible, suggesting a lower level of POM in the control compared to treatment pockets in autumn and summer. In contrast, higher amounts of POM were found in the control compared to treatment in winter and spring, which may result from different quality of DOC (Hendricks, 1996).

Also, no statistical differences were revealed for TPC between control and treated pockets. However, a general trend exists where the mean TPC is lower in treated pockets. A study by Franken et al. (2001) reported that the sediment protein content was highest in places with high DOM, water nutrient concentrations, and temperature. Total protein content is a reasonable estimate for bacterial biomass and was lower in sediments exposed to PET. This phenomenon was confirmed in an extensive systematic review by Gerassimidou et al. (2022), showing that 150 food contact chemicals may be leaching from

PET bottles, e.g., antimony (Westerhoff et al., 2008). The chemicals or their compounds could be toxic to bacteria present and could inhibit their biomass.

The lack of significant differences in particulate organic matter (POM) and biofilm biomass (TPC) between the seasons was most likely because clear seasonal patterns are blurred in the hyporheic zone due to its hydrogeomorphological complexity in comparison to rivers and streams (Boulton et al., 1998; Boulton et al., 2010). However, microbial aerobic respiration correlates highly with available oxygen (Storey & Dudley Williams, 2004). For example, Simčič and Brancelj (2009) determined that ETSA in sediments primarily depends on the amount of oxygen, which increases early in spring due to the mixing of lake water. ETSA decreases in summer when oxygen decreases in the hypolimnion. Germ and Simčič (2011) also found the highest levels of ETSA in lake sediment in July and September.

While the oxygenation of lakes can vary widely depending on their size, depth, and environmental conditions, rivers generally have a higher oxygenation rate due to their flowing nature. In the case of the HZ, it also depends on the hyporheic exchange. Simčič and Mori (2007) explored the intensity of mineralization in a pre-alpine river and found that ETSA levels remained consistent between sampling dates. However, in this study, ETSA was significantly lower in the treatment than in the control pockets. As ETSA is highly correlated to available oxygen (Storey & Dudley Williams, 2004), it seems that PET sheets altered the hyporheic exchange, the primary source of oxygen coming to the HZ (Boulton et al., 2010; Nogaro et al., 2010) acting as a subsurface physical clogging agent, due to physical characteristics of the PET sheet used - thick wowing. Internal or subsurface clogging occurs when fine sediment gets trapped within the gravel framework through various processes. This clogged layer often appears in the sub-armour layer and is called depth filtration. It is not visible from the surface but is extensively studied, given its long-term effects (Dubuis & De Cesare, 2023). The microbial activity response was previously linked to depend on resource supply, e.g., oxygen, nitrate, and DOC (Nogaro et al., 2010; Nogaro et al., 2013).

Substrate utilization (CLPP) depends on the season, being most efficient in summer in both control and treatment pockets. Although the effect of treatment was significant, explaining about 0.6 % of the variation in total substrate utilization, seasons have a more significant effect, explaining about 4.5%. Boivin et al. (2007) investigated the algal-bacterial interactions in a gradient of metal-contaminated natural sediments. They did not find a robust correlation between bacterial community composition and metal content, but they linked the changes in CLPP to the oxygen concentration of the water layer above the sediment and with the clay content.

In other studies, Liang et al. (2022), who examined the genetic structure of the microbial community (DNA analysis), observed seasonal fluctuation, which was highest in summer and lowest in spring. Their results suggest that bacteria, characterised by fast growth, high food demands and a short generation time, can maintain viable populations in highly dynamic and polluted environments. They can also handle stress as long as they can complete their lifecycle in between two periods of extreme stress (Klok & Kraak, 2008). However, although the biomass appears unaffected, species diversity varies (Liang et al., 2022). Zhang et al. (2019) also reported a higher variation, but in this case, seasonal variation rather than different pollution sources. As they also used DNA analyses, the next logical progression would be to conduct DNA analyses on hyporheic samples.

It is essential, however, to highlight that for biofilms in proximity to PET, microbial functioning was consistently suppressed across all seasons, while for bacteria from sediment pockets, all measured values were generally higher than in the treated pockets (POM, TPC, ETSA, CLPP). PET was found to leach over 150 food contact chemicals (Gerassimidou et al., 2022), which can explain the inhibition of microbial functioning. Furthermore, Miao et

al. (2019a) reported that MP decreases the richness and diversity of biofilms in comparison to biofilms formed on natural substrates.

Significant differences between seasons were observed for bacteria on PET sheets. Microbial biomass (TPC) was significantly higher in summer than in winter, and ETSA was significantly higher in summer than in autumn. This finding suggests PET sheets may have trapped higher-quality organic matter when nutrient levels were high (Summer), creating a more favourable environment for bacteria with increased nutrient availability (Franken et al., 2001), which can lead to higher bacterial biomass and increased bacterial activity.

Spatial patterns in hyporheic biofilm response to pollution with PET fibres

The significantly lower POM, TPC and ETSA values observed for the treatment sediments compared to control sediments at most sites indicate that PET suppresses the microbial community's function.

The amount of POM indirectly influences the abundance, biomass and activity of bacteria (Franken et al., 2001). Given that the POM was significantly lower at KBR, the microbial biomass and activity were expected to be significantly lower at KBR compared to other sites. However, significant differences occur not only when comparing the pristine site (KBR) to other sites but also when comparing sites affected by WWTP effluent (KBB and LJU) and those heavily regulated (GRA).

Studies suggest that microbial communities already exposed to specific materials or pollutants tend to have established metabolic pathways for degradation (Matjašič et al., 2021b). While the KBB and LJU environment is markedly influenced by its surroundings, it does not experience the same level of urbanization and confinement as the GRA. The most significant divergence between urban and non-urban streams appears to be the frequency and speed of ascending and descending flow events and higher temperatures (Walsh et al., 2005).

Urban streams are often engineered for water transfer and are commonly viewed as drains, gutters, ditches, and pipes (Meyer et al., 2005). Consequently, although the inflow of nutrients is high, they struggle to retain organic matter. Also, despite the elevated temperatures and accelerated leaf decomposition, the organic matter is swiftly carried away due to higher scour. Microbial activity, however, is heavily contingent on organic matter availability and in highly altered river channels, where a reduction of shifting sands is typical, this translates to a decrease in microbial degradation of organic matter (Fischer et al., 2002a; Fischer et al., 2002b; Sobczak & Findlay, 2002; Zlatanović et al., 2017). This fact is also reflected in GRA's lower TPC and ETSA despite its higher POM content than KBB and LJU.

In this study, the TPC was significantly lower in KBR than in other rivers, likewise, GRA, where TPC was significantly lower than in KBB and LJU. Feris et al. (2003) found heavy-metal contamination did not affect microbial biomass, but individual phylogenetic groups differed along the contamination gradient. Song et al. (2020) showed that microbial biomass decreased significantly in the presence of 5 and 50 mg g^{-1} of multi-walled carbon nanotubes. The authors assumed that the decrease in biomass resulted from an antibacterial effect, which can also be the case in this study.

Organic matter, water content (hyporheic exchange) and sediment structure are also important drivers of microbial metabolic activity (Romaní & Sabater, 2001; Muri & Simčič, 2004; Nogaro et al., 2007; Simčič & Mori, 2007; Mori et al., 2017). The ETSA was significantly lower in treatment than in the control pockets in this study. KBR and GRA ETSA were also significantly lower than KBB and LJU sites. ETSA is highly variable, following the heterogeneity among habitat types (Mori et al., 2017). For example, in oligotrophic lakes, they measured low microbial activity, and the activity was reduced by

half in sediment layers below 15 cm (Simčič & Mori, 2007). It was, however, generally consistent in a study between two contrasting floodplains, where it was lower in coarse channel and gravel-bar sediments compared to finer soils from islands, riparian forests and grasslands, which were all also rich in organic matter content (Mori et al., 2017). It was also shown by Simčič and Mori (2007) that the ETSA is lower on the surface compared to interstitial water of the hyporheic sediments. In addition, Nogaro et al. (2013) also found that ETSA was significantly different between the three rivers examined in their study.

The river sites featured in this study differed based on the inflow of nutrients, their fluvial system characteristics (pre-alpine, karstic), and land use (rural–agricultural, urban), which explains the statistical differences observed between the rivers. Specifically, the differences between the sites are that in the KBR, the hyporheic zone is a cold, oligotrophic environment with harsh hydrological conditions, while the LJU site is a polluted site below the Ljubljana city and WWTP.

At polluted sites, biofilms are characterised by gram-negative bacteria, part of α-proteobacteria (Feris et al., 2003). Several sequences obtained by Feris et al. (2003) were unrelated to known organisms, providing little indication of species-level identification. Microbial communities inhabiting the hyporheic zone are relatively unknown (Findlay & Sobczak, 2000; Feris et al., 2003). For this reason, a metagenomics analysis in the future would be advisable.

The differences between locations were also observed when looking at functional microbial diversity (using CLPP) visualized by heatmaps. The LJU site stood out with the highest substrate utilization compared to other rivers, closely followed by KBB. However, substrate utilization in treatment pockets seemed to be less efficient in comparison to control pockets. However, the opposite was true for KBR, where higher utilization of some substrates (LAsp, LArg) was observed in the treatment pockets instead of control pockets. It seems that the proximity of PET fibres diminishes the community composition. However, spatial difference seems to have a higher effect than treatment in our study. CLPP in various EU sites seems specific for land use, soil management and soil texture (Rutgers et al., 2016). However, Zhang et al. (2019) reported a lower impact of the pollution gradient compared to seasonal variation in river sediments.

Our finding that location has a greater effect on microbial community function than seasonality agrees with published studies. For instance, Akinwole et al. (2021) conducted a study in the US comparing microbial community structures across two watersheds. The authors employed genomic DNA analysis of sediment alongside phospholipid analysis. Their findings revealed a distinct regional-level spatial variability in the community structure. Notably, variations were also observed in the total microbial community structure of the sediments.

Similarly, Zhang et al. (2020a) arrived at comparable conclusions, highlighting the dominance of spatial heterogeneity over seasonality in predicting microbial community diversity. Like Akinwole et al. (2021), their investigation relied on DNA analysis. Findlay et al. (2008) compared nine streams grouped into three biomes, with samples taken from a 2 cm sediment depth. Utilizing DNA-based analysis, they found a distinct biome-level biogeography in the bacterial community structure, even when confronted with substantial within-stream and among-stream variations. In another study from the USA, Xueqing et al. (2005) scrutinised sediment samples collected from nine streams between June and August of 2001. Their findings similarly highlighted differences in bacterial communities among those streams. However, all these studies predominantly focus on DNA-based analyses, whereas the present study depended on differences in the microbial metabolism of individual river biofilms. My findings also align with the observations discussed in studies relying on DNA-based analyses.

The biofilm on PET between different rivers showed no significant differences regarding TPC and ETSA. It appears that geomorphological differences do not influence the biofilm on PET sheets, as the PET sheets most probably served as a new habitat for bacterial assemblages, commonly referred to as plastisphere. Additionally, pollution makes the biofilm community structure more uniform (Zhang et al., 2019), lowering microbial diversity (McCormick et al., 2014; Seeley et al., 2020; Miao et al., 2021b). For example, McCormick et al. (2014) reported less diverse bacterial assemblages colonizing MPs that significantly differ in taxonomic composition from those in the water column, potentially representing unique bacterial assemblages.

The fate of PET fibres in the hyporheic zone over 2-years

The $\delta^{13}C$ data did not yield significant results. Nevertheless, a noticeable trend of increasing $\delta^{13}C$ values, indicating deterioration over the years, was observed, particularly at the Kamniška Bistrica locations (KBR and KBB), while it remained relatively stable in LJU and even slightly decreased in GRA. Interestingly, a comparison between the untreated sample (Figure 30) and the $\delta^{13}C$ values of samples from all rivers revealed signs of deterioration. The changes became visible when considering SEM micrographs (Figure 31), as the reference material appeared smooth with minimal bumps and crevices, presenting a noticeable contrast to the material collected from the river. Specifically, the materials exposed to environmental elements for two years exhibited visible signs of damage, such as dents, grooves, and loose pieces. However, based on visual inspection alone, it was impossible to determine any differences in the fibres between rivers. Despite this, the results agree with the conclusions of a previous study by Matjašič et al. (2021a).

Conclusions

This two-year investigation into hyporheic biofilm responses to PET fibres in diverse river systems produced interesting results. For example, PET fibres influenced biofilm growth and metabolic structure, favouring already adapted biofilms while reducing metabolic biodiversity among less adaptable types, manifesting in generally lower microbial biomass, microbial activity, and altered physiological profiles. The effect of the study site was more pronounced than seasonal influences. Nevertheless, the pollution of hyporheic sediments with PET fibres shaped biofilm structure and functionality across distinct study river sites, influenced by varying pollution levels and unique geomorphology. Seasonal variations of POM and TPC were insignificant, most probably due to the hydrogeomorphological complexity of the HZ. ETSA was significantly lower in the sediment pockets treated with PET fibres, most probably due to the PET fibres causing clogging and possibly the leaching from PET of inhibitory chemical compounds. However, such a hypothesis needs to be tested.

