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Distribution of microplastics in river sediments and consequences on bioturbation associated ecosystem processes

Mohammad Wazne

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**THESE de DOCTORAT DE
L'UNIVERSITE CLAUDE BERNARD LYON 1**

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**Distribution of microplastics in river
sediments and consequences on
bioturbation associated ecosystem
processes**

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Résumé en français

Les microplastiques constituent une préoccupation de plus en plus importante dans les écosystèmes d'eau douce, mais l'absence d'approches cohérentes et de protocoles normalisés entrave l'évaluation précise des risques qu'ils représentent. De plus, la recherche sur l'impact des microplastiques sur le fonctionnement des écosystèmes reste limitée. Par conséquent, cette thèse de doctorat vise à combler ces lacunes en abordant la présence et les conséquences des microplastiques dans les environnements d'eau douce. La thèse commence par aborder les défis liés à l'étude des microplastiques, en mettant l'accent sur les avancées méthodologiques. Elle met en évidence le manque de normalisation et les limitations de débit des méthodes existantes d'extraction et d'échantillonnage des microplastiques. Ainsi, cette étude optimise un protocole d'extraction actuel et compare différentes approches d'échantillonnage des microplastiques dans les sédiments des cours d'eau pour faciliter les comparaisons inter-études. De plus, cette recherche présente une méthodologie fiable pour l'identification spécifique des polymères synthétiques à l'aide de la coloration au rouge Nil, qui permet de distinguer les matériaux plastiques des matériaux non plastiques en fonction de leur intensité de fluorescence. Cette recherche étudie ensuite le transport et le destin des microplastiques dans le lit des rivières, en mettant l'accent sur le rôle de l'interface sédiments-eau dans la rétention temporaire des débris plastiques. L'influence des facteurs hydrologiques et sédimentaires, en particulier les échanges hydrologiques entre les eaux de surface et les eaux souterraines à l'interface sédiments-eau (c'est-à-dire les échanges hyporhéiques), sur la distribution des microplastiques est examinée. Des variations significatives dans les concentrations de microplastiques à travers différents échanges hyporhéiques et profondeurs de sédiments sont observées. Enfin, cette thèse examine l'effet des microplastiques sur la bioturbation et les processus biogéochimiques à l'interface sédiments-eau. Les activités de bioturbation de *Tubifex tubifex* (*Oligochaeta*, *Tubificidae*), un important ingénieur de l'écosystème, sont étudiées sous différentes concentrations de microplastiques dans les sédiments. La recherche révèle des réponses au stress oxydatif et une réduction des activités de bioturbation, ce qui entraîne une diminution de la minéralisation de la matière organique et des flux de nutriments. Ces résultats mettent en évidence les implications écologiques potentielles à long terme de la pollution par les microplastiques sur le fonctionnement des écosystèmes, un aspect qui a été peu étudié dans les recherches précédentes.

Abstract

Microplastics are a growing concern in freshwater ecosystems, but the lack of consistent approaches and standardized protocols hampers accurate assessments of their risks. Additionally, there is still limited research on the impact of microplastics on ecosystem functioning. Therefore, this PhD manuscript aims to bridge these gaps by addressing the presence and consequences of microplastics in freshwater environments. The manuscript begins by addressing challenges in investigating microplastics, with an emphasis on methodological advancements. It highlights the lack of standardization and throughput limitations in existing methods for microplastic extraction and sampling. Thus, the study optimizes a current extraction protocol and compares different sampling approaches for microplastics in streambed sediments to facilitate reliable inter-study comparisons. Additionally, this research presents a reliable methodology for synthetic polymer identification using Nile red staining, which distinguishes between plastic and non-plastic materials based on fluorescence intensity. This research then investigates the transport and fate of microplastics in streambed rivers, with a focus on the role of the sediment-water interface in temporarily retaining plastic debris. The influence of hydrological and sedimentary factors, particularly hydrological exchanges between surface and ground water at the sediment-water interface (i.e., hyporheic exchanges), on microplastic distribution is examined. Significant variations in microplastic concentrations across different hyporheic exchanges and sediment depths are observed. Finally, the manuscript examines the effect of microplastics on bioturbation and biogeochemical processes at the sediment-water interface. The bioturbation activities of *Tubifex tubifex* (Oligochaeta, Tubificidae), an important ecosystem engineer, are studied under varying microplastic concentrations in sediments. The research reveals oxidative stress responses and reduced bioturbation activities, resulting in decreased organic matter mineralization and nutrient fluxes. These findings highlight the potential long-term ecological implications of microplastic pollution on ecosystem functioning, an aspect that has received limited attention in previous research.

Thesis framework

This thesis was carried out as a part of an academic collaboration between Université Claude Bernard Lyon 1 (UCBL1) - Laboratoire d'Ecologie des Hydrosystèmes Naturels et Anthropisés (UMR 5023 LEHNA), and University of Birmingham - School of Geography, Earth and Environmental Sciences.

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***« C'est le temps que tu as perdu pour ta rose
qui fait ta rose si importante. »***

Antoine de Saint-Exupéry

Chapter I

General introduction

1. Introduction

1.1. The history of plastic

The use of natural polymers by humankind dates back to the earliest civilizations. The Egyptians used resin to varnish the sarcophagi, while the Greeks fashioned amber into ornaments and jewelry (Bijker et al., 1989). Semi-synthetic polymers were initially developed by a metallurgist and inventor from Birmingham, England, named Alexander Parkes in 1862. Parkes introduced a cellulose-based polymer called Parkesine, which could be chemically modified from rigid to flexible or even soft and rubberlike. Parkes research paved the way for the American inventor John Wesley Hyatt to improve the production of Celluloid (i.e., chemically modified cellulose) as an alternative for ivory in billiard balls and other innovative uses (Bijker et al., 1989; Lintsen et al., 2017). The emergence of fully synthetic plastics was not recorded until 1907, when the Belgian chemist, Leo Hendrik Baekeland, mixed two common chemicals (i.e., phenol and formaldehyde) under pressure and heat in a sealed autoclave, giving rise to the first synthetic plastic known as Bakelite (Bijker et al., 1989; Wagner, 2011). Having successfully proven its efficacy during the war, Bakelite, which was later famously known as plastic, greatly expanded after World War II (Bijker et al., 1989; Freinkel, 2011). Plastic products and markets increasingly flourished at the expense of traditional materials, replacing steel in cars, paper, and glass in packaging, and wood in furniture (Freinkel, 2011). Today, thanks to their properties as versatile, lightweight, strong, durable, and cheap materials, plastics are present in every aspect of our modern daily life (Andrady and Neal, 2009). Nevertheless, their inexpensive and disposable nature makes them easily and carelessly discarded in the environment, leaving behind a tremendous amount of plastic waste that we are suffering to mitigate (Borrelle et al., 2020).

1.2. Chemical composition and types of plastics

Plastic production throughout the world has skyrocketed over the last decades, increasing by 230-fold from 2 million tonnes (Mt) in 1950 to an estimated 460 Mt in 2019. Yet, it is expected that in 2060 it will reach 1,231 Mt, almost triple (OECD, 2022). Globally, only about 9 percent of discarded plastic has been recycled. The remaining waste is either incinerated, deposited into the ocean, or buried in landfills (OECD, 2022; Ritchie and Roser, 2018). Recently, during the

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COVID-19 pandemic, synthetic plastic waste has increased by 370% due to the increasing usage of personal protective equipment (PPE), such as face masks and medical plastics (Patrício Silva et al., 2021). Moreover, due to safety concerns, the demand for single-use plastics for food packaging applications increased by 40% (Prata et al., 2020). Today, as a result of the increased production and demand for plastics, the environment pays the highest price, with plastic debris omnipresent in almost every ecosystem, serving as a geological indicator for the proposed Anthropocene period (Allen et al., 2019; Andrady, 2011; Frei et al., 2019; Huang et al., 2020; Zalasiewicz et al., 2016).

Plastics are complex materials derived from fossil hydrocarbons and composed of a variety of constituents (Wiesinger et al., 2021). The primary constituent is a synthetic polymer matrix that is composed of a repeating organic monomer unit. Based on this polymer matrix, plastics can be classified as thermoplastics (e.g., polyethylene, polypropylene; approximately 90% of plastics) or thermosets (e.g., epoxy, polyurethane; approximately 10% of plastics). Thermoplastics are a variety of plastic that can be deformed at a certain elevated temperature and reform when cooled (i.e., they can be recycled multiple times), whereas thermoset plastics cannot be reformed after the initial heating (Alauddin et al., 1995; Kazemi et al., 2021). The thermoplastic polymers polypropylene (PP) and polyethylene (PE), including both low- and high-density polyethylene (PE-LD and PE-HD, respectively), are among the most produced and highly consumed plastic polymers worldwide. Polyvinyl chloride (PVC), Polyurethane (PUR), Polyester (PET), and Polystyrene (PS) often come next (Geyer et al., 2017; Plastics Europe, 2022).

In most applications, the resin polymer cannot be used on its own; instead, it is mixed with a cocktail of chemicals that together make up the bulk of the plastic material. These chemicals make up a significant portion of the overall weight of the material and contribute to enhancing plastic performance (Rochman et al., 2019). Such chemicals are called additives, and they include a variety of inorganic fillers such as carbon and silica that reinforce the material, as well as plasticizers to make the material more flexible (e.g., phthalates and bisphenol group), thermal and UV stabilizers, flame retardants (e.g., polybrominated diphenyl ethers and tetrabromobisphenol A), and colorants (Hermabessiere et al., 2017; Thompson et al., 2009a). Currently, the plastics industry uses resin codes to identify the main type of chemical compound

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used to create the product. However, despite the fact that there are only seven resin codes (**Figure 1**), there are thousands of different types of plastic. Different dyes and additives can be added to the basic resin to create various plastic products. This causes variations in melting points and other physical properties within a given resin code, making it difficult to recycle plastics bearing the same code (ASTM International, 2020; Jiang et al., 2022; Recycling, 2009).















| | | | | | | |
|--|--|---|--|---|---|---|
|  |  |  |  |  |  |  |
| PET | HDPE | PVC | LDPE | PP | PS | Other |
| Polyethylene Terephthalate | High-Density Polyethylene | Polyvinyl Chloride | Low-Density Polyethylene | Polypropylene | Polystyrene | Other |
| Soda and water bottles | Shampoo and detergent bottles | Plastic pipes and food container | Bread bags and plastic films | Yogurt tubs and plastic straws | Styrofoam cups and take-away containers | CDs and food containers |
|  |  |  |  |  |  |  |

Figure 1 Different types of plastic, some of their applications, and their resin codes.

The variety of polymers and chemicals used in the production of plastic products enabled them to possess unique properties such as being lightweight, strong, durable, corrosion-resistant, and having high thermal and electrical insulation properties, thereby making them an incredibly versatile material that could be easily produced for a variety of applications at a low cost of production (Andrady, 2011; Andrady and Neal, 2009). However, the same properties that make plastics so versatile have created an emergent environmental threat, as millions of metric tons of plastic particles accumulate through ecosystems and threaten biodiversity (Hughes, 2022; Kubowicz and Booth, 2017; Shah et al., 2008; Thompson et al., 2009b). Nowadays, in an attempt to foster a more sustainable society and address plastic waste management crises, there is a growing demand for eco-friendly plastics (Iwata, 2015). Under this category, bio-based and

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biodegradable plastics are the two most prominent types. These two classes cause frequent confusion because they are believed to be identical, despite their conceptual differences. Bio-based plastics, in contrast to conventional petroleum-based plastics, are produced from biomass feedstocks or renewable resources (such as starch, cellulose, hemicellulose, lignin, or plant oil). Biodegradable plastics are materials that are degraded or decomposed to carbon dioxide and water by microorganisms in the environment (Oever et al., 2017; Thompson et al., 2009a; Wang and Praetorius, 2022). In light of their primary materials and biodegradability, plastics can now be divided into four categories: (1) oil-based non-biodegradable plastics; (2) oil-based biodegradable plastics; (3) bio-based non-biodegradable plastics; and (4) bio-based biodegradable plastics (Iwata, 2015) (**Figure 2**).

Once released into the environment, plastics exhibit a resistance to degradation that may last hundreds or even thousands of years (Barnes et al., 2009). Although they are still not well understood, several mechanisms interfere in breaking down large plastic debris into tiny size particles or even into individual

polymers. These mechanisms include physical (e.g., photo-oxidative and thermal degradation), chemical (e.g., hydrolysis and chemical fragmentation), and biological processes (e.g., microbial and enzymatic decomposition) (Boyle and Örmeci, 2020; Singh and Sharma, 2008). The degradation of larger plastic particles produces several size-based classifications of plastic debris in the environment, which include: megaplastic (>1 m in diameter), macroplastic (<1 m), mesoplastic (<2.5 cm), microplastic (<5 mm), and nanoplastic (<1 μm) (Chatterjee and Sharma, 2019; Gigault et al., 2018; Kershaw and Rochman, 2015; Wang et al., 2018).

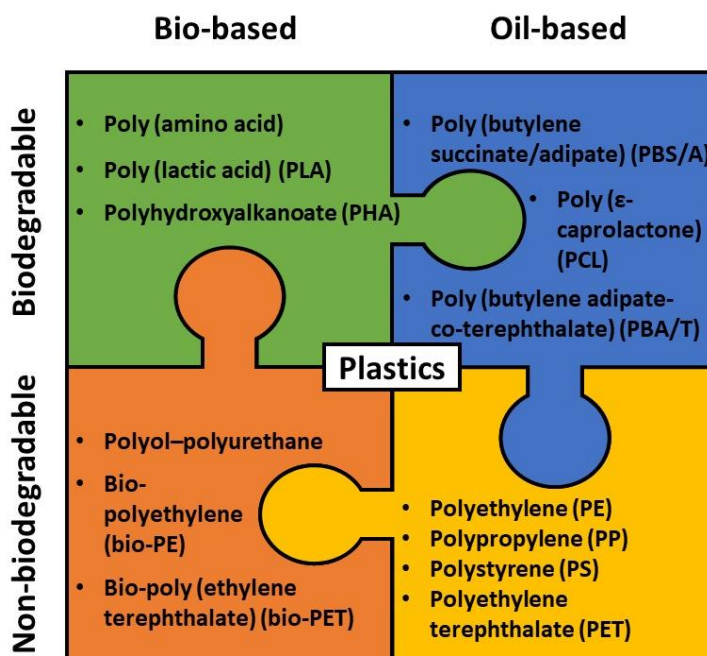


Figure 2 Schematic figure showing examples of plastic polymers categorized based on their primary materials, bio-based vs oil-based (i.e., petrochemical), and biodegradability, biodegradable vs non-biodegradable plastics.

1.3. Microplastics

In 2004, Richard Thompson coined the term "microplastics" to refer to plastic debris smaller than 20 μm (Thompson et al., 2004). This term was later defined as particles and fragments less than 5 mm in size (Arthur et al., 2009). The vast majority of microplastics are anthropogenically synthesized as primary microplastics for specific applications, such as cosmetics and cleaning products. On the other hand, they can also be produced as secondary microplastics as a result of the decomposition of larger plastic wastes in the environment (Duis and Coors, 2016; Wu et al., 2019). Currently, these polymeric particles are ubiquitous in the environment (Lambert and Wagner, 2018), posing a threat to organisms (Krause et al., 2021; Kukkola et al., 2021; Kwak and An, 2021) and ecosystems alike (de Souza Machado et al., 2018; Galgani and Loisel, 2021; Rillig et al., 2021; Seeley et al., 2020).

Due to their micro-size scale, microplastics are bioavailable to a wide range of fauna and flora, and their ingestion results in several negative impacts on organisms (Barnes et al., 2009; Santana et al., 2016; Sussarellu et al., 2016; van Weert et al., 2019). It has been widely reported that terrestrial and aquatic organisms can ingest microplastics (Duis and Coors, 2016; Vázquez and Rahman, 2021). In aquatic environments, where plastic debris are transported and dumped (Eriksen et al., 2014; Horton and Dixon, 2018; Sandgaard et al., 2023), the uptake of microplastic has been shown by numerous species, spanning from protozoans to aquatic mammals (Cole et al., 2013; Hollman et al., 2013; Kukkola et al., 2021; Naidoo and Glassom, 2019; Wagner et al., 2014; Wright et al., 2013). Ingested microplastic may result in a variety of severe effects, such as obstruction of the digestive tract, reduced growth rates, adverse effects on the feeding rate, and diminished energy reserves. As a result, growth, survival, fecundity, and reproduction rate are negatively impacted, which affects the overall fitness of the organism (Besseling et al., 2013; de Sá et al., 2018; Jemec et al., 2016; Lei et al., 2018; Redondo-Hasselerharm et al., 2018; Straub et al., 2017; Ziajahromi et al., 2018).

Upon ingestion, microplastic can directly damage the organism either physically through the internal abrasion and clogging caused by the plastic structure within the organs and tissues (Wu et al., 2019), or chemically through the transfer and release of the toxic chemicals (through

degradation or leaching of the plastic additives) of which they are made, resulting in a variety of adverse effects, including endocrine-disrupting effects (Teuten et al., 2009). Besides, due to their high surface-to-volume ratio, microplastics can act as vectors for pathogen microorganisms and viruses, and organic and inorganic chemical pollutants that can adsorb and concentrate at the surface of microplastics, causing indirect toxicity (Cole et al., 2011; Krause et al., 2021; Rochman et al., 2013). The extent of the damage caused by microplastics, both directly and indirectly, is still poorly understood. The absorption and toxicity of plastic particles are determined by a number of parameters that include the microplastic concentration, exposure time, particle size, particle shape, and polymer type. Additionally, the outcome of plastic particle exposures is significantly influenced by the species studied, the developmental stage and the sex of the organism, the quantity of food available, and the likelihood of exposure to other contaminants within a particular environmental condition (Kögel et al., 2020; Malli et al., 2022).

1.4. Microplastics in freshwater ecosystems

In comparison to freshwater ecosystems, marine environments have been the focus of the majority of plastic pollution studies (Blettler et al., 2018). However, the prevalence of microplastic pollution in freshwater is comparable to or even greater than that reported in marine waters (McCormick et al., 2016). More than 5 trillion plastic particles have been identified in the ocean (Eriksen et al., 2014), making it the final destination for microplastics, where 80% of these particles were transported by rivers (Horton et al., 2017; Meijer et al., 2021). It is estimated that rivers act as a primary conduit for plastic litter, contributing to the annual transport of 1.15 to 2.41 million tonnes of plastic to the ocean (Lebreton et al., 2017). It is particularly observed in aquatic ecosystems in urban areas because microplastic contamination of land waters is closely related to population density (Xue et al., 2020). Therefore, microplastic pollution from inland water deserves further attention.

Several human and industrial activities, including swimming, boating, and fishing, as well as storm drains, wastewater treatment plant (WWTP) effluents, and the degree to which plastic factories are located near watersheds, contribute to the introduction of plastic waste into freshwater systems (Dendievel et al., 2023; Kooi et al., 2018; Margenat et al., 2021). Yet, sewage sludge,

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which may contain synthetic fibers and microplastics from household or personal care products, may be a significant source of plastic pollution that can enter rivers from land-based sources (Best, 2019; Hale et al., 2020). Once in freshwater systems, the buoyancy of the plastic particles renders them to disperse easily (Molazadeh et al., 2023; Paduani, 2020).

When moving throughout the riverine systems, similar to natural particles, microplastics are not uniformly transported. Their particle properties, such as size, shape, density, surface roughness, as well as hydrodynamic conditions (e.g., aggregation, settlement, and resuspension) and the hydrological factors (e.g., stream flow), play a key role in controlling the mechanisms and timescales of their transport (Kumar et al., 2021; Li et al., 2023). Microplastics can be translocated from surface water to streambed sediments due to gravitational settling (i.e. sedimentation) and/or vertical water fluxes at the sediment-water interface, which are governed by the exchange of surface water and groundwater (i.e., hyporheic exchanges) (Drummond et al., 2022, 2020; Frei et al., 2019). Hence, riverbed sediments will act as a temporary sink for microplastics, increasing the likelihood of plastic particles being ingested by benthic organisms. This will therefore threaten interstitial fauna, as well as the sedimentary microbial communities, thus negatively affecting the sediment-water interface ecosystem functioning (Frei et al., 2019; Krause et al., 2021; Sandgaard et al., 2023; Seeley et al., 2020; Silva et al., 2022).

Currently, the absence of standardized or harmonized analytical approaches and experimental protocols for studying microplastics introduces uncertainty in the assessment of their concentration, transport, and distribution in rivers; consequently, the severity of the ecological risks associated with microplastic pollution may be misunderstood if the improper methodology is implemented (Mitrano et al., 2023; Razeghi et al., 2022). In light of this, and given the disproportionate focus on marine ecosystems compared to freshwater systems, as well as the presence of various factors governing plastic mobility processes in riverine systems, particularly at the sediment-water interface, efforts should continue to improve methodologies that allow for a more thorough understanding of the transport, distribution, and impacts of microplastics in riverbed sediments. This is critical for gaining deeper insights into the ecological consequences of microplastics, developing effective mitigation strategies, and formulating informed policies

and regulations to address this crisis more efficiently (Barcelo and Pico, 2020; Provencher et al., 2020; Rochman, 2016).

1.5. Ecosystem functioning at the sediment-water interface

In aquatic ecosystems, the sediment-water interface is a dynamic zone that regulates the exchange of organic matter, nutrients, and pollutants between the surface water, sediments, and ground water. The abiotic features of sedimentary ecosystems and the activity of resident macro – and microorganisms interact in complex ways at this interface in order to regulate essential ecological and biogeochemical processes (Krause et al., 2009; Lewandowski et al., 2019; Mermillod-Blondin, 2011).

Bioturbation is one of the most important ecological processes that take place at the sediment-water interface and maintain the stability of the aquatic ecosystem. According to Kristensen et al., (2012), bioturbation is defined as *“all transport processes carried out by animals that directly or indirectly affect sediment matrices”*. Bioturbation-induced transport impacts both solids (i.e., particle reworking) and solutes (i.e., ventilation), having profound effects on sediment geochemistry, matter exchange across sediment-water interfaces, and thus, ecological functioning of aquatic sediments (Gautreau et al., 2020; Nogaro et al., 2009). The process of bioturbation is regulated by ecosystem engineers (i.e. bioturbators), which create biogenic structures (i.e. galleries) in sediments that modify biogeochemical processes at the sediment-water interface (Kristensen et al., 2012; Mermillod-Blondin et al., 2018). Bioturbators contribute to ecosystem services by stimulating organic matter degradation, nutrient cycling, and microbial activities (Pignoret et al., 2016) (**Figure 3**). However, the presence of plastic pollutants in sediments may threaten their growth and the ecological services they provide (Galloway et al., 2017; Green et al., 2016; Huang et al., 2021; You et al., 2020).

Despite the existing evidence that microplastic can adversely impact the feeding, growth, reproduction, physiology, and survival of bioturbators (Besseling et al., 2013; Green et al., 2016; Redondo-Hasselerharm et al., 2018; Urban-Malinga et al., 2022; Van Cauwenberghe et al., 2015; Watts et al., 2015; Wright et al., 2013), only a handful of studies have addressed the impact of microplastics on bioturbation and ecosystem functioning, leading to a gap of knowledge, in

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particularly in freshwater ecosystems (Bour et al., 2018; Redondo-Hasselerharm et al., 2018). Moreover, the mechanism of bioturbation itself can alter the distribution of microplastics at the streambed sediments affecting their bioavailability to benthic fauna, but this is poorly understood (Näkki et al., 2019).

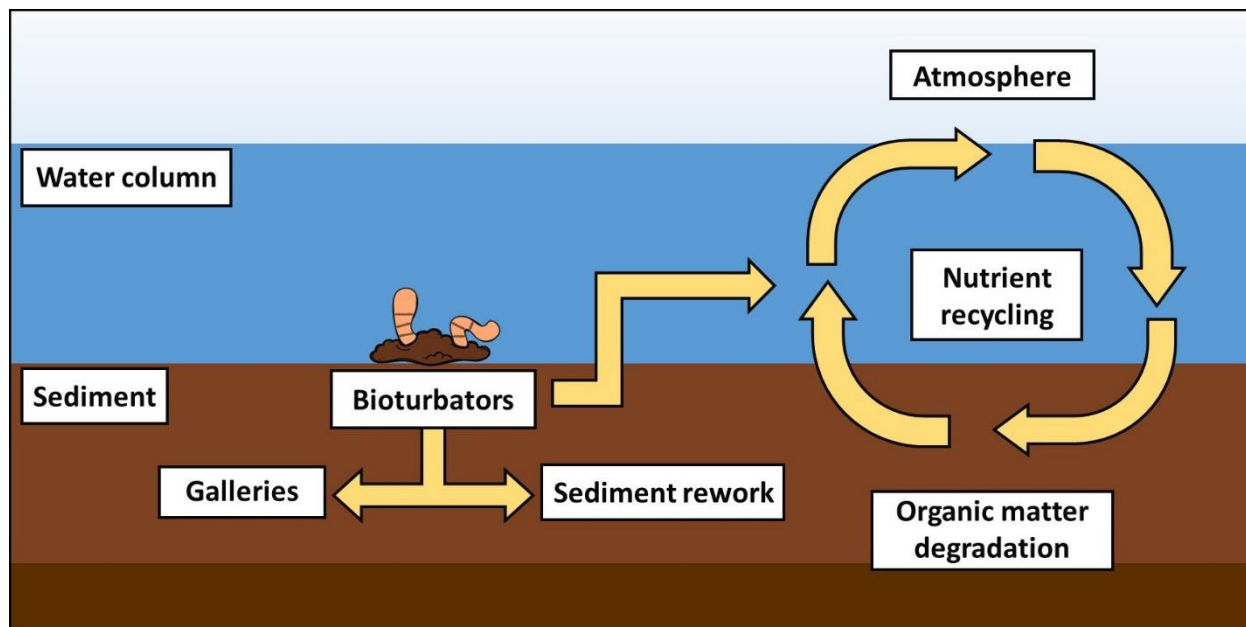


Figure 3 Schematic figure demonstrating the bioturbation activity regulated by ecosystem engineers (i.e., bioturbators), and impacting biogeochemical processes at the sediment-water interface.

1.6. Objective of the thesis

In order to better understand the transport and presence of microplastics in streambed sediments and their consequences on the organisms and ecosystem functioning, my objectives in this PhD are to (1) develop and ameliorate suitable tools needed to accomplish microplastic extraction and identification from several matrices, (2) improve our understanding to the sampling regimes used to collect microplastics from streambed sediments and the different outcomes resulted from each approach, (3) studying the distribution of microplastics in riverbed sediments with respect to hydro-sedimentological processes, and (4) understanding the ecotoxicological impacts of microplastics on the freshwater ecosystem engineers and their consequences for the ecological functioning of river systems.

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Four main chapters make up the remaining part of the thesis manuscript (chapters II through V). In chapters II through IV, I will focus on the four goals mentioned above (**Figure 4**). The following three chapters are mainly written in the form of articles. Some of the data have already been published, while others have been submitted, and the remainder is currently being finalized for submission in the near future.

Chapter II is a methodological study conducted in both indoor and outdoor laboratories to achieve our first two PhD objectives. This chapter is divided into three sections. The first section is an article describing the development of the protocol for extracting microplastics from various matrices, particularly sedimentary matrices. The second section is another article based on controlled outdoor laboratory experiments that shows how various sampling techniques (i.e., scooping, coring, freeze coring, resuspension, and piezometer sampling) influence the recovery of microplastics from streambed sediments. The third and final section of this chapter is a concise report outlining the efforts established to develop the tools necessary to physically (i.e., number, size, shape, and color) and chemically (i.e., polymer type) characterize the collected plastic particles, using a fluorescent stereo microscope and Fourier transform infrared (FTIR) spectroscopy.

The technical advancement covered in Chapter II is applied in Chapter III to achieve the third goal of the PhD by examining how streambed sediments act as significant sinks that can temporarily entrap microplastics. Chapter III is a fieldwork carried out at the lower Ain River, which is a large tributary of the Rhône River (France). This research aimed to better understand the role that hydrological exchanges play in determining the concentration and distribution of microplastics in streambed sediments.

After studying through Chapter III the role of streambed sediments in the temporary trapping of microplastics and consequently acting as a source of exposure for benthic organisms, ecosystem engineers (i.e., bioturbators) that inhabit this sediment-water interface were studied in Chapter IV. This chapter targets the last objective of the PhD. It is a laboratory experiment investigating the impact of sediment contamination with microplastics on survival, the physiological state, and the bioturbation activity of freshwater deposit-feeding bioturbators. This work has been

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published in the Environmental Science & Technology scientific journal (American Chemical Society) under the following DOI: <https://doi.org/10.1021/acs.est.2c05662> (Environ. Sci. Technol. 2023, 57, 3042–3052).

Finally, chapter V provides (i) a general discussion of the results and their implications in the field of microplastics, and (ii) an overall conclusion followed by recommendations for future research.

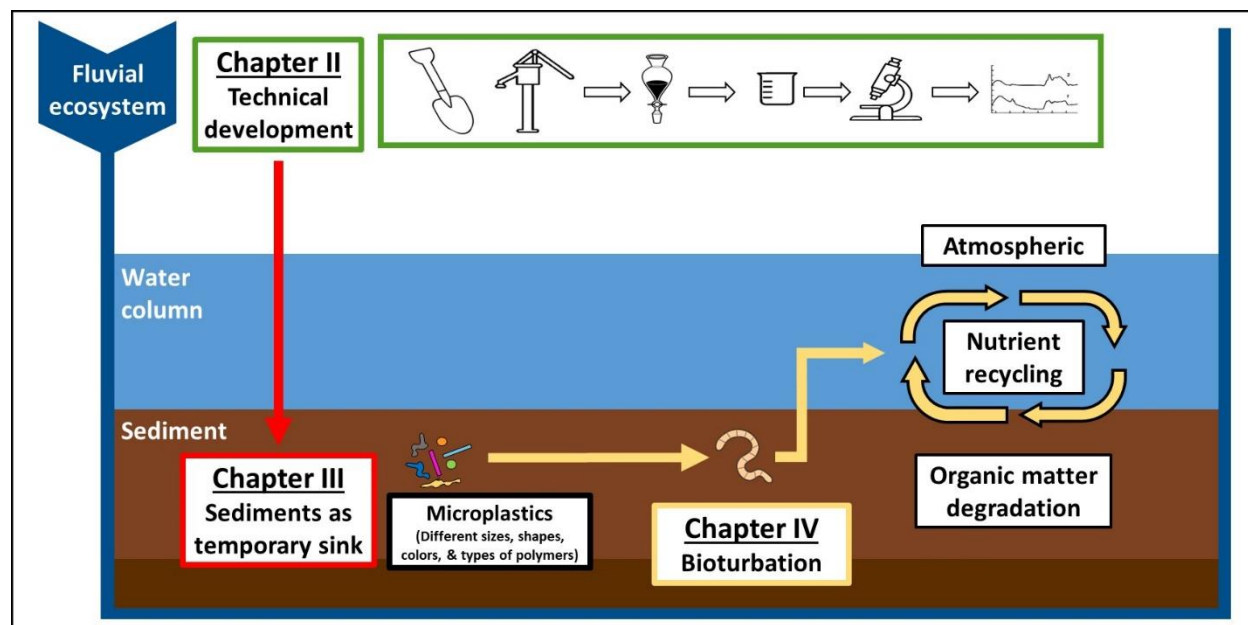


Figure 4 Schematic figure showing the three different chapters (chapter II through IV) that will be discussed in the manuscript and that targets the four different objectives of the PhD.

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Chapter II

Methodological developments

Section 1: Extraction of microplastics from sediment matrices:

As indicated in the introduction, Chapter II is divided into three sections, each of which discusses technical advancements that had been developed and enhanced to enable extraction, sampling, and characterization of microplastics in sedimentary matrices.

*The next section describes improvements made to facilitate the extraction of microplastics from sedimentary matrices using conventional glass separation pathways. This part constitutes the manuscript of an article submitted for publication in the journal *MethodsX*.*

1. Optimization of glass separating funnels to facilitate microplastic extraction from sediments

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1.1. Abstract

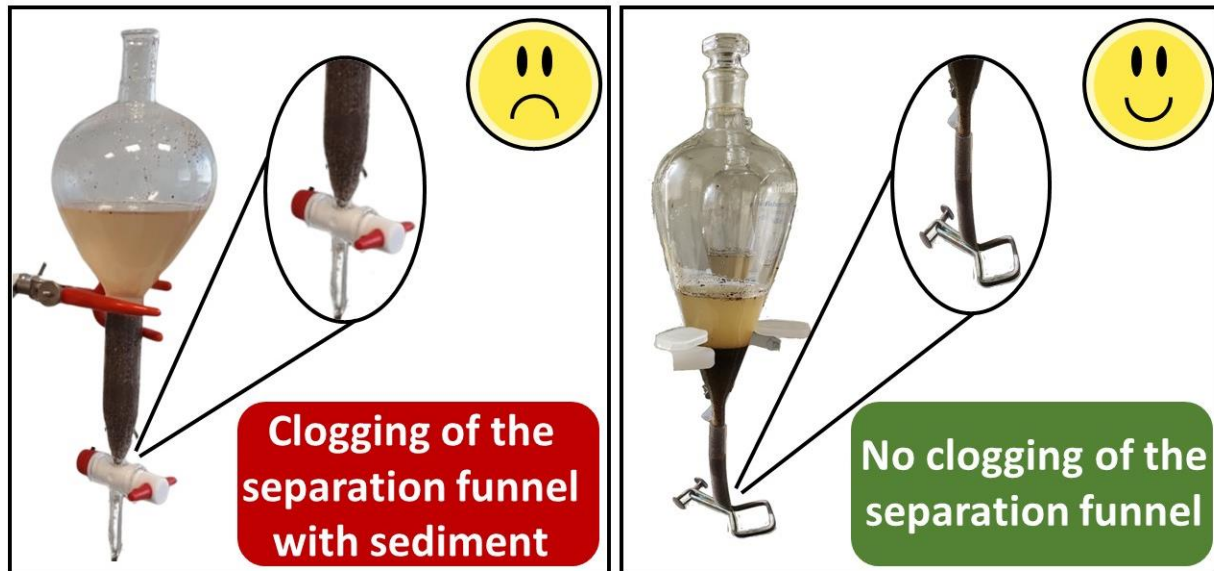
Our ability to understand the distribution, fate, and transport of microplastics and mitigate their impact on the environment is hampered by the lack of standardization of sampling and extraction protocols that allow adequate throughput of samples. In particular, our ability to extract microplastics has often been proven to be a time-limiting step, also causing challenges in adaptability and comparability between different environmental substrates such as soils or sediments. Recent studies have deployed a variety of different methods and devices for the density separation of microplastics from sediments. Instrument-specific limitations have been noted, including the fact that some of these devices are expensive, difficult to handle, or/and made entirely of plastic, thus increasing the risk of additional contamination. This research builds on the adaptation of existing techniques for separating microplastics from sediments by introducing important adjustments to the commonly used conical shape glass separating funnels. These adjustments presented here proved efficient in resolving critical clogging problems that are often observed in density separation units, allowing the microplastic extraction process to be more efficient and leading to a 90% recovery rate of microplastics. In order to demonstrate the validity of these new method adjustments, an experiment was conducted on sand-based sediment previously spiked with fragments of polyamide. Future studies should take into account naturally heterogeneous substrates since the recovery efficiency might depend on the nature of the sediment matrices.

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Two points describe the adjustment of the glass separation funnels:

- Stopcocks were removed from the separation funnels
- Silicon tubes and Mohr clamps were instead used to control the release of sediments from the separation funnels

1.2. Graphical abstract



1.3. Keywords

Density separation, aquatic systems, sediments, polymers, recovery rate, stereo microscope.

1.4. General context

Microplastic pollution of the world ocean was first reported in the 1970s, with the earliest concerns arising from North America (Carpenter et al., 1972; Carpenter and Smith, 1972). Only a few years later, a number of resin pellets were found on the beaches of New Zealand, Lebanon, Spain, Canada, and Bermuda (Gregory, 1977, 1978; Shiber, 1979, 1982; Gregory, 1983). By the early 21st century, Thompson provided evidence that microplastics, defined as plastic particles smaller than 5 mm (Arthur et al., 2009), do not simply vanish in marine ecosystems, but rather break down into smaller particles and eventually settle in sedimentary habitats (Thompson et al., 2004). Since then, microplastics have also been documented in most major surface waters,

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including lakes and rivers (Wagner et al., 2014). Recent research has proven that river corridors and their sediments represent long-term reservoirs of microplastics alongside marine sediments (Anderson et al., 2016; Drummond et al., 2022, 2020).

Microplastics in sediments pose a threat to the functioning of ecosystems (Wazne et al., 2023), particularly as they can be ingested by benthic species and therefore ascend to the food chain and enter human diets (Du et al., 2021; Smith et al., 2018). For this reason, the development of an effective approach for detecting the distribution and concentrations of microplastics in sediments is indispensable for understanding their availability in aquatic systems and, therefore, the threat presented to aquatic animals and related ecological processes. The separation of microplastics from sediment is complicated by the presence of organic matter, which can mask microplastic particles and might mislead scientists during the identification procedures (Nel et al., 2021). Moreover, the size, shape, and density of microplastics and sediments may make them challenging to manage. Smaller microparticles, for instance, may be more difficult to separate than larger ones, particularly in finer sediments where they might adhere to the surface of microplastics, increasing their densities and reducing the efficacy of density separation techniques (Constant et al., 2021; Quinn et al., 2017). To overcome these obstacles, a range of different methods for digesting organic materials (such as oxidation, enzymatic digestion, and acid–alkaline digestion) and separating inorganic particles (such as elutriation and density separation) have been adopted (Razeghi et al., 2022).

One of the most frequently applied methods for extracting microplastics from sediments is density separation (Chubarenko et al., 2016). In this method, sampled materials are separated based on their density differences using dense intermediate solutions such as sodium chloride (1.20 g cm⁻³), sodium iodide (1.6-1.8 g/cm³), zinc chloride (1.5-1.8 g/cm³), and zinc bromide (1.7 g cm⁻³) (Campanale et al., 2020; Razeghi et al., 2022). To achieve this separation, a number of different devices and designs have been developed and deployed. Nevertheless, these devices have shown drawbacks that affect research outcomes and thus the comparability between studies (Prata et al., 2019). Despite the fact that glass beakers are often used for this as they are readily available, the adhesion of microplastics to the glass wall might lead to a loss of plastic particles during the pouring of the supernatant. In addition, the resuspension of settled sediment

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is highly possible (Masura et al., 2015). This resuspension can be avoided when deploying glass funnels, yet only a little amount of sediment may be handled at once (Masura et al., 2015). Because of this, conical shape separating funnels are also deployed, which allow for far larger sample volumes to be treated in a single operation. However, this device is prone to frequent clogging, especially when dealing with fine sediment substrates (Enders et al., 2020; Tophinke et al., 2022). The stainless-steel Munich Plastic Sediment Separator (MPSS) represents another option. It recovers a high percentage of microplastics (95.5%), but it is expensive, large in size (1.75 m high), and challenging to handle (Imhof et al., 2012). Coppock et al. overcame the cost and handling issues by developing a low-cost PVC portable device (Coppock et al., 2017). These sediment microplastic isolation (SMI) units, however, contaminate the samples by introducing PVC shavings (Nel et al., 2019). Another small portable device that can be used and is made entirely of two glass plates, is the Japan Agency for Marine-Earth Science and Technology (JAMSTEC) microplastic-sediment separator (JAMSS). Despite its high rate of microplastic recovery, this apparatus should be handled with caution as the sliding of the two glass plates can cause resuspension of the sediments from its lower to the upper tube. Microplastics have also been reported to get entangled in the silicone grease used to lubricate the plates, reducing the efficiency of recovering the plastic particles (Nakajima et al., 2019).

