The distribution and effects of microplastics in freshwater ecosystems

A thesis submitted for the degree of Doctor of Philosophy

by

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Summary

Plastic is essential in modern society, yet over-use and improper disposal have increased microplastic pollution dramatically over the past century. Microplastics pose unique physical, chemical, and biological threats to the environment, with particular concern for freshwater ecosystems due to their proximity to microplastic sources, importance for biodiversity, and role in human well-being. Despite growing research on microplastic distribution, load, and ecological effects in freshwater systems, substantial gaps remain. Major challenges include: (i) both the dynamic nature of freshwater ecosystems and complex behaviour of microplastic particles; (ii) poorly resolved interactions between microplastics and organisms over space and time; and (iii) inconsistencies in research methodologies.

This thesis empirically assesses some ecological risks of freshwater microplastic pollution at the global, catchment, and reach scale in four steps. First, published studies are reviewed to identify trends in microplastic distribution in freshwater ecosystems across a hierarchy of spatial and temporal scales. Next, methodologies to sample, extract, quantify and characterise microplastic are evaluated to determine how varying protocols might influence estimated loads and trends. Third, recommended protocols are used to sample microplastic comprehensively across the whole River Taff catchment, Wales, as a model river system with varying land use. Lastly, ecological interactions and impacts of microplastic from point sources are assessed experimentally in field stream mesocosms.

Globally, the results reveal that freshwater microplastic pollution is associated with urban sources and poor waste management, though variations amongst sources and hydrodynamics lead to site-specific exceptions. The review of methods reinforces the need for harmonised protocols. In the model catchment, sampling shows the widespread but patchy distribution of microplastic in freshwater sediment and invertebrates. At the reach-scale, microplastic addition can have limited ecological effects either reflecting limited interaction with organisms or limited immobilisation of microplastic under natural stream conditions. These novel findings contribute toward improved risk assessment.

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Chapter 1: General Introduction

1.1 Introduction

Plastics are useful and ubiquitous synthetic materials that benefit humans in modern society. Derived mostly from fossil fuels, plastics are formed by linking carbon, hydrogen, and oxygen monomers into long polymer chains. Around twenty chemically distinct polymers serve as fundamental building blocks, including polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC), which are combined with additives to achieve specific properties. Additives include fillers, hardeners, flame retardants, UV and thermal stabilisers, antimicrobial agents, surfactants, synthetic dyes, and pigments, creating over 80,000 plastic formulations available commercially (Andrady 2015). Since their invention early 20th century, plastics have radically changed society. The material can be moulded into virtually any shape, enabling their use in a wide range of applications across industries including packaging, construction, electronics, healthcare, and transportation (Andrady and Neal 2009). Plastics' durability ensures products have a long lifespan and perform reliably in diverse environments. Lastly, their light weight and cost-effectiveness compared to alternative materials like metals or glass, reduces their carbon footprint, energy consumption, and costs associated with transportation and production (Amienyo et al. 2013; Stefanini et al. 2021). Yet, these precise advantages bring some equally harmful pitfalls.

Plastic production has burgeoned since mass production began in the 1950s, reaching an annual total of 400.3 million metric tonnes (Mt) in 2022, with China alone responsible for a third of this output (PlasticsEurope 2023). Concomitantly, the worlds annual plastic waste production was 353 Mt in 2019, 22 Mt of which leaked into the environment through inadequate waste collection and disposal (OECD 2022). This leakage is estimated to rise to 90 Mt/year under 'business as usual' by 2030 or 53 Mt/year even if ambitious mitigation commitments are met by governments (Borrelle et al. 2020). Most plastics do not biodegrade easily and instead, may persist in the environment for hundreds to thousands of years (Chamas et al. 2020). Degradation through mechanical, chemical, and biological processes produces a range of material categorised by size (macro-: > 25 mm, meso-: 5-25 mm, micro-: 0.0001-5 mm, and nanoparticles: < 100 nm) and shape

(fragments, pellets, spheres, films, and fibres) (Arthur et al. 2009; GESAMP 2015). While plastic particles of all size classes carry environmental risks (Jâms et al. 2020), the smaller micro- and nano-scale size fractions have attracted particular interest, in part because they account for over 90% of plastic waste in the marine environment (Eriksen et al. 2014). Microplastics are classified into two broad groups: primary microplastics manufactured at this size, such as nurdles and microbeads for cleaning products and cosmetics (Zitko and Hanlon 1991), air blasting, and medicinal drug vectors (Patel et al. 2009); and secondary microplastics degraded from larger plastic material. Microplastic has widespread documented presence across all environments (Akdogan and Guven 2019; Windsor et al. 2019a), including air (O'Brien et al. 2023), soil (Yang et al. 2021), and water bodies (Du et al. 2021). It has even been detected in the deep ocean (Van Cauwenberghe et al. 2013), on the highest mountain (Napper et al. 2020), and inside the human body (Ragusa et al. 2021).

Microplastic pose physical, chemical, and biological threats to organisms and ecosystems. Physically, microplastic may entangle small organisms including invertebrates and cause gut obstruction when ingested, which can limit energy intake and produces inflammatory and stress responses (Wright et al. 2013a; Wright et al. 2013b; Steer and Thompson 2020). Chemically, microplastic may act as a source of metal ions and persistent organic pollutants (POPs) from their incorporated additives, degradation products, or chemicals sorbed from the environment (Teuten et al. 2007; Andrady 2011; Vieira et al. 2021). This leads to non-toxic and toxic effects, for example through endocrine, carcinogenic, and mutagenic pathways (Nobre et al. 2015; Hermabessiere et al. 2017; Li et al. 2018; Verla et al. 2019). Biologically, microbial communities can colonise microplastic surfaces and change the likelihood of their ingestion (Zettler et al. 2013; Wang et al. 2021c), while facilitating gene-exchange and antimicrobial resistance (Arias-Andres et al. 2018; Yang et al. 2019b). Consequential toxicity and impacts on individual growth and performance (Anbumani and Kakkar 2018) threatens populations and trophic-level energy transfer, potentially reducing the function of communities and ecosystem (Ma et al. 2020b; Ockenden et al. 2021; Amaneesh et al. 2023). Conversely, some literature suggested potential benefits of environmental microplastic by diluting POPs

in aquatic environments and reducing their bioaccumulation within organisms (Besseling et al. 2013; Koelmans et al. 2013; Wang et al. 2020c).

This high abundance, widespread environmental presence, and potential risks create global concern for microplastic pollution. Research has historically focussed on the marine environment (e.g., Cole et al. 2011; Ivar do Sul and Costa, 2014; Alomar, Estarellas and Deudero, 2016), encompassing 87% of peer-reviewed publications from 1980 to May 2018 (Blettler et al. 2018). However, freshwater ecosystems, including lakes, ponds, rivers, streams, and wetlands, act as both major sinks (Free et al. 2014; Nava et al. 2023) and conduits of microplastic from the terrestrial to marine environment (Rech et al. 2014; Lebreton et al. 2017; Meijer et al. 2021). Suggested sources to freshwaters include landfill and urban area runoff, Wastewater Treatment Plant (WWTP) and sewage system effluent, agricultural runoff (equipment and machinery, sewage sludge application), tyre wear particles (TWPs), industry pollution, and atmospheric deposition of airborne particles (Wagner and Lambert 2018). Freshwater ecosystems are highly important, being hotspots of biological diversity (Dudgeon et al. 2006) and providing critical ecosystem services including provision of drinking water, flood/erosion buffering, energy production, and cultural values (Postel and Carpenter 1997). This has led to a recent surge in freshwater microplastic investigation (Sarijan et al. 2021). Furthering our understanding of microplastic dynamics in freshwater environments is therefore relevant to preserving functions of freshwater ecosystems as well as cross-ecosystem subsidies.

Notable research gaps in freshwater microplastic pollution include the limited understanding of microplastic sources and transport mechanisms into and within freshwater ecosystems. Addressing this knowledge gap requires detailed studies on the occurrence and distribution of microplastics across different spatio-temporal scales to capture the dynamic and complex nature of both freshwater ecosystems and microplastic particles. Furthering our understanding of microplastic transportation and interaction with flow dynamics is crucial for predicting their unique distribution and accumulation in different freshwater systems, which will inform risk assessments and the development of effective management and mitigation strategies. The impact of microplastics on freshwater ecosystems also warrants more attention. Research is needed to understand how microplastics affect the

health and behaviour of freshwater organisms that are crucial to ecosystem function. Environmentally relevant conditions are essential to bridge the gap between laboratory-based studies and real-world scenarios, ensuring that research on microplastics accurately reflects the complex interactions and impacts in natural freshwater systems. Additionally, the interplay between microplastic pollution and other environmental stressors, such as climate change and shifts in water quality, requires further investigation to understand how these factors compare to or influence microplastic distribution and effects. Lastly, the effect of inconsistent and unstandardised methods for extracting, quantifying, and characterising microplastics is poorly understood.

1.2 Aim of the thesis

The overarching aim of this thesis was to identify sources, fluxes, and endpoints of microplastic pollution in freshwater ecosystems at different spatial and temporal scales, while assessing the influence of methodological technique. The overall thesis hypotheses are: (i) microplastics occur widely in freshwater ecosystems from the local to the global scale, and interact with organisms to affect individuals, communities, and ecological processes; and (ii) clarity over understanding these effects is affected by methodological challenges. Data were collected through systematic literature reviews, secondary datasets, and primary data collection from the field and constructed mesocosm experiments. Analyses ranged in spatial coverage from global freshwater ecosystems to individual catchments (e.g., River Taff, South Wales) to local habitats (e.g., stream leaf litter). The thesis is divided into several chapters for which, the background, aims and objectives are as follows.

Chapter 2: Bridging the gap between the spatio-temporal distribution of microplastics in freshwater ecosystems and biological exposure.

Data on both spatial and temporal trends of microplastic in freshwater ecosystems are scarce, especially across different hierarchical scales. This chapter, therefore, provides systematic review and meta-analysis of field data on microplastic in freshwater matrices to assess spatial patterns at local, catchment and global-scales, and temporal trends over hours to years. Analysis includes geographical links

with economic development and land-use, as well as evaluation of abiotic and biotic variables that influence reported microplastic loads and characteristics. By summarising current research and identifying knowledge gaps, the synthesis presented provides context and reasoning for subsequent primary data chapters.

Chapter 3: A global review and analysis of microplastic extraction and analytical methods in freshwater ecosystems.

Wide variation in reported microplastic concentrations within the freshwater environment creates concern about the influence of research methodology on reported loads. Moreover, lack of methodological standardisation complicates the determination of the most effective and accurate protocol. This chapter, therefore, builds and complements *Chapter 2* by appraising techniques to sample, extract, and characterise microplastic particles from different freshwater matrices. The analysis enables the evaluation of advantages and disadvantages to different equipment and protocols, leading to recommendations for best practice. The synthesis presented in this review provides methodological context for subsequent primary data chapters as well as summarised criteria to identify and characterise microplastic particles.

Chapter 4: Microplastic in the sediments and invertebrates of an urban river system.

Stratified sampling of microplastic distribution across whole freshwater catchments is limited, considering the variability in spatial trends of freshwater microplastic and effects of site-specific hydrodynamics investigated in *Chapter 2*. Moreover, freshwater microplastic research is biased towards the water column, reducing understanding of factors influencing contamination of sediment and biota. This chapter sampled microplastic contamination in sediment and invertebrates at 38 sites across a whole river catchment, using the River Taff in Wales as a model. This aims to identify trends in microplastic distribution from upstream to downstream and associations with land-use variables, whilst attempting to identify major point sources. Sampling four different feeding guilds of macroinvertebrates enables the investigation of microplastic entry into freshwater food webs, with the aim to identify microplastic bioindicators. Sediment sampling will provide further evidence into its role as a microplastic sink in freshwater ecosystems.

Chapter 5: Comparing methods to extract microfibres from leaf litter decomposing in fresh water.

Lack of standardisation in methods to sample freshwater media and extract, quantify, and characterise microplastic makes experimental design difficult when investigating microplastic loads in poorly researched media. This chapter develops, describes, and compares methods to extract microplastic from submerged leaf litter. The aim is to identify methodological pitfalls and validate methods through microplastic recovery assessment. Addressing this research gap is important in the context of the thesis and in particular, methodological development to support assessment of microplastic capture by submerged leaf litter in *Chapter 6*.

Chapter 6: Microplastic addition has minimal effects on invertebrate communities or litter decomposition in stream mesocosms of differing pH.

Research into the effects of microplastic on freshwater ecosystems has primarily been conducted on individual organisms in controlled, single-species laboratory experiments with unnatural microplastic concentrations. Concern is growing over increased release from either wastewater treatment or spills from Combined Sewer Overflows (CSOs), where microplastic is not removed from wastewater. This chapter investigates the interaction of microplastic from point and pulse sources with allochthonous material and aquatic invertebrates, with the aim to assess potential effects at the population (macroinvertebrate density), community (diversity; abundance of different feeding guilds), and ecosystem level, specifically affecting ecosystem processes (leaf litter decomposition). By utilising field mesocosms at contrasting pH, the aim is to create environmental realism. Addressing this research gap in association with pH supports environmental risk assessments and effective management when multiple stressors are at play.

Chapter 7: General discussion.

The final chapter synthesises the outcomes from *Chapters 2-6*. This discusses how microplastic distributional trends and knowledge gaps were identified and research methods were synthesised, to inform catchment-scale and local-scale assessment of microplastic fate, flux, and effect in running freshwater ecosystems. Research design is reviewed to draw upon general caveats of thesis results and

comment on the potential efficacy of environmental microplastic monitoring to inform regulation and policy. The chapter finishes by discussing future research directions that stem from this thesis, including the required harmonisation of microplastic extraction methodology and necessary developments required to understand fully the risk presented by microplastic to freshwater ecosystems.

Chapter 2: Bridging the gap between the spatio-temporal distribution of microplastics in freshwater ecosystems and biological exposure

2.1 Abstract

Although evidence of microplastic transport in freshwater ecosystems is growing rapidly, available data are fragmentary. This chapter presents a quantitative review of microplastics in freshwater ecosystems from 300 field studies to appraise (i) spatial patterns at local, catchment, and global-scales and (ii) temporal trends over hours to years. This review extends previous work in using a uniquely hierarchical approach in space and time to identify research needs, as well as improving our understanding of organism exposure.

Reported microplastic concentrations in water and sediment are aggregated in distribution and range over eleven orders of magnitude. Values are greater in urban regions and in less developed compared to more developed countries, but data are skewed to the global North and patchy across spatial scales. Temporal effects such as hydrological events influence microplastic concentrations, such as the increase in concentration during winter, but have been assessed in less than a quarter of available studies and seldom over complete hydrological events. Over both space and time, microplastic concentration in freshwater follows one of two opposing mechanisms: (1) resuspension and (2) dilution, respectively increasing or decreasing with greater water flow. Microplastic data from freshwater organisms are limited and biased towards fishes. Studies indicate the lowest microplastic abundance per individual in bivalves and greatest in ray-finned fishes, and suggests a rise in microplastic abundance per unit mass with increasing body size. Multiple biotic variables have varying influence on microplastic uptake across biota, which may change over space and time, yet more research is needed to further this understanding.

Integrated, interdisciplinary studies are needed to link microplastic distribution to biological exposure including: (i) micro-distributional patterns where organisms occur; (ii) catchment-scale variations; (iii) temporal dynamics from hydrological events to interannual trends; and (iv) interactions with other pressures, for example

climate change. Gaps for freshwater biota include: (i) variations in exposure across levels of organisation; (ii) effects of contrasting life-history traits; (iii) characterisations of polymer types and additives most likely to affect organisms; (iv) the role of organisms as biological indicators; and (v) microplastic effects on ecological processes. Previous interdisciplinary studies of other freshwater stressors at multiple scales provide models to advance understanding on freshwater microplastic pollution.

2.2 Introduction

Microplastic has widespread documented presence across all environments (Akdogan and Guven 2019; Windsor et al. 2019a), persisting for hundreds to thousands of years (Chamas et al. 2020) and posing physical, chemical, and biological threats to organisms and ecosystems (Issac and Kandasubramanian 2021; Dissanayake et al. 2022). Research into microplastics has increased rapidly over the last decade, but there is still considerable imbalance both across and within ecosystems in knowledge of their occurrence, behaviour, and potential effects. Within the aqueous environment, most literature focused on marine ecosystems (e.g., Galloway et al. 2017; Tsang et al. 2017; Duncan et al. 2019). More recently, attention has turned to standing and running freshwater ecosystems for three principal reasons. Firstly, their proximity to terrestrial sources of microplastic (Auta et al. 2017) means freshwater ecosystems are at significant risk of microplastic pollution. These include point sources of industry outflows, Wastewater Treatment Plants (WWTPs), and Combined Sewer Overflows (CSOs) (Woodward et al. 2021), that can be traced to a single origin. Microplastics are also easily transported into freshwater by wind or surface water runoff (Windsor et al. 2019a). These diffuse sources include sewage sludge (Hatinoğlu and Sanin 2021), plasticulture (Mormile et al. 2017), litter, urban dust, and tyre wear (Wagner et al. 2018). As a result, standing waters can be significant stores of microplastic (Vaughan et al. 2017), while flowing waters could contribute up to 80% of the plastic entering marine systems (Cole et al. 2011; Jambeck et al. 2015; Akdogan and Guven 2019; Lebreton and Andrady 2019). Secondly, physico-chemical conditions differ between freshwater and marine environments in ways that influence microplastic occurrence and behaviour. Differences in salinity (<1% and ~3.5%, respectively), water density (1 g/ml and

1.025 g/ml, respectively), extent, depth distributions, bed substrata, hydraulic character and currents may all affect plastic buoyancy and dispersion, while differences in temperature and UV penetration are likely to affect plastic degradation (Simpson et al. 2005; Eerkes-Medrano et al. 2015). Thirdly, evidence has accumulated to illustrate how freshwater organisms are now exposed to microplastics either directly (Atici 2022) or through food-web transfer (D'Souza et al. 2020).

Despite the increasing volume of data, however, information on microplastics in freshwater ecosystems is still fragmentary, and few available reviews provide a systemic, quantitative assessment of microplastic distribution (e.g., Cera et al. 2020; Li et al. 2020). Reviews specifically assessing sources, fluxes, fates, and biological interactions involving microplastics are more qualitative (Eerkes-Medrano et al. 2015; Lu et al. 2021; Sarijan et al. 2021). These circumstances prevent the accurate assessment of freshwater microplastic loads, making it difficult to compare different regions or sources and track changes over time. This limits our understanding of exposure risk to organisms and prevents evidence-based management options in freshwater ecosystems.

This systematic, quantitative review of field data on microplastic in freshwater matrices aimed to: (i) summarise current knowledge on the abundance and distribution of microplastic over different spatial and temporal scales, including geographical links with economic development; (ii) evaluate abiotic and biotic variables that influence reported microplastic loads and characteristics; and (iii) review knowledge gaps and deficits in order to recommend future research particularly with respect to organismal exposure. This provides a more quantitative insight than previous reviews that are also based on an array of freshwater microplastic studies (Eerkes-Medrano et al. 2015), and answers seminal calls for expanded effort and prioritisation of microplastic research in the context of freshwaters. In contrast to previous reviews, this work takes an explicitly scale-dependent and hierarchical approach to distribution and exposure patterns in space and time. Lastly, knowledge gaps are identified to provide context for subsequent primary data chapters and future research.

2.3 Methods

2.3.1 Data sources

Literature was collected following procedures recommended by the 'Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA)' approach (Moher et al. 2009, Figure 2.1). Peer-reviewed, primary literature written in English were retrieved from the Web of Science and Google Scholar databases up to 3rd August 2022 by searching titles, abstracts and keywords with combined search terms including 'microplastic*' or 'micro-sized plastic*' or 'micro sized polymer*' or 'synthetic polymer*' and 'freshwater' or 'river' or 'lake' or 'water' or 'sediment', producing 3,059 results. Secondary references from these sources were also searched. These articles were screened, refined, and accepted if they: (i) sampled a freshwater environment (river, stream, canal, lake, pond, reservoir, wetland, and surface runoff); (ii) sampled water, benthic sediment, and/or biota associated with freshwater; and (iii) extracted and quantified microplastic particles (< 5 mm in size and any shape). Studies reporting meso- and macro-plastics or those sampling marine or estuarine environments were excluded. The final sample of 300 articles (Table A1) covered the period 23rd November 2010 (Moore et al. 2011) to 19th September 2022 (Zhang et al. 2022b), with almost three quarters published from 2019 onwards and numbers expected to grow further (Figure 2.2). While this sample cannot be exhaustive given the current rates of publication, it is viewed as a representative sample within the above definitions and at the timepoint prior to subsequent primary data chapters. Limitations also arise from the different definitions of microplastic, including their size and shape (Frias and Nash 2019), while methods of sampling environmental matrices, extracting particles, and identifying polymers also vary between studies (Li et al. 2018; Stock et al. 2019). For example, methods using finer sieves, larger sampling volumes, and/or accurate spectroscopic polymer identification methods would capture more particles, whilst methods of contamination control prevent particle overestimation.

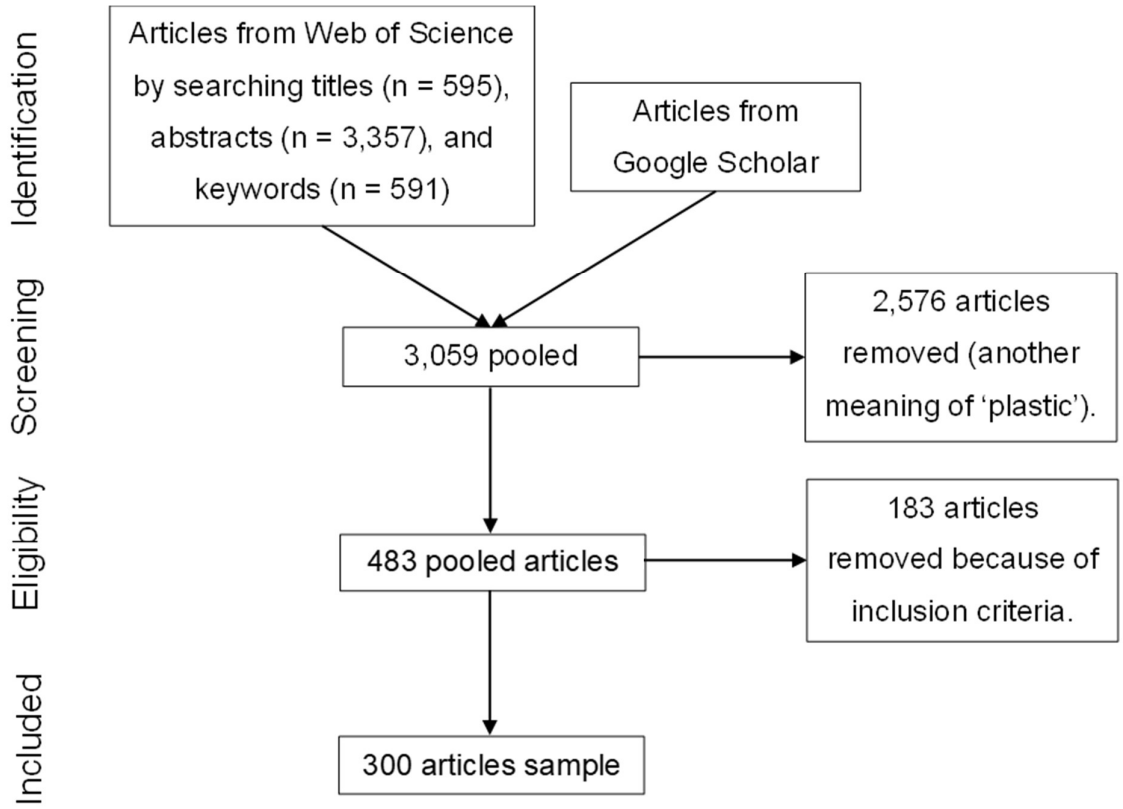


Figure 2.1 PRISMA literature search flow diagram (Moher et al. 2009) stating the number of studies identified, screened, retained and discarded.

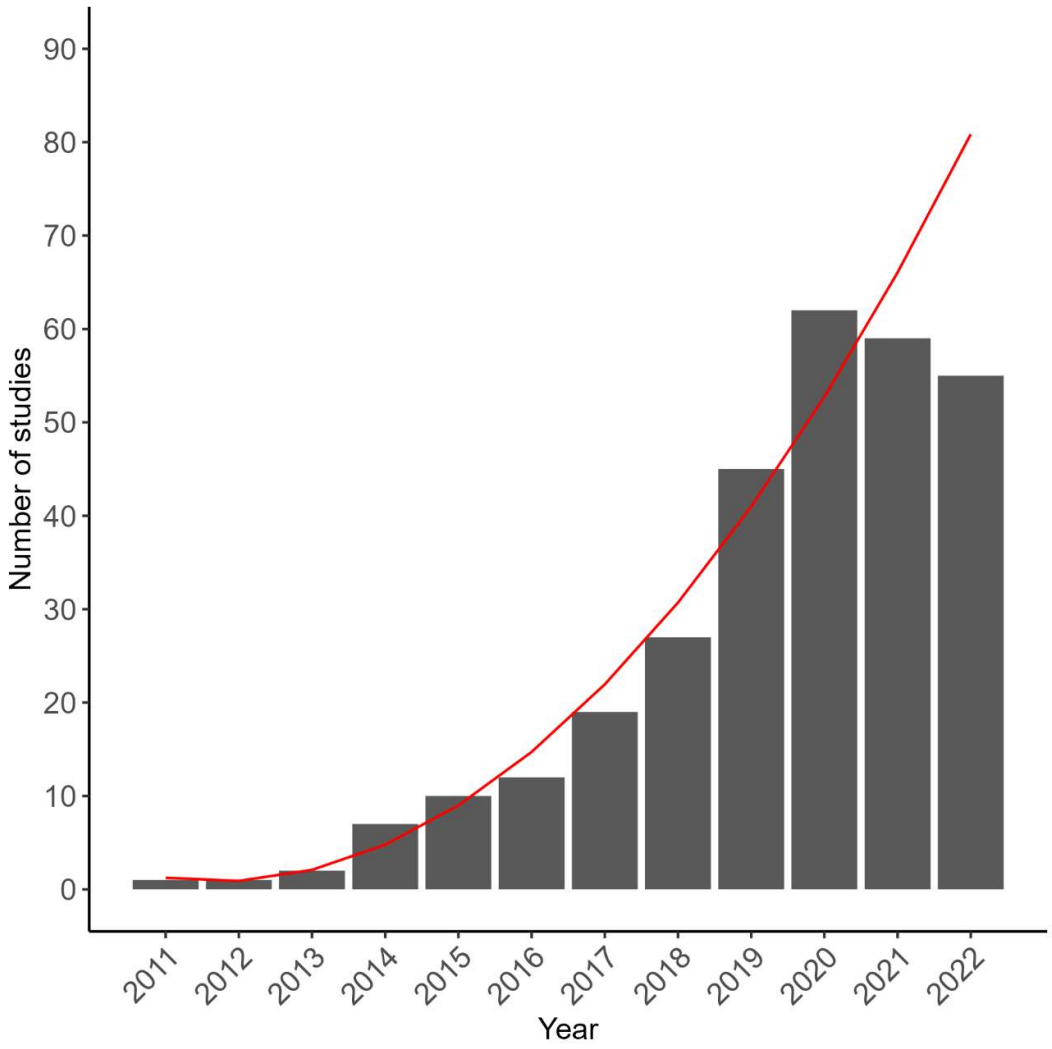


Figure 2.2 Frequency of published freshwater microplastic studies (bars) with non-linear polynomial regression (red line; $F(2, 9) = 198.4$, $R2 = 0.888$, $p < 0.005$).

Qualifying studies were classified by freshwater ecosystem type (flowing: river (e.g., Dris et al. 2025), canal (e.g., Leslie et al. 2017), stream (e.g., Dahms et al. 2020), creek (e.g., Moore et al. 2011), surface runoff (e.g., Grbic et al. 2020); standing: pond (e.g., Turner et al. 2019), lake (e.g., Xia et al. 2020), reservoir (e.g., Lin et al. 2021), stormwater retention pond (e.g., Liu et al. 2019a), wetland (e.g., Abbasi 2021)) and by the matrix sampled: water (surface (e.g., Mao et al. 2020a), column (e.g., Park et al. 2020b, bed (e.g., Kay et al. 2018)), suspended particulate matter, sediment (benthic (e.g., Lenaker et al. 2021), shore (e.g., Jiang et al. 2018)) and biota (with taxon and species where available: Table 2.1). Eighteen studies investigated both standing and flowing freshwaters, and 103 studies sampled more than one type of environmental matrix (Table 2.1). Snow, ice, and suspended particulate matter were only sampled in three studies and were excluded from further evaluation. Information was extracted, where possible, on sample location (country), sample time, sample matrix, abundance of microplastics reported (as range and means of concentration by mass, particles number or percentage prevalence), and qualitative spatial and temporal relationships. This allowed analysis of freshwater microplastic concentration across space at a global, catchment, and local scale, and through time at an inter-annual, intra-annual, and circadian scale. To assess the global distribution of microplastic freshwater ecosystems, standardised average concentrations from selected studies were converted to arithmetic means per continent and per country.

Table 2.1 Number of studies sampling static and flowing freshwater ecosystems, sub-categorised by freshwater matrices at two levels. L1 and L2 are level 1 and level 2, respectively. Data from n = 300 reviewed studies.

		Sample matrix			
Sample system		**L1**		**L2**	
Flowing	211	Water	147	Surface	140
				Column	10
				Bed	3
		Sediment	89	Benthic	76
				Shore	13
		Organism	57	Insecta	9
				Malacostraca	7
				Gastropoda	6
				Bivalvia	3
				Clitellata	4
				Actinopterygii	42
				Amphibia	0
				Aves	2
				Angiospermae	0
				Mammalia	1
				Biofilm	1
				Zooplankton	0
Static	113	Water	74	Surface	71

		Column	6
		Bed	1
Sediment	62	Benthic	48
		Shore	16
Organism	26	Insecta	1
		Malacostraca	2
		Gastropoda	3
		Bivalvia	1
		Clitellata	0
		Actinopterygii	16
		Amphibia	3
		Aves	3
		Angiospermae	1
		Mammalia	0
		Biofilm	0
		Zooplankton	0

2.3.2 Standardisation

Differences in sampling techniques and limited standardisation among studies (see *Chapter 3*) resulted in 69 different forms of units to describe microplastic concentration in water, sediment, and organisms (Table A2). Where possible, reported microplastic concentration in water were converted to concentrations in particles/m^3. This required the assumption that 1 item/m^2 = 1 particle/m^3, 1 item/km^2 = 10^{-6} particles/m^3, and 1 particle/L = 10^3 particles/m^3, further assuming an average water depth per unit area of 1 m (Chen et al. 2020b). Only microplastic concentration in sediment recorded in particles/kg were assessed, as this was the most common

recording unit. However, some studies did not state whether sediment concentrations were recorded on a wet- or dry-weight basis and thus, all sediment concentrations were used in analysis, whether dried or not.

2.3.3 Statistical analysis

Global increase in plastic production and consumption led to the hypothesis of higher microplastic concentrations in freshwater ecosystems in more economically developed countries and in urban areas. Average microplastic concentration was therefore compared between countries categorised as less developed (n = 26) and more developed (n = 34) , where the UN Human Development Index was < 0.8 and > 0.8, respectively (United Nations 2019). Country-averaged microplastic concentrations in water (particles/m^3) were not normally distributed (Shapiro-Wilk test: W = 0.201 and 0.244 for less developed and more developed countries, respectively, both with $p < 0.01$), although more developed country data could be normalised by Ordered Quantile transformation (best transformation based on Pearson P test). Concentrations in economically less developed and developed countries were therefore compared using the non-parametric Mann-Whitney-Wilcoxon test. Country-averaged microplastic concentration in sediment (particles/kg) were both normally distributed after Ordered Quantile normalising transformation (W = 0.999 and 0.997 for less developed and more developed countries, respectively, both with $p < 0.01$) and thus, were compared using unpaired 2-sample *t*-test. Furthermore, general linear regression modelling (GLM) was used to test whether country-averaged freshwater microplastic concentration (particles/m^3) could be predicted by social measures obtained from United Nation's 2021 International Statistical Yearbook (Department of Economic and Social Affairs, Statistics Division 2021). Variables were transformed to ensure normal distribution (Table A3), creating normally distributed residuals (Shapiro-Wilk test: W = 0.965, p = 0.443) with uniform variances.

Average microplastic concentration in water and sediment were compared independently between static and flowing ecosystems. All datasets initially had distributions departing significantly from normality (W = 0.119, 0.153, 0.139, 0.093, for static water, flowing water, static sediment, and flowing sediment, respectively, all

with $p < 0.01$) and so were transformed using Ordered Quantile Normalisation (best transformation based on Pearson P test: $p = 1.207, 1.247, 1.560, 1.546$, for static water, flowing water, static sediment, and flowing sediment, respectively) resulting in Normal distributions ($W = 0.988, 0.997, 0.996, 0.999$, for static water, flowing water, static sediment, and flowing sediment, respectively, all with $p > 0.05$) allowing comparison using unpaired 2-sample *t*-test.

2.4 Spatial pattern

2.4.1 Global scale

Published microplastic data on freshwater ecosystems comes mostly from Asia (n = 127; 68% of which are in China), then Europe (n = 88), North America (n = 54), Africa (n = 17), South America (n = 11), and Oceania (n = 4) (Table A1). Currently less than one-third of all 195 countries (n = 59) have been involved, with water, sediment and biota studied in 25% (n = 49), 21% (n = 40), 16% (n = 31) of all countries, respectively. Most investigations by county have been in China (n = 86), followed by the USA (n = 31), while there have been proportionately fewer freshwater studies in African (n = 17), South American (n = 11), and Oceanian countries (n = 4), and none in Antarctica. This disparity reduces the reliability of global-scale estimates of freshwater microplastic abundance, for example in relation to ecoregions, land-use, culture, and development.

For surface waters, average published microplastic concentrations rank in the order Asia > North America > Europe > Africa > Oceania > South America (Table A4). Site-specific concentrations ranged over eleven orders of magnitude from 2.8×10^{-5} particles/m^3 in Lake Veeranam, India (Bharath et al. 2021) to 4.0×10^{6} particles/m^3 in the Xiangjiang River, China (Shen et al. 2021) (Figure 2.3), with an overall median of 14 (IQR = 1,477) particles/m^3. In freshwater sediments, average concentrations rank in the order Europe > Asia > North America > South America > Africa (Table A5), and ranged over three orders of magnitude from 0.81 ± 0.37 particles/kg ww in dammed and constructed fish ponds, Hungary (Bordós et al. 2019) to 980 particles/kg dw in Lake Ontario, Canada (Ballent et al. 2016) (Figure 2.3), with an overall median ($\tilde{x}$) of 287 (IQR = 1,027) particles/kg.

Less developed countries had significantly greater median microplastic concentration in freshwater ($\tilde{x}$ = 1,324, IQR = 6,113.77 particles/m^3) than developed countries ($\tilde{x}$ = 1.98, IQR = 69.08 particles/m^3; Mann-Whitney-Wilcoxon: W = 9,301.5, $p < 0.05$), but there was no corresponding difference for sediment (2-sample t-test: $t(128) = 0.009$, $p = 0.993$) (Figure 2.4). Chen et al. (2020b) similarly found significantly greater microplastic concentration in freshwaters of less developed compared to developed countries, when reviewing 37 locations. Estimated microplastic outputs from European rivers modelled by Siegfried et al. (2017), also related to socio-economic development and wastewater treatment technology. It is likely that limited infrastructure and wastewater treatment in less developed countries reduces pollution control (Blettler et al. 2019). However, this global-scale assessment is constrained by incomplete coverage of the world's countries, as well as factors influencing microplastic concentration over smaller spatial scales, such as local land-use and hydrological conditions (see sections 2.4.2 Catchment scale and 2.4.3 Local scale).

The attempt to model microplastic concentration by country revealed some relationships with socio-economic predictors. GLM regression of country-averaged freshwater microplastic concentration using respective human population density (per km^2; 2021), proportion of population with access to a safe water supply (2020), and tourist numbers (2018; Department of Economic and Social Affairs, Statistics Division 2021) had the lowest Akaike Information Criterion (AIC) value, no collinearity between independent variables (Figure A1), and no obvious autocorrelation in the residual sequence (Durbin-Watson test: D-W = 1.969, p = 0.906), indicating best fit. This model approached formal significance (R^2 = 0.246, $F(28, 25) = 2$, $p = 0.054$), but the relationship with human population density (per km^2 in 2021) was stronger (β = 0.461, p = 0.034). Chen et al. (2020b) previously reported a similar effect in 37 waterbodies, which was ascribed to greater demand for plastic products coupled with greater waste output. This relationship is also observed at smaller spatial scales, with population density and land use influencing microplastic concentration in 29 Japanese rivers (Kataoka et al. 2019), across four estuarine tributaries of Chesapeake Bay, USA (Yonkos et al. 2014), and in the Biobío River, Chile (Correa-Araneda et al. 2022) (see sections 2.4.2 Catchment scale and 2.4.3 Local scale). For freshwater sediment, country-averaged microplastic concentration was not

significantly related to any socio-economic predictors (R^2 = 0.163, F(17, 14) = 2, p = 0.374).

This may relate to the different dynamic forces acting on freshwater sediments and surface waters. Water flow immediately influences the particles carried by surface waters, whereas particles in sediment may be more stable over time and thus, microplastic loads in sediment may reflect historical, rather than current socio-economic conditions. This may also be true for lakes as, despite being classed as static systems, many are affected by currents, seiche effects, and turbulent mixing at a range of water depths, which all influence microplastic fate (Eriksen et al. 2013; Xiong et al. 2018). When comparing mean microplastic concentration across sampled lakes and rivers identified in the literature search, no clear differences were observed either in surface water (3,163 ± 21,210 particles/m^3 in lakes vs 61,748 ± 375,152 particles/m^3 in rivers; Student's 2-sample *t*-test: $t(401) = 0.051$, $p = 0.959$) or sediment ($\tilde{x}$ = 249, IQR = 886 particles/kg and $\tilde{x}$ = 336, IQR = 1,198 particles/kg, respectively; Mann-Whitney: $U = 1{,}850.5$, $p > 0.05$), but these estimates were characterised by large variability. This may indicate re-suspension of particles in "static" lake systems. Potential differences between lakes and rivers have, however, been detected at more local scales across a single catchment. For example, fibrous and fragmented microplastic particles were up to three-fold higher in lentic compared to fluvial habitats of the Biobío River, Chile (Correa-Araneda et al. 2022).

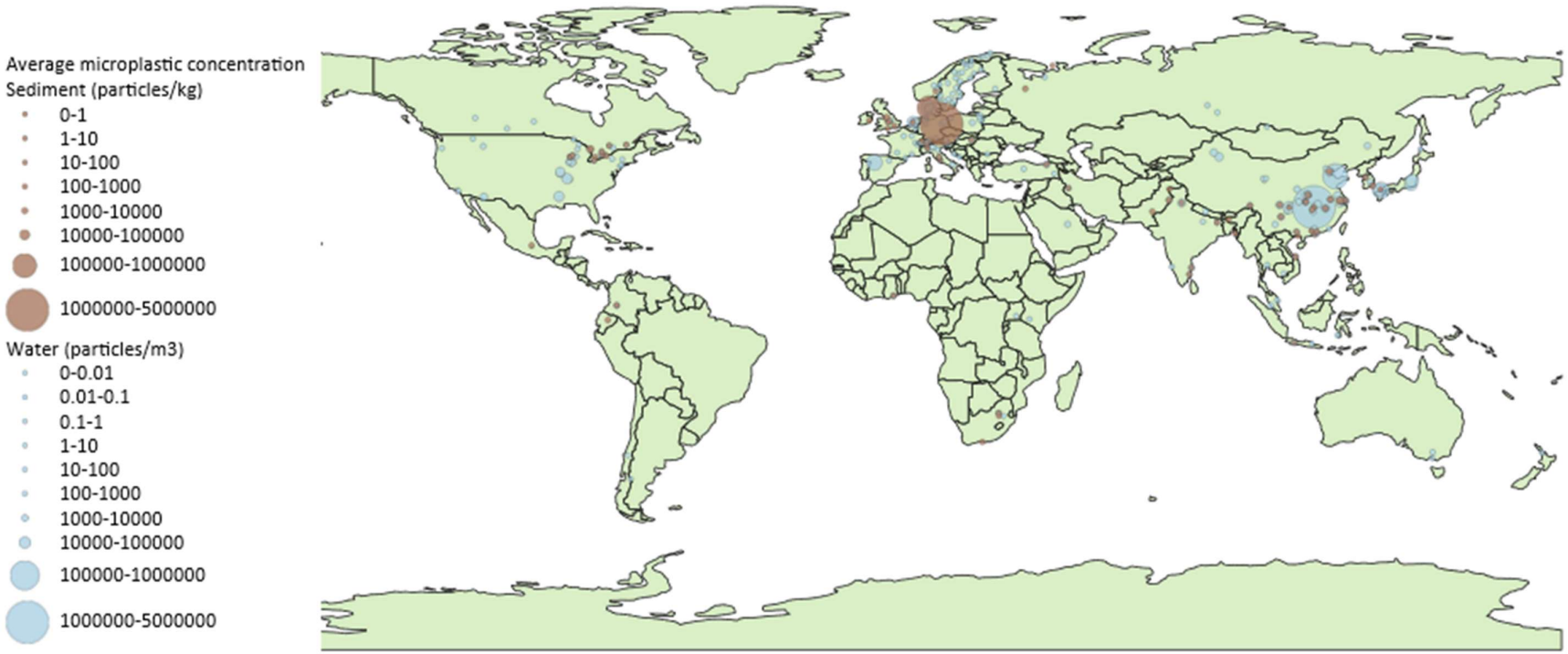
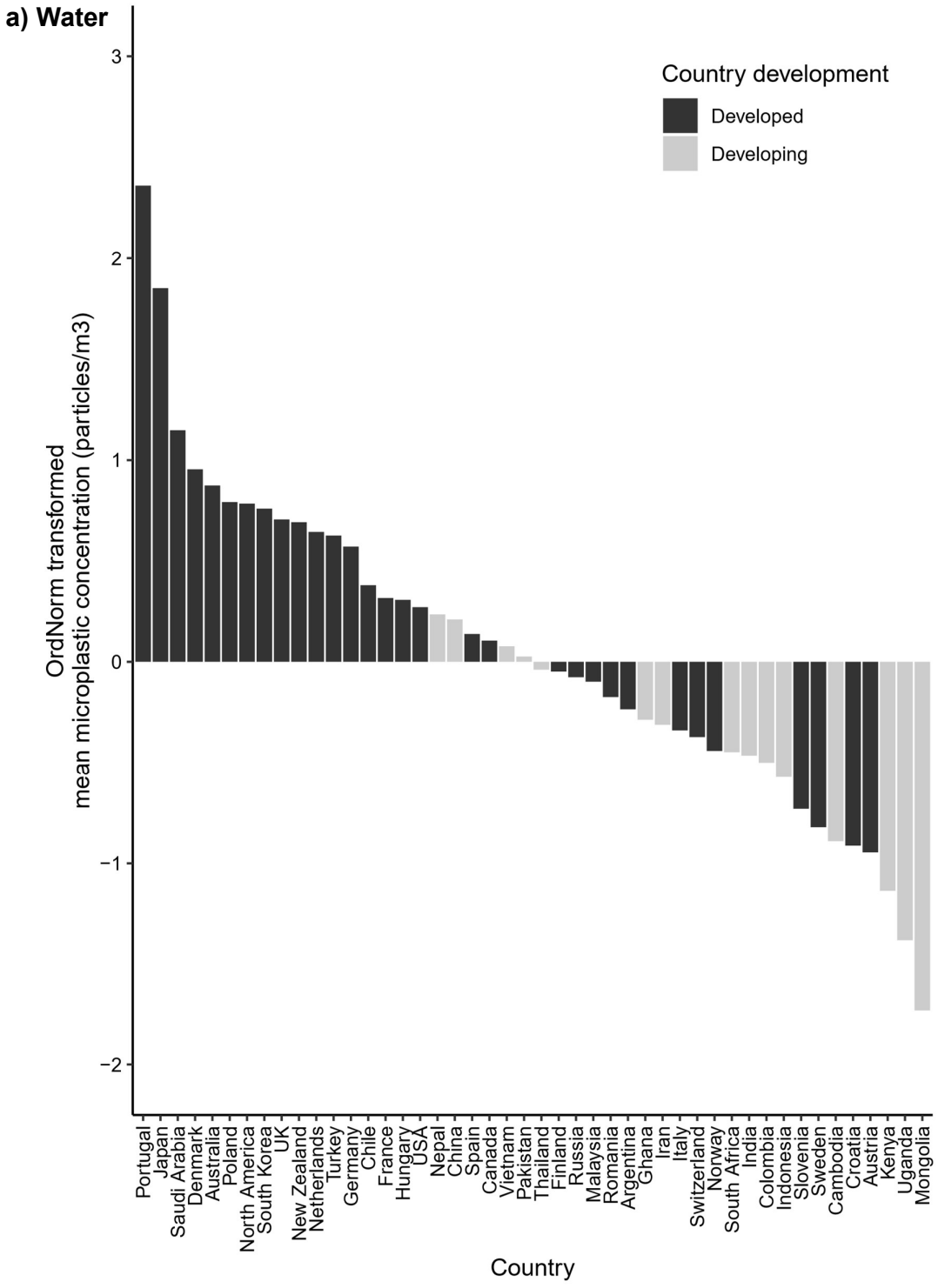


Figure 2.3 Global microplastic (<5 mm) average concentrations in water and sediment of freshwater environments. World map at 1:110 m scale, obtained from Natural Earth (https://www.naturalearthdata.com/).


a) Water
Country development
Developed
Developing
OrdNorm transformed
mean microplastic concentration (particles/m3)
3
2
1
0
−1
−2
Portugal
Japan
Saudi Arabia
Denmark
Australia
Poland
North America
South Korea
UK
New Zealand
Netherlands
Turkey
Germany
Chile
France
Hungary
USA
Nepal
China
Spain
Canada
Vietnam
Pakistan
Thailand
Finland
Russia
Malaysia
Romania
Argentina
Ghana
Iran
Italy
Switzerland
Norway
South Africa
India
Colombia
Indonesia
Slovenia
Sweden
Cambodia
Croatia
Austria
Kenya
Uganda
Mongolia
Country

b) Sediment

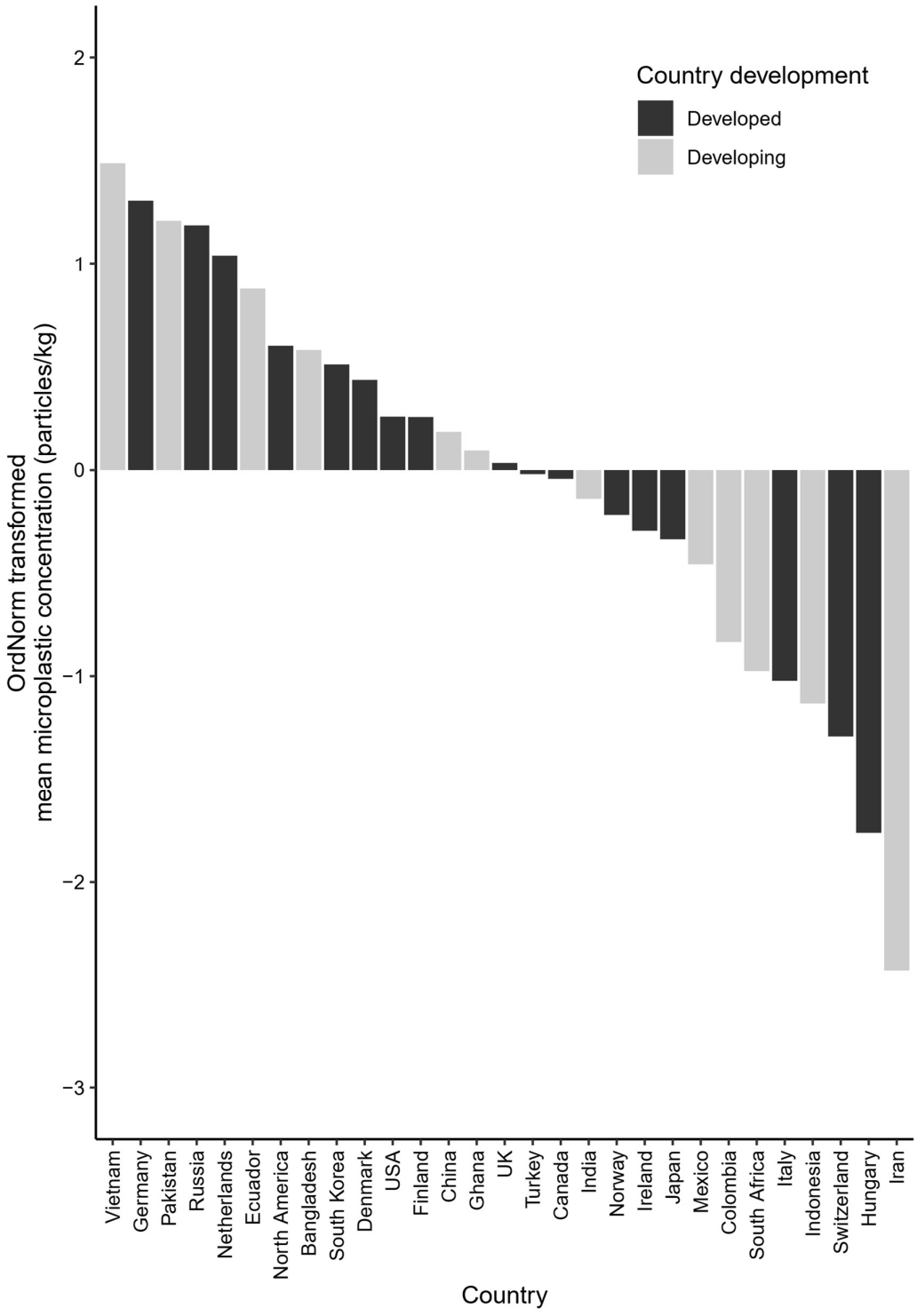


Figure 2.4 Ranked Ordered Quantile normalising transformed mean microplastic concentration in **a)** water (particles/m^3) and **b)** sediment (particles/kg) of freshwater ecosystems per country, marked by level of country development.

2.4.2 Catchment scale

Freshwater catchments are highly connected networks varying in area and surrounding land-use, while also being characterised by hydrological dynamism and heterogeneity in hydraulic conditions. Assessing catchment-scale microplastic distribution, therefore, ideally requires samples from multiple locations to best capture all major sources of variability. This can also identify putative pollutant sources as well as physical conditions influencing microplastic retention or dispersal. So far, much of the focus in catchment studies has been on land use, and there is strong evidence that microplastic concentration increases with urbanisation (34 reviewed studies, e.g., Tibbetts et al. 2018; Yuan et al. 2022). A combination of increased population density, resource use, plastic demand, greater cover of non-permeable surfaces (increasing surface runoff), poor waste disposal, and increased discharge from wastewater or industry are all likely to be implicated (Yonkos et al. 2014; Baldwin et al. 2016; Fan et al. 2019; Kataoka et al. 2019; Townsend et al. 2019; Mbedzi et al. 2020; Zhang et al. 2020a; Bertoldi et al. 2021; Fan et al. 2021; Sekudewicz et al. 2021; Belontz et al. 2022; Correa-Araneda et al. 2022; Dai et al. 2022; Li et al. 2022a; Ma et al. 2022). Less obvious sources – such as tourism - can also increase microplastic release (Jin et al. 2022). In combination, these factors lead often to an increase in microplastic concentrations from upstream to downstream of rivers following a rural-to-urban gradient (Han et al. 2020; Napper et al. 2021; Prata et al. 2021; Wicaksono et al. 2021; Murphy et al. 2022; Park et al. 2022; Rakib et al. 2022; Yuan et al. 2022). However, Tibbetts et al. (2018) and Mao et al. (2020b) report greater microplastic concentration in respective urban upstream reaches of River Tame (UK) and Yulin River (China), compared to rural downstream sites, consistent with the role of urban areas as overriding sources of pollution rather than downstream microplastic accumulation.