Seasonal effects primarily impacted the biofilm on PET sheets, while spatial heterogeneity (different sites) impacted the sediment-pockets values. However, heatmaps revealed differences due to seasonality and between rivers while highlighting distinct variations in substrate utilization capabilities among treatments (control, PET polluted). The CLPP is valuable for assessing community structure since it provides insight into community metabolic functioning. However, bacterial biomass can stay consistent while species composition changes, mainly when new species are introduced (for example, during floods). Therefore, the logical next step is to conduct complementary DNA analysis for a more comprehensive understanding of microbial dynamics. Also, despite little change in the stable isotopic composition of PET, SEM micrographs demonstrated structural alterations and damage induced by biofilm colonization, offering insights into degradation mechanisms.

In summary, this study revealed that PET fibres can negatively impact hyporheic biofilm structure and functioning in different river systems. PET pollution can lead to the adaptation of biofilms through reduced microbial functional diversity and alter the biofilm's structure. Other significant findings are that site-specific factors outweigh seasonal variations, and PET sheets can act as a clogging agent and potentially harbour unique bacteria. Most importantly, this work highlights the importance of understanding local conditions in assessing the ecological significance of PET pollution.

3.3.3 Scientific article in preparation: "Resilience of hyporheic biofilms to non-flow events and PET pollution"

Introduction

Freshwaters are dynamic and complex environments exposed to various natural and anthropogenic stressors. Amongst these, climate change is one of the most pressing challenges of this century. Climate models predict decreased precipitation in the Mediterranean and central EU, leading to longer dry spells and droughts (Pörtner, 2022). Consequently, perennial streams and rivers will increasingly fall dry, affecting surface and subsurface habitats (Milly et al., 2005; Pörtner, 2022; Zhang et al., 2023).

In intermittent streams, the hyporheic zone is exposed to three major hydrological conditions with consequences on the solute exchange: "flowing" (i.e. 3-dimensional water and solute exchange), "stagnant" (solute exchange only via diffusion), and "unsaturated" (no solute exchange) (Coulson et al., 2021). However, even during unsaturated conditions, the hyporheic zone (HZ) typically maintains a moisture content that protects hyporheic biofilms from drying out completely (Bruno et al., 2020; Coulson et al., 2021). Thus, studies have not yet observed huge impacts of drying on the microbial community of the hyporheic zone (Coulson et al., 2021; Coulson et al., 2022a; Coulson et al., 2022b), depending on the harshness of the unsaturated condition.

Drought conditions can have a notable impact on microbial communities. For example, they expose decomposing leaf litter, potentially disrupting organic recycling processes (Duarte et al., 2017) and reducing efficiency (Ledger et al., 2013). In fluvial ecosystems, stream biofilms are the primary sites for nutrient processing and carbon cycling, crucial ecological processes in freshwater environments (Boulton et al., 1998; Battin et al., 2003). Moreover, dry conditions can also affect the structure of microbial communities. Species adapted to wetter conditions may decline in abundance, and the composition of these communities may be more similar to those typically found in terrestrial soil ecosystems (Arce et al., 2019; Gionchetta et al., 2019). Droughts can also lead to temporary increases in specific microbial processes, such as the decomposition of DOM and respiration (Harjung et al., 2019; Coulson et al., 2021).

However, investigations of the effects of different hydrological conditions on hyporheic biofilms are rare. Specifically, interactions with other stressors, such as pollution, have not been investigated. Emerging pollutants, such as MP (Yang et al., 2021), concern stream systems' future integrity and functioning. MPs can accumulate in the HZ in large amounts (Frei et al., 2019; Yang et al., 2021; Matjašič et al., 2023), where they can influence microbial activity and biogeochemical cycles (Matjašič et al., 2021a). The presence of MP alters the microbial community and impairs the decomposition of organic matter and nutrient cycling via community, but also metabolic changes (Li et al., 2020; Seeley et al., 2020; Matjašič et al., 2021a; Miao et al., 2021b).

Microplastics can exhibit a wide array of effects on microorganisms. They can have a selective effect on microorganisms, promoting the growth of specific bacterial strains (Li et al., 2020; Matjašič et al., 2020). A diverse microbial community that colonises plastic particles is referred to as the "plastisphere", and it has been shown that this community differs from the surrounding surface water (Zettler et al., 2013). Furthermore, the presence of MP can change the composition of sediment microbial communities and influence nitrogen cycling processes (Seeley et al., 2020).

The specific outcomes from MP contamination depend on the type of MP present. For example, PUF and PLA particles in sediments can enhance nitrification and denitrification processes, while PVC particles inhibit both (Seeley et al., 2020). The potential impact also

varies with particle size and surface modification (Miao et al., 2019b). Also, alpha diversity in MP-associated communities is lower than that of natural substrates. However, functional profiles indicate increased metabolic pathways related to amino acid processing in biofilms on MP substrates (Miao et al., 2019c). Moreover, biofilms forming on PET surfaces demonstrate reduced capacities, carbon metabolism rates, and lower diversity indices than those on glass and PVC substrates (Miao et al., 2021b). Moreover, PET has been found to inhibit biofilm activity and impact community-level metabolic profiles (Matjašič et al., 2021a).

The potential interaction between flowing conditions and MP can lead to enhanced transport. For instance, MPs and their leachates may be transported more rapidly and over greater distances (Horton & Dixon, 2018; Hoellein et al., 2019; Waldschläger et al., 2020). Furthermore, high flow conditions have the potential to remobilise the MP from sediments into the water column (Nizzetto et al., 2016) and expose MP particles to mechanical stress, leading to further fragmentation (Wong et al., 2020; Dai et al., 2023; Matjašič et al., 2023).

Additionally, MP can potentially harbour toxic chemicals or harmful microorganisms and, with high flow, disperse them over long distances (Mato et al., 2001; Caruso, 2019; Gorman et al., 2019; Laganà et al., 2019; Wu et al., 2019b). In stagnant or low-flow areas, MP can accumulate, leading to higher local concentrations, and can create localised high MP-influenced areas (Tibbetts et al., 2018; Drummond et al., 2022). Microbes in these areas may experience prolonged contact with MPs, potentially leading to more pronounced inhibitory effects (Matjašič et al., 2021a).

In unsaturated conditions, with limited water in the form of moisture, the interaction between MPs and microbes may be reduced and more localised around the individual MP particle. Here, microbes face desiccation stress and decreased nutrient availability (Febria et al., 2012; Coulson et al., 2021), potentially impacting their ability to colonise and degrade the MP. Research suggests that biofilms may be more successful in biodegrading MP when other readily available nutrients are present (Tribedi & Sil, 2013; Santisi et al., 2015; Karimi & Biria, 2019; Matjašič et al., 2021b). To summarise, flow conditions can influence the release rate and dispersion rate of MP and its leaching substances: high flow increases the release rate and dispersion, while low or no flow conditions mean more effects on the substances released.

Aims and objectives

The overarching goal of this study was to investigate the interplay between water regime variations, the presence of PET microplastics in the form of textile fibres, and their combined effects on selected functional attributes and bacterial abundance of hyporheic biofilm communities through controlled experiments using vertical flume reactors.

The specific objectives were to analyse the effects of PET fibres and flow interruption on overall metabolic activity (ETSA), community-level physiological profiles (CLPP), microbial biomass (assessed as TPC – total protein content), and the bacterial abundances of hyporheic biofilms to reveal both the effects of the individual stressors and the type of stressor interaction.

The study provides a deeper understanding of the biogeochemical processes occurring in river hyporheic zones contaminated with MPs in the context of climate change and, consequently, its impact on water quality.

Hypothesis

1. The presence of PET fibres in sediments of the hyporheic zone will result in a significant reduction in metabolic activity, biomass production, and bacterial

abundance and will cause a shift in the metabolic profile of the microbial communities during all experimental water regimes (flow, stagnant, unsaturated).

2. Biofilm exposure to stagnant conditions will result in higher metabolic activity while reducing biomass and bacterial abundances compared to unsaturated conditions. Similarly, the microbial metabolic function will be inhibited due to a gradual shift to anoxic and low nutrient condition.
3. The presence of PET fibres has a measurable effect on biofilm metabolic activity, biomass production, and bacterial abundances in stagnant and unsaturated conditions than in flowing conditions.

Methodology

The preliminary experiments and the sediment and PET preparation were carried out at the National Institute of Biology in Ljubljana, Slovenia, while the flume reactor experiment was conducted at the WasserCluster Lunz in Austria.

Preliminary tests

The preliminary experiments were aimed to determine the optimal sediment-to-buffer ratio and also identify the minimum amount of PET fibres that would still produce a response resembling natural values. The biofilm response was measured by determining the total protein content (TPC), issuing a fast, low-cost and safe colourimetric method that relies on the reaction between proteins and the BCA protein assay reagent—the colour change results from the alkaline reduction of cupric ions.

Initially, tests were conducted with varying sizes of sediment grains (1 – 2 mm, 2 – 4 mm, 1 – 4 mm). Grain size is crucial when estimating biofilm activity (Mori et al., 2017). The amount of sediment (8 g, 6 g, and 3 g) and buffer volume (deionised water; 1.5 ml, 2 ml, 3 ml, 4 ml) were also varied to determine the optimal balance. The aim was to ensure complete sediment coverage in the centrifuge tube while maintaining sufficient material for analyses (TPC and ETSA) without excessive dilution and to obtain background values of TPC. Given the larger grain size, which deviates from the TPC protocols used in previous studies (Matjašič et al., 2021a), optimal conditions for TPC were explored while closely adhering to the manufacturer's instructions for all other aspects of the procedure.

Samples containing the different sediment-to-buffer ratios were transferred to a series of centrifuge tubes and sonicated using an ultrasonic homogeniser 4710 (Cole-Parmer, Vernon, IL, USA) for 2 minutes (with mixing after 1 minute) in an ice bath. The samples were centrifuged at 0°C at 10,000 rpm for 4 minutes. After centrifugation, 25 µL of supernatant was transferred to two separate microplate wells, to which 200 µL of reagent was added. The microplate was then incubated for 30 minutes at 37 °C, and the absorbance measured at 562 nm. The most favourable response was observed with 6 g of sediment and 1.5 ml of buffer, yielding positive results across all fractions (Figure 32).

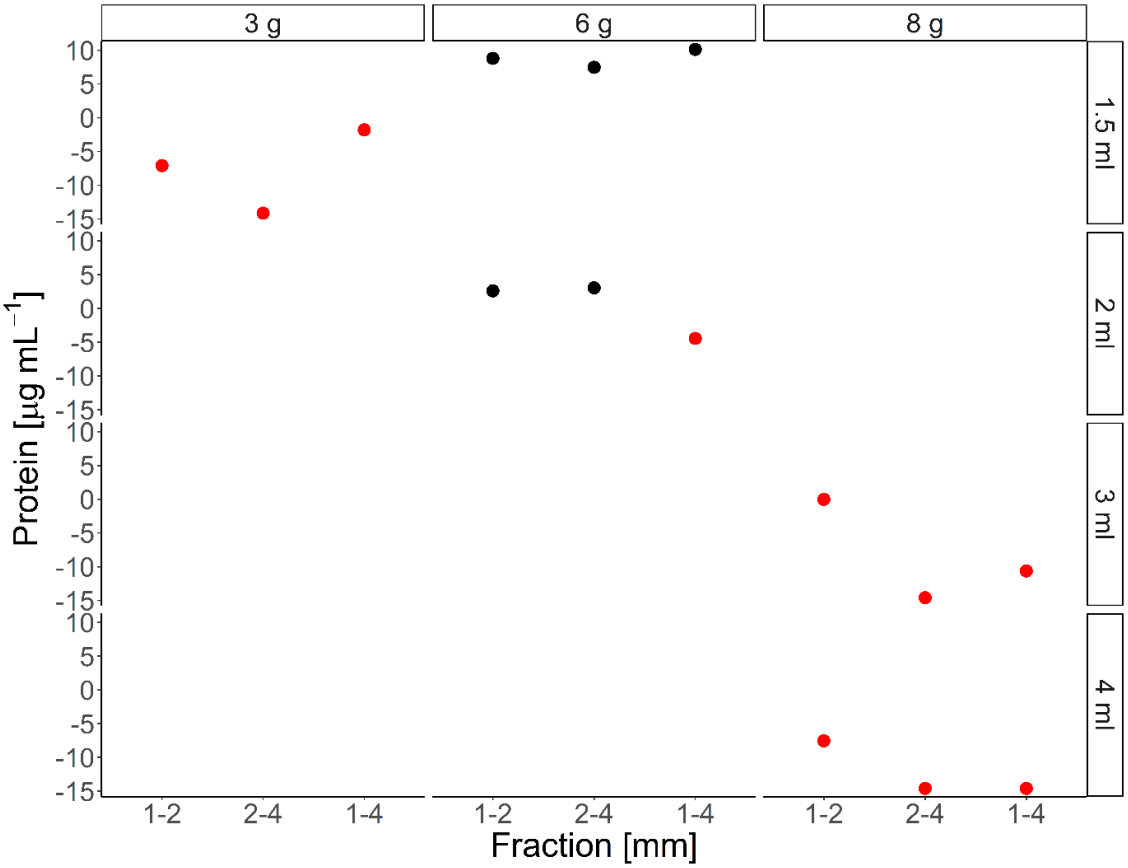


Figure 32: Scatterplot showing different combinations of sediment-to-water ratios (3 g, 6 g, 8 g to 1.5 ml, 2 ml, 3 ml, and 4 ml, respectively) across various fractions (1-2 mm, 2-4 mm, 1-4 mm). Negative values are represented by red dots.