In this work, we build on existing devices for extracting microplastics from sediments; by introducing technical adjustments to the commonly used conical shape separating funnels that will allow for easier device manipulation and yield high microplastic recovery rates. These adjustments were made in order to improve the overall efficiency of the microplastic extraction process.

1.5. Method modification

The conical-shaped separating funnels used here are completely made out of glass to avoid contamination. They are low-cost and portable and can process a significant volume of sediment all at once. However, the presence of a tap with a 0.2 cm pore size opening that allows the passage of the funnel content (i.e., sediments and liquids) makes it difficult to manipulate and causes frequent clogging. This, in turn, results in a disturbance of the sediments after they have

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settled, which makes it harder to separate microplastic particles from the sediment, which is the main aim of the entire separation step.

Because of these shortcomings of the existing approach, we designed here a 500 ml cone-shaped glass separation funnel without any stopcocks. The top opening, through which we sample materials are added for separation has a diameter of 2 cm, while the bottom opening, through which material is removed has a 0.8 cm diameter (**Figure 5 A**). In the new design, a silicone tube (with a diameter of 0.9 cm and a length of 12 cm) is attached at the base of the funnels, whose opening and closure is manually controlled by a Mohr clamp (**Figure 5 B**). The clamp also makes it possible to regulate the amount of sediment removed from the funnel during each opening of the Mohr clamp by either moving the clamp vertically upward so that sediment is ejected from the bottom portion of the silicone tube or moving the clamp down so that sediment is blocked in the tube (**Figure 5 C**). This design allows to handle the sediment calmly and avoid turbulence, even with clay and other fine-grained sediments.

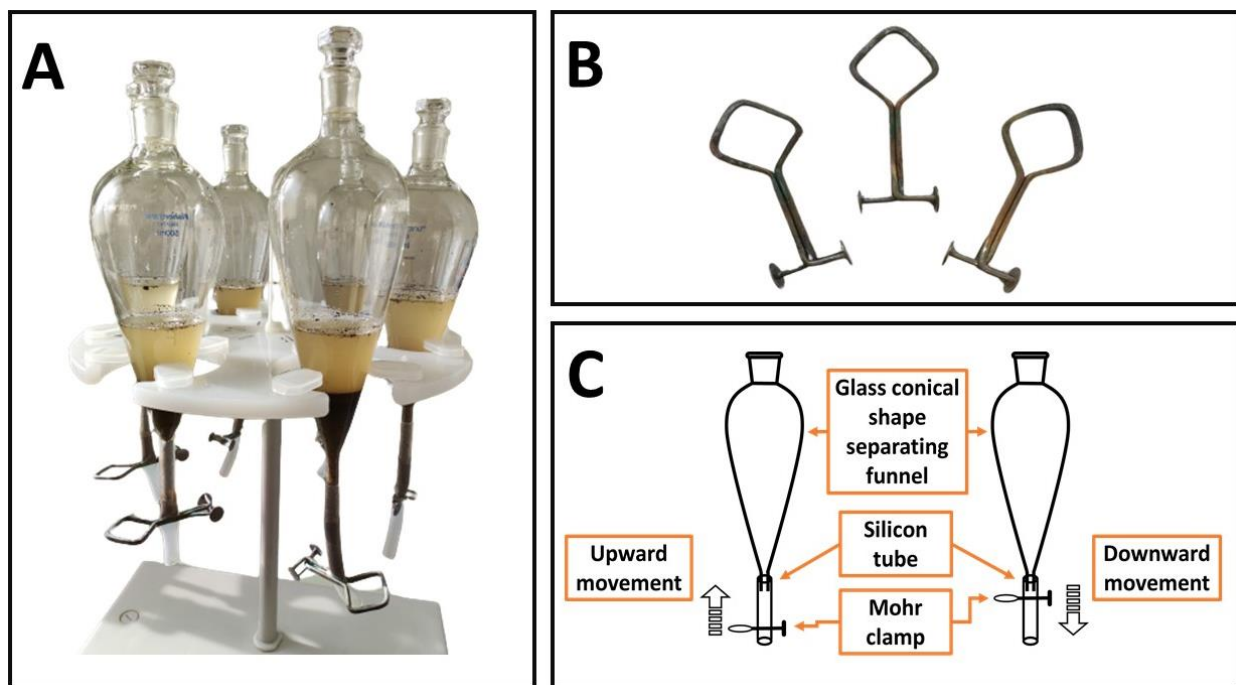


Figure 5 The adjusted design of cone-shaped glass separation funnels with silicone tubes and Mohr clamps (A), Mohr clamps (B), and an illustrating schema about the upward and downward movement of the Mohr clamps attached adjusted to the glass funnels (C).

1.6. Method validation

To validate if the proposed modifications to the separation funnel design will help to enhance the handling of the manipulation with the glass separation funnels and thus obtain a sufficiently high rate of microplastic recovery, a simple but efficient test was undertaken:

First, polyamide (PA) pellets were acquired (Resinex Ltd., High Wycombe, United Kingdom), frozen at -80°C for 72 hours, and then pulverized with liquid nitrogen for 20 minutes in a Fritsch Pulverisette 0 ball mill to form PA fragments. Then using stainless steel sieves, the resultant PA powder was sieved to retain particles smaller than $1000\ \mu\text{m}$ and bigger than $250\ \mu\text{m}$. After confirming the size and shape of the PA particles using a digital microscope (Keyence VHX-7000), coupled with a Keyence VHX-7020 camera connected to a Keyence VH-ZST lens (**Figure 6 A**), the particles were dyed with high concentrations of Nile Red ($> 0.01\ \text{mg ml}^{-1}$) overnight and then oven-dried at $55\ ^{\circ}\text{C}$ for 48 hours (**Figure 6 Erreur ! Source du renvoi introuvable.B**), yielding fragments of PA that can be easily identified and recovered at the end of the experiment.

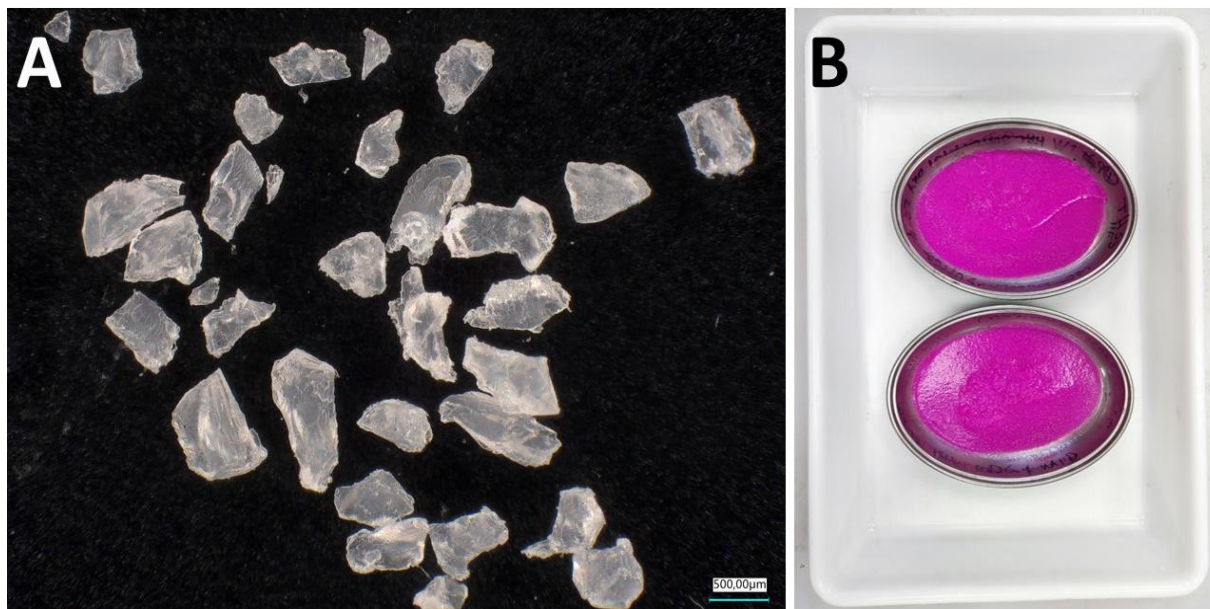


Figure 6 Particles of polyamide fragments observed under a digital microscope (A), and then colored with Nile Red (B).

In a second step, samples of streambed sediment were taken at the Canal de Miribel on the Rhône River in the South-East of France ($45^{\circ}48'14.2''\text{N } 4^{\circ}53'50.4''\text{E}$). The particle size distribution of the sediment was determined using a laser granulometer (Mastersizer, 2000, Malvern Instrument) on three replicate samples previously treated with ultrasounds (50–60 Hz, 1 minute)

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to eliminate non-stable particle aggregates (Gette-Bouvarot et al., 2014). In addition, the organic matter content was quantified by the loss on ignition method, calculating the weight difference of the same sample after drying for 48 hours at 55 °C and burning at 550 °C for 5 hours. Our results indicated a dominance of fine sand particles (**Figure 7**) and the presence of 0.96 % of organic matter content in mass.

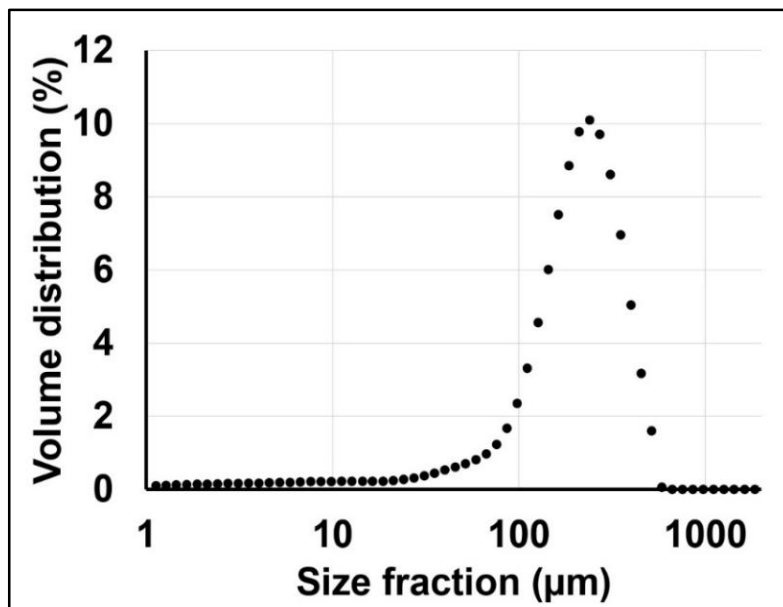


Figure 7 Grain size distribution of the sediment used in this experiment.

After this, a sediment mixture containing a microplastic concentration of 500 particles Kg^{-1} sediment dry weight (DW) was created by spiking the sediment with colored PA fragments. Seven replicates were assembled, each containing 30 g of DW sediment injected with 15 particles of the PA fragments counted under a stereo microscope (Nikon SMZ1270) to achieve the desired concentration.

This spiked sediment was used to generate environmentally realistic samples so that the efficiency of the performed adjustments to the glass conical shape separating funnels could be evaluated. PA polymers were chosen as they are some of the most common plastic polymers found in the environment (Hale et al., 2020), and in particular, PA fragments have been widely reported (Bikker et al., 2020) in different environmental substrates. Furthermore, MP concentrations of around 500 particles Kg^{-1} DW can be considered ecologically realistic and have been found in a wide range of environmental samples (Klein et al., 2015).

The microplastic extraction protocol (**Figure 8**) started by oven-drying sediment matrices for two days at 55 °C. Sediment samples were then placed into glass separating funnels that had already been filled with about 100 ml of a zinc chloride solution (ZnCl_2 ; 1.5 g cm^{-3}). After that, zinc

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chloride solution was added until each funnel was filled to about 200 ml. To ensure that plastic particles were well homogenized and mixed with the solution, the sediment matrices and the zinc chloride solution were gently agitated and shaken several times before letting the sediment settle for 24hr.

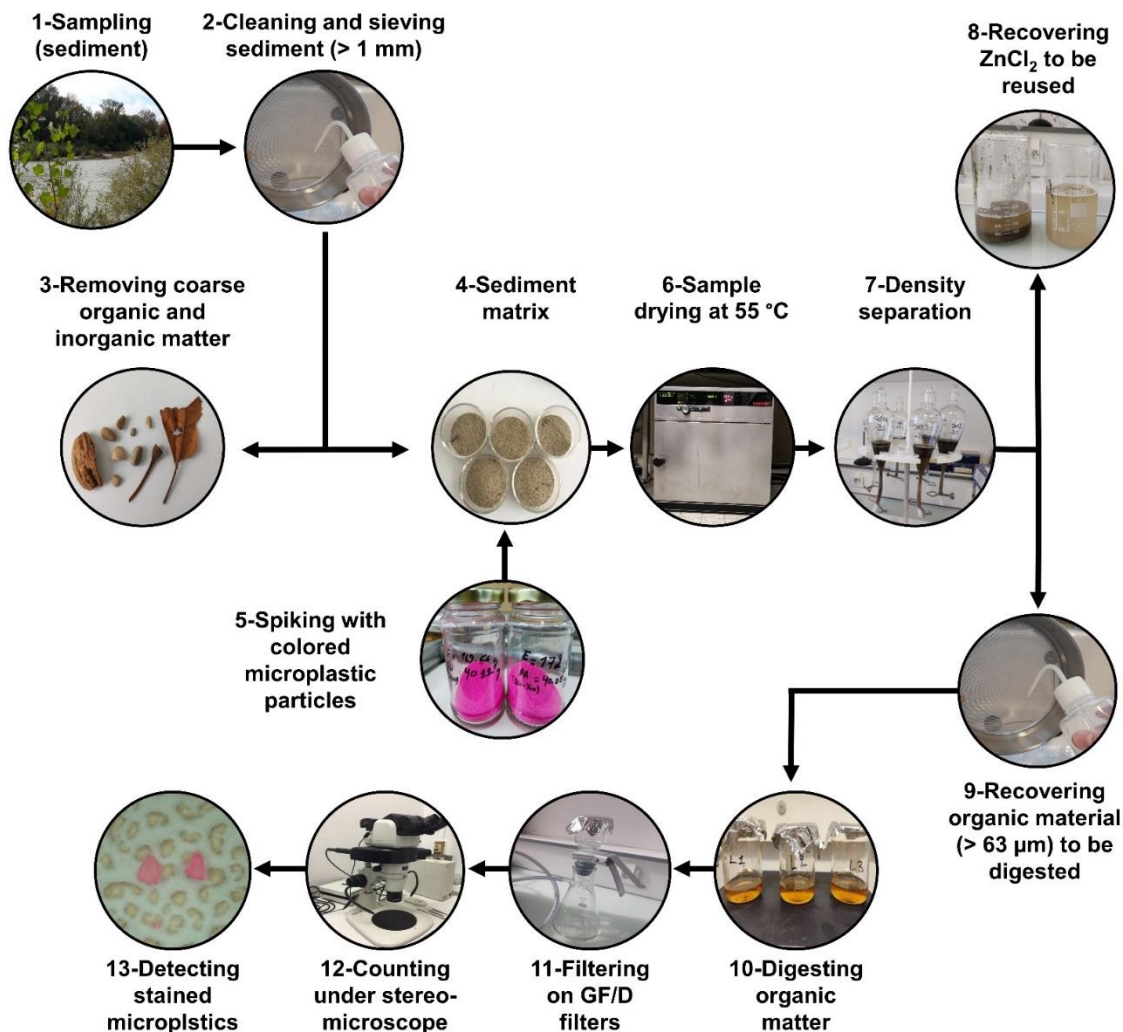


Figure 8 An overview of the steps of the extraction protocol of microplastics from sediments.

After letting the sediments settle for a full day, the Mohr clamps were gently opened and closed to release the precipitated sediments from the funnels. Without producing any turbulence, sediments were gently passed through the silicon tubes and drained off the funnels. Moving the Mohr clamp up- and downward allowed for an easy regulation of sediment discharge from the funnels. After releasing the precipitated substrate, PA particles, and organic materials were left floating in the zinc chloride solution in each funnel. Following this density separation step, the

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remaining solutions were poured onto a 63 μm mesh. The funnels were rinsed three times with ultra-pure water previously filtered through Whatman GF/D glass fiber filters (2.7 μm in porosity; 47 mm diameter) to ensure that all organic materials and PA particles were well collected and recovered effectively. It is necessary to point out that the use of 63 μm mesh was adopted specifically for this experimental setup since the size fraction of the spiked PA particles was greater than 250 μm . Nevertheless, microplastic particles smaller than 250 μm may be extracted from environmental field samples using meshes with smaller pores.

Particles collected on the mesh were transferred onto a beaker using between 50 - 100 ml of 30% H_2O_2 . The beakers were then filled with 0.05 M Fe(II) solution in a 10/1 ml proportion. Digestion was then left to proceed for 24hr. On the following day, digested samples were poured again through a 63 μm mesh and then recovered with filtered ultrapure water into new clean beakers. These samples were then filtered through Whatman GF/D glass fiber filters (2.7 μm in porosity; 47 mm diameter) and recovered PA particles were counted under a stereo microscope (Nikon SMZ1270 fluorescent stereo microscope) where fluorescent mode was not needed since the used PA particles were already colored and easily identified under the bright field mode.

The recovery rate (%) of the PA particles collected on the seven GF/D filters was 90.5 ± 6.5 %. The particle recovery rate was calculated by dividing the number of recovered particles by the number of particles added initially in each sediment replicate (**Table 1**). The majority of the spiked PA plastic pieces were successfully recovered in our research using the extraction process. The modifications to the glass conical shape separating funnels made it possible to operate these density separation units without clogging or disturbing the settled sediments.

Table 1 Number of polyamide particles recovered from the seven replicates of the spiked sediments.

| Replicates | Particles recovered out of 15/30 g DW sediment | Recovery rate (%) | Average | Recovery rate (%) | Standard deviation (%) |
|------------|--|-------------------|---------|-------------------|------------------------|
| R1 | 13.0 | 86.7 | | | |
| R2 | 15.0 | 100.0 | | | |
| R3 | 13.0 | 86.7 | | | |
| R4 | 15.0 | 100.0 | 13.6 | 90.5 | 6.5 |
| R5 | 13.0 | 86.7 | | | |
| R6 | 13.0 | 86.7 | | | |
| R7 | 13.0 | 86.7 | | | |

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Our research found a high recovery rate for PA particles, with an average extraction efficiency of $90.5\% \pm 6.5\%$. However, this recovery rate was still lower than that detected by Coppock et al., (2017) (92%-98% recovery), Nakajima et al., (2019) (94%-98% recovery), and Imhof et al., (2012) (95.5% for microplastics smaller than 1 mm). This may be due to the fact that our study assesses the recovery rate of the plastic particles after going through the steps of the entire extraction technique (**Figure 8**), while earlier studies typically just tested the newly built density separation unit. Hence, it is possible that microplastic could have been lost during the digestion stage, the filtering process, or the phases of collecting PA particles on the 63 μm mesh. Today, there is a lack of thorough assessments of all stages of the extraction protocol in the literature; instead, the enhanced step often stands out independently and receives more attention than the combination of all steps. However, the recovery test should not cease at the phase of modification; rather, the whole extraction protocol should be taken into account to help in the design of reliable and harmonised protocols by which researchers may refer for monitoring the actual microplastic concentrations in riverine systems.

1.7. Methodological recommendations

To better understand the complexity of microplastic contamination in the environment, it is essential to optimize and validate existing approaches employed for sampling, extracting and characterizing microplastics. In this technical note, our goal was to validate the adjustments made to the glass funnels by using one type of sediment matrix with only one polymer type and shape that could be easily detected under a stereomicroscope. Given that recovery rates decrease with decreasing size of microplastic particles, future studies should investigate extraction procedures with small particle fractions, particularly those smaller than 100 μm (Enders et al., 2020). In addition, other types of polymer sizes, densities, and shapes must be considered, especially since fibers are usually underestimated in the sediments because of technical limitations (Constant et al., 2021; Rebelein et al., 2021). Composing a matrix that contains a heterogeneous mix of several plastic sizes, shapes, densities, polymer types, and morphologies is greatly needed.

1.8. Acknowledgments

We are grateful to our colleagues Félix Vallier, Lara Konecny-Dupré and Florian Malard (Univ. Lyon 1, CNRS, UMR 5023 LEHNA, France) for their precious help and advices during the course of developing the extraction protocol in the lab. We also thank Brice Mourier and André-Marie Dendievel (Univ. Lyon 1 and ENTPE, UMR CNRS 5023 LEHNA, France) for offering the laser granulometer platform (Mastersizer, 2000, Malvern Instrument). We appreciate the fruitful discussion that took place with our colleagues Holly A. Nel, Anna Kukkola, and Uwe Schneidewind (School of Geography, Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK). This work has been funded by the IDEXLYON of Université de Lyon (UdL) within the program "Investissements d'Avenir" (ANR-16-IDEX-0005), and was performed within the frameworks of the EUR H2O'Lyon (ANR-17-EURE-0018) and the Rhone Basin Long Term Environment Research (LTSER ZABR).

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Section 2: Sampling microplastics in sediment matrices:

Within the context of Chapter II, which discusses the technical advancements investigated for the PhD thesis, the section that follows describes some of the most widely used microplastic sampling techniques in the literature. The part aims to investigate the influence of these various sampling approaches (i.e., scooping, coring, freeze coring, resuspension, and piezometer sampling) on the recovery of microplastics from streambed sediments.

This section is written as a journal article and was submitted to Environmental Science & Technology Letters - American Chemical Society publications.

2. The choice of sampling method defines results of microplastic contamination in streambed sediments

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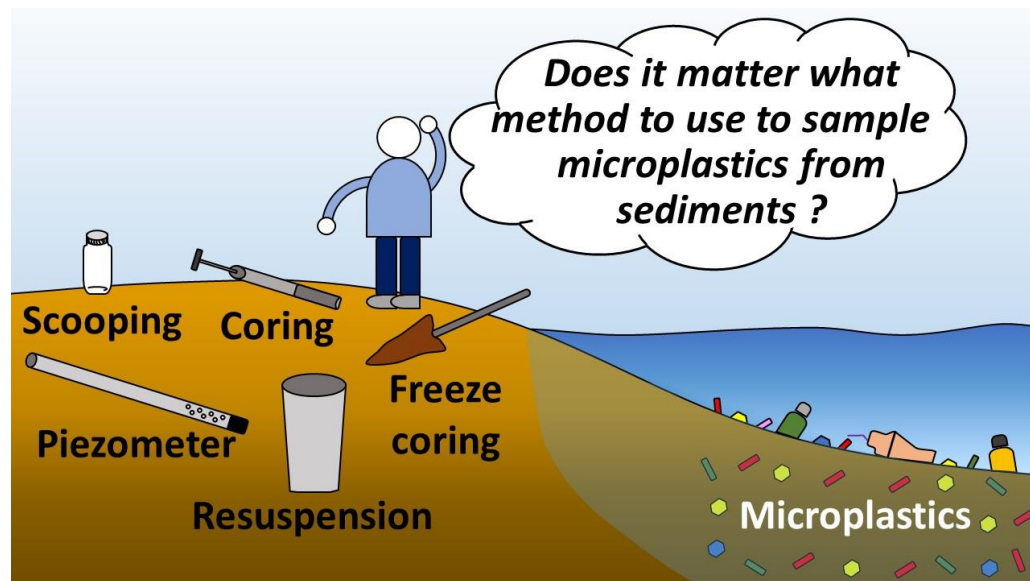
2.1. Abstract

Approaches for sampling microplastics from streambed sediments vary depending on hydrological flow conditions and streambed sediment type. To date, no inter-comparison of the variety of different sampling methods applied worldwide has been conducted, preventing liable inter-study comparisons. To address this critical knowledge gap, this study compares five commonly applied sampling methods (scooping, coring, freeze coring, resuspension, and piezometer sampling). A mesocosm experiment was developed to compare concentrations and particle size distributions of microplastics recovered by the five sampling methods. Therefore, two types of sediment mixtures (sand and gravel) were spiked with a known concentration of polyamide fragments. Our results demonstrate that the scooping method is highly efficient for quantifying microplastics in bulk surface sediments, lending itself to large sample numbers due to its simplicity. Sediment coring and freeze coring extend the understanding of bulk sediment concentrations, providing information on vertical distributions. In contrast, the resuspension and piezometer sampling methods provide crucial information on different fractions of mobilizable microplastics under different forcing from pore water (piezometer) or the entire sediment-water

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interface (resuspension). Our results underline the need for harmonization and standardization in microplastic research and support the inter-comparability of data collected by different methods, thus, enabling targeted mitigation and intervention practices.

2.2. Graphical abstract



2.3. Keywords

Freshwater ecosystems, grain size distribution, mesocosm, harmonization.

2.4. Synopsis

Understanding the ramifications of microplastic sampling methodologies helps to make informed decisions for targeted sampling and improves our ability to compare results across studies.

2.5. Introduction

A solid scientific evidence base is needed to guide environmental policies and inform decision-making on how to address global plastic pollution (Rochman, 2016; Sedlak, 2017). Targeted strategies to combat the plastic crisis require evidence of the spatial distribution and temporal dynamics of microplastics (particles < 5 mm in length) (Arthur et al., 2009) in the environment (Zhang et al., 2019), covering the diversity of natural environmental settings and conditions (Waldman and Rillig, 2020; Waldschläger et al., 2022). Previous studies have shown that streambeds represent a significant temporary repository for microplastics (Drummond et al.,

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2022, 2020; Waldschläger et al., 2020), but conclusive evidence about the spatial organization of microplastics in relation to potential sources and transport pathways is still missing. Despite increasing evidence of microplastic contamination in streambed sediments, it is currently difficult to obtain accurate information concerning the interactions between microplastic distribution and environmental conditions due to the lack of tested and validated laboratory and field approaches (Provencher et al., 2020).

The absence of standardized protocols and the availability of a wide range of methodologies considerably limit the comparability of quantitative information on microplastic particles (MPs) among existing studies (Adomat et al., 2022; Cowger et al., 2020). In addition to the variations induced by the different lab-based analytical methods and processing steps including MP extraction, digestion, separation, counting, and polymer characterization, the use of different field sampling techniques while sampling for MPs should also be considered (Adomat et al., 2022; Prata et al., 2019). For instance, 160 microplastic particles/kg of sediment dry weight (DW) were measured by using a stainless steel scoop to collect sediment samples at depths of 5 to 10 cm in the upper River Tame catchment (Tibbetts et al., 2018), whereas 70 000 particles/kg DW were reported from the same river catchment after sampling by sediment resuspension (Hurley et al., 2018). Although this large difference in MP concentration could simply be the result of sampling at slightly different locations and times within the same catchment, it is prudent to hypothesize that the respective field sampling technique at least to some degree also influences measured MP concentrations. As microplastics have been sampled from streambed sediment using a variety of techniques in the past (Adomat et al., 2022; André-Marie et al., 2023; Frei et al., 2019; Lai et al., 2021), we argue that it is imperative to study how these different sampling techniques impact the MP recovery efficiency from field samples to better understand their advantages and limitations.

To address this critical knowledge gap, we conducted controlled outdoor laboratory experiments using known sediment conditions and microplastic particles to determine the MP recovery efficiency of five commonly used streambed sampling methodologies; (i) sediment scooping (Dikareva and Simon, 2019; Kelleher et al., 2023), (ii) resuspension method (Hurley et al., 2018), (iii) sediment coring using augers (Lai et al., 2021), (iv) freeze coring (Frei et al., 2019), and (v)

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porewater extraction using piezometers (Severini et al., 2022). We analyze how the obtained results reflect on the individual sampling technique as well as the respective sample support volume. We discuss the specific strengths and limitations of the five sampling methodologies and aim to support researchers and practitioners in making informed decisions with respect to their choice of sampling method applied in field campaigns, as well as during the interpretation of their sampling results across different field sites and studies.

2.6. Materials and methods

2.6.1. *Microplastic preparation*

Polyamide (PA) pellets (Resinex Ltd, High Wycombe, United Kingdom) were ground into smaller fractions using liquid nitrogen and Fritsch Pulverisette 0 ball mill after being frozen at -80°C to create a stock of PA fragments used in the controlled sampling experiments of this study. Stainless steel sieves were used to size fractionate the obtained PA powder, retaining fragments smaller than $1500\ \mu\text{m}$ and larger than $200\ \mu\text{m}$. These proportions resemble a diversity of microplastic size fractions found in the environment and are frequently used in laboratory experiments (Lim, 2021). Later, the size and morphological properties of the PA fragments were examined under bright field mode using a fluorescent stereo microscope (Nikon SMZ1270, [Supplementary Figure 1](#)). Prior to their use in the experiment, the PA fragments were stained overnight with high concentrations of Nile Red ($> 0.01\ \text{mg mL}^{-1}$) (Nel et al., 2021), then oven-dried at $55\ ^{\circ}\text{C}$ for 48 hours in order to create appropriately colored fragments that could be readily tracked throughout the experiment ([Supplementary Figure 2](#)).

2.6.2. *Mesocosm setup*

Two conically shape plastic containers (i.e., mesocosms) with a height of 50 cm, a top inner diameter of 81 cm, and a bottom inner diameter of 89 cm were set up as mesocosms in the Environmental Change Outdoor Laboratory (ECOLAB) facility of the University of Birmingham, UK ([Supplementary Figure 3 A and B](#)). Two sediment mixtures of different substrates were used to fill each mesocosm (**Table 2**) to test for a potential influence of sediment grain size distribution on microplastic recovery. Each mesocosm was wet-packed with 342 kg of sediment and uniformly mixed with the previously stained PA particles using metal hand shovels to reach a concentration

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of 2140 particles/kg (DW) ([Supplementary Table 1](#)), which represents a realistic environmental concentration (Huang et al., 2021; Klein et al., 2015). Afterward, both mesocosms were topped up with 100 L of tap water. Before the beginning of the experiment, the mesocosms were left to equilibrate for two days to ensure the settling of sediment and plastic particles. Except during samplings, mesocosms were covered with a blue, waterproof Polytuf Medium Duty Tarpaulin (MAYO, high-density polyethylene).

Table 2 Grain size fractions (%) of sediment mixtures A and B used in the ECOLAB mesocosm experiments.

| Grain size fractions (% in dry mass) | Silt & Clay < 63 μm | 63 μm < Sand < 2 mm | 2 mm < Gravel < 8 mm |
|--------------------------------------|--------------------------------|--------------------------------|----------------------|
| Sediment A | 7.1 | 71.2 | 21.7 |
| Sediment B | 5.7 | 57.7 | 36.6 |

2.6.3. Sampling techniques

Five commonly reported microplastic sediment sampling techniques were used to assess the recovery of fluorescently stained PA microplastic particles from the sediment mixtures of the mesocosms. To account for possible inhomogeneities during packing three samples were collected from each mesocosm using each sampling technique. These three samples were then mixed and gently homogenized using a metal spatula to create a single composite representative sample (Adomat and Grischek, 2021a). The five sampling methods were performed sequentially as described subsequently, with the techniques that sample at the surface being carried out first, and all samples were collected at different positions within the mesocosms across the sampling techniques. Prior to each round of sediment sampling, the absence of PA fragments in the water column from each mesocosm was confirmed by examining three replicates of 20 mL of water samples under the stereo microscope. Besides, the equipment was thoroughly cleaned with deionized (DI) water in between rounds of sampling.

Scooping

Using a 325 mL glass jar pre-washed with deionized (DI) water, sediment surface samples were collected from the top 5 cm of each mesocosm by pushing the jar at a 45° angle into the sediment,

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thereby collecting the maximum amount of sediment possible, sealing the glass, and then removing it from the mesocosm (**Figure 9**).

Resuspension method

For this purpose, a hollow black conical plastic container with a height of 54 cm, a top inner diameter of 30 cm, and a bottom inner diameter of 38 cm, was positioned at the center of each mesocosm and pushed into the top 5 cm of the sediment. Then the water column inside the cylinder was stirred with a wooden rod for 10 sec to resuspend the sediment. Following this procedure, 325 mL of a mixture of water and re-suspended sediment was collected using a glass jar (**Figure 9**).

Sediment coring

A metallic hand corer was pushed into the sediment to collect sediment cores. Cores of 13 cm in length and 5 cm in diameter were collected from three locations in each mesocosm (**Figure 9**).

Freeze-coring of sediment

A hollow metallic rod of 3 cm in diameter with a hole on one side and a sharp closed end on the other side was used for freeze-coring. The closed portion of the rod was inserted 16 cm into the sediment and the rod was filled with a mixture of 97% ethanol and dry ice to freeze the sediment in contact with the metallic rod. After about 10 min, the metallic rod and the frozen sediment core were removed from the mesocosm (**Figure 9**). This procedure was repeated at three locations in each mesocosm to obtain three frozen cores per mesocosm.

Piezometer porewater sampling

For each mesocosm, three piezometers were installed into 17 cm deep pre-drilled sediment holes. Each piezometer consisted of a hollow plastic tube of 3 cm in diameter, sealed at the bottom. The tube was perforated by 0.5 cm diameter holes (20 holes) drilled into its walls along its bottom section from 0 to 12.5 cm to permit the collection of porewater. This type of tube is commonly used by ecologists to sample fauna and study community organizations in streambeds and groundwater (Brunke and Gonser, 1999; Malard et al., 1997). After installation, the piezometers were left to settle for three days. Then, samples (a mixture of porewater and

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sediments) were extracted with a peristaltic pump (Solinst 410, flow rate of 29.4 mL s⁻¹) (Figure 9).

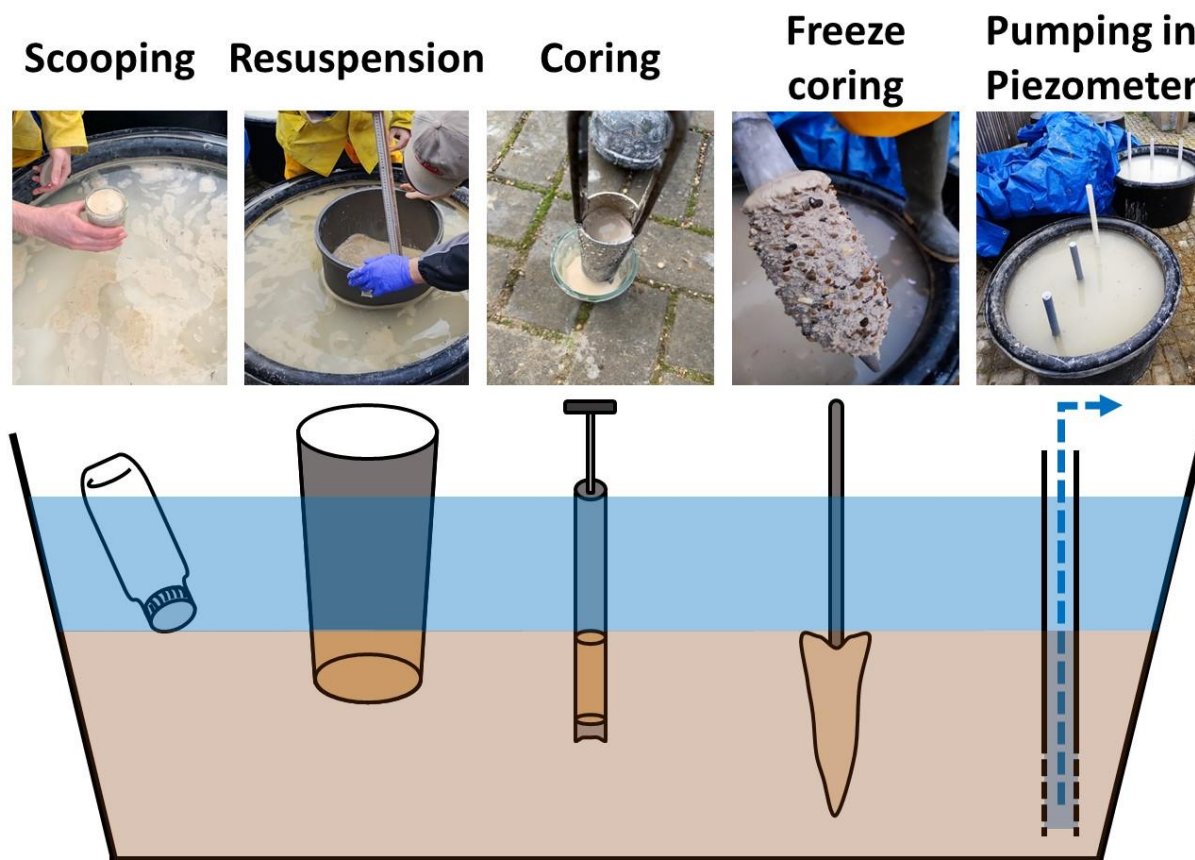


Figure 9 Schematic figure representing the five techniques used for assessing microplastic contamination in sediments.

2.6.4. Laboratory processing of samples

The obtained samples were oven dried at 50 °C for one week. From each composite sample (10 in total, 5 per mesocosm), three subsamples of about 35 g each were used to extract MPs by applying Sediment Microplastic Isolation (SMI) units and following the protocol of Coppock et al. (2017) (Coppock et al., 2017), modified by Nel et al. (2019) (Nel et al., 2019). The PA fragments were density-separated from sediments using a solution of zinc chloride (ZnCl_2 ; 1.5 g cm⁻³, [Supplementary Figure 4](#)). Following an overnight soak, PA particles were decanted from the SMI units onto a 63 µm mesh and then washed with DI water onto Whatman GF/D glass fiber filters (pore size of 2.7 µm; 47 mm in diameter, [Supplementary Table 2](#)), placed in clean polypropylene

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petri dishes, and dried for at least 24 h at 50 °C. As no meaningful amounts of organic matter were present on filters, no digestion had to be carried out for our sediment samples.