Relationships between microplastics and land use are not always straightforward. Di et al. (2019) observed greater microplastic concentrations in the water column of China's Danjiangkou Reservoir near urban areas, but the pattern was not apparent in sediment. More notably, other studies have revealed no clear influence of urbanisation (Barrows et al. 2018; Wen et al. 2018; Alfonso et al. 2020; Wang et al. 2020b; Wang et al. 2021d) or human population density (Miller et al. 2017; Kapp and Yeatman 2018; Tibbetts et al. 2018; Dikareva and Simon 2019;

Alfonso et al. 2020; Frank et al. 2021; Li et al. 2022b) on freshwater microplastic concentrations, indicating other site-specific influences on plastic flux, fate, or distribution might sometimes be greater. These include microplastic point sources such as industrial effluent (Ziajahromi et al. 2016; Chan et al. 2021; Lofty et al. 2022) or the resuspension of plastic particles from sewage sludge spread to land (Schell et al. 2022b). Microplastic movement through freshwater ecosystems might also be influenced by factors such as morphology and flow dynamics. For example, in China's Lake Dianchi, microplastic concentration in surface water correlated negatively with bed roughness, relief, and slope gradient, with particles diffusing more over rougher topography (Deng et al. 2022). In China's Yulin River, microplastic concentration in water negatively correlated with channel width (Mao et al. 2020b), indicating diffusion across a greater area. Therefore, freshwater microplastic concentrations can vary with downstream progression of microplastic sources and hydrodynamics (Mani et al. 2015; Troyer 2015; Lestari et al. 2020; Winkler et al. 2022).

Apparent variability in the distribution of microplastics across catchments raises the need for more extensive studies designed with sufficient detail to appraise natural and anthropogenic influences on microplastic distribution. Such studies are still scarce, with only eight described explicitly as 'catchment-scale' freshwater microplastic investigations. These studies involved 10-72 sites where samples were collected to assess different land-use effects and microplastic sources across whole basins, ranging in size from 522 to 1,808,500 km^2. In one of the best examples to date, Hurley et al. (2018a) sampled freshwater sediment from forty sites across ten tributaries of the Irwell and Mersey rivers in northwest England, which drains into the Irish Sea. The work identified urban hotspots, for example in the Greater Manchester tributary, River Tame, microplastic concentration exceeded 40,000 particles/kg of sediment, with greater contamination immediately downstream of WWTPs and CSOs (Hurley et al. 2018b). Catchment-scale work by He et al. (2020) and Mao et al. (2020b) respectively in the Brisbane River, Australia, and Yulin River, China, also reported correlations between microplastics and anthropogenic activity, with concentrations greatest in upstream residential, commercial, and industrial areas, and lower concentrations downstream with mixed land use. Similarly, Yuan et al. (2022) noted a general increase in microplastic concentration with downstream

progression across 38 sites in China's Yangtze River, co-attributed to distance to urban areas, population density, urbanisation rate, and land use across 38 sites. Despite this, however, and given the limited distribution of microplastic assessments across the world (Figure 2.3), there remains a significant need for representative and stratified studies to account for natural and anthropogenic influences on the sources, fate, and flux of microplastics in contrasting river catchments.

2.4.3 Local scale

As well as their emergent structure at coarser scales, freshwater catchments are also characterised by internal heterogeneity at scales ranging from subcatchments to reaches, biotopes, patches, and individual substratum. This is described sometimes as a 'catchment hierarchy' or network over which there are large variations in physico-chemical conditions including velocity, turbulence, bed roughness, frictional drag, depth, sedimentation, temperature, intra-bed flow, redox potential, and dissolved gasses. At these finer scales, microplastic concentration in freshwater ecosystems over space and time reflects two opposing mechanisms: (1) resuspension and (2) dilution. Multiple freshwater studies record sites with relatively higher flow velocity to have greater microplastic concentrations in water and reduced concentrations in sediment (Tibbetts et al. 2018; Ding et al. 2019; Mani et al. 2019a; Dahms et al. 2020; Migwi et al. 2020; Tien et al. 2020; Feng et al. 2021b). High velocity and turbidity may disturb sediment and resuspend microplastics into the water column, whereas low velocity may facilitate microplastic settling into sediment (Eo et al. 2019; Liu et al. 2022b) and finer sediment associated with these conditions can trap more microplastics (Fischer et al. 2016; Dikareva and Simon 2019). Increased flow is also associated with precipitation, which flushes microplastics from land into freshwater, increasing local microplastic concentration. In contrast, some freshwater studies report higher microplastic concentration where flow velocity is relatively lower (Kapp and Yeatman 2018; Xiong et al. 2019; Huang et al. 2021a; Sekudewicz et al. 2021), also seen behind dams (Zhang et al. 2015; Watkins et al. 2019a; Weideman et al. 2019; Gopinath et al. 2020; Wang et al. 2020b; He et al. 2021b), indicating a dilution effect, where more water disperses microplastic particles.

Fluvial processes also differ across a river's perpendicular axis, causing microplastic concentration to differ up to 10-fold across a river's cross-sectional profile (Wong et al. 2020). Tang et al. (2021) sampled fewer microplastics in surface water of the centre of Songhua River, China, where river flow is higher, compared to the riverbank. In Rhine River, Germany, Mani et al. (2015) found more microplastic at the right riverbank where the Main River enters, compared to middle and left bank. In sediment, Corcoran et al. (2020) sampled fewer microplastics along straight courses of the Thames River, Canada, compared to inner and outer bends. Nonetheless, Barrows et al. (2018), Constant et al. (2020), and He et al. (2021b) report no variation in microplastic concentration across a river's perpendicular axis. These local-scale impacts of fluvial processes suggest observed concentrations reported by freshwater studies may be biased by the specific sample location within a site.

Particle characteristics and polymer type can indicate the origin of sampled microplastics from local sources, but how far they travel within freshwater catchments is poorly known. Using Global Positioning System (GPS) and satellite technology, a 500 ml poly(ethylene terephthalate) drinks bottle was recorded to travel 2,845 km through the Ganges River, Bangladesh, over 94 days (Duncan et al. 2020). Microplastic has been found in remote locations including ocean gyres far from land sources (Moore et al. 2001), but methods of tracking microplastic have yet to be developed. With knowledge on sediment and allochthonous material movement in freshwater (Newbold et al. 1982; Webster et al. 1999; Drummond et al. 2014) and hydrodynamic behaviours of microplastic (Möhlenkamp et al. 2018; Lofty et al. 2023), microplastic transport in freshwater catchments can be modelled to predict particle fate and thus, the location of potential impact. Nizzetto et al. (2016) and Besseling et al. (2017) simulated the first such model, accounting for advective transport, particle aggregation, polymer degradation, sedimentation, and resuspension. Interestingly, particle size was found to override any effect of polymer density on modelled microplastic fate, with particles > 0.2 mm more likely to be retained in sediment and settlement of smaller particles being dependent on hetero-aggregation (Nizzetto et al. 2016; Besseling et al. 2017). However, this model did not account for particle shape nor biofilm growth, which can impact the rate of microplastic settlement in water (Nguyen et al. 2022). Microplastic biofouling occurs rapidly in freshwater ecosystems (Semcesen and Wells 2021; Nava et al. 2022) and

increases particle density, with smaller biofouled microplastics expected to sink before larger biofouled plastics (Semcesen and Wells 2021). A 3D model by He et al. (2021a) suggests polymer density does affect microplastic dispersal in river sediment, with denser particles having lower mobility and higher river flow velocity moving particles further from their source. The fate of tyre wear particles (TWPs) in Göta River, Sweden, modelled by Bondelind et al. (2020), also suggests higher density and larger sized particles settle in sediment and therefore, accumulate near their source.

Regarding freshwater lakes, instantaneous microplastics movement can be influenced by currents (Eriksen et al. 2013; Xiong et al. 2018). Daily and Hoffman's (2020) 3D lentic model predicts turbulent mixing retains microplastics in water, but ignore particle size and shape, density changes with aggregation, biofouling, or polymer degradation, overestimating particle resuspension. Lastly, Hoffman and Hittinger's (2017) model suggests proximity to large population centres has a greater impact on microplastic distribution in lakes compared to gyre patterns. This variety of model predictions reiterates the complexity of microplastic flux within the dynamic freshwater environment.

2.5 Temporal dynamics

Studies into temporal variations contribute around a quarter of identified freshwater microplastic studies (n = 78), with patterns attributed to trends in production through time, hydrodynamics such as flow velocity and discharge, weather seasonality, and meteorological events (Talbot and Chang 2022a). These all occur at different scales and several effects are still poorly resolved.

2.5.1 Inter-annual scale

Long-term data is crucial for assessing whether microplastic pollution is increasing, decreasing, or remaining stable over multiple years. Analyses of historic microplastic samples from freshwater ecosystems is limited, but sediment cores have provided some temporal assessments. Here, vertical columns of sediment are extracted from the bottom of a water body and divided into layers, each of which are

radiocarbon dated to identify distinct time periods, and examined for microplastic contamination. Cores from Hamstead pond (UK), Lake Uluabat (Turkey), and Wujiang River (China) all show very low microplastic concentrations in the early 20th century before acceleration in the 1960s and continued accumulation into the 21st century (Turner et al. 2019; Almas et al. 2022; Wu et al. 2022a). Cores from Lake Mjøsa (Norway) showed increasing microplastic concentration and richness from the 1980s to 2017 (Lusher et al. 2018; Clayer et al. 2021). These studies show an almost immediate occurrence of plastic pollution in the environment after industrial production began mid-20th century (Geyer et al. 2017). Few studies, however, have assessed current trends in freshwater microplastic concentrations between years. Lechner et al. (2014) detected an apparent reduction in plastic density and composition in the Danube River, Austria, from 2010 to 2012, but Anderson et al. (2017) found no change in the microplastic concentration in Lake Winnipeg, Canada, from 2014 to 2016. Inter-annual scale research is important for identifying recent sources of microplastic pollution, evaluating the impact of regulations and management practices, and raising public awareness about the issue to emphasise continued efforts to reduce plastic waste. Therefore, this is a clear area where expanded activity is needed.

2.5.2 Intra-annual scale

Within a year, changes in freshwater microplastics have been observed over different seasons, which has been associated with weather and rainfall (Xia et al. 2020). Similarly to observed local-scale spatial trends (see section 2.4.3), there are contrasting patterns in the available data that either show increases in microplastic transport and higher concentrations at times of high flow, or dilution when discharge increases. Studies reporting increased microplastic transport in rivers during wetter periods and/or sedimentations during times of reduced flow are numerous (Moore et al. 2011; Yonkos et al. 2014; Faure et al. 2015; Lasee et al. 2017; Hurley et al. 2018b; Nel et al. 2018; Schmidt et al. 2018; Alam et al. 2019; van Emmerik et al. 2019; Eo et al. 2019; Mai et al. 2019; Campanale et al. 2020b; Constant et al. 2020; Gerolin et al. 2020; Park et al. 2020a; Wong et al. 2020b; Woodward et al. 2020; Bujaczek et al. 2021; Chen et al. 2021; Haberstroh et al. 2021a; Munari et al. 2021; Shen et al. 2021; Dahms et al. 2022; Winkler et al. 2022). For example, 71% of

microplastic transported by the Nakdong River, South Korea, throughout 2017, occurred during the wet season (Eo et al. 2019). Greater microplastic concentrations were sampled by citizen scientists across 72 sites along the Gallatin River, USA, during the wetter March 2016, with reductions in the drier June 2016 (Barrows et al. 2018). Similarly, He et al. (2020) found higher concentrations of microplastics in the Brisbane River, Australia, in December and September during the wet season in Queensland State, with lower concentrations in March during the dry season.

Contrasting patterns from those described above come from studies reporting either dry season peaks (Eo et al. 2019; Woodward et al. 2020; Haberstroh et al. 2021a) or reductions in microplastic concentrations during high flow or wetter months (Dris et al. 2015; Mani et al. 2015; Barrows et al. 2018; Rodrigues et al. 2018a; Fan et al. 2019; Liu et al. 2019b; Watkins et al. 2019b; Han et al. 2020; Mbedzi et al. 2020; Scircle et al. 2020; Wang et al. 2020a; Wong et al. 2020a; Wu et al. 2020b; de Carvalho et al. 2021; Huang et al. 2021b; Liu et al. 2021a; Napper et al. 2021; Wang et al. 2021b; Wicaksono et al. 2021; Aslam et al. 2022; Malla-Pradhan et al. 2022; Talbot et al. 2022b). In yet further contrasts, other studies have detected no seasonal variations or influences of discharge on microplastic concentration (Su et al. 2016; Peller et al. 2019; Weideman et al. 2019; Constant et al. 2020; Mani and Burkhardt-Holm 2020; Stanton et al. 2020; Chanpiwat and Damrongsiri 2021).

These differences among available data have a range of putative explanations including confounding influences from urbanisation or other microplastic sources, the ability of different sampling methods to detect plastics that are saltating, floating or in suspension, and potential effects from antecedent conditions. A case of the latter effect arose from the UK's River Irwell which experienced a 70% reduction in catchment-wide microplastic concentrations in river sediment after winter flooding (2015/16) - in many cases by an order of magnitude (Hurley et al. 2018b; Woodward et al. 2020). Export effects of this type would reduce potential benthic reservoirs of microplastic that could be mobilised in high-flow periods. There are also instances where reductions in river wetted perimeter during low flow reduce the ratios between water volume and the contaminated surface area of sediment, possibly leading to higher microplastic concentrations in samples collected in shallower flows (Wicaksono et al. 2021). A more unusual explanation for changing microplastic fluxes between winter and summer arose from Browne et al.

(2011), who suggested that higher microplastic fibre releases into freshwater during colder months reflected changes in clothing use that caused household washing machine usage to rise by 700%. Such effects would vary between regions with different climates.

2.5.3 Event scale

Environmental dynamics and variations between seasons could also reflect changes in microplastic concentration over shorter timescales, down to hours and minutes, such as during hydrological events or pollution incidents. So far, however, there are few comprehensive studies – particularly examining microplastic behaviour over entire storm hydrographs. Watkins et al. (2019b) investigated microplastic variation in tributaries to an American lake over 24-hours during low (August 2016) and high (April 2017) flow conditions, by sampling every three hours. No significant change was observed over each 24-hour period, but concentrations at high flows were consistently more variable than those at low flows (Watkins et al. 2019b). Dris et al. (2018b) sampled surface waters in the Seine River, France, over a 2-hour and 12-hour period during high flow (March 2015) and low flow conditions (July 2015), respectively. Again, greater variability in microplastic concentration occurred at high flows, but this might have reflected shorter sampling periods at high flow compared to low flow. Cheung et al. (2019) reported an order-of-magnitude reduction in microplastic concentration in the Lam Tsuen River, China, even over a brief rainfall event of 2-hours. In contrast, Chen et al. (2021) observed a 3-fold increase in microplastic concentration of the Langat River, Malaysia, in response to flooding over a 24-hour period, whilst de Carvalho et al. (2022) reported up to 8-fold increase during flood episodes in France's Garonne river. In other instances, effects of hydrological events can be more delayed or prolonged and in Lake Donghu, China, and Lake Chiusi, Italy, microplastic concentration in water increased over days following rainfall (Fischer et al. 2016; Xia et al. 2020).

As well as in-channel mobilisation during events, heavy rainfall induces runoff from adjacent land that may increase microplastic transport into freshwater systems both directly (Dikareva and Simon 2019; Shen et al. 2021; Warrier et al. 2022) or indirectly through WWTPs, CSOs, or septic tanks (Grbić et al. 2020; Park et al.

2020a; Liu et al. 2022a; Warrier et al. 2022; Zhang et al. 2023). These effects can be mediated by local topography, such as steep slopes or impervious surfaces (Yonkos et al. 2014). Moreover, rainfall may transfer microplastic from the atmosphere to freshwater through wet deposition, by adhering to raindrops (Dris et al. 2015; Dris et al. 2016; Xia et al. 2020; Xiong et al. 2022). This diversity of influential mechanisms reflects the observed contrasting temporal trends.

2.6 Consequences for freshwater biota

Organisms can be directly exposed to microplastic through ingestion or respiration, and through food-web transfer to predators (Kim et al. 2018; D'Souza et al. 2020). Field studies investigating microplastic exposure in freshwater biota so far are outnumbered by assessments in non-biological media by around 3.7-4.3 times (Table 2.1). Microplastic presence was investigated in 11 classes, with over 70% of studies sampling fish (Actinopterygii; n = 54) (Figure 2.5). Other taxa investigated, in decreasing study frequency, include insects (Insecta; n = 10), gastropods (Gastropoda; n = 8), crustaceans (Malacostraca; n = 8), annelid worms (Clitellata; n = 4), bivalves (Bivalvia; n = 4), birds (Aves; n = 4), amphibians (Amphibia; n = 3), mammals (Mammalia; n = 1; O'Connor et al. 2022), microorganismal biofilm (n = 1; Huang et al. 2021), and monocot plants (Angiospermae; n = 1; Yin et al. 2021) (Figure 2.5). Quantification of microplastic in freshwater zooplankton was not identified in this literature search, but has since been published by Lawrence et al. (2023). Thirteen studies investigated microplastic in multiple organism classes, with Garcia et al. (2021) sampling six classes in Garonne River, France.

The skew towards microplastic investigation in freshwater fish limits our understanding of microplastic load in organisms at lower trophic levels that make up freshwater communities and support top predators. Windsor et al. (2019a) called for more research on microplastic ingestion in freshwater macroinvertebrates, which act as entry points of microplastic to freshwater food webs and now make the second most studied organismal class for microplastic ingestion (Figure 2.5). The dominance of fish in literature also heavily skews the trends described in subsequent sections, which should be accounted for when drawing conclusions. The sections that follow first summarise field data on biological factors affecting the occurrence of

microplastics in freshwater organisms, before discussing current understanding of their exposure in relation to spatio-temporal variations in microplastic distribution.

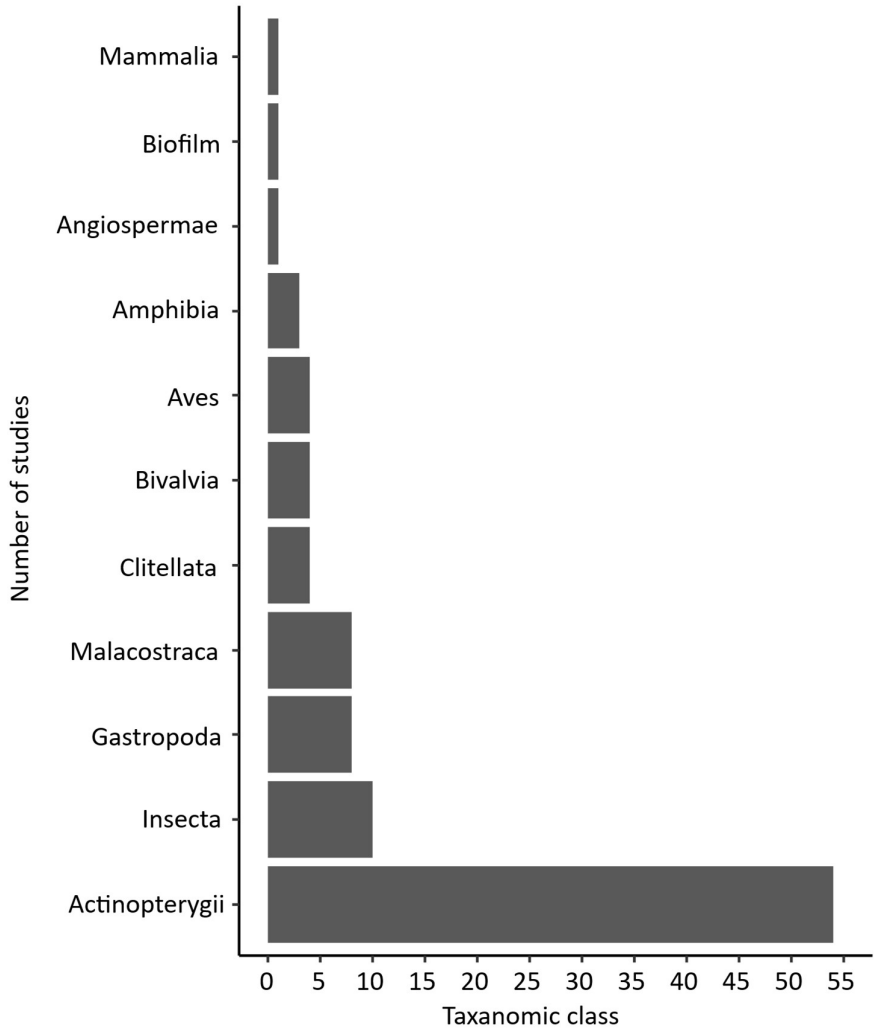


Figure 2.5 Number of studies investigating microplastic abundance in organisms associated with freshwater, per taxonomic class.

2.6.1 Biotic influences on microplastics in organisms

Organismal contamination by microplastics is likely to reflect biotic factors such as their taxonomic group, body size, energetic demands, feeding guild, trophic position, or even individual behaviour. In all these cases, however, data are inevitably preliminary in part because studies are still relatively few and results sometimes conflict.

From a taxonomic perspective, available data reveal microplastic prevalence is greatest in freshwater mammals, ray-finned fish (Actinopterygii), gastropods, and insects, where around half of individuals sampled contain microplastic (Table 2.2). However, this is limited by scarce evidence, with fewer than 10 studies representing each of these taxonomic groups. This 50% proportion also implies that some individuals contain no plastic raising questions even at the level of individual behaviour. Considering class averaged concentrations, ray-finned fish (Actinopterygii) have the highest microplastic abundance per individual (5.6 ± 8.7 particles/individual), but relatively low microplastic abundance per gram of tissue (0.3 ± 0.5 particles/g) (Table 2.2), likely due to their larger size relative to other investigated species. Conversely, insects have the highest microplastic abundance per gram (265.8 ± 372.9 particles/g) and comparatively lower abundance per individual (1.3 ± 1.4 particles/individual; Table 2.2), possibly due to their relatively smaller size. Differences in overall number of particles per individual will have a greater influence on the particle abundance to body size ratio for smaller individuals. In reviewed papers, contamination peaked at over 1,200 particles in individual Brown trout (*Salmo trutta*) and Brook trout (*Salvelinus fontinalis*) from the Kinnickinnic River, USA (Simmerman and Wasik 2020), while concentrations were greatest (19,023 particles/g wet weight) in individual Asellidae (Isopoda) from River Dommel, Netherlands (Pan et al. 2021). This reveals opposing relationships between body size and microplastic concentration, depending on the unit of measurement.

The influences of body size within taxa were explored in fish from freshwaters of South Korea (Park et al. 2020a; Park et al. 2022) and the Orontes River, Syria (Kılıç et al. 2022). In all cases, microplastic burden increased with individual weight and length, possibly related to greater food consumption increasing microplastic uptake and retention. A similar relationship with body size was observed in freshwater crustaceans in laboratory studies (Burns 1968; Zánkai 1994), as well as in the marine environment (Pegado et al. 2018; Hossain et al. 2019). However, other fish studies have found no difference in microplastic ingestion among species of different size or mass in freshwater (Campbell et al. 2017; Parvin et al. 2021; Dahms et al. 2022; Kılıç et al. 2022; Parker et al. 2022a; Pittura et al. 2022), nor in marine environments (Lusher et al. 2013; Neves et al. 2015; Pazos et al. 2017). Independently of size, Horton et al. (2018) observed greater microplastic uptake in

female common roach (*Rutilus rutilus*) compared to males, in River Thames, UK, attributing this to increased energy demands of spawning causing increased food consumption and thus, microplastic uptake.

With respect to organismal feeding traits and trophic position, data are again conflicting. For example, some studies have shown how omnivorous and insectivorous fishes contain more microplastics than carnivorous fishes (Park et al. 2020a; Wang et al. 2020b; Zhang et al. 2020b; Parvin et al. 2021; Park et al. 2022). Whereas McNeish et al. (2018) and Tien et al. (2020) reported greater microplastic uptake in zoobenthivorous fishes at higher trophic levels than in herbivorous/omnivorous fishes. In fishes from China's Dafeng River (Liu et al. 2021a) and the UK's River Bourne (Parker et al. 2022a), tropic level had no apparent influence. Among invertebrates, Parker et al. (2022) found lower microplastic concentrations in macroinvertebrates from higher trophic positions. Studies by Akindele et al. (2020), Pan et al. (2021), and Bertoli et al. (2022) revealed significantly higher microplastic concentrations in collector-gatherer gastropods and other invertebrates in Nigerian, Dutch, and Italian rivers, respectively, compared to other trophic groups. However, feeding mechanisms among invertebrate primary consumers in the River Usk and River Taff, UK, had no reported influence on individual microplastic loads (Windsor et al. 2019b). Some speculation suggests these organisms may mistakenly identify microplastic as food or incidentally consume microplastic from the environment and/or contaminated organisms (Park et al. 2022). Such incidental ingestion is likely to be the case in filter-feeders that sometimes contain higher microplastic concentrations than other taxa (Reynolds and Ryan 2018; Wu et al. 2022b), with encounter rates likely to increase with volume of water filtered. These differences among studies illustrate some of the current uncertainties in understanding processes such as encounter rates, behavioural aspects of prey selection, trophic transfer, or biomagnification (Gouin 2020).

Table 2.2 Mean averaged microplastic concentration and prevalence in freshwater associated biota by class, in descending order, from 70 reviewed studies. Data displayed as available, and averages include recordings of zero particles. n = sample size.

Class	**Average particles/ individual (n)**	**Class**	**Average particles/g tissue (n)**	**Class**	**Average % prevalence (n)**
Actinopterygii	5.57 ± 8.665 (*68*)	Insecta	265.78 ± 372.94 (*8*)	Gastropoda	58 (*5*)
Magnoliopsida	4.90 ± 2.600 (*1*)	Amphibia	259.26 ± 78.65 (*3*)	Mammalia	57 (*1*)
Malacostraca	4.51 ± 8.462 (*4*)	Malacostraca	35.13 ± 44.66 (*3*)	Actinopterygii	50 (*50*)
Amphibia	2.73 ± 0.780 (*1*)	Gastropoda	28.28 ± 78.65 (*5*)	Insecta	49 (*6*)
Clitellata	2.33 ± 3.269 (*2*)	Bivalvia	1.44 ± 2.00 (*2*)	Bivalvia	36 (*2*)
Aves	2.31 ± 2.821 (*2*)	Actinopterygii	0.29 ± 0.47 (*19*)	Aves	8 (*3*)
Insecta	1.30 ± 1.398 (7)	Biofilm	0.02 (*1*)	Clitellata	2 (*1*)
Gastropoda	1.18 ± 1.348 (7)	Clitellata	0.0004 (*1*)	Malacostraca	2 (*1*)
Bivalvia	0.01 ± 0.004 (*2*)				

2.6.2 Varying organism exposure in space and time

Previously discussed variations in microplastic distribution from local to global spatial scales and over timescales ranging between events, seasons, or years are likely to affect interactions with organisms, because they too, have distributions, life cycles, or behaviours that vary over time and space. Contrasting geographical ranges, different habitat occupancy, movements among habitats, changing patterns of prey use, breeding cycles, and seasonal or even circadian migration are all examples of factors that could change microplastic exposure. Although effects like these are well understood for other pollutants – which the most useful indicator organisms integrate through time – there is still a need for considerable advance to identify robust and reliable biological indicators for microplastics.

At global or continental scales, no systematic studies were identified that compare microplastic contamination in freshwater organisms with sufficiently comparable taxonomic identity, feeding niche, or trait character to allow assessments that would not be confounded by other biotic effects. Such inter-continental comparisons have been made for other pollutants where similar species from the same genus occupy different geographical ranges (Morrissey et al. 2010).

At catchment scales, species-specific assessments of microplastic contamination are more feasible and have provided some of the clearest spatial assessments of organismal exposure to date. For example, samples from multiple sites along land use gradients have shown how microplastic ingestion increases in organisms such as fish and river birds at the most urbanised locations (Peters and Bratton 2016; Silva-Cavalcanti et al. 2017; D'Souza et al. 2020). Departures from these clear trends include instances where microplastic occurrence is patchy or at low frequency among organisms (e.g., Windsor et al. 2019c; O'Connor et al. 2020), where there are confounding effects between land use and other variations among sites (Wardlaw and Prosser 2020), or where relationships between microplastics in environmental media and organisms are not correlated (Parker et al. 2022a).

Smaller-scale variations in microplastic distribution can also affect the exposure of organisms, but once again, there are conflicting data. In the Han River, South Korea (Park et al. 2022) and the Orontes River, Syria (Kılıç et al. 2022), higher microplastic concentrations were observed in pelagic fish, followed by benthopelagic,

then demersal species, consistent with marine environments (Rummel et al. 2016). More specifically, highly dense polytetrafluoroethylene (PTFE) is more concentrated in bottom-feeding fish species of Han River compared to surface-feeders (Park et al. 2020b). However, these patterns were less clear in fish from Taihu Lake, China (Jabeen et al. 2017), freshwaters in Bangladesh (Parvin et al. 2021), and in Fengshan River, Taiwan (Tien et al. 2020). Moreover, field studies on a range of taxa have found no clear effect of habitat selection or ecological niche on microplastic uptake (Holland et al. 2016; Windsor et al. 2019b; Akindele et al. 2020; Liu et al. 2021a; Pan et al. 2021; Bertoli et al. 2022b).

Turning to variations through time, organism exposure varies between shorter-term patterns linked to changing hydraulic conditions influencing local microplastic behaviour, and longer-term trends resulting from increasing plastic pollution (Kowalski et al. 2016; Besseling et al. 2017; Khatmullina and Isachenko 2017; Lenaker et al. 2019; Waldschläger and Schüttrumpf 2019). In the latter case, historic biological specimens have provided an important indicator of the advent of microplastic occurrence in freshwater ecosystems (Lusher et al. 2018). Hou et al. (2021) used freshwater fish specimens collected at various intervals between 1900 and 2018 in Chicago, to illustrate how no fish were contaminated prior to the 1950s, but concentrations significantly increased thereafter as plastic production became industrialised (Geyer et al. 2017). This matches trends observed in water and sediment (see section 2.5.1). Nevertheless, there were variations among individuals and species (Hou et al. 2021).

On intra-annual timescales, studies have shown greater microplastic burdens in otters, fish, and biofilm in winter than summer (O'Connor et al. 2022, Wu et al. 2022b, and Huang et al. 2021b, respectively). The pattern in biofilm was consistent with increased microplastic concentration in water and sediment during the winter (Huang et al. 2021b), which was previously suggested may result from greater microfibre release in wastewater outflows during colder months, due to the 700% rise in household washing machine usage (Browne et al. 2011). Similarly with invertebrates, Nel et al. (2018) observed 2.5 times greater maximum microplastic loads in *Chironomus* (Diptera) collected in winter (5.04 particles/mg wet weight) compared to summer (1.44 particles/mg wet weight), as well as a greater prevalence of microplastic in winter (98%) over to summer (75%) samples. This occurred

independently of environmental changes and was attributed to concurrent increases in sediment microplastic concentration between seasons (Nel et al. 2018). Interestingly, mosquitofish sampled post-flood from Santa Cruz River, USA, contained more microplastics than those sampled at baseflow, possibly due to an increased encounter rate and/or an impaired ability to distinguish prey from microplastic during turbid high flows (Eppehimer et al. 2021). In direct contrast, however, planktivorous fish in Lake Chaohu, China, were less contaminated with microplastic in the wet versus dry season (Wu et al. 2022b). These findings highlight the complex and variable nature of microplastic contamination across different species and environments, underscoring the need for further research to understand the driving factors behind seasonal variations in microplastic exposure.

2.7 Knowledge gaps and deficits

Against expanding research and knowledge of microplastics in freshwater ecosystems, this review of field data has revealed a range of knowledge gaps, deficits, and uncertainties – including contrasting results with respect to microplastic distribution and behaviour. This includes considerable patchiness among continents and nations in data availability as well as substantial variability in measured microplastic concentrations in water and sediments by 11 and 3 orders of magnitude, respectively. At catchment scales, urban areas appear to be important sources of microplastic pollution, but this pattern is not universal. Moreover, there have been few stratified field surveys to assess or account for other sources of variation, for example from other land uses or natural hydro-morphological differences among survey sites. At more local scales, small-scale variations in microplastic distribution in relation to biotopes, hydraulics, or fine-scale geomorphology are understudied, but could be important in informing catchment-scale designs as well as understanding microplastic dynamics. Assessments of microplastics through time are substantially outnumbered by spatial studies, with longer-term trends or short-term dynamics during whole hydrological events scarce among available data. Variations with discharge have produced contrasting patterns that suggest the need to reduce confounds, improve sampling design, and assess the effects of antecedent conditions. Interactions with other stressors, notably climate change and other pollutant sources, require expanded attention.

Assessments of microplastics in freshwater organisms are outnumbered by studies of abiotic media by a factor of around four and skewed towards fish. Additional data will further our understanding of potential effects on ecosystem processes, food-web transfer, community interactions, and individuals. This includes bolstering understanding of how different life-history traits affect exposure - notably feeding methods, trophic level, habitat use, movement pattern and lifespan. Better integration between laboratory assessments of exposure effects and consequences for free-living populations and communities is a general concern in ecotoxicology and applies also to microplastics (Windsor et al. 2018).

One of the greatest needs apparent from this review is for interdisciplinary work that integrates spatio-temporal patterns in microplastic distribution with organismal exposure and potential ecological impacts at all scales. For example, at the global scale, data from regions in Africa, South America, and Oceania would aid understanding of microplastic exposure in some of the world's most biodiverse regions while also bolstering eco-regional comparisons (Li et al. 2020). At the catchment scale, further work is needed to resolve apparent uncertainties in microplastic distribution relative to the distribution of organisms and communities whose occurrence also varies naturally at this scale. At finer scales within catchments, reaches, patches, and microhabitats, distributions in microplastic are still poorly described despite their likely importance to individual behaviours, life histories and ecological processes that vary at these scales. There is a particular need to boost data on interactions between microplastics and organisms in freshwater sediments and benthic environments, which are likely to be microplastic sinks that support large densities of organisms expected to mediate microplastic entry into food webs (e.g., Windsor et al. 2019c; D'Souza et al. 2020). This also implies a need to assess the temporal dynamics of organismal exposure relative to the movement, fluxes, and changing concentrations of microplastics at all scales from events to inter-annual trends.

The above challenges are not unique to microplastics, meaning that approaches used to address similar large-scale problems could serve as models. At the largest spatial extents, for example, emerging pollution problems such as pharmaceuticals have been addressed through global surveys (Wilkinson et al. 2022). In other cases, such as nutrient pollution, data syntheses have integrated

smaller-scale reductionist studies with landscape- or catchment-scale information to advance understanding (Heathwaite 2010). At national to continental scales, co-ordinated research programmes have linked models with hierarchically-scaled and multi-disciplinary field investigations in time and space, coupled with experiments that also straddle different scales from laboratory to mesocosm (Hering et al. 2015; Birk et al. 2020). These suggestions are not exhaustive, and it is important to stress that full understanding of pollution problems requires substantial investment and often accumulated data from multiple studies with consistent methods (e.g., Haase et al. 2023).

2.8 Conclusion

This systematic, quantitative review builds on previous assessments of microplastics in freshwater matrices by summarising current knowledge and identifying patterns across different spatial and temporal scales. A valuable next step would be to quantify how many studies contribute to the review at each spatial and temporal scale. This would help to identify research gaps and support the development of more standardised, scalable approaches for future monitoring. While highlighting the extent to which freshwater ecosystems act as major sinks and pathways of microplastics from terrestrial to marine environments, the data reviewed here also reinforce earlier assessments by illustrating how research coverage remains fragmented, with persistent gaps and uncertainties – particularly concerning organismal exposure and resulting impacts on ecosystem processes (Eerkes-Medrano et al. 2015). These findings add weight to widespread concerns about the lack of methodological consistency across literature and the inconsistent units in which microplastic data are reported. These caveats pertain to disparities revealed here in microplastic concentrations across water, sediment, and organisms, which span over 11, 3 and 6 orders of magnitude, respectively, as well as variation among countries at different stages of economic development. Similar caveats apply to the current understanding of interactions between flow velocity, discharge, hydrological events, and the transport of microplastic particles.

Collectively, these issues raise questions about (i) the representativeness of studies conducted to date; (ii) the limitations in the spatio-temporal coverage of

available data; and (iii) the effects of variations in sampling, processing, quantification, and characterisation of microplastics in freshwater. Attention is drawn to the potential for interdisciplinary research, similar to those used in addressing other pressures on freshwater ecosystems, which integrates the environmental distribution and behaviour of microplastics with the exposure pathways affecting organisms, which has been a central concern in the broader context of plastic pollution.

Chapter 3: A global review and analysis of microplastic extraction and analytical methods in freshwater ecosystems

3.1 Abstract

Reported freshwater microplastic concentrations range up to 11 orders-of-magnitude, leading to growing concern about the standardisation of methods and units in which microplastic data are reported. However, variation between freshwater media necessitates the use of diverse techniques for effective microplastic extraction. A systematic, quantitative literature review was used to appraise variability in methods of sampling, extracting, enumerating, and characterising microplastic particles polluting freshwater ecosystems from 300 studies. Water and sediment have been sampled either via volume-reduction techniques using nets, or in bulk using containers, grab samplers, or corers, with the latter technique used more frequently for sediment sampling. Nets range in dimension (11.6-725 cm^2), aperture (5 – 2,000 µm), and volume of water filtered (0.0003-522 m^3). Freshwater biota are investigated as whole specimens except for fish, where the gastrointestinal tract (GI) tract, liver, muscle, or gills are dissected. Samples have been processed with combinations of sieving, density separation, chemical digestion, and filtration, with filtration aperture, chemicals, and experimental conditions varying amongst studies. Fine-mesh and multiple stacked sieves are used by one-third of studies processing through sieving. Sodium chloride (NaCl) is used by over half of studies processing through density separation. Oxidative digestion with hydrogen peroxide (H_2O_2) is most commonly used amongst studies processing through digestion (84%). Filtration typically occurs through glass fibre filters and using a pore size of 0.45 µm. Finally, microplastics were quantified and characterised using microscopes, where particles per unit volume is recommended as the most appropriate reporting unit. Polymer type was investigated using spectroscopy and spectrometry techniques, mainly using infrared (IR), with polypropylene (PP) and polyethylene (PE) occurring most frequently. Key areas for research expansion including: (i) microplastic vertical distribution through the water column; (ii) microplastic contamination of biota other than fish; (iii) the influence of equipment and methodology choice on observed microplastic loads; and (iv) the harmonisation of freshwater microplastic research.

3.2 Introduction

With the exponential growth in plastic production (Geyer et al. 2017) and growing evidence for its harmful impacts (Gola et al. 2021), it is vital that microplastic abundance and characteristics in freshwater environments are assessed. However, methodologies used to extract, quantify, and characterise microplastic in environmental media are not consistent between studies. This not only limits comparison of reported loads in different environments, but suggests potential biases in different techniques, and makes experimental design difficult for new researchers.

This chapter builds on and complements *Chapter 2* by explicitly appraising: (i) techniques used to sample, extract, and characterise microplastic particles from different freshwater matrices; (ii) advantages and disadvantages of the different equipment and methodologies used in order to make recommendations for best practice; and (iii) knowledge gaps and data deficiencies that require further research. This work contributes to the growing literature reviewing methods of microplastic detection in freshwater (Prata et al. 2019b; Cutroneo et al. 2020; Fok et al. 2020; Lu et al. 2021), contributing information to support methodology standardisation, and reviews of microplastic occurrence across freshwater ecosystems (Eerkes-Medrano et al. 2015; Lu et al. 2021; Sarijan et al. 2021). These aforementioned reviews only cover literature published up to October 2020, and investigate 49, 67, 74, and 183 studies, respectively. Fok et al. (2020) focus on studies sampling in China and Cutroneo et al. (2020) focuses on studies sampling the marine environment. The intention in this Chapter was to review a more extensive array of freshwater microplastic studies than previous accounts, covering 300 studies published up to August 2022, to assess knowledge gaps, research questions, and implications for current understanding.

3.3 Methods

See section 2.3 of *Chapter 2* for methods on how data were sourced. Where available, data were collected on methods of: sample collection (apparatus); sample processing, including sieving (aperture), density separation (chemicals used),

digestion (chemicals used, temperature, time, and mixing rate), and filtration (pore size, filter type); quality control; particle quantification (apparatus, recovery testing, reporting units); and particle characterisation (distinguishing microplastic from organic and inorganic plastics, shape classification, polymer analysis). For consistency, 'aperture' is used throughout to describe the opening size of both nets and sieves, while 'pore size' refers to the nominal retention size of filter paper.

3.4 Sample collection

Microplastics have been sampled from the aquatic environment either thorough bulk, volume-reduced, or selective sampling techniques (table 3.1). Bulk sampling is where media is collected as a whole, whereas volume-reduced sampling reduces the sample volume *in situ* and preserves material for *ex situ* processing (Hidalgo-Ruz et al. 2012). The mean *in situ* aperture used in volume-reducing and bulk sampling equipment were compared using Student's 2-sample *t*-test (results below). The distribution of both datasets differed significantly from normality (Shapiro-Wilk test: $W = 0.871$ and 0.307 for volume-reduced and bulk sampling, respectively, both with $p < 0.01$) and thus, data were transformed using Ordered Quantile normalising transformation (best transformation based on Pearson P test: $p = 1.744$ and 1.376 for volume-reduced and bulk sampling, respectively). Bulk and volume-reducing sampling can be selective of certain media; for example, at certain timepoints or locations, or under certain conditions. However, hereafter, selective sampling refers to the direct collection of individual organisms from the environment. Variations of these techniques used for each sample matrix are described below.

3.4.1 Water sampling

Microplastic within the water matrix of freshwater ecosystems was investigated by 205 reviewed studies, with 147 and 74 studies sampling fluvial and lentic systems, respectively, indicating overlap in multiple studies. Most of the selected studies (96%; n = 196) sampled microplastic in surface waters, with only fourteen and three studies sampling sub-surface and benthic water, respectively. However, microplastic buoyancy is affected by particle size, shape, surface-area to volume ratio, and density, which themselves are influenced by homo- and

heteroaggregation and biofouling (Kowalski et al. 2016; Nizzetto et al. 2016; Besseling et al. 2017; Khatmullina and Isachenko 2017; Waldschläger and Schüttrumpf 2019). For example, the ratio of heavier polymers to lighter polymers tends to increase with depth (Lenaker et al. 2019; Liu et al. 2021c), yet high-density polymers have been found in surface water (e.g., Bordós et al. 2019; Park et al. 2020a,b; Bertoldi et al. 2021) and low-density polymers occur in benthic sediment (e.g., Sruthy and Ramasamy 2017; Gopinath et al. 2020; Oni et al. 2020; Lenaker et al. 2021). Therefore, the skew towards surface water sampling may bias overall estimates of microplastic abundance, as well as limit risk assessment of benthic habitats.

A few studies sampled microplastic at multiple depths across the water column to investigate their vertical distribution within the same location, generally reporting greater loads in surface waters (McCormick et al. 2016; Lin et al. 2018; Lenaker et al. 2019; Lestari et al. 2020; Xu et al. 2022a). However, Dris et al. (2018b) found insignificant variability in microplastic concentration across three depths in Marne River, France, likely due to vertical mixing of microplastics via hydrodynamics (Wagner and Lambert 2018). Interestingly, Lenaker et al. (2019) observed slightly negative relative percent differences between water surface and depth-weighted microplastic concentrations in Milwaukee River, USA, suggesting underestimation of water column concentrations using surface-only sampling. Conversely, relative percentage differences were positive at lake and estuarine sites (Lenaker et al. 2019). Overall, sampling throughout the water column would provide a more complete understanding of microplastic presence in freshwaters.

Water was sampled by volume-reducing or bulk collection techniques, each used by a similar number of studies (n = 106 and 111, respectively). Table 3.1 lists the number of studies using different water sampling techniques. Volume-reducing techniques mainly involved trawl nets (Plankton, Neuston, Manta, drift, streambed sampler; n = 100), whilst few used sieves (n = 6). Nets used ranged in dimension (11.6-725 cm^2), sample depth (0-15 m), volume of water filtered (0.0003-522 m^3), and aperture (see below) between studies, and were deployed from bridges (centrally and at either side of rivers), riverbanks, docks, boats, or by wading (Kapp and Yeatman 2018). Nets are easy to use and sample large volumes of water but have disadvantages. Firstly, flowing water or a boat is required for sample collection,

with boats, tow ropes, air, and the plastic nets themselves potentially contaminating samples (Prata et al. 2019b). Secondly, nets are subject to clogging depending on water quality and length of time in the water. Lastly, nets come in a range of shapes and sizes that are not standardised across studies, changing their behaviour in water. For example, the side 'wings' of Manta nets provide lift, but hinder their function in rough water (Anderson et al. 2017). Net type may influence the microplastic sampled as Constant et al. (2020) found significantly higher mass concentrations in Rhône River, France, from conical net samples compared to Manta trawl samples, but no difference in numerical concentration.

Bulk sampling collected water using containers made of stainless steel, glass, or plastic (n = 80) or a pump (n = 32), which was then filtered *in situ* or *ex situ*. Containers such as buckets and jars have small volumes and thus, sampling is time consuming and requires multiple replicates to account for local-scale spatial variation (Zhang et al. 2018). Moreover, seven studies used plastic containers to sample water (Vermaire et al. 2017; Schmidt et al. 2018; Watkins et al. 2019a; Weideman et al. 2019; Crew et al. 2020; Irfan et al. 2020a; Nan et al. 2020), risking sample contamination but taking blanks to account for this. Pumps sample larger volumes more easily and allow more choice of filtration aperture, increasing the chance of microplastic detection (Prata et al. 2019b). However, the large equipment and requirement for an energy source can be limiting in the field. Lastly, remotely operated vehicles have been used to continuously sample marine water at different depths up to 1,000 m (Choy et al. 2019), but may not be suited to relatively shallower freshwater environments and water with high flow velocity.

Disparity in water sampling technique has research implications, as apparatus type influences the microplastic load sampled. Felismino et al. (2021) collected over 500 times more microplastic per volume of water in Lake Simcoe, Canada, with grab samples compared to manta trawls. Kapp and Yeatman (2018) collected 355 times more microplastics from Snake River, USA, using 1.85 L glass jars compared to a 100 µm mesh plankton net with a mean collection volume of 3,207 L. Barrows et al. (2017) found an over three orders of magnitude greater microplastic concentration in Maine coast, USA, from a 1 L bulk sample compared to a 335 µm neuston net sample, in addition to a significantly greater proportion of small (100 µm to 1.5 mm) and non-fibrous microplastic and significantly narrower microfibres compared to net

samples. These differences indicate that sampling water using nets may underestimate microplastic load, even when sampling greater volumes, possibly due to missing particles that do not float or are too small to be retained. In contrast, collecting water via pumps may overestimate microplastic load due to the accelerated water flow field enhancing collection of surrounding microplastic (Zhang et al. 2021a). Sample technique may also influence the type of microplastic sampled. For example, Zhang et al. (2021) only observed granules and foam in plankton net samples and not bulk samples from Lijiang River, China.

Water samples were often filtered *in situ* with nets and sieves, with aperture reported in 155 reviewed studies. Net aperture differed by three orders of magnitude from 5 µm (Simon-Sánchez et al. 2019) to 5,000 µm (Xu et al. 2022b) (Figure 3.1); even nets of the same type had different apertures. The most frequently used net apertures were 333 µm (n = 33) and 330 µm (n = 18). Sieve aperture ranged from 10 µm (Liu et al. 2019) to 5 mm (Tien et al. 2020; Xu et al. 2022b), with 45-50 µm used most frequently (n = 17). Mean aperture of volume-reduced ($\bar{x}$ = 234 ± 145 µm) and bulk ($\bar{x}$ = 321 ± 996 µm) sampling techniques were not significantly different (Student's 2-sample *t*-test: $t(159) = -0.059$, $p = 0.953$), indicating that both techniques filter water to the same degree of precision. Sampling apparatus with smaller apertures are known to collect more microplastic particles. For example, the probability of sampling microfibres from River Seine water, France, was 250 times greater using 80 µm mesh compared to 330 µm mesh (Dris et al. 2018b). In coastal water, overall microplastic concentration was 2.5 and 10 times greater in samples collected using 100 µm mesh compared to 333 and 500 µm mesh, respectively (Lindeque et al. 2020). Figure 3.1 shows no trend in microplastic concentration in water sampled with different filtration apertures. With the majority of microplastic being less than 300 µm (Eo et al. 2019; Kooi et al. 2021) and their abundance widely reported to increase with decreased particle size (Baldwin et al. 2016; Mani et al. 2019a; Park et al. 2020a,b; Lenaker et al. 2021), filters with apertures larger than 300 µm should be avoided to prevent underestimating microplastic abundance (Wang et al. 2018b). Sieve aperture must be as low as possible to maximise the number of microplastics sampled, whilst accounting for water quality to avoid blockage. As results depend on the method and apparatus used for sample

collection, biases associated with each technique require further understanding and a standardised method is required for future microplastic detection in water.

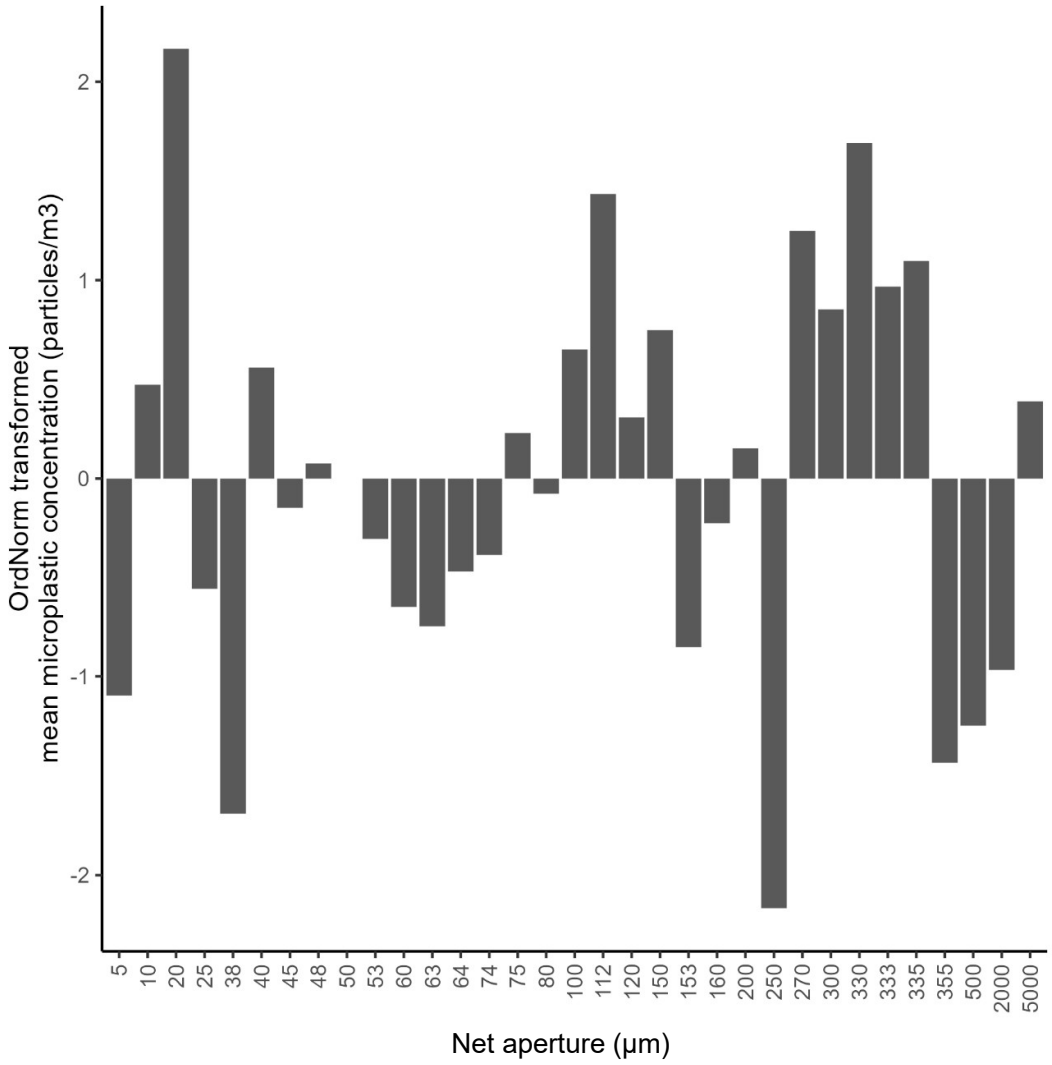


Figure 3.1 Ordered Quantile normalising transformed mean microplastic concentration in water (particles/m^3) of freshwater ecosystems sampled with different net apertures.