A series of samples containing varying PET-to-sediment ratios (1:1000, 1:100, 1:50, 1:40, 1:30, 1:20, Table 8) were analysed to identify the minimum amount of PET fibres still eliciting a response from HZ biofilms. Different buffer volumes were also tested (8 g and 4 ml, 8 g and 3 ml, 6 g and 2 ml, 6 g and 1.5 ml, and 3 g of sediment and 1.5 ml, respectively) to determine the optimal volume needed while ensuring sufficient material for TPC and ETSA analysis. As in the previous experiment, the chosen quantities were selected to match natural environmental conditions, taking into account technical limitations.

The pre-washed and sun-exposed PET fabric, previously cut into pieces, was combined with a predetermined quantity of sediment (grain size 2 – 4 mm, Table 8) sourced from the Kamniška Bistrica River. This mixture was then placed into PVC tubes (15 cm x 3.5 cm in diameter), closed at one end, each equipped with an "air stone" diffuser at the base. Air was supplied using an air pump (AquaPro AP-120 and Trixie). The pump was operated at its lowest setting to avoid disturbing the sediments.

The tubes plus the sediment-PET mixture were placed in a temperature-controlled chamber at 18°C in the dark for four weeks (Figure 33). During incubation, tap water was periodically added to compensate for evaporation. Sediment samples were collected time = 0 and 4 weeks. All samples were stored at -80 °C for a minimum of one week before analysis.

Table 8: The amount of PET fibres, sediment and their ratio in individual treatments (grain size = 2-4 mm).

	T1	T2	T3	T4	T5	T6
PET fibres (g)	0.16	1.6	3.2	4	5.3	8
Sediment (g) (grain size: 2-4 mm)	160	160	160	160	160	160
ratio	1:1000	1:100	1:50	1:40	1:30	1:20

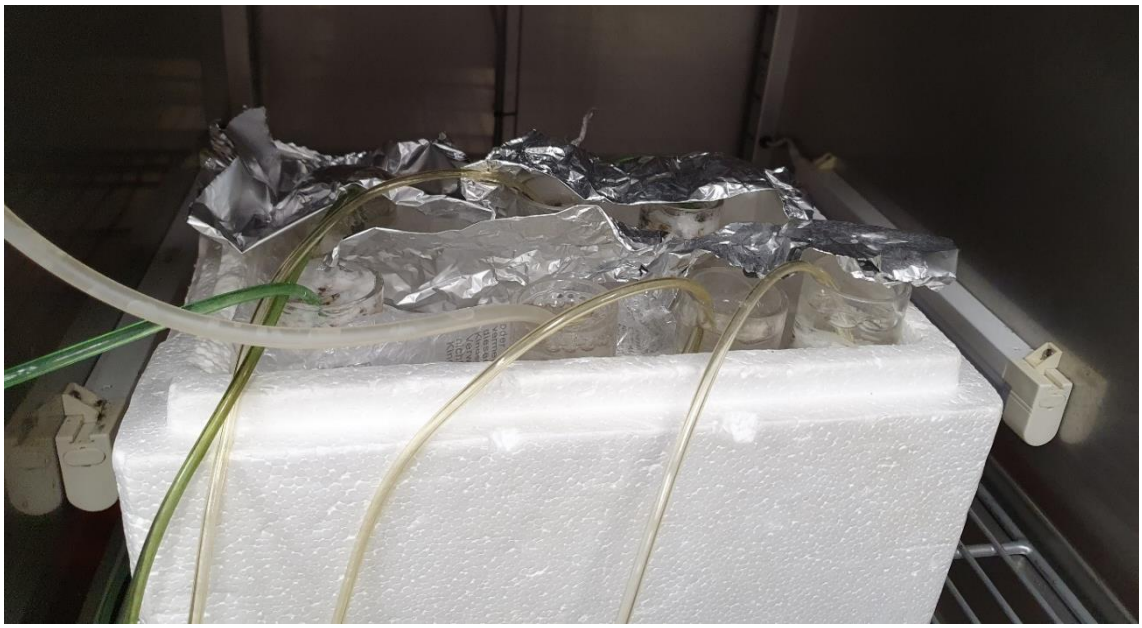


Figure 33: Photo showing preliminary test tubes containing sediment-PET mixture in the temperature-controlled chamber.

The preliminary test was important from two aspects. First, to determine the optimal grain size, amount of sediment, and buffer volume. Second, to establish the optimal amount of PET for the mesocosm experiment. The aim was to strike a balance that would not overly suppress microbiota while closely resembling natural conditions.

The TPC was measured across all three fractions using a sediment-to-buffer ratio of 6:1.5 (Figure 32). This ratio was adopted for all subsequent analyses.

Negative TPC values were obtained after four weeks of incubation (Figure 34, a). This result was likely due to the known inhibitory effects of PET. Three additional standards were introduced to address this issue, resulting in final concentrations of BSA of 62.5, 12.5, and 6.25 μg ml^{-1}. The manufacturer's protocol recommends nine standards within the working range of 20 and 2000 μg ml^{-1}, with the lowest concentrations being 25, 125, and 250 μg ml^{-1}. By including these standards, positive values were obtained (Figure 34, b).

Furthermore, sediments acclimating to semi-natural conditions and later used in the mesocosm experiments were also tested. After one week at -80 °C, all the tested samples produced positive results, with a mean value of 13.45 ± 3.90 μg ml^{-1}.

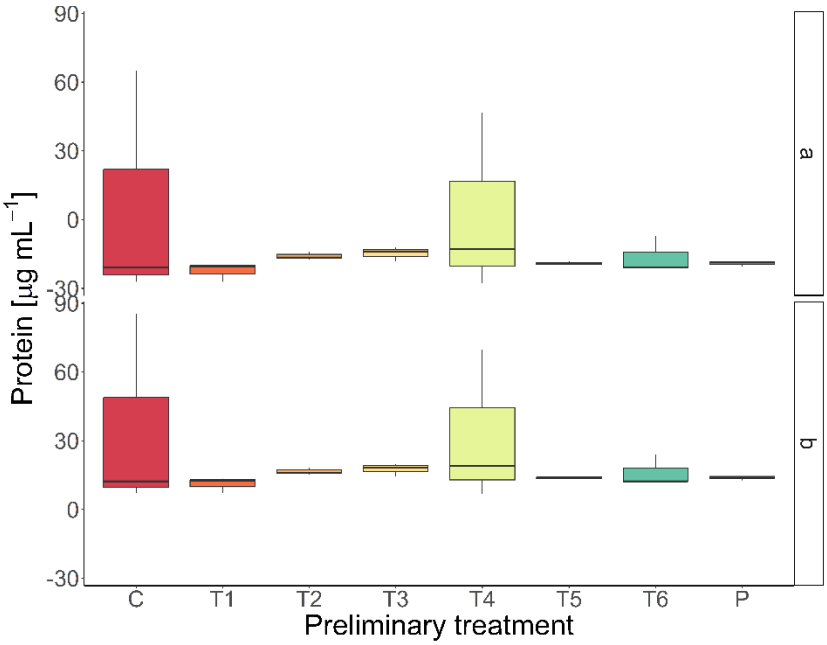


Figure 34: Boxplots of TPC of preliminary testing of different ratios of sediment vs. PET fibres before (a) and after (b) addition of three additional standard concentrations.

Based on the results, a sediment: MP ratio of 160:4 (T4) was used. This ratio was used due to higher TPC values observed in the preliminary test.

Sediment colonization and pre-experiment adaptation

Mesocosm/column experiment sediments were collected on 6th April 2022 from the Kamniška Bistrica River, Slovenia (coordinates: 46.247677, 14.602315), at 20-40 cm depth. The riverbed sediments were sieved to obtain about 40 L of sediment with a 2 to 4 mm particle size distribution (Minor, Endecotts, 2 – 4 mm) and 10 L of sediment with a 1 to 2 mm particle size distribution. The sediments were washed with river water on site and rinsed with deionised water in the laboratory to remove particulate organic matter. Similarly, 5 L of sediment was collected from the Kamniška Bistrica River (46.088449, 14.626049). The sediments were washed with the river water to remove organic matter but retain the natural microbial biofilm. The samples were sieved to obtain a 1 – 2 mm particle size distribution and fused as a "seed" to establish the hyporheic microbial community. The biofilm was collected next to the Wastewater treatment plant Domžale-Kamnik outflow.

Figure 35: The containers with the incubated sediments. On top of sediment the green tubes, connected to water pump are visible.

The collected sediments were allowed to adapt to semi-natural conditions in the laboratory for one month. The sediments were mixed in a ratio of 2:1, i.e., 12 L (2 to 4 mm): 6 L (1 to 2 mm) and divided equally into two containers (35 x 55 x 15 cm each) (Figure 35). Only 2 L of the smaller fraction was used as seed and collected fresh the day before, while the other 4 L were collected two weeks prior.

River water from the previous day was added to cover the sediments, and a water pump was installed to circulate over the sediment. The containers were kept at 18 °C in the dark. The water was replaced weekly with fresh river water, and the tubing and pumps were cleaned with tap water.

Nutrient broth was added every two weeks to ensure biofilm survival and growth and simulate nutrient input from leaf litter. The nutrient broth was prepared by taking 30 g of poplar and 30 g of alder, adding 600 ml of tap water and leaving the mixture to soak for 48 hours. The leaves were filtered out, and the filtered broth was autoclaved at 121.5 °C for 15 min.

At the same time, 10.5 m^2 of clean commercial PET cloth was exposed to sunlight for one month. After one month of exposure, it was cut into small squares (12 x 12 cm) weighing approximately 1 g. These were grounded in a mill (SM 300, Retsch, Germany) to produce fibres (< 5 mm in length), meeting the definition of microplastics (particles < 5 mm).

Experiment setup

The up-flow reactors (Figure 36) used in the experiment consisted of transparent acrylic glass cylinders (50 cm x 5 cm Ø). Fifteen of the 32 reactors (15) contained only sediment, while another 15 contained a mixture of sediments and MP (Table 9). Two additional reactors were used as the controls and were filled with muffled sediments.

Based on the preliminary tests, the ratio between sediments and added MP weight was 40:1. In both sediment and sediment+MP reactors, two pieces of punctured aluminium foil were placed at the bottom and in the centre to disperse water throughout the sediment. The reactors were wrapped with opaque plastic sheeting and aluminium foil to simulate conditions in the hyporheic zone.

Each reactor was then installed upright and filled with water collected from Oberer Seebach, a largely pristine, well-oxygenated headwater stream with low nutrient status and turbidity. A separate 20 L tank was used to supply (with and without MPs) water to each reactor to prevent fibre contamination between reactors. Water quality was regularly checked in the tanks. Water flow through the reactors was 1 mL min^{-1} in a bottom-to-top direction using a peristaltic pump (Watson Marlow 205S, Thermo Fisher Scientific, USA).

All reactors and tubes used in the experiment were disinfected by soaking in a 5% bleach solution. This step was crucial to prevent contamination from pre-existing microbes within the tubes. Initially, the tubes were immersed in the disinfectant and allowed to soak for 30 minutes while the pump operated at 8 rpm to facilitate thorough circulation. Subsequently, the tubes underwent an hour-long rinse with tap water. The rinsing process utilised the same water that was intended for use in the experiment. The primary objective of this rinsing was to ensure the complete removal of any residual bleach. This measure was undertaken to eliminate the risk of interference or bactericidal effects posed by lingering traces of bleach during the subsequent experiment.

Sediments in the reactors were first exposed to a two-week adaptation period. During the adaptation, leachate from Alder leaves (five leaves in 200 mL water) was added to the tanks to stimulate biofilm growth. The experimental design comprised five control reactors (sediment) and five MP reactors (sediment plus PET fibres), all subjected to three hydrological treatments. The reactors were drained and left unsaturated in the first batch for one month. In the second batch of reactors, water flow was halted, subjecting the biofilms to stagnant conditions for one month. For the third batch, the reactors remained under continuous flow conditions. Biofilm samples were collected after one month without rewetting them.