2.6.5. *Microplastic measurements*

Using a Nikon SMZ1270 fluorescent stereo microscope equipped with an Intensilight C-HGFIL Lamp (130w) and 0.75 X objective lens, the quantity and size of PA fragments were measured. All measurements were performed at a 15 X overall magnification. Since the PA particles were previously stained and no other plastic of the same color was present during the experiment, bright field mode was sufficient for distinguishing the particles ([Supplementary Figure 5](#)). While each GF/D filter was counted in full, the microplastic size distribution on each filter was assessed for 50% of the filter area.

2.6.6. *Statistical analysis*

As the number and size fraction of microplastics did not match the normality criterion ($p < 0.05$ for Shapiro-Wilk tests), non-parametric tests were applied to our dataset. The effects of sampling methods (scooping, resuspension, sediment coring, freeze-coring, and piezometer porewater sampling) and sediment mixture type (Sediment A and Sediment B) on the number of recovered microplastic particles were tested using the Scheirer-Ray-Hare (SRH) test. Two Chi-squared nonparametric tests were conducted to examine the influence of each sampling method on the size distribution of microplastics recovered from the two sediment types by (1) comparing the size distribution of the recovered PA fragments with those introduced in the containers for each sampling method, and (2) studying the relationship between the sediment mixture and the size distribution of the recovered PA fragments from each sampling method. These statistical analyses were done with the RStudio (R 4.2.2) software (“R Development Core Team, 2018,” 2018).

2.7. Results and discussion

As microplastics are likely to be unevenly distributed in natural sediments because of their physical properties (shape, size, and density) and environmental factors controlling their

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deposition and resuspension (e.g., hydrological conditions) (Adomat and Grischek, 2021b; Campanale et al., 2020; Prata et al., 2019), the present methodological study was performed under laboratory conditions to control for the homogeneous distribution of microplastic particles in the sediment. Applying a thorough mixing of PA particles with sediments allowed us to compare the various tested sampling strategies, regardless of the sampling area or depth.

2.7.1. Microplastic recovery

Unlike the type of sediment mixture, the applied sampling methods had a significant impact on the results of the recovered microplastic particles (Scheirer-Ray-Hare test, $p < 0.05$ for the effect of the sampling method, **Figure 10**). When applying scooping, coring, and freeze coring methods, the concentrations of recovered PA fragments collected from both sediment mixtures were of the same order of magnitude (i.e., from Sediment A: 1655 ± 484 fragments/Kg DW of a sample using scooping, 4577 ± 670 using coring, 3326 ± 440 using freeze coring, and from Sediment B: 2235 ± 217 using scooping, 2043 ± 443 using coring, 1547 ± 428 using freeze coring) as the original spiked microplastic concentrations introduced to the mesocosms at the beginning of the experiment (2140 fragments/kg DW, **Figure 10**). In contrast, the microplastic concentrations per sampled sediment dry mass obtained with the resuspension and piezometer techniques were 3 to 9-fold higher than the original spiked microplastic concentrations for the two sediment mixtures (**Figure 10**). Despite results showing that estimated PA concentrations tended to be higher in Sediment A compared with Sediment B for four out of the five of the sampling methods (**Figure 10**), no significant difference in microplastic concentrations was measured between the two sediment mixtures (Scheirer-Ray-Hare test, $p = 0.08$).

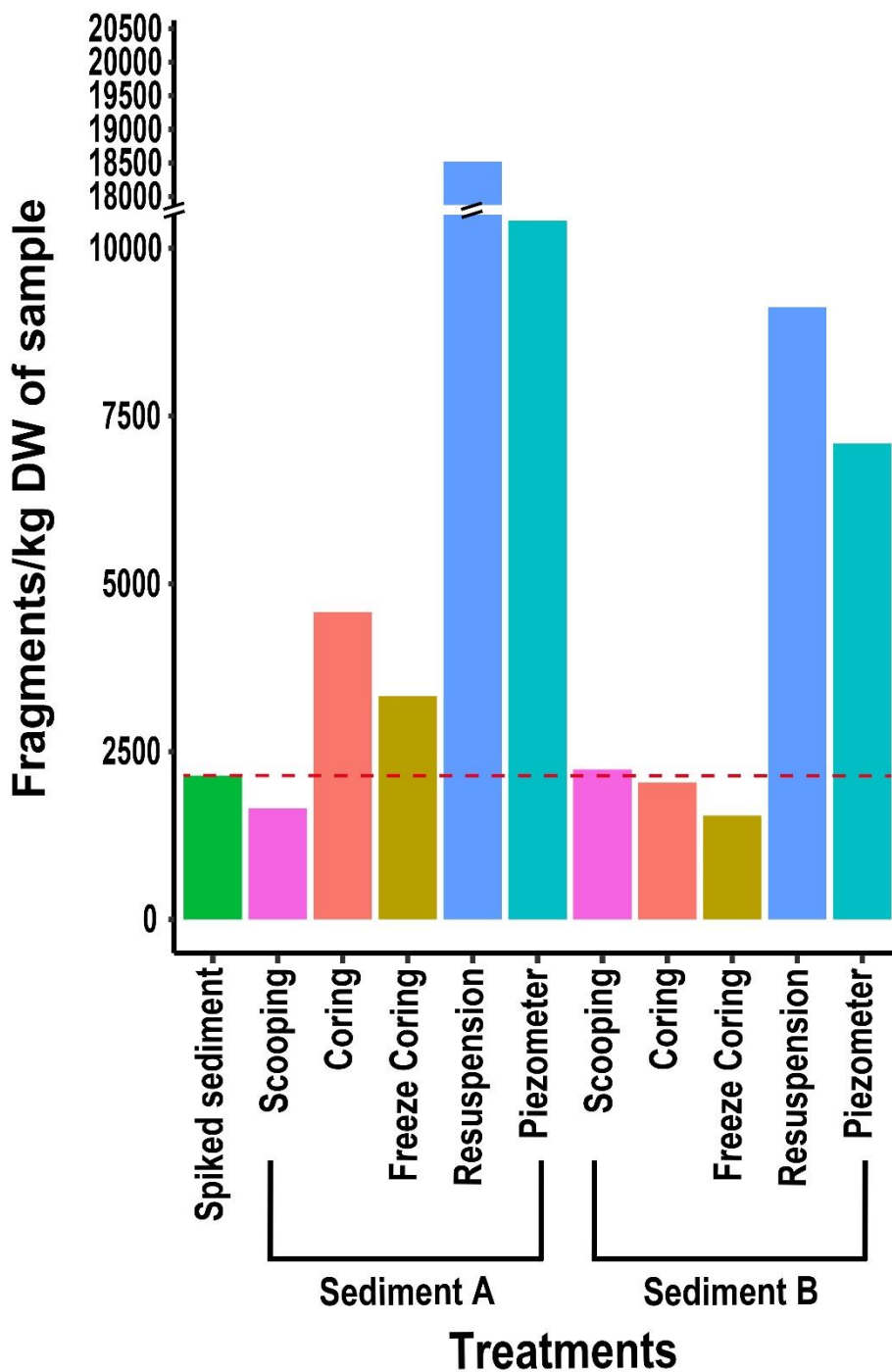


Figure 10 Number concentration of polyamide fragments recovered by each of the tested sampling methods for each sediment mixture (see Table 1 for the grain size distributions of Sediment A and Sediment B).

When attempting to target the concentration of microplastics at the first 5 cm of the sediment surface for scooping, and the first 10 cm for coring and freeze coring approaches, scooping, coring, and freezing coring yield results that are in the range of expected concentrations

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(Dikareva and Simon, 2019; Frei et al., 2019; Lai et al., 2021; Tibbetts et al., 2018). The nature of the scooping method, however, requires extra caution to ensure that only a small amount of surface water is retained in the containers; otherwise, microplastics can be either lost or resuspended in the water column (Tibbetts et al., 2018). If the area of interest concerns sediment layers deeper than 5 cm, coring and freeze coring offer specific advantages as they permit the investigation of microplastics at varying sediment depths (Brander et al., 2020; Frei et al., 2019; Lai et al., 2021). However, freeze coring provides distinct advantages over traditional corers when seeking to minimize the disturbance induced on the material during extraction. Although traditional corers seek to provide samples of intact sediment layers (Brander et al., 2020; Lai et al., 2021), it has been observed that extracted sediment samples in the stainless steel tubes or plastic liners used in the field can be compressed or loose which leads to difficulties in assigning the correct depths (Glew et al., 2001). Also, using traditional corers, an underestimation of MP concentrations may occur when the corer is extracted and water and fine sediment drain from the open tube (Lai et al., 2021). On the contrary, the frozen-sliced sediment layers obtained by freeze coring are well-preserved and more resistant to disturbance (Frei et al., 2019). However, performing freeze coring in the field is often more complicated as dry ice or other cooling solutions such as liquid nitrogen is required to produce sufficient temperature gradients between the streambed and the metal rod.

While coring and freeze-coring methods permitted us to estimate microplastic abundance and distribution in the whole sediment bed (i.e., from surface to depth profile), the resuspension and piezometer approaches largely overestimated the abundance of microplastics present in the two sediment mixtures (**Figure 10**). The two later methods are known to preferentially sample water and suspended material rather than sediments. Consequently, they are efficient to collect low-density microplastics easily remobilized by water flow (Belkhiri et al., 2022; Hurley et al., 2018; Lewandowski et al., 2019; Severini et al., 2022). Therefore, the overestimation of microplastic concentrations by these two methods might be largely due to the mobilization and collection of the lighter particles (including microplastics and fine particles of sediment) rather than the heavier particles. These techniques, hence, end up including more microplastics while excluding denser sediment particles. As a result, the ratio of microplastic particles to total sediment mass

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is overestimated compared with the concentrations of microplastics in the sedimentary environment (**Figure 10**). Because of this bias, researchers are strongly encouraged to use a grain size normalization to obtain microplastic concentrations in the sedimentary environment by assuming that microplastic sampling would be comparable to the sampling of fine sediment particles present in the sedimentary environment (Dendievel et al., 2023).

Although we have only tested one type of piezometer with rather large openings (5 mm) as is typical for interstitial fauna sampling (Brunke and Gonser, 1999; Malard et al., 1997), we are aware that the design of the filter opening in the screened piezometer section will also influence on MP recovery rates. As several different piezometer types are in use in hyporheic zone research (Anibas et al., 2016; Kalbus et al., 2006; Robinson et al., 2022), the future investigation might be needed to better understand variability in recovery rates among different designs.

2.7.2. Size distribution of microplastics

The particle size distributions of PA fragments recovered through the 5 sampling methods were comparable to those of the PA introduced in Sediment A (**Figure 11**), whereas for Sediment B, this was only true for the freeze coring approach (Chi-square = 10.009, df = 16, p-value = 0.8662, Figure 3, [Supplementary Table 3 A](#)). The particle size distributions of PA recovered by scooping, coring, resuspension, and pumping were significantly different than the distribution of PA introduced into Sediment B. More precisely, the PA size distributions recovered by scooping, coring, resuspension, and piezometer sampling from Sediment B underestimated the lowest size fractions of PA particles (**Figure 11**, [Supplementary Table 3 B and C](#)).

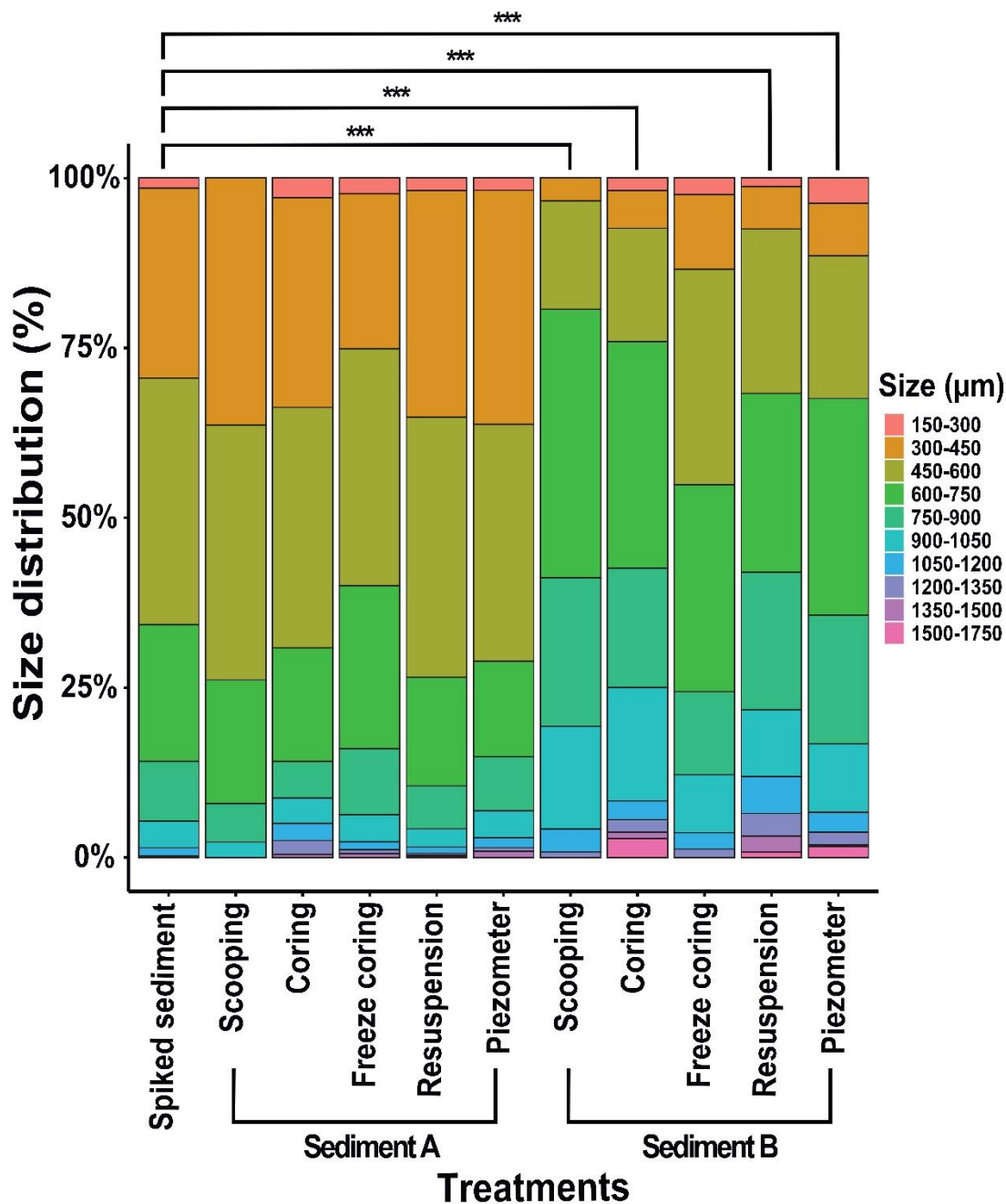


Figure 11 Size distribution of microplastics spiked at the beginning of the experiment and recovered at the end of the experiment (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, see Table 1 for the grain size distributions of Sediment A and Sediment B).

These results are in agreement with the study of Adomat et al. (Adomat et al., 2022) which reported the impact of sediment characteristics on the microplastic size fraction estimated by several sampling methods. Their work showed that the use of grab samplers, corers, and shovels to collect light microplastic particles made them more likely to be lost in the water column in the

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case of gravelly sediments. In contrast, denser microplastic particles were not excluded from the extracted sample because their large sizes and weights reduced their remobilization from the sedimentary matrix (Adomat et al., 2022). In addition, a variety of factors besides physical forces affect how plastic particles aggregate and are attached to natural sediments. Few studies on the relationship between microplastic abundance and fine sediment particles (< 63 µm) have been reported (Enders et al., 2019; Strand et al., 2013). Governed by hydrophobic interactions and electrostatic forces, microplastics can attach to suspended sediments forming heteroaggregates that can increase the sedimentation rate of microplastic particles (Paduani, 2020). The type of polymer and its shape has a significant impact on this heteroaggregation, and therefore, it influences the sampling efficiency of microplastic particles (Adomat et al., 2022; Paduani, 2020). Thus, obtaining a successful and representative microplastic sample largely depends on both the sediment composition of the sampled environment and the physical properties of the microplastics. Selecting an appropriate sampling method should be done with care and, based on the present study, freeze coring appears as a promising tool for identifying microplastics in streambed sediments, especially when interactions between sediments and microplastic particles reduce the extraction efficiency for the tiniest microplastic fractions (Adomat et al., 2022; Frei et al., 2019).

2.8. Environmental implications and future recommendations

The present study aimed, for the first time to explain how different sampling methods provide different insights into the distribution of microplastics in streambed sediments. Our results highlight that sediment microplastic sampling strategies must be carefully chosen and guided by the scientific question being addressed in order to optimize the desired outcome and ensure the correct interpretation of the nature of the samples origins.

While coring and freeze coring methods are helpful for understanding the vertical distribution of microplastics in sediments, other methods such as scooping, resuspension, and piezometer pumping provide depth-integrated information. When focusing on microplastics in surface sediment, scooping appeared as a pertinent method to estimate microplastic concentrations regardless of sediment type. This approach would be very suitable for sampling in aquatic

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ecosystems characterized by low flow velocities where depositional mechanisms of microplastic and sediment particles occur on the sediment surface. In contrast, this method would be less appropriate in gravel bed rivers where microplastic particles can be transported by water exchange in the hyporheic zone until they are trapped deeper into the streambed sediment, and whenever possible, freeze coring should be preferred in gravel bed rivers for sampling microplastics to obtain their vertical distribution (Drummond et al., 2020; Enders et al., 2019; Frei et al., 2019; Paduani, 2020).

Consequently, evaluating the concentrations of microplastics in rivers with gravel beds requires evaluating the vertical distribution of microplastics using techniques such as coring and freeze coring (Adomat et al., 2022; Frei et al., 2019). Nevertheless, special attention needs to be paid to the lowest microplastic size fractions that are prone to loss during coring. In comparison with other methods, the resuspension and piezometer pumping methods considerably accelerate the resuspension of light PA fragments from the sediment, giving an assessment of the mobility of microplastics in porewater and at the sediment-water interface (e.g., potential re-suspension during floods). Of the methods described here, freeze-coring is the most applicable for determining representative concentrations and size distributions of microplastic particles in streambed sediments because the sediment and microplastics are preserved in the extracted, undamaged frozen cores. However, when used in the field, this method may be subject to restrictive regulations and is more challenging to implement. To assess the environmental consequences of plastic pollution, it is necessary to understand how the particles are dispersed and accumulated in the environment. The five tested methods provide different forms of insight that allow different risk assessments that could be representative of specific environmental events. For example, the sediment resuspension method would be highly suitable for estimating local sediment-water interface exposures and assessing the threat of microplastic mobilization, which would increase their uptake by biota in the water column during flood events (Hurley et al., 2018).

In order to improve the representativeness and reproducibility of sampling for microplastics, we recommend the following:

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- 1- The selection of an appropriate sampling method should be guided by the specific demands of the respective scientific question and site/situation specific conditions, with our results demonstrating the different values, and capacities of the different sampling methods.
- 2- These approaches must be harmonized to precisely assess microplastic distribution and concentrations in sediments, ensure representativeness and comparability across studies, and enable monitoring choices for preserving freshwater ecosystems.
- 3- Although we did not detect significant differences in microplastic concentrations between the two sediment types across the five sampling methods, our tests did not concern a wide gradient of grain size distributions. Thus, tests on larger settings of sediment mixtures are clearly needed to evaluate the potential impact of the sedimentary context (grain size distribution of sediments, organic matter content, porosity, ...) on the microplastic concentrations recovered from sediments by distinct sampling methodologies.

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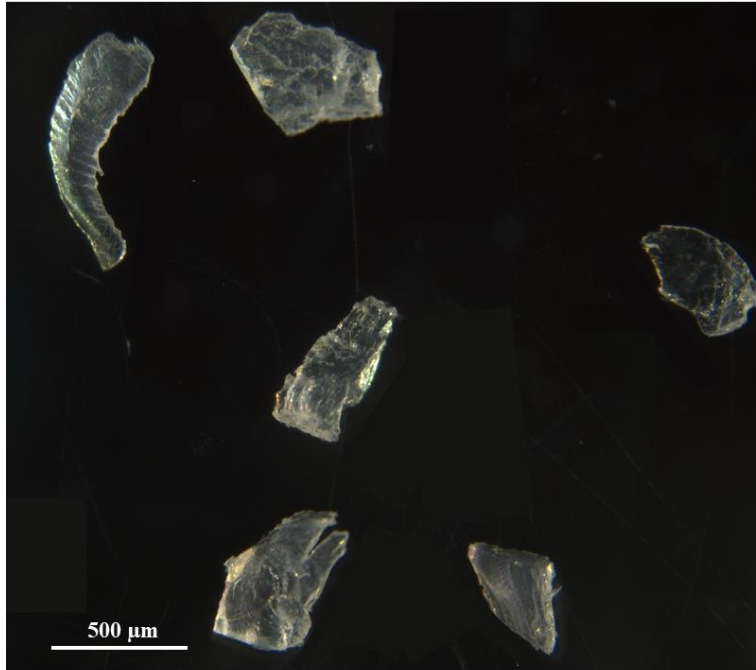
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2.11. Supplementary materials

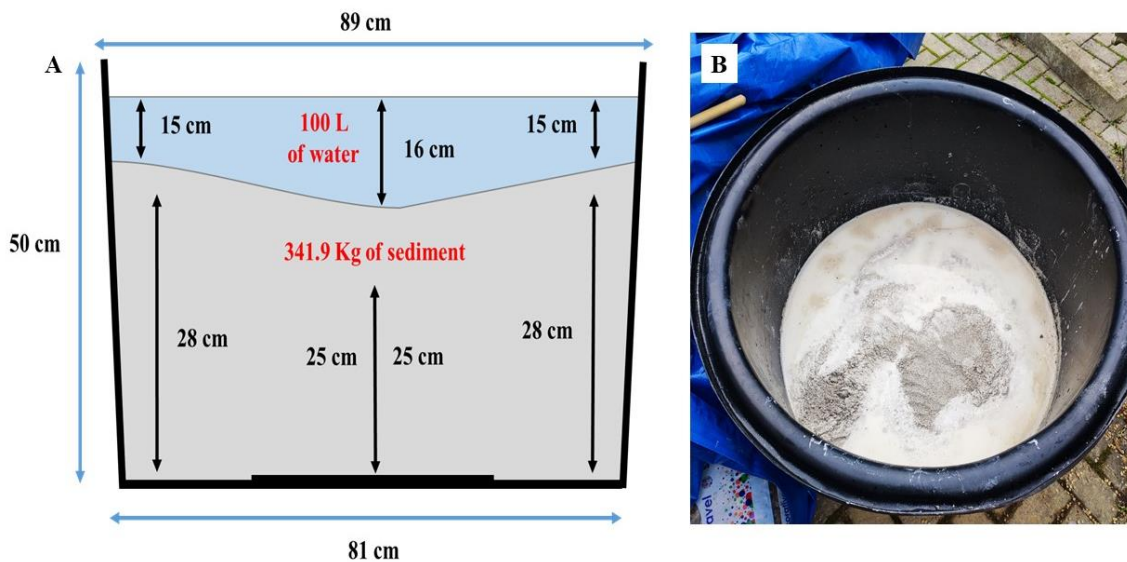


Supplementary Figure 1 Stereo microscopic images for polyamide fragments observed under bright field mode using a fluorescent stereo microscope (Nikon SMZ1270).



Supplementary Figure 2 Colored Polyamide fragments after being stained with Nile Red solution and oven-dried at 55 °C for 48 hours.

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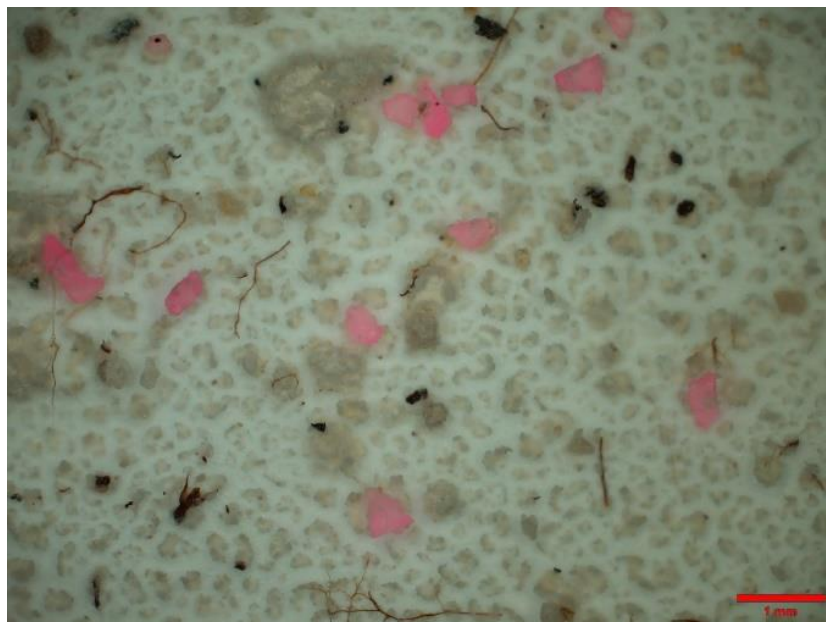


Supplementary Figure 3 Schematic diagram (A) and an image of the plastic containers (mesocosms) (B) that were set up in the Environmental Change Outdoor Laboratory (ECOLAB) facility of the University of Birmingham, UK.



Supplementary Figure 4 Density separation of the polyamide fragments from sediments using Sediment Microplastic Isolation (SMI) units and zinc chloride (ZnCl_2 ; 1.5 g cm^{-3}).

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Supplementary Figure 5 Stained polyamide fragments observed under bright field mode using a Nikon SMZ1270 fluorescent stereo microscope.

Supplementary Table 1 Weight (mg) of 100 particles from each polyamide (PA) fragments (A), the weight of the sediment added in each mesocosm (B), and the calculation done for each mesocosm to obtain the 2140 particles/Kg DW concentration used in the experiment.

| Supplementary Table 1 - A | | |
|---------------------------|-------------|---------------------|
| Microplastics | Weight (mg) | Number of particles |
| PA fragments | 7.47 | 100 |

| Supplementary Table 1 - B | | |
|---------------------------|-----|-----|
| Sediment per microcosm | Wet | Dry |
| Weight (Kg) | 342 | 311 |

| Supplementary Table 1 - C | Number of PA | Weight of PA |
|-----------------------------------|--------------|--------------|
| For 1 Kg of sediment dry weight | 2140 | 159.78 |
| For 311 Kg of sediment dry weight | 665540 | 49715.84 |

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Supplementary Table 2 Number of polyamide fragments recovered from the containers.

| Sediment mixtures | Treatments | Sediment dry weight (g) | Number of fragments/filter | Number of fragments/Kg DW | Mean of the three composite samples |
|-------------------|---------------|-------------------------|----------------------------|---------------------------|-------------------------------------|
| Sediment A | Scooping | 35.5 | 40 | 1125.7 | 1655 |
| | | 35.2 | 73 | 2075.9 | |
| | | 35.2 | 62 | 1763.2 | |
| | Resuspension | 35.1 | 560 | 15932.2 | 18518 |
| | | 34.4 | 673 | 19574.2 | |
| | | 35.0 | 702 | 20047.4 | |
| | Coring | 35.1 | 143 | 4078.8 | 4577 |
| | | 35.0 | 187 | 5339.0 | |
| | | 35.0 | 151 | 4314.3 | |
| | Freeze coring | 35.1 | 134 | 3818.1 | 3326 |
| | | 35.0 | 104 | 2969.8 | |
| | | 35.1 | 112 | 3190.2 | |
| | Piezometer | 35.5 | 346 | 9743.2 | 10410 |
| | | 35.6 | 388 | 10893.1 | |
| | | 35.3 | 374 | 10594.3 | |
| Sediment B | Scooping | 35.3 | 87 | 2462.4 | 2235 |
| | | 35.5 | 72 | 2030.0 | |
| | | 35.2 | 78 | 2214.0 | |
| | Resuspension | 35.0 | 341 | 9738.1 | 9121 |
| | | 35.1 | 321 | 9151.6 | |
| | | 35.2 | 298 | 8473.4 | |
| | Coring | 35.1 | 70 | 1994.1 | 2043 |
| | | 35.1 | 57 | 1626.2 | |
| | | 35.1 | 88 | 2507.3 | |
| | Freeze coring | 35.3 | 69 | 1954.5 | 1547 |
| | | 35.3 | 56 | 1586.8 | |
| | | 35.4 | 39 | 1100.5 | |
| | Piezometer | 35.4 | 277 | 7822.2 | 7090 |
| | | 35.4 | 193 | 5453.7 | |
| | | 35.7 | 285 | 7993.5 | |

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Supplementary Table 3 Chi-squared test to compare the size distribution of the recovered PA fragments with those introduced in the containers for each sampling method (A) and the relationship between the sediment mixture and the size distribution of the recovered PA fragments from each sampling method (B), and Pearson residuals (C) (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

| Supplementary Table 3 – A: | | | | | |
|----------------------------|----------------------------|----------------------------|---------------|-----------------------------|-----------------------------|
| Chi-squared test | | | | | |
| Sampling technique | Scooping | Coring | Freeze coring | Resuspension | Piezometer |
| Chi-squared | 41.825 | 58.687 | 10.009 | 131.79 | 86.238 |
| df | 16 | 18 | 16 | 18 | 18 |
| p-value | 4.191×10^{-4} *** | 3.329×10^{-6} *** | 0.8662 | $< 2.2 \times 10^{-16}$ *** | 6.792×10^{-11} *** |

| Supplementary Table 3 – B | | | | |
|---------------------------|------------------|-------------|----|-----------------------------|
| Sampling technique | Sediment mixture | Chi-squared | df | p-Value |
| Scooping | Sediment A | 2.1385 | 8 | 0.9735 |
| | Sediment B | 38.478 | 8 | 6.142×10^{-6} *** |
| Coring | Sediment A | 10.195 | 8 | 0.2516 |
| | Sediment B | 49.121 | 9 | 1.575×10^{-7} *** |
| Freeze coring | Sediment A | 2.8085 | 8 | 0.9458 |
| | Sediment B | 7.6644 | 8 | 0.4669 |
| Resuspension | Sediment A | 8.0355 | 9 | 0.5306 |
| | Sediment B | 95.348 | 9 | $< 2.2 \times 10^{-16}$ *** |
| Piezometer | Sediment A | 9.3865 | 8 | 0.3107 |
| | Sediment B | 68.076 | 9 | 3.625×10^{-11} *** |

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| Supplementary Table 3 – C | | | | | | | | | | | |
|---------------------------|--------------------|-----------|-----------|-----------|-----------|-----------|------------|-------------|-------------|-------------|-------------|
| Pearson residuals (r) | Size fraction (µm) | | | | | | | | | | |
| Sampling method | Mix | [150-300] | [300-450] | [450-600] | [600-750] | [750-900] | [900-1050] | [1050-1200] | [1200-1350] | [1350-1500] | [1500-1750] |
| Scooping | Spiked | 0.338 | 0.531 | 0.491 | 0.583 | 0.519 | -0.704 | -0.168 | -0.171 | 0.068 | / |
| | Sediment A | -0.626 | 1.005 | 0.225 | 0.362 | 0.672 | -0.576 | -0.613 | -0.25 | -0.125 | / |
| | Sediment B | -0.728 | 2.849 | -2.03 | 2.491 | 2.52 | 3.129 | 1.157 | 0.854 | -0.146 | / |
| Coring | Spiked | -0.34 | 0.387 | 0.459 | 0.155 | -0.05 | -0.734 | -0.47 | -1.058 | -0.543 | -0.909 |
| | Sediment A | 0.845 | 0.646 | 0.045 | 0.742 | 1.042 | -0.343 | 0.789 | 2.013 | 0.62 | -0.345 |
| | Sediment B | 0.075 | 2.481 | 1.868 | 1.715 | 1.751 | 3.395 | 0.668 | 1.154 | 1.207 | 4.086 |
| Freeze coring | Spiked | 0.207 | 0.54 | 0.127 | -0.41 | 0.176 | -0.24 | -0.1 | -0.369 | -0.321 | / |
| | Sediment A | 0.405 | 0.574 | 0.139 | 0.515 | 0.168 | -0.042 | -0.072 | 0.466 | 1.107 | / |
| | Sediment B | 0.34 | 1.594 | -0.37 | 1.094 | 0.546 | 1.141 | 0.557 | 0.983 | -0.169 | / |
| Resuspension | Spiked | 0.139 | 0.762 | 0.489 | 0.183 | 0.736 | -0.597 | -1.005 | -1.477 | -1.37 | -0.945 |
| | Sediment A | 0.411 | 2.473 | 0.986 | 1.547 | 2.001 | -1.487 | -1.129 | -0.847 | -0.65 | -0.256 |
| | Sediment B | 0.325 | 4.932 | 2.312 | 1.855 | 4.214 | 3.224 | 3.477 | 3.956 | 3.477 | 2.125 |
| Piezometer | Spiked | 0.663 | 0.702 | 1.021 | 0.218 | -0.94 | -0.98 | -0.58 | -1.068 | -0.951 | -1.133 |
| | Sediment A | 0.082 | 2.137 | 0.253 | 1.937 | 0.921 | -0.524 | -0.065 | 0.078 | 1.656 | -0.653 |
| | Sediment B | 1.495 | 4.071 | 2.456 | 2.81 | 3.096 | 2.698 | 1.299 | 2.152 | -0.008 | 3.177 |

Section 3: Characterization of microplastics:

After discussing the extraction and sampling of microplastics in the previous sections of this chapter (i.e., Chapter II), some of the methods used to characterize microplastics at the physical and chemical levels are discussed below in the next section.

Therefore, these three sections together target the technical developments made in this PhD study.

3. Characterization of microplastics using fluorescence stereo microscope and Fourier transform infrared (μ FTIR) spectroscopy

3.1. Introduction

In the first two sections of this chapter, a complete technical development was established for extracting microplastics, particularly from sediment matrices, based on sieving the samples, removing the inorganic material with a density separation process using zinc chloride solution (ZnCl_2 ; 1.5 g cm^{-3}), and digesting the organic matter using Fenton reagent (30% H_2O_2 plus 2 ml of aqueous 0.05 M Fe (II) solution). It was also investigated how different sediment sampling approaches can provide us with various insights into the concentration and size distribution of the microplastics extracted. Following these two technical studies, it is necessary to identify, quantify and characterize the collected plastic particles in order to better understand and monitor microplastic contamination in the environment.

Although there is a growing body of literature on microplastics, their identification and quantification in environmental samples have not yet been standardized (Christian and Köper, 2023; Rochman et al., 2017). Some studies rely on the visual identification provided by microscopy, while others use approaches like Fourier transform infrared (μ FTIR) spectroscopy, Raman spectroscopy, and pyrolysis-gas chromatography-mass spectrometry for chemical identification of the polymer type (Lenz et al., 2015; Nel et al., 2021; Picó and Barceló, 2020). In contrast to these expensive techniques, microscopy instruments are considered to be the most accessible and cost-effective methods and are hence most often employed for quantifying and identifying microplastics (Shim et al., 2017). Nevertheless, they are subject to observer and technical bias, which increases the likelihood of misidentification (Joana Correia Prata et al., 2019). Moreover, using microscopy, the smaller the particle, the more challenging it is to obtain a correct identification (Song et al., 2015). Besides, synthetic polymers and naturally occurring organic and inorganic particles of comparable sizes and forms may be difficult to distinguish, leading to subsequent errors (Shim et al., 2017). Consequently, it is often common that when

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utilizing visual identification as the sole approach for microplastic quantification, results are frequently prone to either underestimation or overestimation (Song et al., 2015).

In an attempt to overcome these challenges, the lipophilic fluorescent dye Nile red has been employed to enhance visual identification (Stanton et al., 2019). Labeling microplastics with fluorescent dyes contributes to facilitating the distinction of plastic particles from other non-plastic substances (Joana C. Prata et al., 2019). However, a potential disadvantage of this staining method is the possibility of producing false positive results due to the staining of biological substances (Maes et al., 2017). To confirm that suspected stained "anthropogenic particles" are, in fact, microplastics, analytical chemistry techniques such as Fourier transform infrared (μ FTIR) spectroscopy based on detecting the type of polymer are fundamentally required (De Frond et al., 2021). On one hand, it is possible to find up to 70% of incorrectly assigned particles by applying chemical identification assessment using analytical chemistry methods (Hidalgo-Ruz et al., 2012). On the other hand, these methods also enable us to determine the type of polymer the particle is made of, a feature that is inaccessible with traditional microscopy techniques (Xu et al., 2019).

During the course of this PhD, visual and chemical identification protocols were needed. For this purpose, two critical objectives were achieved. First, to obtain a robust methodology using Nile red staining, a polymer-specific parameter was tested that helps in determining whether the observed stained particle is plastic or not. This parameter relies on the fluorescence intensity or pixel brightness of stained microplastic particles and aims to assess microplastic particle pixel brightness (PPB) before and after staining with Nile red (Nel et al., 2021). Therefore, any increase in microplastic particle intensity is monitored and recorded with respect to particle polymer type and size. This offers us a "PPB library" for different microplastic polymers and size fractions, which acts as a reference to our research and helps in primarily identifying whether the measured brightness of a particle observed under a fluorescence stereo microscope is a plastic or biological matter. Second, in order to develop a reliable methodology for obtaining accurate Fourier transform infrared (μ FTIR) spectroscopy outcomes, tests of quality control were conducted on pristine plastic particles.

3.2. Methodology

3.2.1. *Adaptation of visual identification*

Nile red staining

For this test, plastics from nine virgin polymers were evaluated: polyamide (PA), nylon (PA6), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), polyethylene terephthalate (PET), polyethylene (PE), polyoxymethylene (POM) and polytetrafluoroethylene (PTFE). Different sizes were selected (i.e., between 20 μm and 1000 μm), and also different colours ranging from white, blue, red, and grey. Our plastic particles were in two shapes, fibers and fragments. Additionally, non-plastic particles were also used. Samples of sediment (i.e., previously burned at 550°C), wood, leaves, and chitin, which can be found in environmental samples, were analyzed to evaluate the intensity of the dye on both inorganic and organic matter. Finally, to obtain robust and reproducible results, three replicates were prepared for each type of particle.

Both plastic and non-plastic particles were stained using a Nile red solution in acetone at a concentration of 5 $\mu\text{g ml}^{-1}$ that was previously prepared from a stock solution of 1 mg ml^{-1} . After 1 hour of staining, the particles were filtered on Whatman GF/D glass fiber filters (2.7 μm in porosity; 47 mm diameter) and then were left to dry in an oven at 50°C for 24 h (Maes et al., 2017; Nel et al., 2021).