3.4.2 Sediment sampling

Microplastic in freshwater sediment (benthic and shore) was investigated by 141 reviewed studies, with 89 and 62 studies sampling sediment in fluvial and lentic systems, respectively. This was 31% lower than the number of studies sampling water. Sediment is a key part of freshwater ecosystems, providing ecosystem

services and habitat for benthic taxa, and can act as a sink to microplastics that will not be transported into the marine environment. Therefore, it is important to consider this matrix in microplastic assessment of freshwater ecosystems.

Table 3.1 lists the number of studies using different sediment sampling techniques. Benthic sediment (bottom of water body) was sampled in 117 studies, mainly by bulk sampling. Half of these studies used a grab sampler (Ekman, Shipek, petite Ponar, standard Ponar, van Veen, and Peterson; n = 57), which ranged in dimension (15-930 cm^2) and sample depth (1-50 cm), even within the same apparatus type (NB not all studies reported equipment dimension). Other sampling equipment included corers (gravity, hammer, sludge, multi-core, Kajak, piston, box, and Dutch auger; n = 30) that ranged in dimension (5-10 cm diameter) and sample depth (0.02-2.12 m), and scoop samplers (shovel, scoop, spatula, spoon, and container; n = 31). Three studies use plastic scoops (Dikareva and Simon 2019; Watkins et al. 2019a; Negrete Velasco et al. 2020), which could contaminate samples.

Hurley et al. (2018a) and Woodward et al. (2021) used the cylinder resuspension technique (Lambert and Walling 1988) to sample fine-grained riverbed sediment in the upper Mersey and Irwell river catchments, UK. This involved placing a large cylinder (height: 69 cm, diameter: 42-45 cm) into the riverbed to a depth of 10 cm, agitating the sediment with a trowel and collecting turbid water (Hurley et al. 2018b; Woodward et al. 2021). Cylinder resuspension can be used across different bed substrates (Duerdoth et al. 2015) and therefore will not be hindered by coarse sediment, a limitation of grab samplers and corers. However, the suspended particulate matter collected with the resuspension technique is mixed with water and thus, could be diluted or contaminated with water associated microplastic. This would similarly happen with grab and scoop samples, as well as the Surber net used by Garcia et al. (2021). To minimise sediment disturbance, sample loss, and contamination, Mani et al. (2019a) used a diving bell to manually sample River Rhine sediment. More microplastic was collected with this technique compared to a bucket and chain dredge (42 and 111 cm depth), but differences were non-significant due to low sample sizes (Mani et al. 2019a). However, this is expensive equipment that is not widely available to researchers. The problems encountered when dredging

sediment are unavoidable but could be controlled for by comparing microplastics loads in sediment to loads in above water.

Shore sediment (riverbanks, lake shores, and beaches) was sampled in 27 studies, again, mainly by bulk sampling, with only three studies sampling both shore and benthic environments (Ballent et al. 2016; Dean et al. 2018; Woodward et al. 2021). Three-quarters of these studies took bulk samples using sediment scoops (2-20 cm deep) (n = 20), three of which employed a quadrat to delineate a sampling area of up to 20 x 30 cm. Bulk shore samples were also taken using a corer (15-30 cm deep; n = 3; Ballent et al. 2016; Dean et al. 2018; Yuan et al. 2022) and an Ekman grab (2-5 cm deep; n = 2; Scopetani et al. 2019; Merga et al. 2020). Only two studies sampling shore sediment took a volume reduced sample; sieving sediment from a 0.25 cm^2 quadrat to a 3 cm depth *in situ* using a 5 mm mesh sieve (Fischer et al. 2016; Blettler et al. 2019). If a study compares the microplastic load of benthic and associated shore sediment, the same sampling apparatus should be used to limit cofounding factors.

3.4.3 Biota sampling

Due to their small size, microplastics can interact with aquatic organisms, being taken up accidentally or actively from water and sediment, posing as a physical, chemical, and biological hazard. Microplastic in freshwater biota was investigated by 76 reviewed studies, with 57 and 26 studies sampling organisms from fluvial and lentic systems, respectively. Table 3.1 lists the number of studies using different biota sampling techniques. The method of sampling biota was reported in all but three studies and varied across classes. Half of fish studies captured specimens in the field using nets (dip, vertical, hook line, fyke, gill, cast, Siene, or trawl; n = 26) and almost half by fishing or electrofishing (n = 24). The remaining studies either purchased specimens from local fishers (n = 8) or used historic specimens (n = 1; Hou et al. 2021). Insects and gastropods were collected by net (dip, kick, Surber, gill, fyke, or cast; n = 17), directly hand-picked from littoral zones (n = 5) or sediment cores (n = 1; Hurley et al. 2017), or purchased (n = 1; Blankson et al. 2022). Within the few bird studies, three sampled faecal material (Reynolds and Ryan 2018; D'Souza et al. 2020; Winkler et al. 2022) and two

sampled whole organisms found dead (Faure et al. 2015; Holland et al. 2016). The single mammal study sampled Eurasian otter faeces (O'Connor et al. 2022). Lastly, reeds (Angiospermae) were hand-collected (n = 1; Yin et al. 2021) and biofilm was sampled with a steel shovel (n = 1; Huang et al. 2021). Unlike water and sediment sampling, techniques used to collect whole organisms from freshwater are unlikely to influence the assessed microplastic contamination, unless plastic equipment fragments and is taken up by organisms.

Most taxonomic classes have been investigated as whole specimens, except for fish. All but one fish study examined microplastics in the gastrointestinal (GI) tract or stomach of individuals after dissection, with muscle, liver (n = 3; Collard et al. 2018; Garcia et al. 2020; Pittura et al. 2022), and gills (n = 7; Garcia et al. 2020; Park et al. 2020b; Liu et al. 2021a; Zhang et al. 2021; Ditlhakanyane et al. 2022; Kılıç et al. 2022; Wu et al. 2022) additionally being examined. Dissecting the GI tract reveals microplastic uptake through ingestion, whereas gills indicate uptake through respiration. Emerging evidence reveals organ-specific localisation of microplastics in fish that differs between species (Jabeen et al. 2017). Ditlhakanyane et al. (2022) found microplastic abundance in banded tilapia fish (*Tilapia sparrmanii*) was 46% higher in their stomach compared to their intestines, with gills contributing to less than one-third of individual microplastic loads. Liu et al. (2021a) and Zhang et al. (2021) also found greater microplastic abundance in fish GI tracts compared to gills. However, Garcia et al. (2020) found similar microplastic concentrations in the gut and gill tissue of two native fish species in Magdalena River, Colombia, indicating similar uptake rates through ingestion and inhalation. Evaluating microplastic uptake on one organ may bias the assessed exposure, especially as different methods of uptake are involved. Furthermore, microplastics found in faecal matter (Reynolds and Ryan 2018; D'Souza et al. 2020; Winkler et al. 2020; O'Connor et al. 2022) reveal the complete passage of particles through organisms, yet little is known regarding any processing, degradation, and/or movement through cell walls, especially for more relevant nanoparticles.

Table 3.1 Number of studies sampling water, sediment, and biota using different sampling techniques. L1 and L2 are level 1 and 2, respectively. Data from n = 300 reviewed studies.

		Sample technique					
Matrix		**L1**		**L2**		**L3**	
Water	205	Bulk	111	Sampler	82	Steel	42
						Glass	21
						Plastic	6
				Pump	32		
		Volume-reduced	106	Net	112	Manta	44
						Plankton	39
						Neuston	16
						Trawl	5
						Drift	2
						Streambed	1
						Kick	1
				Sieve	6		
Sediment	140	Bulk	137	Grab	59	Van Veen	27
						Ponar	11
						Peterson	8
						Ekman	7
						Shipek	2
				Sampler	50	Steel	42
						Plastic	3
				Corer	32	Gravity	5
						Box	4

						Kajak Brinkhurst	3
						Piston	2
						Splitspoon	2
						Multi	1
						Hammer	1
						Sludge	1
						Glass	1
						Dutch Auger	1
				Cylinder resuspension	2		
		Volume-reduced	3	Sieve	2		
				Net	1	Surber	1
Organism	76	Selective	76	Dissected	57	GI tract	54
						Gills	16
						Muscle	3
						Soft tissue	3
				Whole	16		
				Spraint	3		

3.5 Sample processing

After field sampling, sample matrices need to be processed in the laboratory to isolate microplastic for quantification and characterisation. This is performed using four techniques: (1) sieving, (2) digestion (removal of organic material), (3) density separation (extraction by density), and (4) filtration (extraction by size).

3.5.1 Sieving

Sieving reduces the volume of samples for subsequent processing and enables microplastic to be extracted from environmental matrices. Samples can be wet sieved or sieved after drying and sieve aperture is used to fraction samples and therefore microplastic, into distinct size categories. Sieving was performed in 100 water, 82 sediment, and seven biota studies, with aperture ranging from 0.5 µm (Aslam et al. 2022) to 5.6 mm (Ballent et al. 2016; Kay et al. 2018; Corcoran et al. 2020). The latter exceeds the 5 mm size limit defined for microplastic particles (Arthur et al. 2009; GESAMP 2015) and thus, is not suitable for microplastic sampling. Sieving techniques used by reviewed freshwater studies, as categorised by Fok et al. (2020), include:

i. Fine-mesh sieve (< 0.5 mm; n = 61)
ii. Small-mesh sieve (≥ 0.5-1.99 mm; n = 15)
iii. Large-mesh sieve (2-5 mm; n = 26)
iv. Two-sieve stack, collecting particles passing through the upper sieve (usually 5 mm mesh) but retained on the lower sieve (n = 35)
v. Multiple (3 to 12) stacked sieves with descending aperture, allowing separation of microplastic into distinct size classes (n = 53).

The percentage use of each sieve type is displayed in Figure 3.2, showing fine-mesh sieves (32%) and multiple stacked sieves (28%) were most commonly used. Smaller apertures isolate smaller microplastic particles but are more likely to be obstructed by organic and inorganic material. Furthermore, smaller apertures are more likely to retain particles, especially microfibres, due to electrostatic attraction, capillary action, and/or cohesive forces increasing surface tension. This can be reduced with gentle agitation or the addition of surfactants (e.g., Tween 20) to samples or rinse water, helping release particles (Oladejo 2017). A glass or metal plate can also be vertically dipped and withdrawn from water samples to capture surface particles via adhesion and surface tension. Ideally, a standardised filtration aperture should be determined to allow comparison between studies and to consolidate the definition of microplastic, taking into account water quality and sediment grain size.

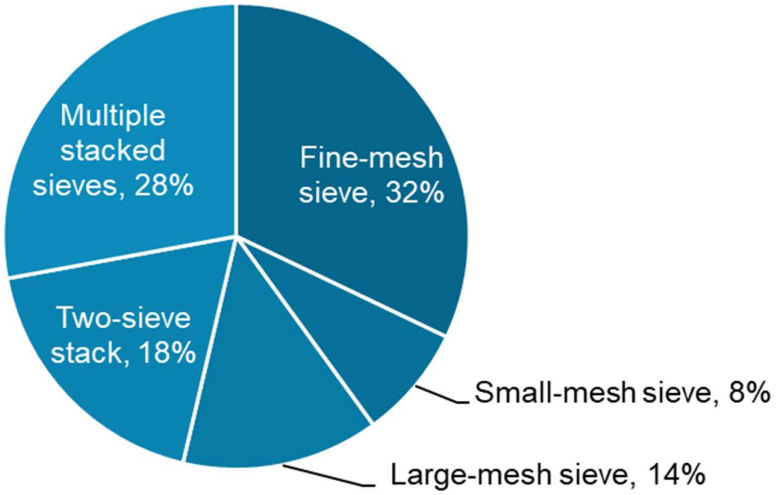


Figure 3.2 Percentage of studies that employed sieving for microplastic extraction (n = 190) that used different sieve types.

3.5.2 Density separation

Plastic polymers range in density from 0.8 to 2.3 g/cm^3 (Hidalgo-Ruz et al. 2012) (Table 3.2), whilst incorporation of additives during manufacturing as well as external biofilm growth (Semcesen and Wells 2021), increases this range. Microplastic can therefore be separated from environmental material based on particle density, as the density of sediment is 2.65 g/cm^3 (Hidalgo-Ruz et al. 2012). Samples are mixed and shaken with a saturated solution, causing sediment to settle and less dense particles to remain suspended or float, with this supernatant being extracted for further processing (Hidalgo-Ruz et al. 2012). This technique was used by 60% (n = 185) of selected studies and commonly applied to sediment (91%; n = 128; Figure 3.3). Only 37% (n = 76) and 22% (n = 17) of water and biota studies used density separation, respectively (Figure 3.3).

Of all selected studies using density separation (n = 185), over half used saturated sodium chloride (NaCl, ρ = 1.13-1.3 g/cm^3; n = 107). This is low-cost, easily available, and non-toxic (Cutroneo et al. 2020; Fok et al. 2020) and thus, is recommended for use by the Marine Strategy Framework Directive technical subgroup of the European Commission Joint Research Centre (European Commission, 2013) and National Oceanic and Atmospheric Administration (NOAA; Mausra et al. 2015). However, NaCl and other applied solutions including canola oil

(n = 1; Crew et al. 2020), castor oil (n = 2; Mani and Burkhardt-Holm 2020; Pol et al. 2022), tap water (1 g/cm^3; n = 2; Vaughan et al. 2017; Lusher et al. 2018), sodium dodecyl sulfate (SDS; 1.01 g/cm^3; n = 1; Cable et al. 2017), and potassium metaphosphate (KO_3P, 0.0055 g/cm^3; n = 1; Tien et al. 2020), are less effective in extracting higher-density polymers (Lusher et al. 2017), especially particles with biofilm. This potentially underestimates observed microplastic concentrations. Density separation via water can recover over 95% of fibres due to their large surface area to volume ratio trapping them in the surface tension film (Alomar et al. 2016; Quinn et al. 2017). Oil has been used due to the oleophilic properties of plastic, with canola oil providing Crichton et al. (2017) with faster microplastic extraction than sodium iodide (NaI) and calcium chloride ($CaCl_2$) and recovering 96% of microplastic from sediment including high-density polyvinyl chloride (PVC). Using oil does require a cleaning step with detergent, but can be combined with saturated solutions to improve recovery rates (Prata et al. 2019b).

Over one quarter of procedures used zinc chloride ($ZnCl_2$, 1.5-1.7 g/cm^3; n = 51) and 10% used NaI (1.6-1.8 g/cm^3; n = 18), which both have higher densities, but are not recommended due to their toxicity and high cost (Cutroneo et al. 2020). Moreover, NaI reacts with cellulose filters used during filtration (see section 3.5.4), turning them black and complicating visual identification (Prata et al. 2019b). Interestingly, most studies using density separation to extract microplastic from sediment used $ZnCl_2$ over NaCl (Figure 3.3), indicating that a higher density solution is needed to separate the organic material. Other applied solutions of higher density are $CaCl_2$ (1.4 g/cm^3; n = 5), sodium tungstate dihydrate (STD: $Na_2WO_4 \cdot 2H_2O$, 1.4 g/cm^3; n = 1; Tsering et al. 2021), potassium formate (HCOOK: K(HCOO), 1.5 g/cm^3; n = 6), sodium polytungstate (SPT: $3Na_2WO_4 \cdot 9WO_3 \cdot {}_2H_2O$, 1.5 g/cm^3; n = 6), NaCl and $ZnCl_2$ combined solution (1.6 g/cm^3, n = 1; Hu et al. 2020), potassium iodide (KI, 1.5-1.75 g/cm^3, n = 1; Zhang et al. 2020b), and zinc bromide ($ZnBr_2$, 1.7 g/cm^3; n = 1; Park et al. 2020a). Zobkov et al. (2020a,b) used HCOOK on sediment to enable further analysis of heavy and trace metals on microplastics, due to its low toxicity.

Fourteen studies (eleven sediment, two water, one biota) used multiple density separation steps with different solutions, either consecutively or with other processing steps in-between, helping to isolate different polymer types. For example,

Hurley et al. (2017) firstly used 1.025 g/cm^3 NaCl, filtering the supernatant through a GF-C glass microfibre filter, then subsequently applied 1.2 g/cm^3 NaCl and 1.8 g/cm^3 NaI for further density separation. Table 3.2 illustrates which density separation solution is effective for different polymer types according to their respective densities. However, it should be acknowledged that microplastic density is further influenced by additive concentration, homo- and hetero-aggregation, and biofouling (Quinn et al. 2017). This can be mitigated by performing chemical digestion (see section 3.5.3) to remove biofilm before density separation. Solutions greater than 1.4 g/cm^3 are recommended to separate microplastics from organic and inorganic material, as this exceeds the density of most polymer types and microplastic recovery increases with increasing solution density (Quinn et al. 2017).

Table 3.2 Density of polymer types and resulting effectiveness of separation solutions, adapted from Stuart (2008) and Prata et al. (2019b).

Polymer	Density (g/cm^3)	Oil	Water	SDS	NaCl	$CaCl_2$, STD	HCOOK, SPT, $ZnCl_2$	KI	NaI, NaCl+$ZnCl_2$	$ZnBr_2$
		0.9 g/cm^3	1 g/cm^3	1.01 g/cm^3	1.2 g/cm^3	1.4 g/cm^3	1.5 g/cm^3	1.52-1.63 g/cm^3	1.6 g/cm^3	1.7 g/cm^3
SR	0.8	+	+	+	+	+	+	+	+	+
PP	0.85-0.92	+	+	+	+	+	+	+	+	+
LDPE	0.89-0.93	+	+	+	+	+	+	+	+	+
HDPE	0.94-0.98	-	+	+	+	+	+	+	+	+
PA	1.01-1.05	-	-	-	+	+	+	+	+	+
PS	1.04-1.1	-	-	-	+	+	+	+	+	+
PAN	1.14-1.17	-	-	-	+	+	+	+	+	+
PMMA	1.09-1.2	-	-	-	+	+	+	+	+	+
PMA	1.17-1.2	-	-	-	+	+	+	+	+	+
PU	1.2-1.26	-	-	-	±	+	+	+	+	+
PC	1.2-1.22	-	-	-	±	+	+	+	+	+
PVC	1.16-1.58	-	-	-	±	+	+	+	+	+
PVA	1.17-1.31	-	-	-	±	+	+	+	+	+

Alkyd	1.24-2.1	-	-	-	-	+	+	+	+	+
PEST	1.24-2.3	-	-	-	-	+	+	+	+	+
PET	1.37-1.45	-	-	-	-	±	+	+	+	+
POM	1.41-1.61	-	-	-	-	-	±	±	±	+
PTFE	2.1-2.3	-	-	-	-	-	-	-	-	-

Key +: separation, ±: possible separation, -: no separation. Separation solutions: sodium dodecyl sulfate (SDS), sodium chloride (NaCl), calcium chloride ($CaCl_2$), sodium tungstate dihydrate (STD: $Na_2WO_4 \cdot 2H_2O$), potassium formate (HCOOK: K(HCOO)), sodium polytungstate (SPT: $3Na_2WO_4 \cdot 9WO_3 \cdot {}_2H_2O$), zinc chloride ($ZnCl_2$), potassium iodide (KI), sodium iodide (NaI), zinc bromide ($ZnBr_2$). Polymers: SR: silicone rubber, PP: polypropylene, LDPE: low-density polyethylene, HDPE: high-density polyethylene, PA: polyamide (nylon), PS: polystyrene, PAN: polyacrylonitrile PMMA: Poly(methyl methacrylate) (acrylic), PMA: poly(methyl acrylate), PU: polyurethane, PC: polycarbonate, PVC: polyvinylchloride, PVA: polyvinyl alcohol, PEST: Polyester, PET: polyethylene terephthalate, POM: polyoxymethylene, and PTFE: polytetrafluoroethylene. Polymer density adapted from Hidalgo-Ruz et al. (2012).

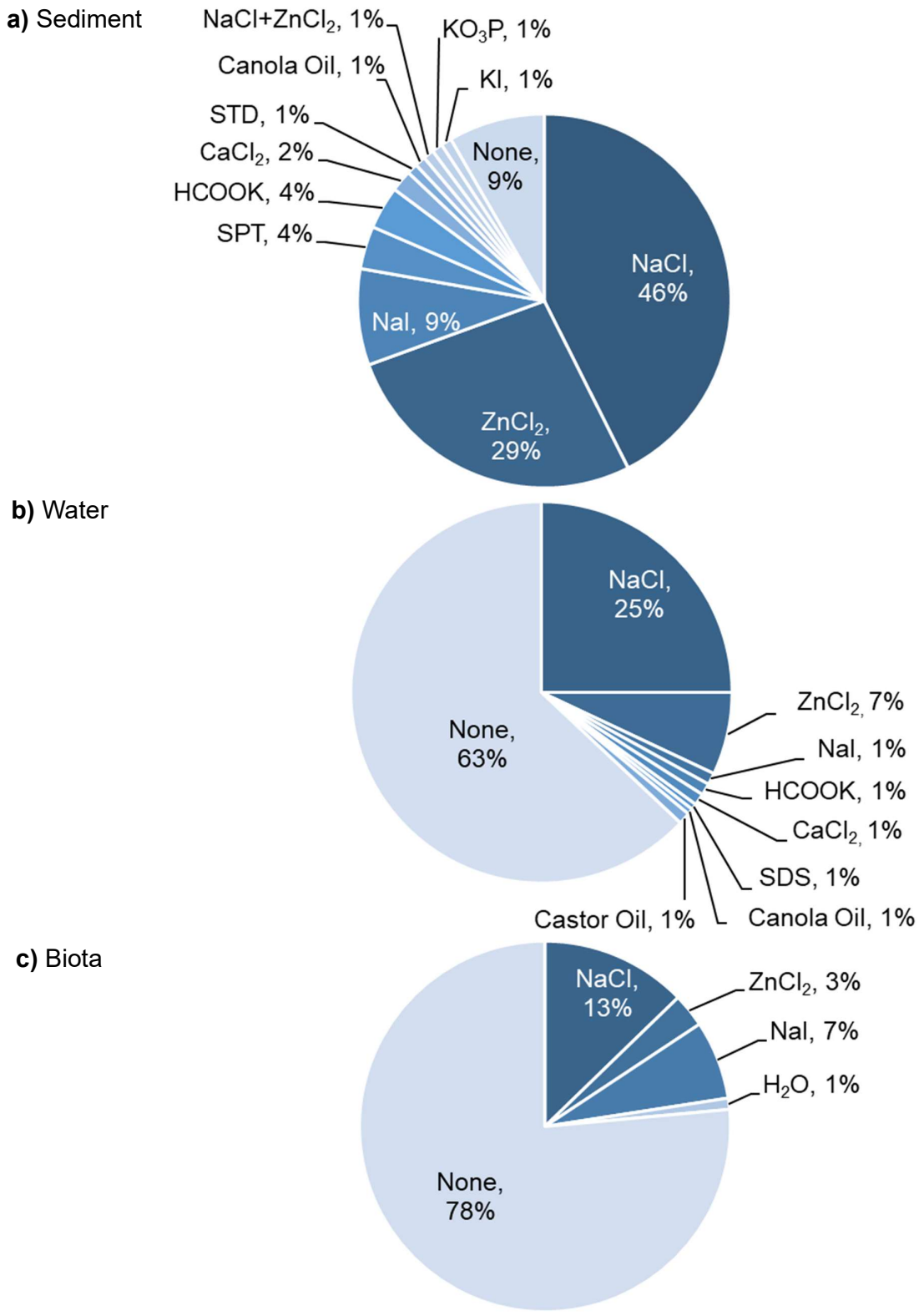


Figure 3.3 Percentage of all freshwater **a)** sediment (n = 141), **b)** water (n = 205), and **c)** biota (n = 76) microplastic studies that used different density separation solutions. Sodium chloride (NaCl), sodium iodide (NaI), zinc chloride ($ZnCl_2$), zinc

($ZnBr_2$), calcium chloride ($CaCl_2$), potassium formate (HCOOK), potassium metaphosphate (KO_3P), sodium polytungstate (SPT), sodium dodecyl sulfate (SDS), sodium tungstate dihydrate (STD), canola oil, castor oil, water (H_2O), or potassium iodide (KI). Eleven studies applied multiple density separation solutions and thus, percentage sums exceed 100%.

3.5.3 Chemical digestion

Chemicals, with stirring and/or heating, have been used to digest organic matter, aiding the isolation of microplastic. This approach was used by three quarters of selected studies (n = 227) and was slightly more common in biota (80%; n = 61) and water (76%; n = 156) studies than sediment studies (63%; n = 89). Four main methods of chemical digestion were used: oxidative, acidic, alkaline, and enzymatic digestion.

Oxidative digestion with hydrogen peroxide (H_2O_2), known as Wet Peroxide Oxidation (WPO), was the most used digestive agent (84% of studies performing digestion), more so in sediment (92%; n = 82) and water (91%; n = 142) studies than biota studies (46%; n = 28) (Figure 3.4). The concentration was typically 30% but ranged from 10% (Cera et al. 2022b) to 50% (Liu et al. 2019a; Olesen et al. 2019). When H_2O_2 is used alone, digestion times range from 15 minutes to 1 week, with 24 hours being used most frequently amongst reviewed studies (n = 28). In many cases, this digestion was accelerated using ferrous iron (Fe(II)) catalysts, known as Fenton's reagent: Fe(II) (n = 43), Fe(II) sulfate ($FeSO_4$; n = 12), Fe(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$; n = 8), Fe(II) chloride ($FeCl_2$; n = 1; Peller et al. 2019), and Fe(III) chloride ($FeCl_3$; n = 1; Zhang et al. 2022). The most frequently used digestion time reduced to 30 minutes with the addition of Fe(II) (n = 7). Acids and bases were often added to Fenton's reagent to alter the pH, including sulfuric acid (H_2SO_4; n = 10), hydrochloric acid (HCl; n = 1; Peller et al. 2019), or sodium hydroxide (NaOH; n = 2; Liu et al. 2019; Olesen et al. 2019). Fenton's reagent is considered optimal as it removes significantly more organic matter than alkaline digestion and does not degrade microplastic (Tagg et al. 2017; Hurley et al. 2018a). Zobkov et al. (2020a) published a detailed method for microplastics extraction from freshwater sediment. This included a preliminary wet peroxide oxidation step with H_2O_2, before filtration and extraction with density separation, followed by wet

peroxide oxidation with Fenton's reagent and chitin and mineral fraction digestion with HCl (Zobkov et al. 2020a).As well as WPO, alkalines were also used to digest soft tissue in biota (48% of studies; n = 30; Figure 3.4c). These include 10-30% potassium hydroxide (KOH; n = 41) and sodium hydroxide (NaOH; n = 6). Digestion time for alkalines ranges from 10 minutes to 3 weeks, with 48 hours being most frequently used amongst reviewed studies (n = 7). When comparing the recovery rate of three different methodologies for extracting microplastic from sediment, Nava and Leoni (2021) concluded digestion with 10% KOH to be optimal due to its simplicity, reproducibility, and affordability, despite recovery rate being greatest with NaCl and NaI density separation. Alkalines have high digestive efficiency (Wu et al. 2020a), but are reported to damage or discolour polyethylene (PE), PVC and nylon (Cole et al. 2014). The more basic and potentially less damaging detergent, sodium dodecyl sulfate (SDS), was used to digest organic matter in water residue (n = 7), mostly for 24 hours (n = 4).

The alternative of acidic digestion was used less frequently: 55-69% nitric acid (HNO_3; n = 4) was used to digest insects (Nel et al. 2018; Stanković et al. 2021), fish tissue (Roch et al. 2019), and water residue (Kaliszewicz et al. 2020), for 15 minutes to 72 hours. 4.5% HCl (calcite digestion) digested water and sediment residue (n = 5) for 24 to 48 hours and sodium hypochlorite (NaClO; n = 2) digested fish (Collard et al. 2018) and water residue (Correa-Araneda et al. 2022) for 24 hours. Acid can also degrade polymers, especially those with a low pH tolerance and low heat deflection temperature (Claessens et al. 2013; Qiu et al. 2016; Prata et al. 2019b) such as polystyrene (PS), poly(ethylene terephthalate) (PET), and nylon. Moreover, HCl is reported to have low digestion efficiency (Wu et al. 2020a) and thus, is sometimes used in combination with WPO (Lenaker et al. 2019; Lenaker et al. 2021; Shen et al. 2021). Some studies mixed digestive chemicals to increase digestion efficiency, including a 1:1 (v/v) mixture of 34.5-36.5% H_2O_2 and 10% KOH (Akindele et al. 2019), a 1:3 (v/v) mixture of 30% H_2O_2 and 69% HNO_3 (Kaliszewicz et al. 2020) and 1:1 (v/v) mixture of 30% KOH and NaClO (Yuan et al. 2022).

Enzymes were used to digest organic material in water (n = 7) and biota (n = 5) (Figure 3.4b,c) and included lipase, protease, amylase, cellulase, chitinase, and carbohydrase. Enzymes are less damaging to both microplastics and the environment (Cole et al. 2014; Courtene-Jones et al. 2017), but are expensive,

operate at small scales, and digestion is time-consuming (Wu et al. 2020). Digestion times ranged from 16 hours to 5 days, with 48 hours being the most frequently used digestion time amongst reviewed studies (n = 4). Furthermore, enzymes are biological substances that require specific activation conditions. Lastly, 26 studies used multiple digestion steps with different solutions, with sieving, filtration, or density separation in between. For example, Liu et al. (2019) digested sieved water samples using 1) SDS, 2) 50% H_2O_2 with a Fe(II) catalyst, 3) Cellubrix and Viscozyme enzymes, 4) Alcalase enzyme, then 5) 50% H_2O_2 with a $FeSO_4$ catalyst and NaOH.

Digestion conditions, including temperature, stirring, and duration, varied among studies. Overall, duration was mentioned by 152 studies, with a large range from five minutes (Rakib et al. 2022; Warrier et al. 2022) to one month (Imhof et al. 2013; Tien et al. 2020), with most digestion procedures running “overnight” or for 24 hours (n = 47). Incubation temperature was mentioned in 146 studies, ranging from 20°C (Li et al. 2021b; Prata et al. 2021) to 100°C (Nel et al. 2018; Mao et al. 2020b). Most operated at room temperature (n = 32), followed by 60°C (n = 30) and 75°C (n = 24). Choice of temperature is partly dictated by the chemical used, but must consider that most plastics melt above 100°C, with some melting below 70°C. Stirring was mentioned in 47 studies, with rotational speed ranging from 30 rpm (Parker et al. 2022a) to 5,000 rpm (Collard et al. 2018). As with density separation (see section 3.5.2), chemical digestion techniques for microplastic extraction are not standardised, which may cofound the comparison of microplastic loads reported across studies. However, the most effective technique must be established based on sample type, quality, and amount of organic and inorganic material.

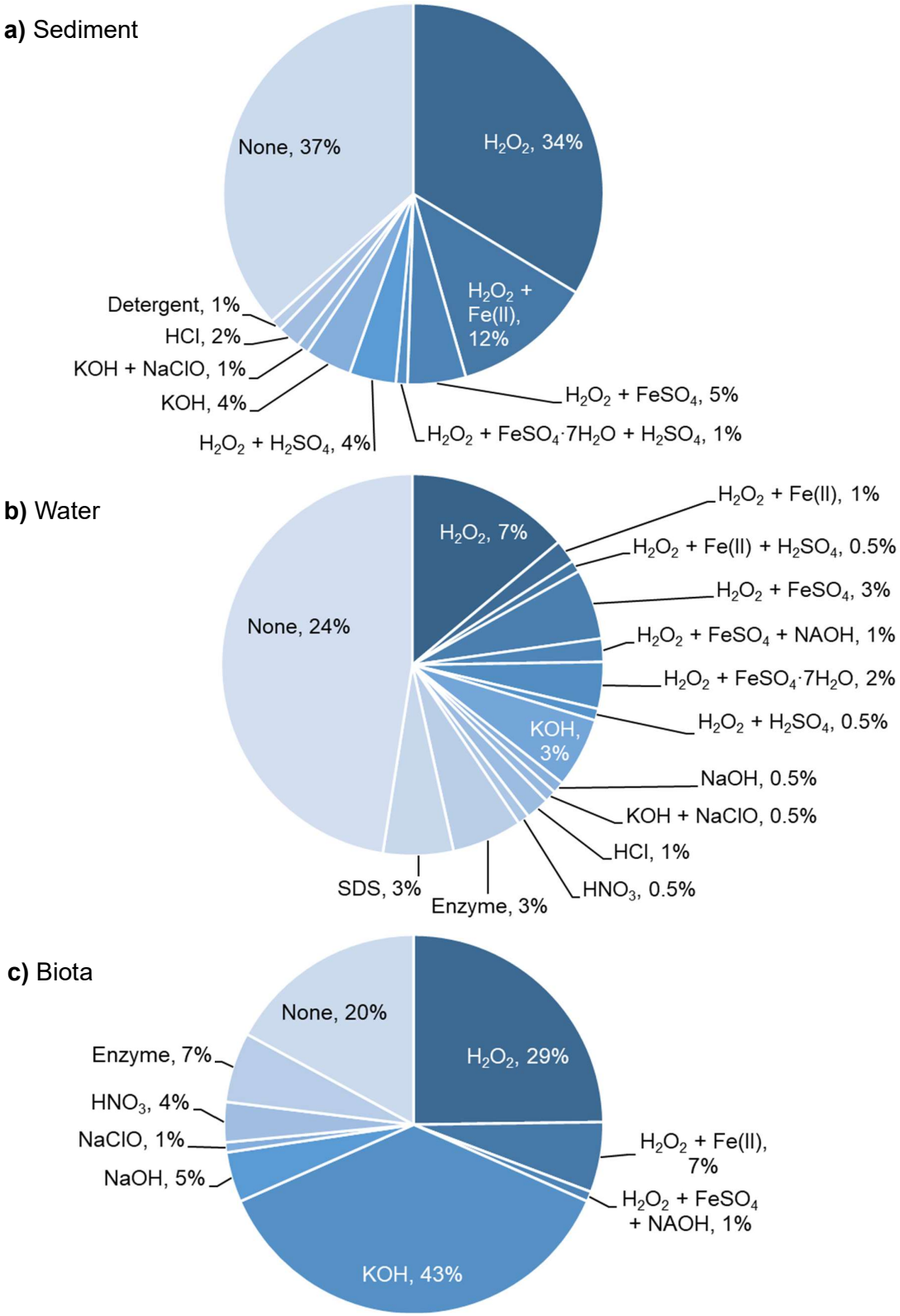


Figure 3.4 Percentage of all freshwater **a)** sediment (n = 141), **b)** water (n = 205), and **c)** biota (n = 76) microplastic studies that used different digesting solutions. Twenty-six studies used multiple solutions and thus, percentage sums exceed 100%.

3.5.4 Filtration

Microplastic was extracted directly from environmental media or from density separated and chemically digested supernatant via filtration, either assisted by gravity or a vacuum. Filtration was performed by 224 studies: 149 water (of which 19% had no pre-treatment), 106 sediment, and 58 biota studies. Peters and Bratton (2016) and Horton et al. (2018) filtered stomach contents of freshwater fish with no pre-treatment. Pore sizes used range from 0.2 µm (Akindele et al. 2019; Bordós et al. 2019; Mintenig et al. 2020; Negrete Velasco et al. 2020; Scherer et al. 2020; Tien et al. 2020; Ajay et al. 2021; Shen et al. 2021) to 500 µm (de Carvalho et al. 2021; Garcia et al. 2021; Haberstroh et al. 2021a), with 0.45 µm being the most used (n = 63). Similarly to sieving (see section 3.5.1), filter paper pore size influences the type and abundance of microplastic extracted from samples, as it limits the size of particles that can be retained. Filters with larger pores are more likely to result in underestimation of microplastic abundance.

Glass fibre filters were most commonly used for filtration (n = 97; Figure 3.5) in studies reporting filter material, ranging in pore size from 0.2 µm (Ajay et al. 2021) to 5 µm (Yan et al. 2021). This was followed by cellulose esters, including cellulose nitrate/nitrocellulose and cellulose acetate (0.22-450 µm; n = 43). Other filter material used include nylon (0.22-500 µm; n = 12), polycarbonate (0.45-10 µm; n = 10), quartz (0.3-2.2 µm; n = 4), stainless-steel (2-45 µm; n = 3), aluminium oxide (0.2 µm; n = 3), inorganic membrane (0.2-25 µm; n = 3), polytetrafluorethylene (0.22-5 µm; n = 3), silver (0.5-5 µm, *n* = 3), “mesh” (5-500 µm; n = 3), nickel copper alloy (Monel; 30 µm; n = 1; Scircle et al. 2020), milling silk (10 µm, n = 1; Stanković et al. 2021), and microline (reverse osmosis; n = 1; Yin et al. 2019) (Figure 3.5). Polymer-based filters, i.e., cellulose esters, polycarbonate, and polytetrafluorethylene, should be avoided when identifying polymers with Fourier-transform infrared spectroscopy (FTIR) (see section 3.6.3), as they are identified in the spectra. This needs to be considered when choosing a filter type, as well as their cost, which can vary greatly.

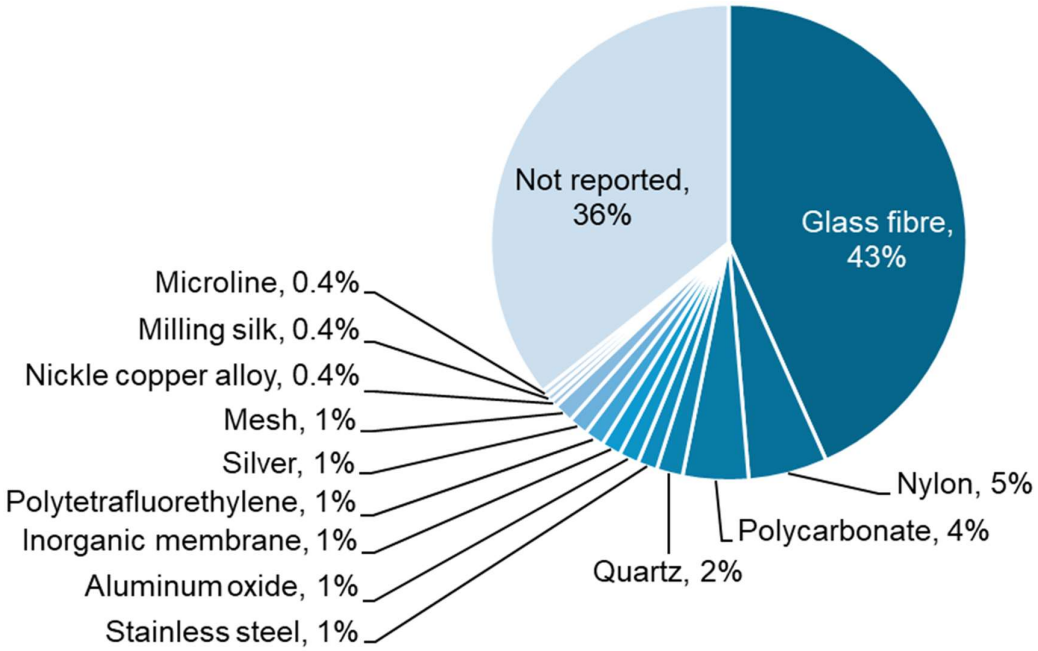


Figure 3.5 Percentage of studies employing filtration for microplastic extraction (n = 224) using different filtering material.

3.5.5 Quality control

Samples can easily become contaminated by particles present in the air *in situ* and *ex situ*, on clothes of workers, in containers and equipment, and by improperly sealed samples (Hidalgo-Ruz et al. 2012). These sources of contamination must be minimised as much as possible to improve accuracy of results and avoid overestimation of microplastics in samples. Control measures include: (1) wearing 100% cotton clothing and laboratory coats when sampling and processing samples; (2) minimising traffic in the laboratory; (3) minimising use of synthetic materials; (4) wearing gloves; (5) processing samples in a laminar flow cabinet; (6) wiping down surfaces before processing; (7) rinsing all equipment before use; (8) filtering solutions before use; (9) storing samples and filters in glass containers where possible; and (10) keeping samples and filters covered whenever possible (Marine & Environmental Research Institute 2015). It is good practice to take a control sample at various stages of sampling and processing to identify microplastic contamination, which can be deducted from the sample microplastic count.

3.6 Microplastic quantification and characterisation

3.6.1 Microplastic identification

Extracted particles were visually sorted to quantify microplastic loads in samples, with 91 studies using no equipment and only human eye. Microscopes were usually employed to aid particle detection (n = 240) up to 1 mm (Song et al. 2015) or 500 µm (Hidalgo-Ruz et al. 2012; Löder and Gerdts 2015b). Light microscopes, including binocular, compound, optical, and stereo (dissecting) microscopes, were most common amongst studies using microscopy (n = 212; 71%). Other microscopes used were scanning electron (SEM; n = 28), which is also coupled with Energy Dispersive Spectroscopy (EDS; see section 3.6.3), UV fluorescence (n = 11), metallographic (n = 7), and laser confocal (n = 1).

Criteria were used to distinguish microplastic from organic and inorganic particles, thereby preventing over- or underestimation of microplastic loads through misidentification. Reported criteria (Norén 2007; Hidalgo-Ruz et al. 2012; Nor and Obbard 2014; Horton et al. 2017a; Vaughan et al. 2017; Barrows et al. 2018; Horton et al. 2018; Tibbetts et al. 2018; Townsend et al. 2019; Khan et al. 2020; Kuśmierek and Popiołek 2020; Mao et al. 2020a; Uurasjärvi et al. 2020; Woodward et al. 2021) were reviewed and summarised as follows:

1) Particles have no cellular or organic structure.
2) Particles have an unnatural shape.
3) Fibres are equally thick throughout their length, are not segmented or twisted flat ribbons, and have 3D bending (i.e., not entirely straight).
4) Particles are not shiny, have clear and homogenous colour and if transparent or white, must be examined under high magnification and fluorescence to exclude organic origin.
5) Particles have a homogenous texture.
6) Particles maintain structural integrity when compressed, without being brittle.

Simple tests were also used to aid microplastic identification. The break test classes particles that do not break when prodded with probes as plastic (Marine & Environmental Research Institute 2015). However, this can still misidentify particles as plastic, notably cotton fibres (Hendrickson et al. 2018), and was only used by two studies (Egessa et al. 2020; Wu et al. 2022b). The hot needle test classes particles

that melt or curl when prodded with a heated needle tip as plastic (De Witte et al. 2014; Marine & Environmental Research Institute 2015) and was used by thirteen reviewed studies.

Staining particles can further aid the sorting process and reduce false positives (Löder and Gerdts 2015b; Pastorino et al. 2021). Nile Red is a lipophilic dye used to stain microplastic and not biological material, and is visualised under fluorescent microscopy (Jee et al. 2009; Andrady 2011; Shim et al. 2016). This was used by six studies (Fischer et al. 2016; Crew et al. 2020; Mao et al. 2020b; Scircle et al. 2020; Simmerman and Wasik 2020; Prata et al. 2021). Rose-Bengal (4,5,6,7-tetrachloro-2',4',5',7'-tetraiodofluorescein) is a bright red stain that dyes biological material (epithelial cells, mucus, fibrous tissue) and not microplastic, and does not require fluorescence microscopy (Feenstra and Tseng 1992a; Ziajahromi et al. 2017b; Pastorino et al. 2021). This was only used by Pastorino et al. (2021) and is limited by the exclusion of red/pink microplastic particles during enumeration, causing underestimation. Interestingly, Lasee et al. (2017) performed no visual sorting or microplastic identification test and determined microplastic mass concentration (mg/L) by dividing the filtrate mass by the grab sample volume, which does not assess whether particles are microplastic.

As well as microplastic enumeration, visual sorting enables microplastic characterisation. Particle shape, size, and colour were used to identify the primary and secondary origin of microplastic particles, environmental residence time, and fragmentation processes. This aided the identification of pollution sources and informed understanding of microplastic behaviour in freshwater. Moreover, particle characteristics drive effects on biota and thus, should be reported to inform exposure experiments (Thornton Hampton et al. 2022a). Particle shape was recorded in 92% of studies (n = 275) and described with 84 unique terms. The latter profusion limits comparison between studies and analysis of trends, with similar microplastic particles potentially classed into distinct categories. Criteria used for microplastic shape classification (Supplementary Information A1) were reviewed and summarised as follows:

1) Fragment – Hard, irregular shaped cube with at least one smooth plane, angular, jagged, incomplete, and 3D.
2) Bead/Pellet – Hard, round, spherical, ovoid discs, cylinders, and 3D.
3) Foam – Lightweight, sponge or bubble-like, and surface is not smooth.
4) Fibre – Thin, fibrous, thread-like, slender, elongated, cylindrical, equally thick throughout (not tapered at ends), not entirely straight, 3D bending, and length is >3 times width.
5) Film – Thin with two smooth planes, 2D, flat, irregular in shape soft, and flexible.
6) Other.

Future studies should use these standardised microplastic identification and shape criteria to aid characterisation and comparison between studies. Visual sorting is time intensive, subject to human error, and dependent on microscope quality and magnification (Löder and Gerdts 2015b). Reported error rates from visual sorting range from 20% (Eriksen et al. 2013) to 70% (Hidalgo-Ruz et al. 2012) and increases with decreasing particle size (Löder and Gerdts 2015b). This suggests that reported microplastic loads are underestimated. Instrumental analysis of particles should be carried out as standard (see section 3.6.3), to confirm their polymer status and limit human error.

3.6.2 Reporting units and freshwater microplastic observations

Different quantitative results were reported based on the approaches adopted in sampling and processing. Microplastic loads were reported as raw values, ranges, and/or averages, mainly as numerical concentration (n = 290; 97% of studies), with a few studies reporting mass concentration (n = 28; 9%), or prevalence, i.e., percentage of individuals or samples contaminated with at least one microplastic particle (n = 49; 16%). However, researchers recommend both counts and mass should be reported as both influence effects in biota (de Ruijter et al. 2020; Thornton Hampton et al. 2022a).

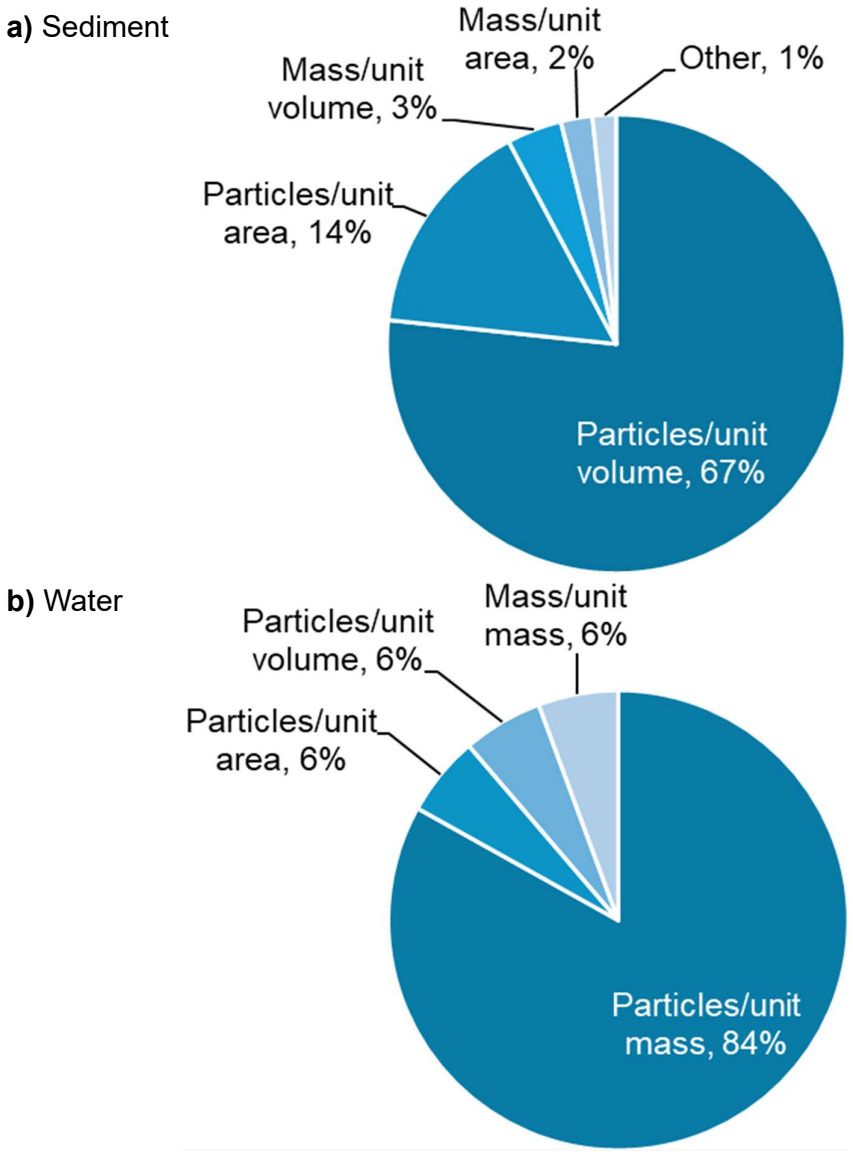
In the 141 sediment studies, microplastic numerical concentration was mainly reported per mass in wet weight (ww) or dry weight (dw) (particles/g or particles/kg; n = 118; 84% of studies), then per unit area (particles/m^2 or particles/km^2; n = 8; 6%)

or per volume (particles/m^3, particles/dm^3, or particles/L; n = 8; 6%) (Figure 3.6a). Microplastic mass concentration in sediment was only reported in eight studies, as particle mass per unit mass of sediment (µg/g or mg/kg). In the 205 water studies reviewed, microplastic numerical concentration was mainly reported per unit volume (particles/m^3, particles/mL, or particles/L; n = 138; 67%), followed by unit area (particles/km^2; n = 28; 14%) (Figure 3.6b). Single studies used unique numerical concentration units including particles/15-minute trawl (Kay et al. 2018), particles/m^3/minute (Bertoli et al. 2022), and particles/sample (Chauhan et al. 2021), which are non-comparable to other studies. Microplastic mass concentration in water was reported almost ten times less than numerical concentration, as mass per volume (µg/m^3, mg/m^3, g/m^3, µg/L, and mg/L; n = 7; 3%) or mass per area (µg/m^2, g/km^2, mg/km^2; n = 4; 2%). All reporting units used in reviewed studies are listed in Table A2.

Inconsistency in reported units of microplastic concentration has research implications as it limits comparison amongst studies, both within and between environmental matrices, and makes meta-analysis less reliable. Unit area is not representative of these matrices as they are inherently 3D, with freshwater hydraulics allowing microplastic to be incorporated in all dimensions. Regarding mass concentration, there was no standardisation of water content in sediment and in many cases, the distinction between wet and dry mass was not reported. Freshwater sediment mass depends on its water content, as well as material type, and grain size and thus, are non-comparable within and between freshwater ecosystems. Future research should report microplastic concentration in as many units as possible to enable comparison to previous studies. To compare microplastic loads between environmental matrices, a standardised reporting unit is required, for which particles per unit volume is the most appropriate. This is because environmental media are 3D and volume is a consistent measure. Moreover, it removes the influence of inconsistent sediment mass and water content between ecosystems on observed microplastic concentrations, and allows the effect of sediment type and grain size on microplastic retention in sediment to be measured.