Table 9: Summary of experimental design (different water regimes and PET contaminations) with number of replicates.

Set	**Water regime**	**Reactor contents**	**Replicates**
1	Unsaturated	Sediment	5
		Sediment + PET	5
2	Stagnant	Sediment	5
		Sediment + PET	5
3	Flowing	Sediment	5
		Sediment + PET	5
Water control	Flowing – Control T	Sterilised sediment	1
Water control	Flowing – Control C	Sterilised sediment	1

Figure 36: Photo showing the mesocosm experimental setup, with covered and upright reactors connected to separate water tanks (control and treatment).

Laboratory work

After one month, the sediment samples were collected from the top and bottom of each reactor to account for spatial variability within the reactors. The PET fibres were left in the sample so as not to alter the reactor conditions. For example, if a difference in density were used to separate the PET fibres from the sediments, the sediments would be re-wetted, which could alter the study results for unsaturated samples.

The bacterial abundance in the reactors was estimated using flow cytometry. The biofilm response was measured by measuring biofilm activity (ETSA) and biomass (TPC) of the sediments and by looking at the biofilm metabolic profile (CLPP with Biolog® Ecoplates).

Flow cytometry and microbial community physiological profiling (CLPP) were performed at WLC. At the same time, the remaining sediment samples were stored at -80°C before being transported to the National Institute of Biology, Ljubljana, to determine total protein content (TPC) and respiratory electron transport system activity (ETSA).

Water analyses

To ensure comparable water quality in both water tanks, NO_3^-, NH_4^+, NO_2^-, PO_4^{3-} and DOC levels were monitored during the experiment. Measurements were taken after adding

nutrients and just before setting up the experiment. Water samples from the flow reactors were also taken weekly during the experiment and at the end.

Bacterial abundance

The sediment samples were analysed using the CytoFlex Flow Cytometer (Beckman Coulter GmbH, Krefeld, Germany). Flow cytometry is a method used to detect the physical and chemical characteristics of a population of cells or particles. The samples are transported through the device to a detection laser, where, ideally, only one cell at a time passes through. The scattered light detected is characteristic of different types of cells and their components. The cells were stained with a Sybr Green II RNA gel stain (200x diluted in DMSO, Invitrogen Molecular Probes Inc., California, USA). The method followed was a standard protocol used at WasserCluster Lunz, modified after Duhamel and Jacquet (2006). This method does not distinguish between living (i.e. membrane intact) and dead cells.

First, 1.32 g of fresh sediment and 3 ml of filtered water were added to a test tube. The samples had been fixed immediately after collection with 37% formaldehyde and stored at 4 °C until analysis. Next, 5 µL of Tween 80 (10%) and 1 ml of sodium pyrophosphate solution (10 mM) were added and sonicated three times for 1 min in a water bath and thoroughly shaken. This step was then repeated a further two times. Following sonification, the test tubes were immersed in an ice bath for 15 minutes, shaken for 1 minute and then centrifuged for 1 minute at 1400 g. Two mL of the supernatant was then filtered through a 5 µm membrane, and then 50 µL of the filtered sample was carefully transferred to a fresh 15 ml tube, followed by the addition of 4950 µL of sterile filtered water. Lastly, 995 µL of the diluted mix was transferred into two eppis, one for the stained and one for unstained samples. In the eppis prepared for stained samples, 5 µL of Sybr Green II was added and then incubated for 15 minutes in the dark at room temperature. After incubation, the samples were measured with the cytometer.

The cytometer records the events detected by the laser. The unstained samples are negative controls used to determine background fluorescence and autofluorescence, which is then subtracted from the stained samples. The number of events is then converted to events per ml to account for the dilution and addition of stain and the volume of the original tube. Bacterial abundance is expressed as cells $x10^6$ $gDWsed^{-1}$.

Total protein content (TPC)

The utilization of total protein content (TPC) is an indirect method for estimating microbial biomass. In the scope of this study, this evaluation was conducted using a spectrophotometric assay, specifically the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). This assay relies on reducing cupric ions by proteins under alkaline conditions, subsequently setting off chelation and the development of colour through the bicinchoninic acid (BCA) reagent. The BCA/copper complex is water soluble and has a robust linear correlation between absorbance at 562 nm and protein concentration. A distinctive aspect is the deliberate inclusion of supplementary standard concentrations and standards prepared according to the manufacturer's instructions. However, an adjustment was needed to account for the lower values obtained during preliminary testing.

Sample preparation consisted of adding 6 g of sediment and 1.5 ml of homogenizing buffer to a centrifuge tube and homogenised for 2 minutes using an ultrasonic homogeniser 4710 (Cole-Parmer, Vernon, IL, USA). The lysate was centrifuged for 4 minutes at 0 °C at 10,000 rpm. Following the manual, 25 µL of the standard (or sample) was mixed with 200 µL of the working reagent. The mixture was incubated at 37°C for 30 minutes. The absorbance was measured at 562 nm using a spectrofluorometer (SynergyMX, BioTek Instruments, USA). The results were presented in micrograms of protein per gram of dry

sediment (µg protein g sed^{-1}). The same procedure was already used in another study from this thesis (Matjašič et al., 2021a).

Respiratory electron transport system activity (ETSA)

The overall microbial respiratory activity was estimated using ETSA. This method detects the activity of dehydrogenases and cytochromes within the electron transport system as a part of the cell respiration cycle. The protocol was adapted from Packard (1971) and described in full in Matjašič et al. (2021a).

Samples were prepared by adding 6 g of sediment to a centrifuge tube. Next, 1.5 ml of homogenising buffer was added, and the mixture was homogenised using an ultrasonic homogeniser 4710 (Cole-Parmer, Vernon, IL, USA) for 2 minutes. The mixture was centrifuged at 0°C at 10,000 rpm for 4 minutes. According to the protocol, 30 µL of the sample was added to two adjacent wells on the microplate, and the third adjacent well was left empty. To each well was added 150 µL of substrate solution and 50 µL of reagent solution. The microplate was incubated in the dark at 20 °C. After 30 minutes of incubation, 50 µL of stopper solution was added. Additionally, 30 µL of the sample was added to the third cell, which was used as a blank. The absorbance was measured with a microplate reader SynergyMX (BioTek Instruments, USA) at 490 nm.

The blank value was subtracted from the average of the duplicates, and the resulting value was used in the formula described in detail in chapter 3.3.1:

$$ETS - activity\ (\mu l\ O_2\ S^{-1}\ h^{-1}) = ABS^{490nm}\ x\ V_r\ x\ V_h\ x\ 60\ x\ \frac{1.30}{V_a\ x\ S\ x\ t\ x\ 1.42}$$

The measurements were presented as the concentration of oxygen used per dry sediment weight during a specific time interval (µL O_2 gDW^{-1} h^{-1}).

Community-level physiological profiling (CLPP)

The community functioning was assessed using the Biolog Ecoplates™ Assay (Biolog, California, USA), which provides information on the community metabolism of 31 different carbon sources, nutrients and redox dyes. It is a quick and cost-effective approach for evaluating microbial functional diversity (Garland & Mills, 1991). The intensity of substrate utilization is measured spectrophotometrically and expressed as absorbance. The raw absorbance of substrate wells is corrected with the absorbance of the blank wells. The absorbance readings after 72 h were used to preserve the comparability to other studies conducted during this thesis.

To each sample, 2 g of sediment or 1 PET sheet was placed in labelled beakers, and 20 ml of chilled Ringer solution was added. The samples were treated in an ultrasonic bath (Elmasonic P, Elma, Singen, Germany) for 1 minute. The contents were shaken and centrifuged at 4°C for 5 min at 800 rpm. Next, 150 µL of supernatant was transferred to microplate wells. The ecoplates are designed (3x32 wells on a 96-well ecoplate) to allow each sample to be added in triplicate. The plates were incubated in the dark at 20 °C between measurements. The absorbances were measured using SynergyMX (BioTek Instruments, USA) at 590 nm at 0, 24, 48 and 72 h. The raw absorbance was corrected by subtracting the blank value (control - first well with water). The substrate utilization measurements after 72 h were used for further data analysis. These were chosen to reduce measurement errors, as most values were less than two, in line with Weber and Legge (2010), and to maintain comparability with previous studies within this thesis.

Statistical analysis

The effects of the water regime (regime: "flowing", "stagnant", and "unsaturated"), the PET treatment (treatment: with PET fibres = "PET"/without PET fibres = "control"), and the position in the reactors (position: "inflow"/"outflow") were tested. The factor "position" corresponds to the spatial heterogeneity within the HZ. The labelling of the factor levels is based on their location within the reactors rather than denoting the actual presence of continuous flow. Specifically, only one batch of reactors (flow) had a consistent water flow throughout the experiment.

The normality of the data was assessed with the Shapiro-Wilk normality test. If the assumptions were achieved, ANOVA and Tukey's Honest Significant Difference (HSD) test were applied. If the data was not normally distributed even after log transformation ($\log_{10}(x+1)$), Kruskal-Wallis and Dunn's post hoc tests were used. The significance level was determined at $p < 0.05$ unless specified otherwise. The data was analysed using R studio (R Core Team, 2021), using the tidyverse (Wickham H et al., 2019), openxlsx (Schauberger & Walker, 2022), vegan (Oksanen et al., 2020) packages.

The CLPP data were normalised with log transformation ($\log_{10}(x+1)$) and investigated using NMDS according to Matjašič et al. (2021a). Here, Bray-Curtis dissimilarity was used to visualise patterns and calculate distance (Figure 41). In addition, an Analysis of Similarities (ANOSIM) was conducted to assess the dissimilarity among groups defined by categorical factors, and a Permutational Multivariate Analysis of Variance (PERMANOVA) to assess the significance of differences between groups or treatments in multivariate data sets.

Results

Water analyses

The nutrients differed after adaptation (signifying the start of the experiment) and at the end of the experiment. In the control reactor water, NPOC increased from 0.884 mg L^{-1} to 1.175 mg L^{-1}, NH_4 from 0 µg L^{-1} to 10.1 µg L^{-1}, NO_2 1.9 µg L^{-1} to 3.4 µg L^{-1}, PO_4 from 10.9 µg L^{-1} to 15.6 µg L^{-1}, and NO_3 from 110.5 µg L^{-1} to 193.2 µg L^{-1}. In the treatment reactor water, NPOC increased from 0.930 mg L^{-1} to 1.105 mg L^{-1}, NH_4 from 0 µg L^{-1} to 4.5 µg L^{-1}, NO_2 from 1.6 µg L^{-1} to 5.9 µg L^{-1}, PO_4 from 0 µg L^{-1} to 12.2 µg L^{-1}, and NO_3 from 53.6 µg L^{-1} to 293 µg L^{-1} (Figure 37). No significant differences between the water from the two tanks for any tested parameters were found.