Microscopic analysis

The PPB measurement were performed using a Nikon stereo microscope (model SMZ1270) equipped with a Nikon long-life metalloid light source and a Nikon DS-Fi3 high definition 5.9-megapixel colour camera. To obtain the fluorescence imaging mode, a GFP-B filter was used (EX450-490 nm, DM500 nm, BA510-560 nm). The plastic and non-plastic particles were observed under the fluorescence conditions of the stereo microscope, where the PPB was calculated by averaging the fluorescence intensity for a line crossing

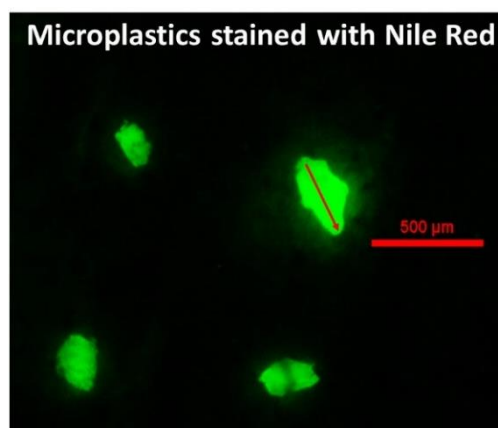


Figure 12 Measuring the fluorescence intensity for a stained microplastic particle. The red arrow represents the measurement path of the fluorescence intensity.

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the entire length of a particle (Nel et al., 2021) (**Figure 12**). This value range between 0 and 255 arbitrary units (a.u.), with background fluorescence < 1.

3.2.2. Adaptation of chemical identification

Microplastics of known numbers and polymer types (i.e., similar to those used previously to adapt the PPB visual identification approach), as well as particles of organic matter (i.e., wood, leaves, and chitin), were analyzed using Fourier transform infrared (μ FTIR) spectrometer (PerkinsElmer Spotlight 400) in transmittance mode (pixel resolution: 25 μ m, spectra resolution: 8 cm^{-1}). These synthetic and natural particles, smaller than 1000 μ m in size, were spiked on alumina filters (Whatman Anodisc: diameter= 25 mm and porosity= 0.2 μ m) to form three main samples: Sample 1 (spiked with white POM, blue PE, red PA, and grey PVC), Sample 2 (spiked with blue POM, white PET, and grey PP), and Sample 3 (spiked with white PS, white PTFE, wood, leaf, and chitin particles). For each sample, three replicates were spiked with the same particles to ensure reproducibility in the results. After treating the spectral map produced from each sample using the siMPle software (version 18/09/2020), the number and polymer types of the plastic and non-plastic particles were determined. Using a probabilistic method, this software makes repeated correlations based on a spectra database to identify the polymer type with an associated confidence/probability value that is provided by the reference database presented by Primpke et al., (2018).

3.3. Results and discussion

Nile red staining and subsequent PPB measurement provided better identification of the particle type using a fluorescence stereo microscope. The constructed PPB library included data on the range of values for each polymer particle brightness, making it easier to distinguish plastic from non-plastic particles. According to the data in (**Table 3**), and in accordance with Nel et al., (2021), PP and PE polymers have the highest and most consistent PPB. Nile red gave a strong stain on PA and PS, as well as POM, however, their PPB was lower than that of PP and PE. Yet, the fluorescence of PVC and PET did not show any enhancement upon Nile red staining. These findings are in line with those of other studies that recommended avoiding the use of Nile red to detect PET and PVC due to weak fluorescence (Erni-Cassola et al., 2017).

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Table 3 Particle pixel brightness (PPB; min - max (median)) for various synthetic polymers of different shapes, sizes and colours at different magnifications.

| ID | Source | Shape | Size (mm) | Colour | Stereo. Magnification | PPB min - PPB max (a.u.) | | PPB \pm s.d. (a.u.) ; (Median) | |
|------|-------------------------------|----------|------------|-------------|--------------------------|--------------------------|------------------|----------------------------------|------------------------|
| | | | | | | With NR stain | Without NR stain | With NR stain | Without NR stain |
| PA6 | Flock Depot (Germany) | Fiber | 0.15 | Red | 15 X | 0 - 17 | / | 5.5 \pm 2.8 (5) | / |
| PA | High Wycombe (United Kingdom) | Fragment | 0.02 - 0.5 | Transparent | 15 X | 127 - 255 | 104 - 255 | 247 \pm 20.8 (255) | 195.6 \pm 48 (182.5) |
| PA | High Wycombe (United Kingdom) | Fragment | 0.5 - 1 | Transparent | 4.725 X | 47 - 157 | 3 - 121 | 99.5 \pm 19.3 (100) | 21 \pm 16.3 (16) |
| PP | Boutyplast (France) | Fragment | 0.02 - 0.5 | White | 15 X | 96 - 255 | 5 - 56 | 247.4 \pm 24.8 (255) | 22.2 \pm 7.5 (21) |
| PP | Boutyplast (France) | Fragment | 0.5 - 1 | White | 4.725 X | 109 - 255 | / | 253.1 \pm 12.4 (255) | / |
| PVC | Boutyplast (France) | Fragment | 0.02 - 0.5 | Grey | 15 X | 16 - 137 | 26 - 98 | 64.4 \pm 21.1 (62) | 66 \pm 10.1 (66) |
| PVC | Boutyplast (France) | Fragment | 0.5 - 1 | Grey | 4.725 X | 2 - 40 | 3 - 42 | 15.2 \pm 5.6 (15) | 14.4 \pm 5 (14) |
| PE | Boutyplast (France) | Fragment | 0.02 - 0.5 | Blue | 15 X | 193 - 255 | 71 - 255 | 253.7 \pm 7.2 (255) | 202.8 \pm 52.5 (224) |
| PE | Boutyplast (France) | Fragment | 0.5 - 1 | Blue | 4.725 X | 107 - 255 | / | 239.4 \pm 25.5 (255) | / |
| PET | Water bottle | Fragment | 0.02 - 0.5 | Transparent | 15 X | 21 - 152 | 18 - 110 | 56 \pm 25.9 (48) | 65.3 \pm 19.6 (67) |
| PET | Water bottle | Fragment | 0.5 - 1 | Transparent | 4.725 X | 2 - 38 | 7 - 71 | 17 \pm 5.8 (16) | 30.7 \pm 12.8 (28) |
| PTFE | Boutyplast (France) | Fragment | 0.02 - 0.5 | White | 15 X | 6 - 92 | / | 24.2 \pm 10.4 (23) | / |
| PTFE | Boutyplast (France) | Fragment | 0.5 - 1 | White | 4.725 X | 0 - 19 | / | 6.8 \pm 3.5 (7) | / |
| PS | Spectroscopy cuvettes | Fragment | 0.02 - 0.5 | Transparent | 15 X | 107 - 255 | 10 - 62 | 251.8 \pm 16 (255) | 26.4 \pm 7.7 (25) |
| PS | Spectroscopy cuvettes | Fragment | 0.5 - 1 | Transparent | 4.725 X | 71 - 252 | 1 - 39 | 194.6 \pm 44.2 (212) | 10.1 \pm 5 (9) |
| POM | Boutyplast (France) | Fragment | 0.02 - 0.5 | White | 15 X | 93 - 255 | 5 - 126 | 245.9 \pm 18.8 (255) | 23 \pm 11.7 (20) |
| POM | Boutyplast (France) | Fragment | 0.5 - 1 | White | 4.725 X | 73 - 225 | 6 - 88 | 160.9 \pm 23.3 (159) | 35.7 \pm 18.2 (33) |
| POM | Boutyplast (France) | Fragment | 0.02 - 0.5 | Blue | 15 X | 124 - 255 | 10 - 51 | 236 \pm 24.5 (243) | 28.9 \pm 7.1 (28) |
| POM | Boutyplast (France) | Fragment | 0.5 - 1 | Blue | 4.725 X | 34 - 167 | 0 - 32 | 101.2 \pm 26.5 (103) | 10.3 \pm 4.3 (10) |

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Unlike the sediment samples (which had previously been burnt at 550°C), chitin, leaves, and wood all fluoresced when stained with Nile red. In spite of this, when comparing the conditions before and after Nile red staining, they showed no sign of fluorescence increase (**Table 4**). Out of these non-plastic particles, chitin demonstrated the highest PPB with a median of 124 and 163 a.u. in the presence and absence of Nile red staining, respectively. Thus, to exclude chitin components when applying visual identification using Nile red staining, microplastic extraction techniques need to include a digestion phase, particularly in the presence of chitinase enzyme (Kallenbach et al., 2021; Maes et al., 2017). Based on the PPB library, a lower threshold limit of 100 a.u. might also be advised after the digestion stage to capture five out of the nine polymers evaluated in our study and avoid overestimation when using the Nile red stain (Stanton et al., 2019). The 100 a.u. could be recognized as the threshold below which a particle is likely to be identified as organic and not plastic.

Table 4 Particle pixel brightness (PPB; min - max (median)) for natural materials at different magnifications.

| Type of matrices | Stereo. Magnification | PPB min - PPB max (a.u.) | | PPB \pm s.d. (a.u.) ; Range (Median) | |
|---------------------------------------|-----------------------|--------------------------|------------------|--|------------------------|
| | | With NR stain | Without NR stain | With NR stain | Without NR stain |
| Sediment (previously burned at 550°C) | 4.725 X | / | 3 - 83 | / | 27.4 \pm 18.8 (23.5) |
| Chitin | 4.725 X | 67 - 216 | 57 - 255 | 125 \pm 35.1 (124) | 152.7 \pm 63.7 (163) |
| Leaves Litter | 4.725 X | 12 - 84 | 10 - 91 | 33.7 \pm 13.9 (31) | 53.3 \pm 16 (51) |
| Wood | 4.725 X | 9 - 80 | 36 - 129 | 36.4 \pm 15.7 (34) | 73.5 \pm 19.7 (71) |

When a more precise identification is required, fluorescence microscopy and PPB analysis are insufficient. Therefore, we utilized FTIR to determine the polymer of microplastic particles extracted from sediments. The quantification, using FTIR, of the different polymers in Samples 1, 2 and 3, showed that the recovery rate expressed by number was around 50%, and was more than 85% when expressed by area (**Table 5**). It is important to note that Polyamide (PA) particles were excluded from the analysis as their spectra are similar to those of natural particles.

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Table 5 Quality control of synthetic and natural particles analysed using μ FTIR.

| Spiked samples | Plastic types and organic matter | MP recovery (n) | MP recovery (area) |
|---|---|------------------|--------------------|
| Sample 1 (n=3) | POM (white), PE (blue), PA (red fibers), PVC (grey) | 41.6 \pm 10.4% | 84.7 \pm 3.3% |
| Sample 2 (n=3) | POM (blue fibers), PET (white), PP (grey) | 67.3 \pm 26.8% | 82.1 \pm 11.5% |
| Sample 3 (n=3) | PS (white), PTFE (white), wood, leaf and chitin | 42.3 \pm 7.1% | 94.8 \pm 4.2% |
| MP recovery on average \approx 50% (n) and \approx 87% (area) | | | |

Comparison of the particle spectrum to that of custom made and/or commercial libraries has always been a challenge in chemical identification of the polymers due to the lack of a single library broad enough to assure extensive success rates (Araujo et al., 2018; De Frond et al., 2021). According to its molecular structure and conformational properties, the same plastic might actually display a variety of spectral features. For instance, crystalline and amorphous polyethylene polymers exhibit different spectral characteristics. In addition, the same plastic type can exhibit spectral variations depending on the environment with which the polymer interacted or was prepared during the extraction or purification process (Xu et al., 2019). To overcome these challenges, and on the basis of the obtained results (i.e., 50% recovery), the level of associated confidence/probability value demonstrated by the reference database published by Pimpke et al., (2018) for siMPle software, was increased (**Table 6**). As a result, the chemical identification analysis became more reliable, and all previously undetected pristine plastic particles from Samples 1, 2, and 3 were re-identified and recovered.

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Table 6 The adjustment of the confidence/probability value demonstrated by the reference database published by on Primpke et al., (2018) for siMPle software.

| Plastic name | Abbreviation | Probability value (Primpke et al., 2018) | Probability value (used in this study) |
|---------------------------------|--------------|--|--|
| acrylates/polyurethanes/varnish | PUR-A-V | 0.67 | 0.75 |
| Cellulose artificial/modified | Cell_AM | 0.65 | 0.65 |
| ethylene-vinyl-acetate | EVA | 0.65 | 0.9 |
| polycarbonate | PC | 0.65 | 0.65 |
| polyester | PES | 0.75 | 0.8 |
| polyethylene | PE | 0.75 | 0.75 |
| polyethylene, chlorinated | PE-C | 0.7 | 0.85 |
| polyethylene oxidized | PEOX | 0.7 | 0.85 |
| polyethylene terephthalate | PET | - | 0.75 |
| polyoxymethylene | POM | 0.65 | 0.65 |
| polypropylene | PP | 0.55 | 0.7 |
| polystyrene | PS | 0.6 | 0.75 |
| polyvinylchloride | PVC | 0.6 | 0.7 |
| rubber type 1 | RUB1 | 0.7 | 0.9 |
| rubber type 2 | RUB2 | 0.7 | 0.9 |
| rubber type 3 | RUB3 | 0.81 | 0.9 |

3.4. Conclusion

The technical improvements presented in this section were crucial to the work presented in the next chapters. Creating the PPB library for materials enhances the reproducibility and precision of identification based only on visual characterization. The PPB library can reduce the common misidentification under fluorescence microscope instruments by providing guidance for distinguishing plastics from non-plastics based on the fluorescence intensity of particles. In order to determine the polymer type of a substance, it is essential to use analytical chemistry methods such as μ FTIR. The use of reported database libraries, such as the one used in this study and adapted by Primpke et al., (2018) is a means of finding a solution in the absence of a universal library that can cover all plastic types and potential spectra. Prior to utilizing such custom-made libraries, however, it is necessary to conduct quality control tests that provide the means to calibrate and adjust the level of accepted probabilities in software like siMPle.

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In future work, we recommend a thorough and detailed search in this concern that include the following:

- Since Nile red is a solvatochromic dye where its fluorescence emission spectrum shifts depending on the polarity of its environment (Maes et al., 2017), the PPB of several polymer types in presence of different solutions (e.g., ZnCl_2 and H_2O_2), must be tested.
- As microplastics in the environment are subjected to various physical, chemical, and biological mechanisms of degradation (Boyle and Örmeci, 2020; Singh and Sharma, 2008), they may become more brittle as a result of additive leaching. This in turn alters the roughness, porosity, polarity, and hydrophobicity of the plastic particle (Fu et al., 2019; Luo et al., 2020). Such modifications can dramatically affect the PPB of a fluorescent particle, thereby increasing the difficulty of using Nile red to identify microplastics sampled from the environment. Therefore, determining the PPB of microplastic particles before and after degradation processes is highly needed.
- Similar to the effect on PPB, plastic degradation in the environment can have a significant effect on spectral changes (Xu et al., 2019). This is why it is emphasized that more work is required to construct a spectral library customized to microplastics.

3.5. References

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Chapter III

Microplastic accumulation in streambed sediments

Microplastic distribution at the sediment-water interface in gravel bed rivers:

In recent years, it has been widely acknowledged that rivers play a crucial role in the transport of microplastics from land to the ocean. Nonetheless, it is becoming increasingly apparent that the sediment-water interface within river networks also plays a significant role in the temporary suspension and retention of plastic debris. A complex interplay of hydrological patterns, sediment dynamics, physicochemical interactions, and biological processes govern the fate and transport of microplastics along river corridors. Not only do these factors affect the vertical and horizontal dispersion of microplastics throughout the water column, but they also determine their ultimate fate at the sediment-water interface. Our understanding of microplastic behavior at this vital interface remains limited despite extensive research efforts aimed at uncovering these complex mechanisms.

This chapter investigates how hydrological exchanges at the sediment-water interface can influence the distribution of microplastic concentrations in streambed sediments. This chapter consists of an article currently being finalized for submission to the Journal of Water Research.

1. Microplastic concentrations in downwelling, upwelling, and flow stagnation zones at the sediment-water interface in gravel bed rivers

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1.1. Abstract

While rivers have previously been seen as primary vectors via which microplastics are transported from land to the ocean, the sediment-water interface of river networks provide also opportunity for the temporary suspension and retention of plastic debris. During their travel through river corridors, hydrological, sedimentary, physicochemical and biological factors govern the fate and transport of microplastics and regulate their vertical and horizontal dispersion across the water column. Although several studies have been dedicated to understand these factors, the mechanisms of microplastic transport and transfer at the sediment-water interface remain poorly understood. In this study, we analyzed how the potential for hydrological exchanges at the sediment-water interface can influence the distribution of microplastic concentrations in streambed sediments. Microplastic concentrations were therefore compared between zones with surface-groundwater exchanges (upwelling or downwelling) and sediment accumulation

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zones along gravel bars situated in the lower catchment of the Ain River (France). Sediment samples were collected from 15 locations (five per hydro-sedimentary condition) at two different depths (i.e., sediment surface and -20 cm). Following the field campaign, samples were processed in the laboratory and microplastics between 20 and 500 μm in size were extracted. Our findings indicate that the concentration of microplastics in the sediment fraction lower than 2 mm is of the same order of magnitude between the three hydrological zones in the surface sediments. At 20 cm depths, however, up-welling zones contain significantly more microplastics than down-welling zones. These higher concentrations are driven by larger numbers of particles at the smallest analysed size fractions. Possible explanations for the observed pattern is that microplastics at the streambed of the gravel bars are undergoing degradation and fragmentation into further smaller sizes, thereby facilitating their mobility within the slow fluxes of the upwellings. Yet, compared to these two zones of water exchange, the first sediment layers in the sedimentation regions exhibit significantly increased microplastic abundance within the dry mass of the total sediments. These higher concentrations may be governed by the presence of fine sediments and organic particulates which stimulate rapid deposition of the plastic polymers. Rivers with coarse sediment bed should thus be given greater attention in future studies as they have the potential to store pore size microplastic particles that could endanger the interstitial fauna, pollute groundwater, and threaten food webs.

1.2. Keywords

Microplastics, sediment-water interface, hyporheic exchanges, gravitational settling, polymers.

1.3. Introduction

Civilizations have flourished along riverbanks throughout human history, benefiting ecologically, socially, and economically (Everard and Powell, 2002). However, river corridors have always been under threat from multiple anthropogenic activities, one of which is the production of plastic (Best, 2019). There is growing concern over the presence of plastic debris in inland waterbodies due to the risks to freshwater organisms and ecosystem functioning (Castro-Castellon et al., 2022; Krause et al., 2021; Kukkola et al., 2021; Seeley et al., 2020; Silva et al., 2022; Wazne et al., 2023). This is particularly pertinent given that rivers are responsible for transporting a substantial

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amount of land-based plastic to the ocean (Schmidt et al., 2017), with 70 to 80% of marine plastics being transported to the sea via rivers (Horton et al., 2017). Microplastic may be designed at their actual size range or result from degradation of larger plastic waste into microplastics (i.e. plastic particles smaller than 5 mm in size, Arthur et al., 2009) and damage marine ecosystem (Guzzetti et al., 2018; Sharma and Chatterjee, 2017). It is therefore crucial to identify key pathways and the mechanisms governing their transport to marine ecosystems (Drummond et al., 2020; Nizzetto et al., 2016; Yan et al., 2021).

While rivers act as the primary transport vectors for microplastics, connecting terrestrial sources to marine environments, streambed sediments are serving as temporary storage zones for these contaminants (Horton and Dixon, 2018; Kabir et al., 2022). The mobilization, transport and deposition of microplastics at the sediment-water interface are largely controlled by the present hydrological conditions and materials properties of the microplastics (Yan et al., 2021). Similar to naturally occurring allochthonous particles (Hoellein et al., 2019), microplastics can aggregate, settle vertically, and are transported horizontally in the water column (Bai et al., 2022; Yan et al., 2021). This behavior is governed by a number of factors, such as precipitation, wind, stream flow (Bai et al., 2022; Chubarenko et al., 2016; Cohen et al., 2019; Margenat et al., 2021), river morphology and the presence of vegetation (Bai et al., 2022; Paduani, 2020), and the existence of artificial structures as dams and bridges (Dendievel et al., 2023; van Emmerik et al., 2018; Weideman et al., 2019). Moreover, the characteristics of microplastics themselves such as polymer density, size, and shape (Kooi et al., 2017; Kowalski et al., 2016), and finally the degree of abiotic and biotic interaction (i.e., through biofilm formation) modifies the density of microplastics in the water column and eventually cause them to sink towards the streambed sediment (Fazey and Ryan, 2016; Van Cauwenberghe et al., 2015).

Due to their often hydrophobic nature properties, microplastics can heteroaggregate with other environmental particles such as organic matter, phytoplankton and suspended sediments and act as surfaces for microbial colonization and biofilm development (Long et al., 2015; Paduani, 2020; Yan et al., 2021). Since heteroaggregation alters the size, shape, and density of microplastics, and hence their buoyancy and settling velocity, it is considered a key factor controlling how microplastics sink and accumulate at the sediment-water interface (Paduani,

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2020; Yan et al., 2021). However, recent studies have revealed that in addition to gravitational settling (sedimentation), hydrodynamically driven mechanisms such as hyporheic exchange (i.e., exchange between surface water and ground water, Krause et al., 2022; Lewandowski et al., 2019; Tonina and Buffington, 2009) have the potential to transport substantial fractions of suspended microplastics into streambed sediments (Drummond et al., 2020, 2022; Frei et al., 2019; Margenat et al., 2021). Sediment-water interfaces perform important ecosystem services where microorganisms and invertebrates such as bioturbators, interact with the sedimentary habitats to promote gas, nutrient, and water exchanges. (Cardenas and Wilson, 2007; Krause et al., 2022, 2017, 2009; Mermillod-Blondin, 2011; Mermillod-Blondin et al., 2004; Nogaro et al., 2009). As a result of bioturbation activity, microplastics that have been deposited and temporarily stored in river sediments may be remobilized once more at the sediment-water interface (Galloway et al., 2017; Malli et al., 2022; Näkki et al., 2019). Moreover, similar to natural particles, microplastics can also be transported back into the water column at the end of hyporheic exchange flow paths (Drummond et al., 2014, 2019, 2020; Phillips et al., 2019).

In this study, we identify the distribution of microplastics with respect to their appearance in streambed environments representative of distinct transport and retention conditions in the streambed sediments of a gravel-bed river. We therefore studied the concentration of microplastics in the sediments of the Ain River in France at contrasting downwelling and upwelling locations indicating the start and end points of hyporheic flow paths as well as sedimentation zones (i.e., sites of stagnant water) where drops in flow velocity facilitate gravitational settling. Similar to the behavior of fine sediments in gravel-bed rivers, we hypothesize that microplastic particles are more prevalent in riverbed sediments of downwelling flows of the surface water than in areas with significant upwelling. The sedimentation zones, however, may be more seriously polluted by microplastics than the other two zones.

1.4. Materials and methods

1.4.1. Studied area

Sediment samples from five locations identified to be upwelling zones and five from downwelling zones were collected from six gravel bars situated along the lower Ain River, a large tributary of

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the Rhône River that originates in the French Jura Mountains (**Figure 13**). The central valley of the Ain river is a prominent center for the plastics industry in France and Europe, which may contribute to a high level of microplastic contamination of the watershed (Dendievel et al., 2023; Dyvrande, 1980). Due to this high microplastic supply and the well-identified sites of hyporheic exchanges (Dole-Olivier et al., 2019), the lower Ain valley provides an suitable model to test our hypotheses regarding hyporheic transfer of microplastic at the sediment-water interface. Alongside these gravel bars, five sites characterized by stagnant water were identified and sampled to obtain sediments from sedimentation zones.

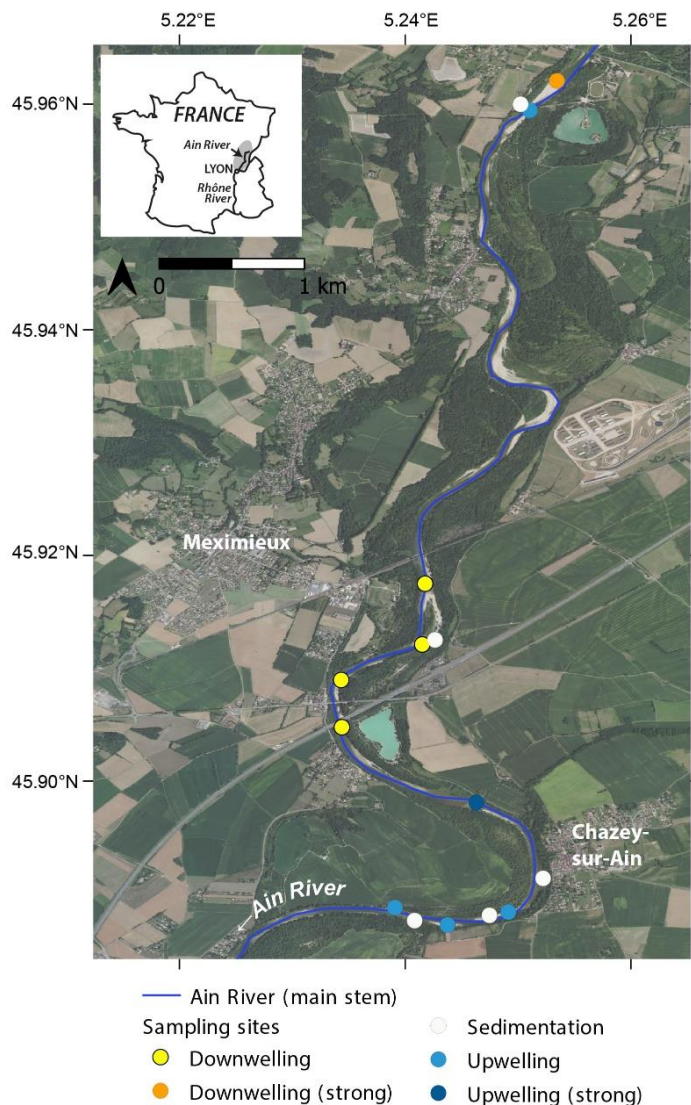


Figure 13 The Studied area of the Lower Ain River and the locations of the sampling sites.

To compare the microplastic concentrations at the sediment-water interface as a function of water exchange flow direction, sediments from each of the 15 sites of the upwelling, downwelling and the sedimentation zones were sampled by scooping with a metal shovel. Three sediment sub-samples were randomly selected and combined to create a composite sample that was representative of each location (Adomat and Grischek, 2021). To study the hyporheic distribution of microplastics in the streambed, a Bou-Rouch pump was installed at a depth range of 20 to 40 cm below the sediment-water interface to collect pore waters and interstitial sediment from the

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5 upwelling and 5 downwelling zones (Bou and Rouch, 1967; Malard et al., 2002). Sediment samples were kept in glass jars and stored in the refrigerator at 4°C for further processing in the laboratory.

Sites of upwelling and downwelling were selected after measuring the vertical hydraulic gradient (VHG) to quantify vertical exchanges between surface and groundwater. To perform this assessment, the hyporheic water level was calculated as the difference between the water level inside and outside the Bou-Rouch tube (functioning as a mini-piezometer) and expressed in % of the sample depth (Baxter et al., 2003; Dole-Olivier et al., 2019).

1.4.2. Characterization of physico-chemical properties of water, sediment, and microplastics

Water quality

During the field work, temperature, electrical conductivity, and dissolved oxygen were measured using a field probe (PONSEL Odeon, from www.aqualabo.fr) for both the surface and the hyporheic water sampled at -20 to -40 cm. A Bou-Rouch pump was used to sample a volume of 10 liters of hyporheic water and then immediately measured.

To obtain a general understanding of the river geochemistry, nutrients (NH_4^+ , NO_3^- , N-NO_2^- and PO_4^{3-}) and chloride (Cl^-) concentrations were analyzed in the samples collected from surface water of upwelling, downwelling, and sedimentation zones, and in water samples obtained from -20 to -40 cm depth, pumped from the upwelling and downwelling zones, using standard colorimetric methods (Voisin et al., 2018) using an automatic analyzer (Smartchem200, AMS Alliance, France).

Sediment particle size distributions

Assuming that microplastics tend to accumulate in depositional areas similar to other fine sediments (Ding et al., 2019; Margenat et al., 2021; Van Cauwenberghe et al., 2015), the overall proportion of sands, silts, and clays (SSC) in the streambed sediments of our sampling sites, particularly the upwellings and downwellings, was evaluated. Bulk sediment samples (approximately 5 kg) were collected from the sediment surface to the depth of -20 cm using a metallic shovel. The sediment samples were then manually sieved using a sieve column meshes from 20 μm to 63 mm, and then dried at 55°C for 1 week. The SSC content in particular represents

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the mass fraction of material found between 2 mm and 20 μm , plus the remaining particles < 20 μm .

1.4.3. *Microplastic extraction and characterization*

In order to extract plastic particles smaller than 500 μm but larger than 20 μm , sediment samples were sieved through 500 μm and 20 μm stainless steel sieve. The sediment samples were then dried in the oven for three to five days at 55°C. Subsequently, a dry mass of about 35 g was weighed from each sample, and then submerged for 24 h in a zinc chloride solution (ZnCl_2 ; 1.5 g cm^{-3}) using glass separating funnels coupled with Mohr clamps (*Wazne et al., MethodsX paper*). This ensures the separation of heavy inorganic particles from the lighter organic material. Following this step, the floating organic materials (fine organic and microplastics particles) were recovered by sieving with vacuum filtered ultra-pure water VFUP (Whatman GF/C filters: porosity= 1.2 μm). Recovered materials were then subjected to wet peroxide oxidation (WPO) using Fenton's reagent (solution of 30% hydrogen peroxide with ferrous sulphate) to remove organic matter (*Vermeiren et al., 2020; Zobkov et al., 2020*). Following digestion, the remaining material from our samples were vacuum filtered on alumina filters (Whatman Anodisc: diameter= 25 mm and porosity= 0.2 μm) for later FTIR spectroscopic analyses. The number of microplastics, their size and the type of polymer (i.e., which allowed us to determine the density of each particle by referring back to literature and database resources, [Supplementary Table 4](#)) were identified using $\mu\text{FT-IR}$ (Fourier-transform infra-red) spectrometer (PerkinsElmer Spotlight 400) in transmittance mode (pixel resolution: 25 μm , spectra resolution: 8 cm^{-1}) in accordance with our previous work where machine settings and quality control tests were set-up (*Dendievel et al., 2023*). The acquired spectral map for each filter was then processed using siMPLe software (version 18/09/2020) that allow us to determine and distinguish natural particles from synthetic ones by referring to the spectra database published by *Primpke et al. (2018)*. Finally, due to the significant likelihood of misinterpretation with spectra associated with natural particles, polyamide (PA) particles were not included in the study.

1.4.4. *Normalisation of microplastic concentrations*

As sediment sampling using Bou-Rouch pump results in the extraction of relatively fine particles of sediments (i.e., size lower than 3 mm), and as we choose to sieve the sediment samples

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between 20 µm and 500 µm, the microplastic concentrations ($[MPs]_{fine\ sediment}$) were expressed as a function of the dry mass of fine sediments (SSC).

The microplastic concentration relatively to the dry mass of the total sediment ($[MP]_{total\ sediment}$) was calculated using the percentage of SSC measured at each location using the following equation:

$$[MP]_{total\ sediment} = ([MPs]_{fine\ sediment} \times SSC) + ([MPs]_{coarse\ sediment} \times \overline{SSC})$$

where $[MPs]_{coarse\ sediment}$ is the concentration of microplastics in the coarse fraction of the sediment, SSC is percentage of sands, silts, and clays at surface down to – 20 cm below the sediment-water interface, and \overline{SSC} is the percentage of the size fractions larger than silts, and clays at surface down to – 20 cm deep below the sediment-water interface. All MPs concentrations are expressed as number of MP/Kg of dry sediments.

However, since microplastics accumulation is particularly correlated with the presence of fine sediment (Ding et al., 2019; Margenat et al., 2021; Van Cauwenberghe et al., 2015), the content of microplastic in the coarse sediment size fraction was assumed to be negligible and was set to 0 in the equation above.

1.4.5. Data treatments

As the concentration of microplastics in the finest sediment fractions (in the top sediment layers) and total sediment fractions (in both upper and bottom sediment layers) did not match the normality criterion ($p < 0.05$ for Shapiro-Wilk tests), non-parametric tests were applied on the dataset. The impact of different hydrological sampling zones (downwellings, upwellings, and sedimentations) on microplastic accumulation was tested using Kruskal–Wallis test. When Kruskal–Wallis test revealed significant differences among treatments (i.e., $p \leq 0.05$), Dunn's post hoc test was performed to evaluate which treatments significantly differed. On the other hand, since normality criterion was verified ($p > 0.05$ for Shapiro-Wilk tests) for the concentration of microplastics in the finest sediment fractions (in the deep sediment layers), and the results of the average size fractions and average densities of the plastic polymers at both depths, 1-Way ANOVA test was applied. In case of significant differences among treatments (i.e., $p \leq 0.05$), the 1-Way ANOVA test was followed by Tukey's post hoc test was performed to distinguish

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treatments that were significantly different. These statistical analyses were done with the R software (R 4.2.2 version, R Development Core Team, 2018).

1.5. Results

1.5.1. *Environmental parameters*

Across all upwelling and downwelling locations, the vertical hydraulic gradient (VHG) ranged from -37.5% to +35% (*Supplementary Table 5*). The average VHG for the five downwelling locations was $-18.3\% \pm 11.0$, whereas at the five upwelling sites it was 13.3 ± 13.1 . In accordance to Dole-Olivier et al., (2019), certain gravel bars had downwelling sites not only at the head but also at the tail (or apex) of the gravel bar. At all sample depths, the VHG of downwelling zones was associated with low conductivity (337 to 379 $\mu\text{S cm}^{-1}$) and high temperature (10.6 to 17.9 °C). However, the VHG of the upwellings was correlated with higher conductivity (362 to 418 $\mu\text{S cm}^{-1}$) but lower temperature (9.3 to 17 °C) (**Table 7**). Since we were unable to use the Bou-Rouch sampling method in sedimentation zones, the VHG was not recorded for these locations, but the very fine sediments present reflect a possible absence of water exchange between surface and groundwater. These sites were characterized by low surface flow velocity and abundant vegetation.

At the downwelling and the upwelling locations, water chemistry didn't vary significantly across all sampling depths. The mean values of the concentrations of inorganic nutrient (NO_3^- , N-NO_2^- and PO_4^{3-}) and chloride (Cl^-) were similar in both zones, except for the N-NH_4^+ , which was almost 13-fold higher in the upwelling zone at the surface to -20 cm and almost 40-fold higher at the -20 to -40 cm depth compared to the downwellings.

The mass fraction (%) of the sediment particle size recovered from the surface of the streambed to a depth of -20 cm revealed that the concentration of sands, silts, and clays (SSC) was nearly five times higher in the downwelling locations than in the upwelling (mean value: 14.8% vs. 2.8%, respectively). The sediment size fraction of the five sedimentation areas was almost entirely composed of SSC.

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Table 7 Environmental parameters measured for the surface water and ground water in addition to the sediment size fraction sampled at the first 20 cm of the streambed sediments of each hydrological condition.

| Environmental parameters | Downwellings | | Upwellings | | Sedimentation | |
|---|---------------------------------------|------------------|----------------|------------------|---------------|-------------|
| | 0 to -20 cm | -20 cm to -40 cm | 0 to -20 cm | -20 cm to -40 cm | 0 to -20 cm | |
| Water physico-chemical variables | VHG (%) | N/A | (-18.3) ± 11.0 | N/A | (13.3) ± 13.1 | N/A |
| | pH | 8.5 ± 0.1 | 8.3 ± 0.2 | 8.1 ± 0.2 | 7.8 ± 0.3 | 8.4 ± 0.3 |
| | Conductivity (µS/cm) | 359.5 ± 5.6 | 355.1 ± 16.1 | 377.4 ± 17.6 | 396.6 ± 18.9 | 361 ± 11.5 |
| | Dissolved oxygen (mg/L) | 12.3 ± 1.6 | 9.8 ± 1.4 | 10.9 ± 1.1 | 9.3 ± 1.1 | 9 ± 4.3 |
| | Dissolved oxygen Saturation (%) | 113.4 ± 8.6 | 95.7 ± 13.8 | 100.3 ± 9.6 | 86.5 ± 6.3 | 95.8 ± 44.9 |
| | Temperature (°C) | 12.7 ± 2.4 | 13.5 ± 2.7 | 11.1 ± 3.2 | 11.9 ± 2.4 | 17.5 ± 0.8 |
| | Cl (mg/L) | 8.4 ± 0.2 | 8.9 ± 0.4 | 6.8 ± 0.2 | 8.7 ± 1.1 | N/A |
| | P-PO ₄ ⁻ (µg/L) | 1.5 ± 0.9 | 2.9 ± 1.5 | 2.8 ± 1.5 | 3.8 ± 1.8 | 2.8 ± 1.5 |
| | N-NO ₂ ⁻ (µg/L) | 6 ± 0.2 | 6.4 ± 2.4 | 6.5 ± 3.6 | 7 ± 2.3 | N/A |
| | N-NO ₃ ⁻ (mg/L) | 0.9 ± 0.0 | 1.1 ± 0.3 | 1.5 ± 0.8 | 2.2 ± 1.1 | 0.83 ± 0.17 |
| N-NH ₄ ⁺ (µg/L) | 10.7 ± 8.4 | 20.4 ± 16.2 | 148.4 ± 95 | 831 ± 1210.3 | 0.01 ± 0 | |
| Mass fraction (%) of the sediment size distribution | 63 mm < Cobbles | 3.7 | N/A | 7.6 | N/A | 0 |
| | 2 mm < Gravels < 63 mm | 81.5 | N/A | 89.5 | N/A | 0.1 |
| | SSC < 2 mm | 14.8 | N/A | 2.9 | N/A | 99.9 |

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1.5.2. *Microplastic concentrations*

In the top 20 cm of the streambed sediments, the [MPs] *fine sediment* was comparable at sites with downwelling, upwelling, and sedimentation (or stagnant water areas) (**Figure 14 A**, Kruskal–Wallis test, $H(2) = 0.22$, $p = 0.89$). The concentration of extracted plastic particles (i.e., $20\ \mu\text{m} < \text{particles} < 500\ \mu\text{m}$) at these three locations ranged from 112 to 2217 particle/kg of DW, with a mean value of 917 ± 755 , 753 ± 664 , and 772 ± 819 particle/kg of DW at the downwellings, upwellings, and sedimentation areas, respectively. When normalised by the sediment grain size, the concentration of microplastics in the total sediment, [MP] *total sediment*, (**Figure 14 B**) varied significantly between the downwellings and upwellings on one side, and the sites of sedimentation on the other side. Hence, in comparison to upwelling and downwelling zones, the sedimentation regions had significantly greater microplastic concentrations in the total sediment (Kruskal–Wallis test, $H(2) = 8.3$, $p < 0.05$, Dunn’s test, $p < 0.05$).