In the 76 studies sampling freshwater biota for microplastic, particle numerical concentration was mainly reported per whole individual (n = 52; 68%), then per unit mass of tissue (particles/g, particles/g ww or particles/g dw; n = 24; 32%), per

individual organ, i.e., GI tract, GI tract content, gill, or gizzard (n = 8; 11%), and per spraint (n = 2; Reynolds and Ryan 2018; O'Connor et al. 2022) (Figure 3.6c). Similarly to water and sediment sampling, microplastic mass concentration in freshwater biota was poorly reported, with only four studies reporting microplastic mass per individual or per wet or dry mass of individuals or tissue (mg/g dw or mg/g ww) (Faure et al. 2015; Olesen et al. 2019; Merga et al. 2020; Pan et al. 2021). Both numerical and mass concentrations are required to study microplastic effects on biota, as particles are insoluble and thus, have physical and chemical effects (Thornton Hampton et al. 2022a). Lastly, over half of reviewed biota studies reported prevalence of microplastic in individuals sampled (n = 43; 57%), which shows the extent of microplastic infiltration into the food web.



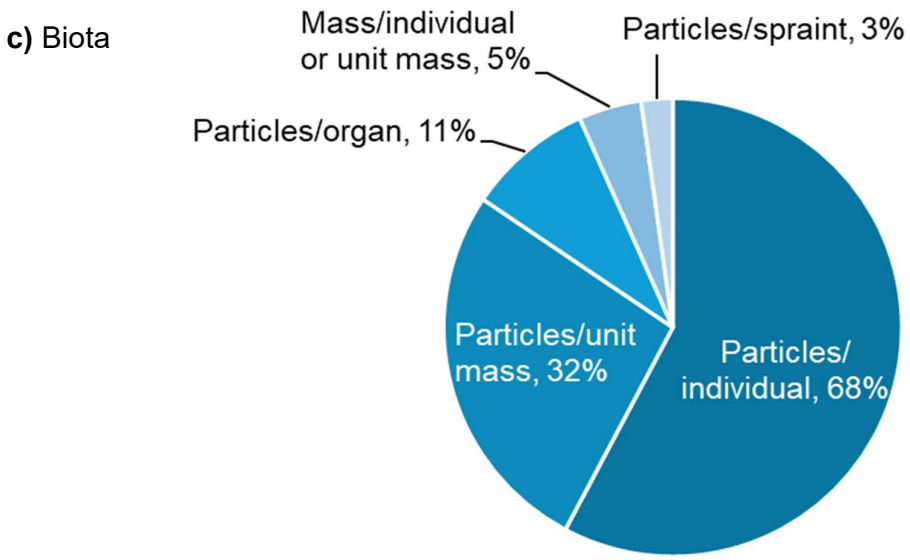


Figure 3.6 Percentage of all freshwater **a)** sediment (n = 141), **b)** water (n = 205), and **c)** biota (n = 76) microplastic studies that used different reporting units.

3.6.3 Polymer analysis and type

To remove false positives identified during visual identification and to assess pollution sources and their impact on organisms and the environment, the polymer type of particles should be assessed. Instrumental analysis is usually performed on a subset of isolated particles and was conducted by 89% of studies (n = 246). Several instruments were employed to identify polymer type, which are grouped into spectroscopy and thermal analysis with mass spectrometry. Figure 3.7 shows the division of these instruments between studies.

Spectroscopy projects electromagnetic radiation at specific wavelengths onto polymers to determine the absorbance or transmittance response of electrons, providing information about specific chemical bonds and functional groups (GESAMP 2019). Unique particle spectra are compared with spectra of known polymers to identify polymer type (Hidalgo-Ruz et al. 2012). Spectroscopy was used in three-quarters of studies using instrumental analysis, mainly with infrared (IR). Fourier-transform infrared spectroscopy (FTIR; n = 44) measures transmittance, reflectance, and attenuated total reflectance (ATR-FTIR; n = 67). Using ATR has a number of advantages over transmission as it does not require IR light to pass through samples and thus, can be used for thick and opaque material (GESAMP

2019). ATR also provides more stable spectra from material with typically irregular surfaces, can detect smaller particles compared to transmission, and has a higher detection rate of natural and synthetic cellulosic fibres (Rayon/Viscose) that are difficult to discriminate with microscopy alone (Comnea-Stancu et al. 2017). However, ATR requires contact between the sample and ATR crystal, which may hinder further analysis (Shim et al. 2017; GESAMP 2019). Smaller particles down to 10 µm can be identified using micro-FTIR (µ-FTIR; n = 55) along with ATR (ATR-µ-FTIR; n = 13) (La Russa et al. 2009; Shim et al. 2017; GESAMP 2019). This microscope is more suitable for polymer analysis of microplastic as it does not require the movement of particles as with macro-FTIR, which is difficult at the micron size.

Automated processing using linear array or Focal Plane Array (FPA) FTIR, can scan and analyse whole sample filters at once, reducing manual identification (Levin and Bhargava 2005; Primpke et al. 2017; Scircle et al. 2020). However, automated techniques are expensive, require clean filters (apart from microplastic), take multiple hours per scan, and produce cumbersome data. This may be more labour intensive than manual scanning, likely resulting in automated-FTIR being used by only one reviewed study (Scircle et al. 2020). Other IR spectroscopy techniques used include near-infrared (NIR; 0.8-2.5 µm; n = 2; Van der Wal et al. 2015; Fiore et al. 2022), short-wavelength infrared (SWIR; 0.9-1.7 µm; n = 1; Schmidt et al. 2018), and laser direct infrared imaging (LDIR; n = 2; Jin et al. 2022; Yan et al. 2022). Compared to FPA-FTIR, LDIR uses a tuneable quantum cascade laser as the IR source to only target particles, not empty spaces (Cheng et al. 2022). It therefore produces stronger signals at a faster speed than FPA-FTIR and does not require liquid nitrogen (Cheng et al. 2022).

Raman mass spectroscopy (RMS) was used by one quarter of reviewed studies analysing polymer type (n = 65). This technique projects a laser beam (500-800 nm) onto a sample, which produces different frequencies of back-scattered light depending on crystalline structure and atoms present, creating a unique spectrum for microplastic identification (Löder and Gerdts 2015b). Both RMS and FTIR are non-destructive techniques, but RMS can detect microplastic down to 1 µm compared to 10 µm using µ-FTIR (Imhof et al. 2013; Shim et al. 2017). Micro-RMS can even be used to identify particles below 1 µm (Löder and Gerdts 2015b), but is a lot slower

than μ-FTIR (GESAMP 2019) and no reviewed studies have used this technique. RMS was also used in combination with FTIR by three studies (Ballent et al. 2016; Dean et al. 2018; Sekudewicz et al. 2021). However, a disadvantage of RMS is that it obtains spectra for additive and pigment chemicals in microplastic, which are subsequently identified rather than the polymer itself (Van Cauwenberghe et al. 2013). Alternatively, energy-dispersive x-ray spectroscopy (EDS) in combination with SEM determines the surface elemental composition of particles, by sending electrons to the sample and detecting x-ray photons. This was used by 20 reviewed studies. X-ray photoelectron spectroscopy (XPS) and x-ray fluorescence (XRF) spectroscopy are two further x-ray spectroscopy techniques, each used by single studies (Ballent et al. 2016 and Mao et al. 2020b, respectively).

Despite its popularity, FTIR is limited by coloured particles that produce poor-quality spectra, and RMS is limited by fluorescence interference from additives, or contaminants within or in the surrounding biofilm (Van Cauwenberghe et al. 2013). An alternative used by eight reviewed studies is thermal analysis, which measures changes in the physical and chemical properties of polymers depending on their thermal stability (Löder and Gerdts 2015b). Differential scanning calorimetry (DSC), used on sediment microplastic by Castañeda et al. (2014) (n = 1), measures heat flow into and out of a sample and compares this to reference material to identify chemical composition (Shim et al. 2017). This technique is relatively simple and fast, but cannot identify different polymers in the same sample (Shim et al. 2017). Pyrolysis gas chromatography-mass spectrometry (Py-GC/MS; n = 7) thermally decomposes microplastic through pyrolysis, then separates components according to their boiling point and polarity (mass spectrometry). These programmes are compared to reference programmes of known polymer samples to identify the polymer type (Shim et al. 2017). However, Py-GC/MS is limited to homogenous samples of 0.5 mg in size, which is unsuitable for environmental samples. Particles also need manual handling, like macro-FTIR, to be inserted into pyrolysis tubes, limiting the lower size of particles that can be analysed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) separates depolymerised samples based on their interaction with stationary and mobile phases. This is used to specifically quantify PET and polycarbonate (PC) only (Yan et al. 2022). Lastly, thermal extraction desorption-GC/MS (TED-GC/MS) completely decomposes samples and

can measure 20 mg of material (Dümichen et al. 2017), but so far has not been applied to freshwater samples. Disadvantages of thermal analysis is the more time- and energy-consuming process that also destroys samples, preventing subsequent analysis (Shim et al. 2017).

Tyre wear particles (TWPs) are estimated as a major contributor of microplastic pollution (Hann et al. 2018). Zinc is an indicator of TWPs (Rogge et al. 1993; Fauser 1999) and thus, collision-inductively coupled plasma-mass spectrometry (ICP-MS) was used by Wang et al. (2017) to assess metal content in microplastic. This uses collision-inductively coupled plasma (energy supplied by electric currents produced by electromagnetic induction) to ionise samples, before analysis with mass spectrometry. Wang et al. (2017) identified 2,414.8-14,815.3 μg of zinc per gram of microplastic sampled from Beijing River sediments, which was one to five orders of magnitude higher than zinc present in plastic bags and screw caps (Imhof et al. 2016) and three to five orders of magnitude higher than zinc found in pre-production microplastic pellets in the marine environment (Ashton et al. 2010; Rochman et al. 2014), possibly indicating TWP presence in Beijing Rivers. TWPs are also identified in environmental samples using other markers including sulphur (Rogge et al. 1993), which like zinc, have other traffic-related sources; styrene-butadiene rubber (SBR) or natural rubber (NR) that must be broken down for identification due to their high molecular weight; and benzothiazoles (Kumata et al. 2002) that leach from TWPs due to their lower molecular weight and higher polarity (Wagner et al. 2018). As shown, TWP markers are limited and individually have issues rendering them unsuitable as markers.

It is becoming essential for researchers to check particles are plastic in origin. However, if researchers do not have access to the described specialised equipment, Rose Bengal is recommended as a low-cost option to ensure counted particles are not organic in origin (see section 3.6.1).

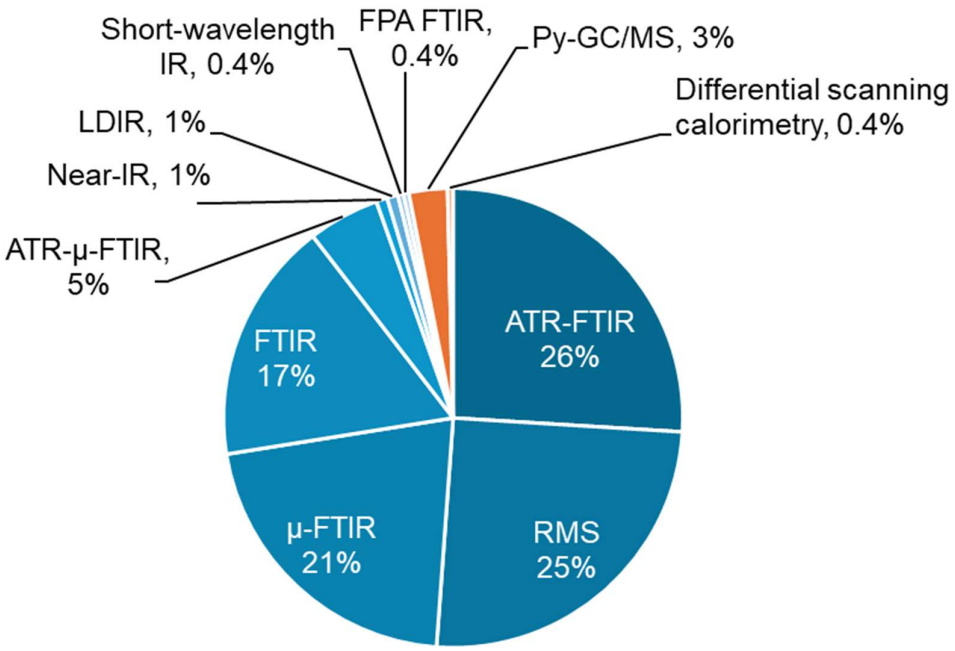


Figure 3.6 Percentage of studies employing instrumental analysis to detect polymer type (n = 246) using different techniques.

Polymer type of sampled microplastics was described in three-quarters of reviewed freshwater studies (n = 225), with a median of 5 (IQR = 4) unique polymers per sample. There was huge disparity in reported polymer type names, limiting understanding and comparison. For analysis, polymer types were standardised into 142 names, including “other”, which are listed in Table A6. The most frequently identified polymers were polypropylene (PP; n = 198/225 studies; 88%), and PE (n = 187/225 studies; 83%), of which 13% and 11% are high-density PE and low-density PE, respectively. Followed by PS (n = 153; 68%), PET (n = 132; 59%), polyamide (PA; n = 110; 49%), PVC (n = 103; 46%) (Figure 3.8). These polymers are commonly used for bags, bottles, containers, trays, toys, and packaging. Freshwater organisms specifically are most commonly contaminated with PP (n = 32/47 studies reporting polymer type in organisms; 68%) and PE (n = 29; 62%), followed by PA, PET, PS, PEST, and PMMA (Figure 3.8). Research into the effects of microplastics on the environment and organisms should focus on these commonly occurring polymers in freshwater and ensure different polymer types are tested to cover the range found in nature.

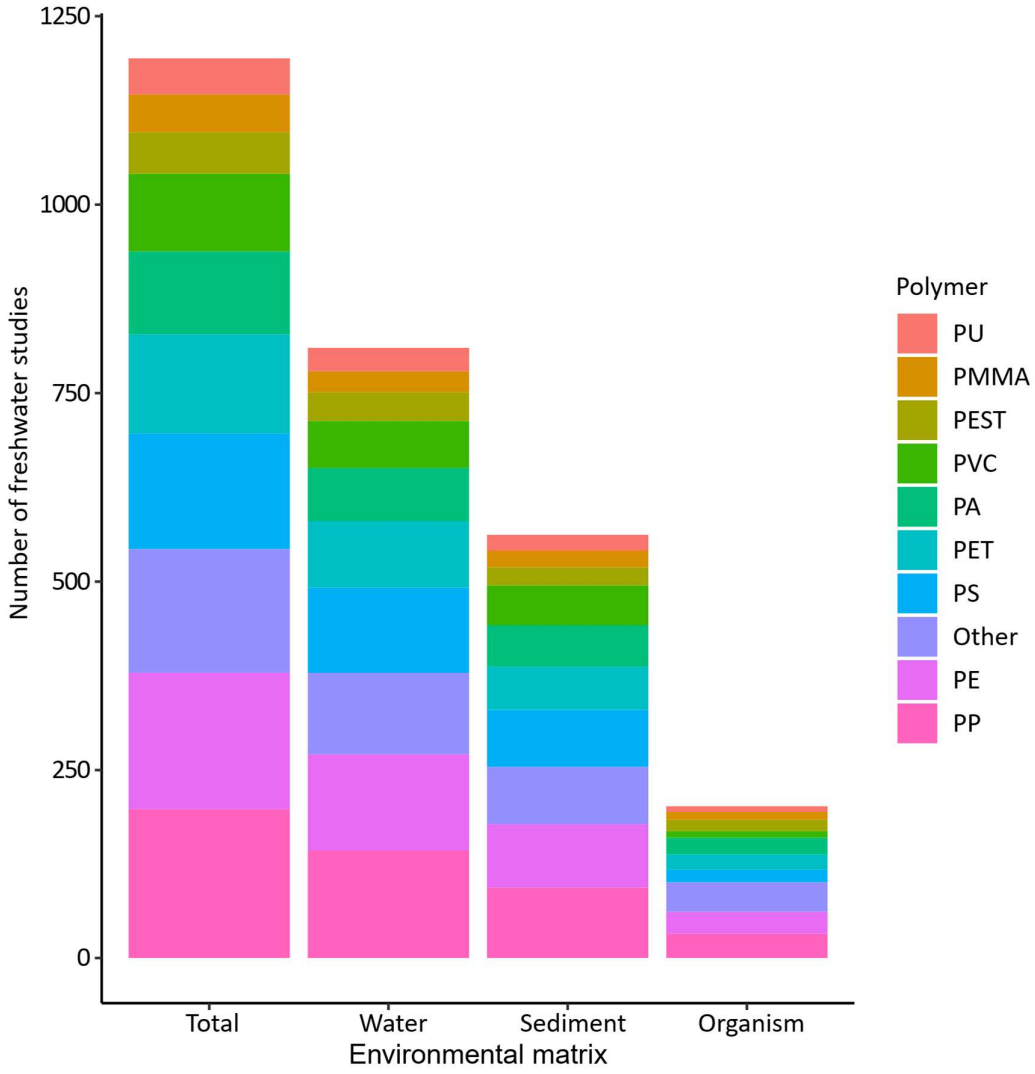


Figure 3.7 Number of studies reporting the occurrence of the top 10 most frequent polymer types in reviewed studies sampling freshwater for microplastic, and specifically in the water, sediment, and organism matrix.

3.6.4 Microplastic recovery testing

As there is no “one-size-fits-all” method of microplastic extraction from environmental media, multiple studies use recovery tests to validate the efficacy of their chosen method in extracting microplastic. Collected media are spiked with known types and amounts of microplastic and running the same extraction method to establish what particles are recovered. This ‘recovery rate’ indicates whether the extraction method underestimates (< 100% recovery) or overestimates (> 100% recovery) environmental microplastic loads. Such inaccuracies are problematic as

underestimation reduces the assessed severity of freshwater contamination, whereas overestimation skews our understanding of microplastic fate in the environment (Way et al. 2022). Therefore, microplastic recovery testing serves as a useful tool allowing true environmental microplastic loads to be estimated. Furthermore, comparing recovery rates between studies indicates which techniques are most effective for different environmental media. However, recovery testing was used sparingly amongst freshwater microplastic studies (e.g., Stolte et al. 2015; Di and Wang 2018) and is often poorly executed (Way et al. 2022).

3.7 Knowledge gaps and research requirements

Despite growing evidence of microplastic contamination in freshwater biota, several critical knowledge gaps and research needs persist. First, current studies predominantly focus on fish, with far fewer investigations into other taxa such as mammals, birds, invertebrates, and plants, limiting our understanding of microplastic exposure across trophic levels. Moreover, most fish studies examine only the GI tract, potentially underestimating total microplastic burden by neglecting other organs like gills, liver, and muscle, which may also accumulate particles via different uptake pathways. Additionally, while some studies report microplastics in faecal matter, little is known about particle degradation, translocation across tissues, or cellular-level interactions, particularly for nanoplastics. The field would benefit from an increase in biota sampling and an expanded taxonomic coverage using standardised methods to enable a more integrated assessment, as biotic microplastic loads reflect uptake over time.

There is a clear deficiency in the representation of subsurface and benthic water in reviewed studies, limiting our understanding of the vertical distribution of microplastic within freshwater ecosystems. Sampling across the water column at the same timepoint would reveal microplastic transport patterns and highlight exposure risk to freshwater biota. Note, sample collection and processing must be consistent at each depth for valid comparison, considering the huge diversity discussed in this chapter. We must also elucidate the interaction between microplastic buoyancy and hydrodynamics to aid prediction of microplastic fate within freshwaters. For freshwater sediment, research on shore and bank material was sparse. Considering

their role as a microplastic sink during high flow and flood events (Woodward et al. 2020; see *Chapter 2*), more should be done to sample microplastics in these environments. Remediation of these environmental matrices after heavy rainfall could help reduce microplastic loads in freshwater ecosystems, which needs investigating.

This review revealed huge heterogeneity amongst freshwater microplastic research in sample collection and microplastic extraction, quantification, and characterisation techniques. Research is required to compare the recovery rate and accuracy of microplastic identification amongst different methodologies throughout the process chain to understand potential under- or overestimation with different techniques. This can be performed using different sampling equipment on the same environmental matrix in the same location and using different processing methods on divisions of the same sample. More reliable measures of microplastic abundance and characteristics would also inform exposure experiments investigating microplastic effects. Many effect experiments use a narrow range of polymer types, and microplastic concentrations up to seven orders of magnitude higher than environmental levels (Lenz et al. 2016), which poorly represents environmental conditions.

Multiple researchers call for standardisation of microplastic research (e.g., Prata et al. 2019b; Campanale et al. 2020; Fok et al. 2020; Skalska et al. 2020) to create reproducible data that is comparable between studies. However, complete standardisation of microplastic research is difficult to achieve as ecosystem characteristics and study media vary considerably within and between freshwater ecosystems. Therefore, a more harmonised approach should be discussed (Lusher et al. 2020b), where chosen methods are dependent on the environmental matrix in question. Since the completion of this literature review, International Standards for the analysis of microplastic present in environmental samples have been published - ISO 24187 (2023). This states that all analytical steps must be undertaken in plastic-free or low-plastic working conditions, using alternative materials, contamination controls, and laminar flow boxes where feasible. Recovery tests and blank value determination are recognised as essential for detecting and accounting for inevitable contamination. For environmental sampling, standards 1, 4, 6, 7, 8, and 17 from the ISO 5667 series are recommended for sampling water, ISO

5667-12 is recommended for sampling freshwater sediment, and ISO 10870 and standards 1-6 from ISO 23611 are recommended for biota sampling alongside consideration of local laws and regulations governing such actions. For sample processing, it is recommended to dry samples at temperatures no higher than 40 °C, remove organic matter in solid samples (not water) via density separation using saturated salt solutions, and remove inorganic matter in solid and water samples at temperatures no higher than 25 °C. The standard does not recommend a single digestion solution, rather it highlights that oxidizing solutions are most frequently used, followed by acids or bases, and enzymatic processing. Furthermore, no specific processing times or volumes are stated. For polymer detection, the standard states that simple and inexpensive (pre-)screening techniques can be sufficient, but also lists multiple more sophisticated instrumentation as options for polymer detection. These include visual sorting, hot needle test, dye with fluorescence microscopy and spectroscopy, FTIR, ATR-FTIR, FPA-FTIR, LDIR, NIR or SWIR, Py-GC/MS, TED-GC/MS, DSC, ICP-MS and LC (see section 3.6.3 for technique descriptions). The lack of specific technique recommendations in ISO 24187 (2023) reflects the complexity in processing different types of media, which require different techniques depending on their make-up and the expected particle number/mass content under investigation.

3.8 Conclusion

Freshwater is an essential resource to life and freshwater ecosystems provide valuable services and thus, it is integral that microplastic pollution within this environment is investigated. This study reviews how freshwater ecosystems are assessed for microplastic pollution, informing researchers of the complex process involved. This begins with sample collection through bulk, volume-reduced, or selective techniques, followed by sample processing using sieving, density separation, chemical digestion, and filtration, to extract microplastic. Particles are then quantified and characterised using visual techniques and polymer analysis. This study reveals a huge variety of methods and equipment used in each step, which limits comparison of reported microplastic loads between environmental matrices and freshwater catchments. Evaluation of each technique informs method development and contributes to the harmonisation of microplastic research.

Moreover, identification of knowledge gaps informs future research to further understanding of microplastic pollution in freshwater ecosystems.

Chapter 4: Microplastic in the sediments and invertebrates of an urban river system

4.1 Abstract

Microplastics are pollutants of concern in freshwater environments, yet knowledge on their distribution and flux throughout continuous riverine catchments is still limited. This information is needed to identify sources of microplastic as well as areas and taxa most at risk. This chapter investigated microplastic concentration, characteristics, and spatial variation along the River Taff, South Wales, as a model river with both rural and urban land use. Sediment and four macroinvertebrate families from different feeding guilds (Hydropsychidae, Leuctridae, Heptageniidae, and Rhyacophilidae) were collected from 38 sites along the river, incorporating differences in land use and potential point sources of microplastic. Microplastics were present in sediments from 71% of sites, with concentration ranging from 73 to 594 particles/kg dry weight. Microplastics were detected in just 5% of invertebrate individuals, with no variation amongst feeding guilds. These concentrations demonstrated a patchy distribution throughout the River Taff catchment, indicating that unique variables of influence may act in different locations. Fibres dominated sediment microplastics (99%), whilst macroinvertebrates contained fragments and fibres (52% and 42%, respectively), with transparent being the dominant colour for all sampled microplastics. Alongside the high prevalence of synthetic cellulose, this suggests that textiles were the major microplastic source in this system. These data confirm the ubiquity of microplastics in the sediments across a river catchment, yet the apparently limited occurrence in macroinvertebrates contrasts with previous data.

4.2 Introduction

Historically, studies of microplastic loads in aquatic ecosystems have focused on the marine environment (Blettler et al. 2018). However, rivers have been increasingly identified as major pathways of microplastics into the oceans (Jambeck et al. 2015; Lebreton et al. 2017), while also being important ecosystems to protect (Dudgeon et al. 2006). This has brought about a recent surge in freshwater

microplastic investigations (Sarijan et al. 2021; see *Chapter 2*). Microplastic is thought to enter freshwaters through point sources including wastewater treatment plants (WWTPs; Windsor et al. 2019b; Schmidt et al. 2020; Montecinos et al. 2022), combined sewer overflows (CSO; Treilles et al. 2020; Gogien et al. 2023), septic tanks (Liu et al. 2022a), and industrial outflows (Chan et al. 2021; Magalhães et al. 2022). Microplastic is also carried in surface water run-off and the atmosphere, creating diffuse sources including urban dust, tyre wear particles (TWPs), litter degradation, and agricultural sludge. Meta-analysis of freshwater microplastic in Chapter 2 revealed various spatial trends, including increases towards urban areas and influences of hydrodynamics.

This apparent variability in the distribution of microplastics across highly connected freshwater catchments raises the need for more extensive, catchment-scale assessments to appraise natural and anthropogenic influences on their distribution. Up to mid-2022, such catchment-wide published studies only equated to eight, with UK examples being particularly scare (see *Chapter 2*). Hurley et al. (2018a) sampled benthic sediment from 40 sites across 10 tributaries of the Irwell and Mersey rivers in northwest England. This allowed identification of urban hotspots where microplastic concentration exceeded 40,000 particles/kg, particularly immediately downstream of WWTPs and CSOs (Hurley et al. 2018b). Elsewhere in the world, catchment-wide assessments of microplastic pollution identify positive trends with urbanisation, transportation, waste management, and agricultural practices (He et al. 2020; Mao et al. 2020b; Yuan et al. 2022; Kunz et al. 2023; Chen et al. 2024).

Microplastic pollution can also vary vertically throughout the freshwater environment, with no common trend in abundance changes with water depth in literature (Lenaker et al. 2019; Liu et al. 2021c; Xu et al. 2022a; Pasquier et al. 2023). This alters the risk of microplastic in distinct habitats. Disparity is likely governed by physical characteristics of particles and hydrodynamics (Kumar et al. 2021). Particle density may influence their position in the water column (Waldschläger and Schüttrumpf 2019), with lighter polymers under 1.1 g/cm^3 (e.g., EVA, PP, PE) dominating surface waters and denser polymers (e.g., PS, PVC, PA, PET) sinking to sediment (Lenaker et al. 2019; Liu et al. 2021c; Chevalier et al. 2023; Pasquier et al. 2023; Wang et al. 2024). Yet, multiple studies report the

occurrence of high-density polymers in water columns and conversely, lower-density polymers in sediments (Lahens et al. 2018; Tibbetts et al. 2018; Eo et al. 2019; Corcoran et al. 2020). This highlights the complexity of microplastic pollution in dynamic freshwater environments.

Microplastics occur in a variety of shapes, including fibres, fragments, films, pellets, and foams (section 3.6.1), each of which reflects different sources and degradation pathways. Fibres are frequently reported as the dominant microplastic shape in riverine environments, due to their widespread sources and high environmental persistence (Li et al. 2020; Lu et al. 2021; Wang et al. 2021f). These fibres primarily originate from the breakdown of synthetic textiles during washing processes, with WWTPs acting as major pathways for their release into aquatic ecosystems (Browne et al., 2011; Dris et al., 2015). Unlike fragments or beads, fibres are more buoyant and can remain suspended in the water column longer, facilitating their transport and eventual deposition in sediments or ingestion by aquatic organisms (Wagner et al., 2014). Their high surface-area-to-volume ratio also increases the likelihood of interaction with biota, leading to greater bioaccumulation in macroinvertebrates, which are often used as indicators of microplastic pollution (Silva et al., 2021).

With the extensive distribution of microplastic in freshwater systems, aquatic organisms are exposed to microplastic likely through direct ingestion, respiration, and indirect food-web transfer (Kim et al. 2018; D'Souza et al. 2020). For freshwater biota, literature is biased towards assessment of microplastic in organisms at higher trophic levels such as fish, with less representation of aquatic invertebrates (see *Chapter 2*). Although controlled exposures (Blarer and Burkhardt-Holm 2016; Redondo-Hasselerharm et al. 2018; Weber et al. 2018) and meta-analysis of published studies on invertebrates indicate relatively few negative impacts (Foley et al. 2019; Castro-Castellon et al. 2022; Doyle et al. 2022), these organisms can act as entry points of microplastic into freshwater food webs (Windsor et al. 2019b) and transfer them to terrestrial food webs (Yıldız et al. 2022), making them ecologically important. Moreover, macroinvertebrates occupy a wide range of feeding guilds and ecological niches (Bonada et al. 2006), are ubiquitous across freshwater ecosystems, and sample the environment over space and time. Therefore, they are often recommended for biological monitoring of pollutants and environmental quality

(Milner and Roberts 1997; Markert et al. 2003). Consequently, further investigation into their microplastic uptake is necessary to adapt and optimise this bioindicator for assessing microplastic pollution.

Microplastic uptake by aquatic invertebrates can be influenced by multiple factors including microplastic presence in the surrounding freshwater environment. For example, higher loads have been observed in macroinvertebrates living downstream of WWTPs compared to those upstream (Hurley et al. 2018a; Grbić et al. 2020; Woodward et al. 2021). Biotic factors also play a role, with microplastic load varying between functional feeding guilds (FFGs). Multiple studies observe greater microplastic loads in collector-gatherers/detritovores (Bour et al. 2018; Nel et al. 2018; Akindele et al. 2020; Pan et al. 2021; Bertoli et al. 2022; Parker et al. 2022b; Di Lorenzo et al. 2023; Khedre et al. 2023). This may be linked to habitat affinity, with collector-gatherers and grazers feeding on material sedimented or deposited on submerged substrata (Berg 1995). Since sediments tend to accumulate more microplastic than surface water, benthic organisms are more likely to interact with microplastic than pelagic organisms (Schell et al. 2022a). However, many macroinvertebrate studies show no influence of habitat affinity nor ecological niche on microplastic uptake (Windsor et al. 2019b; Akindele et al. 2020; Pan et al. 2021; Bertoli et al. 2022b). Another explanation could be association of microplastic with material consumed by different FFGs. For example, leaf litter may capture and accumulate microplastic (López-Rojo et al. 2020; Bertoli et al. 2023a; Bertoli et al. 2023b). Moreover, collector-gatherers and grazers may discriminate food items less than shredders and scrapers that selectively shear organic material and attached algae (Schmid-Araya and Schmid 2000). Lastly, predators obtain microplastic through trophic transfer and thus, their comparatively lower loads may result from microplastic egestion in their prey (Cole et al. 2013; Windsor et al. 2019b).

Laboratory exposure experiments and literature review (see *Chapter 2*) also indicate that within freshwater macroinvertebrate species, feeding rate, life stage, and body size of individuals can influence their microplastic uptake (Burns 1968; Burns 1969; Zánkai 1994; Scherer et al. 2017). For example, within the same species, microplastic uptake per individual is shown to negatively correlate with body size (Windsor et al. 2019c; Garcia et al. 2021; Ng, 2023), which has also been found in fish (Kılıç et al. 2022; Park et al. 2020a, 2022) and crustaceans (Pegado et al.

2018; Hossain et al. 2019). This may result from lower food consumption by smaller individuals, reducing microplastic uptake and retention relative to larger individuals. Given this variability in exposure related to biological traits, assessing microplastic uptake across different FFGs within a single freshwater catchment is essential to disentangle the relative influence of ecological and physiological factors on ingestion patterns.

Aims and hypotheses

The overarching aim of this study was to assess microplastic contamination across a whole river catchment rather than single sites to appraise point and diffuse sources, including land use. The intention was to assess microplastic contamination in bed sediment and benthic macroinvertebrates of four different feeding guilds (shredder, grazer, filter feeder, predator), which could potentially be developed as microplastic bioindicators in freshwater ecosystems.

Specific hypotheses were:

1) Microplastic concentrations in sediment and macroinvertebrates (particles/individual) increase from upstream to downstream.
2) Microplastic hotspots in sediment and macroinvertebrates occur downstream of WWTP outflows.
3) Macroinvertebrates of all feeding guilds are contaminated with microplastic, but concentrations (particles/individual) are greatest in grazers and lowest in predators, irrespective of body weight.
4) Macroinvertebrate microplastic concentration (particles/individual) decreases with body size.
5) High-density microplastics and fibres dominate microplastic occurrence in bed sediments and benthic macroinvertebrates.

An important objective in the work was to standardise sampling techniques with consistent reporting units, to enable comparison with previous studies.

4.3 Materials and methods

4.3.1 Sample sites and environmental characterisation

The River Taff (Afon Taf) is a temperate river in south Wales, UK, which was historically grossly polluted by coal mining, sewage, and gasification plants (Scullion and Edwards 1980; Windsor et al. 2019a), but has since recovered (Vaughan and Ormerod 2012). It is 67 km long and begins as two main stems (Taf Fawr and Taf Fechan) in the central Brecon Beacons that join at Merthyr Tydfil, before flowing south towards the River Severn Estuary in Cardiff Bay. Over half of the catchment comprises of four major tributaries: the Taff Bargoed, Cynon, Nant Clydach, and Rhondda. Its elevation drops 11 m every 1 km, and rainfall ranges from 950 mm at Cardiff to 2400 mm in the Brecon Beacons (Williams and Simmons 1999). Land-use in the catchment ranges from rural to heavily urbanised and river water has been historically contaminated with litter (Williams and Simmons 1999), macronutrients, xenobiotic pollutants (Kasprzyk-Hordern et al. 2008; Morrissey et al. 2013a; Morrissey et al. 2013b; Windsor et al. 2019c), and microplastic (Windsor et al. 2019a; D'Souza et al. 2020).

Quantum Geographic Information System (QGIS) software (version 3.26, QGIS Association 2021) was used to map the river catchment (Figure 4.1). Secondary data was collected to: (i) environmentally characterise the catchment, aiding sample site identification; and (ii) investigate the influence of freshwater microplastic sources identified in literature (see *Chapter 2*) on observed River Taff microplastic occurrence. Land-use (urban, agricultural, grassland, heathland, and woodland) was obtained from UK Centre for Ecology and Hydrology's (UKCEH) 'Land Cover Map 2021' data (UKCEH 2022). Urban and agricultural land are known sources of microplastic pollution to freshwaters (Qiu et al. 2020; Hatinoğlu and Sanin 2021; Li et al. 2023a) and impermeable surfaces aid microplastic transport through surface runoff (Sun et al. 2023). Rural land was separated into grassland, heathland, and woodland, to test the influence of their different surface runoff capabilities (Archer et al. 2012; Han et al. 2020). Roads and railways were obtained from ©OpenStreetMap contributors (Ordnance Survey, 2021), as vehicles produce microplastic through tyre wear (Wagner et al. 2018; Kitahara and Nakata 2020; Rødland et al. 2020).

Potential point sources were also mapped, including landfill sites obtained from NRW Waste Sites (Resource identifier 116331; Lle.gov.wales, 2013) and WWTP and CSO outlets obtained from The Rivers Trust (provided by Dŵr Cymru/Welsh Water). Five WWTPs treat wastewater and discharge effluent into River Taff, though three of these are very small (Dŵr Cymru/Welsh Water; Table B1). Two serve the headwaters of the Taf Fechan: Ponticill Houses (1 residential/non-residential person served from ONS 2021 census (PE)) and Ponticill (360 PE). One serves Taf Fawr headwaters: Llwyn-On Houses (44 PE). The remaining two serve the mid-catchment: Cilfynydd (76,521 PE) and Cynon (68,434 PE). Details of treatment types are found in Table B1. The Taff catchment also has 309 CSOs (2021), calculated from spill Event Duration Monitoring (EDM) by Dŵr Cymru/Welsh Water (The Rivers Trust 2022) and NRWs database of consented discharges to controlled waters (The Rivers Trust 2021). These monitored CSOs spilled on average 38 ± 41 times (range: 0-172) in 2021, for an average of 9.6 ± 18 days (range: 0 – 120.7 days) in total. No data are available on the volumes of wastewater discharged.

A total of 38 sampling sites were selected *a priori* to represent the whole River Taff network and to reflect different land uses, accounting for river accessibility (Figure 4.1; Table B2). Sample sites were mapped in QGIS and snapped to the River Taff network obtained from a 50 m Digital Terrain Model (DTM; OS Terrain 50, Ordnance Survey, 2021). Overlapping subcatchments of each site were delineated using flow direction derived from the DTM and PCRaster tools in QGIS and employed to calculate percentage land use, counts of point sources, and road and rail length (Table B3). Human population density and vehicle density were also calculated based on local authority 2021 population size (Office for National Statistics 2021) and vehicle counts (Department for Transport 2021), respectively (Table B3).

During sample collection at each sample site, stream chemistry was assessed through spot measurements of pH, electrical conductivity (EC; µS/cm), and water temperature using HI-9813-5 potable meter (Hanna Instruments, UK), and river flow velocity (m/s) was measured using a magnetic-inductive flow meter (OTT MF pro Meter, OTT HydroMet, US) and wading rod (average of 3 measurements) (Table B2).

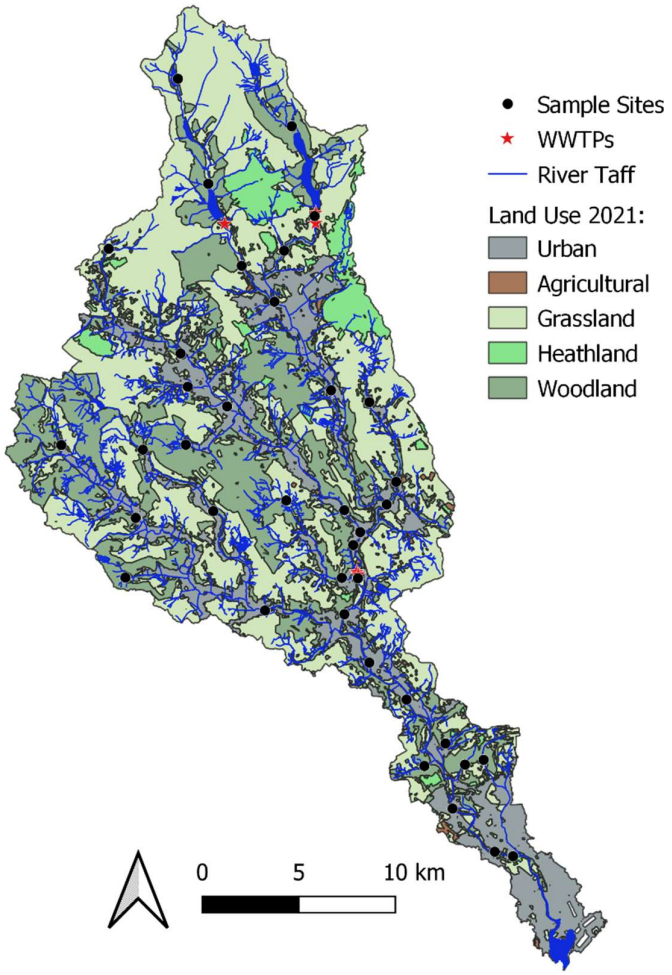


Figure 4.1 Map of River Taff catchment with 38 sample sites (black dots), WWTPs (red stars), river (blue line) and land-use (urban – grey, agricultural – brown, grassland – light green, heathland - green, woodland – dark green).

4.3.2 Macroinvertebrate and riverbed sediment sampling

Macroinvertebrate nymphs and larvae of four widespread families from three orders (Trichoptera, Plecoptera, and Ephemeroptera) were investigated: Leuctridae (Stonefly; shredder), Heptageniidae (Mayfly; grazer), Hydropsychidae (net-spinning caddisfly; filter feeder), and Rhyacophilidae (free-living caddisfly; predator), each of a different FFG (Cummins 1973). Samples were collected between 12th April and 20th May 2022 using kick sampling (Freshwater Biological Association hand net, 1 mm aperture) (Bradley and Ormerod 2002). Net contents were emptied into a bamboo tray filled with river water and five individual larvae (n = 5) of each family were

identified and placed into glass vials using stainless steel forceps. No Leuctridae were found at sites 5 and 10, while Rhyacophilidae were absent from sites 6, 14, 15, 21, 23, 25, 31, 32, 33, 34, and 37, precluding these taxa from microplastic analyses. Fourteen individuals were excluded from analysis after being identified as different insect families (see section 4.3.3), producing a final count of 681 macroinvertebrate individuals. Glass vials were immediately filled with 70% ethanol to fix specimens and prevent degradation and excretion of gut contents. Microplastics are not affected by ethanol (Courtene-Jones et al. 2017; Herrera et al. 2018). The samples were stored at 4°C until processing.

Fine riverbed sediment was sampled at 35 sites (samples from sites 3, 21, and 22 were excluded due to human error) simultaneously to macroinvertebrate collection. At each site, three < 50 ml samples (n = 3) were taken from depositing areas using triple-rinsed, 50 ml plastic centrifuge tubes, and stored at ~5°C until processing. One sediment sample was excluded from site 2 due to human error, producing a final count of 104 sediment samples. Section 3.5.5 recommends using controls throughout environmental microplastic assessments. In this case, blank filter papers placed on the ground during sampling would have measured potential atmospheric contamination of samples. This was not performed as lids were placed on samples immediately after they were taken, to limit environmental contamination.

4.3.3 Microplastic extraction from macroinvertebrates

Macroinvertebrate identifications to family were confirmed under a GXMXTL3 Stereo Microscope (GT Vision Ltd) using identification guides (Croft 1986; Pawley et al. 2011). Under laminar air-flow conditions, individuals were triple-rinsed with filtered deionised water (FDW) to remove any externally attached material including microplastics (Nel et al. 2018), then wet weight was measured to ± 0.1 mg using an analytical microbalance.

Microplastics were extracted from macroinvertebrates using methods adapted from Windsor et al. (2019b), shown in Figure 4.2a. Washed individual specimens were placed into individual triple-rinsed Eppendorf tubes filled with filtered 30% hydrogen peroxide (H_2O_2) solution and left to digest at room temperature for up to 48 hours. 30% H_2O_2 digests organic particles more optimally compared to 37%

hydrochloric acid (HCl) and 20-50% sodium hydroxide (NAOH), and does not degrade polymers (Nuelle et al. 2014; Gulizia et al. 2022), whilst higher H_2O_2 concentrations can degrade polymers (Karami et al. 2017). This digestion technique, known as Wet Peroxide Oxidation (WPO), was most commonly used amongst studies reviewed in *Chapter 3*. A 48-hour digestion period at room temperature was used in accordance with the protocol described by Windsor et al. (2019b), which was found to be sufficient for the effective breakdown of soft tissues in similar macroinvertebrate taxa. Remaining macroinvertebrate exoskeletons were pulverised using a triple-rinsed pestle and mortar for one minute to enable microplastic extraction, as performed by Windsor et al. (2019b). This method was selected for its effectiveness in liberating microplastics from biological tissue without the need for harsher chemical treatments or high-energy mechanical disruption, which may pose a greater risk to particle integrity. While thermal or mechanical degradation of particles is possible, leading to overestimation of microplastic concentration, the short duration and manual nature of the grinding minimised this risk. Conversely, potential loss of material could result in underestimation of microplastic concentration. This was mitigated by rinsing the mortar thoroughly after each sample and combining rinsates with the main extract to ensure maximum recovery. The procedure was applied consistently across all samples to ensure comparability.

The resulting material was vacuum filtered onto gridded cellulose nitrate membrane filters (47 mm diameter, 0.45 µm) for particle quantification. Although cellulose nitrate filters were second most popular in studies reviewed in *Chapter 3*, the most popular glass fibre filters are more expensive and are not gridded, which aids visual counting of particles. However, the filter pore size of 0.45 µm was most commonly used by reviewed studies (*Chapter 3*). Wet filters were dyed with ~2 ml Rose Bengal solution (200 mg/l; 4,5,6,7-Tetrachloro-2′,4′,5′,7′-tetraiodofluorescein disodium salt, Sigma-Aldrich, Dye content 95% with FDW) to stain organic material (Feenstra and Tseng 1992b) and thus, not microplastic. After five minutes, the dye was filtered away and washed with FDW (Liebezeit and Liebezeit 2014). Filters were placed in plastic petri dishes with the desiccant agent Silica gel (Fisher Scientific) to dry, then sealed with parafilm to prevent contamination. Plastic laboratory equipment, such as Eppendorf tubes, Petri dishes, and parafilm, was used during sample processing due to its wide availability, low cost, and compatibility with

standard microplastic analysis protocols. The use of plastic equipment in freshwater microplastic studies occurs throughout literature (e.g., Schmidt et al. 2018; Weideman et al. 2019; Watkins et al. 2019b; Dikareva and Simon 2019; Nan et al. 2020; Crew et al. 2020; Negrete Velasco et al. 2020; Irfan et al. 2020a). To minimise the risk of contamination from these materials, strict quality control measures were implemented as described at the end of section 4.3.5.

4.3.4 Microplastic extraction from sediment

The method for extracting microplastic from sediment is shown in Figure 4.2b. Sediment samples were weighed before drying in a 90°C drying oven (10% fan) for 24 hours, after which dry weight (dw) was measured. This oven temperature is recommended by Mausra et al. (2015) and is below the threshold temperature for melting and decomposition of common plastics (Kandola et al. 2014). Filtered 30% H_2O_2 was added to samples inside a fume hood to digest organic material for 48 hours or until bubbles no longer formed. Digested material was sieved through triple-rinsed 500 µm and 63 µm stacked sieves, and material captured by the latter was dried at 90°C and 10% fan for 24 hours.

Suspected microplastics were then extracted through density separation and centrifugation using methods adapted from Grause et al. (2022). Specifically, 10 ± 0.3 g of dried material from each sample was placed in a 50 mL centrifuge tube with 40 mL of filtered potassium iodide solution (KI; ρ = 1.52-1.63 g/cm^3) solution and centrifuged at 4,000 rpm for ten minutes. KI was used despite Chapter 3 identifying only one freshwater microplastic study using KI (Zhang et al. 2020b), due to multiple reason. Firstly, KI is generally considered to be less toxic, more environmentally benign, and less corrosive than commonly used alternatives like zinc chloride ($ZnCl_2$) and sodium iodide (NaI) (Gago et al. 2019). Therefore, it poses fewer risks to the researcher, the environment, and laboratory materials during its use and disposal. Secondly, KI is low cost, available in the study laboratory, and its solutions can be filtered and reused multiple times, further improving its cost-effectiveness and sustainability over time. Lastly, KI can reach relatively high densities, which is sufficient to float most of the common plastic polymers (Table 3.2), as well as dense polymers such as PET (1.3-1.4 g/cm^3) and PVC (1.1-1.6 g/cm^3) (Van Cauwenberghe

et al. 2015; Quinn et al. 2017) and oleophobic fibres (Crichton et al. 2017). A disadvantage of a higher density solution is reduced settling of fine sediment. This was compensated by centrifugal separation rather than gravitational separation. A centrifugation time of 10 minutes was chosen because, although Grause et al. (2022) observed a 3% reduction in microplastic recovery rate when increasing centrifuge time from one minute to ten to twenty minutes (98 wt% to 95 wt%), short times do not ensure sufficient settling of fine sand particles, which obscure microplastic identification after subsequent filtration. To complete microplastic extraction, supernatants were filtered, stained, and stored with the same procedure as macroinvertebrate samples (section 4.3.3).

4.3.5 Microplastic quantification, characterisation, and polymer analysis

Each filter was systematically scanned along the grid lines under a stereo dissecting microscope (GXMXTL3) at 4.5x magnification, following the Marine and Environmental Research Institute's 'Guide to Microplastic Identification' (2015). Suspected microplastic particles were recorded if they met criteria detailed in Supplementary Information B1 and were characterised by shape (fibre, fragment, film, bead, foam; criteria in Supplementary Information B2) and colour (black, blue, brown, grey, white, cream, transparent). These criteria were established in *Chapter 3* after systematic literature review. Red/pink particles were excluded as organic material was dyed red by Rose Bengal stain. To confirm identified particles as plastic, subsamples (n = 112 of 1,606 and n = 66 of 318 for macroinvertebrates and sediment, respectively) were analysed using μ-Fourier transform infrared (FT-IR) spectroscopy (Perkin Elmer Spotlight 400 FT-IR Imaging System) to determine their polymeric structure. Subsamples covered the range of colour-shape combinations of extracted particles and reflected the time and funds available for analysis. Particle analysis was carried out in transmission mode, using a diamond compression cell to hold the particle, producing high quality spectra compared to reflectance mode (Löder et al. 2015a).

Spectra were collected over a broad spectral range (600-4,000 cm^{-1}) at a resolution of 4 cm^{-1} from an average of 16 sample scans and corrected for background variation prior to further analysis. Using PerkinElmer Spectrum software

(version 10.5.4.738), FT-IR spectra were compared to a spectral database from nine commercial polymer libraries (adhes.dlb, Atrpolym.dlb, ATRSPE~1.dlb, fibres.dlb, IntPoly.spl, poly1.dlb, polyadd1.dlb, POLYMER.dlb and Microplastics.spl) and one in-house library (Greenpeace Research Laboratories, UK), allowing exclusion of common laboratory contaminants. For each particle, the top ten matches were checked to verify reliability of identification, only accepting match qualities >70% with the average match quality being 86.27%. Black fragments were excluded as they cannot be analysed by μ-FT-IR due to colour absorbance (Zhu et al. 2020). Analysed particles identified as non-plastic (methyl cellulose, chipboard/cellulose+lignin, methylergonovine maleate, cyanox, acetoacetyl coenzyme A trisodium salt, acetylmuramyl-l-alanyl-D-isoglutamine, fructose/glucose, and zein/poly(n-methyl acrylamide) film) were excluded. Analysed particles identified as cellulose, methyl cellulose, or cellophane were assumed synthetic if they were unnaturally coloured (blue, black, grey, white). Microplastic count data consisted of analysed particles identified as plastic and non-analysed particles corrected to reflect the proportion of analysed particles identified as plastic per colour-shape combination (Woodward et al. 2021).