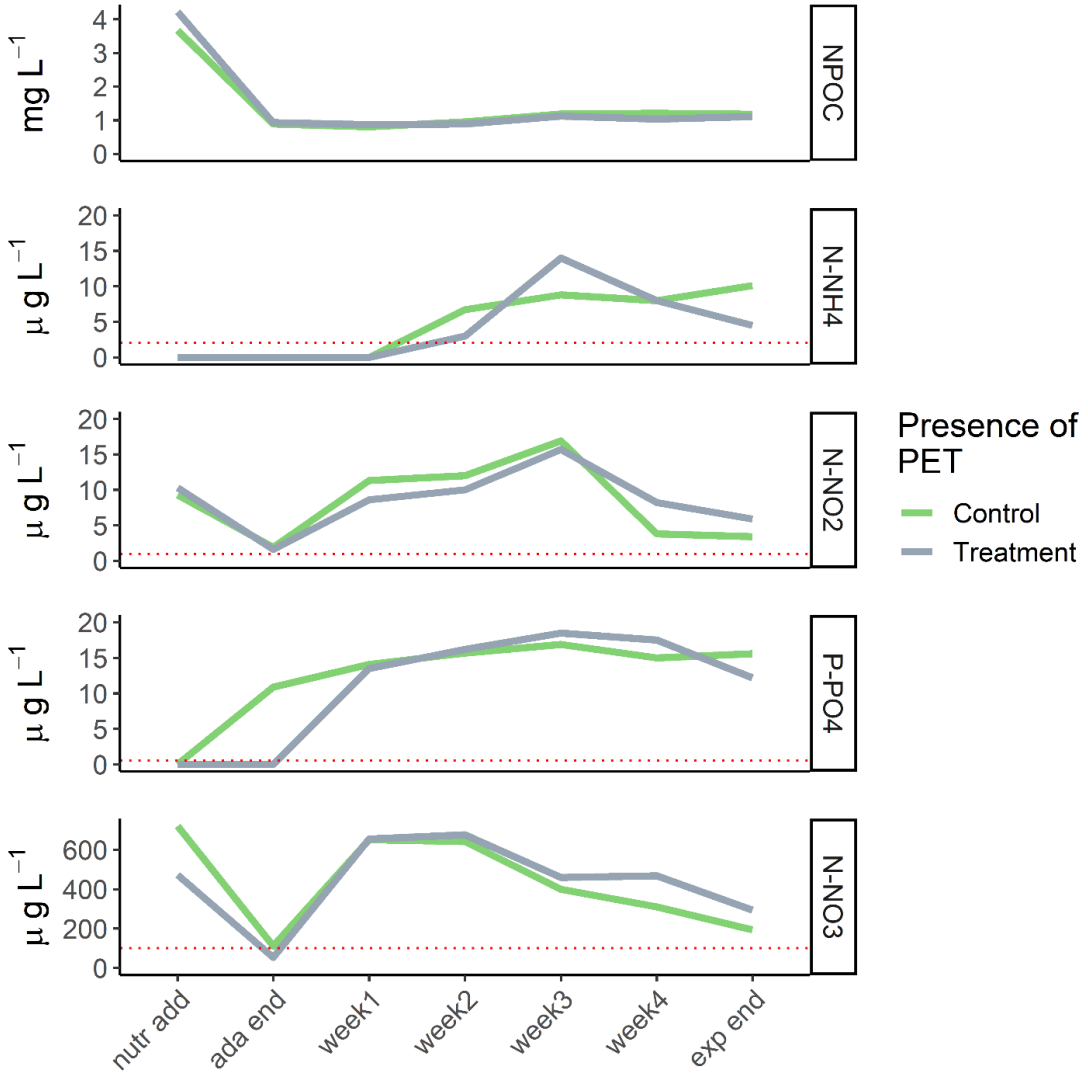


Figure 37: Graph showing changes in water chemistry measured in both water canisters during biofilm adaptation and mesocosm experiment (i.e, flow reactor). The red dotted line is the detection limit for individual compounds. Nutr add = nutrient added; ada end = adaptation end; exp end = experiment end.

Bacterial abundances

Bacterial cell counts were consistently higher in sediment samples without PET fibres. Moreover, a general trend indicated that the lowest cell counts were typically observed in stagnant reactors (Figure 38).

Bacterial cell counts in the control group ranged from 10.13 ± 6.45 x10^6 gDWsed^{-1} (inflow of saturated reactors) to 21.40 ± 12.04 x10^6 gDWsed^{-1} (outflow of unsaturated reactors) and were consistently higher in the control than in samples with PET fibres. In the PET treatments, bacterial count ranged from 6.06 ± 4.06 x10^6 gDWsed^{-1} (inflow of flowing reactors) to 19.99 ± 9.86 x10^6 gDWsed^{-1} (outflow of unsaturated reactors).

The data did not significantly deviate from a normal distribution (Shapiro-Wilk, p = 0.0823). Consequently, a 3-way ANOVA was used, with the factors “PET treatment”, “position”, and “regime”. All three factors exhibited statistical significance ($p_{treatment}$ = 0.0131, p_{regime} = 0.0464, $p_{position}$ = 0.0126).

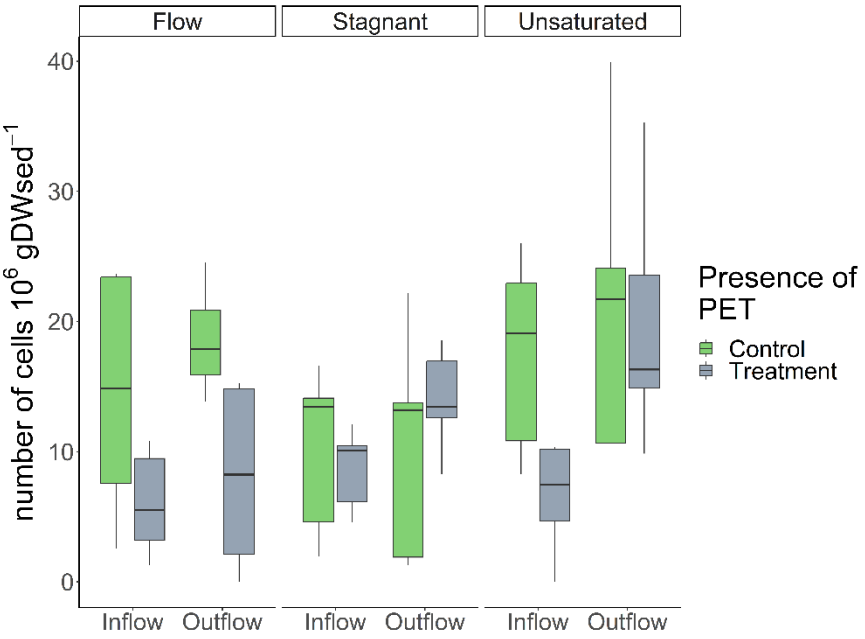


Figure 38: Boxplot comparison of bacterial abundances between different water regime (Flow, Stagnant and Unsaturated) and sediment treatments (control and treatment) after one month (N = 2 treatments x 3 regimes x 2 positions x 5 replicates). Inflow and outflow indicate the position of the samples collected from the reactors, indicating the direction of flow during the experiment or in the reactors during the adaptation stage of the experiment.

Total Protein Content (TPC)

In general, higher TPC was observed in the control compared to treatment with PET fibres. Similarly, elevated TPC was noted at the inflow sections of the reactors, as opposed to the outflow sections. Additionally, a trend showed that TPC was at its lowest in the saturated reactors (Figure 39).

The range of TPC within the control reactors spanned from 9.97 ± 2.89 µg prot g sed^{-1} in the outflow of the flow reactors to 18.15 ± 5.76 µg prot g sed^{-1} in the inflow of the flow reactor. The TPC in the treatment reactors ranged from 2.97 ± 2.36 µg prot g sed^{-1} at the inflow of saturated FRs to 17.90 ± 3.59 µg prot g sed^{-1} in the inflow of the flow reactors.

The results of the Shapiro test yielded a p-value of 0.05036, which prompted a confirmation of the 3-way ANOVA results through the Kruskal-Wallis test. The outcomes of the two tests aligned in terms of significance; hence, only the ANOVA results are presented. Only "treatment" ($p = 0.0247$) and "position" ($p < 0.0001$) showed any statistical significance, while the factor "regime" did not exhibit any significant variation ($p = 0.4157$). However, noteworthy interactions were observed between factors, including interactions incorporating the "regime".

Tukey's post hoc analysis identified the "treatment" and "position" factors as significant. The inflow to the control reactors exhibited notably higher TPC levels than the outflow ($p = 0.018$). Moreover, the outflow of the treated reactors exhibited lower TPC levels than the inflow ($p < 0.0001$). The interactions between "regime" and "position" identified two significant relations. The outflow of the flow reactors had significantly lower TPC than the inflow ($p = 0.0042$). Similarly, the outflow section of the stagnant reactors had significantly lower TPC than the inflow section ($p = 0.0049$). All three factors were significant for the stagnant reactors. The treated outflow had significantly lower TPC than the control inflow ($p = 0.0101$) or treated inflow water ($p = 0.0287$).

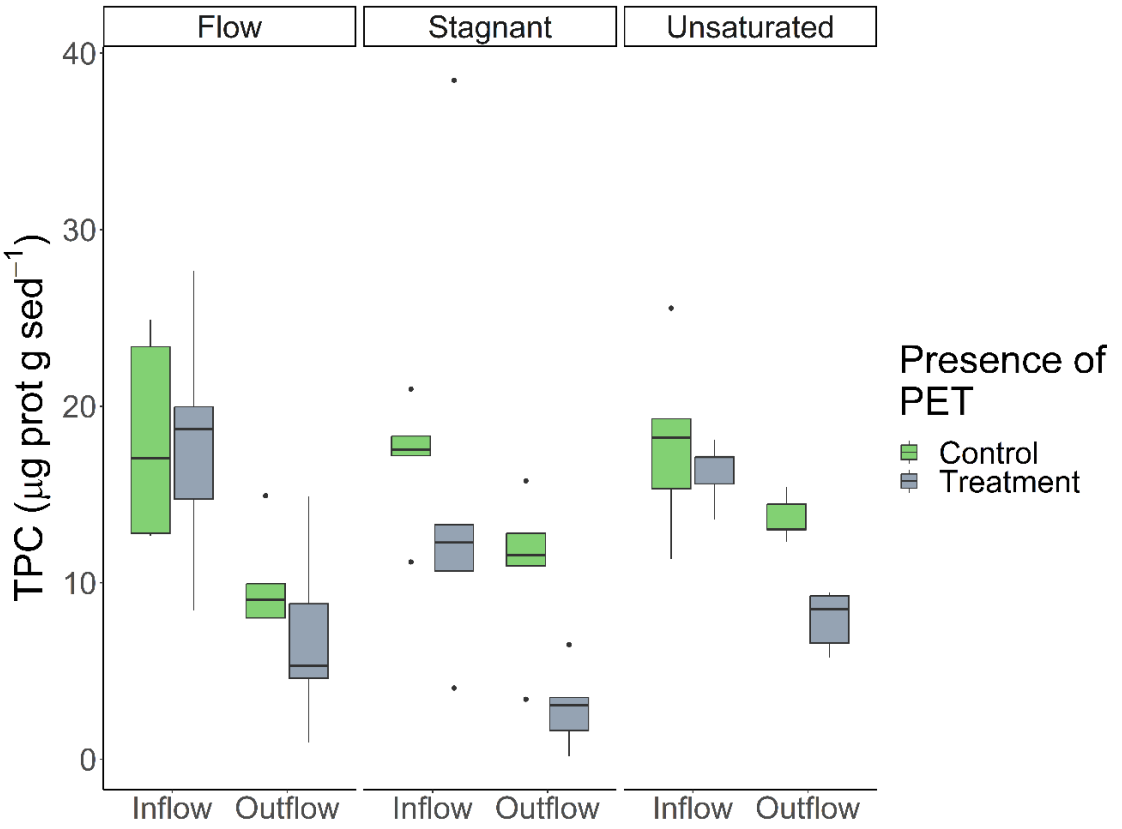


Figure 39: Boxplot comparison of TPC between different water regime treatments (Flow, Stagnant, Unsaturated) and sediments (control and treatment) after one month (N=60). Inflow and outflow indicate the position of the samples collected from the reactor, indicating existing flow direction during experiment or flow direction in the reactors during the adaptation phase of the experiment.

Respiratory electron transport system activity (ETSA)

ETSA appears to be higher in the treatment FRs than in the control. Additionally, ETSA is higher at the inflow than outflow, though no apparent differences are observed between the water regimes (Figure 40).

The mean ETSA in the control FRs ranged from -0.19 ± 0.56 µL O_2 gDW^{-1} h^{-1} (outflow unsaturated) to 0.32 ± 0.06 µL O_2 gDW^{-1} h^{-1}(outflow saturated). The mean ETSA for the treatment FRs ranged from 0.16 ± 0.27 µL O_2 gDW^{-1} h^{-1}(outflow saturated) to 0.44 ± 0.20 µL O_2 gDW^{-1} h^{-1}(inflow flow).

The distribution of ETSA data underwent assessment for normality using the Shapiro test. The results indicated a non-normal distribution ($p < 0.001$), even after applying a $\log_{10}(x+1)$ ($p < 0.001$). Therefore, the Kruskall-Wallis test was employed. The results revealed the significance of the factor "position" ($p = 0.0014$), while "treatment" exhibited borderline significance ($p = 0.0613$). Conversely, "regime" was insignificant ($p = 0.1649$).