Microplastics from depth of - 20 cm to -40cm were compared between the downwellings and upwellings. The concentration of microplastics extracted from the finest sediment fraction of the upwelling zones is significantly greater than that from the downwellings (**Figure 14 C**, 1-way ANOVAs, $p = 0.05$). The average microplastic concentration extracted from the upwellings (1793 ± 1151 particles/kg DW of the fine sediment) was almost three folds and a half greater than the concentration extracted from the downwellings (505 ± 489 particles/kg DW of the fine sediment). However, when these concentrations were normalized to the total sediment size fraction, significance was not detected (**Figure 14 D**). Microplastic concentrations extracted from downwellings and upwellings were comparable, with 75 ± 72 and 53 ± 34 particles/kg DW of the total sediment, respectively.

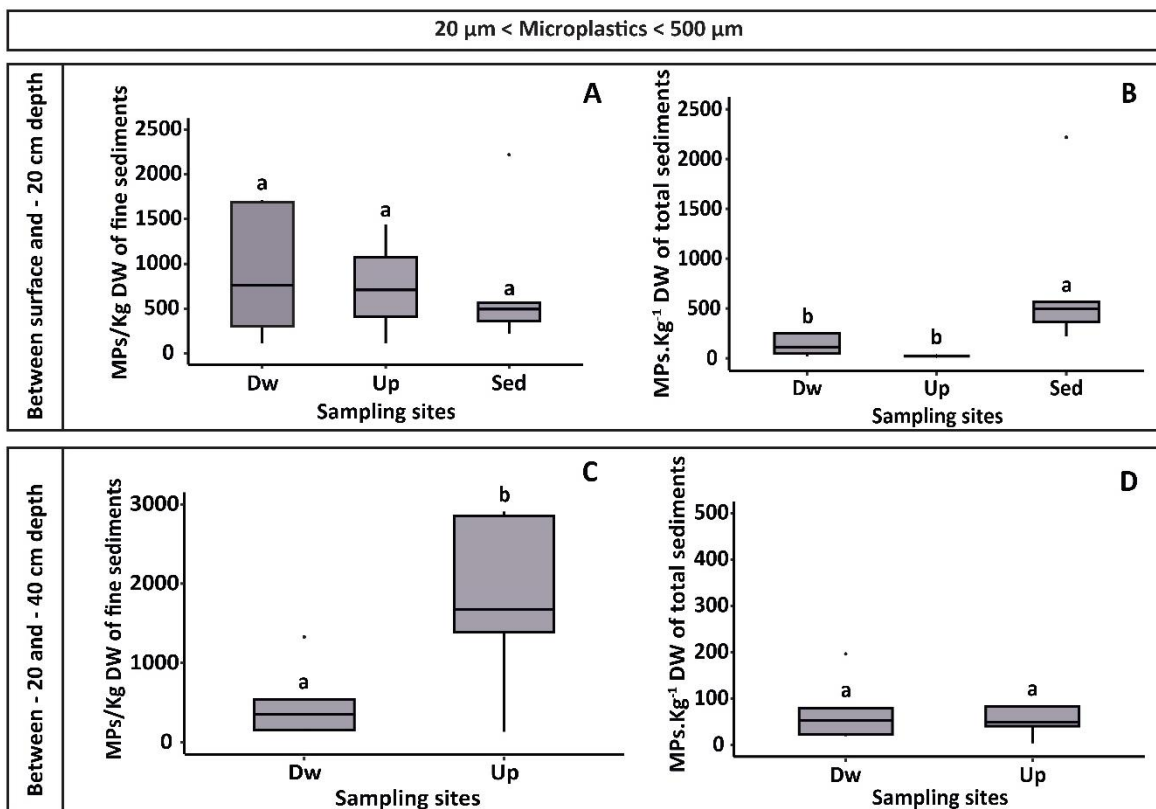


Figure 14 Microplastic concentrations (MPs) in the dry mass (DW) of the fine sediments at the top and bottom sediment layers (A and C), as well as the concentrations of microplastics in the dry mass of the total sediment content at the top and bottom sediment layers (B and D) of the three hydrological zones (Dw = downwellings, Up = upwellings, Sed = sedimentation zones). For each treatment, data are presented as means \pm SD ($n = 5$). Different letters indicate significant differences among sampling zones (For parametric test: Tukey test at $p \leq 0.05$, and for non-parametric test: Dunn test at $p \leq 0.05$).

Downwelling and upwelling locations with low VHG showed the highest concentration of microplastics in sediments between -20 and -40 cm depth. The highest concentration of microplastics in the fine sediments tended to accumulate in weak downwelling locations (i.e., VHG = - 12.5%) and weak upwelling locations (i.e., VHG = 5%) with concentration of 1,329 and 2,908 particles/kg DW, respectively (**Figure 15 A**). In the contrary, microplastic concentrations were found to be lowest in regions that had the highest VHG. Microplastics extracted from fine sediments showed the lowest concentration at sites with strong downwelling (i.e., VHG = - 37.5%) and strong upwelling zone (i.e., VHG = 35%), with 140 and 134 particles/kg DW, respectively.

Similar to microplastic concentrations, the content of fine sediments (i.e., sands, silts, and clays) is likely to be influenced by the water fluxes between surface water and ground water (**Figure 15 B**). Although it is not significant, among the 10 gravel bars addressed in our study, the fine

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sediment concentrations in the zones of low VGH, indicating potential weak flows, tended to be high, harboring nearly 10% and 18% at the zones where VHG is 5% and -16%, respectively. Nevertheless, under high VHG such as 35% and -37%, the content of SSC tended to decrease to 2 and even almost 0%, respectively.

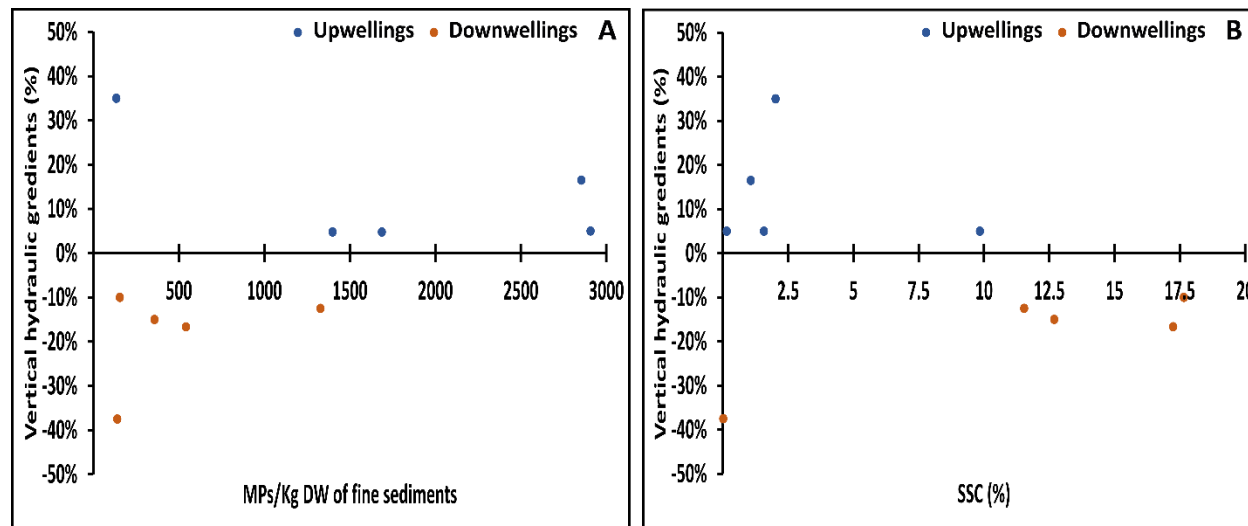


Figure 15 Vertical hydraulic gradient as function of the microplastic concentrations (MPs) in the dry mass (DW) of the fine sediment fractions bottom sediment layers (A) and as function of the percentage of sands, silts, and clays (B) in each of the two hydrological hyporheic conditions of downwelling and upwelling.

1.5.3. *Microplastic properties*

The mean size fraction of the plastic particles determined by FTIR analyses was significantly higher in downwelling zones than in upwelling zones (**Figure 16 A**, 1-way ANOVAs, $p < 0.05$, Tukey's HSD post-hoc tests, $p < 0.05$). The average diameters of microplastics extracted from the surface sediments of downwelling locations were $239 \pm 34 \mu\text{m}$ (median = $223 \mu\text{m}$), whereas the average microplastic diameters in upwelling areas were on average 20% smaller (mean value = $180 \pm 13 \mu\text{m}$, median = $184 \mu\text{m}$). Nonetheless, particle size distributions of microplastics extracted from both areas of water exchange were comparable to those collected from sedimentation zone (mean value $199 \pm 13 \mu\text{m}$, median = $189 \mu\text{m}$, **Figure 16 A**).

At -20 to -40 cm depth, although the size fraction of the microplastic particles at the downwelling and upwelling zones were not significantly different (**Figure 16 C**, 1-way ANOVAs, $p = 0.67$), the size distribution of microplastics extracted from the upwellings (mean value = $251 \pm 154 \mu\text{m}$,

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median = 179 μm) had the tendency to be smaller than those from the downwellings (mean value = $289 \pm 116 \mu\text{m}$, median = 274 μm).

There were no significant variations in the average density of plastic particles collected in the sediments (**Figure 16 B and D**), with an average of $0.96 \pm 0.01 \text{ g cm}^{-3}$ calculated for all the plastic particles collected from all of the three examined zones and from both depths.

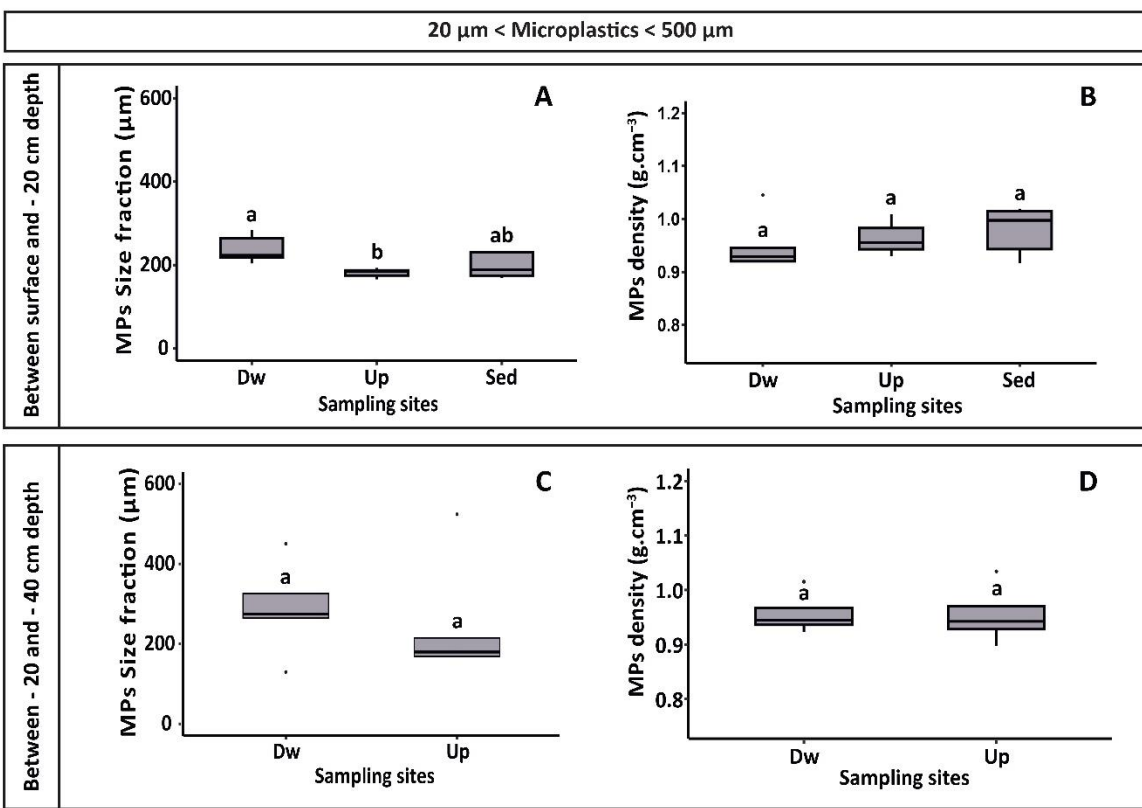


Figure 16 The size fraction (μm) of microplastics extracted at the top and bottom sediment layers (A and C), as well as their average density ($\text{g}\cdot\text{cm}^{-3}$) (B and D) within the three hydrological zones (Dw = downwellings, Up = upwellings, Sed = sedimentation zones). For each treatment, data are presented as means \pm SD ($n = 5$). Different letters indicate significant differences among sampling zones (For parametric test: Tukey test at $p \leq 0.05$, and for non-parametric test: Dunn test at $p \leq 0.05$).

1.5.4. *Polymer identification*

Out of the extracted microplastics, 16 different polymers were identified in the upper sediment layers (from the downwelling, upwelling, and sedimentation areas), and deep sediment layers (downwellings and upwellings) that varied in their concentrations (**Figure 17**). Out of these polymers, polypropylene, polyethylene oxidized, polyethylene, and polyethylene chlorinated

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were the most common polymers found in the top sediment layers of the three zone (**Figure 17 A**). In fact, polypropylene and polyethylene (i.e., polyethylene based materials) are two of the most widely manufactured polymers in the world, and the high relative abundance of these materials found at the investigated area is probably due to the fact that they are heavily used in everyday production and living (Geyer et al., 2017). Nevertheless, the chemical composition analysis indicated that polypropylene was more dominant (more than 50%) in the downwelling and upwelling zones than it was in the sedimentation sites (where polypropylene was less than 20%). The proportion of synthetic rubber detected in this study was twice as high in the sedimentation zones (more than 20%) than it was in the downwelling and upwelling zones (less than 10%). Nevertheless, it is essential to be aware that identifying and quantifying synthetic rubbers (e.g., as particles from automobile tires) in the environment is challenging (Mengistu et al., 2019).

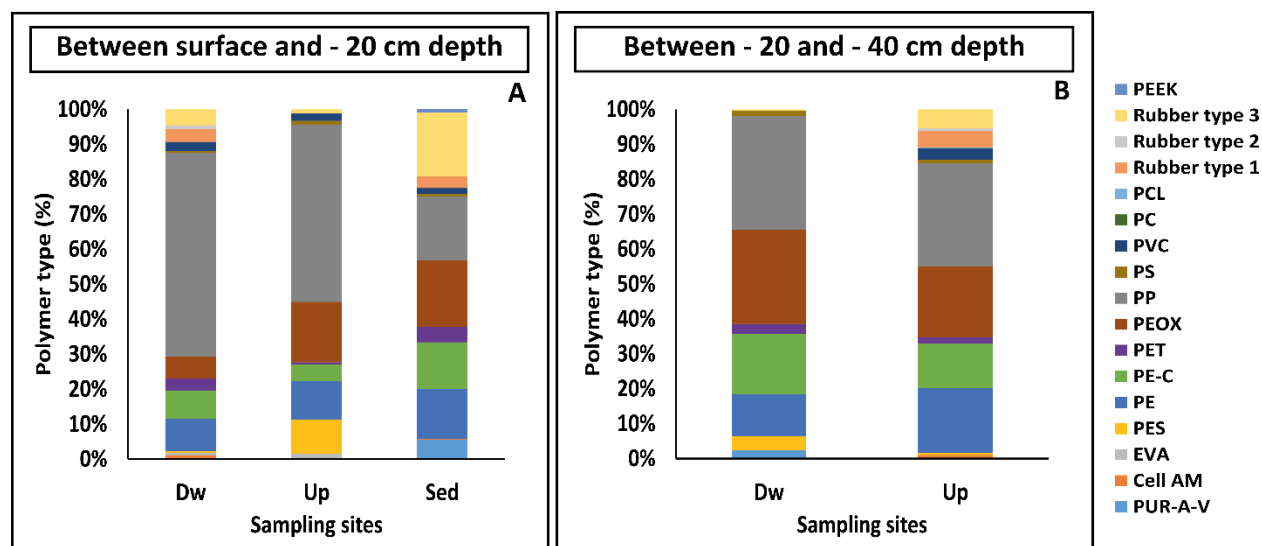


Figure 17 Polymer type compositions of the microplastics extracted from the different hydrological conditions (Dw = downwellings, Up = upwellings, Sed = sedimentation zones), from the first 20 cm layers (A) and the bottom (B) of the streambed sediments. (PUR-A-V = acrylates/polyurethanes/varnish, Cell AM = cellulose artificial modified, EVA = ethylene-vinyl-acetate, PES = polyester, PE = polyethylene, PE-C = polyethylene chlorinated, PET = polyethylene terephthalate, PEOX = polyethylene oxidized, PP = polypropylene, PS = polystyrene, PVC = polyvinylchloride, PC = polycarbonate, PCL = polycaprolactone, Rubber type 1, Rubber type 2, Rubber type 3, PEEK = polyetheretherketone).

Similar to the top sediments, deeper sediments (**Figure 17 B**) at downwelling and upwelling zones revealed the presence of the same four polymers that were prevalent in the top sediments of

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these locations. However, compared to the top sediments, the proportion of polypropylene polymers at the deeper sediments in downwelling and upwelling locations was lower (almost 30%), while the proportions of polyethylene oxidized, polyethylene, and polyethylene chlorinated were at least two times higher.

1.6. Discussion

While microplastics in marine environments have been studied at a greater detail for several decades, the fate and transport of microplastics in freshwater ecosystems is still poorly understood.

Similar to the findings of (Dole-Olivier et al., 2019), the coarse bed river located adjacent to the six gravel bars was characterized by hyporheic exchanges between surface water and ground water forming either downwelling or upwelling hydraulic gradients varying from -37.5% to +35% ([Supplementary Table 5](#)). Whilst upwelling zones were usually located at the apex of each gravel bar, downwelling zones were not always constrained to the heads of the bars and were also present at their tails. In contrast to downwelling zones, upwellings were characterized by high electric conductivity (362 to 418 $\mu\text{S cm}^{-1}$) but lower temperature (9.3 to 17 °C), providing another indicator of upwelling groundwater that is colder in summer than stream water. Yet, high ammonium concentrations were recorded in these cold water patches (**Table 7**). Compared to downwellings, it is likely that the mineralization of organic materials was greater in these cold water patches, where organic nitrogen was converted into ammonium in the presence of microorganisms (Covatti and Grischek, 2021; Krause et al., 2009; Weatherill et al., 2018). Our assumption is in line with previous findings that showed that the accumulation of microplastics can have either a positive or negative effect on sediment nitrification (Seeley et al., 2020; Zhu et al., 2022). Accumulation of microplastics in riverbed sediment can alter sediment physical properties by clogging sediment pores, thereby altering the microbial composition, which in turn impacts riverbank nitrification and decreases ammonium removal (Chen et al., 2022; Cluzard et al., 2015; Shen et al., 2022). In our study, the high ammonium concentration detected in the upwelling zones is associated with high microplastic concentrations (i.e., 20 μm < microplastics <

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500 μm) particularly in the finest sediment fraction of the deep sediment layers (**Figure 14 C**, 1-way ANOVAs, $p = 0.05$).

Only in the fine sediments, microplastic content at – 20 to – 40 cm depths of the upwelling zones were three folds and a half higher than the concentration extracted from the downwellings. Yet, comparable quantities of microplastics were detected at the top sediment layers within these two hydrological exchanges (**Figure 14 A and B**). The average densities (**Figure 16 B and D**) and compositions of polymer type of the upwellings and downwellings (**Figure 17**) were similar, suggesting that probably similar plastic particles derived from the same origin, are transported from surface water and retained in both hyporheic zones. However, our data revealed that the upwellings contained significantly smaller particle size fractions than the downwellings did (**Figure 16 A and C**). This suggests that, during advective transfer, microplastics transported from the surface flow into the streambed sediments within the downwelling zones (Frei et al., 2019) experience physical abrasion in the coarse gravel bars of the streambed caused by the fine grain sizes (Waldschläger and Schüttrumpf, 2019), which facilitates the fragmentation of the plastic particles into smaller size fractions and their remobilization under upwelling fluxes. Earlier studies reported that microplastics exceeding the pore size of the sediment are more susceptible to be trapped and retained in the streambed than the smaller particles (Hoellein et al., 2019; Waldschläger et al., 2020). Hence, via hyporheic porewaters, these smaller particles are transported into the streambed where they remain retained in the sediments until being resuspended back into the water column (Drummond et al., 2018, 2017; Hoellein et al., 2019). In our research, we found that the Bou-Rouch pump was more effective to capture small plastic particles trapped in the finest sediment fraction of the upwellings than the bigger plastic particles retained in the streambed of the downwellings. While porous media like streambed sediments have previously shown that pore size microplastics are mobile (Drummond et al., 2020), the Bou-rouch pumps clearly augment their mobility.

In addition to the size property of microplastics that affect their hydrodynamic regime in the sediment-water interface, the mobility of plastic debris is highly controlled by the hydraulic conditions that influence the suspension, transport, settlement and resuspension of microplastic particles. According to our results, microplastic concentrations in the surface water are lowest in

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regions with highest VHG (i.e., strong downwellings and strong upwellings, **Figure 15 A**) and highest in regions with a low VHG (i.e., weak downwellings and weak upwellings, **Figure 15 B**), thus mimicking the distribution of fine sediment particles (**Figure 15 B**), and similar to that seen by Hoellein et al., (2019). These findings are in accordance with (Zhang et al., 2017) who reports a negative correlation between microplastic abundance and flow velocity in the sediments, indicating that a decrease in river flow velocity promotes the deposition of dense plastic particles. According to their research, when nutrient levels are relatively high, poor hydrodynamic conditions promote the fast formation of biofilm on the surface of plastic debris, which results in biofouling and causes the particles to become denser and to sink before breaking down in the streambed sediment (Fazey and Ryan, 2016; Kooi et al., 2017).

Additionally, the high microplastic concentrations found in the dry mass of total sediment of the first 20 cm layers of the sedimentation locations (**Figure 14 B**) could be explained by the fact that, like all fine-grain sediments, fine microplastics favor low-energy environments (Enders et al., 2019) and that, at small size scales, they flocculate and settle with natural fine-grain suspended sediments in riverine systems (Laursen et al., 2023). Studies have revealed that deposits of microplastics are more likely to occur in regions rich in both organic matter and fine sediment fractions (Ding et al., 2019; Paduani, 2020; Strand et al., 2013). To illustrate, the five sedimentation regions included in this research were characterized by streambed sediments that were mostly composed of fine sediment particles (**Table 7**) and abundant in vegetation. In comparison to the sites of hyporheic exchanges, these two key variables may stimulate heteroaggregation with plastic particles and trigger their gravitational settlings in sites of lower flow velocity (Frei et al., 2019; Yan et al., 2021). Moreover, sedimentation regions are considered to be sites where, during high flow events, microplastics are re-mobilized, transported from the streambed sediments, and then settle in vegetated and low-energy sites further downstream (Hurley et al., 2018). Therefore, compared to locations with likely more substantial fluxes across sediment-water interfaces, larger concentrations of microplastic are anticipated in these areas.

Our study revealed a high relative abundance of polypropylene and polyethylene based materials in comparison to other synthetic polymer types. This was expected given our previous work demonstrating that these polymers are dominant in this river catchment. Polypropylene is

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considered as one of the most widely used hard plastics produced by the industries situated upstream of the Chazey meander (Dendievel et al., 2023). Nevertheless, sedimentation areas were distinguished from the zones of hyporeic exchanges by entrapping higher concentrations of synthetic rubbers. It is possible that these synthetic polymers are carried to the deposition areas by faster fluxes during flood events. Future work should aim to determine how the transport behavior of different polymer types are affected hydraulic conditions and sediment properties.

1.7. Conclusion

Our findings suggest that riverbed sediments act as temporal sinks for fine sediments as well as microplastics that follow similar transport and deposition mechanisms. Additionally, similar to the behavior of fine sediments, the retention and remobilization of plastic particles in streambeds seem affected by hydrological exchanges at the sediment-water interface. Contrary to our expectations, microplastic concentrations in downwelling zones were not significantly higher than in upwelling zones. Following the results of the size distribution of microplastic which revealed smaller size fractions in upwelling locations compared to downwelling zones, we speculate that during their flow in the pore water of the streambed, plastic particles are being degraded by the tiny sediment grains and other form of physical, chemical and biological degradation. Degradation has the potential to increase mobility of the smaller particles in the pore water under low upwelling fluxes rendering them easily sampled using a pump.

We recommend that future research should continue to elaborate the following:

1. To expand our knowledge on hydro-geomorphological conditions and microplastics contamination in streambed sediments, further studies in more contrasting sedimentological settings are required (e.g., considering sandy rivers).
2. Since they are physically abraded in streambed sediments, data on microplastics residing in the pore water of hyporheic zones is also required to determine if they could potentially pose a possible environmental threat to benthic organisms, groundwaters, and freshwater food webs; thus, necessitating the need for appropriate mitigation measures.

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1.10. Supplementary materials

Supplementary Table 4 Polymer types identified by siMPLe software and their densities in g. cm⁻³.

| Polymer type | Density (g cm ⁻³) | Density (g cm ⁻³) |
|--|----------------------------------|--|
| acrylates/polyurethanes/varnish | 1.28/1.20/ 0.99 | 1.16 (i.e., average of acrylates/polyurethanes /varnish) |
| cellulose artfical modified | 1.28 | 1.28 |
| ethylene-vinyl-acetate | 0.95 | 0.95 |
| polyester | 1.38 | 1.38 |
| polyethylene | 0.97 | 0.97 |
| polyethylene. chlorinated | 0.93 | 0.93 |
| polyethylene terephthalate | 1.38 | 1.38 |
| polyetyhlene oxidized | 0.94 | 0.94 |
| polypropylene | 0.92 | 0.92 |
| polystyrene | 1.05 | 1.05 |
| polyvinylchloride | 1.41 | 1.41 |
| polycarbonate | 1.2 | 1.2 |
| polycaprolactone | 1.145 | 1.145 |
| polyetheretherketone | 1.32 | 1.32 |
| rubber type 1 (isoprene (2-methyl-1 3- butadiene)) | 0.681 | 0.681 |
| rubber type 2 (1,3-butadiene) | 0.615 | 0.615 |
| rubber type 3 (chloroprene (2-chloro- 1,3-butadiene)) | 0.958 | 0.958 |

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Supplementary Table 5 The latitude and longitude of the sampling sites with the percentage (%) of vertical hydraulic gradient (VHG) measured from each location.

| Locations with surface-groundwater exchanges | Name of each sampling location | Latitude (DD, °N) | Longitude (DD, °E) | VHG (%) |
|--|--------------------------------|-------------------|--------------------|---------|
| Upwelling | Up1 | 45.8981 | 5.246317 | 35 |
| Upwelling | Up2 | 45.8884 | 5.249133 | 5 |
| Upwelling | Up3 | 45.887267 | 5.24375 | 5 |
| Upwelling | Up4 | 45.8888 | 5.2391 | 5 |
| Upwelling | Up5 | 45.95945 | 5.25104 | 16.5 |
| Downwelling | Dw1 | 45.912117 | 5.241517 | -16.7 |
| Downwelling | Dw2 | 45.9175 | 5.241767 | -10 |
| Downwelling | Dw3 | 45.904783 | 5.2344 | -12.5 |
| Downwelling | Dw4 | 45.908967 | 5.234333 | -15 |
| Downwelling | Dw5 | 45.96206 | 5.25345 | -37.5 |
| Sedimentation | Sed1 | 45.959989 | 5.250228 | |
| Sedimentation | Sed2 | 45.912522 | 5.242651 | |
| Sedimentation | Sed3 | 45.89139 | 5.252222 | |
| Sedimentation | Sed4 | 45.888114 | 5.247467 | |
| Sedimentation | Sed5 | 45.887647 | 5.240842 | |

Chapter IV

Microplastics impact on freshwater ecosystem functioning

Microplastics impact on ecosystem functioning at the sediment–water interface:

After studying the function of streambed sediments in temporarily storing microplastics and acting as a source of exposure for benthic organisms in chapter III, the function of ecosystem engineers (i.e., bioturbators) that reside at this sediment-water interface was explored in chapter IV. In this following chapter, the effects of sediment contaminated with microplastics on the survival, physiology, and bioturbation activity of freshwater deposit-feeding bioturbators were investigated in a controlled laboratory setting.

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1. Microplastics in freshwater sediments impact the role of a main bioturbator in ecosystem functioning

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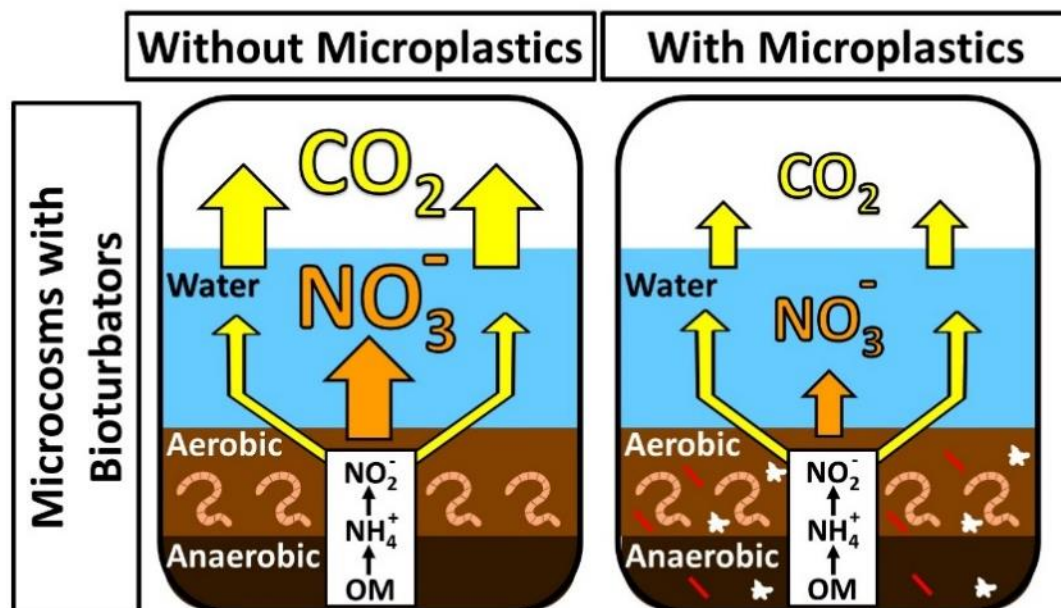
1.1. Abstract

While microplastic transport, fate and effects have been a focus of studies globally, the consequences of their presence on ecosystem functioning have not received the same attention. With increasing evidence of the accumulation of microplastics at sediment-water interfaces there is a need to assess their impacts on ecosystem engineers, also known as bioturbators, which have direct and indirect effects on ecosystem health. This study investigated the impact of microplastics on the bioturbator *Tubifex tubifex* alongside any effects on the biogeochemical processes at the sediment-water interface. Bioturbators were exposed to four sediment microplastic concentrations: 0, 700, 7000 and 70000 particles kg⁻¹ sediment dry weight. Though no mortality was present, a significant response to oxidative stress was detected in *tubificid* worms after exposure to medium microplastic concentration (7000 particles kg⁻¹ sediment dry weight). This was accompanied by a reduction in worm bioturbation activities assessed by their ability to rework sediment and to stimulate exchange water fluxes at the sediment-water interface. Consequently, the contributions of tubificid worms on organic matter mineralization and nutrient fluxes were significantly reduced in the presence of microplastics. This study demonstrated that environmentally realistic microplastic concentrations had impact on

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biogeochemical processes at the sediment-water interface by reducing the bioturbation activities of tubificid worms.

1.2. Graphical abstract



1.3. Keywords

Microcosms, tubificid worms, exposure, sediment-water interface, biogeochemical fluxes.

1.4. SYNOPSIS

Lethal and sub-lethal endpoints for microplastics toxicity tests should not cease at the level of organisms, they must include the impacts of contaminants on ecosystem functioning.

1.5. Introduction

Despite the global attempts to manage plastic waste, humanity is still far from confronting the plastic crisis (Shen et al., 2020a, 2020b). Even with efforts to reduce plastic pollution, the amount of mismanaged plastic waste expected to be released in aquatic ecosystems by 2030 may reach 53 million metric tons (Mt), twice the amount estimated for 2016 (Borrelle et al., 2020). Microplastics (plastic particles ≤ 5 mm in size)(Arthur et al., 2009) are part of this plastic waste, and are transported to the ocean via coastal runoff and river discharge (Kooi et al., 2018; Malli et al., 2022). Once in the river environment, depending on their density (Drummond et al., 2022),

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microplastics can float in the water column, or sink into sediment beds (Kaiser et al., 2017; Sandgaard et al., 2023; Waldschläger et al., 2020) where they can be temporary trapped (Horton and Dixon, 2018). Microplastic concentrations estimated in European riverbed sediments range from 18 up to 75000 particles kg⁻¹ dry weight (Hurley et al., 2018; Rodrigues et al., 2018), with the majority of particles detected being polyethylene, polypropylene, and polystyrene polymer types (Klein et al., 2015) in a wide range of sizes and shapes (fragments, fibers, and spherical forms) (Horton et al., 2017; Hurley et al., 2018; Klein et al., 2015).

The sediment-water interface is a hotspot of biological activity in streams and rivers, which play a fundamental role in ecosystem functioning through biogeochemical processes involved in carbon (C) and nitrogen (N) cycles (Bardini et al., 2012; Krause et al., 2022, 2017). Although these biogeochemical processes are driven mainly by microbial activities (Arora-Williams et al., 2018; Krause et al., 2022, 2011), benthic invertebrates acting as bioturbators can significantly influence these microbial processes (Kristensen et al., 2012) by sediment reworking, biogenic structure building (e.g., burrows), and bioirrigation (i.e., the action of benthic organisms flushing their burrows with overlying water). These processes can modify pore water chemistry, which will in turn have consequences on microbial communities and associated biogeochemical processes (Cariou et al., 2021; Svensson and Leonardson, 1996; Svensson et al., 2001). Chironomid larvae (Diptera, Chironomidae) and tubificid worms (Oligochaeta, Tubificidae) have been identified as important bioturbators in freshwater ecosystems (Baranov et al., 2016; Gautreau et al., 2020; Pignoret et al., 2016). Despite this recognized importance of bioturbators on organic matter processing and nutrient recycling at sediment-water interfaces of freshwater ecosystems, the consequences of microplastics exposure of sediments on these bioturbation-driven processes remain unexplored.

Bioturbators like tubificid worms are deposit feeders that can be exposed to microplastics trapped in river bed sediments (Frank et al., 2023; Hurley et al., 2017; Kukkola et al., 2021; Nel et al., 2018; Scherer et al., 2017; Van Cauwenberghe et al., 2015). Yet, it is still unclear how this exposure may impact their physiology, and activities in sediments. Although there has been little research on this topic, a reduction in the survival rates of deposit feeding organisms like *Chironomus tepperi* (Ziajahromi et al., 2018) and *Caenorhabditis elegans* (Lei et al., 2018) exposed

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to microplastics has been previously reported. In contrast, other exposure studies did not find any adverse effects on growth and survival (Redondo-Hasselerharm et al., 2018; Scopetani et al., 2020; Urban-Malinga et al., 2022). In aquatic benthic organisms, it has been shown that microplastics could significantly decrease energy reserves (Bour et al., 2018; Wright et al., 2013), increase lipid peroxidation (Oliveira et al., 2018), trigger oxidative enzymes (Cole et al., 2020), and generate neurotoxicity (Urban-Malinga et al., 2022). However, it has also been reported that exposing deposit feeders to microplastics did not affect their energy storage (Van Cauwenberghe et al., 2015) nor cause any oxidative damage (Scopetani et al., 2020). The feeding activity of benthic macro-organisms could also be reduced by the presence of microplastics in sediments (Foley et al., 2018), although this is also not universally accepted and may vary with respect to microplastic concentration, size, shape, polymer type, exposure time and species sensitivity itself (Bour et al., 2018; Van Cauwenberghe et al., 2015).

The present study aims to fill this gap by investigating the impacts microplastic exposure may have on the tubificid worm *Tubifex tubifex* and on the ecosystem services they offer, by assessing changes to survival, physiological state and bioturbation activity (Kang et al., 2016; Mermillod-Blondin et al., 2018; J. Yang et al., 2020). Laboratory experiments were conducted in microcosms at four sediment microplastic concentrations. Health (survival, energetic reserves and oxidative stress) of tubificid worms and their potential consequences on bioturbation activities (sediment reworking and bioirrigation rate) and ecological processes (nutrient and CO₂ fluxes) at the sediment-water interface were assessed.

1.6. Materials and methods

1.6.1. Collection and characterization of sediments used

Sediments were collected using a metal shovel and metal buckets from the streambed of the Lone des Pêcheurs, a dead arm of the Rhône river in the South-east of France (45°48'41.1"N, 5°6'1.6"E). Collected sediments were sieved (< 3.6 mm) to exclude any large debris and homogenized in a large glass container using a metal scoop. Sediments were then stored at – 20°C to kill any macro-organisms present. In addition, sediment samples were analyzed to determine their particle size distribution, total organic carbon (TOC) and total nitrogen (TN)

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content. Particle size distribution was obtained using laser granulometer (Mastersizer, 2000, Malvern Instrument) as reported by Gette-Bouvarot et al. (2014) (Gette-Bouvarot et al., 2014). Three measurements (i.e. three replicates) were carried out for the sediment after ultrasound treatment (50–60 Hz, 1 min) to eliminate non-stable particle aggregates (Gette-Bouvarot et al., 2014). TOC and TN contents were determined by high-temperature *in situ* combustion on pre-acidified (i.e., HCL 2 mol/L) dry samples (60°C, 48hr) for three replicates of 15 mg each, using an elemental analyzer (FlashEA, Thermo Electron Corporation). The microplastic content of the sediment was assessed following an approach modified from those of Frei et al. (2019) (Frei et al., 2019). Briefly, after drying at 50 °C for 24 hours, a sediment sample of 50 g was suspended in zinc chloride (ZnCl₂) solution (density 1.7 g cm⁻³) for 24 hours to collect microplastic particles and organic components which were later degraded with 30% H₂O₂ in the presence of 0.05 M Fe²⁺ (i.e., aqueous). The remaining material was then stained with Nile Red solvent (Maes et al., 2017; Nel et al., 2021), filtered, and microplastics > 20 µm sizes were quantified under Nikon SMZ1270 fluorescent stereo microscope (**Table 8**).