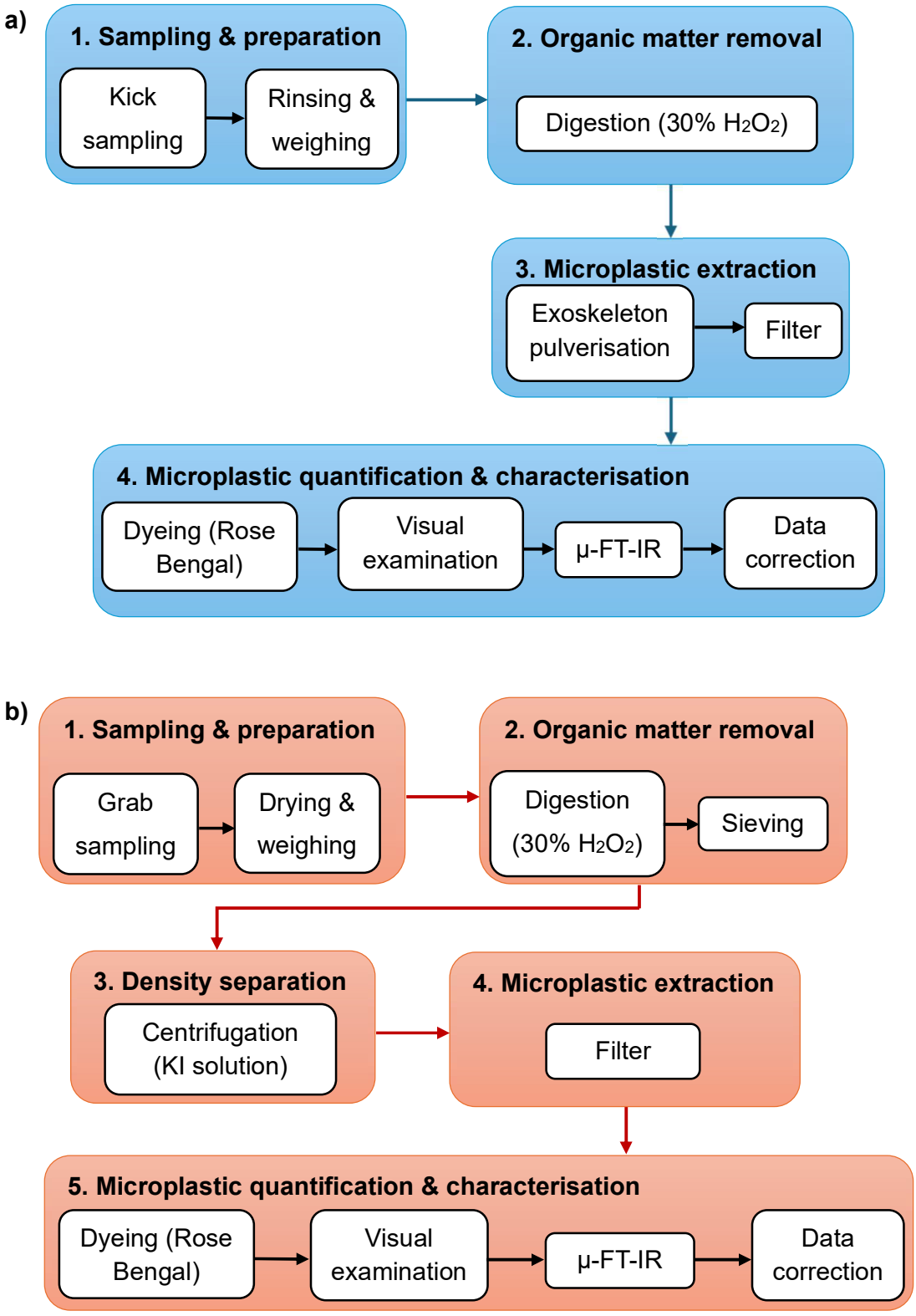


Figure 4.2 Flow chart of the separation and quantification of microplastics from **a)** macroinvertebrates and **b)** riverbed sediment.

Samples are at risk of plastic contamination from external sources in the air and solutions used (Prata et al. 2020b; Kernchen et al. 2022). Measures to prevent this include pre-filtering all solutions through 0.45 µm cellulose nitrate filter paper, processing samples in a laminar flow cabinet, triple-rinsing all equipment before and during sample processing with FDW, and wearing 100% cotton clothing and nitrile gloves. Remaining exogenous contamination was assessed using procedural blanks of ethanol (5 for macroinvertebrates, 20 for sediment), processed in the same way as respective environmental samples. On analysis (see section 4.4.4), blank samples did not consistently produce values of zero. Instead, they contained low numbers of primarily transparent, cellulosic fibres (n = 17 of 27), likely originating from textiles. Macroinvertebrate sample blanks contained a mean average ± standard deviation (SD) of 1.8 ± 1.92 transparent fibres, 0.8 ± 0.84 black fibres, 0.2 ± 0.45 white fibres and 0.2 ± 0.45 blue fibres. Sediment sample blanks contained on average 0.40 ± 0.68 transparent fibres and 0.25 ± 0.44 black fibres. These values were not directly subtracted from sample values. Instead, a more rigorous method was used, following the approach recommended by Bråte et al. (2018) and Dawson et al. (2023), whereby the Limit of Detection (LOD; mean + 3x SD) and Limit of Quantification (LOQ; mean + 10x SD) were calculated for each particle shape based on blank sample means and standard deviations. Sample values corrected by µ-FT-IR analysis (see section 4.3.5) that exceed the LOQ were reported as quantified microplastic counts. Values falling between the LOD and LOQ were treated as detectable but not reliably quantifiable and were therefore excluded from reported total. Values below the LOD were considered indistinguishable from background contamination and were also excluded. This method is described in Supplementary Information B3.

4.3.7 Statistical analysis

All statistical analyses were performed in ‘R’ software (version 4.3.0; R Core Team 2023). A paired samples t-test was conducted to compare observed microplastic concentration and µ-FT-IR and blank corrected concentration (see sections 4.3.4 and 4.3.5). For both sediment and macroinvertebrates, observed microplastic concentrations (mean ± 1 SD: 0.14 ± 0.19 particles/g and 0.55 ± 1.06 particles/individual, respectively) were significantly greater than their corrected

concentration (mean ± 1 SD: 0.07 ± 0.12 particles/g and 0.04 ± 0.22 particles/individual, respectively) ($t(102) = 6.36$, $p < 0.001$ and $t(673) = 13.24$, $p < 0.001$, respectively). Further analysis was therefore conducted on the μ-FT-IR and blank corrected occurrence (binomial, 0-1) and concentration of microplastic.

Microplastic concentration data were sequentially assessed for outliers, normality, heteroscedasticity, and collinearity (Zuur et al. 2010). Due to the positive skew and large sample size of dependent variables (microplastic presence and concentration in sediment and macroinvertebrates), removing high outliers would reduce the mean relative to the true mean (Miller 1991). To avoid this bias, concentration outliers were identified and eliminated using Van Selst and Jolicoeur's (1994) modified recursive procedure with a criterion cut-off of 3.5 standard deviations above the mean. This correction was performed on the subset of data comprised of contaminated samples only, to avoid being restricted by zero-inflation. Both dependent and independent variables were then assessed for normality using Shapiro-Wilks test (Shapiro and Wilk 1965) and visual inspection of histograms and Q-Q plots. Microplastic concentration in sediment and macroinvertebrates, and distance upstream, subcatchment area, and mean river flow rate in outlier removed macroinvertebrate data were all non-normally distributed, even after transformation (Shapiro-Wilk test: $p < 0.0001$). The ‘orderNorm’ transformation from the ‘bestNormalize’ package in R (Peterson and Cavanaugh 2020) was applied to macroinvertebrate individual wet weight and distance upstream, subcatchment area, and mean river flow rate in outlier removed sediment data, to achieve a normal distribution (Shapiro-Wilk test: $p > 0.05$). All independent variables were scaled using ‘scale’ function from the ‘base’ package in R (Becker et al. 1988; R Core Team 2023), which transforms data to have a mean of zero and a standard deviation of one, aiding comparison between variables. Lastly, homogeneity of variances across groups were assessed via Levene’s test (Levene 1960) for normally distributed data and Filgner-Killeen’s non-parametric test (Fligner and Killeen 1976) for non-normally distributed data. Variance of microplastic concentrations in sediment and macroinvertebrates were equal across sites (Fligner-Killeen test: $\chi^2 = 30.892$, $df = 34$, $p = 0.621$ and $\chi^2 = 45.527$, $df = 37$, $p = 0.159$, respectively). Variance of transformed macroinvertebrate wet weight was not equal (Levene test: $F = 2.002$, $df = 37$, $p < 0.001$).

Generalised linear models (GLMs) were used to test hypotheses 1, 3, and 4 (respective effects of distance upstream, invertebrate family and individual wet weight), and identify covariates (land-use, potential point sources of microplastic). Significantly correlated covariates according to Pearson and Spearman correlation tests for normally and non-normally distributed variables, respectively, were excluded from investigation (see section 4.4.1 for correlating covariates). Specifically, Bernoulli GLMs with a clog-log link function were used to model presence/absence of microplastic to accommodate the binomial distribution, with p-values for individual variables determined using Wald z-statistics. The Akaike Information Criterion (AIC; Akaike 1974) was used for stepwise model refinement. Goodness of fit was evaluated by assessing the distribution and variance of deviance residuals, and identifying potentially influential observations according to diagnostic measures including Cook's Distance, leverage, and covariance ratio (Cook 1977; Montgomery et al. 2021). The model of microplastic presence in sediment (presence ~ distance upstream + subcatchment area + mean flow velocity) had deviance residuals no larger than two with homogeneous variance across the range of fitted values and only three potentially influential observations, indicating a good fit. The model of microplastic presence in macroinvertebrates (presence ~ family + individual wet weight + distance upstream + subcatchment area + mean flow velocity) had some observations with a deviance residual larger than two, alongside 83 potentially influential observations. This indicates a lack of fit, likely due to the extreme zero-inflation (95%).

To accommodate the continuous and positively skewed microplastic concentration data, Inverse Gaussian and Gamma error families with square root link function were independently used to model the data, adding a small transformation of +0.0001 to move the distribution away from zero. For sediment data, models were over dispersed according to respective overdispersion parameters of 6,667 and 17, and standardised residuals had a distribution significantly different to normal (Shapiro-Wilk test: $W = 0.732$, p-value < 0.001 and $W = 0.638$, p-value < 0.001, respectively). This indicates that observed variance in data was greater than the variance expected by the models. The same was true for macroinvertebrate models (overdispersion statistic of 9,256 and 11, respectively; Shapiro-Wilk test for normal distribution of standardised residuals: $W = 0.121$, p-value < 0.001 and $W = 0.175$,

p-value < 0.001, respectively). This overdispersion probably reflected zero-inflation and thus, a compound Poisson-Gamma GLM with square root link function was used via the glmmTMB R package (version 1.1.7; Magnusson et al. (2021) to account for positively skewed, continuous, and zero-inflated data (Zuur and Ieno 2021). Again, AIC was used for stepwise model refinement (Akaike 1974). Model validity was assessed by checking the model assumption of normally distributed residuals with homogenous variances, following approaches of Thomas et al. (2017). The final models for sediment (microplastic concentration ~ distance upstream + subcatchment area + mean flow velocity) and macroinvertebrates (microplastic concentration ~ individual wet weight + distance upstream + subcatchment area + mean flow velocity) still showed overdispersion but to a smaller degree (overdispersion parameter: 0.3 and 2.4, respectively), with residuals distributed significantly differently to normality (Shapiro-Wilk test: W = 0.638, p-value < 0.001 and W = 0.200, p-value < 0.001, respectively). This may have implications for the validity of results.

Hypothesis 2 was assessed by comparing microplastic presence and concentration upstream and downstream of WWTPs. Sites 37 and 33 were respectively upstream and downstream of Pontsticill and Pontsticill Houses WWTPs, sites 36 and 32 were respectively upstream and downstream of Llwyn-On Houses WWTP, and sites 13 and 11 were respectively upstream and downstream of Cynon and Cilfynydd WWTPs. These sites were ~2-6 km from respective WWTPs and upstream WWTPs were all a minimum of 5 km downstream of proximal upstream point-sources. These WWTP clusters differ greatly in the population size served (Table B1), likely influencing the amount of microplastics in their effluent. For this reason and with limited replication of paired sites, no statistical test could be performed. A two-samples Wilcoxon rank test (non-paired) was used to compare microplastic concentrations in sediment and macroinvertebrates between sites devoid of CSOs upstream to sites with CSOs upstream. This analysis considered their cumulative impact, as all except the most upstream were located downstream of CSOs.

Finally, hypothesis 5 was tested by evaluating polymer distribution amongst subsampled particles analysed by μ-FT-IR, and shape (fibre, fragment, film) and colour (blue, black, brown, grey, white, transparent) distribution amongst corrected

particle counts. To assess the role of aquatic invertebrates as biological indicators of microplastic in freshwater ecosystems, the distribution of shape and colour amongst microplastic in macroinvertebrates was compared to that in sediment using Fisher's exact tests (Mehta and Patel 1983), as sample sizes were low. A regression analysis was also used to test the relationship between microplastic abundance in macroinvertebrates and microplastic abundance in sediment. As the linear model was not normally distributed (Shapiro-Wilk test: $W = 0.839$, p-value < 0.001) and GLMs with either Inverse Gaussian or Gamma error families, and 'inverse' link function, were over dispersed (respective over-dispersion statistics: 4,847 and 8), a negative binomial GLM with 'log' link function was used.

4.4 Results

4.4.1 Catchment characterisation

Distance upstream positively correlated with percentage of the subcatchment covered by grassland (r(36) = 0.59, p < 0.001; Pearson Correlation Test) and negatively correlated with percentage cover of urban (rho(36) = -0.43, p < 0.001), suburban (r(36) = -0.66, p < 0.001), and agricultural land (rho(36) = -0.48, p < 0.001), as well as population density (r(36) = -0.54, p < 0.001). Subcatchment area positively correlated with road length (r(36) = 0.93, p < 0.001), 2021 vehicle count (r(36) = 0.91, p < 0.001), 2021 vehicle density (per km) (r(36) = 0.33, p < 0.001), rail length (rho(36) = 0.85, p < 0.001), area of all categorised land uses (see section 4.3.1; all correlation coefficients > 0.75, p < 0.001), and counts of CSOs (rho(36) = 0.87, p < 0.001), WWTPs (rho(36) = 0.77, p < 0.001), treated controlled discharge (rho(36) = 0.88, p < 0.001), untreated controlled discharge (rho(36) = 0.93, p < 0.001), plastic waste sites (rho(36) = 0.82, p < 0.001), and sludge distribution sites (rho(36) = 0.67, p < 0.001). Subcatchment area positively correlated with 2021 population size (r(36) = 0.83, p < 0.001), but negatively correlated with 2021 population density (r(36) = -0.32, p < 0.05). These patterns were all consistent with increased urbanisation downstream (see Figure 4.1).

4.4.2 Occurrence of microplastics in sediment

After data correction, microplastics were observed in 35.9% of sediment samples collected (37 of 103), contaminating 71% of sites (25 of 35) across the

River Taff. In contaminated sediment, mean microplastic concentration was 196.9 ± 129.8 particles/kg dw (73.1 – 593.5 particles/kg dw; Figure 4.3a). Both presence and concentration (particles/kg dw) of microplastic did not vary significantly with distance upstream, subcatchment area, nor mean river flow velocity (Table 4.1; Figure 4.4). These models had weak Nagelkerke's R^2 values (e.g., R^2 for microplastic presence model = 0.04) and the latter model was over-dispersed (see section 4.3.7). Therefore, they account for only a small proportion of the variation in microplastic concentration, indicating the model did not capture certain factors.

A mean average of 197.6 and 296.3 microplastic particles/kg of sediment dw occurred at sites 37 and 33, respectively upstream and downstream of both Pontsticill and Pontsticill Houses WWTPs serving a total of 361 people in 2021. No microplastic particles were observed in sediment sampled from sites 13 and 11, respectively upstream and downstream of both Cynon and Cilfynydd WWTPs serving 144,955 people in 2021. A mean average of 128.7 and 148 microplastic particles/kg of sediment dw occurred at sites 36 and 32, respectively upstream and downstream of Llwyn-On Houses WWTP serving 44 people in 2021. Microplastic concentration did not differ significantly between sites without CSOs upstream and all sites downstream of CSOs (two-samples Wilcoxon rank test: W = 1,382, p = 0.517).

Table 4.1 Regressions of associations between microplastic **a)** presence and **b)** concentration (particles/kg dw) in sediment collected across 35 sites in River Taff, UK, and abiotic effect measurements, using a binomial GLM. ß = estimate, SE = standard error, CI = confidence interval, and df = degrees of freedom. Stars indicate levels of statistical significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

a)

Effect	ß	SE	z-value	p-value	95% CI
Intercept	-0.83	0.170	-4.889	1.01e-06***	[-1.19, -0.52]
Distance upstream (m)	0.27	0.181	1.475	0.140	[-0.08, 0.63]
Subcatchment area (km^2)	-0.002	0.190	-0.012	0.990	[-0.37, 0.39]
Mean flow velocity (m/s)	0.18	0.168	1.051	0.293	[-0.16, 0.51]

df residual = 99

b)

Effect	ß	SE	z-value	p-value	95% CI
Intercept	8.38	1.327	6.312	<2.76e-10***	[5.78, 10.98]
Distance upstream (m)	0.77	1.454	0.531	0.596	[-2.08, 3.62]
Subcatchment area (km^2)	0.63	1.303	0.486	0.627	[-1.92, 3.19]
Mean flow velocity (m/s)	-0.02	1.658	-0.015	0.988	[-3.27, 3.22]

df residual = 97

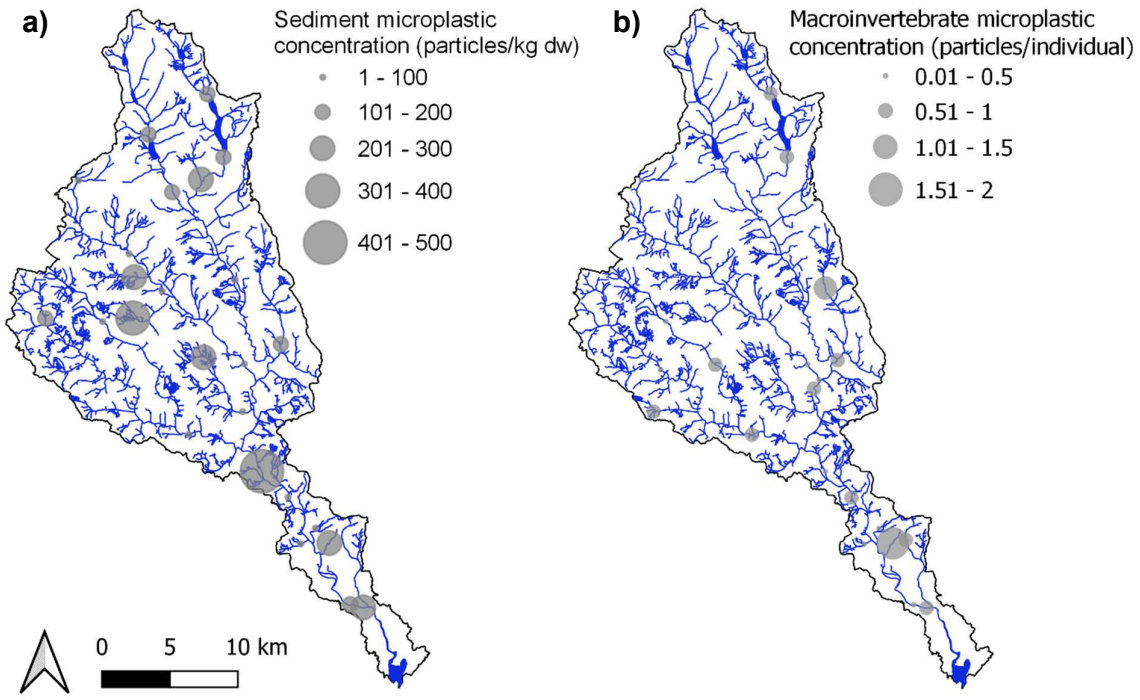


Figure 4.3 Map of the River Taff catchment, South Wales, with microplastic concentrations observed in **a)** sediment and **b)** macroinvertebrates collected from 35 and 38 sample sites, respectively.

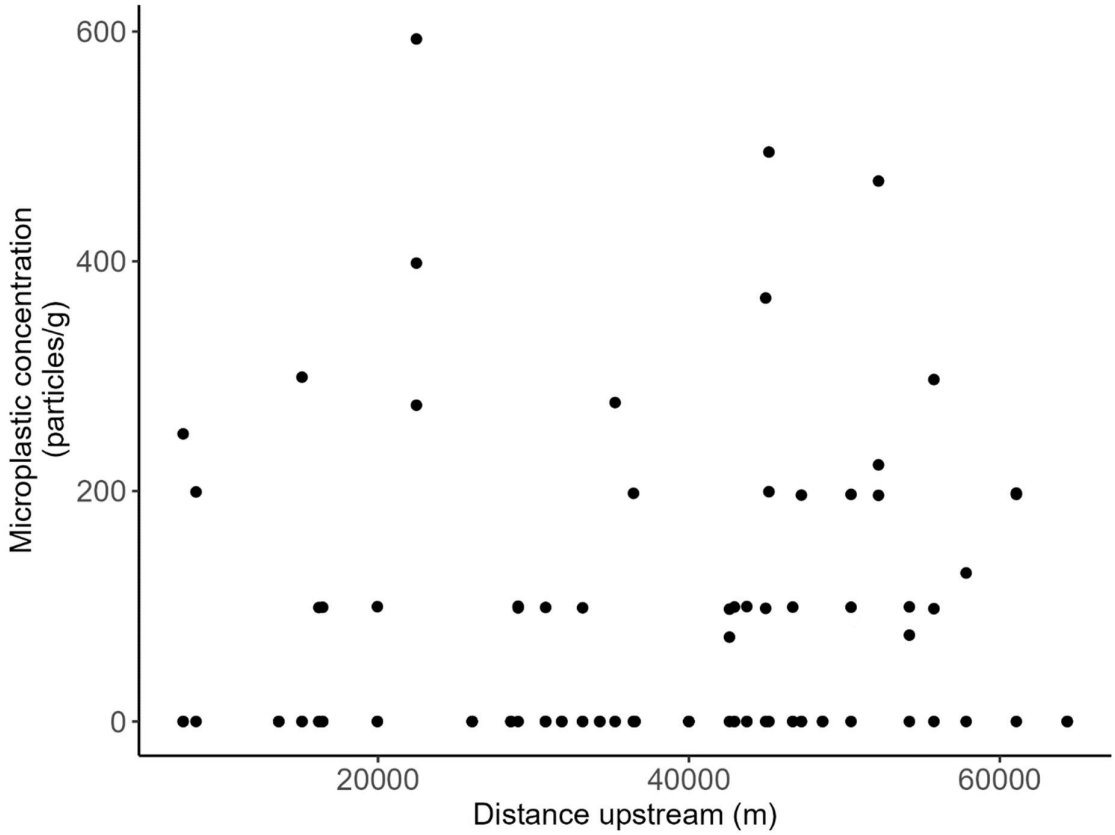


Figure 4.4 Relationship between microplastic concentration in sediment (particles/kg dw) and distance upstream (m) of River Taff.

4.4.3 Occurrence of microplastics in macroinvertebrates

Microplastics were observed in 5% of macroinvertebrate individuals (32 of 679) across 55% of sites (21 of 38) on the River Taff. Of those contaminated, mean microplastic concentration was 0.8 ± 0.3 particles/individual (0.5-1.6 particles/individual) and 96.4 ± 150.5 particles/g (7-833.3 particles/g) (Figure 4.3b). Macroinvertebrates of all feeding guilds were contaminated with microplastic, and neither family nor individual wet weight significantly affected microplastic presence and concentration by individual or gram of tissue (Table 4.2). Microplastic presence in macroinvertebrates was not significantly affected by mean river flow velocity nor subcatchment area, but there was some tendency for presence to decline with distance upstream ($p = 0.07$; Table 4.2a). Models indicated that microplastic concentration (particles/individual) was significantly positively affected by distance upstream and subcatchment area, and significantly negatively affected by mean flow velocity (Table 4.2b). However, substantial zero-inflation in the data limited model fits

and reduced their ability to explain variation (e.g., R^2 for microplastic presence model = 0.04).

Microplastic was observed in one individual upstream of Cynon WWTP (site 13), two individuals upstream of Ponticill WWTP (site 37) and one individual downstream of Ponticill WWTP (site 33), all with the same concentration. Microplastic concentration did not differ significantly between sites without CSOs upstream and all sites downstream of CSOs (two-samples Wilcoxon rank test: W = 57,749, p = 0.673). Lastly, no significant relationship was observed between microplastic concentration in sediment and in macroinvertebrates from the same site (Negative Binomial GLM: ß = -3.85 e-04, SE = 0.002, *z*-value = -0.166, 95% CI [-0.005, 0.004], p = 0.868).

Table 4.2 Regressions of associations between microplastic **a)** presence and **b)** concentration (particles/individual) in macroinvertebrates collected across 38 sites in River Taff, UK, and biotic and abiotic effect measurements, using a binomial GLM. ß = estimate, SE = standard error, CI = confidence interval, and df = degrees of freedom. Stars indicate levels of statistical significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

a)

Effect	ß	SE	z-value	p-value	95% CI
Intercept	-3.32	0.425	-7.797	6.36e-15***	[-4.27, -2.58]
Family: Hydropsychidae	0.44	0.519	0.856	0.392	[-0.55, 1.53]
Family: Leuctridae	0.22	0.644	0.344	0.731	[-1.01, 1.53]
Family: Rhyacophilidae	-0.20	0.655	-0.312	0.755	[-1.58, 1.07]
Wet weight (mg)	-0.34	0.256	-1.343	0.118	[-0.85, 0.15]
Distance upstream (m)	-0.38	0.207	-1.812	0.070	[-0.78, 0.04]
Subcatchment area (km^2)	0.02	0.181	0.104	0.917	[-0.34, 0.37]
Mean flow velocity (m/s)	-0.04	0.184	-0.197	0.844	[-0.41, 0.32]

df residual = 671

b)

Effect	ß	SE	z-value	p-value	95% CI
Intercept	0.74	0.070	10.559	<2e-16***	[0.60, 0.88]
Wet weight (mg)	0.09	0.053	1.744	0.081	[-0.01, 0.20]
Distance upstream (m)	0.80	0.081	9.923	<2e-16***	[0.64, 0.96]
Subcatchment area (km^2)	0.59	0.063	9.234	<2e-16***	[0.46, 0.71]
Mean flow velocity (m/s)	-0.23	0.046	-4.968	6.76e-07***	[-0.32, -0.14]

df residual = 671

4.4.4 Particle characteristics

Polymer analysis revealed 78.8% (n = 52) of the 66 subsampled particles extracted from sediments were synthetic polymers, 10.6% (n = 7) were composed of non-plastic materials, specifically natural cellulose (lignin) or their derivatives (glucose, zein), and 10.6% (n = 7) were unidentifiable by μ-FT-IR. The most abundant synthetic polymer found in sediment was synthetic cellulose (n = 39), followed by polyester (n = 12) and poly(ethylene:vinyl acetate:vinyl chloride) (n = 1). Out of 112 subsampled particles extracted from macroinvertebrates, polymer analysis revealed 37.5% (n = 42) were synthetic polymers, 36.6% (n = 41) were composed of non-plastic materials, including natural cellulose (lignin), organic salts (methylergonovine maleate and acetoacetyl coenzyme A trisodium salt), and a polymer additive (CYANOX® antioxidant), and 25.9% (n = 29) were unidentifiable by μ-FT-IR. The most abundant synthetic polymer found in macroinvertebrates was synthetic cellulose (n = 36), followed by polyvinyl acetate (n = 2) and polyamide (nylon; n = 2), then polyester (n = 1) and epoxy resin (n = 1).

After correcting the data to account for non-plastic particles and sample contamination (see sections 4.3.5 and 4.3.6), 194 out of 318 particles extracted from sediment samples were classified as microplastic residing in sediment. Almost all of these microplastics were fibres (n = 192; 99%), with 1% (n = 2) being fragments (Figure 4.5a). Transparent was the dominant colour (n = 119, n = 61.3%), followed by black (n = 47; 24.2%), white (n = 15; 7.7%), blue (n = 10; 5.2%), and grey (n = 4; 2.1%; Figure 4.5b). For macroinvertebrates, 35 out of 1,606 extracted particles were classified as ingested microplastic, which were either fragments (n = 18; 51.9%) or fibres (n = 15; 42.3%), with a small number of films (n = 2; 5.8%) (Figure 4.5a). Transparent was the dominant colour (n = 19; 54.3%), followed by blue (n = 14; 40%), white (n = 3; 8.6%), and brown (n = 1; 2.9%; Figure 4.5b). Hydropsychidae was the only family to have ingested brown microplastic and Leuctridae was the only family to ingest film. Microplastic from macroinvertebrates significantly differed in both shape and colour distribution compared to microplastic from sediment (Fisher's exact test: $p < 0.001$ for both shape and colour; Figure 4.5).

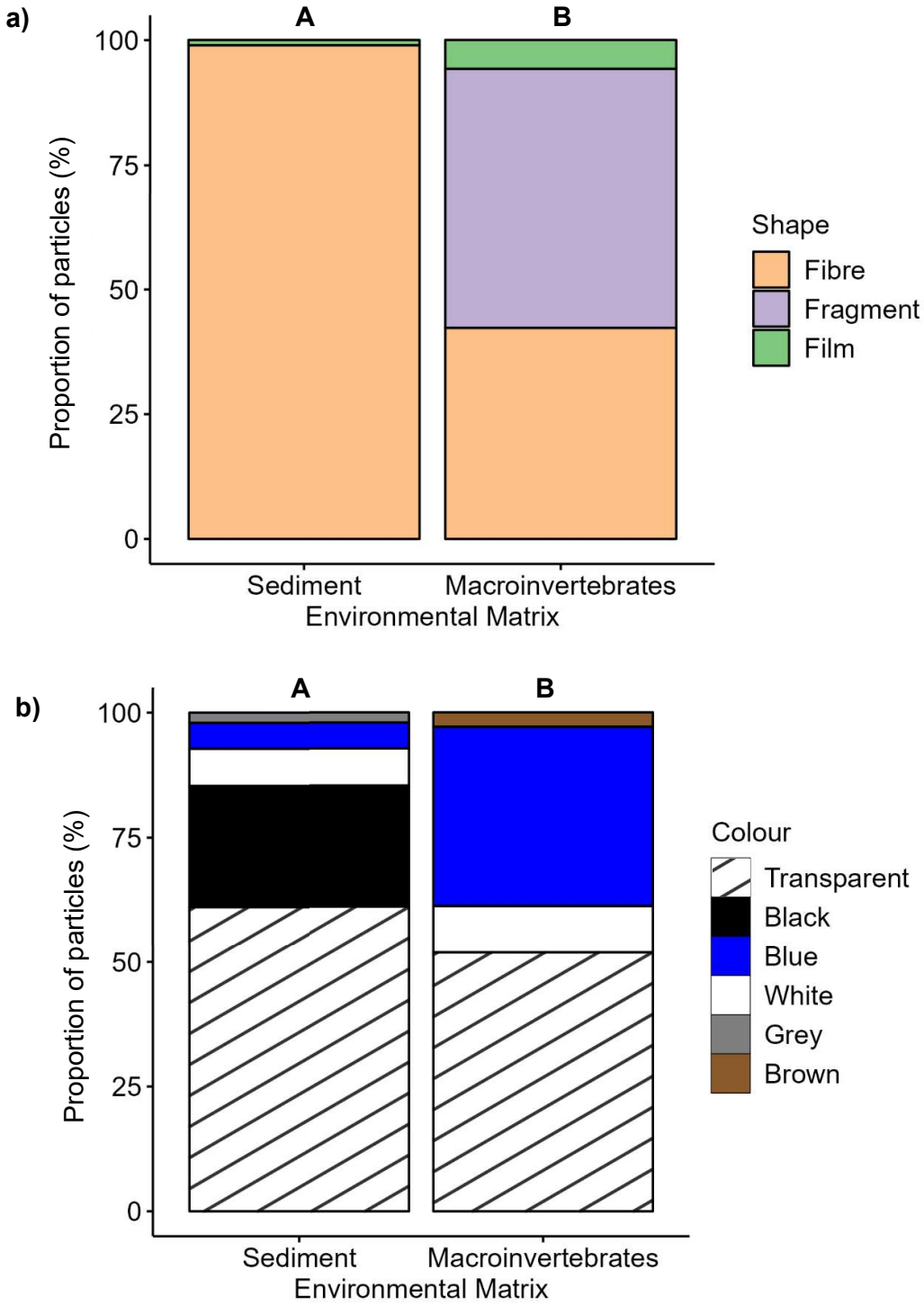


Figure 4.5 a) Shape and **b)** colour distribution amongst microplastic particles extracted from sediment and macroinvertebrates sampled across 38 sites along River Taff, UK, after data correction (see sections 4.3.4 and 4.3.5). Total particle count after correction: sediment = 194, macroinvertebrates = 35. Different capital letters above bars indicate significant difference (Fisher's exact test: $p < 0.001$).

4.5 Discussion

This is the first study to systematically evaluate microplastic pollution across an entire river catchment in Wales, to determine how land use, putative point sources, and hydrodynamics influence microplastic concentration and type in freshwater ecosystems. Sampling both sediment and aquatic invertebrates offers dual insight into microplastic distribution and uptake into food webs, enabling the study of microplastic flow through different environmental matrices. The results did not support the hypothesised changes among locations and with taxonomic identity. Moreover, the results did not identify microplastic hotspots in River Taff, but instead, reveal a patchy distribution. The sections that follow first outline the inevitable caveats and limitations of correlational field studies such as this. Next, the occurrence of microplastic in River Taff sediment is discussed in relation to potential catchment influences compared with studies elsewhere. Lastly, patterns in the occurrence of plastic in invertebrates are described in relation to feeding guild, body size, and the microplastic found in surrounding benthos.

4.5.1 Study limitations

The patchy distribution and limited occurrence of microplastics in these data from the Taff, particularly from macroinvertebrates, made analysis of microplastic distribution challenging. This spatial heterogeneity, combined with robust microplastic identification criteria, meant that there were many zero counts (i.e., zero-inflation), complicating the modelling process. Despite efforts to address these challenges - such as excluding zeros when calculating outliers and summary statistics, and using a compound Poisson-Gamma distribution specifically designed to handle such data - the models still deviated from ideal performance. The presence of numerous data points with high statistical influence, unmet model assumptions, overdispersion, and weak R^2 values all suggest models were unable to accurately capture the variability in microplastic concentrations. These issues raise important caveats about the results, but similar challenges may arise in other regions where microplastics are patchily distributed or have low prevalence, as observed in the River Taff. This study emphasises the importance of critically evaluating performance and fit of statistical

models, particularly in the context of ecological and environmental research, where data can be highly variable and complex.

These issues also raise important methodological caveats. Firstly, there are multiple pathways where microplastic concentrations may have been inflated. Despite measures to minimise sample contamination (section 4.3.6), the use of plastic equipment and materials may have introduced exogenous microplastic. While procedural blanks were used to account for contamination, any inherent background noise may still remain, particularly in low-concentration samples. The decision not to use field blanks (e.g., exposed filter papers) during sample collection also limits the ability to fully quantify atmospheric contamination. Finally, manual crushing of exoskeletons may result in microplastic degradation, inflating particle counts.

There are also multiple pathways leading to potential underestimation of microplastic concentration. The 500 µm sieve used to remove large material also excluded microplastics between 0.5 mm and 5 mm – despite the standard definition of microplastics as particles < 5 mm (Arthur et al. 2009; GESAMP 2015). Meanwhile, the 63 µm sieve likely retained microfibres due to electrostatic attraction, capillary action, and/or cohesive forces increasing surface tension of water, despite triple-rinsing, excluding particles from quantification. Sample agitation or the addition of surfactants (e.g., Tween 20) to rinse water could have been used to reduce surface tension and improve microfibre removal (Oladejo 2017). Also, sample digestion relied solely on H_2O_2, which may be less effective at breaking down lipid-rich or proteinaceous material including macroinvertebrate exoskeletons, compared to enzymatic treatments. This remaining biological material could have blocked microplastic visualisation through the stereo microscope. Furthermore, density separation may have been incomplete, as the maximum density of KI solution (1.75 g/cm^3) is insufficient at separating the densest polymers or undigested-biofouled particles. On the contrary, incomplete settling of fine sediment due to the high density of KI solution, may have also obscured microplastic isolation and visual detection.

The second hypothesis, examining the impact of WWTP, lacks statistical power due to minimal replicates (only three pair site clusters), rendering the findings anecdotal. Additionally, sampling was conducted during a single spring period (April-

May 2022), constraining the temporal generalisability of observed patters of microplastic distribution, concentration, and composition. As shown in Chapter 2, seasonal variables such as rainfall, flow rates, and stormwater runoff - typically higher in autumn and winter – can substantially influence microplastic loads, especially via WWTPs and CSOs. Finally, the study did not assess microplastics in the water column, limiting insights into their vertical distribution and bioavailability to aquatic organisms.

These difficulties in accurately modelling microplastic distribution are compounded by challenges in distinguishing between plastic and non-plastic particles. Separating plastic synthetic fibres (e.g., polyester), non-plastic synthetic fibres (e.g., cellulosic), natural fibres (e.g., cotton or wool), and fibrous organic material (e.g., plant fibres or chitin from organisms) through visual and polymer analysis is notoriously difficult (Primpke et al. 2018; Lusher et al. 2020a). This likely creates uncertainty in microplastic concentrations recorded throughout literature. In this study, from the subsample of particles initially classed as plastic under a stereo microscope using visual criteria in this study (Supplementary Information B1), polymer analysis revealed only 11% were plastic and 42% were non-plastic synthetic particles, whilst 27% were natural fibres and 20% were unidentifiable by μ-FT-IR. This suggests that microplastic detection using a stereo microscope overestimated microplastic loads in the River Taff by approximately 89%. Distinction between plastic and non-plastic microfibres has also been made in other freshwater studies (e.g., Miller et al. 2017; Dris et al. 2018b). For instance, Stanton et al. (2019) reported that 93.8% of textile fibres extracted from River Trent water, UK, were non-plastic, closely aligning with the overestimation observed in this study. Natural and non-plastic synthetic fibres used in the textile industry are still processed with dyes, chemicals including flame retardants, and known carcinogens (Schreder and La Guardia 2014), and are often coated in resin that prolongs environmental degradation (Li et al. 2010; Zambrano et al. 2021). Therefore, non-plastic particles may not be exempt to causing physical, chemical, and biological damage to the environment as plastic does (Huang et al. 2021c). The inclusion of all synthetic particles in ecotoxicology studies would improve understanding of the environmental effects of fibres and particles, potentially also being extended to include non-plastic particles.

4.5.2 Microplastic in sediment

Microplastics contaminated sediment in over 70% of sample sites, indicative of the widespread distribution of litter within river catchments (Barrows et al. 2018; Wang et al. 2021f) and the role of sediment as a microplastic sink (Castañeda et al. 2014; Scherer et al. 2020). The concentration of microplastic in River Taff's sediment fell at the lower end of the range reported in freshwater systems globally (5 – 3.35 ± 6.6 million particles/kg dw; see *Chapter 2*). The one order of magnitude variation within the catchment (lowest non-zero: 73.1 particles/kg dw, highest: 593.5 particles/kg dw) has similarly been found in other freshwater systems, including the catchment-scale study of Brisbane River, Australia (He et al. 2020), but concentrations can range up to four orders of magnitude (Belontz et al. 2022). Compared to freshwater systems in UK, microplastic burden in the River Taff sediment was similar to that documented in the River Bourne (Parker et al. 2022a: 0 – 0.36 particles/g), River Kelvin (Blair et al. 2019: 161 – 432 particles/kg dw), River Thames (Horton et al. 2017: averages of 18.5 – 66 particles/100 g), and River Tame (Tibbetts et al. 2018: 2 – 35 particles/100 g, when scaled by weight. Variance between rivers likely stems from disparities in pollution sources and river characteristics. The River Bourne is one-tenth the length of the River Taff and flows partly through rural area. In contrast, the Tame, Thames, and Irwell rivers flow through the top three largest cities in UK (London, Birmingham, and Manchester, respectively), with greater populations and built-up areas than Cardiff. This follows the association between microplastic load and urbanisation observed in *Chapter 2*, whilst highlighting the need to treat every freshwater river as unique systems when evaluating microplastic burden.

Far greater microplastic loads have been observed in the River Tame by Woodward et al. (2021) (2,400-138,400 particles/kg dw), as well as the Irwell and Mersey rivers by Hurley et al. (2018a) (0 – 72,400 particles/kg). This may be influenced by their unique use of cylinder resuspension to sample sediment, which involves agitating sediment into suspension within a large cylinder and sampling turbid water. This approach samples sediment to greater depths compared to grab samples, and microplastic suspended in the water column may get incorporated into the sample. This extends comparison of sampling techniques in *Chapter 3*, but further experimental work is required to compare microplastic recovery rates from

sediment collected via grab sampling and via the cylinder resuspension technique. This will contribute to the harmonisation of microplastic sampling techniques for future research.

The first hypothesis proposed that microplastic concentrations would increase with distance downstream due to cumulative effects, increased drainage area, and intensified human land-use in the River Taff catchment. However, microplastic concentrations in River Taff sediment did not correlate with distance downstream nor subcatchment area. This contrasts with multiple studies reporting accumulation downstream (Mani et al. 2015; Eo et al. 2019; Mani et al. 2019b; Wagner et al. 2019; Gallitelli et al. 2020; Mani and Burkhardt-Holm 2020; Mao et al. 2020b; Huang et al. 2021b; Schell et al. 2021; Tamminga et al. 2022; Treilles et al. 2022) and microplastic pollution associated with local urbanisation (e.g., Tibbetts et al. 2018; Wagner et al. 2019; de Carvalho et al. 2021; Rakib et al. 2022; Tamminga et al. 2022; Yuan et al. 2022). However, these results conform with studies reporting no influence of urbanisation (Barrows et al. 2018; Wen et al. 2018; Alfonso et al. 2020; Wang et al. 2020b; Wang et al. 2021d) or human population density (Miller et al. 2017; Kapp and Yeatman 2018; Tibbetts et al. 2018; Dikareva and Simon 2019; Alfonso et al. 2020; Frank et al. 2021; Li et al. 2022b).

Instead, microplastic occurrence in River Taff sediment demonstrated a patchy distribution. Such spatial trends have also been documented across the Scheldt River, Belgium (Troyer 2015), Yongjiang River, China (Zhang et al. 2020b), and River Tame sediment, UK (Woodward et al. 2021), pointing towards WWTPs and industrial areas as microplastic sources. However, the second hypothesis of microplastic hotspots at WWTP outlets cannot be confirmed by results in this study. Despite slight increases in microplastic concentration downstream of some WWTPs, no microplastic was observed immediately downstream of Cynon and Cilfynydd WWTPs, which serve the greatest number of people relative to other WWTPs in the catchment (144,955 in 2021). This may be due to the high effectiveness of local WWTPs at removing microplastic from wastewater. Lofty et al. (2022) reported a 100% removal rate of microplastic from the local Nash WWTP in Newport, South Wales, and Johnson et al. (2020) reported an average removal of 99.99% of microplastics in six WWTPs across England and Wales. Three meta-analyses reported most WWTPs around the globe remove over 90% of microplastics from

inputs, but depending on treatment type, removal can be as low as 4% (Cristaldi et al. 2020; Iyare et al. 2020; Liu et al. 2021b). Moreover, untreated wastewater from CSOs and stormwater drains are likely to be laden with microplastic (0.0093 – 5,195.8 ± 425.5 particles/L; Dris et al. 2018a; Treilles et al. 2020; Sun et al. 2023). For example, heavy microplastic contamination in River Tame sediment, UK, was attributed to routine discharge of untreated wastewaters into low river flows (1.69-4.09 m^3/s), where microplastic deposits to sediments (Woodward et al. 2021). However, no cumulative effect of CSOs on microplastic loads in River Taff sediment was observed. This may be due to the relatively higher water flow of up to 15 m^3/s during sample collection (eight gauging stations on River Taff described in Table B4; National River Flow Archive; UKCEH 2024). The high ratio of river flow to effluent discharge in River Taff may dilute this microplastic source and limit sedimentation and bioavailability (Dris et al. 2015).

Local point sources may have also been masked by diffuse sources including urban dust, tyre wear particles, stormwater, and surface runoff from sewage sludge used as soil fertiliser. For example, Lofty et al. (2022) reported 96% of microplastic entering a Welsh WWTP ends up in sewage sludge used as soil fertiliser, leading to an estimated maximum application rate of 4.8 g or 11,489 particles of microplastic/m^2/year. However, in the year prior to sample collection, WWTP digested biosolids were only delivered to two locations within the River Taff catchment, over 7 km upstream of any sample site. Thus, this is not considered a major pollution source in the River Taff catchment.

Alternatively, the analytical approach of studying microplastic distribution across the whole river catchment may have limited the isolation of point sources, despite the high coverage of sampling sites. This may be captured over smaller geographic distances by sampling directly above and below postulated point sources. Instead, results suggest widespread but diverse sources of microplastic within the River Taff catchment that do not accumulate into longitudinal trends. The patchy distribution may also relate to the complex suspension and deposition cycles of microplastic within fluvial systems, influenced by hydrodynamics and variation in particle characteristics (Hoellein et al. 2019; Waldschläger and Schüttrumpf 2019; Skalska et al. 2020; Waldschläger et al. 2020; Yan et al. 2021a; Range et al. 2023); see *Chapter 2*). This underscores the multifaceted nature of microplastic pollution.

The dominance of transparent fibres in River Taff sediment and identification of synthetic cellulose, polyvinyl acetate, nylon, and polyester, all major components of textiles (Oerlikon 2010), suggests wastewater is the main source of microplastic pollution to the River Taff. Synthetic microfibres are released from domestic washing into wastewater (De Falco et al. 2018; Yang et al. 2019a), which enters the environment through untreated outlets (Habib et al. 1998). The dominance of synthetic cellulose supports the fifth hypothesis that predicted a prevalence of dense microplastic particles in bed sediment, as it has a greater density (1.5 g/cm^3) compared to the other polymers identified by μ-FT-IR (poly(ehtyleve:vinyl acetate:vinyl chloride): ~1.15 g/cm^3 and polyester: 1.37 g/cm^3; British Plastics Federation 2023) and thus, are more likely to sink through the water column and deposit onto bed sediment.

4.5.3 Microplastic in macroinvertebrates

The observation of microplastics in River Taff aquatic macroinvertebrates contributes to evidence of plastic particles entering freshwater food webs from basal levels (Nel et al. 2018; Windsor et al. 2019b; Akindele et al. 2020; Dahms et al. 2020; Simmerman and Wasik 2020; Garcia et al. 2021; Pan et al. 2021; Pastorino et al. 2021; Stanković et al. 2021; Corami et al. 2022; Ribeiro et al. 2022; Thamsenanupap et al. 2022; Winkler et al. 2022; Stanković et al. 2024). The 5% contamination rate is similar to that in Garonne River, France (2%; Garcia et al. 2021), but is far lower than the 100% contamination rate of macroinvertebrates sampled across Danube River (Stanković et al. 2024). Occurrence in the present study is also lower than the 50% macroinvertebrate contamination rate recorded previously across the Rivers Taff, Wye, and Usk in south Wales (Windsor et al. 2019b). Moreover, maximum microplastic load per contaminated individual was around four times lower (1.6 particles after corrections) than that observed by Windsor et al. (2019b) (6 particles). This inconsistency within the same river system could indicate a dramatic decrease in microplastic pollution over six years between the two sampling events. However, global plastic production, generation, and disposal have continuously increased since mass production started in 1950 (Geyer et al. 2017), with freshwater sediment cores from Japan, Thailand, and Malaysia revealing concurrent temporal increases in microplastic contamination of water

bodies (Matsuguma et al. 2017). Instead, the greater scale of sampling effort in this study (38 compared to 5 sites in Windsor et al. 2019b) may have diluted macroinvertebrate contamination rates due to the capture of a patchy distribution.

Alternatively, this disparity could be attributed to climatic differences between sampling events. Precipitation increases stormwater runoff, a flux of land-based microplastics into freshwater environments (Shruti et al. 2021), and high freshwater microplastic concentrations have often been observed after rain events (Schmidt et al. 2018; Piñon-Colin et al. 2020; Wong et al. 2020a; Xia et al. 2020). Local rainfall in May and June 2016 prior to sample collection by Windsor et al. (2019b) averaged at 109.7 mm per month, double the monthly average of 53.4 mm experienced in March to May 2022 during sample collection for this study (Met Office 2024; Cardiff Bute Park, latitude: 51.488, longitude: -3.187). This may have led to relatively higher microplastic loads in the River Taff during Windsor et al. (2019b) sampling compared to this study, creating a greater exposure risk for macroinvertebrates. Simultaneously, daily flow rate of the River Taff over eight gauging stations (Table B4) was greater in June and July 2016 (mean ± 1 SD: 2.6 ± 4.2 m^3/s, range: 0.1-53.4 m^3/s), compared to April and May 2022 (mean ± 1 SD: 1.5 ± 2 m^3/s, range: 0.2-15 m^3/s) (National River Flow Archive; UKCEH 2024). Greater flow may have created more unfavourable conditions for macroinvertebrates during sample collection by Windsor et al. (2019b), where individuals may have been less able to decipher between microplastic and their natural food source and/or required more energy and thus, were less selective about the items they ingested.

Conversely, however, macroinvertebrate microplastic loads within this study were greater in sites with lower flow velocity. Amongst literature, low flow velocity has been associated with microplastic sedimentation (Kapp and Yeatman 2018; Tibbetts et al. 2018; Tien et al. 2020; see *Chapter 2*), whilst high flow events such as flooding, have reduced microplastic loads in freshwater sediment (Hurley et al. 2018a; Liu et al. 2019b). Therefore, within the catchment, benthic macroinvertebrates may be more at risk of sediment microplastic at low flow sites. This discussion suggests a complex relationship between microplastic uptake by invertebrates and hydraulic processes, as found in *Chapter 2*. Further work is needed to decipher the spatial scale at which dynamic hydraulic processes influence microplastic flux, to better predict their fate in different environmental matrices.

Sparse observation of contaminated individuals in this study constrains investigation into predictor variables. Results suggest that while fewer upstream individuals contained microplastic, those contaminated had greater microplastic loads compared to downstream individuals. This suggests that upstream areas may have fewer sources of microplastic pollution, but where contamination does occur, it might be from a concentrated or localised source. Conversely, however, sites with larger subcatchment areas also exhibited higher microplastic concentrations, despite being located downstream. This may be attributed to differences in microplastic sources between river tributaries, but likely results from the weak and poorly fitting statistical models created by zero-inflated data, as discussed in section 4.5.1.

Regarding biotic predictor variables, Windsor et al. (2019b) reported greater microplastic concentration in Heptageniidae compared to Hydropsychidae and Baetidae from Welsh rivers, and attributed this to their greater mass. In this study, however, neither feeding behaviour nor individual biomass influenced microplastic concentration. This refuted the third and fourth hypotheses, but matched patterns in Garonne River (Garcia et al. 2021), Danube River (Stanković et al. 2024), and Bourne Stream, UK (Parker et al. 2022a), invertebrates. With macroinvertebrates at over half of sample sites ingesting microplastic, these results underscore the widespread bioavailability of microplastics in freshwater environments and universal exposure risk within aquatic invertebrates, whilst expanding the identified potential pathways for microplastic transfer through the food web.

Lastly, multiple studies report an association between microplastic in freshwater organisms and sediments, suggesting that benthic feeders ingest microplastic from surrounding sediment and thus, act as indicators of microplastic pollution (Su et al. 2016; Hurley et al. 2017; Hu et al. 2018; Nel et al. 2018; Su et al. 2018; Yuan et al. 2019; Merga et al. 2020; Park et al. 2020a; Hou et al. 2021; Kallenbach et al. 2022). However, only 13 of 35 sites had microplastic in both invertebrate and sediment samples, with contaminated invertebrates being less widespread than contaminated sediment. This lack of association has also been observed in European rivers (Garcia et al. 2021; Parker et al. 2022a; Winkler et al. 2022), African streams (Dahms et al. 2020), and a Bangladesh river (Haque et al. 2023). In a similar vein, particle characteristics differed between microplastic extracted from these invertebrates and sediment samples. Fibres dominated in the

Taff's sediment, consistent with other studies in rivers (e.g., Horton et al. 2017; Vermaire et al. 2017; Blair et al. 2019) and lakes (e.g., Clayer et al. 2021; Felismino et al. 2021; Min et al. 2023). In macroinvertebrates, both fragments and fibres were prominent, as found in freshwater fish (Andrade et al. 2019; Olesen et al. 2019; Roch et al. 2019; Garcia et al. 2020; Wang et al. 2020b). Results, therefore, suggest that Hydropsychidae, Leuctridae, Heptageniidae, and Rhyacophilidae are not reliable bioindicators of microplastic contamination in River Taff.