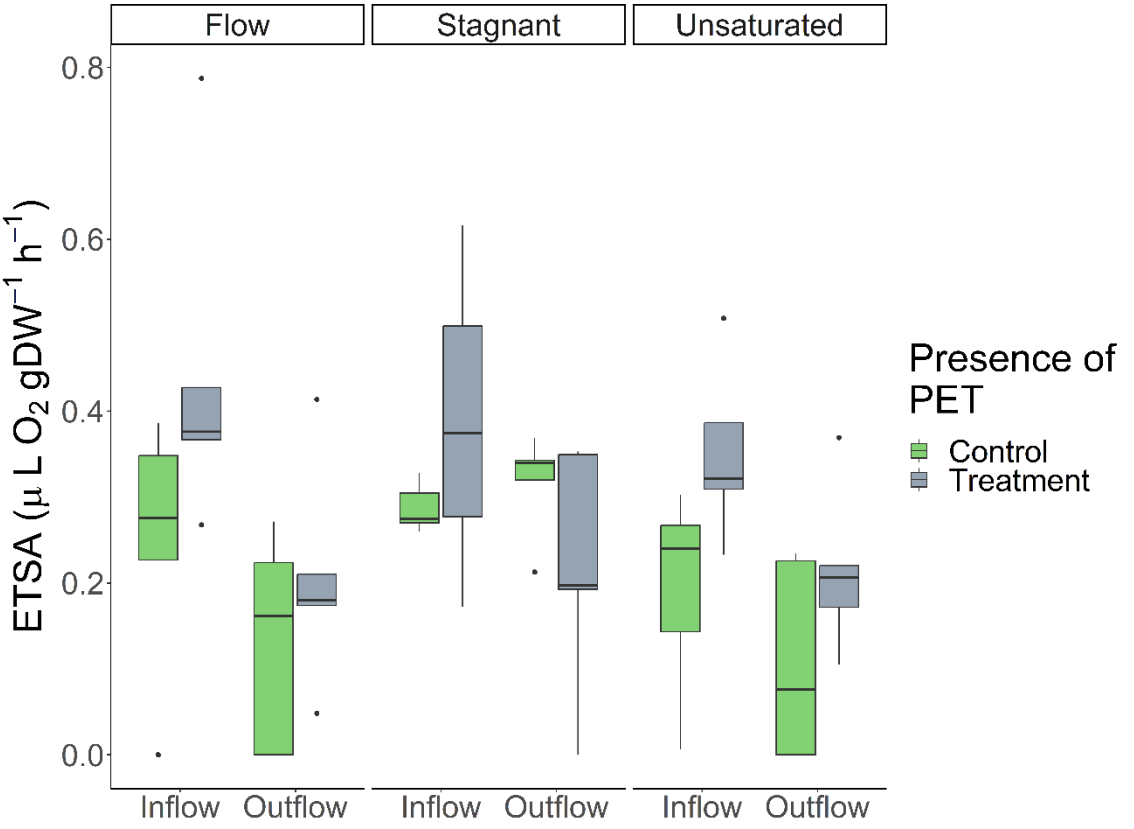


Figure 40: Boxplot comparison of ETSA between different water regime treatments (Flow, Stagnant and Unsaturated) and sediments (control and PET polluted) after one month (N=60). Inflow and outflow indicate the position of the samples collected from the reactor, indicating the direction of flow during the experiment or the reactors during the adaptation phase.

Community-level physiological profiling CLPP

Overall, the biofilms in the PET treatments, except for the stagnant ones, show suppressed metabolic functions across all the reactors (Figure 41). While the difference in inflow between the control and treatment for stagnant reactors seems minimal, greater diversity is observed in the biofilm from the treatment reactors at the outflow end. ANOSIM was performed for all factors, each yielding low p-values ($p = 0.001$ for all), indicating statistically significant differences among the three factors.

Likewise, the PERMANOVA analysis indicates that the factors "treatment", "regime", and "position", as well as their interactions, significantly contribute to explaining the dissimilarities between groups. The factor "treatment" ($F= 5.4185$, $p = 0.001$) explains 2.44% of the total variation, "regime" ($F=4.4791$, $p = 0.001$) explains 4.04 %, and "position" ($F = 18.7088$, $p = 0.001$) 8.43 % of the total variation, collectively accounting for 15% of the explained total variation.

Interactions between factors also prove to be significant: the combined influence of "treatment" and "regime" ($p = 0.007$), "treatment" and "position" ($p = 0.001$), and "regime" and "position" ($p = 0.001$). The interaction of all three factors demonstrates a trend toward significance ($p = 0.061$), suggesting that their combined influence has some impact on dissimilarity, though not reaching statistical significance.

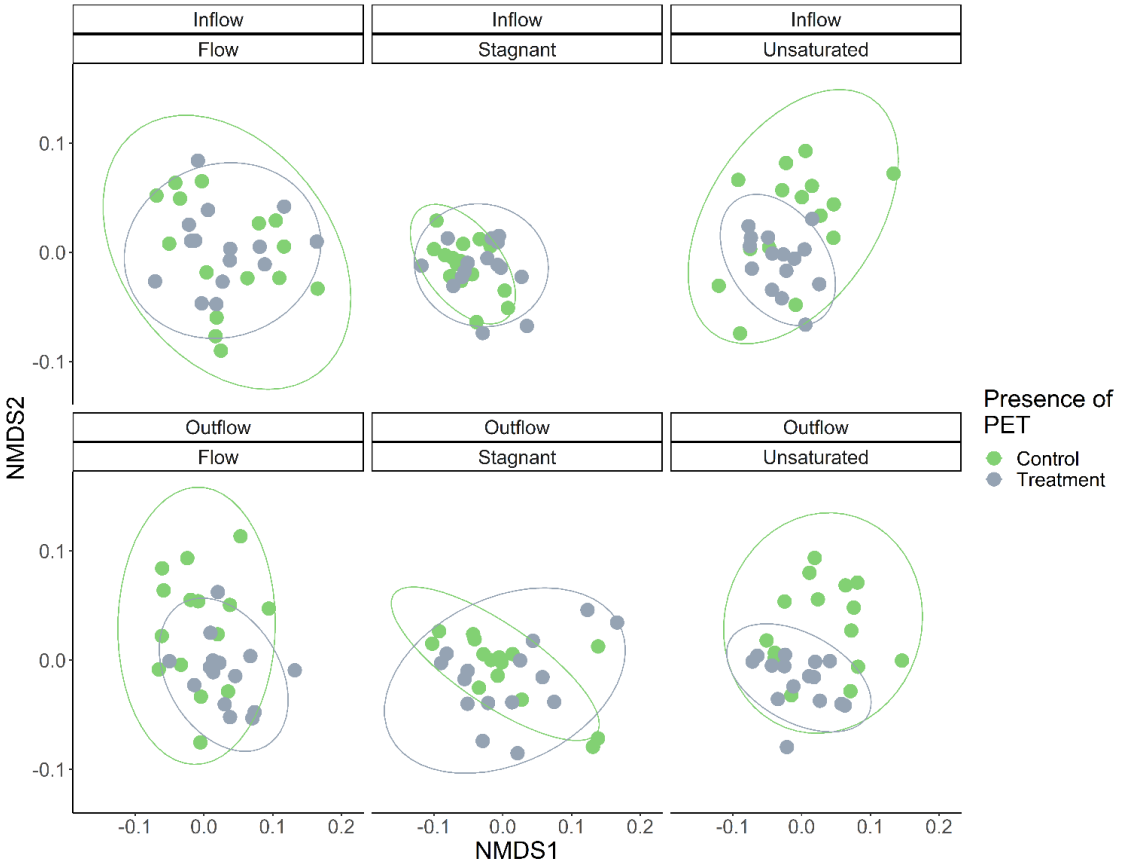


Figure 41: NMDS ordination diagrams based on data (N= 2 treatment x 3 regime x 2 positions x 5 replicates) on substrate utilization during mesocosm experiment (stress = 0.19) with factors: water regime, presence of PET fibres (treatment), and the position of the sample in the experimental reactor (inflow and outflow).

The heatmaps revealed distinct patterns (Figure 42). In the control group, the biofilms exhibited a greater capacity to utilise the substrates. Notably, L-asparagine was particularly efficiently utilised within the control setting. Within the control group, the biofilms sampled at the outflow displayed a trend where those from the stagnant reactors demonstrated higher success in utilizing most substrates, while their unsaturated counterparts exhibited lower success rates. A contrasting trend emerged for the biofilms within the unsaturated reactors at the outflow of the treatment reactors, where they exhibited a notably heightened utilization of substrates compared to the control.

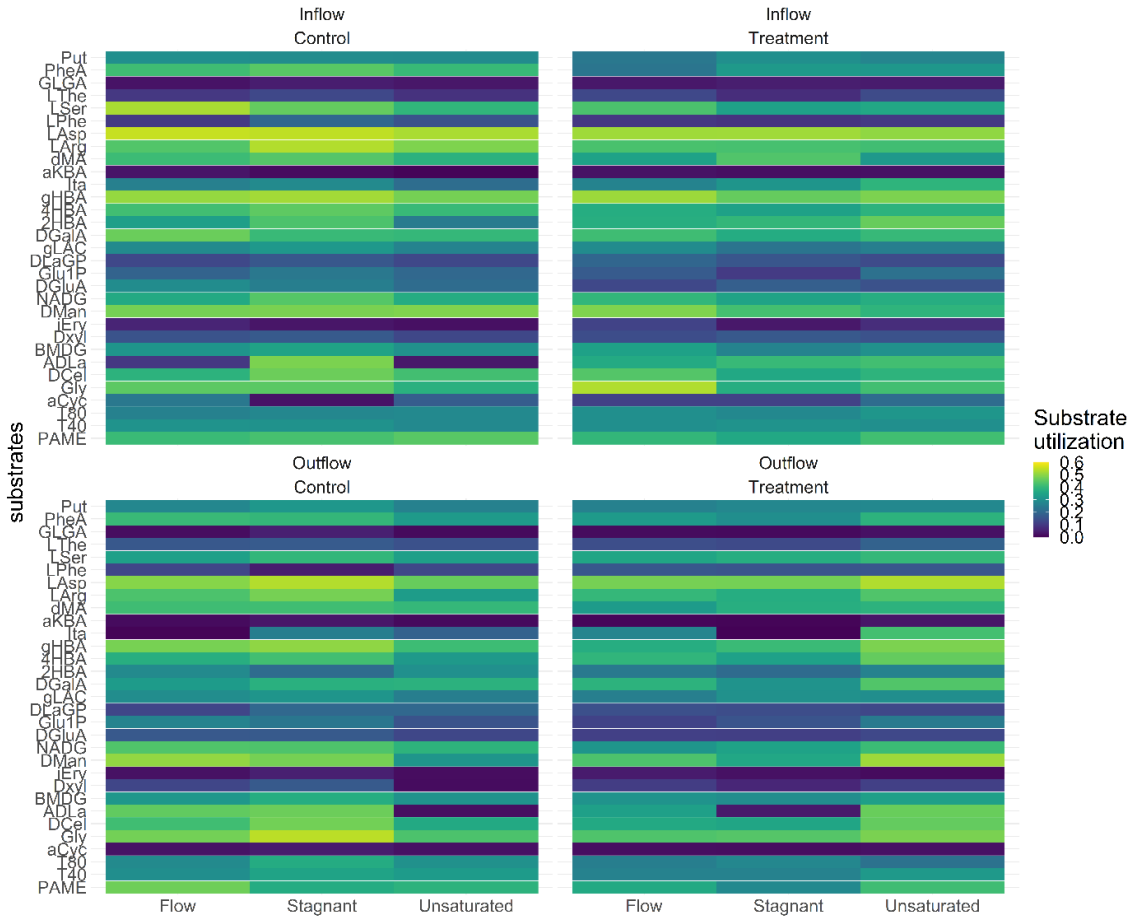


Figure 42: Heatmaps visualizing the intensity of substrate utilization. The visual comparison depicted was made between all three factors: “treatment”, “position” and “regimes”.

The factor “location” displayed the most substantial count of significant substrates (n = 22), with the “regime” closely following (n = 19) and “treatment” having a comparatively lower count (n = 6). When assessing their combined effects, the combination of “treatment” and “position” had the most influence on the number of substrates (n = 12), followed by the interaction of “position” and “regime” (n = 10), and the interaction of “treatment” and “regime” (n = 5) (Table 10).

Furthermore, the simultaneous impact of all three factors notably influenced seven distinct substrates, which included glycogen, symbolising the polymer group. However, most substrates with significance were lower within the Carbohydrates category. Notably, “position” influenced all four constituents of the polymer group, adding to its significance.

Table 10: The 3-way ANOVA results based on CLPP. Treatment - control or treatment; Regime - water regimes unsaturated, flow, saturated; position - inflow or outflow. The sign ":" indicates the combination of effects. Significance codes: '***' ≤ 0.001, '**' ≤ 0.01, '*' ≤ 0.05, '.' ≤ 0.1.

Substrate groups	**abbr**	**Eco**	**Treatment**	**Regime**	**Position**	**Treatment: Regime**	**Treatment: Position**	**Position: Regime**	**Treatment: Position: Regime**
Carbohydrates	BMDG	A2			**	*	***		
	PAME	B1		*					**
	Dxyl	B2		*	**				
	iEry	C2	***	***	***	.		***	**
	DMan	D2		*				***	
	NADG	E2		*			***	**	*
	DCel	G1		*	*			***	
	Glu1P	G2		***	***	.			
	ADLa	H1	***	***		***		*	
	DLaGP	H2		.	*	***	***		
Polymers	T40	C1			***				
	T80	D1			***		***		
	aCyc	E1			***			***	
	Gly	F1		*	***		***		*
Carboxylic and Ketonic Acids	gLAC	A3	*						**
	DGalA	B3					*		
	gHBA	E3	.		**		**		
	DGluA	F2	***	*	***		***		
	Ita	F3	.	*	**	.			
	aKBA	G3			***			**	
	dMa	H3	***	**			**	*	*
Phenolic compound	2HBA	C3		**	***	*	**		*
	4HBA	D3		*	*	.		.	
Amino Acids	LArg	A4	.		*				
	LAsp	B4		**	**			.	
	LPhe	C4		**		*	*	.	
	LSer	D4	.		***				
	LThe	E4		**	***		*	*	
	GLGA	F4		**	***	.			
Amines/ Amides	PheA	G4	***	***	**			***	
	Put	H4	.						