Table 8 Physical characteristics of the sediment (mean ± SD, n = 3).

| Measurements | | |
|---|---|------------|
| Grain size distribution (%) | 64 µm < Sand < 2 mm | 35.9 ± 5.8 |
| | 2 µm < Silt < 64 µm | 60.2 ± 2.9 |
| | Clay < 2 µm | 3.9 ± 0.3 |
| Organic matter composition (% sediment dry mass) | Total organic carbon | 2.4 ± 0.10 |
| | Total nitrogen | 0.2 ± 0.02 |
| Microplastic concentration | 3140 particles / Kg dry weight of sediments | |

1.6.2. Microplastics preparation

A heterogeneous mixture of two types of microplastic fragments (polystyrene (PS) and polyamide (PA)) and one type of microplastic fibers (PA) was used in this study. The PS particles were made-in-house by cutting cuvettes (VWR) into small pieces and then ground in a Retsch ball mill MM 400 at 30 Hz for 30 s with liquid nitrogen. The resulting powder was sieved to exclude polystyrene

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fragments > 1000 μm using a stainless steel sieve mesh. To obtain multiple shapes of PA particles, fibers (L = 500 μm , W = 15 μm) were obtained from Flock Depot (Stuttgart, Germany) and pellets were sourced from Resinex Ltd (High Wycombe, United Kingdom). The pellets were subsequently frozen at -80°C for 72hr and ground using a Fritsch Pulverisette 0 ball mill with liquid nitrogen for 20 minutes. The resulting PA powder was size fractionated by stainless steel sieves to exclude PA fragments > 1000 μm . Prior to their use in the controlled experiments, the particle size distribution of PS and PA particles (i.e., both fragments and fibers) was measured by laser granulometry with the same method and equipment than those used previously to measure sediment grain size distribution ([Supplementary Figure 6](#)). The size and morphological features of microplastic particles were investigated with a fluorescent stereo microscope (Nikon SMZ1270) without fluorescence under the brightfield mode. Overall, the microplastic particles used in the experiment presented a wide variety of shapes and sizes varying from 15 to 1500 μm ([Supplementary Figure 7](#)), including particles that could be easily ingested by tubificid worms (< 63 μm) (Hurley et al., 2017).

1.6.3. Bioturbation model organisms

The tubificid worm, *Tubifex tubifex* was chosen to investigate the impacts of microplastics on freshwater bioturbators as this species has a key role in the biogeochemistry and ecology of freshwater benthic habitats (Mermillod-Blondin et al., 2018), and due to its wide use in measuring the ecotoxicity of sediments (Hurley et al., 2017; Paris-Palacios et al., 2010). Tubificid worms were bought from a commercial breeder (GREBIL & Fils, Arry, France) and acclimatized to experimental conditions (i.e., temperature of 20°C , sediments collected from Lone des Pêcheurs, aerated synthetic water, and under a 16:8 h light: dark cycle) for two weeks prior to the start of the experiment.

1.6.4. Experimental design and preparation

A complete randomized block design was used to test the impacts of the addition of four microplastic concentrations (0 particles/kg sediment dry weight (DW) (control); 700 particles/kg sediment DW (low); 7000 particles/kg sediment DW (medium); 70000 particles/kg sediment DW (high)) on microcosms with or without tubificid worms. By measuring the interaction between microplastic treatments and worm presence, this experimental design aimed to evaluate

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whether the presence of microplastics impacted the role of tubificid worms in sediments compared with the direct impact of microplastics on microorganisms (treatments without worms). The four microplastic treatments were chosen based on reports of actual environmental realistic concentrations reported from Europe to China (Huang et al., 2021; Hurley et al., 2018; Klein et al., 2015). These investigations indicated the presence of hundreds to thousands of plastic particles per kilogram of dry sediment, with the greatest concentration of microplastics discovered in the riverbeds of the Irwell River, with up to 75,000 particles per kilogram of dry sediment (Hurley et al., 2018).

A total of eight treatments (4 microplastic concentrations * 2 worm conditions) was tested with five replicates per treatment ($N_{\text{total}} = 40$). Each replicate was made up of a 2 L glass bottle (internal diameter: 13 cm, height: 25 cm) filled with 8 cm of sediment (i.e., 0.738 kg DW) (*Supplementary Figure 8*) previously prepared by mixing microplastic particles with sediments to obtain the four desired concentrations. The mixing process took place one week before filling the 2 L glass bottles by using one glass container (L = 50 cm, W = 20 cm, H = 25 cm) per microplastic treatments (4 glass containers were used for the initial preparation of the 4 tested microplastic concentrations, *Supplementary Table 6*). After a week of homogeneous mixing of sediment with the desired concentration of microplastics (for each treatment, microplastics and sediments were thoroughly mixed in a glass container for 30 min every day during 1 week using a stainless steel spoon), sediments were transferred to 2 L glass bottles (i.e. microcosms), with 10 glass bottles per microplastic treatment. By homogeneously mixing sediment and microplastics, we used a simplified distribution of microplastics in sediments compared with heterogeneous distributions of microplastics in freshwater environments (Drummond et al., 2022; Frei et al., 2019), but it was the best way to control the exposure of tubificid worms to microplastic particles. After sediment addition, 10 cm of overlying synthetic freshwater ($96 \text{ mg L}^{-1} \text{ NaHCO}_3$, $39.4 \text{ mg L}^{-1} \text{ CaSO}_4 \cdot 2\text{H}_2\text{O}$, $60 \text{ mg L}^{-1} \text{ MgSO}_4 \cdot 7\text{H}_2\text{O}$, $4 \text{ mg L}^{-1} \text{ KCl}$, and $6.4 \text{ mg L}^{-1} (\text{CH}_3\text{CO}_2)_2\text{Ca} \cdot \text{H}_2\text{O}$; pH = 7.5) (Weber, 1991) was added to each microcosm. Synthetic water was gently added to each microcosm to avoid any sediment resuspension. Microcosms were aerated to maintain a dissolved oxygen (DO) concentration between 7.5 and 8.5 mg L^{-1} in the water column throughout the full duration of the experiment. The experiment was conducted at a constant room temperature of 20°C under

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a 16:8 h light: dark cycle. After microcosm preparation, 180 individuals of tubificid worms having lengths ranging between 1 and 3 cm were introduced into each microcosm dedicated to treatments with worms (half of the microcosms), representing a density of 13,274 individuals per m² commonly observed in the braided arms of the Rhône river (Martinet, 1993).

Then, the experiment started for 77 days, an experimental duration which was long enough to observe long-term impacts on tubificid worms without having a depletion in sedimentary organic matter that could impair food availability for tubificid worms.

During the last four weeks of the experiment, the bioturbation activities (surface sediment reworking and bioirrigation rate) of worms and biogeochemical processes (CO₂, CH₄, and nutrient fluxes) were assessed to evaluate the influences of microplastic contaminated sediments on individuals and associated ecosystem processes. At the end of the experiment (i.e., day 77), microcosms were dismantled and sediments were sieved on a 250 µm mesh sieve to recover tubificid worms. After collection, tubificid worms were counted to determine their survival rate in each microplastic treatment. Surviving worms were collected with each worm being examined to see whether it moves or not. Then worms from each microcosm were separated in two groups: a first group of around 130 individuals was used to evaluate the physiological state of worms and a second group of around 50 individuals was used to determine the quantity of microplastics ingested by worms. In microcosms where less than 180 individuals were alive, individuals were divided into two groups while maintaining the same ratio of division.

To avoid contamination with microplastics from laboratory equipment, we used glass material for handling of particles, manipulating tubificid worms and for exposure experiments. Materials were covered to reduce airborne contamination.

1.6.5. Exposure impact analysis

Physiological analyses

The first group of collected worms (130 individuals per microcosm) were kept for 48hr in glass beakers filled with 0.7 µm filtered synthetic water for depuration. Afterwards, tubificid worms were freeze-dried and weighed collectively. These freeze-dried 130 individuals were then divided randomly into four small subgroups, each subgroup being used to perform a given physiological

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analysis (2 analyses associated with energy body stores and 2 analyses associated with oxidative stress). Energy body stores were determined in the whole animal by extracting and measuring the amount of triglycerides (Sigma Aldrich, T2449 and F6428) and glycogen (Sigma Aldrich, G3293) as described in Hervant et al. (1999) and Salin et al. (2010) (Hervant et al., 1999; Salin et al., 2010). Two markers of oxidative stress were measured: malondialdehyde (MDA) content which is a biomarker of lipid peroxidation (LPO) and the antioxidant enzyme superoxide dismutase (SOD) activity which is induced by the formation of reactive oxygen species (ROS). The thiobarbituric acid (TBARS) assay (Cayman chemicals, 10009050) was used to determine the MDA content (Lawniczak et al., 2013) and the SOD activity (Cayman chemicals, 706002) was estimated according to Flohe (1984) and Lawniczak et al. (2013) (Flohe, 1984; Lawniczak et al., 2013). MDA contents were expressed in nanomoles (nmol) of MDA mg⁻¹ dry mass, whereas SOD catalytic activities were expressed as U g⁻¹ dry mass, where one unit was defined as the quantity of enzyme that reduced 50% of oxidized cytochrome c (which is oxidized under superoxide production).

Microplastics ingestion analysis

The second group of collected worms (50 individuals combined per microcosm) were cleaned from external debris using vacuum filtered ultrapure (VFUP) water (i.e., filtered through Whatman GF/F filters (0.7 µm)) without any depuration phase. Then, microplastics were extracted from tubificid worms according to Hurley et al. (2017) with some modifications (Hurley et al., 2017). Fresh tubificid worms were weighed and placed into glass tubes previously washed three times with VFUP water. Worms were digested with 30% H₂O₂ in the presence of 0.05 M Fe²⁺ (i.e., aqueous) catalyst solution at an ambient room temperature for 24hr. After digestion, samples were washed with VFUP water then filtered through Whatman GF/F filters (0.7 µm). The number of microplastics were determined using a Nikon SMZ1270 fluorescent stereo microscope after staining with Nile Red of 5 µm mL⁻¹ as final concentration (Maes et al., 2017; Nel et al., 2021). The stereo microscope was fitted with Intensilight C-HGFIL Lamp (130w) with a 0.75 X objective lens. All measurements were done at an overall magnification of 15 X. Three procedural blanks were prepared simultaneously. These blanks were assumed to indicate background microplastic contamination, and as such, blank values were subtracted from the final results.

Sediment reworking analysis

As microplastic contamination has been shown to reduce the egestion of fecal pellets by the marine worm *A. marina* (Green et al., 2016), we also expected that microplastic exposure could reduce the production of fecal pellets at the sediment surface by tubificid worms. To assess this potential effect, the impact of microplastic exposure was measured on the surface sediment reworking (SSR) process induced by tubificid worms during 5 days in the last week of the experiment (from day 70 to day 74, [Supplementary Figure 9](#)) using the method of De Nadaï-Monoury et al. (2013) (De Nadaï-Monoury et al., 2013). For each microcosm, a 2 mm thick layer of a particulate tracer (white sand of Fontainebleau) was uniformly distributed at the surface of the sediment seven days before the end of the experiment. Translocation of the tracer due to the fecal deposition by tubificid worms was monitored by taking pictures every 12 hours for five days. Pictures were analyzed using ImageJ software (Schneider et al., 2012). Automatic pixel counts were performed based on colors (sandy tracer was white whereas fecal pellets appeared in dark, [Supplementary Figure 10](#)) to estimate the surface covered by tracer. The remaining area covered by fecal pellets was calculated as the difference between total surface area of microcosms minus surface occupied by the tracer. SSR rate was calculated and expressed in % of area recovered by fecal pellets per hour.

Bioirrigation activity

A dissolved conservative tracer (i.e., KCl) was added to the water column (as the methodology used by Mermillod-Blondin et al. (2004) (Mermillod-Blondin et al., 2004) with Br⁻ as conservative tracer) to quantify water fluxes from the water column to sediment. This measurement was done for 1 day, two weeks before the end of the experiment (from day 63 to day 64, [Supplementary Figure 9](#)), by monitoring the concentration shift over time of the dissolved tracer. More precisely, for each microcosm, water was replaced with synthetic freshwater enriched with KCl to obtain a concentration of 37 mg L⁻¹ of Cl⁻, a concentration which was not a stress for *Tubifex tubifex* (Soucek et al., 2011), and commonly measured in freshwater environments (Voisin et al., 2018). Using acid-washed 100 ml syringes, water samples were collected immediately after water column replacement and 1 day after for each microcosm. Water samples were filtered through Whatman GF/F filters (0.7 µm) and Cl⁻ concentration were analyzed using a sequential analyzer

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based on colorimetric methods (Smartchem 200, AMS Alliance, France). The decrease in Cl^- concentrations between the two days was used to evaluate the water fluxes at the sediment-water interface in each microcosm. Based on changes in Cl^- concentrations and the water volume in microcosms, these fluxes were expressed in L of water exchanged per day and m^{-2} .

Biogeochemical analysis

The influence of microplastic contamination on biogeochemical processes (organic matter mineralization and nutrient cycling) was estimated by measuring the CO_2 and CH_4 fluxes and the fluxes of nutrients (N-NH_4^+ , N-NO_3^- , N-NO_2^- and P-PO_4^{3-}) at the sediment-water interface for each microcosm (Mermillod-Blondin et al., 2005; Pigneret et al., 2016).

CO_2 and CH_4 fluxes were measured for 5 days according to Pigneret et al. (2016) (Pigneret et al., 2016), three weeks before the end of the experiment (from day 56 to day 60, [Supplementary Figure 9](#)). Each microcosm was tightly sealed with a lid connected to a pump and a greenhouse gas analyzer (G2201i PICARRO). CO_2 and CH_4 fluxes were measured by monitoring the increase in concentrations every 2 hr during 10 hr at 20 °C for each microcosm. Then, CO_2 and CH_4 fluxes were expressed in mmol per hour and m^2 of sediment-water interface.

For nutrient fluxes, 2/3 of the water column was replaced in each microcosm four weeks before the end of the experiment (from day 49 to day 53, [Supplementary Figure 9](#)). For five days, water samples (100 ml) were collected in the water column of each microcosm at daily intervals. Water samples were taken using acid-washed 100 ml syringes, filtered through Whatman GF/F filters (0.7 μm), and analyzed for nutrient concentrations within 24hr using a sequential analyzer based on colorimetric methods (Smartchem 200, AMS Alliance, France). The fluxes of N-NH_4^+ , N-NO_3^- , N-NO_2^- and P-PO_4^{3-} at the sediment-water interface were calculated from the linear changes over time of the concentration of each nutrient in the water column. Then, nutrient fluxes were expressed in mg per day and m^2 of sediment-water interface. Positive fluxes indicated an efflux of nutrients from sediments to the water column whereas negative fluxes indicated an influx of nutrients from the water column to sediments.

1.6.6. Statistical Analyses

For the three physiological variables measured on tubificid worms (glycogen content, triglyceride content, SOD activity), the influence of microplastic treatments (control, low, medium, and high) was tested using one-way analyses of variance (1-way ANOVAs) with microplastic concentrations as the fixed factor. A one-way ANOVA was also used to test the influence of microplastic treatments on the surface sediment reworking rate due to tubificid worms. For chloride, nutrient fluxes, CO₂ and CH₄ fluxes, the effects of microplastic treatments, tubificid worm treatment and their interactions were tested using two-way analyses of variance (2-ways ANOVA) with microplastic concentrations and tubificid worms as fixed factors. When 2-ways ANOVA revealed significant differences among treatments (i.e., $p < 0.05$), Tukey's post hoc tests were performed to evaluate which treatments significantly differed. For all variables, the normality and the homoscedasticity of the residues were verified using the Shapiro-Wilk's test and the Bartlett's test, respectively. As for survival rates and MDA contents the requirements for normality and homoscedasticity were not met, non-parametric Kruskal-Wallis test were performed. All statistical analyses were done with the RStudio software ("R Development Core Team, 2018," 2018).

1.7. Results and discussion

1.7.1. Survival and physiology of *Tubifex tubifex*

After 77 days of exposure, the survival rates of tubificid worms were higher than 75% in all microplastic treatments (**Figure 18 A**). Survival rate was not significantly affected by microplastic concentrations (Kruskal–Wallis test, $H(3) = 3.8$, $p = 0.28$) although it tended to decrease for individuals exposed to the medium microplastic concentration in comparison with the control treatment. Interestingly, this trend was associated with a significant increase in SOD activity in tubificid worms exposed to the medium microplastic concentrations (**Figure 18 B**, 1-way ANOVA, $p < 0.05$) with a mean value of $12.8 \pm 6.2 \text{ U g}^{-1}$ dry mass which was 16-fold higher than in the control treatment ($0.8 \pm 1.9 \text{ U g}^{-1}$ dry mass). These differences in SOD activity among microplastic treatments were not associated with significant changes in MDA concentrations among tubificid worms exposed to different microplastic treatments (**Figure 18 C**, Kruskal–Wallis test, $H(3) = 2.49$, $p = 0.48$). Observed increase in SOD activity showed that tubificid worms exposed to

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microplastics produced antioxidant defense enzymes to catalyze the dismutation of superoxide into molecular oxygen and hydrogen peroxide (Piddington et al., 2001; Pignoret et al., 2016). Thus, the physiology of tubificid worms was influenced by reactive oxygen species (ROS) generated by microplastic exposure, especially for the medium microplastic concentration. In general, the presence of ROS causes lipid peroxidation (LPO) damage that leads to an increase in MDA which is a by-product of LPO (W. Yang et al., 2020). Nevertheless, the lack of significant increase in MDA concentrations in tubificid worms despite an increased production of SOD enzymes indicated that tubificid worms succeeded in neutralizing both ROS production and oxidative damage to maintain their homeostasis (a mechanism described by Trestrail et al. (2020)) (Trestrail et al., 2020). This mechanism has never been detected in invertebrates but has been already observed in the freshwater fish *Oreochromis niloticus* by Ding et al. (2018) (Ding et al., 2018). These authors found that the presence of polystyrene microplastics significantly increased SOD activity in the fish without affecting its MDA content (Ding et al., 2018). Thus, in the present experiment, it is likely that oxidative stress provoked by microplastic exposure was efficiently managed by antioxidative defenses of tubificid worms, preventing cellular damages caused by high lipid peroxidation levels (Trestrail et al., 2020).

In addition, the presence of microplastics at different concentrations did not significantly affect energy reserves in tubificid worms (**Figure 18** D, E, 1-way ANOVAs, $p = 0.2$ for glycogen and $p = 0.1$ for triglyceride concentration). These findings are in agreement with Van Cauwenberghe et al. (2015) who observed that polystyrene microspheres had no significant impact on the energy reserves of the lugworm *Arenicola marina* (deposit feeder) or the blue mussel *Mytilus edulis* (filter feeder) (Van Cauwenberghe et al., 2015). Theoretically, the antioxidative defenses activated in tubificid worms when exposed to microplastics should have generated a significant consumption of energy reserves, leading to lower stocks of glycogen and triglycerides in worms exposed to medium microplastic concentrations (Lu et al., 2016; Xia et al., 2020). However, the present study indicated that tubificid worms did not rely on their energy stores to manage oxidative stress induced by microplastics exposure.

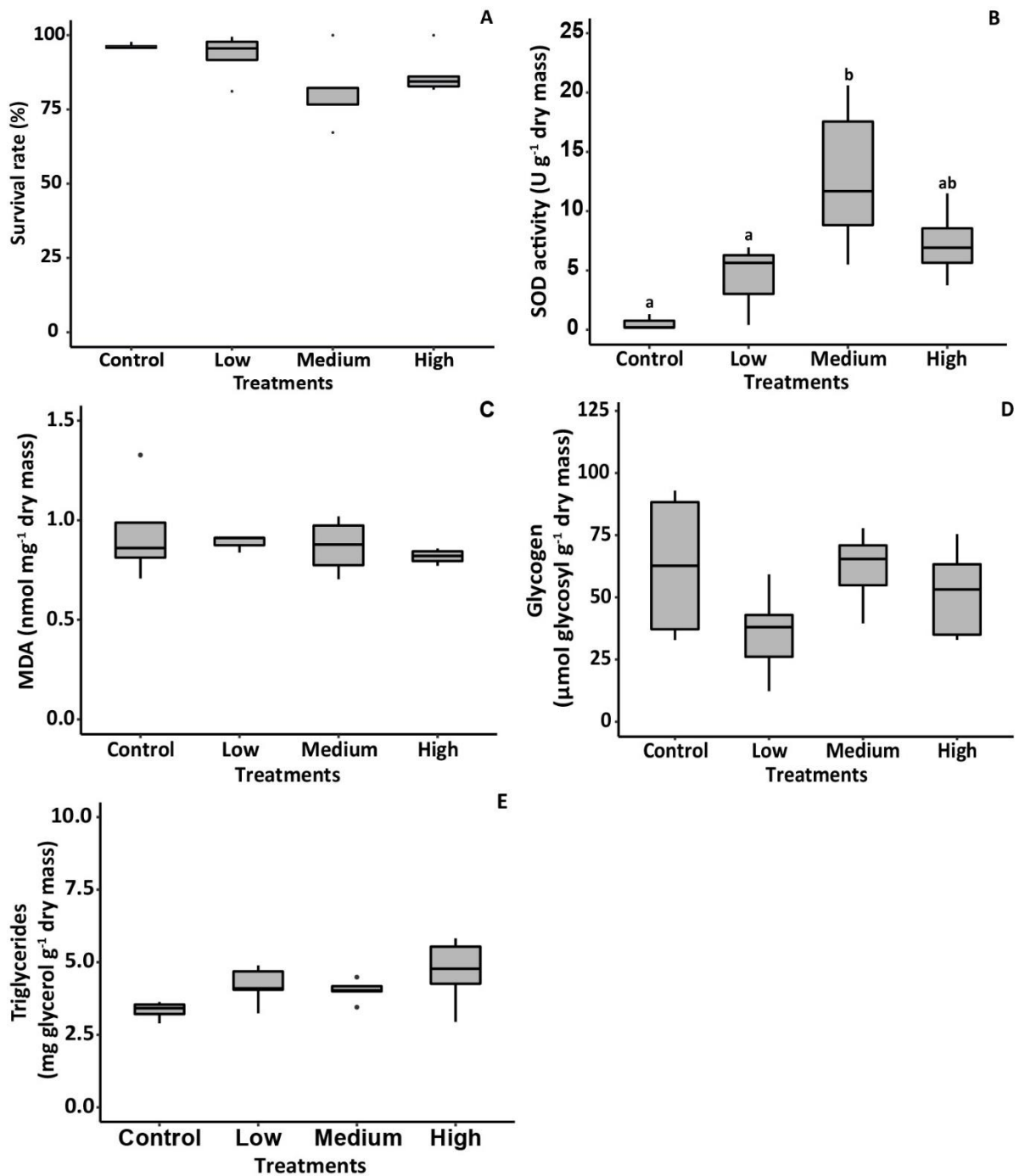


Figure 18 Survival rates (A), superoxide dismutase activity (B), malondialdehyde concentration (C), concentration of glycogen (D), and triglyceride (E) of *Tubifex tubifex* after seventy-seven days of microplastic exposure in sediments. For each treatment, data are presented as means \pm S.D. ($n = 5$). Different letters indicate significant differences among microplastic treatments (Tukey test at $p \leq 0.05$). The absence of letters indicates no significant difference among treatments (Tukey test at $p \geq 0.05$).

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1.7.2. Ingestion of microplastics

Although the microplastic mixture contained particles that could be easily ingested by tubificid worms (< 63 µm) (Hurley et al., 2017) and despite the presence of high microplastic concentrations in sediments (up to 70000 particles/kg DW), no significant numbers of microplastic particles (neither fragments nor fibres) were detected by fluorescence microscopy in the 50 organisms digested with Fenton reagent at the end of the experiment ([Supplementary Table 7](#)). This lack of ingested microplastics could partly explain the lack of observed effects on energy reserves in tubificid worms. For example, Wright et al. (2013) reported that longer gut residence time of ingested microplastics was positively correlated with the consumption of energy reserves in marine worms (*Arenicola marina*) (Wright et al., 2013). As we did not detect microplastics particles in tubificid worms tissues, tubificid worms were more likely able to exclude the microplastics when feeding on sediments, thereby avoiding a large oxidative stress and the high energy expenditure associated with defense against oxidative damages. This interpretation is supported by Rodriguez et al. (2001) who showed that tubificid worms could selectively feed on fine organic rich particles in sediment (Rodriguez et al., 2001). Selective feeding excluding microplastic particles has been also reported by other aquatic deposit feeders as polychaetes of the genus *Marenzelleria* (Näkki et al., 2019). Therefore, the observed elevation in SOD activity in tubificid worms exposed to microplastics could be the result of external physical damage induced by plastic particles rather than due to ingestion (Yardy and Callaghan, 2020). It is also worth noting that the physiological impacts induced by microplastic exposure on biota have been reported to be species-specific (Bour et al., 2018). In the present study, it can be concluded that the benthic species *T. tubifex* was relatively tolerant to the microplastic exposure applied, explaining the low impact on MDA and worm survival. Such conclusion was consistent with other field studies that have reported that *T. tubifex* is one of the most pollution-resistant species living in freshwater environments (Chapman et al., 1982; Milbrink, 1987).

1.7.3. Bioturbation activities of *Tubifex tubifex*

While surface sediment reworking (SSR) was absent in the treatments without tubificid worms, SSR rapidly occurred after the addition of the white Fontainebleau sand (non-fluorescent tracer) in microcosms with *Tubifex tubifex* (**Figure 19 A**). The mean surface reworked by tubificid worms

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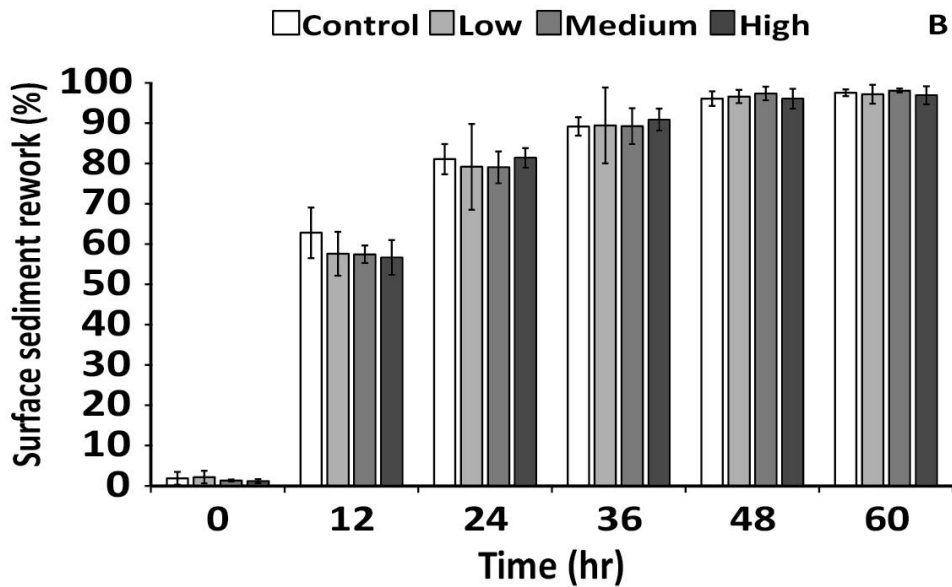
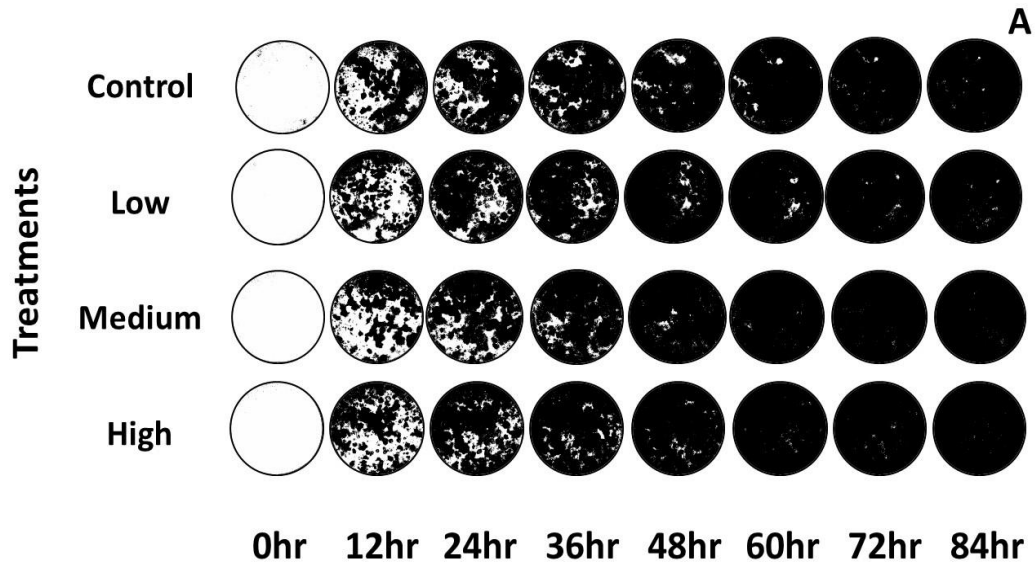
exceeded 50% of microcosm surface for all treatments after 12hr (**Figure 19 B**). The surface of sediment reworked increased strongly within the first 12hr to reach values higher than 50% (**Figure 19 A**). As most of the sediment surface was already reworked within these first 12hr, SSR rates decreased after this time (**Figure 19 B**). For this reason, only the areas occupied by fecal pellets within the first 12hr were used to compare treatments (**Figure 19 C**). Although statistical analyses were only marginally significant (1-way ANOVA, $0.05 < p < 0.1$), SSR due to fecal pellet deposition by worms tended to decrease with the increasing concentration of microplastics added to sediments. After 12hr of tubificid worm bioturbation, the SSR was $60.9 \pm 5\%$ in the treatment without microplastic (control) and was reduced by around 12% in the treatment with the highest microplastic concentration.

Tubificid worms significantly increased the water flux between water and sediments in the microcosms (2-way ANOVA, worm effect, $p < 0.05$) regardless of the microplastic treatment (2-way ANOVA, interaction between worm treatments and microplastic treatments, $p = 0.8$). For all microplastic treatments, the exchange of Cl^- measured at the sediment-water interface was around two-fold higher in treatments with tubificid worms than in treatments without worms (**Figure 19 D**). Nevertheless, it is worth noting that no significant difference in water fluxes was detected between treatments with and without worms at medium microplastic concentrations (Tukey's test, $p = 0.1$) whereas significant differences between treatments with and without worms were measured for all other microplastic concentration treatments (Tukey's tests, $p < 0.05$).

SSR measurements indicate that the presence of microplastics tended to decrease the feeding activity and the deposition of fecal pellets by tubificid worms at the sediment surface. In contrast, the bio-irrigation process generated by tubificid worms and assessed by water flux measurements was not strongly affected by microplastic treatments. Thus, microplastic contamination did not affect similarly bioirrigation and feeding activities of tubificid worm, highlighting the complex influences of microplastic contaminations on bioturbators. Remarkably, the different impacts of microplastics on bioirrigation and sediment reworking processes could have different effects on ecosystem functioning because these two bioturbation processes are

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known to differently stimulate nutrient fluxes and mineralization processes in sediments (Anschutz et al., 2012; Braeckman et al., 2010; Gautreau et al., 2020; Stief, 2013).



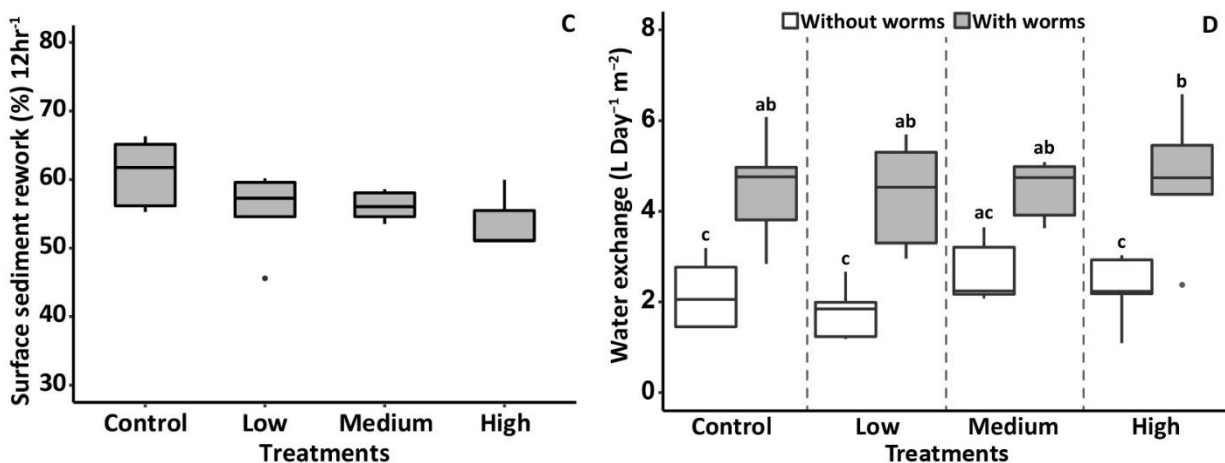


Figure 19 Sediment surface images taken from four replicates of each microplastics treated microcosm in the presence of tubificid worms. White pixels represent the white Fontainebleau sand tracer disappearing within 84hr through the addition of fecal pellets (A). Surface sediment reworking within 60 hr (B), percentage of surface sediment reworking in first 12hr (C) and bioirrigation (D). For each treatment, data are presented as means \pm S.D. (n = 5). Different letters indicate significant differences among treatments (Tukey test at $p \leq 0.05$). The absence of letters indicates no significant difference among treatments (Tukey test at $p \geq 0.05$).

1.7.4. Biogeochemical Fluxes

No significant CH₄ fluxes were measured from all microcosms, whereas significant CO₂ fluxes were calculated from linear increases of CO₂ concentration over time ([Supplementary Figure 11 A](#), and B). Overall, tubificid worms significantly increased CO₂ fluxes (**Figure 20 A**, 2-way ANOVA, worm effect, $p < 0.05$) depending on microplastic concentrations in sediments (2-way ANOVA, statistical interaction between worm treatments and microplastic treatments, $p < 0.05$). CO₂ fluxes were around 2.5-fold higher with worms ($0.47 \text{ mmol h}^{-1} \text{ m}^{-2} \pm 0.2$) than without worms ($0.19 \text{ mmol h}^{-1} \text{ m}^{-2} \pm 0.1$) in the control treatment (**Figure 20 A**, Tukey's HSD, $p < 0.05$). In contrast, CO₂ fluxes were similar between control microcosms and microcosms with worms in the presence of low and medium microplastic concentrations (**Figure 20 A**, Tukey's HSD, $p > 0.5$). Surprisingly, this inhibition of the effects of tubificid worms on CO₂ fluxes was not observed in the treatment with the highest microplastic concentration where tubificid worms stimulated by two-fold the CO₂ fluxes emitted from microcosms (**Figure 20 A**, Tukey's HSD, $p < 0.05$). It is also worth noting that microplastic contamination did not have a direct effect on microbial production of CO₂ as CO₂ fluxes were comparable among the four microplastic treatments without tubificid worms (**Figure 20 A**, Tukey's HSD, $p > 0.05$).

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Nutrient flux measurements did not report any linear increase in N-NH_4^+ and P-PO_4^{3-} concentrations with incubation time, indicating no significant fluxes of these two nutrients from sediments to water column (*Supplementary Figure 11 C*, and *D*). The absence of N-NH_4^+ and P-PO_4^{3-} fluxes associated with the lack of measured CH_4 flux (associated with methanogenesis processes) indicate that no strong anoxic conditions occurred in the sediment used for the experiment. More precisely, aerobic conditions in sediments probably inhibited the fluxes of PO_4^{3-} released to the water column by facilitating the adsorption of P with Fe(OH)_3 and Mn(OH)_2 (Gautreau et al., 2020). Such aerobic conditions could also have minimized the release rates of NH_4^+ from sediments by facilitating a rapid nitrification of NH_4^+ produced by organic matter mineralization into N-NO_3^- (Hedin et al., 1998). In line with this facilitated nitrification process, N-NO_x ($\text{N-NO}_3^- + \text{N-NO}_2^-$) concentrations linearly and significantly increased during the incubation, indicating a significant release rate of NO_3^- from sediments to the water column (*Supplementary Figure 11 E*, and *F*). Overall, tubificid worms significantly increased the NO_x fluxes from sediments to the water column in microcosms (**Figure 20 B**, 2-way ANOVA, worm effect, $p < 0.05$). However, this stimulation of NO_x fluxes due to the presence of worms was significantly reduced in presence of microplastics at low, medium and high concentrations (**Figure 20 B**, 2-way ANOVA, interaction “worm*MPs” effect, $p < 0.05$, Tukey’s HSD post-hoc tests, $p < 0.05$). As observed for CO_2 fluxes, NO_x fluxes were comparable among the four microplastic treatments without tubificid worms (**Figure 20 B**, Tukey’s HSD, $p > 0.05$), suggesting no direct influence of microplastics on microbial processes involved in N cycling.

These results clearly highlighted that microplastic exposure significantly reduced the contribution of tubificid worms on organic matter processing and nutrient cycling at the sediment-water interface. To our knowledge, this is the first evidence of an adverse impact of microplastics not only on organisms, but also on their contributions to ecosystem functioning.

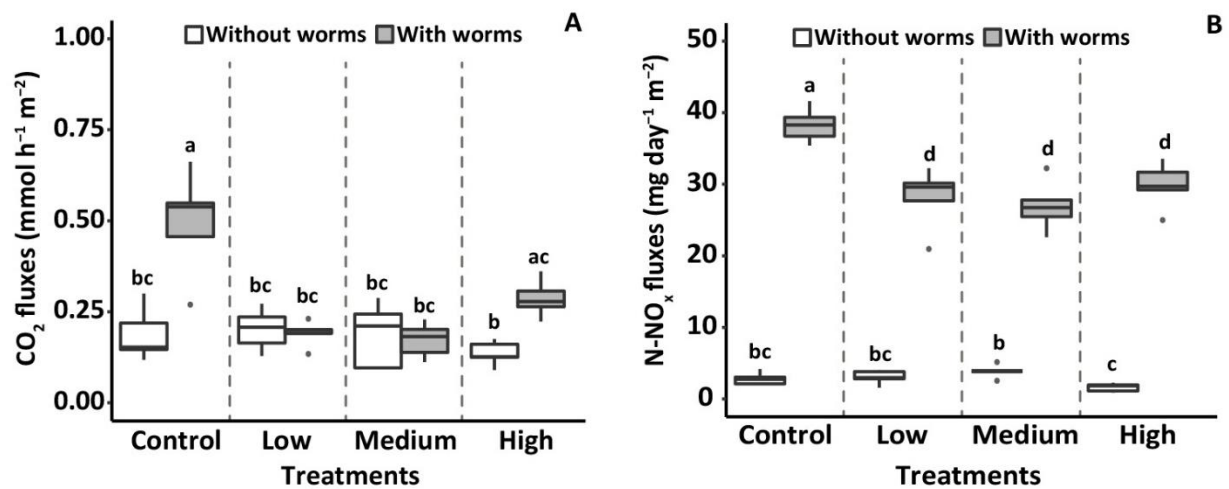


Figure 20 CO₂ (A) and N-NO_x (N-NO₃⁻ + N-NO₂⁻) fluxes (B) measured in the water columns. For each treatment, data presented as means ± S.D. (n = 5). Different letters mean significant differences among treatments (Tukey test at p ≤ 0.05).