4.6 Conclusion

With reports of microplastic in freshwater environments increasing, this study expands evidence of catchment-wide contamination of microplastic as one of the few temperate studies of distribution across an entire, connected riverine system. This provides important environmental contamination levels to inform local pollution risk assessments and mitigation policy, as well as wider laboratory exposure experiments. Microplastics were found in sediment at over 70% of sites, but their concentration was apparently unaffected by land use or likely point sources. Instead, an observed patchy distribution suggests that other factors are involved, including hydrodynamics of fluvial systems or channel storage relative to catchment sources. The data illustrate microplastic uptake by aquatic invertebrates of different feeding guilds, which leads to particle entry into freshwater food webs. However, prevalence was low (5%) compared to 2016 sampling, likely resulting from the greater sampling effort capturing a patchy distribution, and relatively lower precipitation and flow rates during sample collection. This highlights the role of hydrodynamics on influencing microplastic distribution over space and time, which needs further investigation over different spatio-temporal scales. Differences in microplastic location, concentration, and characteristics amongst invertebrates and sediment sampled across the River Taff suggests that the families studied ingest microplastic from surrounding water and/or allochthonous material rather than sediment, or sample particles at different spatio-temporal scales to those influencing sedimentation. This reduces their use as microplastic bioindicators in freshwater ecosystems. Recognising these complex dynamics is essential to developing more accurate models of microplastic behaviour in freshwater systems and informing targeted, effective management strategies.

Chapter 5: Comparing methods to extract microfibres from leaf litter decomposing in fresh water

5.1 Abstract

The occurrence of microplastic in freshwater ecosystems is well documented, yet their accumulation in specific microhabitats, such as accumulated leaf litter, and consequences for ecological processes are poorly understood. In part, this reflects the lack of standardised methods to extract microplastic from environmental media. This chapter develops, describes, and compares two methods to extract microplastic from ethanol-preserved leaf litter: (1) density separation with potassium iodide solution and (2) digestion with hydrogen peroxide followed by density separation. Fluorescent microfibres stained with Nile Red were used to assess recovery rates. No fluorescent microfibres were recovered by either method, suggesting spiked microfibres were degraded or lost during sample processing. However, non-fluorescent microfibres were recovered in apparently larger quantities when using density separation alone, indicating reduced microfibre recovery following digestion. As these microfibres were homogeneous in appearance, their presence confirms recovery of spiked microfibres rather than external contamination of samples. The lack of fluorescent signal suggests loss of stain from spiked microfibres. This suggests that microplastic recovery studies using Nile Red may have false negatives and underestimate recovery rate. Methods employing density separation alone appear favourable for extracting microplastic from leaf litter, but must be combined with permanent or reliable staining techniques to isolate microplastic from fine sediment that is not removed by density separation alone. Options include polymer staining with Nile Red, or organic matter staining such as with Rose Bengal. This study further identified the challenge of removing ethanol used to fix and store samples, for which evaporation techniques were not viable. Removal through sieving is recommended, but risks microplastic loss if trapped on the sieve even after rinsing, leading to the underestimation of microplastic loads. This study offers insights into methods of microplastic extraction from organic media and how this can be optimised.

5.2 Introduction

The flux, dispersion, and fate of microplastic in freshwater ecosystems is determined by multiple factors acting over different spatial and temporal scales (see *Chapter 2*). Current investigation into microplastic contamination of freshwater ecosystems is primarily focussed on material transported or suspended in water, followed by sediment and biota (see *Chapter 3*). However, there have been few investigations of microplastics interacting with other freshwater components including submerged leaf litter, benthic biofilm, or other natural sediments that could interact with food webs or ecosystem processes (Sanpera-Calbet et al. 2012; Lofty et al. 2023). Microplastic adherence to submerged leaf litter has been suggested after microplastic was extracted from leaf litter packs installed into the Vipacco River, Italy (Bertoli et al. 2023a,b), and spiked microplastics were recovered from leaf litter in static freshwater mesocosms (Redondo-Hasselerharm et al. 2018; Weber et al. 2018; López-Rojo et al. 2020). Microplastic on submerged leaves could interact with aquatic organisms that use them for energy and habitat, leading to toxic and non-toxic effects (Rakib et al. 2023). Furthermore, shifts in the activity, survival, and diversity of fungal and macroinvertebrate detritovores caused by micro- and nano-plastics has been shown to negatively affect leaf litter decomposition (Seena et al. 2019, 2022; López-Rojo et al. 2020; Batista et al. 2022; Du et al. 2022; Ockenden et al. 2022; Silva et al. 2022; Trabulo et al. 2022; Borges et al. 2024). This ecosystem function is crucial to freshwater health and thus, there is a call to develop our understanding of microplastic adherence to leaf litter in the real-world and subsequent influence on decomposition. Addressing this research gap is important in the context of the thesis and in particular, methodological development to support assessment of microplastic capture by leaf litter in *Chapter 6*.

There is an extensive list of published methods for isolating microplastic from different environmental matrices of freshwater ecosystems (see *Chapter 3*). Procedural differences in sample processing include filtration size, digestion and density separation solutions, and mass calculation. This reflects the unique challenges that stem from both the make-up of samples as well as equipment availability to researchers. Microplastic recovery studies can be used to assess potential under- or over-estimation of environmental microplastic loads due to the method of extraction (Way et al. 2022). One option for such work involves spiking

matrices of interest with known types and concentrations of microplastic, running the extraction method, and assessing the amount of spiked microplastic recovered. Meta-analysis of published recovery efficiencies suggests that variation in reagents used for microplastic extraction affects observed microplastic loads (Way et al. 2022). Lack of standardisation and comparative approaches compromises comparison of loads reported in different environments using different extraction procedures. Performing a recovery test alongside microplastic extraction from environmental media allows researchers to adjust observed microplastic loads to account for methodological biases. This could be used as a tool to standardise reported microplastic loads without the potentially inappropriate approach of standardising extraction methods. However, recovery testing has rarely been used in microplastic research to date (Way et al. 2022).

Density separation is a major component of microplastic extraction techniques (see section 3.5.2 of *Chapter 3*), but many biological materials have lower densities than the solutions employed in separation processes. Consequently, biological material may float alongside microplastic and contaminate filtered samples, making isolation challenging (Herrera et al. 2018). Additionally, microplastic may be imbedded in organic material, preventing their isolation based on density alone (Herrera et al. 2018). Digestion of organic matter is therefore another major component of microplastic extraction techniques (see section 3.5.3 of *Chapter 3*). Oxidative digestion with hydrogen peroxide (H_2O_2) was most commonly used in freshwater studies analysed in *Chapters 2* and *3* and is cheap and relatively less hazardous compared to other reagents. On the other hand, alkaline and acidic digestion both damage or discolour certain polymers and enzymatic digestion is expensive, time-consuming, operates at small scales, and require specific activation conditions.

5.2.1 Aims

The aim of this study was to devise and assess a methodology to extract microplastic from leaf litter following submergence in freshwater and subsequent recovery. Existing techniques used on other environmental matrices were tested to identify potential pitfalls associated with extracting microplastic from leaf litter, while

also verifying and validating methods by assessing microplastic recovery rate. The latter entailed spiking leaf litter with known types and amounts of microplastic to evaluate any under- or over-estimation of environmental microplastic concentrations.

5.3 Methods

5.3.1 Microplastic preparation

Microplastic recovery from prepared leaf litter packs (see section 5.3.2) was tested with precision cut 1,000 X 1.1 µm (1.1 dtex), polyamide (PA) microfibres purchased from Barnet Europe. Fibres are frequently reported as the dominant microplastic shape in riverine environments, due to their widespread sources and high environmental persistence. These fibres primarily originate from the breakdown of synthetic textiles during washing processes, with WWTPs acting as major pathways for their release into aquatic ecosystems (Browne et al., 2011; Dris et al., 2015). Unlike fragments or beads, fibres are more buoyant and can remain suspended in the water column longer, facilitating their transport and eventual deposition in sediments or ingestion by aquatic organisms (Wagner et al., 2014). Their high surface-area-to-volume ratio also increases the likelihood of interaction with biota, leading to greater bioaccumulation in macroinvertebrates (Silva et al., 2021). PA has large-scale production and extensively used in textile industry (Wesolowski and Plachta, 2016). Microplastic recovery can be calculated as the difference in mass between spiking loads and recovered loads (Grause et al. 2022). However, preliminary testing in preparation for this chapter showed this to inflate microfibre recovery rates beyond 100%, likely due to inorganic material such as fine sediment remaining in the filtrate. Therefore, microplastic recovery was assessed by comparing recovered microfibre counts against estimated counts of spiking microfibres. To identify spiking microfibres, they were dyed with Nile Red (9-(diethylamino)-5H-benzo[α]phenoxazin-5-one), with which PA has a high affinity to. This makes fibres fluorescent under ultraviolet light (blue fluorescence, λex = 300–405 nm), blue light (λex = 405–500 nm), or green light (λex = 500–600 nm; Shruti et al. 2022).

Microfibre staining was performed as per Maes et al. (2017). Specifically, a 5:1 ratio of staining solution (10 µg/mL filtered acetone) to microfibres (Karakolis et

al. 2019) was stirred at room temperature for 30 minutes (Galvão et al. 2023) prior to vacuum filtration, triple rinsing with filtered acetone to remove excess Nile Red, and rinsing once with filtered deionised water (FDW) to remove acetone. Stained microfibres were left to dry in a laminar flow hood for 24 hours, before 96.6 mg was weighed and suspended in 100 mL of FDW. This created a spiking solution containing ~19 microfibre particles per 1 mL. Figure 5.1 shows the fluorescence signal from dyed microfibres compared to un-dyed microfibres with no autofluorescence, using Olympus BX61 under green light at 4x magnification. Aggregated microfibres were separated for individual quantification to determine the spiking solution concentration. To test Nile Red leaching from dyed microfibres, spiking solutions were centrifuged at 4,000 rpm for 10 minutes. No fluorescent signals were observed in the supernatant (Figure 5.2).

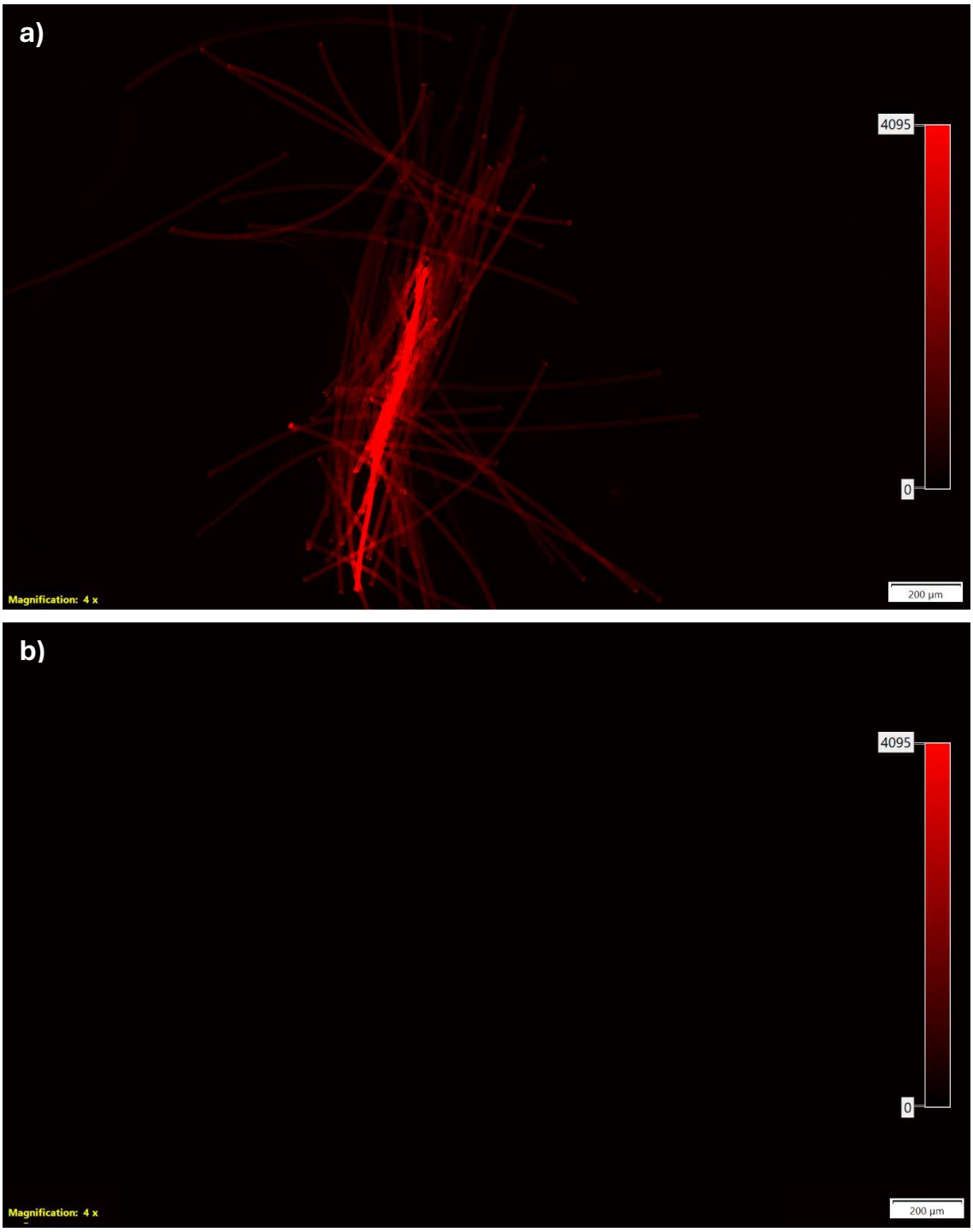


Figure 5.1 Fluorescence images: **a)** Fluorescent signal of PA microfibres stained with Nile Red. **b)** Un-stained microfibres with no autofluorescence. Images taken with Olympus BX61 under green light at 4x magnification.

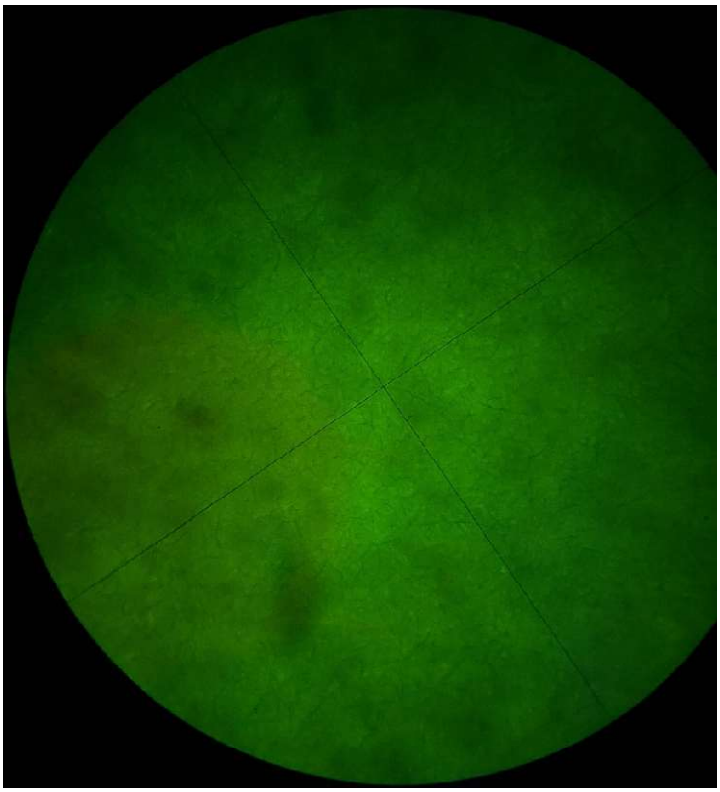


Figure 5.2 Fluorescence leaching test for microfibres dyed with Nile Red in filtered deionised water (19 particles/mL), centrifuged at 4,000 rpm for 10 minutes. Green background was observed under green light at 4x magnification with Olympus BX61, with no fluorescent signals to indicate dye leachate.

5.3.2 Leaf litter

Oak (*Quercus robur*) leaves were collected from the study area (Llyn Brianne Observatory, Cambrian Mountains, mid-Wales) after abscission and cleaned with FDW before drying at room temperature for 48 hours (flipped halfway). Groups of leaves were weighed to 3 ± 0.1 g and placed in 10 x 10 cm mesh bags with 5 mm aperture, subsequently referred to as leaf packs. These leaf packs were placed in individual plastic tubs with 3.5 mL of spiking solution (mean ± 1 SD = 19 ± 8.4 fibres/mL), equivalent to ~66 microfibres. This concentration was selected to reduce the margin of error in recovery rate, whilst ensuring the number of microfibres was manageable for manual visualisation and enumeration. Leaf packs were covered with filtered 100% ethanol to fix samples and prevent decomposition, and gently shaken.

5.3.3 Method development

Two methods of extraction were tested: (1) density separation with saturated potassium iodide solution (KI; ρ = 1.52-1.63 g/cm^3); and (2) digestion with 30% H_2O_2 then density separation with saturated KI solution. Each method was tested with 12 replicate leaf pack samples. As Bertoli et al. (2023a,b) digested leaf litter for microplastic extraction, in preparation for this chapter, dead Oak leaves were digested using 30% hydrogen peroxide (H_2O_2) for 72 hours. This is longer than the 48-hour digestion period used to digest macroinvertebrates and sediment in Chapter 4, due to the high cellulose and lignin content of leaves, which are more difficult to breakdown. After digestion, leaves faded in colour but remained intact, indicating that 30% H_2O_2 was ineffective at breaking down dead leaf litter. Therefore, microfibres were extracted from the storage solution rather than the leaves themselves.

Under laminar flow, leaves were carefully removed from their mesh bags and individually triple rinsed with FDW to remove any adhered microfibres, before being disposed. An attempt was made to examine leaves for fluorescent microplastic before being disposed of, using a blue light and yellow filter, but this did not show any fibres. Leaves could not be examined under the microscope due to their size and curved shape. Ethanol was removed via a triple-rinsed 63 µm sieve, and retained material was triple-rinsed into triple-rinsed and labelled 50 mL tubes for method (1) and triple-rinsed and labelled 50 mL glass beakers for method (2). Samples were dried at 60°C in a fan-assisted oven for 72 hours to remove water. For method (1), dried tubes were topped with filtered saturated KI solution and centrifuged at 4,000 rpm for 10 minutes, before the supernatant was vacuum filtered onto 0.45 µm cellulose-nitrate gridded filter paper. For method (2), dried beakers were topped with filtered 30% H_2O_2 in a fume hood for 48 hours to digest organic material. H_2O_2 was subsequently removed through a 63 µm sieve and retained material was triple-rinsed with FDW into triple-rinsed and labelled 50 mL tubes, before performing the same drying, density separation, and filtering as used in method (1). Each filter paper was systematically scanned along the grid lines using an Olympus BX61 under green light at 4x magnification.

5.3.4 Contamination control

Samples are at risk of plastic contamination from external sources in the air and reagents used (Prata et al. 2020b; Kernchen et al. 2022). To prevent external contamination during microplastic staining and sample processing, all laboratory processes were conducted under a laminar flow hood. All reagents were pre-filtered through 0.45 µm cellulose nitrate filter paper, except for acetone which was filtered through 0.7 µm glass microfibre filter paper. All equipment was triple rinsed with FDW before use and researchers wore 100% cotton clothing and nitrile gloves.

By staining microfibres with Nile Red, only recovered spiking microfibres were intended for quantification, assuming external microplastics do not fluoresce under green light. To test the latter, airborne background contamination was assessed with one air blank per extraction method tested. For this, a wetted 0.45 µm cellulose-nitrate filter was placed into a glass petri dish next to sample processing and later inspected for fluorescence microfibres as above. No fluorescent microfibres were observed on air blank filters.

5.4 Results

No fluorescent microfibres were observed on filters from method (1) and method (2). Non-fluorescent microfibres were observed on all filters, in far greater abundance on method (1) compared to method (2) filters. The abundance in the former was so great that they could not be accurately counted by human-eye. For these reasons, recovery rate could not be calculated. Fine sediment was present on method (1) filters but not method (2) filters.

5.5 Discussion and recommendations

This study utilised reported methods of microplastic extraction from environmental material (see *Chapter 3*) to establish protocols to extract microplastic from leaf litter decomposing in fresh water. The aim of identifying pitfalls in extraction methods that are not described in literature was fulfilled. A challenge arises where field samples have been fixed in ethanol which must be removed prior to the addition of digestion and density separation reagents. Preliminary testing in preparation for

this chapter showed evaporation of ethanol in both a 50°C fan-assisted drying oven and a rotary evaporator at 80-220 rpm and 60-90°C, were not viable because of slow evaporation rates. This is likely due to the hydrogen bonding of ethanol molecules with water molecules disrupting the ability of ethanol molecules to escape into the vapor phase (Nishi et al. 1995). Freeze drying (lyophilisation) may be an alternative solution to remove ethanol and water, but could not be tested due to time and equipment availability constraints. Therefore, in this thesis, reagents were removed through sieving (Mai et al. 2018), despite this invariably leading to some loss of microplastic (Nakajima et al. 2019). A sieve aperture of 63 µm may have allowed 1.1 µm diameter microfibres to pass through, or may have retained microfibres even after triple-rinsing. This could be due to electrostatic attraction, capillary action, and/or cohesive forces increasing surface tension of water. Literature suggests the addition of surfactants (amphiphilic composites) to rinsing water to reduce surface tension and limit microfibre adherence to equipment (Oladejo 2017). Sieves could not be examined to quantify potential microfibre retention as the particles are too small for the human eye, and the sieve does not fit under a microscope.

The aim of quantitatively verifying and validating extraction methods could not be fulfilled, as only non-fluorescent microfibres were recovered, and their abundance was too high to be accurately counted by the human eye, even after attempts to separate fibres. This suggests that not all spiking microfibres were dyed, possibly due to the degradation of Nile Red dye over time before its use in this study. A fluorescence leaching test demonstrated that Nile Red-stained fibres maintained fluorescence when incubated in artificial freshwater and seawater for 24 hours (Ma er al. 2020a). However, the presence of surfactants like Tween-20 affected the fluorescence retention, indicating that chemical environments can influence staining stability (Ma er al. 2020a). Alternatively, Nile Red is known to protonate in very acidic environments (pH < 4) (Sturm et al. 2021). Therefore, the use of 30% H_2O_2 with a pH of 3.5-4.5 may have protonated the Nile Red stain in microfibres and reduced their fluorescence intensity. Prata et al. (2020a) reported a complete loss of Nile Red fluorescence from low-density polyethylene (LDPE) microplastics washed with 30% H_2O_2 with 0.05 Fe(II) or acetone. However, Porter et al. (2023) used 30% H_2O_2 to digest marine worms exposed to Nile Red dyed PA microfibres and successfully recovered fluorescent fibres, refuting loss of fluorescence. Our results suggest that

previous studies focussing only on fluorescent microplastic recovery may have underestimated recovery rates, as non-fluorescent particles were overlooked. This skews reported microplastic loads and limits previously used methods of microplastic extraction. Underestimation of environmental microplastic loads underrates the severity of this pollution, which may lead to lack of mitigation.

Recovery rate could not be calculated as the initial spiking load was based on fluorescent microfibres only. This limits the calculation of under- or over-estimation of microplastic concentration in leaf litter samples. The homogeneity in the appearance of recovered microfibres and the clear blank controls confirming no external contamination, indicates that microfibres can be recovered from leaf packs. Despite the visible reduction of non-fluorescent microfibres with the additional digestion step, the lack of recovery rate quantification means no conclusions can be drawn.

Chapter 6: Microplastic addition has minimal effects on invertebrate communities or litter decomposition in stream mesocosms of contrasting pH

6.1 Abstract

Microplastic occurs throughout freshwater environments and is ingested by organisms. Previous research has mostly focused on individual interactions with microplastics, yet little is known about effects at higher levels of ecological organisation or on ecosystem processes. The magnitude of any such effects relative to other stressors is also poorly understood. For example, leaf-litter decomposition might be sensitive to changes in invertebrate communities caused by microplastics either alone or in conjunction with other pollutants and/or environmental changes, and therefore susceptible to the multiple-stressor effects typically seen in real ecosystems. This study, therefore, assessed the effects of environmentally realistic pulse-injections of microplastic (1,000 x 1.1 µm polyamide microfibres dyed with Nile Red) on the density, diversity, and community composition, alongside leaf litter decomposition, in experimental mesocosms of contrasting pH (acid versus circumneutral). Macroinvertebrate density and family diversity was hypothesised to be lower downstream of microfibre addition and in acidic conditions. This was expected to reduce leaf-litter decomposition as decomposition rate positively correlates with macroinvertebrate density.

Few microfibres were recovered from macroinvertebrates and leaf litter, suggesting limited immobilisation of spiked fibres within mesocosms and/or limited interaction with biological media. Changes in macroinvertebrate density, family diversity, and community composition were all limited, and microplastic had no effects on leaf decomposition downstream of microfibre pulse-injection. Acidic mesocosms had greater macroinvertebrate densities of benthic communities compared to circumneutral mesocosms, with Leuctridae being more dominant at lower pHs. Overall, variance in population, community, and ecosystem functional responses were poorly explained by either microfibre addition or pH treatment. These findings suggest that the negative effects of microplastics sometimes observed in laboratory systems may be overridden by other processes in dynamic

freshwater environments and thus, highlight the need for more realism in future microplastic experiments to decipher potential impacts at different ecological scales.

6.2 Introduction

Microplastic, as defined in previous chapters, is widespread in freshwater ecosystems. Catchment-scale assessments of rivers have detected microplastic at 83% of sites in Wu River, Taiwan (Kunz et al. 2023), and up to 100% of sites across the Huangpu, Wei, Yangtze, and Yellow rivers, China (Chen et al. 2020a; Yuan et al. 2022; Chen et al. 2024; Zhao et al. 2023; Zhang et al. 2024; Zong et al. 2024), Chapora River, India (Kalangutkar et al. 2024), Brisbane River, Australia (He et al. 2020), and the upper Mersey and Irwell rivers, England (Hurley et al. 2018b). In Wales, polluted rivers such as the River Taff contained microplastic in sediment and macroinvertebrates from 71% and 55% of 38 sites, respectively, during 2022 (*Chapter 4*). Microplastic enters freshwaters through both diffuse and point sources (Ding et al. 2021). As detailed in earlier chapters, diffuse sources are widespread and include urban runoff (Wang et al. 2022), agricultural drainage (Hatinoğlu and Sanin 2021), and atmospheric deposition (Su et al. 2020; Tan et al. 2022), where microplastics are transported by wind and rain from a variety of dispersed locations. Point microplastic sources include specific, identifiable origins such as Wastewater Treatment Plant (WWTP) outflows (Ziajahromi et al. 2016; Kay et al. 2018), Combined Sewer Overflows (CSOs; divert excess wastewater and stormwater; Dris et al. 2018a; Parizi 2021), and industrial discharges (Bitter and Lackner 2020; Chan et al. 2021). These point sources could act as “pulse-injections” of microplastic, with average CSO spill duration in England being 5.8 hours in 2022 (The Rivers Trust 2023). Most WWTPs investigated by researchers removed over 85% of microplastic (Cristaldi et al. 2020; Iyare et al. 2020), whereas CSOs are not treated and thus, could form a considerable source of microplastic to freshwater ecosystems (Woodward et al. 2021). With point microplastic sources being easier to identify compared to diffuse sources, understanding their environmental impact will support direct management, regulation, and mitigation of this pollution.

One possible sink for microplastic in freshwater environments is ingestion by organisms. At the individual level, microplastic has caused reduced energy intake,

oxidative stress, genotoxicity, and neurotoxicity (Ma et al. 2020b; Li et al. 2023b; Rakib et al. 2023; Harmon et al. 2024), with subsequent changes in growth, activity, and survival having the potential to affect populations (Foley et al. 2018; Martins and Guilhermino 2018). However, most information about the effects of microplastics has arisen from acute toxicity tests on single species, with microplastic concentrations in organisms seldom observed in the field (Ockenden et al. 2021; Harmon et al. 2024). This lacks environmental realism and limits our knowledge on the impact of microplastic on different trophic guilds, the composition of whole communities, and freshwater ecosystem function (Ma et al. 2020b).

Among a wide range of ecosystem processes, leaf-litter decomposition is crucial to freshwater ecosystem function and is mediated by a combination of microorganisms (dominantly fungi) and invertebrates, particularly leaf shredders (Willoughby 2012). The process releases essential nutrients into water, adds organic matter to sediment which maintains its structure and fertility, and increases downstream fluxes of particulate carbon used by filter-feeding organisms (Cummins et al. 1989; Wallace et al. 1997; Willoughby 2012). Leaf litter breakdown is used as a functional indicator of freshwater ecosystem health (Gessner and Chauvet 2002; Young et al. 2008). Microplastic adherence to freshwater leaf litter has been suggested by Straub et al. (2017), Redondo-Hasselerharm et al. (2018), Weber et al. (2018), López-Rojo et al. (2020), and Bertoli et al. (2023a, c). Concomitant shifts in the feeding behaviour and survival of macroinvertebrate shredders have been shown to have negative effects on the leaf-litter processing under experimental conditions (López-Rojo et al. 2020; Ockenden et al. 2022; Silva et al. 2022; Borges et al. 2024). Reduced fungal activity, sporulation, and diversity due to micro- and nano-plastic also limits leaf-litter decomposition (Seena et al. 2019, 2022; Batista et al. 2022; Du et al. 2022; Trabulo et al. 2022). Similar trends have been observed with other pollutants including heavy metals (Sridhar et al. 2001; Duarte et al. 2008; Roussel et al. 2008; Fernandes et al. 2009; Moreirinha et al. 2011; Bergmann and Graça 2020) and pesticides (Rasmussen et al. 2012; Magali et al. 2016; Rossi et al. 2018; Sumudumali et al. 2022). In contrast with other pollutants, however, the effects of microplastic on litter decomposition are still poorly understood – presenting an important gap in understanding (Wu et al. 2024).

Leaf-litter decomposition can also be affected by changes in abiotic conditions of freshwater ecosystems, both directly, and indirectly by the alteration of detritivore activity and community composition (Webster and Benfield 1986; Taylor and Chauvet 2014). Surface-water acidification, for example, can result from acidic groundwater discharge from abandoned mine workings (Johnson and Hallberg 2005), or ammonia (NH_3) emission from agriculture (Skinner et al. 1997). More geographically extensive acidification also arose previously due to the emission and deposition of acidifying compounds from fossil fuel combustion (Singh and Agrawal 2006; Rice and Herman 2012). This issue was further exacerbated locally in base-poor landscapes by afforestation (Ormerod et al. 1989; Gagkas et al. 2008). Freshwater acidification decreases pH, alters base cation fluxes, and increases concentrations of toxic metals, notably monomeric aluminium (Schindler et al. 1980; Herrmann et al. 1993; Prakash et al. 2023). This has been observed to reduce leaf-litter decomposition due to concomitant changes in invertebrate community composition (Meegan et al. 1996; Dangles and Guérold 1998; Dangles and Guérold 2001a; Simon et al. 2009; Pye et al. 2012), activity of shredding invertebrates (Meegan et al. 1996; Dangles and Guérold 2001a; Dangles and Guérold 2001b; Cornut et al. 2012; Ferreira and Guérold 2017), and microbial activity and biomass (Griffith et al. 1995; Meegan et al. 1996; Dangles et al. 2004; Simon et al. 2009; Clivot et al. 2013), particularly in hyphomycete fungi (Iqbal and Webster 1977; Shearer and Webster 1985; Griffith and Perry 1994; Dangles et al. 2004; Baudoin et al. 2008; Cornut et al. 2012; Seena et al. 2019, 2022). Although many streams in affected areas are now substantially recovered chemically (Ormerod and Durance 2009; Whelan et al. 2022), in some systems, biological recovery is delayed or partial due to continued episodes of acidity during hydrological events (Carr et al. unpublished data; Kowalik et al. 2007).

In the real-world, environmental stressors (physical, chemical, or biotic entities that move biological systems out of normal range; Segner et al. 2014) do not operate in isolation, including within freshwater ecosystems (Ormerod et al. 2010). The impact of co-occurring stressors can be additive (net effects = sum of single effects) or interact in a synergistic or antagonistic manner (net effects respectfully > or < the sum of single effects) (Johnson and Penaluna 2019; Pirotta et al. 2022). Interactions of these types are theoretically possible between acidification and microplastics, for example, because pH can alter the environmental behaviour of microplastics.

Acidification has been suggested to lead to: (i) aggregation of certain microplastic particles due to weakened electrostatic repulsive force (Lu et al. 2018; Wang et al. 2021e); (ii) modulation of microplastic degradation by affecting microorganism survival and activity (Auta et al. 2018); (iii) enhancement of photodegradation (Ariza-Tarazona et al. 2020); (iv) increased leaching of anion and heavy metals (Cui et al. 2019); and (v) reduced adsorption of trace metals (Turner and Holmes 2015; Llorca et al. 2018). These interactions could change the availability of microplastic and associated pollutants to detritovores, enhancing their impact in acidic environments. Alternatively, the well-described adverse effects of acidification on freshwater communities and ecosystem function (Muniz 1990; Gray et al. 2016) may override any impacts of microplastic suggested in laboratory studies.

To date, potential effects of microplastic in freshwater ecosystems have not been studied at contrasting pH. In marine environments, this combined stress has inhibited digestive enzymes in mussels while only slightly affecting their oxidative responses (Wang et al. 2020d), and did not affect bacterial and algal growth rate (Piccardo et al. 2020). However, ocean acidification (0.1 pH unit reduction since the industrial revolution; Pachauri et al. 2014) is far smaller than freshwater acidification (1-2 pH units), implying the potential for more deleterious effects in freshwaters. Bolstering the available information on ecosystem function effects of microplastic and acidification would aid ecological risk assessment and subsequent development of appropriate control strategies (Fleeger 2020).

Laboratory microcosm experiments are important in ecotoxicology as they allow for control of environmental variables (Diamond 1986), enabling researchers to isolate specific processes or interactions with pollutants. Compared to field studies, microcosms are often more cost-effective and accessible (Carpenter 1996) and minimise disturbances to natural ecosystems. However, the oversimplification of complex natural systems can lead to results that do not fully represent real-world dynamics over different spatial and temporal scales, of both abiotic variables (e.g., hydrodynamics, habitat etc.) and biotic interactions (e.g., predation, competition, symbiosis etc.), often using single species (Diamond 1986). Moreover, laboratory conditions themselves may induce stress in organisms (Calisi and Bentley 2009).

This gap between controlled laboratory microcosms and the complex real world can be bridged using mesocosms: bounded and partially enclosed outdoor experimental setups (Odum 1984). Mesocosms can be replicated and controlled to a certain extent, while still maintaining a level of realism that is not achievable in laboratory systems, making them valuable for studying the impacts of environmental stressors (e.g., Graney et al. 1994; Stewart et al. 2013). Yıldız et al. 2022 conducted the first in situ community-level mesocosm experiment testing the effects of microplastic on a model aquatic food web, including herbivorous, predator, and detritivore invertebrates, in a static system. Microplastic particles were added at two different concentrations in a single pulse to the water surface, water column, and sediment. Yıldız et al. 2022 observed a variation in microplastic ingestion according to particle size, invertebrate body size, and feeding guild. Microplastic ingestion by zooplankton was limited, but microplastic presence in faecal pellets of predators indicate trophic transfer (Yıldız et al. 2022). Comparing such results to a fluvial mesocosm would aid understanding of flow effects.

This novel study therefore uses field mesocosms to investigate the potential independent and interactive effects of microplastic and pH on stream organisms at the population (macroinvertebrate density), community (diversity; abundance of different feeding guilds), and ecosystem function levels (leaf litter decomposition). Additionally, ingestion of microplastic by macroinvertebrates and adherence to leaf litter was estimated to establish any entry of microplastic into the freshwater food web, and to connect microplastic exposure to potential density and trait-mediated effects on ecosystem functioning. The experiment aimed to test the following hypotheses:

1) Pulse-injected microplastics are ingested by macroinvertebrates and adhere to submerged leaf litter.
2) Leaf-litter decomposition positively correlates with macroinvertebrate density.
3) Macroinvertebrate density and family diversity, and leaf-litter decomposition decrease downstream of microplastic input versus upstream.
4) Macroinvertebrate density and family diversity, and leaf-litter decomposition is lower in acidic versus circumneutral mesocosms irrespective of plastic addition.

6.3 Methods

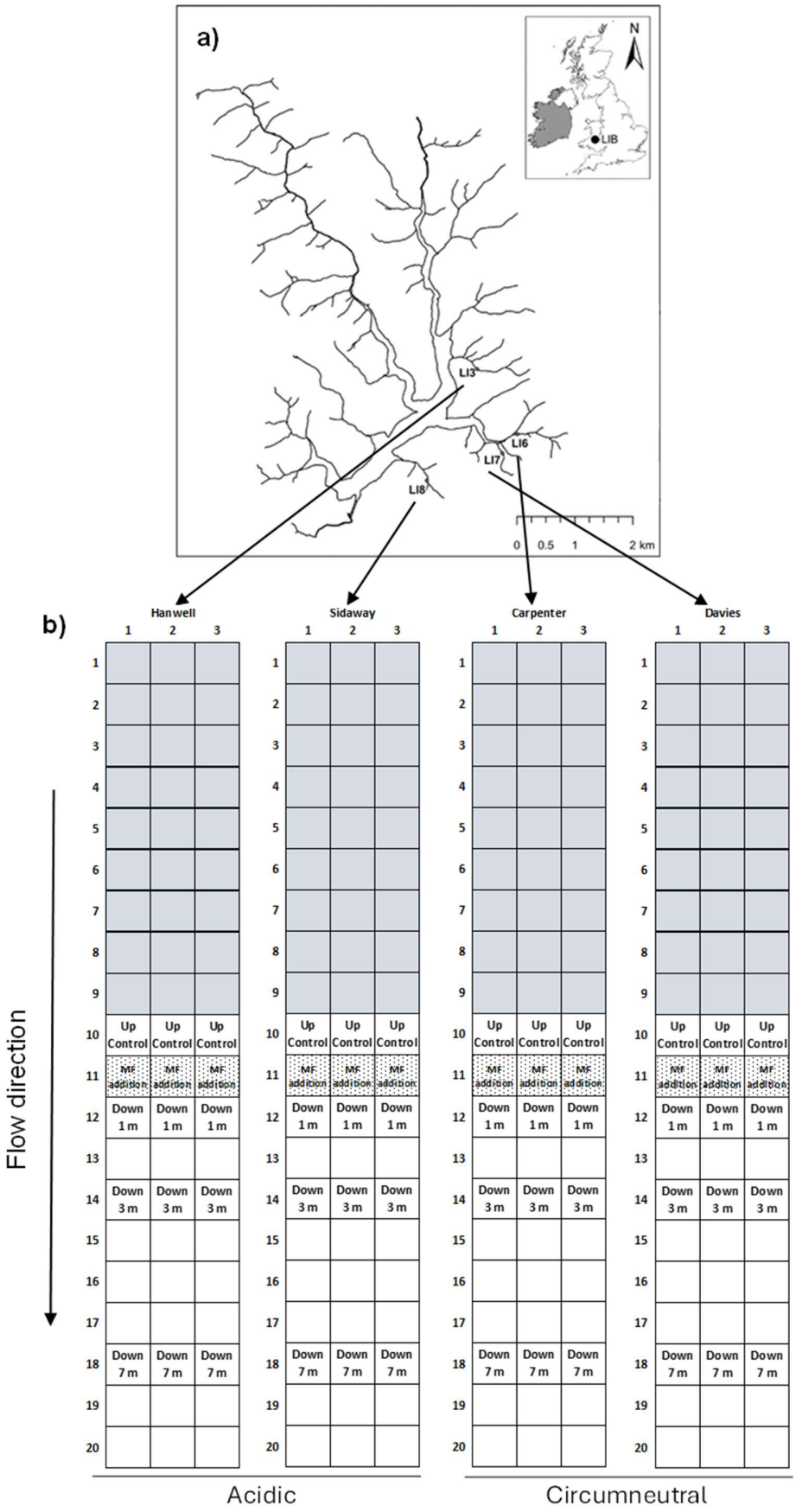
6.3.1 Study area and stream mesocosms

The study was conducted at the Llyn Brianne Stream Observatory (52°8′ N 3°45′ W; Figure 6.1a), where a series of replicate upland streams receive drainage from *c*. 300 km^2 of the upper catchment of the upper Afon Tywi. The area experiences an oceanic climate with stream temperatures ranging 0-16°C, a mean annual precipitation of *c*. 1900 mm, and mean solar radiation of 7.85 MJ/m^2/d (Weatherley and Ormerod 1990) - see Weatherley and Ormerod (1987) and Edwards et al. (1990) for detailed descriptions of the Observatory.

Four of the streams have been fitted with mesocosms, each consisting of three, parallel cascading steel channels (0.2 x 0.2 x 20 m) one-fifth of the size of adjacent streams (Figure 6.1b,c). The mesocosms have a coarse benthic substrate layer of mixed cobbles (D_{50} = 5 cm; Seymour et al. 2018) and receive water and naturally occurring particles directly from their respective streams, making them physiochemically and ecologically representative. Mesocosm water reaches a depth of ~0.05 m above the substrate (cross-sectional area: A = 0.05 x 0.2 m) and flows at a rate of 1.1 L/s (Q). This suggests an average flow velocity (v) of ~0.1 m/s, using the equation $v = Q / A$. In the month prior to this experiment, a preceding experiment exposed mesocosm channels to different drought treatments (100%, 50%, or 10% flow). Moreover, apparatus issues during this experiment caused short-term drying and re-wetting of different channels. Flow was limited in Davies mesocosm in the left and right channels for 3 weeks, and the middle channel for 4 weeks, whilst Hanwell, Carpenter, and Sidaway had limited flow in their left and right channels for 1 week. As mesocosms were visited once a week, the duration of limited flow is unknown could have ranged from 1 to 6 days. The effects of this are discussed in section 6.5.

Two mesocosms (Carpenter and Davies) receive water from first order circumneutral streams (LI6 and LI7, respectively) that drain rough, sheep-grazed moorland and have respective pH ± SD of 6.7 ± 0.03 and 6.8 ± 0.04. Two mesocosms (Hanwell and Sidaway) receive water from acidic streams (LI3 and LI8, respectively) that drain through plantations of Sitka spruce *Picea sitchensis* Carr. with lodgepole pine *Pinus contorta* Doug and have respective pH ± SD of 5.9 ± 0.07 and 5.35 ± 0.07 (pH data recorded in 2018; Seymour et al. 2018). Differences in pH

result in different invertebrate communities among the streams, with fewer grazers occupying acidic mesocosms Hanwell and Sidaway (Ormerod and Durance 2009). This was measured using invertebrate density, Shannon's index (Shannon 1948) and Bray-Curtis dissimilarity (see section 6.3.5), to test hypothesis 4. Replicating mesocosms and their channels enhances both statistical power and the reliability of results. This approach helps to account for the limited control of environmental variables in field settings, which is more precisely managed in laboratory microcosms (Diamond 1986).


a)
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b)
Hanwell
Sidaway
Carpenter
Davies
1 2 3
Up Control
MF addition
Down 1 m
Down 3 m
Down 7 m
Flow direction
Acidic
Circumneutral

Figure 6.1 Study area and stream mesocosms with treatments. a) Location of Llyn Brianne Stream Observatory, UK, consisting of four mesocosms: acidic Hanwell (adjacent to stream LI3) and Sidaway (adjacent to steam LI8), and circumneutral Carpenter (adjacent to stream LI6) and Davies (adjacent to stream LI7). **b)** Experimental design and microplastic treatment – grey = non-experimental sections, spotted = microfibre (MF) addition, white = experimental sections (including upstream control with no microfibre exposure and 3 downstream sections exposed to microfibres at increasing distances from the pollution source). **c)** Photos of mesocosm design, consisting of three, parallel cascading channels, each made of twenty 0.2 x 0.2 x 1 m stainless steel sections. White arrows represent flow direction and white lines represent dimensions of a single section within a mesocosm channel. Red circles show how leaf packs were installed into mesocosms and were fully submerged.

6.3.2 Microplastic preparation

Polyamide (PA) microfibres precision cut to 1,000 µm x 1.1 µm (1.1 dtex) were purchased from Barnet Europe. Microfibres were used due to their extensive occurrence in freshwater environments (see *Chapter 2*) and dominance in point source effluent (Šaravanja et al. 2022; Parashar and Hait 2023). Fibres also have longer gut retention times and slower egestion than spherical particles, resulting in more severe effects on individuals (Au et al. 2015; Ziajahromi et al. 2017a; Qiao et al. 2019). PA was chosen due to its large-scale production and extensive use in the textile industry (Wesolowski and Plachta 2016). PA are easily dispersed in freshwater due to their density (1.01-1.05 g/cm^3) and are reported to have a widespread distribution in the freshwater environment (see *Chapter 2*).

To identify recovered particles and assess their distribution, microfibres were dyed with the lipophilic dye, Nile Red (9-(diethylamino)-5H-benzo[α]phenoxazin-5-one). Nile Red is detected as blue, green, or red fluorescence under ultraviolet light (blue fluorescence, λex = 300–405 nm), blue light (λex = 405–500 nm), or green light (λex = 500–600 nm; Shruti et al. 2022). PA microfibres have a high affinity to Nile Red dye (Erni-Cassola et al. 2017; Prata et al. 2019a; Savage et al. 2022), which helped distinguish added microfibres from inorganic matter (e.g., sediment), organic matter (e.g., macroinvertebrates), and external microplastic contamination. Microfibres were dyed as per Maes et al. (2017), which is described in section 5.3.1 of *Chapter 5*.

6.3.3 Leaf litter preparation

The leaf pack technique (Petersen and Cummins 1974) was used to assess environmental endpoints of microplastic and detrital processing by macroinvertebrates. This technique mimics the natural accumulation of vegetal organic matter in freshwater ecosystems, serving as a trophic resource and refugia for macroinvertebrates. Submerged leaf litter may also act as a retention structure for microplastics transported by aquatic flow. This technique was used by Bertoli et al. (2023a, c) to assess microplastic adherence to leaf litter submerged in the Vipacco River, Italy, verifying that mesh bags with a 5 mm aperture collect and retain microplastics including PA after 45 days of submersion.

In this study, Oak (*Quercus robur*) leaves were collected after abscission from the study area in June 2023, cleaned with water, and left to air dry at room temperature for 48 hours (flipped halfway). Groups of leaves were weighed to 3,000 ± 0.1 mg and placed in 10 x 10 cm mesh bags with a 5 mm aperture, making them accessible for biofilm development as well as fungi, invertebrates, and microfibres. Three leaf packs (n = 3) were installed into sections 10, 12, 14, and 18 (Figure 6.1b,c) at increasing distances from the intended microplastic injection point at the start of the experiment (T0), using plastic cable ties. Oak leaves were chosen due to their abundance within the study area making them a likely source of organic matter in mesocosms, as well as being preferred to bryophytes by local aquatic invertebrates (Johnston et al. 2015). Non-woody plant leaves also breakdown faster than woody plant leaves (Webster and Benfield 1986).

6.3.4 Experimental design and microfibre exposure

The experiment was conducted over the most downstream 10 m of the mesocosms, with a spiking solution of stained microfibres added in section 11 of each channel (Figure 6.1b) on days T0, T7, T14, T21, T28, T35, and T42. The suspension of spiking microfibres in the water column of mesocosms was not tested as the appropriate equipment was not available. Each weekly spiking solution contained 655.2 ± 1 mg of dry, dyed microfibres suspended in 500 mL of filtered deionised water (FDW). This represented pulse injections that would occur in a typical river from a point source such as a CSO. Few measurements of concentration exist for such sewer overflows, but based on microfibre concentrations in Paris CSO effluent between 2014 and 2016 (200 fibres/L; Dris et al. 2018a), mesocosm flow rate (1.1 L/s), and average CSO spill duration in England, 2022 (5.8 hours; The Rivers Trust 2023), ~4,593,600 fibres would be expected to enter mesocosms during a single CSO spill event. In this study, the spiking solution added weekly to each mesocosm channel contained ~4,363,920 microfibres, 95% of the expected quantity. The scale of a CSO spill relates to the capacity, dispersal, and dilution in the system receiving it. If all spiking microfibres settled equally into the treatment reach (2 m^2), the average microfibre concentration in sediment would be 2,181,960 microfibres/m^2. To date, freshwater sediment microplastic concentration

reported per unit area ranges from 67 (Xiong et al. 2018) to 517,142 (Hurley et al. 2018b) particles/m^2, with an average of 44,224 particles/m^2.

The experiment ended after 48 days, when environmental samples (macroinvertebrates and leaf litter) were collected from sections 10 (control = no microfibre addition), 12 (treatment = 1 m downstream of microfibre addition), 14 (treatment = 3 m downstream of microfibre addition), and 18 (treatment = 7 m downstream of microfibre addition) (Figure 6.1b). Benthic macroinvertebrates were sampled via three 71.3 cm^2 Hess samples (Hess 1941) collected across each sampling section. These were stored in individual sealed plastic bags with filtered 99% ethanol at room temperature (n = 48). Leaf packs (total 144) with leaf-dwelling macroinvertebrate colonisers were stored in individual sealed plastic pots with filtered 99% ethanol at room temperature. Ethanol was used to preserve the organic material and prevent gut content excretion of macroinvertebrates (Windsor et al. 2019b).

This is a control vs treatment experimental design, i.e., within each mesocosm, sections 10 with no microfibre addition are compared to sections 12, 14 and 18 subject to microplastic addition. This allows direct comparison at the same time point, isolating any effect of microplastic from temporal or environmental changes. However, differences in pH between mesocosms result in different invertebrate communities among the streams, causing different starting conditions. This was accounted for by the three replicate channels within each mesocosm, and two replicate mesocosms for each pH type. A before-and-after experimental design, where the system is measured at two time points, before and after microplastic addition, would have controlled for pH variability between mesocosms. Although this does not control for temporal or other environmental changes, sampling before microplastic addition would have aided the investigation of effective variables.

6.3.5 Measuring microfibre uptake and effect on macroinvertebrates

Benthic and leaf-dwelling macroinvertebrate individuals were identified to family level under a GXMXTL3 Stereo Microscope (GT Vision Ltd) using identification guides (Croft 1986; Bouchard Jr 2004; Sundermann et al. 2007; Pawley et al. 2011; Eversham 2013; Hackston 2019) and assigned to a Functional Feeding

Guild (FFG) following Merritt et al. (1996; 2002; 2017) and Moog (2002). Family diversity was calculated using Shannon's Index (Shannon 1948) and community composition was compared between samples using Bray-Curtis dissimilarity (see section 6.3.8).

Under laminar flow, individuals were triple rinsed with FDW to remove any externally attached material including fluorescent microfibres (Nel et al. 2018), and wet-weighed to ± 0.1 mg using an analytical microbalance. Microfibres were extracted from all macroinvertebrate individuals as described in section 4.3.3 of *Chapter 4*. This includes digestion with 30% H_2O_2, which does not degrade PA (Gao et al. 2023) nor fluorescence intensity of Nile Red (Wang et al. 2021a; Gulizia et al. 2022; Gao et al. 2023). As in *Chapter 4*, exoskeletons of every macroinvertebrate individual remained after this digestion (quantification was not recorded), which may have interfered with microplastic quantification.