Discussion

This experiment analysed the single and combined effects of water regimes and PET microplastics on the abundance, biomass, and activity of hyporheic biofilms. Treatment with PET fibres showed significant inhibitory effects on microbial abundances and biomass (TPC) but not on ETSA, while the "regime" had a significant effect only on bacterial abundances. Microbial metabolic profiles (CLPP) in different water regimes seem to be

most affected by the presence of PET in unsaturated and flow regimes reactors, while there seems to be a minimal difference (inflow) or even greater diversity (outflow) in the stagnant regime reactors with PET fibres. The differences between the three factors ("treatment", "regime", and "position") were also shown by ANOSIM and PERMANOVA analysis. Similarly, the differences in heatmaps between control and treatment are the most pronounced under stagnant conditions.

Impact of the presence of PET fibres on hyporheic biofilms

The bacterial abundance was lower in the treatment reactors where PET was present. In a study conducted by Sathicq et al. (2022), the impacts of tire wear particles (TWP) and PET particles on the bacterial community were compared across a gradient of relative abundance, ranging from 100% PET to 100 % TWP over one month. The initial bacterial community used in their study was sourced from the coastal surface water of an oligotrophic subalpine lake. This community was then augmented with water from a WWTP before the final disinfection stage, resulting in a mix of 80 % lake water and 20 % WWTP water.

The findings indicated that TWP, unlike PET, encouraged bacterial growth. Microscopic analysis revealed that on PET, limited biofilm formation occurred. After one month, PET surfaces still showed early colonization stages despite greater morphological and taxonomic diversity. PET hosted a higher number of genera compared to water and TWP from the treated WWTP effluent. In essence, PET particles served as ecological refuges for potential pathogens from the WWTP, supporting their maintenance in aquatic systems without actively promoting their growth. The results agree with our study, as PET also inhibited bacterial growth. However, some other studies reported higher microbial growth on PET substrates.

In a study by Wang et al. (2008), the growth of bacteria sourced from groundwater was compared between glass and PET bottles, and significantly higher microbial growth was observed in PET bottles. They explained that PET bottles were pre-sterilised before use by gamma irradiation, which may have increased the release of carbon by PET bottles, stimulating the growth of PET bacteria. Similarly, Muthukrishnan et al. (2019) reported that PET samples showed the highest average bacterial counts, but the sampling location was heavily influenced by anthropogenic activity (the marina), with plenty of carbon sources.

PET inhibited microbial biomass development, assessed here via TPC, though other studies can use different approaches. In a study by Muthukrishnan et al. (2019), researchers also compared total bacterial biomass on different surfaces, including wood, steel, PET and PE, by weighing the biomass developed on each substrate. Plastic surfaces yielded less than half the biomass of wood or steel, with PET displaying the lowest biomass. Similarly, Song et al. (2023) demonstrated lower total biomass (evaluated through crystal violet staining of biomass – a mixture of living and dead cells, and EPS – extracellular polymeric substances) on PET compared to a biodegradable PLA.

Interestingly, PET and PLA biofilms exposed to river water had significantly higher total biomass than those from the HZ, but bacterial diversity was higher when exposed to HZ water. The PET surfaces were less impacted than PLA, displaying fewer grooves, shallower cracks, and cavities, resulting in fewer secure attachment options for biofilms. Furthermore, more EPS accumulated on the PET surface, explaining the higher biomass but lower bacterial abundance observed in the study. Previous research (Matjašič et al., 2021a) and the present study (Chapter 3.3.2.) noted lower biomass when exposed to PET.

The overall ETSA measured in our study remained notably low, registering at less than 0.5 μL O_2 gDW^{-1} h^{-1}. The water was collected from a pristine stream, so low respiration was expected. The low ETSA aligns closely with reported ETSA values for the coarse sediment fraction (ranging from 100 μm and 5 mm) within the riverbed and gravel bars,

as reported by Simčič and Mori (2007). Similarly, in the earlier studies, the microbial activity was inhibited in the presence of PET (Matjašič et al., 2021a) and in Chapter 3.3.2. The ETSA seemed to be subject to the physical and chemical attributes of the stream water, which in this case demonstrated a largely pristine condition.

With absorbance readings from the Ecoplates, a comparison between individual substrate utilization between treatments was visualised with heatmaps. Generally, biofilms from the control were better at utilizing the substrates than the treatment reactors. Although there was significance for several substrates utilised, there were also many substrates near significance, indicating a trend, possibly proving significant with more time. Matjašič et al. (2021a) drew similar conclusions that the presence of PET suppresses the capacity of the microbial community to degrade specific substrates. In this study, however, the polymers do not seem to be utilised more intensely, as in Matjašič et al. (2021a). The main difference between studies is the availability of the nutrients; more nutrients were available in the previous study, with the surrounding land use being mostly agricultural land, as opposed to the oligotrophic stream used in the current study. It again seems that nutrient availability is a significant factor to consider in studies on the effects of MP on biofilms.

Plastic particles are known biofilm inhibitors, as shown by various studies (Miao et al., 2019c; Seeley et al., 2020; Matjašič et al., 2021a; Miao et al., 2021b; Shen et al., 2021; Trabulo et al., 2022). However, bacteria prefer small particles in nutrient-rich environments (Duong et al., 2023). Without any additional carbon, the PET particles act as a niche and a refuge for taxonomically diverse bacteria but can promote growth when an easily attainable nutrient source is available.

Water regimes and hyporheic biofilms

The different water regimes had limited effects, primarily because the flow in the HZ is exceptionally slow. In the flow reactors, a continuous flow was maintained, but organic matter was restricted due to the use of oligotrophic stream water. Stagnant reactors relied solely on solute transport through diffusion, with no external addition of nutrients or oxygen. In unsaturated reactors, the moisture content was sufficiently high to sustain biofilm.

Although Amalfitano et al. (2008) established a significant correlation between bacterial abundance and sediment water content, demonstrating a 74% decrease in total abundance from wet to dry sediments, more recent studies suggested otherwise. Similar to the findings in this study, Harjung et al. (2019) observed an increase in bacterial cell concentration across all sampled locations during drying periods. Pohlon et al. (2013) noted an initial dip in bacterial abundances, followed by a subsequent recovery phase marked by higher abundances of Gram-positive bacteria more resilient to low water content. Similar conclusions were drawn by Gionchetta et al. (2019).

In the aftermath of short-term drought, bacterial communities tended to transition towards compositions typical of soil ecosystems, a trend that could potentially align with the findings of this study. A similar inference could be drawn for bacterial communities within stagnant water conditions. It is plausible that the community structure gradually shifted, potentially moving towards those characteristics of lake environments or even anoxic waters. The difference between the stagnant water regime and the flow and the unsaturated flow was also evident in the NMDS and the heatmaps.

Another plausible explanation would consider an important microhabitat characteristic: the moisture content within the hyporheic sediment. Since the water was drained from the unsaturated reactors without undergoing additional drying treatments, moisture levels likely remained sufficiently high to sustain biofilm survival. A study by Coulson et al. (2021) supports this notion, affirming that substantial residual moisture within the HZ

facilitated consistent microbial functioning during drought periods, irrespective of duration. Similarly, Harjung et al. (2019) found that HZ can provide refuge for microbes when surface water flow decreases or stops altogether. However, these conclusions should be subjected to further validation, considering the absence of bacterial species identification.

Additionally, in the study by Amalfitano et al. (2008), the biomass was significantly higher in wet compared to dry conditions, and the bacterial total biomass exponentially decreased with increasing dryness while also temporarily limiting metabolic activity. Low enzymatic activity (a measurement of microbial activity) in unsaturated conditions were also reported in several studies (Sabater & Romani, 1996; Rees et al., 2006; Pohlon et al., 2013; Ann, 2015; Harjung et al., 2019) and linked to DOM availability. The microbial biomass and activity in this study also did not differ significantly. The reason is likely the absence of nutrient supplementation during the experiment. In cases of carbon limitation, microbial metabolism typically shifts toward autotrophic production (Humphries & Baldwin, 2003). However, in this study, simulating hyporheic conditions in the dark led to constrained nutrient availability, preventing such a shift.

The interactions between factors

Subjecting microbial assemblages to varying water regimes and the presence of PET fibres appears to primarily impact bacterial abundances, where a trend was indicated ($p = 0.784$) that could be significant with more prolonged exposure. Bacterial biomass and activity were not significant. On the other hand, CLPP (among them glycogen as a representative of the Polymers group) and PERMANOVA showed significant interactions between factors, while NMDS and heatmaps visualised their interactions.

Without additional nutrients present, bacteria colonise the PET particles at a slow pace. The unsaturated reactors with no water flow also meant that PET leachates did not reach the bacteria, or the exposure was minor. As a result, these reactors had the highest bacterial abundance and generally lower but consistent microbial biomass and activity. In contrast, bacterial abundance was lowest in the treatment flow reactors, where water containing PET fibres and leachates is repeatedly circulated, resulting in continuous exposure of the microbial communities.

At the inflow, the bacterial biomass and activity were still higher than at the outflow, where most substances concentrate before being diluted in the water tanks. A similar explanation could account for variation in microbial metabolic function; flow reactors constantly remove inhibitory substances, while in unsaturated ones, they are not redistributed or recirculated. In the stagnant waters, the microbial biomass was notably lower at the outflow, but the microbial activity was highest, probably due to lower oxygen content (no external input of oxygen), resulting in the unloading of nutrients from dead bacteria.

Microbial metabolic function showed the most suppression in stagnant reactors, with no difference between control and treatment reactors. However, treated stagnant reactors exhibited higher variability at the outflow than the control. In stagnant reactors, water was present but with a smaller volume, leading to concentrated inhibitory substances, creating an even more toxic environment for un-adapted bacteria. They probably did not survive, again, providing nutrients for other more adapted bacteria. Additionally, anoxic conditions were established due to the lack of oxygen. However, the connection between the plastisphere and anaerobic pathways remains unexplored (Rogers et al., 2020).

In natural environments, stressors often exhibit intricate interconnections (Romero et al., 2019). However, the overall patterns in EU rivers can be categorised as hydromorphology (including riparian use) as the most influential, followed by nutrients and toxic substances (Lemm et al., 2021), which seem to agree with this thesis's findings, especially considering PERMANOVA results. In addition, the study by Lemm et al. (2021) reported

that toxic substances affect all river types, irrespective of their size, except for large rivers, which is probably linked to surrounding land use and WWTP effluents, as well as better dilution capacity, and integration over large and heterogeneous catchment areas of large rivers. Hydrological conditions are integral drivers of the river and hyporheic ecology, influencing physicochemical features (Creuzé des Chatelliers, 1991; Boulton et al., 1998; Mermillod-Blondin et al., 2000; Simčič & Mori, 2007).

Influence of spatial heterogeneity in hyporheic biofilms: inflow vs. outflow

The factor "position", featuring the inflow and outflow levels, represented spatial heterogeneity within the HZ and impacted bacterial abundance, biomass, and activity. Specifically, bacterial abundance was significantly lower at the inflow position, while bacterial biomass and activity were significantly higher at the outflow. Also, "position" seems to affect the stagnant reactors the most (Figure 41); the microbial metabolism at the inflow was much more similar between the control and treatment reactors compared to the outflow, where the microbial metabolism in treatment reactors exhibited even greater variety than those from control reactors.

Water flowed from the bottom to the top of the vertical FRs during the adaptation and experimental phases. This deliberate approach was adopted to control flow velocity. As such, samples collected at the inflow are more similar to those near the surface, while samples at the outflow are similar to those commonly found deeper within the HZ. The progression of depth typically translated to lower levels of organic matter and nutrients, a set of factors recognised as limiting agents influencing bacterial growth and metabolic activity (Battin & Sengschmitt, 1999; Krause et al., 2011; Nogaro et al., 2013; Zhou et al., 2014). This difference in organic matter likely contributed to the formation of a gradient driven by the direction of water (bottom to top) and proximity of the water source.

Perujo et al. reported on decreasing bacterial abundance with depth in their published works (Perujo et al., 2017; Perujo et al., 2019), though their primary focus centred on the distinction between coarse and fine sediments. Nonetheless, they did observe substantial bacterial biomass concentration at considerable depths with coarse sediments but a low proportion of live bacteria.