1.8. Main conclusions and perspectives

The present study highlighted a significant impact of microplastic exposure on bioturbation-driven processes (organic matter mineralization and nutrient fluxes). These effects were probably associated with a reduction of bioturbation activity of tubificid worms monitored by fecal pellet production at the sediment-water interface. However, the obtained results were only marginally significant because more than 50% of the sediment surface area was already covered by fecal pellets at the first time of analysis (12hr). Under these conditions, it was difficult to observe significant results among treatments. Thus, for future experiments, monitoring fecal pellet production at higher frequencies (every 3hr for example) would permit more pertinent calculations of fecal pellet deposition rates. Furthermore, the use of luminophore tracer distribution in the sediment column at the end of the experiment (Ciutat et al., 2005; Gerino et al., 1998; Lagauzère et al., 2009) or techniques such as computed tomography (CT) scanning (Capowiez et al., 2021) could be promising approach to evaluate more precisely the impacts of microplastic exposure on bioturbation process.

Interestingly, we also found that the effects of microplastics on the stimulation of biogeochemical processes by worms were not positively correlated with microplastic concentrations. First, the positive effects of tubificid worms on CO₂ fluxes were totally inhibited at the lowest

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concentration of microplastics in sediments. Similarly, the role of tubificid worms in N-NO_x fluxes from sediment to water column was also impacted in the presence of low microplastic concentrations. These results indicate that concentrations exceeding 700 particles/kg sediment DW and observed in a wide range of freshwater environments (Frei et al., 2019; Hurley et al., 2018; Klein et al., 2015; Lin et al., 2018) may damage the role played by bioturbators on ecosystem functioning. Second, medium microplastic concentrations (7000 particles/kg sediment DW) induced stronger adverse impacts on tubificid worm physiology (higher SOD activity) and activities (worm effect on CO₂ fluxes) than the highest concentration of microplastics in sediments (70000 particles/kg sediment DW). This surprising result could be due to the ability of tubificid worms to better detect and then to avoid microplastic particles at their highest concentration (may be because they are less embedded with sediment particles than for lower concentrations). This mechanism of microplastic avoidance has been already reported in other invertebrate species which were able to detect the physical presence of plastic particles and/or chemical cues associated with microplastics (Carrasco et al., 2019; Yardy and Callaghan, 2020). Under these conditions, high concentrations of microplastic particles might have modified the burrowing behavior of tubificid worms (as suggested by change in fecal pellet production) and the gallery network produced by worms in sediments. For example, it has been largely observed that contaminants could influence the structure of biogenic structures produced by bioturbators in sediments (Lagauzère et al., 2009; Pignoret et al., 2016) and soils (Capowiez et al., 2006). Therefore, change in gallery network would have modulated the solute fluxes at the sediment-water interface by modifying the exchange area between sediments and surface water (Pischedda et al., 2008), impacting the biogeochemical processes occurring in sediments (Aller and Aller, 1998). Further experiments based on precise quantifications of tubificid worm activities in sediments (creation of biogenic structure, sediment reworking using luminophores) are needed to decrypt these complex interactions among microplastic contaminations, tubificid worm bioturbation and biogeochemical processes at the sediment-water interface of freshwater ecosystems.

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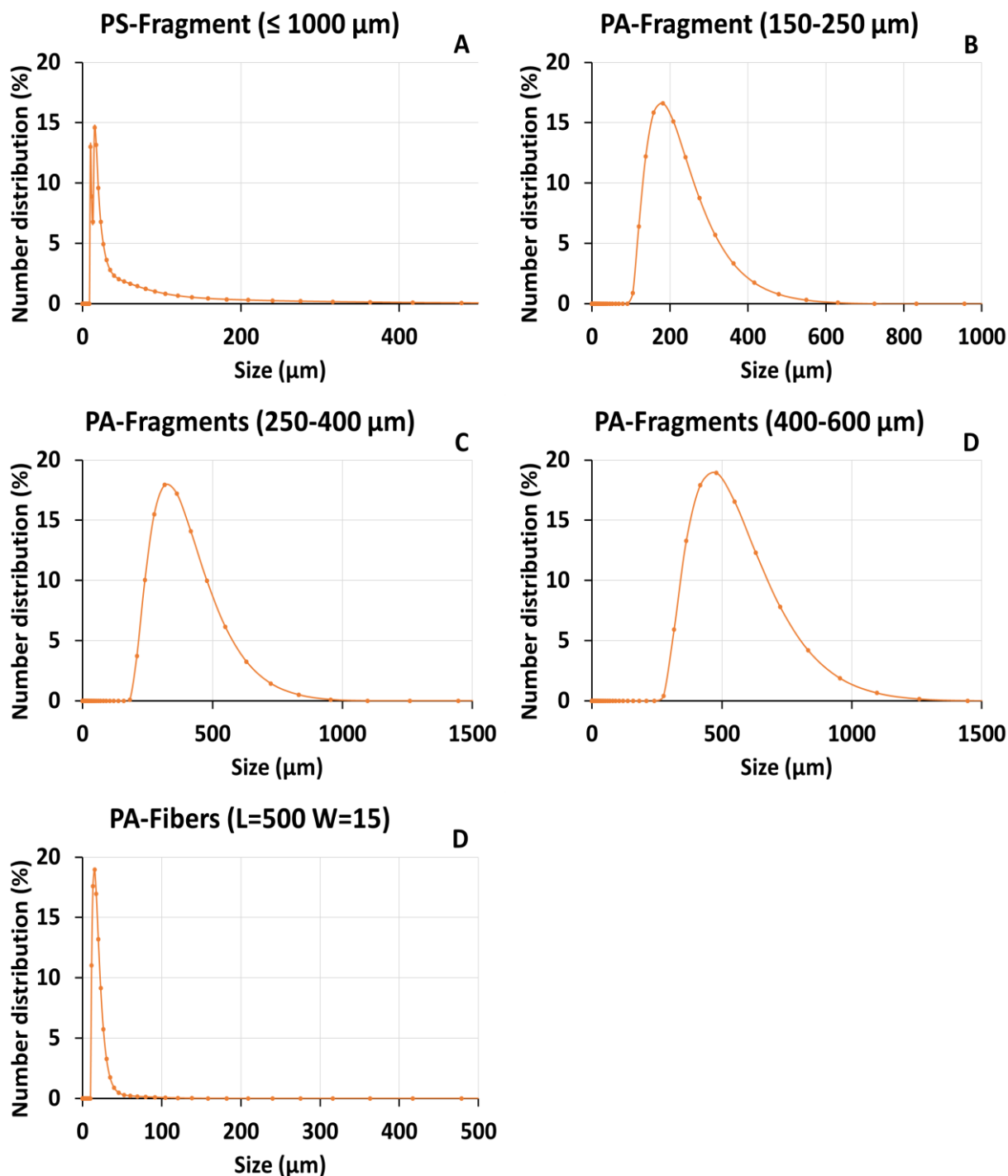
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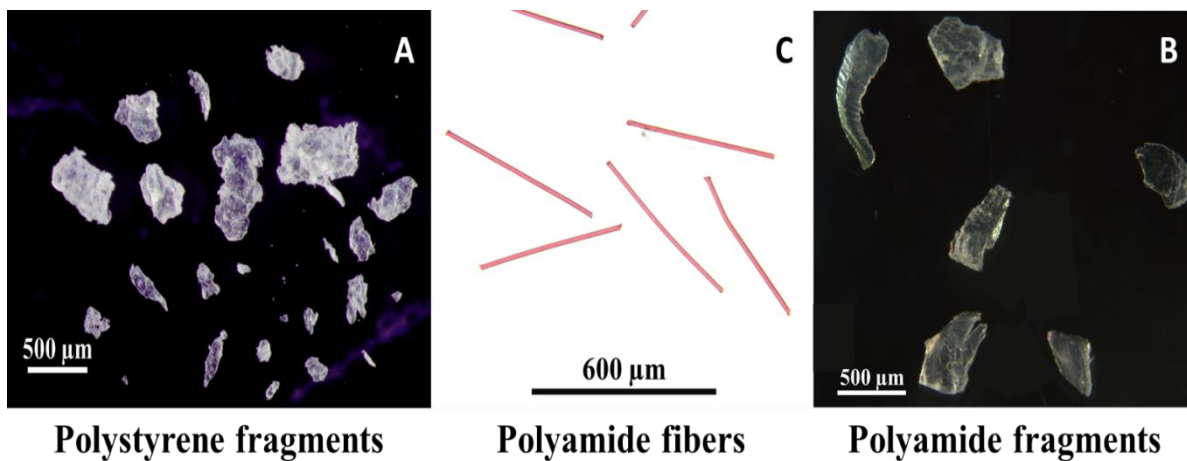
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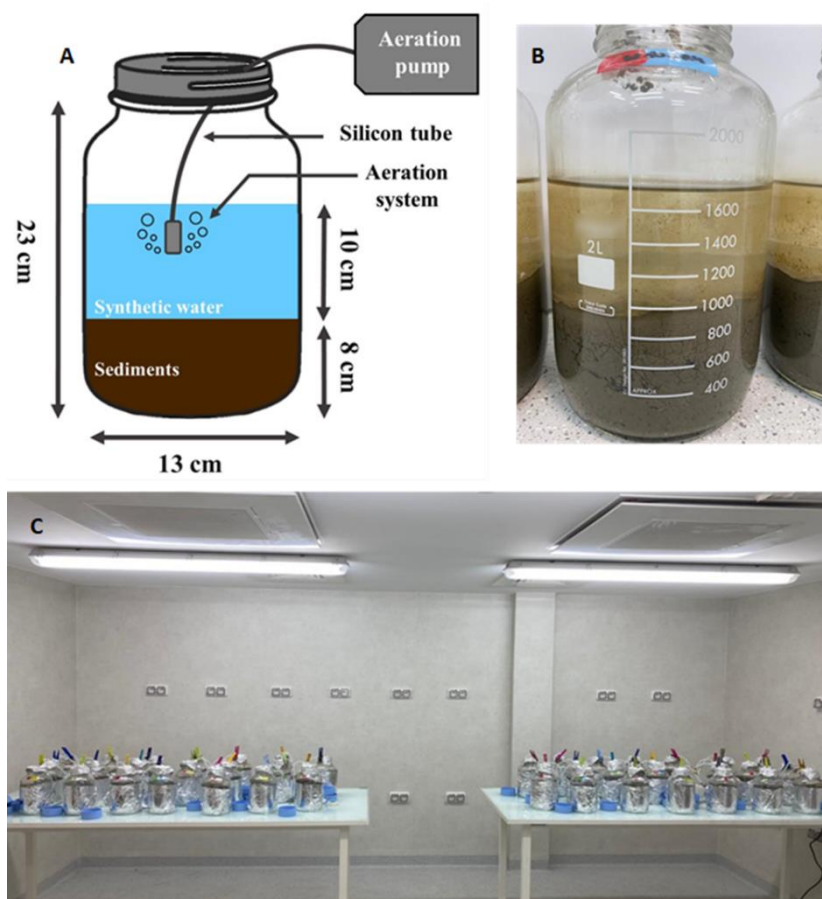
1.11. Supplementary materials



Supplementary Figure 6 Particle size distributions (PSD) based on the number of particles of the PS fragments (A), PA fragments sieved at 150–250 μm stainless sieve (B), PA fragments sieved at 250–400 μm (C), PA fragments sieved at 400–600 μm (D), and PA fibers (E).

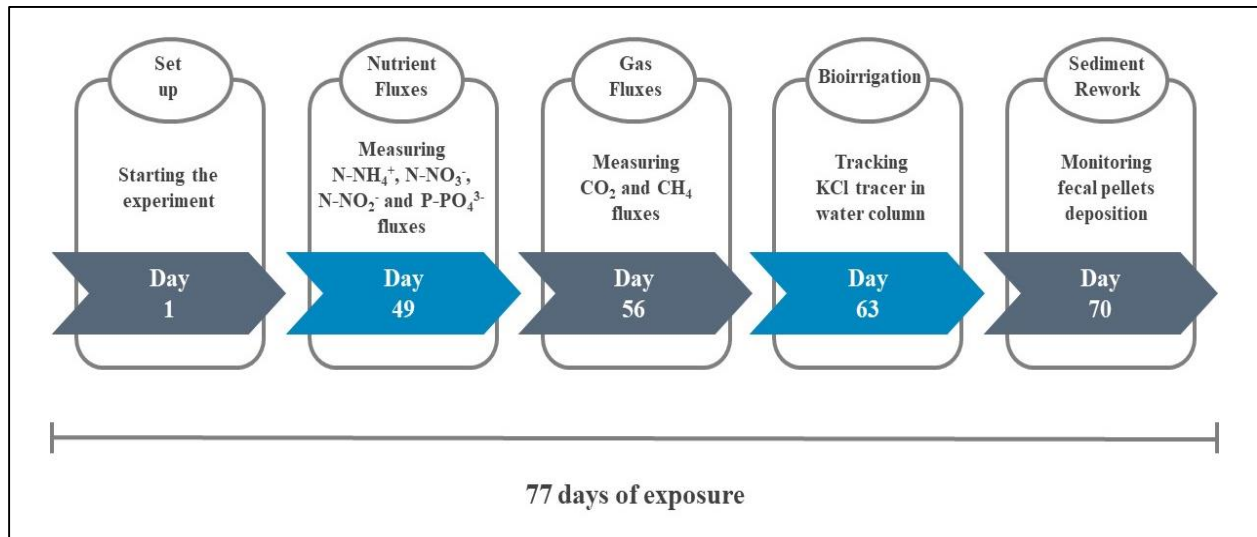


Supplementary Figure 7 Stereo microscopic images for PS fragments (A), PA fibers (B) and PA fragments (C), representing a polymeric plastic particles mixture of different colors, shapes and sizes.

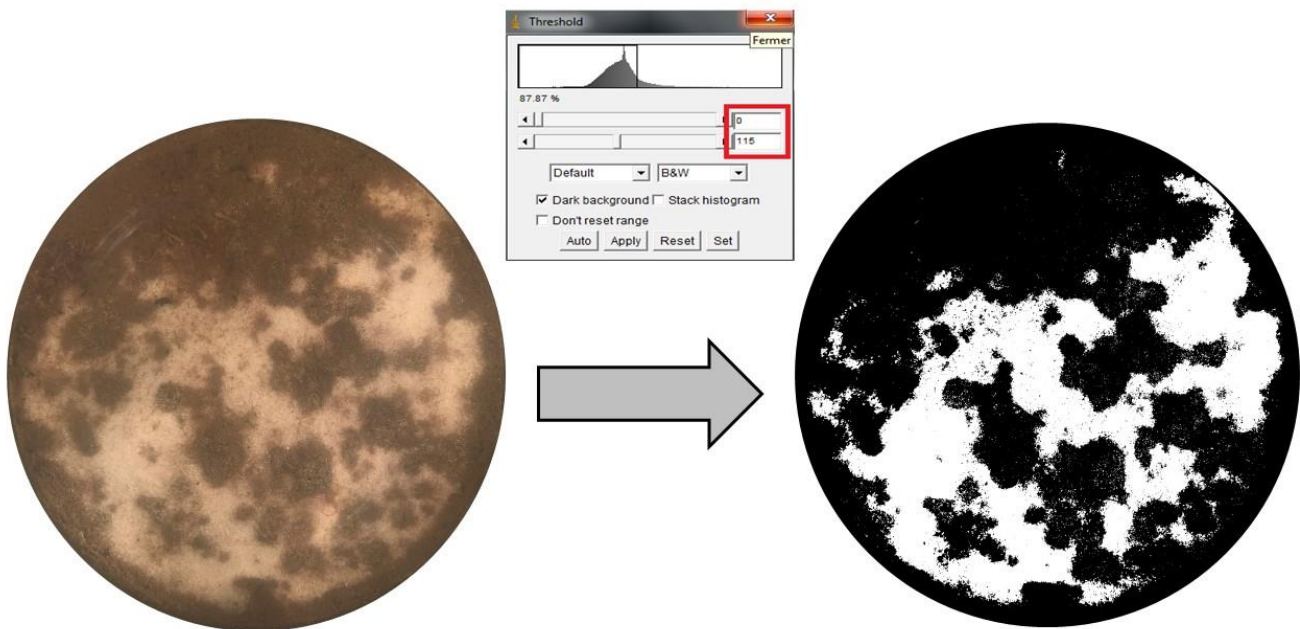


Supplementary Figure 8 Schematic diagram (A) and images of the microcosms jars (B and C) used for exposing Tubifex worms to microplastic concentrations at constant room temperature of 20°C under a 16:8 h light: dark cycle.

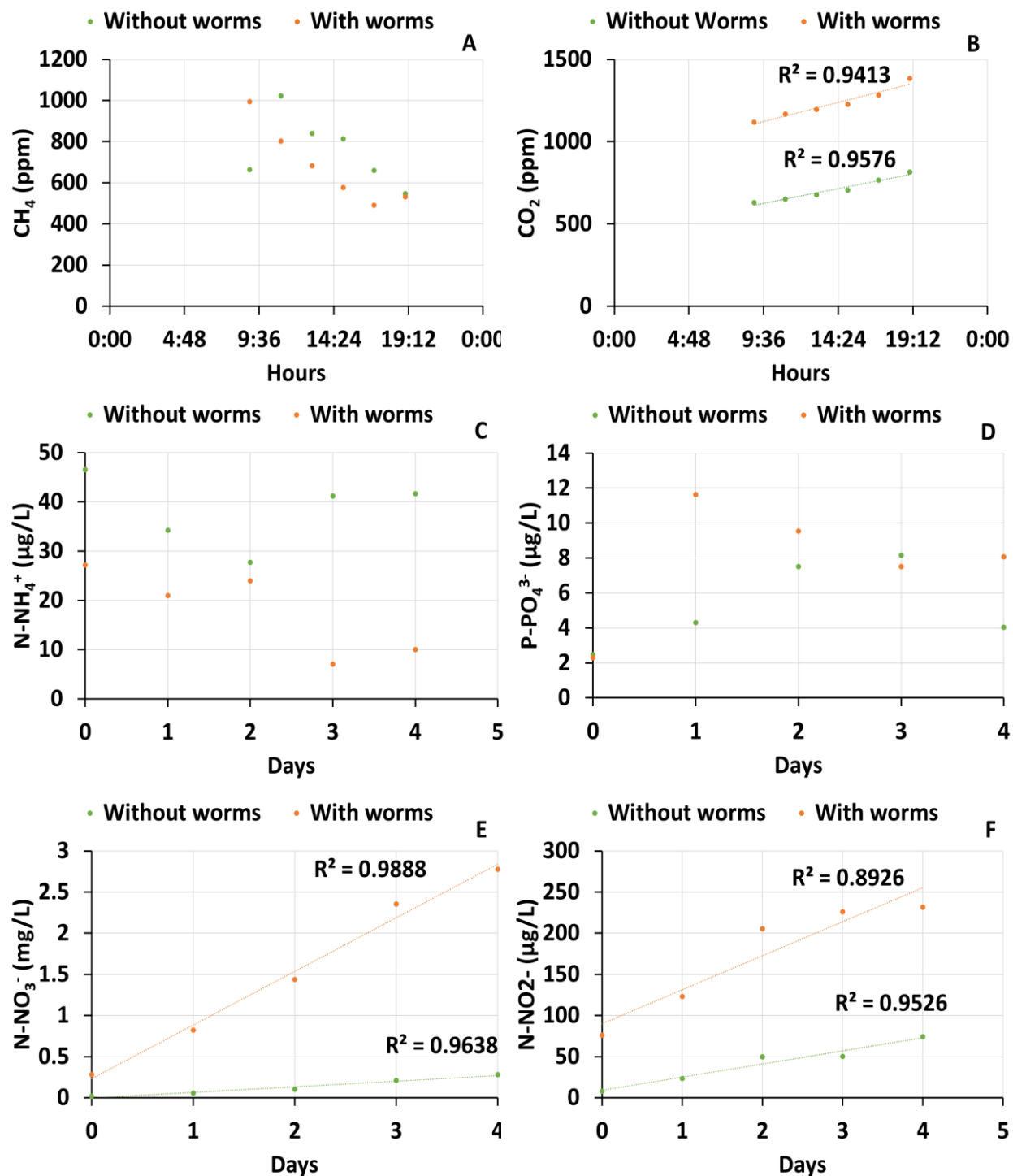
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Supplementary Figure 9 Schematic image showing parameters that were analyzed at different days during the time of the experiment.



Supplementary Figure 10 Sediment surface image taken from a microcosm with tubificid worms and exposed to microplastics. The image was treated using ImageJ software. The threshold was adjusted to fit to the original photo (Min. Threshold = 0, Max. Threshold = 115). White pixels represent the white sand of Fontainebleau (non-fluorescent tracers) and the black pixels represent the fecal pellets produced by the tubificid worms.



Supplementary Figure 11 Examples of temporal changes in concentrations of CH₄ (A), CO₂ (B), N-NO₃⁻ (C), N-NO₂⁻ (D), N-NH₄⁺ (E), and P-PO₄³⁻ (F) during incubations for measuring fluxes. Data were obtained from one unique microcosm.

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Supplementary Table 6 Weight (mg) of 100 particles from each microplastic type (A), weight of the sediment added in each microcosm (B) and the calculation done for each microcosm to obtain the four treatments used in the experiment (C).

| Table 1 - B | | | |
|--------------------|-----|-------------|-------|
| Sediment microcosm | per | Wet | Dry |
| | | Weight (Kg) | 1.352 |

| Table 1 - A | | |
|---------------|-------------|---------------------|
| Microplastics | Weight (mg) | Number of particles |
| PA fibers | 0.01 | 100 |
| PA fragments | 1.88 | 100 |
| PS fragments | 0.05 | 100 |

| Table 1 - C | | | | |
|---|---------|-----------------------------|-------------------------------|---------------------------------|
| | | Number of Particles | | |
| PA fibers | 0 | 251 | 2510 | 25096 |
| PA fragment | 0 | 2460 | 24600 | 246000 |
| PS fragments | 0 | 2460 | 24600 | 246000 |
| Number of particles (PA fibers + PA fragment + PS fragments) per one glass container (containing amount of sediments enough for 10 microcosms) | 0 | 5171 (251+2460+2460) | 51710 (2510+24600+24600) | 517096 (25096+246000+246000) |
| Number of particles per one microcosm | 0 | 517 (5171/10 microcosms) | 5171 (51710/10 microcosms) | 51710 (517096/10 microcosms) |
| Number of particles per Kg dry sediment (Number of particles per one microcosm ÷ 0.738 Kg) | 0 | 700.6 (517/0.738) | 7006.7 (5171/0.738) | 70067.7 (51710/0.738) |
| Treatments | Control | Low | Medium | High |

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Supplementary Table 7 Number of microplastics ingested by the Tubifex worms from each of the four treatments (mean \pm SD, n = 50).

| Treatments | 0 particles/kg sediment dry weight | 700 particles/kg sediment dry weight | 7000 particles/kg sediment dry weight | 70000 particles/kg sediment dry weight | Blank (negative control - no worms) |
|---|------------------------------------|--------------------------------------|---------------------------------------|--|-------------------------------------|
| Number of microplastics per treatment (i.e., 50 individual/microcosm) | 6 \pm 3 | 16 \pm 7 | 13 \pm 12 | 10 \pm 5 | 16 \pm 4 |

Chapter V

Conclusion and perspectives

1. Conclusion and perspectives

The thorough research conducted in this PhD project has provided a comprehensive understanding of the transport and presence of microplastics in streambed sediments, as well as their effects on organisms and ecosystem function. Through addressing four main objectives, the study successfully (1) developed and improved approaches for microplastic extraction and identification, (2) enhanced our understanding of sediment sampling regimes and their impact on shaping the findings of microplastic concentration and size fractions, (3) investigated the distribution and transport of microplastics in riverbed sediments in relation to hydro-sedimentological processes, and (4) studied the ecotoxicological impacts of microplastics on freshwater ecosystem engineers and their implications for river system function.

The methodological advancements discussed in Chapter II provided a framework for the subsequent chapters and contributed to the overall reliability and accuracy of the PhD findings. In fact, improving current techniques used for sampling, extracting, and characterizing microplastics are key aspects that need to be addressed in order to improve the harmonization of methodologies. As a result, the gap within the representativeness and comparability of results across studies may be closed. Only then may we better understand the outcomes in this field and reduce uncertainty, which in turn allows for monitoring decisions to preserve the environment. However, it is necessary to realize that the methods used should always be shaped by the research question at hand. For example, when attempting to comprehend the concentrations of microplastics endangering interstitial fauna, information on the various fractions of mobilizable microplastics in pore water is essential. Therefore, the piezometer pump technique is favored over other alternatives. Besides, the Bou-Rouch pumping method, which ecohydrologists frequently use to collect faunal samples, was utilized for the first time in this PhD study to collect microplastic samples. To better understand the effect of this pumping technique on recovering and remobilizing microplastics from sediments and pore water, additional tests must be conducted, and comparison of this instrument with other conventional sampling tools is mandatory. Today, the field of microplastic research is continuously evolving, and further refinements and advancements in extraction, sampling and analysis techniques are still needed

Chapter V *Conclusion and perspectives*

to overcome existing and emerging challenges, such as the detection of smaller nanoplastics or the discrimination between plastics and other organic or inorganic materials under visual and/or analytical chemistry methods.

Based on the methodological tools developed in Chapter II, Chapter III investigated how streambed sediments function as sinks for microplastics and how hydrological exchanges between surface water and ground water affect their transport, distribution and concentration. The research was conducted in the field, in the lower Ain River (France), and provided insights into the behavior of microplastics at the sediment-water interface, and highlighted the importance of considering hydro-sedimentological conditions when assessing microplastic contamination. The presence of microplastics in streambed sediments was observed to be higher in sedimentation zones (i.e., sites of stagnant water), where fine sediments accumulate. However, similar to the behavior of fine sediments, the retention and remobilization of plastic particles in streambeds seem affected by hydrological exchanges at the sediment-water interface. Understanding these patterns is essential for identifying hot spots for microplastic accumulation and implementing targeted mitigation strategies. However, it is essential to consider the temporal and spatial variability of hydrological processes, as they can impact the dynamics of microplastic deposition and redistribution in river systems. For this reason, future research should focus on capturing these temporal and spatial variations to obtain a more comprehensive understanding of microplastic transport dynamics.

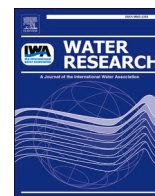
Following the results described in Chapter III which provided evidence for the accumulation of microplastics at the sediment-water interfaces, the effects of microplastics on ecosystem engineers and the associated biogeochemical processes occurring at this interface were investigated in Chapter IV. In this chapter, the ecotoxicological effects of microplastics on the freshwater ecosystem engineers *Tubifex tubifex* were examined, with a particular focus on bioturbation activity and its implications for ecological functioning. The results of the laboratory experiment showed that the significant effects of microplastic exposure extended beyond the level of organisms and included potential disruption of critical biogeochemical processes at the sediment-water interface by impacting bioturbation-driven processes (i.e., as organic matter mineralization and nutrient fluxes). Bioturbators play a vital role in maintaining sediment

Chapter V *Conclusion and perspectives*

structure, enhancing oxygenation, and facilitating nutrient exchange between sediment and water column. The reduction in bioturbation activity due to microplastic exposure can have cascading effects on other organisms and ecosystem processes. For instance, reduced bioturbation can lead to increased sediment compaction, decreased oxygen transportation into sediments, and altered nutrient fluxes, ultimately affecting the overall health and functioning of the river ecosystem. These findings emphasize the need to consider not only the direct physical impacts of microplastics on organisms but also their indirect effects on ecological interactions and processes. Future studies should further investigate the long-term consequences of microplastic exposure on ecosystem structure and function, considering multiple trophic levels and potential cascading effects within food webs. The bioaccumulation and biomagnification of microplastics in benthic invertebrates and freshwater food webs remain poorly understood due to analytical limitations. To address this knowledge gap, the use of metal-doped microplastic tracers combined with metal analysis techniques such as synchrotron micro X-ray Fluorescence (μ XRF) can provide valuable insights on the ecotoxicological risks of microplastics at the level of individual organisms and within trophic interactions. Finally, conducting comparable experiments on organisms from various ecosystems is highly recommended to understand the global consequences of microplastic pollution. For instance, future studies should examine the effects of microplastics on bioturbators in freshwater, marine, and terrestrial ecosystems using a consistent and similar approach. This comparative studies will improve our understanding into the ecological implications of microplastics across different ecosystems.

In summary, these chapters provide valuable insights into the complex dynamics of microplastic contamination in streambed sediments and its ecological implications. This PhD project highlight the need for harmonized methodologies that ensure accurate assessments of microplastic distribution and concentration, site-specific investigations, and a holistic approach to assess the ecological impacts. The research also underscores the role of hydrological processes and ecosystem engineers in shaping the fate and impact of microplastics in river systems. By shedding light on the transport, distribution, and ecological consequences of microplastics, this research contributes to the broader effort of mitigating freshwater ecosystems from the pervasive threat of plastic pollution.

Annex article



Environmental and land use controls of microplastic pollution along the gravel-bed Ain River (France) and its “Plastic Valley”

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ABSTRACT

Understanding microplastic particles (MPs) accumulation and transport along rivers represents a major task due to the complexity and heterogeneity of rivers, and their interactions with their wider corridor. The identification of MPs hotspots and their potential sources is especially challenging in coarse-bed rivers transporting a wide range of particle sizes with a high degree of variability in time and space. This research focuses on the gravel-bed Ain River (Rhône River tributary, France) which is managed by means of various dams and also hosts one of the major plastic production centres in Europe (Oyonnax and Bièvre Plastic Valleys). In this research, (i) Geographical Information Systems (GIS) were used to locate plastic factories and to characterise the land use of the Ain River watershed. (ii) On the field, sediment samples were extracted from the hyporheic zone (HZ) of mobile gravel bar heads, while hydro-sedimentary settings were measured in order to describe site conditions. Sampling sites were especially established in downwelling areas (i.e. where the surface water entered the hyporheic zone), upstream and downstream of dams and plastic factories. (iii) After density separation and organic matter digestion of sediment, MPs were characterised with a μ FTIR device followed by data processing via the siMPLe software. This work highlighted the trapping efficiency of alluvial bars for MPs. The highest MPs concentrations were found along the Plastic Valleys (up to 4400 MPs/kg), while the lower river was less contaminated by MPs. After grain-size correction, a significant breakpoint was identified in the area of the main dams, revealing their major influence on MPs distribution. The variability in MPs concentrations and types suggested a local origin for most of MPs. A particular feature was the dominance of polypropylene (PP) which appears as a critical industrial heritage as the studied region is specialised in the manufacturing of hard plastics. Indeed, multivariate analyses also revealed that MPs concentrations and types were mostly driven by the vicinity of plastic factories and urban areas. This relationship between the land use, the presence of dams and MPs characteristics provides key results for the MPs assessment and the improvement of management issues along coarse-bed rivers.

1. Introduction

Over the last century, the manufacturing, use and release of plastics into the environment hugely increased and became significantly more diversified (Borrelle et al., 2020; Zalasiewicz et al., 2016). Plastics and their chemical makeup, including additives, are so ubiquitous in the environment that recent studies refer to the Plastic Cycle, for the transfer

of microplastic particles (MPs) between environmental envelopes: atmosphere, hydrosphere, lithosphere, and biosphere (Brahney et al., 2021; Zhu, 2021; Bank and Hansson, 2019). Rivers and freshwater ecosystems have been recognised as playing a key role in the transport and accumulation of plastics from the uplands to the oceans (Krause et al., 2021; Lebreton et al., 2017; Siegfried et al., 2017; Jambeck et al., 2015). Plastics are found in river systems in very different sizes, from

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macro- to micro- and nano-plastics, in various shapes and polymer compositions (van Emmerik and Schwarz, 2020). Especially for MPs which are lower than 5 mm (Arthur et al., 2009), rivers can receive plastic particles from urban and industrial areas (wastewaters, runoff, and intentional releases), from agricultural areas (sewage sludge application, degradation of mulching films and tarpaulins, etc.) and from the atmosphere (Allen et al., 2019; Dris et al., 2018; Hurley and Nizzetto, 2018; Rochman, 2018; Cole et al., 2011).

The assessment of the contamination level of aquatic ecosystems with MPs is an important challenge as MPs can be transported and deposited along rivers, reworked by floods or human activities, diluted by tributary inputs along the watercourse or even accumulated in the hyporheic zone (Drummond et al., 2022; Laermanns et al., 2021; Waldschläger and Schüttrumpf, 2019). Understanding the distribution of MPs in sediments along rivers is a topic of growing importance. For instance, research works achieved along the Elbe, Rhine or Inde Rivers in Germany (Laermanns et al., 2021; Lechthaler et al., 2021; Scherer et al., 2020; Klein et al., 2015; Mani et al., 2015), as well as on the Tame River (Trent River tributary) in the United Kingdom (Tibbetts et al., 2018) were pioneering in terms of MPs assessment (counting and classification). More recently, MP estimates from aquatic sediment developed along several rivers worldwide: on the Maozhou, Pearl and Wei Rivers in China (Wu et al., 2020; Ding et al., 2019; Yan et al., 2019), on Kaveri, Killa Chinthamani, Tiruchirappalli, and Ganga Rivers in India (Maheswaran et al., 2022; Sarkar et al., 2019), or on Namibian Rivers in Africa (Faulstich et al., 2022), as well as along the Upper Garonne River in France (Reis de Carvalho et al., 2021) or along the Barrow River in Ireland (Murphy et al., 2022). These studies confirmed the presence of MPs in various concentrations and types around urban-industrial areas. However, the effect of land use, dams and other hydro-sedimentary settings on MPs dynamics remains ambiguous. For instance, some studies indicated a decrease in MPs downstream of dams (Watkins et al., 2019), while others did not show any influence (Weideman et al., 2019). Thus, further research needs to question the effect of dams and other river barriers, land use aspects (including in the presence of plastic factories), and hydro-sedimentological settings (grain size, hyporheic exchanges) on the distribution of MPs along a river (Miller and Orbock Miller, 2020; Nel et al., 2018; Baldwin et al., 2016).

To address these questions, this research aims (i) to characterise longitudinal concentrations of MPs along a coarse-bed river and (ii) to discuss the MPs distribution (concentrations and types) in relation to hydro-sedimentological and socio-environmental factors (grain size, flow/sediment obstacles, land use and waste water treatment plants). This study focuses on sediment from the Hyporheic Zone (HZ) because it is a key zone of water and particle exchanges between surface water and groundwater (Dole-Olivier et al., 2019; Vervier et al., 2009; Malard et al., 2002a). Moreover, downwelling sites - i.e. where the surface water enters into the HZ - along coarse-bed rivers have been demonstrated as suitable environments for the accumulation of MPs during a specified season (Drummond et al., 2022; Frei et al., 2019; Lewandowski et al., 2019). In this research, we focused on the Ain River, which is a gravel-bed tributary of the Rhône River (France), offering a heterogeneous land use with the notable presence of plastic production sites and major dams, thus a representative case study of managed coarse bed-rivers worldwide.

2. Material and methods

2.1. The Ain River continuum: geomorphological and land use insights

The Ain River is a 190 km-long meandering river flowing from the French Jura Mountains to the Rhône River, upstream of the city of Lyon (Fig. 1-A). Near the confluence with the Rhône River, the Ain presents a coarse bed load (D50 = 26-15 mm) and transports annually ca. 15,000 m³ of sediment (Szewczyk et al., 2022; Rollet and Piégay, 2013; Piégay et al., 2008; Bravard, 1986). The river course presents three distinct

sections (Fig. 1-A): (i) a hilly upper section where the river is meandering, (ii) a middle section constrained into gorges (100-300 m width) with a succession of dams, and (iii) the Lower Ain River, downstream of Pont-d'Ain (PDA), which corresponds to a pebble floodplain (1 to 1.5 km width). Two tributaries supply the Middle Ain River (Fig. 1-B): the Bienne (69 km long) and the Lange-Oignin (LAO) Rivers (ca. 40 km long). Gravel bars are typical geomorphic features occurring along the whole hydrosystem, which are also key zones of water, sediment, oxygen and temperature exchanges, critical for the biota (Dole-Olivier et al., 2019; Wawrzyniak et al., 2016; Mermillod-Blondin et al., 2015). Water downwellings along these gravel bars have been demonstrated as key sites for particle accumulation and transfer from riffle-pool sequences (Mermillod-Blondin et al., 2000) to a greater scale (Datry et al., 2008).

In terms of land use, the Upper Ain river section is dominated by forestry and agricultural activities, mainly pastures for milk and cheese production. Then, the Middle Ain Valley presents 22% of urban and industrial areas, especially along the LAO and Bienne Rivers, at Oyonnax, Nantua and Saint-Claude (Fig. 1-C). This area is suspected to offer a high potential for microplastic pollution due to the historical presence of plastic factories since 1889: this region is still a leading centre for the plastics industry in France and Europe (Desgouttes and Bertrand, 2015; Dyvrande, 1980; see also the 'Haut-Bugey tourist office' and 'Oyonnax town' websites). The Middle Ain River also hosts major dams, such as the Vouglans arch dam (height: 103 m), while run-of-the-river dams are established at Coiselet, Cize-Bolozon (CB) and Allement (ALL) on the Ain River itself, and at Charmines and Etables on the LAO and Bienne Rivers, respectively (Fig. 1-A and 1-B). The Middle and the Lower Ain Valley also offer recreational water zones for fishing, whitewater canoeing, swimming and hiking, where waste and pollution issues can be very harmful for water quality.

2.2. Geographical Information System (GIS) approach

A Geographical Information System (GIS) approach was used to collect socio-environmental data on the Ain catchment area. We especially focus on four data available at the scale of the river catchment which are likely to influence the release and accumulation of microplastics: the land use (coming from Corine Land Cover, v. 2018), location and nameplate capacity of waste water treatment plants (WWTP) (SysTraitementEauxUses database, v. 10/12/2021, available at: www.sandre.eaufrance.fr), the presence of dam and other river obstacles (>1 m high; OBS database from: www.sandre.eaufrance.fr), as well as the distribution of plastic factories. This last item required a particular methodological development. The number of plastic factories that have historically been active in the plastic sector was assessed by collecting data from corporate directories (Plastipolis and Polyvia for 2000s and 2010s), historical mentions (from Georisques), and current street views. Based on this review, density maps were computed using QGIS (v. 3.16.2).