6.3.6 Measuring microfibre uptake and effect on leaf litter

Under laminar flow, leaf litter was carefully removed from individual mesh bags before bags were triple rinsed with FDW to remove attached microfibres. Leaves were triple rinsed with FDW and placed in a pre-weighed paper bag. To determine leaf litter decomposition rate and mass loss, leaves were dried at 50 °C for 24 hours and weighed to ± 0.1 mg. Microfibres were extracted through density separation and centrifugation, as determined in *Chapter 5*. Firstly, the leaf rinsate and preserving solution was poured through a triple-rinsed 63 µm sieve to remove ethanol and capture the 1,000 µm long microfibres. Captured debris was triple-rinsed with FDW into triple-rinsed 50 mL polypropylene centrifuge tubes (autoclave-safe) and dried at 50°C and 10% fan for 10 days to remove water. Tubes were then filled with filtered potassium iodide solution (KI; ρ = 1.6-1.66 g/cm^3) and centrifuged at 4,000 rpm for ten minutes (reasoning discussed in section 4.3.4 of *Chapter 4*), after which the supernatant was vacuum filtered onto gridded cellulose nitrate membrane filters (47 mm diameter, 0.45 µm pore size). All filters were systematically visually examined along the grid lines for fluorescent microfibres using an Olympus SZX12 Stereo Microscope under the Green Fluorescent Protein (GFP) filter, which were counted manually.

6.3.7 Contamination control

Samples used in microplastic investigations are at risk of plastic contamination from external sources in the air, equipment, and solutions used (Prata et al. 2020; Kernchen et al. 2022). By staining microfibres with Nile Red, only the microfibres added and recovered were quantified, assuming external microplastics do not fluoresce under green, fluorescent light. To prevent external contamination during microfibre staining and sample processing, all laboratory processes were conducted under a laminar flow hood. All solutions were pre-filtered through 0.45 µm cellulose nitrate filter paper, except for acetone which was filtered through 0.7 µm glass microfibre filter paper. All equipment was triple rinsed with FDW before use and researchers wore 100% cotton clothing and nitrile gloves. Remaining external contamination was assessed using negative blanks of a dampened filter paper (n = 3). These contained a low number of particles that do not fluoresce under green, fluorescent light. Microfibre recovery tests in *Chapter 5* act as positive controls to show the capability of detecting microfibres extracted from leaf litter.

6.3.8 Statistical analysis

All statistical analyses were performed in 'R' software (version 4.3.0; R Core Team 2023). Per mesocosm channel, data outliers were iteratively identified and removed if more than 1.5-times the interquartile range (IQR) below the first quartile or above the third quartile (Tukey 1977). Data were then assessed for normality using Shapiro-Wilks test (Shapiro and Wilk 1965) and visual inspection of histograms and Q-Q plots. Macroinvertebrate density and Shannon's Index in both benthic and leaf-dwelling communities were not normally distributed (e.g., Shapiro-Wilk test on benthic macroinvertebrate density data: $W = 0.727$, $p < 0.0001$), even after transformation. The 'orderNorm' transformation from the 'bestNormalize' package in R (Peterson and Cavanaugh 2020) was applied to leaf litter decomposition rate to achieve a normal distribution (Shapiro-Wilk test: $W = 0.999$, $p = 1$). Homogeneity of variances across groups were assessed via Levene's test (Levene 1960) for normally distributed data and Filgner-Killeens non-parametric test (Fligner and Killeen 1976) for non-normally distributed data. Variances of macroinvertebrate

density in both communities were not equal across mesocosms, i.e., heterogeneous (Fligner-Killeen test: χ^2 = 15.414, df = 3, p < 0.01 and χ^2 = 9.759, df = 3, p < 0.05 for benthic and leaf-dwelling communities, respectively). Variance of leaf litter decomposition was not equal (Levene test: F = 2.776, df = 3, p < 0.05). Variances of Shannon's Index of both communities were equal, i.e., homogeneous (χ^2 = 3.630, df = 3, p = 0.303 and χ^2 = 2.283, df = 3, p = 0.516 for benthic and leaf-dwelling communities, respectively).

To examine hypothesis 2, correlation between both macroinvertebrate density and Shannon's Index and leaf litter decomposition rate were analysed using Kendall's Tau correlation. This accounts for the distribution of macroinvertebrate density and Shannon's Index being different to normal, as well as "tied" observations.

Hypotheses 3 and 4 were examined as follows. The effect of position along mesocosms (i.e., upstream or downstream microfibre input) and pH on macroinvertebrate density and Shannon's Index, independently for benthic and leaf-dwelling macroinvertebrate communities, were all assessed with Generalised Linear Mixed-effect Models (GLMMs). This accounted for the non-normal distribution in data. GLMMs included channel nested within mesocosm as random effects to account for correlations within these groups at each level of the hierarchy. GLMMs were fitted with a Gamma error distribution and log link function, adding a small transformation of +0.0001 to move the distribution away from zero. Density models with no overdispersion according to the dispersion parameter (variance-to-mean ratio) were selected, as this indicates a better fit to the data (Thomas et al. 2017). However, these models had a singular fit and non-normally distributed residuals, indicating poor reliability of estimated random effect parameters. Shannon's Index data of both benthic and leaf-dwelling communities were zero-inflated, with 28% and 21% of data being zero, respectively. A Shannon's Index value of zero indicates a single-family community. This led to under-dispersed GLMMs and thus, excess zeros were accounted for by setting the 'zeroInflation' parameter to "TRUE" in the glmmadmb() model-fitting function, which removed under-dispersion and singular fit. However, these models had non-normally distributed residuals.

Differences in community composition in relation to position along mesocosms and pH was measured using Bray-Curtis dissimilarity based on square

root transformed taxa abundance (to reduce variation), and analysed using a two-way PERMANOVA (Anderson 2001; McArdle and Anderson 2001). Prior to this, multivariate homogeneity of group dispersions was checked using PERMDISP2 (Anderson 2006). Similarity Percentage (SIMPER) analysis was applied to the data matrix to identify the taxa that contributed the most to the significant differences highlighted by the PERMANOVA (Clarke 1993).

The effect of position along mesocosms and pH on leaf decomposition was assessed with a GLMM that included channel nested within mesocosm as random effects, as GLMMs with Gamma error distribution were severely under-dispersed. The model was fitted with Restricted Maximum Likelihood (REML) using the Laplace approximation method. Residuals were normally distributed (Shapiro-Wilk test: W = 0.990, p = 0.470), but the model was slightly under-dispersed according to a dispersion parameter of 0.517, with 1 indicating “normal” dispersion (Thomas et al. 2017).

6.4 Results

6.4.1 Macroinvertebrates

Microplastic uptake

Fluorescent microfibres were observed in 0.5% (5 of 1,043) of benthic macroinvertebrate individuals, three of which were Leuctridae sampled from acidic Sidaway mesocosm and two were Heptageniidae sampled from circumneutral mesocosms. All contaminated individuals were sampled downstream of microfibre input, 40% from 3 m downstream, and 60% from 7 m downstream. Zero fluorescent microfibres were observed in all leaf-dwelling macroinvertebrates. Zero non-fluorescent microfibres were observed in any samples, as observed in *Chapter 5*.

Density

Across the four sites of cascading mesocosms, 1,049 benthic and 891 leaf-dwelling aquatic macroinvertebrate individuals were collected via Hess sampling and leaf packs, respectively. Benthic macroinvertebrate density was significantly greater in acidic (mean ± 1 SD: 1,243 ± 1,322 individuals/m^2) than circumneutral mesocosms

(501± 503 individuals/m^2; $p < 0.01$; Table 6.1a), but there was no effect of microfibre addition: in no mesocosms did benthic density differ between dosed and non-dosed sections ($p > 0.05$; Table 6.1a,b; Figure 6.2a). There was no difference between acidic (mean ± 1 SD: 2.6 ± 2.5 individuals/g final leaf-litter) and circumneutral mesocosms (3.8 ± 3.4 individuals/g final leaf-litter) in the number of leaf-dwelling macroinvertebrates and again, there was no effect from microfibres ($p > 0.05$; Table 6.1b; Figure 6.2b). Marginal R^2 values for the lognormal distribution were 0.2605 and 0.1117 for the benthic and leaf-dwelling community model, respectively. Both models had a singular fit and thus, conditional R^2 values equalled marginal R^2 values.

Table 6.1 Effect of microfibre addition (1 m upstream versus 1 m, 3 m, and 7 m downstream of spiking) and pH (acid versus circumneutral) on density of **a)** benthic (individual/m2) and **b)** leaf-dwelling macroinvertebrate communities (individuals/g final leaf litter), accounting for random effects within channels nested within mesocosms. Colon indicates interaction. SD = standard deviation, ß = estimate, SE = standard error, and df = degrees of freedom. Stars indicate levels of statistical significance: p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***).

a) Benthic macroinvertebrates

Random effects	**Variance**	**SD**
Channel:Mesocosm	0	0
Mesocosm	0	0
Residual	1.002	1.001

Number of observations: 134.

Number of groups: Channel : Mesocosm: 12, Mesocosm: 4.

Fixed effects	ß	SE	t-value	p-value
Intercept	6.7666	0.3566	18.975	<2e-16 ***
pH circumneutral	-0.8474	0.3031	-2.796	0.00518 **
1 m downstream	0.1055	0.4256	0.248	0.80416
3 m downstream	0.5818	0.4350	1.337	0.18108
7 m downstream	0.5043	0.4290	1.175	0.23980

df residual = 126

b) Leaf-dwelling macroinvertebrates

Random effects	Variance	SD
Channel:Mesocosm	0	0
Mesocosm	0	0
Residual	0.7876	0.8875

Number of observations: 136.

Number of groups: Channel : Mesocosm: 12, Mesocosm: 4.

Fixed effects	ß	SE	t-value	p-value
Intercept	1.13236	0.27630	4.098	4.16e-05 ***
pH circumneutral	0.39422	0.24995	1.577	0.115
1 m downstream	-0.03968	0.35245	-0.113	0.910
3 m downstream	-0.32820	0.35693	-0.920	0.358
7 m downstream	-0.42917	0.35288	-1.216	0.224

df residual = 128

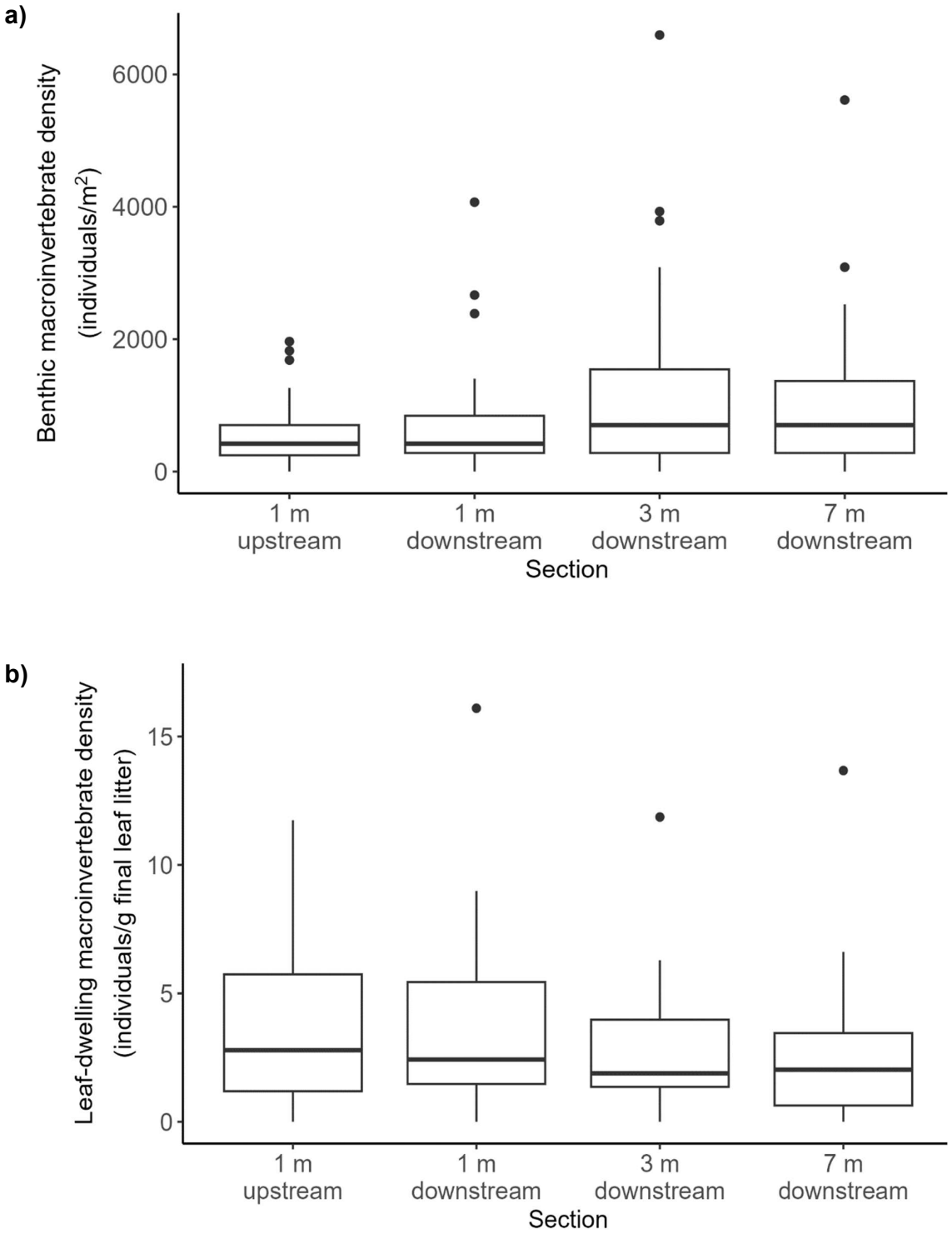


Figure 6.1 Effect of microfibre addition (1 m upstream versus 1 m, 3 m, and 7 m downstream of spiking) on macroinvertebrate density of **a)** benthic (individuals/m^2) and **b)** leaf-dwelling (individuals/leaf pack) communities. GLMMs showed no significant difference between downstream and upstream mesocosm sections.

Community composition

Macroinvertebrates collected by Hess sampling and leaf packs belonged to 26 families dominated by leuctrid Plecoptera (42.1% of all individuals; shredders), followed by chironomid (10.2%; gathering collectors and shredders), simuliid (8.2%; filtering collectors), and Thaumaleid (6%; shredder) dipterans. Community type (benthic or leaf-dwelling) and FFG were significantly associated (Cochran-Mantel-Haenszel test: $\chi^2(4, N = 1{,}934) = 194.39$, $p < 0.001$ after controlling for pH), with gathering collector families being more dominant in leaf packs (22.7% of individuals) than in the benthos (6.9% of individuals), largely because of chironomids and nemourids in the former (Table C1). A two-way PERMANOVA confirmed significant differences in both benthic and leaf-dwelling communities with pH (Table 6.2; Figure 6.3), mainly due to greater Leuctridae dominance in acidic (60.3% of benthic individuals and 42.8% of leaf-dwelling individuals) than circumneutral mesocosms (23.5% of benthic individuals and 28.7% of leaf-dwelling individuals), while Heptageniidae (grazers/scrapers) were only dominant in benthic communities in circumneutral mesocosms (34.2-36.7% of individuals) (Table 6.2, Table C1). Simuliidae (filtering collectors) were most dominant in acidic Hanwell mesocosm (34.9% of individuals) (Table C1). Other than these variations, however, microfibre addition had no effect on community composition (Table 6.2).

Despite some variations in community composition, there were few variations in diversity, and none involved microfibre addition ($p > 0.05$; Table 6.3). Shannon's Index per section ranged from 0 – 1.55 for benthic macroinvertebrates and 0 – 2.02 for leaf-dwelling macroinvertebrates. There was no difference between acidic and circumneutral mesocosms ($p > 0.05$; Table 6.3), although the effect of pH on leaf-dwelling communities was almost significant ($p = 0.063$; Table 6.3b), reflecting higher values in circumneutral (1 ± 0.527) than acidic mesocosms (0.595 ± 0.451). Marginal R^2 values for the lognormal distribution were 0.0033 and 0.0533 for the benthic and leaf-dwelling community model, respectively. Both models had a singular fit and thus, conditional R^2 values equalled marginal R^2 values.

Table 6.2 Results of two-way PERMANOVA and SIMPER test based on macroinvertebrate community (benthic versus leaf-dwelling, represented below as type), at varying distances from microfibre addition (1 m upstream versus 1 m, 3 m, and 7 m downstream of spiking, represented below as section), across two acidic and two circumneutral mesocosms (represented below as pH). For SIMPER test, only taxa with contributions higher than 2% are reported. df = degrees of freedom, SS = sums of squares. Colon indicates interaction. Stars indicate levels of statistical significance: p-value < 0.05 (*), p-value < 0.01 (**), p-value < 0.001 (***).

Two-way PERMANOVA

Source	df	SS	R^2	*F*-value	*p*-value
Type	1	5.321	0.225	30.795	0.001 ***
pH	1	1.594	0.068	9.224	0.001 ***
Section	3	0.382	0.016	0.737	0.802
Type:pH	1	1.465	0.062	8.481	0.001 ***
pH:Section	3	0.235	0.010	0.453	0.987
Type:Section	3	0.368	0.016	0.710	0.833
Type:pH:section	3	0.426	0.018	0.823	0.698
Residual	80	13.822	0.585		
Total	95	23.613	1.000		

SIMPER

Taxon	Average dissimilarity (± 1 SD) %	Contribution of variability %	Cumulative %
Leuctridae	17.43 ± 12.56	27.9	27.9
Chironomidae	7.69 ± 4.51	12.3	40.2
Heptageniidae	6.97 ± 7.58	11.1	51.3
Simuliidae	5.26 ± 5.14	8.4	59.7
Nemouridae	5.20 ± 3.22	8.3	68
Thaumaleidae	4.56 ± 2.56	7.3	75.3
Baetidae	2.69 ± 2.29	4.3	79.6
Scirtidae	1.76 ± 1.89	2.8	82.4
Lymnaeidae	1.58 ± 1.78	2.5	84.9
Oligochaeta	1.43 ± 0.87	2.3	87.2
Pediciidae	1.32 ± 1.02	2.1	89.3
Rhyacophilidae	1.22 ± 1	2	91.3

Table 6.3 Effect of microfibre addition (1 m upstream versus 1 m, 3 m, and 7 m downstream of spiking) and pH (acid versus circumneutral) on Shannon's Index of **a)** benthic (individual/m^2) and **b)** leaf-dwelling macroinvertebrate communities (individuals/g final leaf litter), accounting for random effects within channels nested within mesocosms. Colon indicates interaction. SD = standard deviation, ß = estimate, SE = standard error, and df = degrees of freedom.

a) Benthic macroinvertebrates

Random effects	**Variance**	**SD**
Channel:Mesocosm	1.177e-07	0.0003431
Mesocosm	1.316e-07	0.0003628

Number of observations: 141.

Number of groups: Channel : Mesocosm: 12, Mesocosm: 4.

Fixed effects	**ß**	**SE**	**z-value**	**p-value**
Intercept	-0.49297	0.32993	-1.494	0.135
pH circumneutral	-0.02625	0.29618	-0.089	0.929
1 m downstream	-0.01532	0.41715	-0.037	0.971
3 m downstream	0.06040	0.41711	0.145	0.885
7 m downstream	0.15680	0.41706	0.376	0.707

df residual = 132

b) Leaf-dwelling macroinvertebrates

Random effects	Variance	SD
Channel:Mesocosm	1.125e-07	0.0003355
Mesocosm	1.125e-07	0.0003355

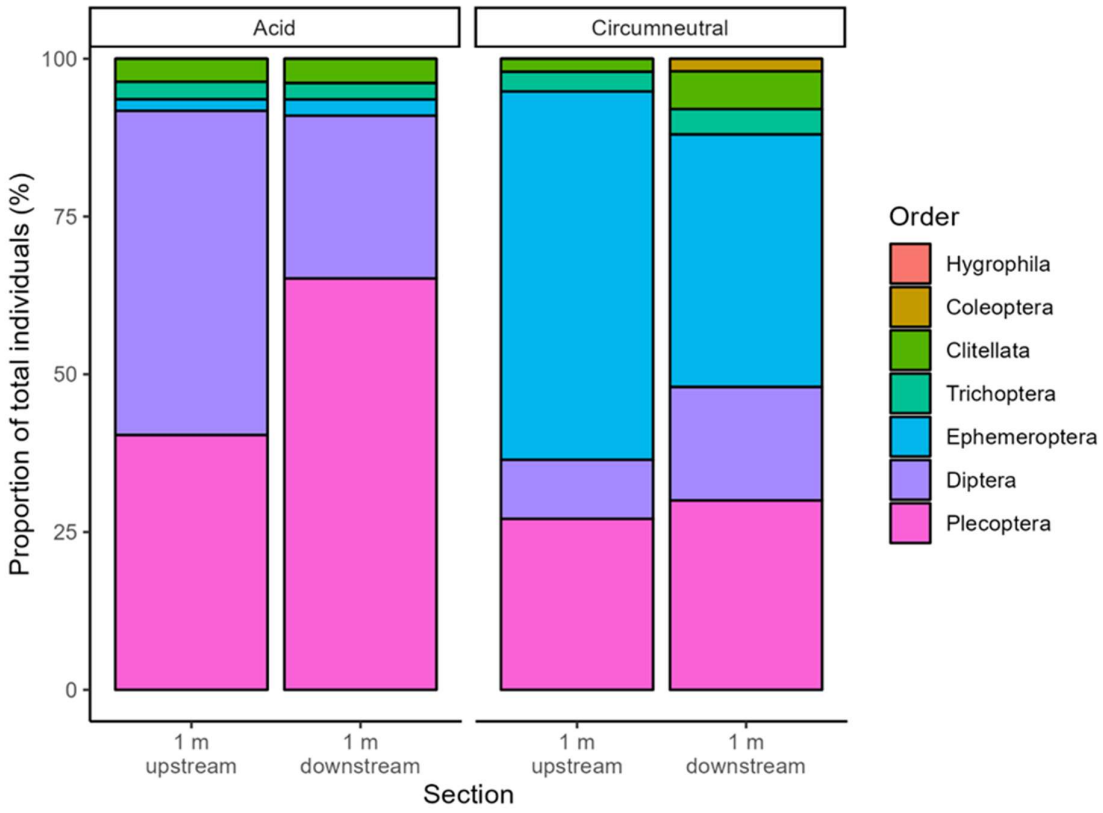
Number of observations: 133.

Number of groups: Channel : Mesocosm: 12, Mesocosm: 4.

Fixed effects	ß	SE	z-value	p-value
Intercept	-0.4460	0.3128	-1.43	0.154
pH circumneutral	0.5221	0.2811	1.86	0.063 .
1 m downstream	-0.0499	0.3895	-0.13	0.898
3 m downstream	-0.1277	0.3979	-0.32	0.748
7 m downstream	-0.1338	0.3980	-0.34	0.737

df residual = 126

a) Benthic macroinvertebrates



b) Leaf-dwelling macroinvertebrates

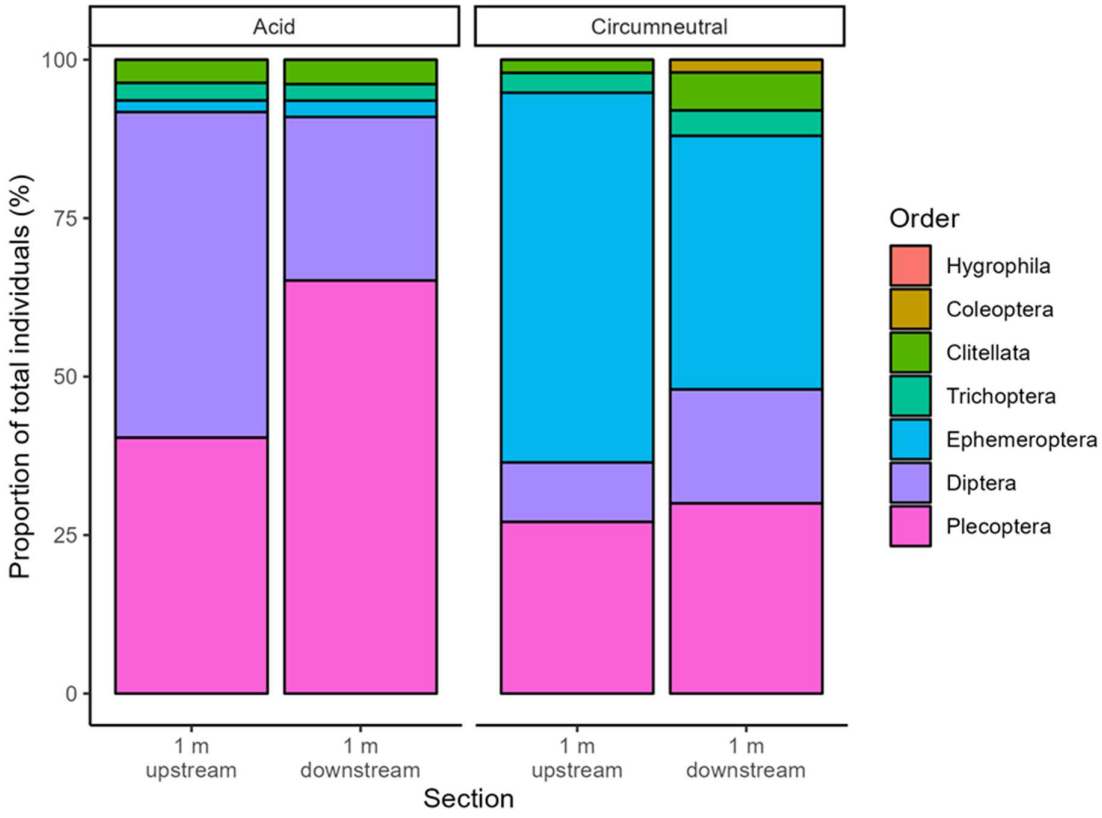


Figure 6.3 Community composition of **a)** benthic and **b)** leaf-dwelling macroinvertebrate communities 1 m upstream and 1 m downstream of microfibres addition, in acidic and circumneutral mesocosms.

6.4.2 Leaf litter

Microplastic adherence

Only one fluorescent microfibre was observed in all leaf litter samples, which was from acidic Sidaway mesocosm, 1 m downstream of microfibre addition.

Decomposition rate

Overall, mean leaf litter decomposition rate was 24.5 ± 5.9 mg/day (± 1 SD). Leaf litter packs lost 35.6-96% of original mass in 48 days, with a mean ± 1 SD loss of 1,177.8 ± 284.8 mg. Decomposition rate was more strongly affected by density of macroinvertebrates in leaf packs compared to the benthos. Decomposition was significantly faster with more leaf-dwelling individuals (Kendall's Tau: $\tau = 0.314$, $Z = 5.364$, $n = 138$, $p < 0.001$), which in turn was positively correlated with both their shredder density ($\tau = 0.611$, $Z = 10.118$, $n = 138$, $p < 0.001$) and family diversity (Shannon's Index) ($\tau = 0.467$, $Z = 7.523$, $n = 128$, $p < 0.001$). Leaf litter decomposition rate did not correlate with benthic macroinvertebrate density ($\tau = 0.063$, $Z = 1.034$, $n = 134$, $p = 0.301$). Otherwise, there was no effect of either microfibre addition or pH.

A GLMM suggested leaf litter degradation rate was 0.3 ± 0.2 mg (± standard error (SE)) lower per day at 1 m downstream of microfibre addition compared to 1 m upstream of addition, but this effect was not statistically significant ($p = 0.142$; Table 6.4; Figure 6.4). Decomposition rate 3 m and 7 m downstream of microfibre addition did not differ significantly to rates observed 1 m upstream ($p > 0.05$; Figure 6.4). Decomposition rate was not significantly affected by pH ($p > 0.05$; Figure 6.4). Overall mean ± 1 SD variability in leaf litter decomposition rate was 0.3 ± 0.6 mg/day between channels and 0.1 ± 0.3 between mesocosms. The model's marginal and conditional R^2 values were 0.0955 and 0.5008, respectively.

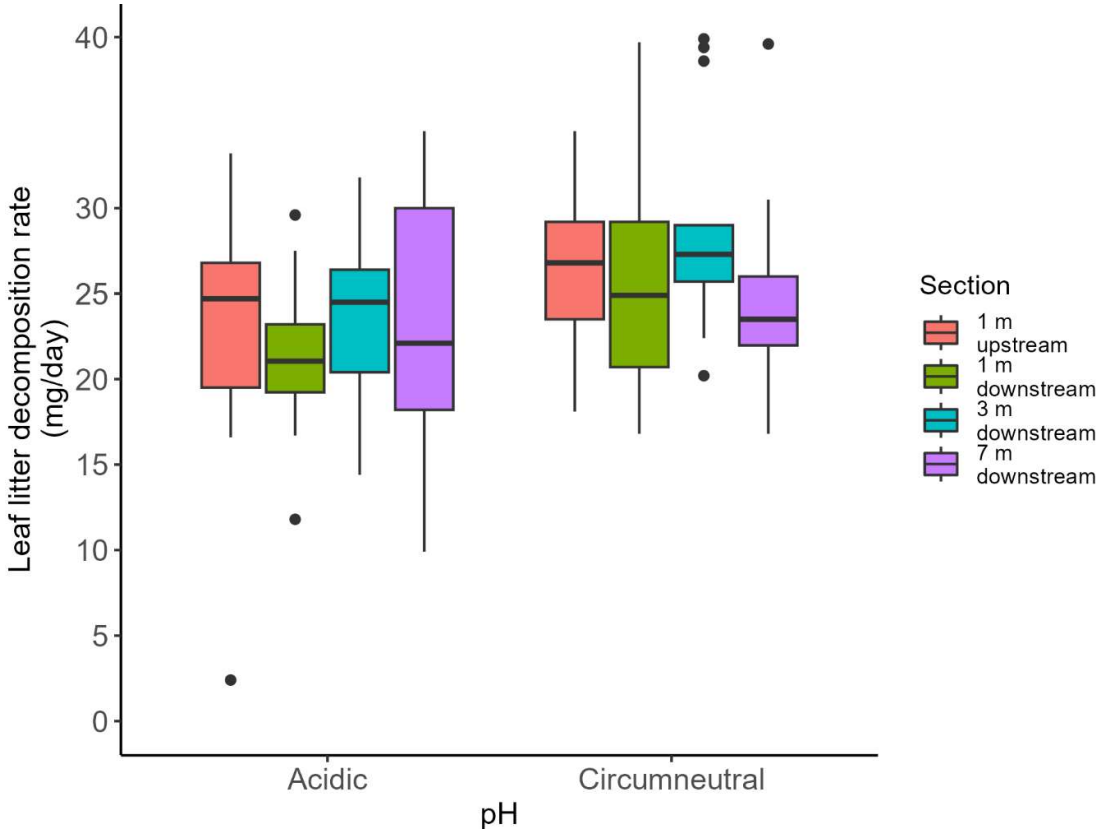


Figure 6.2 Effect of microfibre addition (1 m upstream versus 1 m, 3 m, and 7 m downstream of spiking) on leaf litter decomposition rate (mg/day).

Table 6.4 Effect of microfibre addition (1 m upstream versus 1 m, 3 m, and 7 m downstream of spiking) and pH (acid versus circumneutral) on leaf litter decomposition rate, accounting for random effects within channels nested within mesocosms. Colon indicates interaction. SD = standard deviation, ß = estimate, SE = standard error, and df = degrees of freedom.

Random effects	Variance	SD
Channel:Mesocosm	0.3647	0.6039
Mesocosm	0.07894	0.2810
Residual	0.54644	0.7392

Number of observations: 136.

Number of groups: Channel : Mesocosm: 12, Mesocosm: 4.

Fixed effects	ß	SE	Df	t-value	p-value
Intercept	-0.1972	0.3470	2.4663	-0.568	0.617
pH circumneutral	0.5406	0.4655	1.9958	1.161	0.366
1 m downstream	-0.2638	0.1783	121.199	-1.479	0.142
3 m downstream	0.1557	0.1797	121.2373	0.867	0.388
7 m downstream	-0.2279	-/1812	121.2646	-1.258	0.211

6.5 Discussion

Pulse-injection of PA microfibres into freshwater mesocosms did not induce changes at the population (macroinvertebrate density), community (diversity, abundance of different feeding guilds), nor ecosystem levels (leaf litter decomposition), falsifying hypothesis 3. However, recovery of added microfibres was extremely limited in macroinvertebrates and non-existent in leaf litter, refuting hypothesis 1. Greater macroinvertebrate density in leaf litter led to faster decomposition rates, supporting hypothesis 2. Nevertheless, as pH only affected benthic community density (greater in acid versus circumneutral mesocosms), decomposition rate did not vary with pH, refuting hypothesis 4. The sections that

follow first describe macroinvertebrate community composition in the mesocosms benthos and leaf litter. Next, reasons for negligible microfibre recovery and effects on measured responses are discussed, before discussing the observed influence of pH.

6.5.1 Macroinvertebrate communities

Community composition of macroinvertebrates in the mesocosms is in line with temperate freshwater environments (Croft 1986; Vaughan and Ormerod 2012). Overall, 25 invertebrate families were observed, belonging to six taxonomic orders. Shredders made up over half of sampled macroinvertebrates (61% and 56% of benthic and leaf-dwelling communities, respectively), dominated by Leuctridae, Nemouridae, and Thaumaleidae (Table C1). Remaining individuals were filtering-collectors (12-19%), gathering-collectors (7-23%), scrapers (4-11%), and predators (3-5%) (Table C1).

Differences highlighted by PERMANOVA and SIMPER analysis are likely related to the feeding habit and acid-sensitivity of different families. Leuctridae was the most abundant family in all mesocosms, accounting for 49% and 34% of total benthic and leaf-dwelling communities, respectively. Although this does not meet the 80% coverage of total litter-dwelling invertebrates observed in mesocosm feeder streams reported by Johnston et al. (2015), it conforms with their observed dominance. This likely results from the generalist feeding habit of Leuctridae, ingesting organic matter, algae, fungi, and bacteria (Feminella and Stewart 1986), and their acid tolerance (Braukmann 2001). However, this contradicts the greater dominance of plecopteran shredders in litter bags compared to benthic samples observed previously in both circumneutral and acidic streams in Llyn Brianne (Pye et al. 2012).

Chironomidae larvae were more dominant in leaf-dwelling (22%) compared to benthic communities (1%), with Bertoli et al. (2023a) also reporting high Chironomidae abundance in leaf packs in Vipacco River, Italy. Despite variation in their FFG (Stout and Taft 1985; Oertli 1993; Callisto et al. 2007), larvae mainly feed on organic material and thus, would be attracted to the leaf presence (Grubbs et al. 1995; Mathuriau and Chauvet 2002; Ligeiro et al. 2010). Conversely, Simuliidae larvae were more dominant in benthic communities (13%) compared to leaf-dwellers

(2%), likely due to their habit of filtering suspended organic particulates through labral fans and attachment to substratum by posterior hooks (Hemphill and Cooper 1983; Zhang and Malmqvist 1997). The presence of Leuctridae, Chironomidae, and Simuliidae in both acid and circumneutral sites conforms to their previously reported acid tolerance (Ormerod and Durance 2009).

Lastly, Heptageniidae larvae were mostly absent from acidic mesocosms, supporting evidence of their acid-sensitivity (Courtney and Clements 1998; Braukmann 2001; Kowalik and Ormerod 2006). In circumneutral conditions, their greater dominance in benthic (36%) compared to leaf-dwelling communities (1%) may be due to their feeding habit of grazing-scraping periphyton (Cummins and Klug 1979), which grows on coarse sediment. Pye et al. (2012) similarly observed depleted grazer-scraper dominance in leaf packs in both circumneutral streams in Llyn Brianne, compared to benthic samples. As with other leaf pack studies, the presence of non-shredding taxa in leaf-dwelling communities could be due to the refugia they provide (Richardson 1992; Mutshekwa et al. 2020), whilst the presence of predators could be related to the high abundance of prey within leaf packs (Karádi-Kovács et al. 2015).

6.5.2 Effect of microplastic

The negligible recovery of added PA microfibres from mesocosms falsifies adherence to leaf litter and subsequent ingestion by macroinvertebrates that was suggested by microcosm experiments (Straub et al. 2017; Redondo-Hasselerharm et al. 2018; Weber et al. 2018; López-Rojo et al. 2020). These studies created a ubiquitous microplastic presence contained within indoor lentic conditions, similar to and exceeding environmental concentrations. Bertoli et al. (2023a,c) reported microfibre adherence to leaf litter in the lotic Vipacco River, but observed lower concentrations in leaf packs at times of high flow. Moreover, high flow regimes are often associated with reduced microplastic pollution in freshwaters (*Chapter 2*; Zhao et al. 2014; Wang et al. 2017a; Peng et al. 2018; Pol et al. 2022). With flow velocity of mesocosms in this study being ~0.1 m/s, it is likely that pulse-injected microfibres were mobilised and removed before adherence to leaves and macroinvertebrate ingestion. Microfibres may have also remained on the water surface due to surface

tension, preventing their interaction with leaf packs and invertebrates. Addition of small amounts of tween (detergent) to the spiking solution, would have broken this surface tension to aid suspension. Sampling the outflow of mesocosms would have indicated the degree of microfibre mobilisation and loss. Despite this, fluorescent microfibres were recovered from three Leuctridae and two Heptageniidae individuals, supporting evidence of microplastic uptake by freshwater macroinvertebrates, especially microfibres (e.g., Windsor et al. 2019b; Stankovic et al. 2024; *Chapter 4*). Egestion of microplastic has also been reported in literature (Cole et al. 2013; Blarer and Burkhardt-Holm 2016; López-Rojo et al. 2020), with Windsor et al. (2019b) and Khedre et al. (2023) observing an almost 50% reduction in average microplastic abundance within 24 hours. In this study, samples were collected six days after the last microfibre addition, by which time, microfibres may have been completely egested from macroinvertebrates, limiting their recovery.

Other artefacts of experimental design may have also prevented microfibre recovery. Any microfibres that did adhere to leaves or were ingested by macroinvertebrates, could have been lost during sample processing through adherence to samples and/or equipment or settlement in precipitate of centrifuged samples (Nakajima et al. 2019). As mentioned in *Chapter 5*, microfibres 1.1 µm in diameter could have passed through the 63 µm sieve, or stay adhered to the sieve due to electrostatic attraction, capillary action, and/or cohesive forces increasing surface tension of water. This may disproportionately reduce recovery rates of smaller microplastic particles (Enders et al. 2020) and fibres due to the higher surface area-to-volume ratio increasing their electrostatic attraction. Another artefact is the potential loss of Nile Red stain or its fluorescence from spiking microfibres as suggested in *Chapter 5*, after recovering non-fluorescent microfibres from leaf litter spiked with Nile Red stained PA microfibres. Ma et al. (2021) reported dye leaching from polyethylene (PE) fibres after centrifugation with 80 rpm at 65 °C. This would prevent visualisation under detection methods, leading to false negatives or data inconsistencies if some fibres retained dye better than others. However, the Nile Red used in *Chapter 5* was one year older than that used in this study, leaving time for the dye to degrade. Moreover, Gao et al. (2022) found strong fluorescence of Nile Red dyed microplastics incubated in freshwater at room temperature, suggesting dye leaching in mesocosms is unlikely. Lastly, a layer of sediment remained on filters of

leaf litter rinsate that was not removed through density separation and centrifugation, and exoskeletons remained on filters due to incomplete digestion. This could have covered microfibres and prevented visual observation of their fluorescence. Adding further digestion steps such as enzymes, could have prevented this limitation, but required specific equipment that was not available.

The mobilisation of microfibres and/or methodological limitations to their recovery are the most plausible reasons for their absent effect on macroinvertebrate abundance, diversity, community composition, and leaf litter decomposition. This prevented the investigation of population or ecosystem effects in real-world lotic systems. In corroboration, Silva et al. (2022) attributed an absent effect of short-term PE particle exposure on leaf litter decomposition in an indoor artificial stream, to an absent change in shredder abundance. Redondo-Hasselerharm et al. (2020) and Stanković et al. (2022) also reported no significant changes in freshwater macroinvertebrate abundance, species richness, and diversity with microplastic exposure, and a meta-analysis by Ockenden et al. (2021) found mortality to be the least responsive effect of microplastic in most freshwater functional groups. In contrast, Borges et al. (2024) observed greater macroinvertebrate density and richness with increased microplastic concentration, and subsequent increased leaf litter decomposition. However, this opposite effect was attributed to large 200-600 µm PE microspheres serving as substrate for biofilm growth and thus, promoting resource availability for invertebrates (Borges et al. 2024), which were far larger than the 1,000 µm x 1.1 µm PA microfibres used in this study. Lastly, in this open mesocosm, potential species dispersal from upstream of microfibre addition to downstream may have compensated any alteration in population or ecosystem level effects (Medina Madariaga et al. 2024).

6.5.3 Effect of pH

The greater density of benthic macroinvertebrates observed in acidic mesocosms and absent change in macroinvertebrate diversity across the pH range, contrasts with negative impacts of acidification on freshwater organisms widely reported in literature (e.g., Townsend et al. 1983; Morris 1989; Courtney and Clements 1998). Acidic conditions are said to be stressful for many organisms,

limiting the number of species that can survive there. Even within acidic streams adjacent to mesocosms, significantly lower invertebrate abundance and family richness and different community compositions have been observed compared to adjacent circumneutral streams (Ormerod and Durance 2009; Pye et al. 2012; Johnston et al. 2015). Dominance of acid-tolerant Leuctridae in both communities suggests they were able to survive in large numbers in acidic conditions due to reduced competition. Moreover, acidic conditions support greater algal growth (Hendrey 1976; Sholar et al. 2015), which was visually observed in the mesocosms. This could have heightened benthic macroinvertebrate density due to a more abundant food source, as well as limited leaf litter colonisation due to blockage of leaf pack apertures. Lastly, the 0.8-1.45 pH unit difference in mesocosm pH may not have been large enough to elicit significant differences in measured responses, despite them falling within the range used by (Ward Jr. 1963) to classify acid-tolerant and sensitive aquatic invertebrates.

The observed increase in leaf decomposition rate with leaf-dwelling macroinvertebrate density and concurrent shredder density, conforms with literature (Benfield and Webster 1985; van Dokkum et al. 2002; Hieber and Gessner 2002; Cornut et al. 2010; Raposeiro et al. 2017; Bertoli et al. 2020; Bertoli et al. 2022a; Bertoli et al. 2023b). Shredders play a major role in leaf degradation, with increases in their density leading to increased leaf litter decomposition (Taylor and Chauvet 2014). Therefore, perturbation in their abundance due to environmental stressors are likely to impact leaf degradation. However, despite benthic community density being greater in acidic mesocosms, leaf-dweller density was no different to circumneutral mesocosms, resulting in similar decomposition rates. These results contrast with multiple studies who observed reduced decomposition with acidification, attributed to reduced shredder invertebrate abundance, biomass and feeding activity (Meegan et al. 1996; Dangles and Guérold 1998; Dangles and Guérold 2001a; Dangles and Guérold 2001b; Simon et al. 2009; Cornut et al. 2012; Ferreira and Guérold 2017). Artefacts of the experimental design may have limited leaf litter decomposition rate and its response to microplastic and pH. For example, Pye et al. (2012) highlight potential decomposition retardation of Oak leaves due to their high polyphenolic content and thus, suggest the examination of multiple species over longer timescales to strengthen our understanding of this ecosystem function effect. Moreover, the

45-day treatment design is short when considering the extended timeframe required for leaf decomposition in freshwater streams, with reported breakdown duration of oak leaves to range from 54 to 255 days (Abelho 2001).

Overall, microplastic and pH treatment only explained 11-26% and 0.3-5% of variance in macroinvertebrate density and diversity, respectively, according to R^2 values. When additionally accounting for sample location within mesocosms and their channels, explanation of variance in leaf litter decomposition rate increased from 10% to 50%. This suggests that other stressors varying across channels and mesocosms either directly influenced or indirectly influenced decomposition rates by affecting detritivores. In the month prior to this experiment, a preceding experiment exposed mesocosm channels to different drought treatments (100%, 50%, or 10% flow). Moreover, apparatus issues during this experiment caused short-term drying and re-wetting of different channels. The negative impact of drought on freshwater ecosystems is well understood, broadly reducing macroinvertebrate abundance and diversity (e.g., Boulton 2003; García and Pardo 2016; Aspin et al. 2019) and leaf litter degradation (Schlief and Mutz 2011; Ferreira et al. 2023). Recovery after drought can be rapid for species that possess strategies to survive drying or are highly mobile, but other taxa take longer to recolonise. Ephemeroptera, Plecoptera, and Trichoptera have been reported as drought-sensitive in temperate climates (King et al. 2016; Storey 2016; Doretto et al. 2018), whereas Diptera may be more drought-tolerant (King et al. 2016). This may have had an overriding impact on macroinvertebrate communities and subsequent leaf litter degradation.

Mesocosm channels may have also experienced different shading regimes due to surrounding vegetation growth, which could have influenced water temperature and dissolved oxygen availability. In ectothermic organisms, metabolic turnover increases with temperature (Hochachka and Somero 2002; Brown et al. 2004; Rezende and Bozinovic 2019) and dissolved oxygen availability (Winter et al. 1996; Lowell and Culp 1999; Connolly et al. 2004). This results in elevated feeding and ventilation rates and greater sensitivity to microplastics (e.g., Jaikumar et al. 2018; Chang et al. 2022; Na et al. 2023; Sanpradit et al. 2024). The could have been controlled for by maintaining the area around mesocosms. However, environmental variables occurring at wider scales, e.g., rain evets and heat waves, change for all mesocosms simultaneously, and simulating complex ecological interactions and

processes that occur in natural environments provides a more realistic setting and result than laboratory experiments.

6.6 Conclusion

This study provides novel evidence to suggest negligible adherence of environmentally relevant pulse-injections of microplastic to leaf litter in lotic freshwater ecosystems, leading to no effect on macroinvertebrate populations and decomposition of leaf litter. Moreover, pH mediated changes in benthic invertebrate abundance and community composition may not always alter leaf litter breakdown when multiple stressors are at play. This does not support reduction in efforts to reduce environmental microplastic, as the complexity of microplastic particle shape, size, density, and sorbed chemicals was not captured in this study and requires further investigation, whilst environmental concentrations of microplastic are likely to increase. A priority for future research is to determine the individual and population level effects of microplastic at environmentally relevant concentration, to understand potential ecosystem function impairment relative to or in addition to other contaminants and stressors.

Chapter 7: General Conclusion

7.1 Overview

This thesis aimed to improve understanding of the sources, fate, and emergent ecological effects of microplastic pollution in freshwater ecosystems, and to review practical methods for extracting, characterising, and quantifying microplastics from freshwater media. In testing two overarching hypotheses about biological exposure and methodological influences on their assessment, the work aimed for an unusual and unique perspective on pattern and process at a wide range of scales to improve risk assessments for freshwater ecosystems generally. Using a combination of empirical and literature-based assessments, this thesis highlights the challenges of researching such a diverse class of pollutants under the dynamic conditions in freshwater ecosystems. The work also underscores ongoing difficulties in extracting microplastics from complex environmental media and accurately estimating environmental loads and relevant impacts. Whilst the findings highlight the extent of microplastic pollution in freshwaters around the globe, analysis suggests that apparent global patterns do not appear to represent universal patterns. Instead, identified patchiness at all addressed spatial scales highlights the need for continued research into microplastic sources, distribution patterns, behaviour in relation to hydrological dynamism, and interactions with individual organisms, communities, and ecological functions. For example, limited biological effects after short-term point-source spills of microplastic appear to reveal that adverse effects are not inevitable, while other stressors could potentially be more important.

This final chapter summarises the main findings within each chapter of the thesis and synthesises interconnections between them to come to an overall view of what has been achieved, what more is needed, and what practical applications might arise. Caveats and assumptions that affect the interpretation of thesis results are also discussed.

7.2 Main findings

In *Chapter 2*, despite the identified patchy spatial distribution in freshwater microplastic quantification around the globe and skew towards the global north, the

data reviewed found greater microplastic loads in all environmental matrices associated with urban areas as well as in less developed countries. This trend was greatly influenced by human population density, suggesting that plastic usage and waste disposal are substantial sources of environmental microplastic pollution. However, this pattern does not always hold true due to the influence that other microplastic sources and hydrodynamics play on site-specific plastic fluxes, fate, or distribution. Chapter analysis revealed contrasting phenomena associated with changes in water flow over both space and time, across their hierarchy of scales and axes. High flows are either reported to increase or decrease microplastic transport and concentration in different freshwater compartments, due to mechanisms including resuspension, sedimentation, dispersal, and dilution. This further complicates patterns associated with hydrological events, with evidence of microplastic loads changing within hours after flooding and high rainfall events. Multiple putative explanations for these differences provided in this chapter, highlight how microplastic transport is unique in each freshwater system, emphasising the need for site-specific research to bolster local risk assessment and regulation.

Another key outcome of this meta-analysis was identification of the research gap in microplastic contamination of freshwater biota, especially lower trophic level communities that support freshwater food webs. Reviewed microplastic loads in organisms generally followed seasonal trends seen in surface water and sediment, yet risk assessment for individuals, populations, and communities is cofounded by a myriad of biotic factors that influence microplastic uptake. This limits our understanding of effects of microplastic on individuals, food-web transfer, community interactions, and potential effects on ecosystem processes, especially as most effect studies have been performed on single species with environmentally irrelevant microplastic concentrations.

The global variation in reported freshwater microplastic concentrations, ranging over 11, 6, and 3 orders of magnitude in surface waters, organisms, and sediment respectively, and inconsistent evidence for spatio-temporal trends identified in *Chapter 2*, adds to widely reported concerns about the standardisation of methods and reporting units in microplastic research. *Chapter 3* identified three microplastic extraction techniques used for freshwater media: volume-reduction, bulk, and selective sampling. Volume-reduction sampling of surface water, the most commonly

sampled freshwater media, was cofounded by complex interactions between an observed array of net dimensions and apertures, and site- and time-specific water quality and flow velocity. Analysis revealed this may lead to under- or over-estimation of environmental microplastic loads and limits comparison both within and between studies. Bulk-sampling was therefore recommended, as subsequent microplastic extraction techniques are easier to standardise when performed *ex-situ*.

Chapter 3 also synthesised the four techniques of microplastic extraction from sampled freshwater media - sieving, density separation, digestion, and filtration - each varying in the way they are implemented. This gave rise to recommendations aiming at harmonising future microplastic research. For example, fine-mesh and multiple stacked sieves were most commonly used for sieving media, but aperture needs to be standardised to enable comparison between studies. Density separation was important in microplastic extraction from sediment, with solutions greater than 1.4 g/cm^3 ideally needed to extract all particle types. Chemical digestion was used to remove organic matter, with hydrogen peroxide being most popular. Finally, filtration of subsequent residues was most commonly performed with glass fibre filters to avoid polymer contamination and interactions with chemicals, with 0.45 µm concluded as the recommended pore size.

Synthesis also led to production of standardised contamination control measures, and criteria for microplastic and particle shape classification. This followed identification of the profusion of unique terms used to describe particle characteristics, polymer types, and concentration reporting units. Particles per unit volume was established as the most appropriate reporting unit for standardisation as (i) environmental media are 3D, (ii) volume is a consistent measure, (iii) it removes factors influencing sediment mass, such as water content, and (iv) it accounts for the effect of sediment grain size on microplastic retention. Due to recognised human error during visual identification and difficulty in standardising methods when working with different media, polymer analysis of particles and recovery tests were recommended as standard practice, despite their limited use in reviewed studies. The effects of identified variations in sampling, processing, quantifying, and characterising microplastics in freshwater, raise questions about: (i) the representativeness of studies carried out so far; and (ii) the limitations in spatio-temporal trends identified by meta-analysis in this thesis and elsewhere.