Generally, biofilm activity and biomass decrease with depth (Freixa et al., 2016; Perujo et al., 2017; Perujo et al., 2019), a pattern also evidenced here. Freixa et al. (2016) reported on distinctions in the degradation of organic matter across different sediment depths, highlighting more significant carbon compound decomposition in surface sediments and enhanced nitrogen compound degradation at greater depths, clearly highlighting the significant spatial heterogeneity within the HZ. A comparable phenomenon could be the explanation in the current study due to the clear inflow and outflow difference.

Conclusion

The presence of PET in the sediments had significantly inhibited bacterial abundance and biomass but not activity. The presence of PET also affected the community-level metabolic profile (CLPP). Here, several tested substrates showed significance in the utilisation rate related to the presence or absence of PET. Among them were carbohydrates, carboxylic and ketonic acids, as well as amines/amides.

Regarding the water regime, microbial abundance was higher in unsaturated reactors than in the stagnant and flow reactors, while the water regime did not significantly affect biomass and activity. The flow in the HZ is low, and the remaining moisture seems sufficient to keep the biofilms active. It could also mean that the community shifts toward soil-like communities in unsaturated, and communities typical for lake or anoxic water develop in stagnant reactors. However, this needs further investigation using metagenomics or other molecular approaches.

The combined effect of PET and different water regimes on hyporheic biofilm response was indicated only for bacterial abundances and did not yield statistically significant effects on TPC and ETSA. Also, the CLPP profile (as visualised by NMDS) of stagnant reactors differed from flow and unsaturated reactors.

Interestingly, spatial heterogeneity (i.e. the distribution of samples within the individual reactor) showed high significance in all analyses and was considered more influential than treatment and regime (PERMANOVA). Bacterial abundance was notably lower at the inflow position, while bacterial biomass and activity were significantly higher than the outflow. Such a difference could be attributed to natural processes like filtration, nutrient transformation, residence time, hydrogeological variability, seasonal factors, and redox potential. Redox potential influences the types of chemical reactions, impacting water chemistry. Downwelling and upwelling zones within the HZ also play an essential role by directing water flow and affecting the exchange of substances, further contributing to differences in water chemistry.

While microbial metabolism showed similar variability between control and treatment at inflow and outflow of flow and unsaturated reactors, this was not the case for stagnant reactors. The microbial metabolism variability was similar for control and treatment at the inflow. The variability of bacterial metabolism from the treatment reactors was greater than that of control reactors, most probably due to a gradual shift in the microbial metabolism profile, distinguishing itself from flow and unsaturated reactors.

These findings collectively underscore the complex interaction of PET fibres, water regimes and spatial heterogeneity in shaping biofilm responses. While some effects were significant, others hinted at trends that warrant further investigation. This holistic understanding advances our comprehension of the multifaceted dynamics governing hyporheic biofilm structure and functioning.

Chapter 4

Conclusions

My PhD thesis provides insight into the presence of MPs in freshwater environments, the relationship between hyporheic biofilms and MP and the response of hyporheic biofilms to MPs pollution, more specifically to pollution with polyethylene terephthalate (PET) fibres in the natural environment.

First, I investigated the scale of MP pollution in Slovenian rivers to assess the level of MP pollution. Importantly, I analysed data on MP pollution in small to mid-range-sized catchments that are part of the larger Danube catchment and compared it to information on global MP pollution gathered through a literature review.

Studies show that the concentration of MP typically increases with distance downstream from the source, and significant variations are observed between the two river compartments: water column and riverbed sediments. However, I found an evident lack of data regarding the size and colouration of the particles present. Also, although different approaches to water sampling are mentioned, using a suction basket to pump water samples appears to be the most efficient method for extracting microplastic (MP) particles from the water column. Other important considerations include choosing an appropriate sample size and filtering the samples before examining them under the FTIR microscope to avoid the need for manual separation and sample contamination.

In the sediments, most MPs are fragmented, while fibres are more common in the water column. Furthermore, PE and PP particles were identified as the primary MP pollutants in the water column and sediments. In fact, of all the anthropogenic fibres selected for the chemical analysis, 75 % were characterised as PET. The colouration of the particles was also highly variable across catchments, study sites and water compartments, indicating various sources of MP pollution. Most particles from the two compartments were in the smallest size categories, 0 to 0.99 mm and 1 to 1.99 mm. Finally, data regarding MP pollution in rivers are scattered, and the lack of standardised methods, laboratory procedures, and reporting makes comparing studies difficult and hampers scientific progress.

Next, the systematic literature review (SLR) was conducted to gain a better understanding of the interaction between MPs and bacteria in natural environments. One hundred and forty-five (145) papers reporting on the synthetic polymer degradation by bacteria were systematically reviewed. The studies varied in terms of the source of bacteria (inoculum) investigated, duration (typically < 6 months), temperature (mostly at room temperature), scale (mostly laboratory scale), and polymer type (primarily PE and its variants, e.g., LDPE).

Several bacterial strains, mainly originating from contaminated sites, have demonstrated the ability to degrade synthetic materials. Moreover, research has focused on understanding the microbial degradation processes involving PE and its variants (for example, LDPE). However, such materials should be pre-treated (exposed to UV) to more accurately simulate environmental conditions. So far, most plastic-degrading bacteria identified belonged to the Proteobacteria and Firmicutes phyla. Pseudomonas was the most typical representative of the Proteobacteria,

Bacillus was prevalent among the Firmicutes, while several studies report a combination of both genera. My analysis found only weak negative correlations between temperature and study duration in these groups. Thus, I recommend that future studies use higher temperatures and shorter incubation times (see (Matjašič et al., 2021b)) combined with Pseudomonas, Bacillus, or both to enhance polymer degradation. Moreover, longer-term studies (> 6 months) are essential to gain a comprehensive understanding of plastic biodegradation, extending beyond superficial degradation (biodeterioration). Once again, the inconsistency in reporting experimental conditions underscores the necessity for international standardization procedures to confirm the proposed biodegradation mechanisms.

To explore the connection between freshwater biofilms in polluted environments, their ability to colonize and degrade plastics, and to validate the findings from the literature, an experiment was conducted exposing commonly used plastic products such as textiles (PET), drink bottles (PET), and shopping bags (HDPE) to microorganisms from activated sludge in a low-carbon medium. After two months of incubation, differences were observed among the biofilms formed on various plastic materials (PET and HDPE), as indicated by SEM micrography. This finding is evidence that the type of plastic material can dictate the characteristics of biofilm formation, most probably due to mechanical and physical characteristics of the colonised surfaces and possible compounds leaching from the plastic substrate.

In subsequent experiments, the effects of PET on biofilm function were further tested *in situ* using artificially pre-prepared substrates. PET fibres were selected because they are among the most common microplastic pollutants in freshwater and research on the effects of PET pollution on bacteria is limited compared to that for PE. This task involved a one-year-long study to observe if and how PET fibres in riverbed sediments affect colonization and seasonal patterns of microbial function. For that purpose, various response variables were selected, including TPC as a proxy of biofilm biomass and ETSA and CLPP as proxies of metabolic functioning.

The CLPP using Biolog Ecoplates™ is a quick, cost-effective way to characterise microbial community metabolism by measuring the utilisation intensity of 31 different pre-prepared substrates contained in the Ecoplate wells. I found that the microbial biomass and activity were suppressed during colonization in the presence of PET, while metabolic functioning differed between treatments, although they were generally lower in the presence of PET compared to the control. Similarly, microbial biomass and activity were generally lower in the presence of PET during the seasonal study. I also observed that biofilms exposed to significant quantities of PET fibres were less susceptible to seasonal changes, proving that PET fibres suppress microbial functioning. Additionally, minimal surface deterioration was observed on the PET fibres, even after one year of in situ conditions. Based on these and other findings, this study significantly increases our understanding of how PET fibres may potentially affect biogeochemical processes within hyporheic biofilms.

To study the effects of PET on hyporheic biofilm function across all seasons and hydrogeomorphologically different sites, I extended the *in situ* study across both temporal and spatial dimensions. Over an additional year (two years total), samples from four sampling sites within two different catchments were collected, while consistent methodologies were used to ensure comparability. The sites differed in geology (alpine, karstic rivers), nutrient inflow, hydrology, land use and degree of pollution, thereby allowing me to investigate the response of their hyporheic biofilms to the presence of PET fibres. The findings indicated that exposure to PET fibres resulted in lower microbial biomass and activity and low metabolic variability, although the inhibition was less pronounced at sites close to the WWTP effluent outflow. At locations that are already highly polluted, the hyporheic biofilms are composed of microorganisms already adapted to such pollution. Hence, their response to PET fibres was minimal compared to the more pristine site.

Time was the most influential factor, indicating significant differences between seasons and years. Most probably, differences in discharge and temperature patterns over the years can

substantially affect the structure and function of hyporheic biofilms. The influence of season on the microbial activity in sediment was also noticeable, while for the biofilm on PET sheets, seasonal variation was observed in all measured variables. This finding is most likely due to the characteristics of the PET sheets, i.e., trapping organic material, increasing the nutrient availability to biofilms, and seasonal changes in temperature and precipitation patterns that drive organic matter and nutrient input from the catchments.

The microbial metabolism variability was highest in spring, probably due to higher precipitation and snow melt, where flow brings new bacterial species to colonise the MP particles. In summer and winter, when there is low flow, the metabolic function is more suppressed, as only those biofilms able to adapt can survive. The factor "rivers" influenced mostly the biofilms formed in the sediment pocket. The differences seem linked to their unique hydrogeomorphology and differences in catchment land use (urban, rural).

The microbial biomass and activity were also generally higher in more polluted locations, with higher nutrient availability, but the microbial metabolic variability was suppressed compared to the more pristine site. However, the difference between locations did not affect the biofilms on the PET sheets, probably because specific microbial assemblages usually colonise plastic particles. In summary, seasonal changes had a more pronounced impact on biofilms on PET sheets, whereas spatial variations were significant for biofilms on sediments (both control and those exposed to PET).

To investigate the impact of microplastic (MP) pollution on hyporheic biofilm functioning under continuous flow, stagnant and unsaturated water conditions, I performed another experiment using a controlled flume reactor. I demonstrated that PET fibres inhibited bacterial abundance and biomass while activity remained unaffected. Regarding microbial metabolic profile, the biofilms in flow and unsaturated reactors were similar: the variability was lower in the presence of PET. On the other hand, the stagnant reactors showed more variability than the other reactors with PET fibres. It is plausible that PET inhibited biofilm abundances, biomass and activity due to the leaching of inhibitory substances, but this needs to be tested. In addition, the variety of microbial metabolic profiles is suppressed in the reactors, where the inhibitory substances stay close to the biofilms. Given the significant impact of climate change on hyporheic flows and hydrological conditions, it is crucial to comprehend how the interaction between water regimes and MP pollution affects the HZ. The results from this part of my thesis indicated that the water regime is an important factor when studying the effect of MP pollution on hyporheic biofilm response.

My research has added valuable ecological insights into the environmental issue of microplastic pollution in the freshwater hyporheic zone. It enhances our understanding of the effects of MPs on biogeochemical processes driven by hyporheic biofilms, which play a crucial role in self-purification processes. This knowledge is essential for addressing the challenges posed by MP pollution and for developing effective mitigation strategies.

Ultimately, using molecular techniques to identify species and explore their functional genomics is vital for gaining a deeper insight into an individual species' ecological traits and functional genes. However, these analyses can be costly and are not always feasible in specific research contexts. For this reason, my approach balances cost and the quality of information obtained.

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Conference Paper

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Biography

Tjaša Matjašič was born on July 17, 1990 in Maribor, Slovenia. She pursued her academic career by studying biology at the Faculty of Natural Sciences and Mathematics (FNM) in Maribor. In 2012, she earned her bachelor's degree in Biology (1^{st} cycle). Later, in 2016, she successfully defended her masters thesis in Biology and Ecology with Nature conservation (2^{nd} cycle; bologna degree). Her thesis was titled "The microbiota from choanae of selected free living birds species". During her studies, she participated in several projects, including collaboration with Šampionka d.d., where she worked on microbiota in industrial vinegar production, and at the Medical Faculty in Maribor, where she researched *Clostridium difficile* proteins.

After one year of working at the Community Health laboratory, she enrolled in the doctoral study programme at the Jožef Stefan International Postgraduate School funded by the Young Researchers Programme of the Slovenian Research Agency under the supervision of Dr. Nataša Mori from the National Institute of Biology in Ljubljana. Within the Erasmus program, she conducted a part of her PhD work in the laboratory of Prof. Dr. Gabriele Weigelhofer at Wassercluster Lunz, Austria, specializing in microbial ecology and the response of hyporheic biofilms to different stressors, particularly microplastics. Presently, she is employed as a project associate at Wassercluster Lunz, working on the DIRT project, which investigates the impact of drought on the remobilization of water pollutants from river sediments.