2.3. Sampling strategy and method

Twelve sampling sites were selected along the Ain River continuum (Fig. 1-A and 1-B; Table 1 and Fig. A.1): (i) on the Upper Ain River, one site is upstream of dams influence (CHA1); (ii) on the Middle Ain Valley, five sites were selected to monitor MPs along the "Oyonnax Plastic Valley" on the LAO River (MAR10, BRI6-7-8, PER5), one site at Dortan (DOR3) at the outlet of the "Bienne Plastic Valley", and two sites downstream of the Coiselet and Allement dams, in the zone influenced by dams (COI4 and ALL2); (iii) three sites are then distributed along the Lower Ain River (PDA1, GEV19, SMG40). The sediment samples were obtained in June 2021 (low water period) at -20 cm deep at the head of mobile gravel bars. In each bar, piezometric heads were measured in the river and in the HZ to select sampling zones with negative vertical hydraulic gradients (VHG) characterising downwelling conditions (see

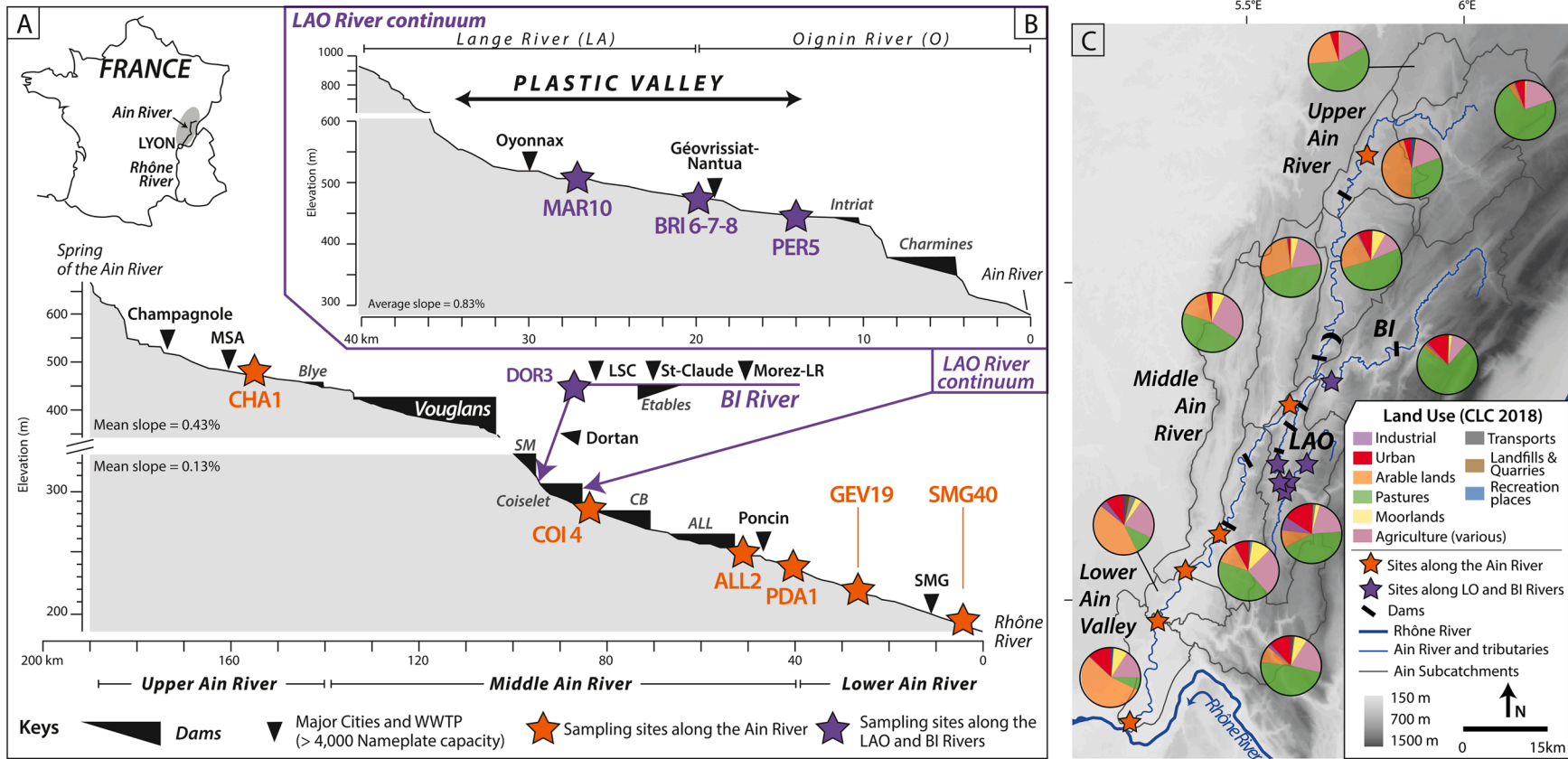


Fig. 1. Physical and land use settings along the Ain River with the location of the sampling sites, dams and the Plastic Valleys. (A) Longitudinal profile of the Ain River. (B) Longitudinal profile of the LAO (Lange-Oignin) River with the location of the "Oyonnax Plastic Valley". (C) Land use for each sub-catchment (based on CLC - Corine Land Corine - 2018, without forests, water and wetland surfaces). Key sites: ALL = Allement dam, BI = "Bienne Plastic Valley" and Bienne River, CB = Cize-Bolozon dam, LR = Les Rousses, LSA = Lavans-Saint-Claude, MSA = Montigny-sur-Ain, SM = Sault Mortier, SMG = Saint-Maurice-de-Gourdans.

Table 1

Location of the sampling sites and associated hydrological settings during the sampling operations. *Discharge measured at Pont-de-Poitte for CHA1; Maillat for MAR10, BRI6-7-8 and PER5; Jeurre for DOR3; Pont-d'Ain for COI4, ALL2 and PDA1; Chazey-sur-Ain for GEV19 and SMG40.

| Site | River (R.) & section | Latitude (DD, °N) | Longitude (DD, °E) | Elevation (m a.s.l.) | Stream width (m) | Discharge (m ³ /s)* |
|--------|----------------------|-------------------|--------------------|----------------------|------------------|--------------------------------|
| CHA 1 | Upper Ain R. | 46.68607 | 5.76592 | 462 | 40 | 11 |
| MAR10 | LAO R. (Lange) | 46.2063 | 5.60466 | 503 | 5 | 1.04 |
| BRI 7 | LAO R. (Lange) | 46.17148 | 5.55054 | 478 | 9 | |
| BRI 8 | LAO R. (Oignin) | 46.17021 | 5.54922 | 480 | 7 | |
| BRI 6 | LAO R. (Oignin) | 46.17214 | 5.54554 | 475 | 18 | |
| PER 5 | LAO R. (Oignin) | 46.2026 | 5.53939 | 437 | 15 | |
| DOR 3 | Bienne R. | 46.33171 | 5.67232 | 307 | 41 | 2.21 |
| COI 4 | Middle Ain R. | 46.2937 | 5.57599 | 294 | 66 | 16.9 |
| ALL 2 | Middle Ain R. | 46.10094 | 5.40809 | 242 | 64 | 16.3 |
| PDA 1 | Lower Ain R. | 46.04589 | 5.33341 | 235 | 140 | 46.3 |
| GEV 19 | Lower Ain R. | 45.96226 | 5.25392 | 221 | 95 | 52.7 |
| SMG40 | Lower Ain R. | 45.80673 | 5.18672 | 194 | 66 | |

Table A.1 for details). After this selection, composite samples were collected at each site based on three samples of pore waters and interstitial sediment collected with the Bou-Rouch pumping method (Bou and Rouch, 1967; Malard et al., 2002b). Temperature, electric conductivity and dissolved oxygen were measured with a portable device (PONSEL Odeon). Bulk sediment samples (ca. 5 kg) coming from the HZ were also collected at -20 cm deep by using a metallic shovel and a stainless steel 100 µm mesh sieve in order to assess the overall proportion of sands, silts and clays (SSC) in the river bed sediments. All samples were stored at 4 °C before analysis.

2.4. Microplastic extraction and characterisation protocol

For MPs extraction, the sediment samples were sorted into two fractions by using a 500 µm stainless-steel sieve and a 20 µm stainless-steel sieve (lower limit). At the lab, the two fractions (>500 µm and <500 µm) were dried for 3 to 4 days at 55°C in an oven and then immersed for 24 h in a ZnCl₂ solution (density: 1.5 kg/L) to separate heavy inorganic materials from lighter particles (Quinn et al., 2017). The floating material was recovered by sieving with vacuum filtered ultra-pure water - VFUP (Whatman GF/C filters: porosity= 1.2 µm). The material was digested for 24 h with Fenton's reagent (30 % H₂O₂ associated with an iron catalyst: H₂SO₄-FeSO₄-H₂O; 0.05 M) to remove the organic matter (Ruggero et al., 2020). After digestion, the remaining material was filtered and placed on alumina filters (Whatman Anodisc: diameter= 25 mm and porosity= 0.2 µm).

MPs counting and polymer type analyses were achieved by using a µFT-IR (Fourier-transform infra-red) spectrometer (PerkinsElmer Spotlight 400) in transmittance mode (pixel resolution: 25 µm, spectra resolution: 8 cm⁻¹). The spectral map obtained for each filter was then treated by using the siMPle software (version 18/09/2020). This software performs multiple correlations based on a spectra database in order to identify the nature of the analysed particles (plastic or natural origin, i.e. plant, animal furs, quartz, and charcoal) through a probabilistic approach (Primpke et al., 2018). Polyamide (PA) particles were excluded from the analysis due to a high probability of confusion with spectra associated with natural particles.

Quality control was based on three steps. (Step 1) A known number of different MPs and organic matter particles (wood, leaves, and chitin) was added in three samples (see Table A.2 for the sample composition). Three replicates for each sample were processed and recovery rates were calculated after the siMPle treatment (Table A.2). (Step 2) In order to consider the background presence of MPs, "negative controls" filtered with VFUP at 20 µm mesh were processed. The number of MPs identified by the software in those "negative controls" corresponds to a false prediction of MPs caused by misinterpretation of the spectra and was integrated in error bars (Table A.3). (Step 3) The level of accepted probabilities in siMPle was increased based on the Step 1 results to improve the robustness of the analysis (see Table A.4 for details).

2.5. Sediment particle size distributions

All sediment samples were dried at 55°C during 1 week. Bulk sediment samples collected at -20 cm deep at each site were manually sieved by using a sieve column (meshes from 20 µm to 63 mm). We assume that this measurement is a reliable representation of the overall proportion of cobbles, gravels and SSC (sands, silts and clays) in the sediment for each site (Table A.5). As the Bou-Rouch pumping method extracted relatively fine sediments (Table A.5) and as MPs were predominantly associated with SSC, the number of MPs was normalised to the proportion of SSC assessed in each site as follows (1):

$$[\text{MPs}]_{\text{in sediment}_{\text{environment}}} = [\text{MPs}]_{\text{in sample}} \times \text{SSC} \quad (1)$$

In the above formula, [MPs] in sediment_{environment} represents the overall concentration of MPs in the river sediment (#/kg dry weight - dw). [MPs] in sample represents MPs concentrations in each sample (#/kg dw) and the SSC represents the percentage of sands, silts and clays at -20 cm deep on each sampled site.

2.6. Data treatments

Based on MPs distribution data (concentrations and types), hydro-sedimentological and socio-environmental data (land use, river obstacles, grain size, WWTP), multiple linear regressions were performed in order to evaluate which hydro-sedimentological and socio-environmental factors can potentially explain MPs concentrations along the Ain River. All statistical analyses were achieved in R (v.4.1.2; R Core Team, 2021). The *mcp* package and functions were used to identify significant breakpoints and to fit linear segments in the data series (Lindelov, 2022). A hierarchical cluster analysis (HCA), using Ward's distance, was also performed in order to identify assemblage groups based on the MPs composition. After re-scaling and centering of the data, a redundancy analysis was performed by using the *vegan* package (Oksanen et al., 2022) to compare the distribution of MPs types with the former variables.

3. Results and discussion

3.1. Densities of plastic manufacturing sites

The distribution of plastic manufacturing businesses and factories within the Ain River catchment was represented by a density map and a histogram (Fig. 2).

More than 550 factories - active in the plastic sector during the 2000s-2010s and/or before according the accessed sources - have been identified on the Ain River watershed. They included compounders, moulding shops, storage areas, transformation and manufacture of finished products (one site may be involved in several activities). A very high density of plastic factories was found along the LAO River, near Oyonnax, which corresponded to the main "Plastic Valley" (Fig. 2-B).

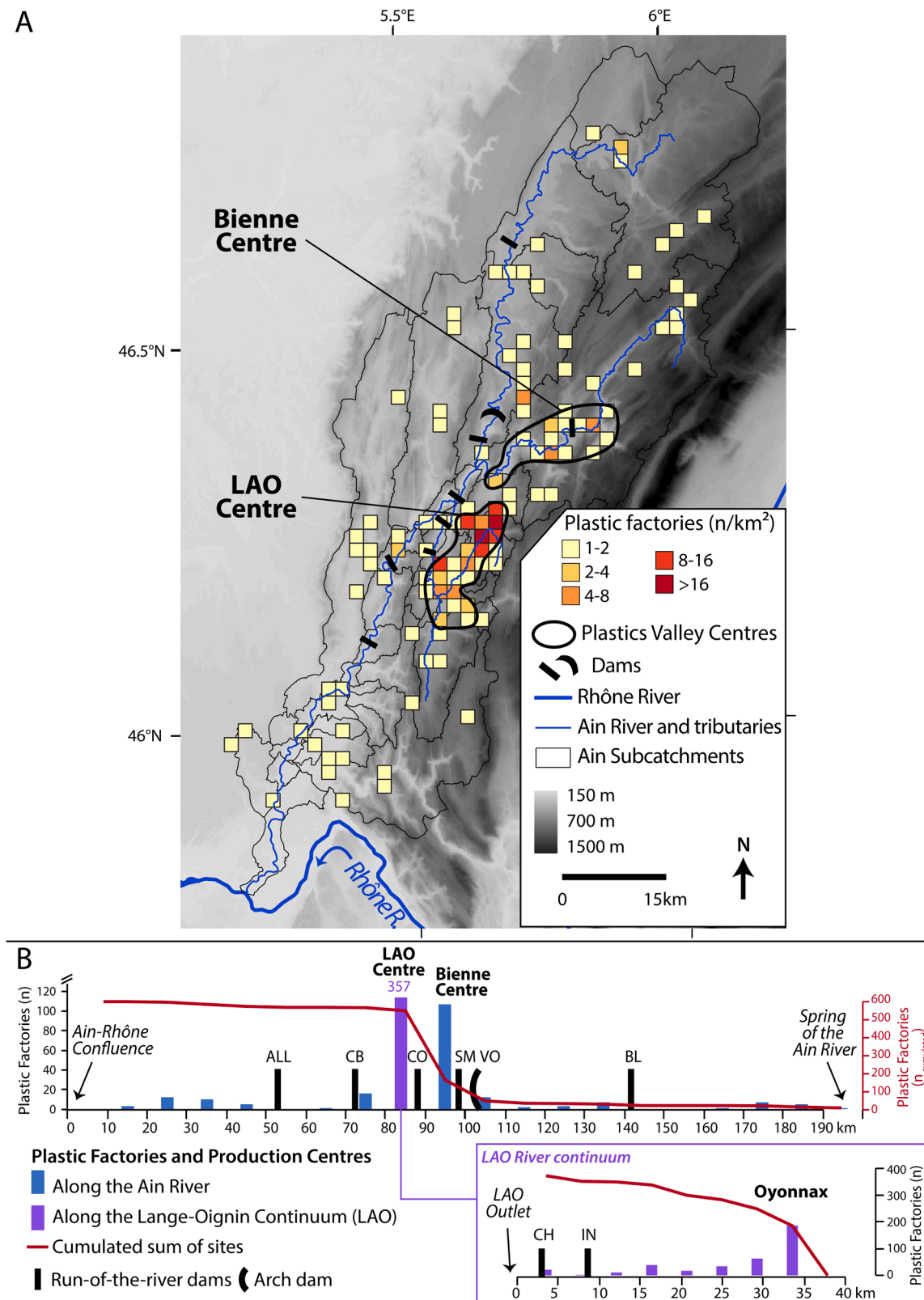


Fig. 2. Plastic factories along the Ain River catchment area: A) Density map; B) Distribution along the Ain River continuum. Dams: ALL = Allement, BL = Blye, CB = Cize-Bolozon, CH = Charmines, CO = Coiselet, IN = Intriât, SM = Sault-Mortier, VO = Vouglans.

This centre comprised 71% of the plastic factories ($n = 393$; average density (d) = 6 factories/km²). The Bienne River also hosted a high density of manufacturing sites with 26% of the listed plastic factories ($n = 145$), with a main centre located between Saint-Claude and Dortan (n

= 45; $d = 3$ factories/km²; Fig. 2-A). In the other parts of the watershed, plastic factories were scattered over the most upstream section near Champagnole and Foncine-le-Bas in the High Jura region ($n = 14$) to the middle and lower sections (27 plastic factories between Clairvaux-les-

Lacs and Moirans-en-Montagne, 30 factories in the Lower Ain River). Following the expected links between the distribution of plastic factories and the MPs contamination, MPs concentrations in sediments are expected to be higher along the LAO and Bienne Rivers than in other sites.

3.2. Spatial distribution of MPs in streambed sediments

The quantification of MPs in streambed sediment samples revealed high numbers of MPs fragments in the finest sediment fraction, i.e. between 20 and 500 μm (Fig. 3). Sediments taken in the Plastic Valley along the LAO and Bienne Rivers (MAR10 to DOR3 sites) had the highest MPs concentrations both in the sediment_{sample} (Fig. 3-A) and in the sediment_{environment} estimates after the grain size correction (Fig. 3-B; see also Table A.5). The main hotspot was MAR10 (ca. 4000 MPs/kg dw) located downstream of the Oyonnax conurbation and its WWTP (Table A.6). Along the "Oyonnax Plastic Valley", MPs concentrations varied between 609 and 1070 MPs/kg dw, while the site located at the outlet of the "Bienne Plastic Valley" (DOR3) also presented 1287 MPs/kg dw. Such concentrations are quite high for coarse-bed river sediments and only a few studies reported comparable concentrations: 1971 ± 62 MPs/kg in sediments from the Nakdong River in South Korea (Eo et al. 2019), or 228 to 3763 MPs/kg in sediments from an urban-industrial section of the Main River in Germany (Klein et al. 2015). Concentrations lower than 600 MPs/kg in general have been measured along other urban-industrial rivers in Canada (Vernaire et al., 2017), in the United Kingdom (Tibbetts et al., 2018) or in Asia (Ding et al., 2019; Sarkar et al., 2019; Wu et al., 2020). Another recent research on the Loire River revealed a median of 1500 MPs/kg (maximal concentration of 7800 MPs/kg) in coarse river deposits (Dhivert et al., 2022). As demonstrated by Drummond et al. (2022), MPs significantly accumulated in the HZ of small rivers - such as the Bienne and LAO Rivers. Such concentrations found in the coarse environments of the Ain River will contribute to the total contamination of the Rhône River by plastic particles (Castro-Jiménez et al., 2019).

Interestingly, MPs concentrations varied strongly along the "Oyonnax Plastic Valley" (Fig. 3) and no uniform decrease was highlighted downstream of the Martignat hotspot (MAR10). It suggests that MPs in the HZ sediment were more likely associated to local contaminations and not due to a unique transport downstream of the Martignat hotspot. For instance, MPs found at PER5 could have been released from the Géovrissiat-Nantua WWTP, from the drainage waters and the runoff

from the Izernore landfill and industrial zone. Such MPs behaviour has been modelled along the Dommel River (Netherlands) where the authors underlined that micro- and millimetre-sized plastics were preferentially retained in the sediment at short distances from their sources (Besseling et al., 2017).

Downstream of PER5 and DOR3 sites, sediments enter in the most intensively managed section of the Ain River with several run-of-the-river dams and reservoirs. COI4 and ALL2 sites displayed the lowest MPs concentrations (Fig. 3-A and Table A.6). The gap with the MPs concentrations observed upstream increases when normalised by the grain size (Fig. 3-B), as LOA and Bienne sediments were richer in sands, silts and clays (SSC) than the Middle and Lower Ain River sections (see Table A.5 for summary). The link between MPs and SSC was also reported along the Wei River, and provided details about the MPs bearing phase (Ding et al., 2019).

Finally, the "Coiselet dam area" - 85 ± 39 km upstream of the Ain-Rhône confluence - was identified as a breakpoint in the MPs data series. It gives a rough location of a change in the MPs distribution and implies that hydro-sedimentological changes due to the presence of dams influenced the MPs distribution. The uncertainty associated with this spatial change was very large (40% of the river length), but this change referred without doubt to the river section from the arch dam of Vouglans to the run-of-the-river dam of Allement (ALL).

After this breakpoint, very low MPs concentrations were measured (250-350 MPs/kg dw). The Lower Ain River has a higher flow rate ($46\text{--}53 \text{ m}^3/\text{s}$) and a greater stream width than upstream (100 m on average; see Table 1). In comparison with upstream sites, the potential storage area in the river bed increases and the flow of small tributaries carrying MPs is diluted into larger volumes. Such dilution mechanism has been already demonstrated by van Emmerik et al. (2018) who highlighted a decrease in MPs concentrations when the discharge increased along the Saigon River (Vietnam). This mechanism also suggests a relatively constant amount and mass of plastic diluted along the Ain River and limited local plastic sources.

3.3. MPs chemical properties and characteristics along the Ain River

Based on MPs types, three groups of sites were clearly identified along the river (Fig. 4).

A first group comprised the Upper Ain (CHA1) and the Plastic Valley sites (MAR10 to DOR3), with mainly PP (42% on average), PE (27%)

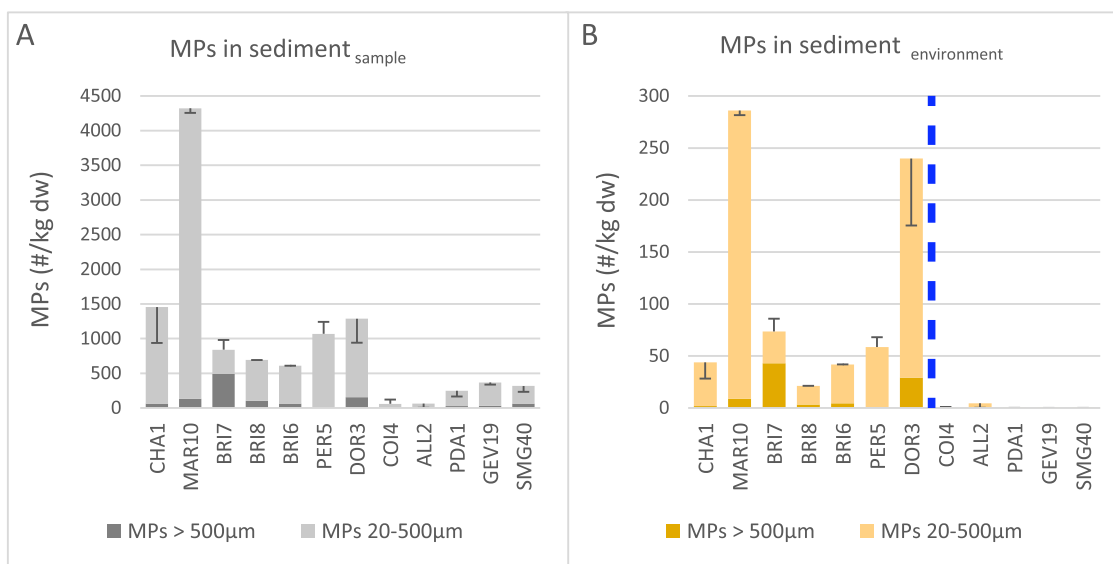


Fig. 3. Microplastics (MPs) concentrations (A) in sediment samples, (B) in the river environment after the grain size normalisation. The blue dashed line in the part B refers to the significant breakpoint identified after grain size correction at 85 ± 39 km upstream of the Ain-Rhône confluence. Error bars consider potential MPs (EVA-PEc-Rubber) in the positive direction, and MPs from negative control samples in the negative direction.

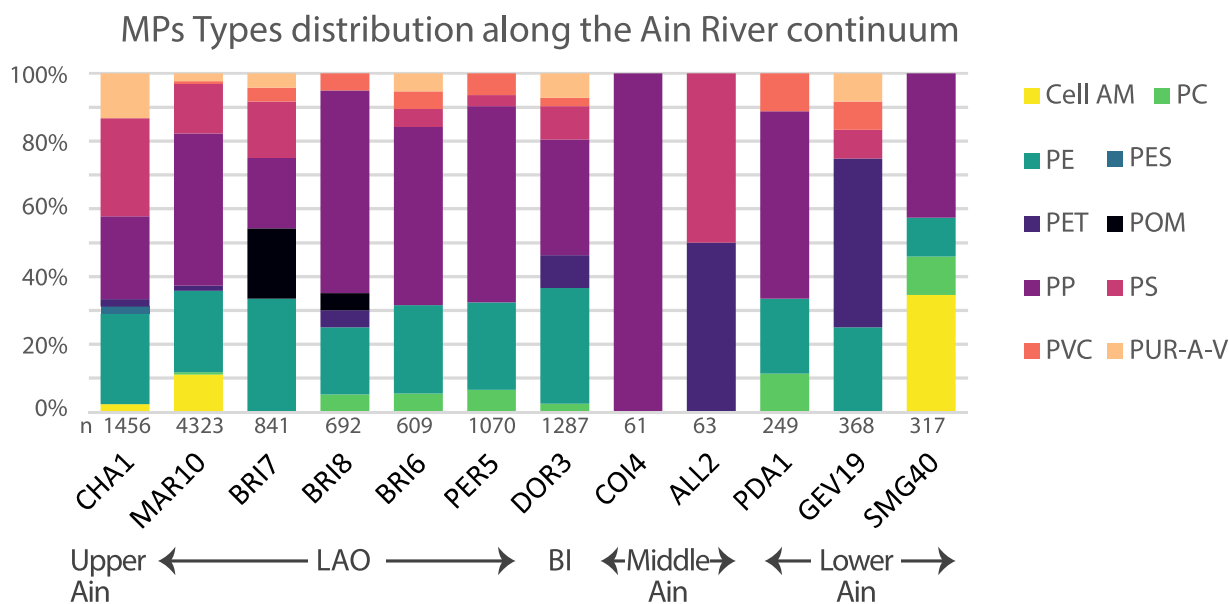


Fig. 4. MPs type distribution along the Ain River continuum. Locations are mentioned at the bottom of the figure: UA = Upper Ain River (CHA1), LAO River = Lange-Oignin sites (MAR10 to PER5), BI = Bienne River outlet (DOR3), MA = Middle Ain River (COI4 and ALL2), LA = Lower Ain River (PDA1, GEV19, and SMG40). Plastic types: Cell AM = Cellulose artificial / modified; PC = polycarbonate; PE = Polyethylene; PES = Polyester; PET = Polyethylene terephthalate; POM = Polyoxymethylene; PP = Polypropylene; PS = Polystyrene; PVC = Polyvinylchloride; PUR-A-V = acrylates/polyurethanes/varnish.

and PS (11%). Such polymers are frequently found in household and food packaging and toys (see for instance <https://plasticoceans.org/7-types-of-plastic/>). The PP-PE-PS triad is well highlighted in other urban-industrial environments; however, the dominance of PP is rather rare. Near Busan (South Korea), the high PP level in the freshwater sediment is interpreted by the authors as a consequence of hydro-sedimentary processes in relation with the polymer densities (Eo et al., 2019). Hydro-sedimentary processes associated with the intrinsic density of polymers have been also interpreted as one of the main factors controlling the MP distribution along the Villerest dam on the Loire River (Dhivert et al., 2022). While, near Durban (South Africa), Govender et al. (2020) link the relative importance of PP in river sediments to urban-industrial releases. Along the Ain River (present study), sediments of group 1 were collected along urban-industrial stretches of the Plastic Valley, which is specialised in manufacturing hard plastics. A likely hypothesis is to consider this MPs assemblage as an industrial heritage, as also evidenced hereafter by statistical analyses (see Section 3.4).

Within group 1, several sites also contained (i) artificial or modified cellulose at CHA1 and MAR10 (2 to 11%). (ii) A significant part of POM - polyoxometalate - at BRI7 and BRI8 (5 to 21%), i.e. near the Lange-Oignin confluence, which might be explained by the local presence of high technology factories (POM being used in electronic circuit boards, for instance). (iii) PC - polycarbonate - downstream of the Lange-Oignin confluence (2 to 6%) which is mainly used in safety glasses, electronics and automobile parts. Polyester polymers (PES/PET), PVC and the PUR-A-V group were also found in low amounts at the sites of group 1. The presence of CHA1 in this group, which is a more rural area, could be explained (1) by the potential influence of plastic factories found near Champagnole, i.e. upstream of CHA1, and (2) by releases from one the biggest WWTP of the river corridor located at a short distance upstream of the sampling site (see Fig. 2).

A second group was identified on the Middle Ain River and characterised by PP (COI4), PS and PET (ALL2). However, as shown in Fig. 3, this group only presented small concentrations of MPs ($n = 124$) and thus it should be considered with caution, or eventually as a sub-group (Table A.6). These low MP concentrations in the HZ of COI4 and ALL2 were probably due to their location between two reservoirs. Dams are known to attenuate sediment supply to downstream locations (Skalac

et al., 2013) and may also limit MPs supply.

A third group is represented by the three Lower Ain sites (Fig. 4). Samples from these sites were characterised by the prevalence of PP and a significant proportion of PE (20% to 33%). Artificialized or modified Cellulose (Cell AM) and PC were also found locally in variable abundance. Apart from this clustering, it is also worth to note that PET fragments were also regularly found at some sites along the Ain (ALL2, DOR3, GEV19, MAR10, BRI8).

3.4. Which factors can potentially explain the MPs distribution along the river?

Multiple linear regressions were run to determine which factors could influence the observed MP distribution along the Ain River. Different combinations of variables that could influence the MPs concentrations were tested, including land use at each sampling site (urban, industrial and transport network areas, arable lands, pasture, moorlands, landfills and quarries, water recreation areas), the number of plastic factories located 10 km upstream of the sampling site, and the nameplate capacity of the closest WWTP. According to this test, the most significant variables were: urban areas (km^2) and the number of plastic factories (Table 2). It was surprising that the presence of river obstacles (dams and weirs >1 m high) was not significant in the analysis. It was probably due to a low statistical value of river barriers which are few and very limited in the dataset. Other parameters, not considered in the present study, such as hydro-geochemical processes and geological changes, affecting the erosion-deposition-transport processes, might also have influenced MPs concentrations in sediments, as demonstrated by Richards et al. (2022) along the Ganga River (India).

To go further about the understanding of the factors explaining the MP distribution along the river, a redundancy analysis (RDA) was

Table 2
Most significant coefficients resulted from the multiple linear regression.

| | Estimate | Std. Error | T value | P-value |
|------------------------------------|----------|------------|---------|---------|
| (Intercept) | -67.35 | 30.27 | -2.23 | 0.05 |
| Urban areas | 2.67 | 0.88 | 3.04 | 0.01 |
| Plastic factories (10 km upstream) | 0.63 | 0.21 | 2.90 | 0.02 |

performed to determine whether the distribution of MPs polymer types could be related to the above-cited land use or hydro-sedimentological settings (Fig. 5). According to this analysis, 64% of the total variance was contained in the two first axes of the RDA (axes RDA1 and RDA2). Plastic factories (10 km upstream of the sampling site) and pastures were the most significant variables according to the RDA analysis ($p < 0.05$). The other variables located on the right side of Fig. 5 influenced the community matrix at a secondary level: SSC, industrial areas and transport networks (Indus_T) in the upper quadrant, and urban areas in the lower quadrant. Three main patterns were observed: (i) PP, PE, and Cell AM were displayed together on Fig. 5 and thus found on common sites, close to plastic factories, industrial zones and transport networks - the most suitable site was MAR10; (ii) PS, PET, and PUR_A_V were associated with urban-Industrial zones and transport networks, these polymers could also be found on pastures in association with PES at CHA1; (iii) PVC, PC and POM which were among the densest polymers identified in this work seemed largely linked to the SSC proportion. The sites that are the closest in Fig. 5 presented most similar MPs assemblages: on the one hand, BRI6-BRI7-BRI8-PDA1-SMG40, and on the other hand, COI4-ALL2-GEV19. Overall, these results provided interesting insights about the contributions of local MP inputs and sources on MPs contamination of riverbed sediments.

4. Conclusions

This research proposed a cross approach combining field measurements (hydro-sedimentary settings), laboratory analyses (grain size, MPs concentrations and types) and socio-environmental information (land use, plastic factories, WWTP nameplate capacity, river flow obstacles) to assess key factors explaining the distribution of MPs accumulated in the hyporheic zone of bar heads along coarse-bed rivers.

As most of MPs was contained in the 20-500 μm fraction, a grain size-based correction was used to discuss the MPs distribution along the river. The sediment sampled in the hyporheic zone (HZ) from gravel bars along the middle section of the Ain River, especially LOA and Bienne Rivers, presented the highest MPs concentrations (up to 4400 MPs/kg dw). This part of the valley corresponds to an urban-industrial corridor, specialised in hard plastic production. MPs seem to enter in the hydro-system at various places along the “Oyonnax Plastic Valley”, as suggested by a large variability in concentrations and types. A significant breakpoint was identified at the end of the Middle Ain River section, where low MPs concentrations were found in the HZ sediment. This breakpoint matched with the presence of major dams. The role of the dams regarding MPs transport and pathways can be questioned because most of them are run-of-the-river dams, while only few structures

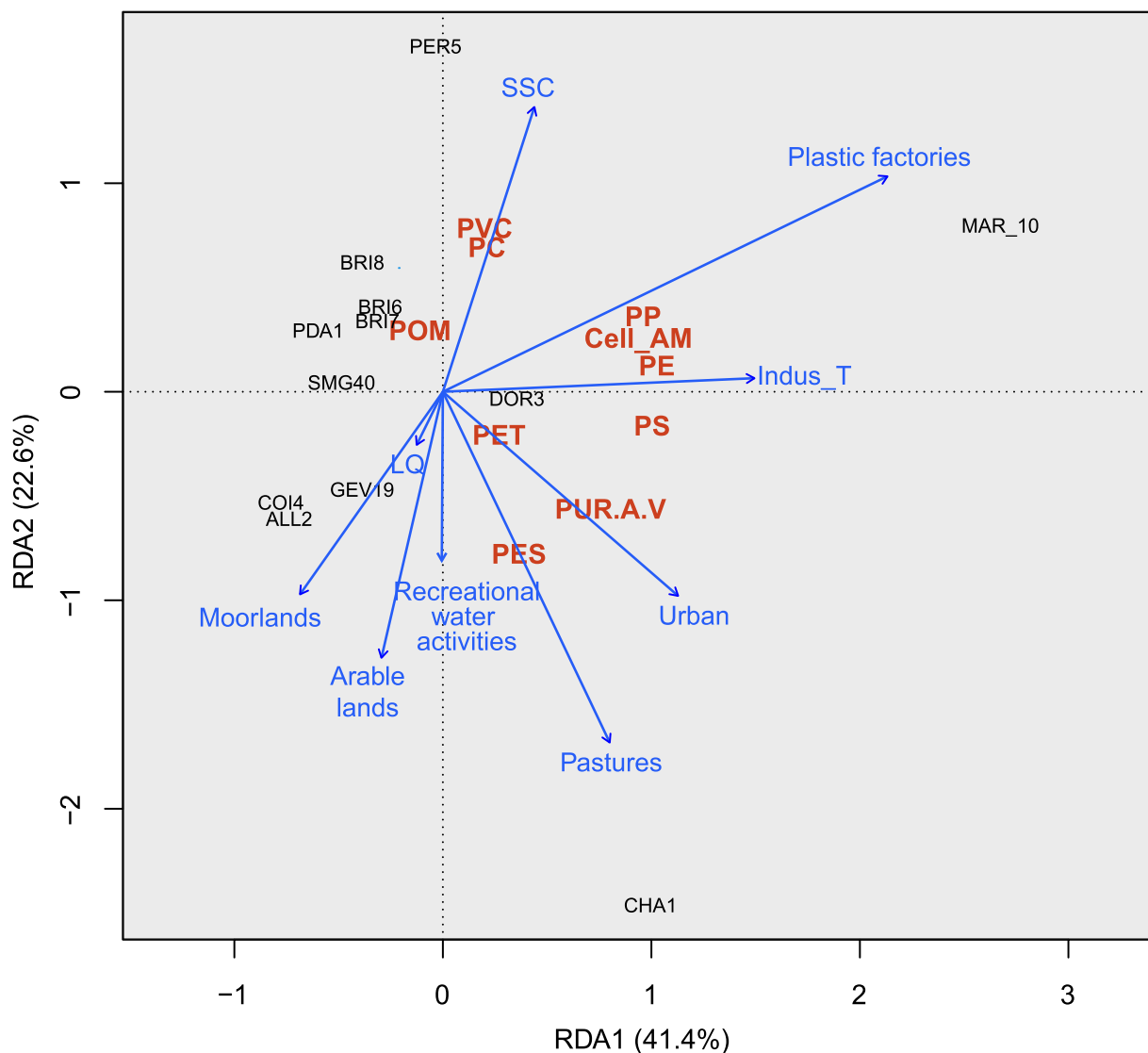


Fig. 5. Redundancy analysis plot achieved after scaling and centring of the data. The two first axes RDA1 and RDA2 explain 41.4% and 22.6% of the total variance, respectively. Individual sites mentioned in black capital letters are given for information. LQ = Landfill and Quarries.

constitute major flow and sediment obstacles, such as Vouglans and Charmines dams. In addition, the decrease in MPs concentrations also occurred where the river bed widens and suggested a dilution effect in the most downstream sections. Thus, the decrease of the MPs distribution along the Ain River corridor was most certainly driven by a combination of factors.

Plastic types were dominated by PP, PE and PS (in order of importance) along the Ain River. The dominance of PP is rare worldwide and can be explained by local industrial history as the Lange-Oignin and the Biemme valleys were specialized in the manufacturing of hard plastics. Moreover, the influence of plastic factories and urban areas was emphasised by the multivariate analysis.

Finally, the methodology developed in the present research (i.e. sampling of HZ sediment in mobile gravel bars along a coarse-bed river) underlined the interest to address, in the future, MPs stocks in other coarse-bed rivers with plastic production centres and/or dams for generalisation purposes.

Supplementary materials (details)

The supplementary material available online contains one plate (Fig. A.1) and six additional tables (Tables A.1 to A.6):

Fig. A.1: Photographs of the sampling sites.

Table A.1: Field measurement detailed for each sampling site.

Table A.2: Quality control based on spiked samples.

Table A.3: Results of the quality control based on negative controls

Table A.4: Plastic types observed in this work and accepted probability thresholds (ProbThreshold).

Table A.5: Summary of the grain size results on the sediment coming from the HZ (20 cm deep).

Table A.6: MPs concentrations in the samples (#/kg dw).

Declaration of Competing Interest

None

Data availability

Data will be made available on request.

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Supplementary materials (availability)

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.watres.2022.119518](https://doi.org/10.1016/j.watres.2022.119518).

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