Chapter 4 built on this observed inconsistency in freshwater microplastic distribution and need to investigate microplastic in freshwater sediment and biota. To this point, systematic evaluation of microplastic pollution in sediment and aquatic invertebrates across entire river catchments were extremely scarce, and the River Taff offered an important model river with both rural and urban land-use. Microplastic contamination of sediment was widespread across the River Taff catchment, indicative of its role as a microplastic sink, but no trends were observed with land-use nor river flow velocity. The observed patchy distribution suggests diversity in microplastic sources within this catchment, which do not accumulate into longitudinal trends at the catchment-scale. This may relate to complex suspension and deposition cycles of microplastic within fluvial systems, influenced by site-specific hydrodynamics and particle characteristics. Results, therefore, underscore the multifaceted nature of microplastic pollution and need for individual risk assessments of unique freshwater systems. Microplastic concentrations in River Taff sediment were comparable to those in other freshwater systems within UK, but were lower than the amounts obtained using cylinder resuspension technique, highlighting methodological variations discussed in *Chapter 3*. With transparent fibres and synthetic cellulose dominating extracted particles, textiles in wastewater were predicted to be the major microplastic source in the River Taff catchment.

Microplastic uptake by aquatic invertebrates in River Taff was limited to 5% of individuals, constraining investigation into potential links to feeding guild or individual biomass. Analysis suggests substantial variation in contamination of Welsh freshwater invertebrates between this study and historic estimates, may be attributed to methodological differences including more accurate identification of false positives in this work, site selection, or differences in hydrological conditions between sampling events. Synthesis reveals little association between microplastic in River Taff sediment and invertebrates, indicating sampled families are not reliable bioindicators of microplastic in this freshwater system.

Turning to the gaps in investigating different freshwater media identified in *Chapter 2* and methodological issues highlighted in *Chapter 3*, *Chapter 5* investigated potential protocols for extracting microplastics from leaf litter decomposing in freshwater. To address the challenge of removing fixing agents from field samples prior to the addition of processing reagents, evaporation tests were

apparently non-viable. Sieving was therefore recommended for reagent removal, as used in *Chapter 4*. The aim to quantitatively verify and validate extraction methods by assessing recovery rate of fluorescent microfibres could not be fulfilled, as only non-fluorescent microfibres were recovered. This suggests that previous studies focussing only on fluorescent microplastic recovery may underestimate recovery rates, underrating estimated pollution severity. Despite the lack of recovery rate quantification, recovery of non-fluorescent microfibres was visibly reduced with the additional digestion step, suggesting density separation alone was the optimal method for extracting microplastics from leaf litter.

Chapters 2 and *4* revealed widespread distributions of microplastic in freshwater river catchments. However, identified patchy uptake by aquatic invertebrates underscores the challenges in assessing microplastic sources across broad spatial scales. Lack of environmental realism in microplastic effect studies is, therefore, a concern in ecotoxicology and impairs risk assessment across all levels of biological organisation. *Chapter 4* also showed differences in microplastic presence, concentration, and characteristics between benthic invertebrates and surrounding sediment across the River Taff catchment, suggesting the families studied ingest microplastic from alternative habitats. Utilising methods developed in *Chapter 5*, *Chapter 6* investigated immobilisation of point source microplastics in leaf litter submerged in streams and potential effects on aquatic invertebrates at various levels of biological organisation, across contrasting pH. Negligible recovery of pulse-injected PA microfibres from mesocosms falsifies adherence to leaf litter, with subsequent limited ingestion by macroinvertebrates suggesting particle mobilisation in streams. This may be explained by potential rapid loss of microplastic in flow water and lack of suspension of microfibres, preventing microplastic exposure. Microplastic, therefore, did not induce changes at the population (invertebrate density), community (diversity, abundance of different feeding guilds), nor ecosystem levels (leaf litter decomposition). pH had a greater influence on community composition compared to microfibre addition, highlighting a need to include multiple stressors in microplastic research. Greater densities of leaf-dwelling invertebrates resulted in faster leaf breakdown, but pH only affected benthic community density and thus, decomposition rate did not vary with pH. Best-fitting statistical models

poorly explained variance in data, suggesting that other stressors varying across mesocosms influenced measured response variables.

7.3 Research design and potential caveats

As with all ecological studies, the findings and outcomes of this thesis are affected by caveats and assumptions that were outlined in individual chapters. Here, however, broader caveats are acknowledged. Firstly, meta-analysis conducted at the start of this thesis reviewed articles published prior to September 2022. This cannot be an exhaustive list given the current rates of publication. Over 200 new studies have since been published that would fit into the selection criteria. *Chapters 2* and *3*, therefore, are viewed as a sample of the state of knowledge on freshwater microplastic pollution at the timepoint prior to subsequent primary data chapters, providing context and reasoning for the thesis. There is no reason to believe the sample to be unrepresentative, but developments are very likely to have moved the field on since the review.

Secondly, the field-based nature of work reported in the thesis means that findings are influenced by spatial and temporal variations across a hierarchy of scales, characteristic of dynamic freshwater systems. These include variations in hydrodynamics, habitat structure, biotic communities, as well as microplastic sources within and between freshwater systems and across seasons. This limits comparison between studies and makes it difficult to pinpoint influential variables. The diversity of sample sites and their land uses within the River Taff catchment allows for comparison among locations, whilst using biological sampling ensures some integration of temporal variation. Studying microplastic effects in stream mesocosms created environmental realism, adding ecological value, whilst replication accounted for uncontrollable environmental differences. Similar dynamism comes from microplastic particles themselves. These physical pollutants range in shape, size, polymer type, and chemical additives, resulting in fluxes, fates, and effects that interact with environmental variation. Moreover, non-plastic particles – such as the cellulose that was detected widely in the Taff system - may still pose similar physical, chemical, and biological threats to ecosystems as plastic particles and thus, should be evaluated in future ecotoxicology studies. Using a singular particle type to test

microplastic effect does not represent the complexity of microplastic pollution in the environment, but provides consistency and control in thesis work.

7.4 Regulatory and policy implications

The findings presented in this thesis lead to several important considerations for regulatory organisations and policymakers. Firstly, the acknowledged environmental dynamism and variation in methodological techniques pose substantial challenges for monitoring microplastic pollution. Effective monitoring programs must account for spatial and temporal scales to accurately assess trends. Additionally, the lack of standardised protocols for microplastic sampling and analysis complicates data comparison across different studies and regions. It is, therefore, essential to evaluate and account for methodological differences when estimating environmental loads.

Secondly, despite the low environmental concentrations of microplastics observed in a Welsh riverine catchment and unidentified ecological effect of point pollution sources, policymakers should not dismiss the potential threat of microplastics. Continued monitoring and research are vital to fully understand the long-term impacts of microplastics on freshwater ecosystems, particularly given the potential for microplastics to affect a wider range of biota. Proactive measures should be taken to mitigate microplastic pollution, considering its persistence in the environment and potential to bioaccumulate in organisms, posing significant risks over time.

Integrating microplastic into existing environmental policies will require adaptive management strategies capable of responding to new information and emerging threats. Policymakers should also consider the cumulative effects of microplastics in conjunction with other pollutants and environmental stressors, ensuring a holistic approach to environmental protection and sustainable management of water resources.

7.5 Future directions

Each chapter of this thesis leverages its findings to highlight specific research needs. The following section outlines the overarching future research directions necessitated by this thesis. Within thesis findings, the distribution of microplastic was relatively widespread but patchy and weakly related to potential sources. It therefore remains unclear whether microplastic occurrence are linked to current environmental contamination or long-term accumulation and remobilisation from sediment sinks and other stores within the catchment. Given the complex interplay between dynamic microplastic sources and environmental variation (Windsor et al. 2019a), it is essential to understand the entire material lifecycle and develop site-specific exposure assessments to manage microplastics across catchments. Further focus should be on analysing diffuse sources of microplastics. Recent work on sustainable drainage systems (SuDS) indicates how achievable town planning can mitigate surface run-off (Rasmussen et al. 2024), yet atmospheric fallout remains poorly understood.

Overall, there is a need to focus on the effect of microplastic on freshwaters. Current research often elicits effects on using single species with limited and pristine microplastic particle types at extreme microplastic concentrations that are not representative of real-world scenarios (Thornton Hampton et al. 2022b). This approach can be problematic, as it may not accurately reflect the actual risks posed by microplastic in natural environments, which may lead to misguided risk assessments and ineffective policy decisions. Further research is, therefore, required to understand the implications of observed individual-level biological effects on populations and ecosystem function, as interactions among individuals and populations may alter food webs and ecological function (e.g., Borges et al. 2024). Ecosystem-scale investigations and effect assessments will, therefore, facilitate risk assessment and identify areas for mitigation.

Lastly, investigating the interactions between microplastic and other pollutants and environmental stressors is vital in determining net ecological effects and prioritising research and mitigation efforts (Nguyen et al. 2023). The impact of microplastic in relation to other stressors is poorly understood, whilst the occurrence of microplastic could alter ecotoxicity of chemical pollutants. A holistic approach is crucial for developing comprehensive regulations that efficiently address the

multifaceted nature of environmental contamination and ecotoxicology. Ongoing research should focus on building data on temporal trends to monitor sources, evaluate regulations and mitigation policies, and increase public awareness to reduce plastic waste.

Success of these future directions in microplastic research is somewhat determined by the optimisation of methods to sample and extract microplastic from the environment. This thesis addresses calls to standardise microplastic research (Horton et al. 2017b; Akdogan and Guven 2019) by formulating classification and quantification criteria with respect to composition, morphology, and reporting units. However, due to the inherent variation with and across environmental matrices, harmonisation of sampling methods and microplastic extraction is a more realistic goal for future research (Lusher and Primpke 2023). These protocols must be adaptable and consider local conditions and specific research requirements (Bakir et al. 2024). This requires continued large-scale collaboration between microplastic researchers to: (i) develop a global agreement on plastic pollution (UNEA-5.2 Resolution); (ii) implement established guidelines (e.g., GESAMP 2019); (iii) follow regulators (e.g., Marine Strategy Framework Directive); (iv) develop sharable national facilities (e.g., Commonwealth Litter Programme); and (v) transfer knowledge. This will optimise protocols and improve research reliability.

Addressing these knowledge gaps will enhance assessments of microplastic distribution and the severity of associated ecological risks, leading to a better understanding of microplastics as agents of global biological change. This will support the 6th and 14th Sustainable Development Goals (SDG) that respectively aim to improve freshwater and marine water quality by reducing pollution, including microplastic (indicator 14.1.1). Additionally, this work aligns with SDG 12, which addresses sustainable consumption and production patterns.

7.6 Thesis conclusion

Microplastic is distributed widely across freshwater ecosystems around the globe, with concentrations increasing over time and in proximity to urban sources. While methodological inconsistencies and caveats pose challenges to research synthesis, they are inherent when dealing with such a diverse and complex physical

pollutant. Data from this thesis indicate limited microplastic loads in a Welsh riverine catchment, yet underscore the persistent exposure within freshwater ecosystems. Although negligible effects of point source injections were observed in environmentally relevant flowing water, the potential threat of microplastics cannot be ignored. Continued research is essential to investigate the diverse types of microplastics, their impact on downstream habitats and sinks, and their long-term accumulation in the environment. Plastic is essential to the function and health of modern society, with a lower carbon footprint than other synthetic materials and thus, should not be demonised. Yet comprehensive and adaptive management strategies are necessary to address the multifaceted nature of microplastic pollution and its potential ecological consequences, with the ultimate goal of eliminating non-natural materials to restore natural environments.

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Supplementary material

Appendix A

Table A1 List of 300 freshwater microplastic articles used for meta-analysis (*Chapter 2* and *Chapter 3)*, grouped by the continent in which data was collected.

Continent	Freshwater microplastic studies
Asia	Free et al. 2014; Zhang et al. 2015; Su et al. 2016; Zhang et al. 2016; Jabeen et al. 2017; Sruthy and Ramasamy 2017; Wang et al. 2017a; Wang et al. 2017b; Zhang et al. 2017; Di and Wang 2018; Hu et al. 2018; Jiang et al. 2018; Lahens et al. 2018; Lin et al. 2018; Peng et al. 2018; Su et al. 2018; Wang et al. 2018a; Wen et al. 2018; Xiong et al. 2018; Alam et al. 2019; Cheung et al. 2019; Di et al. 2019; Ding et al. 2019; Eo et al. 2019; Fan et al. 2019; Jiang et al. 2019; Kataoka et al. 2019; Li et al. 2019; Liu et al. 2019b; Luo et al. 2019; Mai et al. 2019; Tan et al. 2019; Wang et al. 2019; Xiong et al. 2019; Yan et al. 2019; Yin et al. 2019; Yuan et al. 2019; Zheng et al. 2019; Deng et al. 2020; Chen et al. 2020a; Gopinath et al. 2020; Han et al. 2020; Hu et al. 2020; Hwi et al. 2020; Irfan et al. 2020a; Irfan et al. 2020b; Lestari et al. 2020; Liu et al. 2020; Mao et al. 2020a; Mao et al. 2020b; Pan et al. 2020; Pariatamby et al. 2020; Park et al. 2020a; Park et al. 2020b; Pico et al. 2020; Tien et al. 2020; Wang et al. 2020a; Wang et al. 2020b; Wong et al. 2020; Wu et al. 2020; Xia et al. 2020; Zhang et al. 2020a; Zhang et al. 2020b; Abbasi 2021; Ajay et al. 2021; Bharath et al. 2021; Chanpiwat and Damrongsiri 2021; Chauhan et al. 2021; Chen et al. 2021; Kabir et al. 2021; Fan et al. 2021; Feng et al. 2021a; Feng et al. 2021b; Frank et al. 2021; Haberstroh et al. 2021b; He et al. 2021b; Huang et al. 2021a; Huang et al. 2021b; Li et al. 2021a; Li et al. 2021b; Lin et al. 2021; Liu et al. 2021; Mai et al. 2021; Napper et al. 2021; Niu et al. 2021; Parvin et al. 2021; Shen et al. 2021; Singh et al. 2021;

	Tang et al. 2021; Tsering et al. 2021; Wang et al. 2021b; Wang et al. 2021d; Wicaksono et al. 2021; Xu et al. 2021; Yan et al. 2021; Yin et al. 2021; Zhang et al. 2021a; Zhang et al. 2021b; Aslam et al. 2022; Bashir and Hashmi 2022; Bian et al. 2022; Dai et al. 2022; Deng et al. 2022; Ghanbari et al. 2022; Jin et al. 2022; Kabir et al. 2022; Kılıç; Li et al. 2022a; Li et al. 2022b; Liu et al. 2022b; Ma et al. 2022; Masoudi et al. 2022; Neelavannan et al. 2022; Park et al. 2022; Rakib et al. 2022; Tang et al. 2022; Tran-Nguyen et al. 2022; Warrier et al. 2022; Wu et al. 2022a; Wu et al. 2022b; Xu et al. 2022a; Xu et al. 2022b; Yan et al. 2022; Yin et al. 2022; Yu et al. 2022; Yuan et al. 2022; Zhang et al. 2022
Europe	Faure et al. 2012; Imhof et al. 2013; Lechner et al. 2014; Sanchez et al. 2014; Wagner et al. 2014; Dris et al. 2015; Faure et al. 2015; Klein et al. 2015; Mani et al. 2015; Stolte et al. 2015; Troyer 2015; Van der Wal et al. 2015; Fischer et al. 2016; Imhof et al. 2016; Horton et al. 2017; Hurley et al. 2017; Leslie et al. 2017; McGoran et al. 2017; Vaughan et al. 2017; Collard et al. 2018; Dris et al. 2018b; Horton et al. 2018; Hurley et al. 2018b; Kay et al. 2018; Lusher et al. 2018; Rodrigues et al. 2018b; Schmidt et al. 2018; Sighicelli et al. 2018; Tibbetts et al. 2018; Akindele et al. 2019; Blair et al. 2019; Bordós et al. 2019; Frei et al. 2019; Liu et al. 2019a; Mani et al. 2019a; Olesen et al. 2019; Roch et al. 2019; Scopetani et al. 2019; Simon-Sánchez et al. 2019; Slootmaekers et al. 2019; Turner et al. 2019; Windsor et al. 2019b; Bosshart et al. 2020; Campanale et al. 2020; Constant et al. 2020; Erdoğan 2020; Kaliszewicz et al. 2020; Karaoğlu and Gül 2020; Kuśmierek and Popiołek 2020; Mani and Burkhardt-Holm 2020; Mintenig et al. 2020; Negrete Velasco et al. 2020; O'Connor et al. 2020; Scherer et al. 2020; Stanton et al. 2020; Uurasjärvi et al. 2020; Winkler et al. 2020; Zobkov et al. 2020; de Carvalho et al. 2021; Clayer et al. 2021; Garcia et al. 2021; Guven 2021; Laermanns et al. 2021; Munari et al. 2021; Pan et al. 2021; Pastorino et al. 2021; Prata et al. 2021; Sekudewicz et al. 2021; Stanković et al. 2021; Tanentzap et al. 2021; Woodward et al. 2021; Almas et al. 2022; Atamanalp et al. 2022; Atici 2022; Atici et al. 2022; Bertoli et al. 2022; Cera et al. 2022a; Cera et al. 2022b;

	Eibes and Gabel 2022; Fiore et al. 2022; Kallenbach et al. 2022; Murphy et al. 2022; O'Connor et al. 2022; Parker et al. 2022a; Pittura et al. 2022; Pol et al. 2022; Winkler et al. 2022; Zhdanov et al. 2022
North America	Moore et al. 2011; Eriksen et al. 2013; Castañeda et al. 2014; McCormick et al. 2014; Yonkos et al. 2014; Corcoran et al. 2015; Phillips and Bonner 2015; Baldwin et al. 2016; Ballent et al. 2016; Estahbanati and Fahrenfeld 2016; Holland et al. 2016; Mason et al. 2016; McCormick et al. 2016; Peters and Bratton 2016; Anderson et al. 2017; Cable et al. 2017; Campbell et al. 2017; Hoellein et al. 2017; Lasee et al. 2017; Miller et al. 2017; Vermaire et al. 2017; Barrows et al. 2018; Dean et al. 2018; Hendrickson et al. 2018; Kapp and Yeatman 2018; McNeish et al. 2018; Forrest et al. 2019; Lenaker et al. 2019; Peller et al. 2019; Ryan et al. 2019; Shruti et al. 2019; Watkins et al. 2019a; Watkins et al. 2019b; Corcoran et al. 2020; Crew et al. 2020; Dias 2020; Grbić et al. 2020; Mason et al. 2020; Scircle et al. 2020; Simmerman and Wasik 2020; Wardlaw and Prosser 2020; Bujaczek et al. 2021; Eppehimer et al. 2021; Felismino et al. 2021; Haberstroh et al. 2021a; Hou et al. 2021; Lenaker et al. 2021; Martinez-Tavera et al. 2021; Munno et al. 2021; Wardlaw 2021; Belontz et al. 2022; Rowenczyk et al. 2022; Talbot et al. 2022b; Xiong et al. 2022
Africa	Biginagwa et al. 2016; Nel et al. 2018; Reynolds and Ryan 2018; Akindele et al. 2019; Toumi et al. 2019; Weideman et al. 2019; Dahms et al. 2020; Egessa et al. 2020; Khan et al. 2020; Mbedzi et al. 2020; Merga et al. 2020; Migwi et al. 2020; Oni et al. 2020; Blankson et al. 2022; Dahms et al. 2022; Ditlhakanyane et al. 2022; Malla-Pradhan et al. 2022
South America	Silva-Cavalcanti et al. 2017; Andrade et al. 2019; Blettler et al. 2019; Alfonso et al. 2020; Garcia et al. 2020; Gerolin et al. 2020; Martínez Silva and Nanny 2020; Bertoldi et al. 2021; Lucas-Solis et al. 2021; Pastorino et al. 2021; Correa-Araneda et al. 2022
Oceania	Dikareva and Simon 2019; Townsend et al. 2019; He et al. 2020; Nan et al. 2020

Table A2 Reporting units of microplastic concentration in 300 freshwater microplastic articles used for meta-analysis (*Chapter 2* and *Chapter 3*), grouped by sample matrix. dw = dry weight, ww = wet weight, GI tract = gastrointestinal tract, *excluded* = data points were excluded from microplastic concentration summaries.

Sample matrix	Original reporting units	Standardised reporting units
Water	particles/m^3, particles/1,000 m^3, microfibres/m^3, fibres/m^3, fragments/m^3, particles/dm^3, particles/ml, particles/10 ml, particles/L, fibres/L, microfibres/L, particles/m^2, particles/km^2, fibres/km^2	particles/m^3
	ug/m^3, g/m^3, mg/m^3, mg/1000 m^3, mg fibres/m^3, mg fragments/m^3, mg/1000 m^3, ug/L, mg/L, ug/m^2, mg/km^2, g/km^2	mg/m^3
	particles/m^3/min, particles/15-minute trawl	*Excluded*
Sediment	particles/kg	particles/kg
	particles/g dw, particles/10 g dw, particles/42.235 g dw, particles/103.241 g dw, particles/kg dw, microfibres/kg dw	particles/kg dw
	particles/g ww, particles/kg ww, fibres/kg ww	particles/kg ww
	particles/m^3, particles/L	particles/m^3
	particles/m^2, microbeads/m^2	particles/km^2
	particles/m^2/year	particles/m^2/year
	mg/kg	mg/kg
	ug/g dw, mg/kg dw	mg/kg dw

Organism	particles/individual, particles/5 individuals, particles/10 individuals, fibres/individual, particles/organism, particles/individual gill, particles/individual GI tract, particles/individual GI tract & gill combined, particles/gizzard, particles/individual stomach contents	particles/individual
	particles/spraint	particles/spraint
	particles/mg, particles/g, particles/kg, particles/g body weight, particles/g GIT content	particles/g
	particles/mg dw, particles/g dw, particles/kg dw	particles/g dw
	particles/mg ww, particles/g ww, particles/kg ww GIT, particles/kg ww gill	particles/g ww
	ug/individual, mg/individual	mg/individual
	ug/g dw, mg/kg dw	mg/g dw
	ug/g ww, mg/kg ww	mg/g ww

Table A3 Best normalised transformations of secondary social data from United Nation's 2021 International Statistical Yearbook (Department of Economic and Social Affairs, Statistics Division 2021), used in meta-analysis (*Chapter 2*).

Independent variable	**Transformation**	**Shapiro-Wilk *p*-value**
Population density per km^2, 2021	Box-Cox	0.464
GDP per capita (US$), 2019	Box-Cox	0.065
Tourist arrivals, 2018	Box-Cox	0.853
Agriculture production index, 2019	Ordered Quantile	1.000
Proportion of population with access to safe water supply, 2020	Ordered Quantile	0.920

*p-values < 0.05 indicate the distribution of data does not significantly deviate from normality (*)

Table A4 Average microplastic concentration in the water matrix (particles/m^3 ± SD) of freshwater ecosystems reviewed in meta-analysis (*Chapter 2*). n = study count. Data are grouped by continent and country and ordered by continent averaged concentration. Averages include original microplastic concentrations in particles/km^2 converted to particles/m^3 using 1 item/km^2 = 10^{-6} particles/m^3 (Chen et al. 2020a). Excludes samples recorded in the following units: particles, particles/15-minute trawl, particles/m^3/minute (three studies).

Continent	n	Microplastic concentration (n)	Country	n	Microplastic concentration (n)
Asia	79	99,815 ± 487,851 (*137*)	China	53	142,524 ± 594,067 (*91*)
			Japan	2	129,436 ± 96,877 (*5*)
			India	5	4,573 ± 8,373 (*6*)
			Saudi Arabia	1	3,200 (*1*)
			Vietnam	1	1,482 (*1*)
			Indonesia	3	1,466 ± 2,922 (*4*)
			Pakistan	2	1,389 ± 655 (*3*)
			Thailand	1	1,108 ± 476 (*4*)
			Malaysia	3	919 ± 1,942 (*5*)
			South Korea	3	675 ± 860 (*9*)
			Iran	1	300 (*1*)
			Russia	2	32 ± 28 (*3*)
			Cambodia	1	5 ± 4 (*2*)
			Mongolia	1	0.02 (*1*)
North America	30	7,249 ± 18,146 (*95*)	USA	19	9,435 ± 20,489 (*71*)
			Canada	9	973 ± 3,512 (*19*)

Continent	***n***	**Microplastic concentration (*n*)**	**Country**	***n***	**Microplastic concentration (*n*)**
Europe	38	4,396 ± 29,696 (*153*)	Portugal	1	231,000 (*1*)
			Denmark	3	90,470 ± 155,479 (*3*)
			Netherlands	3	20,304 ± 44,553 (*5*)
			Germany	5	5,414 ± 8,424 (*5*)
			Poland	3	5,223 ± 5,270 (*5*)
			Turkey	3	2,244 ± 3,687 (*3*)
			Switzerland	3	578 ± 1,146 (*9*)
			UK	2	463 ± 825 (*4*)
			Finland	2	57 ± 97 (*3*)
			France	5	31 ± 25 (*19*)
			Hungary	1	14 (*1*)
			Italy	9	4 ± 8 (*21*)
			Spain	1	3.5 (*1*)
			Romania	1	1 (*1*)
			Norway	1	0.79 ± 0.65 (*9*)
			Sweden	2	0.50 ± 0.80 (*36*)
			Croatia	1	0.47 ± 0.59 (*3*)
			Austria	2	0.31 ± 0.36 (*9*)
			Slovenia	1	0.28 (*1*)
Africa	6	516 ± 861 (*13*)	Nepal	1	2,235 ± 1,025 (*2*)
			Ghana	1	490 ± 481 (*2*)
			South Africa	2	251 ± 273 (*5*)

Continent	***n***	**Microplastic concentration (*n*)**	**Country**	***n***	**Microplastic concentration (*n*)**
			Kenya	1	2 ± 2 (*2*)
			Uganda	1	0.43 ± 0.43 (*2*)
Oceania	2	266 ± 179 (*3*)	Australia	1	363 ± 89 (*2*)
			New Zealand	1	72 (*1*)
South America	3	85 ± 70 (*5*)	Columbia	1	134 ± 27 (*1*)
			Chile	1	22 (*1*)
			Argentina	1	0.90 (*1*)

Table A5 Average microplastic concentration in the sediment (particles/kg ± SD) of freshwater ecosystems reviewed in meta-analysis (*Chapter 2*). n = study count. Data are grouped by continent and country and ordered by continent averaged concentration. Averages include original microplastic concentrations in dry and wet sediment. Excludes samples recorded in the following units: particles, particles/m^3, particles/km^2, particles/m^2/year (172 studies).

Continent	**n**	**Microplastic concentration (n)**	**Country**	**n**	**Microplastic concentration (n)**
Europe	23	114,483 ± 560,596 (*38*)	Denmark	2	275,000 ± 671,732 (*2*)
			Russia	1	2,189 (*1*)
			Netherlands	1	2,071 (*1*)
			Finland	1	396 (*1*)
			UK	7	374 ± 7,158 (*17*)
			Germany	2	359 ± 1,933,564 (*3*)
			Turkey	1	226 (*1*)
			Norway	1	200 (*1*)
			Ireland	1	172 (*1*)
			Italy	4	39 ± 89 (*6*)
			Hungary	1	1 (*1*)
			Switzerland	1	0 ± 31 (*3*)
Asia	41	1,740 ± 3,594 (*56*)	Vietnam	1	6,120 (*1*)
			Pakistan	1	1,318 ± 1,899 (2)
			Bangladesh	1	1,177 (*1*)
			South Korea	2	657 ± 899 (*3*)
			Japan	1	167 (*1*)
			Indonesia	1	30 (*1*)

Continent	n	Microplastic concentration (n)	Country	n	Microplastic concentration (n)
			India	6	4 ± 751 (*7*)
			Iran	1	4 (*1*)
			China	27	2 ± 4,143 (*39*)
North America	10	1,485 ± 4,284 (*26*)	Mexico	1	20 ± 65 (*4*)
			Canada	5	10 ± 333 (*9*)
			USA	3	5 ± 6,168 (*12*)
South America	2	487 ± 842 (*4*)	Ecuador	1	1,748 (*1*)
			Colombia	1	15 ± 43 (*3*)
Africa	4	192 ± 164 (*6*)	Ghana	1	188 ± 18 (*2*)
			South Africa	3	2 ± 81 (*4*)

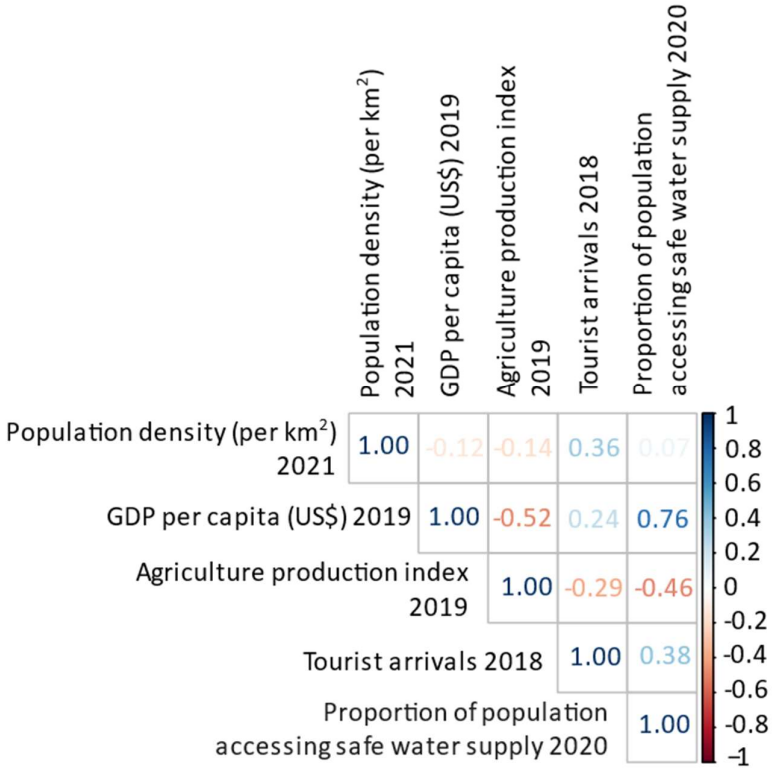

Figure A1 Correlation coefficients between independent variables in the statistical model assessing the effect of social factors on country-averaged freshwater microplastic concentration from meta-analysis (Chapter 2).

Supplementary Information A1 Reference articles used to create microplastic shape classification criteria (*Chapter 2*).

Castañeda et al. 2014; Free et al. 2014; Wagner et al. 2014; Dris et al. 2015; Phillips and Bonner 2015; McCormick et al. 2016; Su et al. 2016; Anderson et al. 2017; Cable et al. 2017; Jabeen et al. 2017; McGoran et al. 2017; Sruthy and Ramasamy 2017; Vaughan et al. 2017; Di and Wang 2018; Hendrickson et al. 2018; Jiang et al. 2018; Pivokonsky et al. 2018; Rodrigues et al. 2018a; Su et al. 2018; Wen et al. 2018; Akindele et al. 2019; Andrade et al. 2019; Ding et al. 2019; Townsend et al. 2019; Watkins et al. 2019b; Watkins et al. 2019a; Yin et al. 2019; Campanale et al. 2020; Constant et al. 2020; Egessa et al. 2020; Hwi et al. 2020; Mani and Burkhardt-Holm 2020; Mao et al. 2020a; Mao et al. 2020b; Martínez Silva and Nanny 2020; Pariatamby et al. 2020; Scircle et al. 2020; Wang et al. 2020a; Wang et al. 2020b; Wardlaw and Prosser 2020; Zobkov et al. 2020; Bertoldi et al. 2021; Bujaczek et al. 2021; Felismino et al. 2021; Garcia et al. 2021; Huang et al. 2021a; Lin et al. 2021; Parvin et al. 2021

Table A6 Standardised polymer name and acronym from articles reviewed in meta-analysis, in alphabetical order.

Polymer name	Polymer acronym
Acrylonitrile butadiene styrene	ABS
Acrylonitrile butadiene	ACNB
Aramid / Aromatic Polyamide	AD
Acrylonitrile	AN
Acrylates / Polyurethanes / Varnish cluster	APV
Alkyd resin / Alkyd varnish	AR
Butadiene rubber	BR
Cellulose acetate	CA
Cellophane	CP
Chlorinated polyethylene	CPE
Cellulose propionate	CPRO
Cellulose triacetate	CTA
Didecyl phthalate plasticizer resin	DIDP
Ethylene/ethylene/ethyl acrylate copolymer	EEA
Ethylene glycol stearate	EG-S
Epoxide / Epoxy Resin	EP
Ethylene propylene diene rubber / Polypropylene-vistalon / Ethylene propylene diene monomer rubber	EPDM
Ethylene propylene diene terpolymer	EPDT
Ethylene-propylene rubber / Ethylene-propylene copolymer	EPR
Expanded polystyrene	EPS
Ethylene propylene	ETP
Poly(ethylene-co-vinyl acetate) / Ethylene-vinyl acetate / Ethylene–vinyl acetate copolymer	EVA
Ethylene vinyl alcohol	EVOH
Fluorinated ethylene propylene	FEP
High density polyethylene	HDPE
Isoprene rubber / Polyisoprene	IR

Polymer name	Polymer acronym
Low density polyethylene	LDPE
Low density polypropylene	LDPP
Melamine-formaldehyde resin	MF
Nitrocellulose	NC
Nitrile rubber	NR
Other miscellaneous/unidentifiable names	Other
Oxidized Polyethylene	OPE
Polyamide / Nylon	PA
Polyacrylic / Poly(acrylic acid)	PAA
Poly(acrylate)	PAC
Polyacetylene	PACL
Polyalkene	PAK
Poly(acrylamide) / Polyacrylamide	PAM
Polyacrylonitrile	PAN
Poly(acrylonitrile: acrylic acid)	PANAA
Polyacrylonitrile Vinyl Chloride	PANVC
Poly acrylate Polyester	PAPE
Poly(acrylate-styrene) / Polyacrylate & styrene co-polymer	PAS
Polyacrylonitrile-Polystyrene-Polymethyl acrylate	PASM
Poly(1-butene)	PB
Poly(butyl methacrylate)	PBMA
Poly(butadiene:acrylonitrile)	PB/PAN
Polybutylene terephthalate	PBT
Polycarbonate	PC
Polycarbonate-Acrylonitrile butadiene	PC/ABS
Polycaprolactone / Polycaprolactone diol	PCL
Polychloroprene / Neoprene	PCP
Polycaprolactam	PCPL
Polysulfide crude rubber	PCR
Poly(cyclohexylenedimethylene terephthalate)	PCT
Polydiene	PD

Polymer name	Polymer acronym
Polydiallyl isophthalate	PDAIP
Polydimethylsiloxane	PDMS
Poly(dimer acid-co-alkyl polyamine)	PDPA
Polyethylene	PE
Poly(ethyl acrylate)	PEA
Poly(ethyl acrylate):st:acrylamide	PEA/AC
Polyether ether ketone	PEEK
Polyethylene glycol	PEG
Poly ethylenimine	PEI
Poly(ethyl methacrylate)	PEM
Polycarbonate-Polyethylene mix	PE/PC
Polyethylene-Polypropylene copolymer	PE/PP
Poly epoxy	PEP
Polyethersulfone	PES
Polyester	PEST
Polyester-Polyamide copolymer	PEST/PA
Polyester-Polyethylene copolymer	PEST/PE
Polyester-Polyethylene terephthalate copolymer	PEST/PET
Polyester-Poly(methyl methacrylate) copolymer	PEST/PMMA
Polyester-Rayon copolymer	PEST/RA
Polyethylene terephthalate	PET
Polyethylene terephthalate-polyurethane copolymer	PET/PU
Polyester urethane	PEUU
Polyethylene vinyl chloride	PEVC
Phenol formaldehyde resin	PF
Propylene glycol monooleate	PGM
Poly(hexadecyl methacrylate)	PHM
Poly(lactic acid) / Polylactide	PLA
Poly(lauryl acrylate) / Poly(dodecyl acrylate)	PLYA
Poly(methyl acrylate)	PMA
Poly(methyl methacrylate) / Acrylic / Acrylic acid	PMMA

Polymer name	Polymer acronym
Polymethyl methacrylate-Polyacrylonitrile	PMMA/PAN
Poly(ethyl methacrylate-co-methyl acrylate)	PMMA/PMA
Polymethyl methacrylate-Polystyrene copolymer	PMMA/PS
Polymethyl methacrylate-Poly(vinyl chloride)	PMMA/PVC
Poly(methylpentene)	PMP
Poly(octyl acrylate)	POA
Poly(octadecyl methacrylate)	POCM
Poly(octadecyl acrylate)	PODA
Poly(oxymethylene)	POM
Polypropylene	PP
Polypropylene/ethylene-propylene / Polypropylene+poly(ethylene:propylene)	PP/EPR
Poly(phenylene sulfide)	PPS
Phenoxy resin	PR
Polystyrene / Poly(styrene) atactic	PS
Polystyrene divinylbenzene	PS/DVB
Polysulfone	PSF
Polystyrene/Polyacrylate copolymer	PS/PAC
Polystyrene Polyacrylonitrile Poly(methyl methacrylate) copolymer	PS/PAN/PMMA
Polystyrene sulfonate	PSS
Polyarylsulphone	PSU
Polystyrene Vinyl Chloride	PSVC
Polysiloxane	PSX
Polyterpene	PT
Polytetrafluoroethylene / Fluoro-polymer/Teflon	PTFE
Poly(tetrafluoroethylene:propene)	PTFE/PP
Poly(trimellitamide imide) / Poly(trimellitic Amide imide)	PTI
Polyurethane	PU
Polyurethane acrylic resin / Polyurethane acrylate	PUAR
Polyvinyl acetate	PVA

Polymer name	Polymer acronym
Polyvinyl acetate / Polyvinyl/vinyl acetate copolymer	PVAC
Polyvinyl acrylonitrile	PVAN
Polyvinyl butyral	PVB
Polyvinyl chloride	PVC
Polyvinyl Chloride:Ethylene	PVC/E
Poly(4-vinylpyridine)	PVD
Polyvinylidene chloride	PVDC
Polyvinyl ester	PVE
Poly(vinyl fluoride) / Polyvinyl fluoride	PVF
Polyvinyl alcohol / Synthetic fibre polyvinil alcohol / Vinylon	PVOH
Poly(vinylpyrrolidone)	PVP
Polyvinyl propionate:acrylate	PVP/A
Polyvinyl stearate	PVS
Polyvinyltoluene:Butadiene	PVT/B
Rayon / Viscose	RA
Styrene acrylonitrile	SAN
Styrene butadiene rubber/ Poly(styrene:butadiene)	SBR
Synthetic cellulose / Chemically modified cellulose	SCL
Synthetic rubber	SR
Tire & bitumen microplastic particles	TBMP
Urethane	U
Urethane alkyd	UA
Urea-formaldehyde resin	UF
Vinyl chloride	VC
Vinyl chloride / Vinyl acetate copolymer	VC/VA

Appendix B

Table B1 Summary of wastewater treatment plants (WWTPs) discharging into River Taff, obtained from Dŵr Cymru/Welsh Water. Population equivalent (PE) represents the number of residential and non-residential persons the plant serves, calculated from 2021 census (Office for National Statistics 2021). Preliminary treatment involves the removal of rags, paper, macroplastics, and metals from wastewater through CopaSac®, escalator, or rake screens, and/or grit removal. Storm settlement involves storage of excess stormwater for later treatment once the storm has passed. Primary and secondary treatment involves sludge settlement with liquid outflow. Biological treatment removes biological pollutants via filter beds (wastewater passes through a bed of coke, gravel, or clinker where surface bacteria, fungi and other organisms digest organic matter) or activated sludge (air pumped into tank of wastewater for bacteria to multiply digest organic matter). Tertiary treatment improves effluent, with reed beds allowing further organic matter digestion from microorganisms. Inflow and outflow data from the year prior to sample collection (12th April 2021 to 20th May 2022) recorded by a MCERT (Monitoring Certification Scheme) flow meter, reported as mean average (± 1 standard deviation); range. There is no recorded flow data for Llwyn-On Houses and Pontsticill Houses WWTPs, due to either flow being less than 50 m^3/day or no Environmental Permit in place requiring flow to be recorded. Missing inflow and outflow data from Cynon and Cilfynydd WWTPs, respectively, is due to only one MCERT at each site.

Name	Asset number	Outlet Latitude	Outlet Longitude	PE	Treatment (trt) process	Inflow (L/s)	Outflow (L/s)
Llwyn-On Houses	30973	51.789281	-3.432257	44	Preliminary trt (CopaSac® screening), primary trt, biological trt (gravity-fed rotating filter bed).	-	-
Pontsticill Houses	31055	51.795154	-3.364071	1	Primary trt, secondary trt (septic tank), biological trt (gravity-fed rotating filter bed).	-	-
Pontsticill	31054	51.790354	-3.363912	360	Preliminary trt, storm settlement, primary trt, biological trt (gravity-fed rotating filter bed), secondary trt (humus tank), tertiary trt (reed bed).	2.29 ± 1.37; 0-5	1.85 ± 1.61; 0.08-20.02
Cynon	30861	51.628263	-3.328372	68,434	Preliminary trt (grit settlement channel, escalator and rake screening), storm settlement, primary trt, biological trt (activated sludge), secondary trt.	-	352.79 ± 131.67; 42.8 – 646.9
Cilfynydd	30843	51.627054	-3.327152	76,521	Preliminary trt (automatic grit removal, escalator screening), storm settlement, primary trt, biological trt (gravity-fed rotating filter bed), secondary trt (humus tanks).	411.26 ± 146.86; 98.4 – 905.5	-

Table B2 Summary of sample sites and water status at time of macroinvertebrate and sediment sampling in April-May 2022. Electrical conductivity (EC; µS/cm), pH, and temperature (°C) were measured using a HI-9813-5 potable meter (Hanna Instruments, UK) and river flow velocity (m/s) was measured using a magnetic-inductive flow meter (OTT MF pro Meter, OTT HYdroMet, US) and wading rod (average of 3 measurements). Site elevation obtained from 50 m Digital Terrain Model (OS Terrain 50, Ordnance Survey). Distance upstream and subcatchment area was calculated using QGIS © OpenStreetMap (Ordnance Survey, 2021) and catchment delineation. Sediment from sites 3, 21, and 22 were not analysed.

Site	Latitude	Longitude	Site elevation (masl)	Distance upstream (km)	Sub-catchment area (km^2)	Sample date	pH	EC (µS/cm)	Temp. (°C)	Mean flow velocity (m/s)
1	51.497102	-3.207883	17	7463.39	500.88	20/05/22	8.0	0.23	15.4	0.24
2	51.499069	-3.221589	17	8290.47	500.17	10/05/22	7.5	0.05	16.5	0.18
3	51.518819	-3.253622	27	12396.38	0.28	20/05/22	7.9	0.23	15.9	0.31
4	51.541798	-3.231026	125	13613.61	1.59	22/04/22	7.8	0.27	10.0	0.36
5	51.539410	-3.244901	83	15106.98	1.82	17/05/22	8.3	0.37	13.3	0.12
6	51.538466	-3.275206	125	16192.06	2.06	28/04/22	8.3	0.25	9.5	0.11
7	51.549117	-3.259740	79	16437.01	7.81	25/04/22	8.0	0.19	11.3	0.09
8	51.569228	-3.289420	41	19957.45	469.28	10/05/22	8.1	0.25	16.7	0.33
9	51.586148	-3.317746	47	22467.89	460.94	10/05/22	8.4	0.24	17.3	0.25
10	51.608484	-3.337020	63	26054.11	342.64	13/04/22	8.4	0.28	17.9	0.27

Site	Latitude	Longitude	Site elevation (masl)	Distance upstream (km)	Sub-catchment area (km^2)	Sample date	pH	EC (µS/cm)	Temp. (°C)	Mean flow velocity (m/s)
11	51.625370	-3.327363	67	28546.36	330.72	13/04/22	8.2	0.32	16.0	0.18
12	51.609517	-3.396408	93	29011.25	101.15	19/04/22	6.3	0.04	9.8	0.50
13	51.640774	-3.331310	83	30769.48	303.79	10/05/22	7.3	0.29	16.0	0.36
14	51.625410	-3.339430	104	30782.25	18.25	14/04/22	7.8	0.10	9.4	0.25
15	51.646880	-3.326300	85	31821.48	103.28	14/04/22	8.3	0.22	11.7	0.30
16	51.656963	-3.338510	91	33160.14	101.75	13/05/22	8.2	0.29	11.0	0.16
17	51.659924	-3.306860	101	34265.82	33.31	10/05/22	7.8	0.27	11.8	0.12
18	51.660940	-3.382085	244	35249.11	7.48	14/04/22	7.8	0.07	10.5	0.30
19	51.670605	-3.300060	150	36432.34	19.85	17/05/22	8.2	0.27	11.4	0.16
20	51.655353	-3.436591	203	36526.31	21.24	28/04/22	7.9	0.16	11.4	0.11
21	51.623553	-3.501314	344	31821.49	1.59	22/04/22	8.3	0.17	11.2	0.21
22	51.651469	-3.494547	154	33160.15	2.77	22/04/22	8.7	0.12	11.5	0.05
23	51.707498	-3.321267	218	39993.54	9.89	27/04/22	7.9	0.32	10.2	0.36
24	51.683331	-3.490218	300	42607.58	11.40	28/04/22	8.0	0.15	12.2	0.31
25	51.712698	-3.350070	146	42926.46	143.81	26/04/22	8.6	0.29	12.2	0.24
26	51.704280	-3.427718	118	43724.22	59.52	27/04/22	8.8	0.21	11.6	0.21
27	51.713080	-3.457495	152	44935.96	8.17	28/04/22	8.2	0.15	11.8	0.43

Site	Latitude	Longitude	Site elevation (masl)	Distance upstream (km)	Sub-catchment area (km^2)	Sample date	pH	EC (µS/cm)	Temp. (°C)	Mean flow velocity (m/s)
28	51.686008	-3.458121	206	45145.17	3.33	27/04/22	7.5	0.05	10.9	0.18
29	51.728471	-3.463451	150	46676.67	41.00	27/04/22	8.5	0.18	11.7	0.23
30	51.684645	-3.551364	214	47235.14	9.78	25/04/22	8.4	0.13	12.1	0.04
31	51.753454	-3.393764	185	48597.18	110.61	26/04/22	8.8	0.11	11.0	0.16
32	51.769901	-3.418894	228	50428.08	54.55	27/04/22	8.3	0.09	10.0	0.15
33	51.777314	-3.387186	163	52193.27	38.58	20/04/22	8.6	0.08	11.4	0.12
34	51.776586	-3.519024	273	54182.42	1.17	25/04/22	8.5	0.18	11.7	0.18
35	51.793688	-3.364854	307	55758.68	34.01	20/04/22	8.1	0.04	11.1	0.35
36	51.807776	-3.445226	270	57832.92	22.75	27/04/22	7.8	0.27	11.8	0.32
37	51.835329	-3.383297	340	61059.82	13.17	20/04/22	7.8	0.06	9.9	0.33
38	51.856376	-3.469610	408	64336.84	3.23	20/05/22	8.2	0.06	10.8	0.09

Table B3 Summary of landscape characteristics and environmental conditions of subcatchments of sample sites.
Population size and density was calculated from population density of local authorities recorded in 2021 census (Office for National Statistics 2021). Vehicle density (vehicles/km^2) was calculated from © OpenStreetMap contributors (Ordnance Survey, 2021). Land-use was calculated as a percentage of subcatchment area, from UK Centre for Ecology and Hydrology's (UKCEH) 'Land Cover Map 2021' data (UKCEH 2022). Counts of WWTPs and CSOs in subcatchments were respectively calculated from Dŵr Cymru Welsh Water (November 2022) and The Rivers Trust (monitored CSOs 2021: The Rivers Trust 2022, unmonitored CSOs 2020: The Rivers Trust 2021). Sediment from sites 3, 21, and 22 were not analysed.

Site	Population size (2021)	Population density (persons/km^2)	Vehicle density (vehicles/km^2)	Land Use (%)							Upstream point sources (count)	
				Urban	Sub-urban	Agricult-ural	Grass-land	Heath-land	Wood-land	Rock	WWTPs	CSOs
1	286,256	572	276,642	0.978	14.019	0.378	48.086	4.363	30.513	0.875	6	234
2	284,448	569	277,091	0.978	13.928	0.379	48.142	4.369	30.547	0.876	6	234
3	713	2,546	157,423	0	93.594	0	5.338	0.000	0	0	6	0
4	2,156	1,356	378,301	0	4.774	0	48.116	0	46.859	0.251	0	0
5	2,825	1,552	169,973	0	8.929	0	18.963	0	61.905	10.204	0	0
6	5,272	2,559	157,743	0	5.379	0	51.149	0	43.472	0	0	0

7	1,012	130	193,628	0	5.130	1.267	39.772	0	53.832	0	0	0
8	230,858	492	294,342	0.801	12.996	0.309	49.305	4.656	30.390	0.806	6	223
9	226,130	491	296,927	0.778	12.727	0.298	49.297	4.739	30.609	0.820	6	220
10	159,994	467	367,821	0.557	11.660	0.389	50.981	6.368	28.094	1.070	6	114
11	152,967	463	368,532	0.574	11.575	0.397	50.347	6.598	28.558	1.054	6	111
12	56,536	559	103,921	1.464	14.530	0.009	44.503	0.012	39.191	0.113	0	80
13	137,875	454	394,254	0.625	11.944	0.408	51.266	7.187	26.471	1.148	4	101
14	10,233	561	98,495	0	5.056	0.077	35.044	0	59.687	0	0	5
15	57,869	560	101,958	0.639	15.811	0.133	45.456	1.078	34.339	2.312	0	68
16	57,013	560	101,815	0.644	15.610	0.135	45.910	1.094	34.037	2.347	0	66
17	18,599	558	686,431	0.107	7.451	1.081	68.736	9.910	11.347	1.252	0	2
18	4,197	561	98,515	0	0.067	0.187	21.036	0	78.376	0	0	0
19	10,702	539	693,906	0.035	3.107	0	71.654	15.347	8.462	1.394	0	1
20	11,903	560	98,505	0.156	8.762	0	47.842	0	42.471	0	0	15
21	1,553	977	102,068	0	0.493	0	11.841	0	87.666	0	0	0
22	1,026	370	98,446	0.164	4.438	0	85.041	0	10.356	0	0	0
23	5,387	545	691,760	0	0.589	0	60.568	29.173	6.870	2.801	0	0
24	6,394	561	98,496	0	0.167	0	45.950	0	52.555	0	0	0
25	48,472	337	505,638	0.767	9.040	0.334	52.473	11.822	23.291	0.467	4	19
26	33,350	560	100,611	0.510	13.353	0	59.716	1.500	20.677	3.990	0	24
27	4,578	560	98,508	0	10.032	0	62.540	0.171	26.829	0.073	0	2

28	1,872	562	98,521	0	1.049	0	42.720	0	56.231	0	0	0
29	22,973	560	100,436	0.017	10.042	0	65.785	1.418	17.123	5.361	0	9
30	4,313	441	118,910	0.000	1.694	0	29.606	0	67.563	1.138	0	0
31	31,337	283	422,154	0.061	3.489	0.182	62.096	9.312	22.535	0.025	4	3
32	16,521	303	313,452	0	0.558	0.092	66.750	7.120	23.639	0.035	1	0
33	5,461	142	420,104	0	0.962	0	62.862	6.743	25.448	0.021	2	1
34	5,486	4,689	98,501	0	2.229	0	89.495	0	4.606	3.670	0	0
35	3,219	95	340,836	0	0.450	0	62.038	6.518	26.494	0	1	0
36	996	44	162,949	0	0.191	0	85.405	0.009	12.645	0	0	0
37	338	26	169,213	0	0	0	70.873	0.230	28.897	0	0	0
38	83	26	169,386	0	0	0	99.288	0.062	0.650	0	0	0

Supplementary Information B1 Criteria used to identify suspected microplastic particles, summarised from Norén 2007; Hidalgo-Ruz et al. 2012; Nor and Obbard 2014; Horton et al. 2017; Vaughan et al. 2017; Barrows et al. 2018; Horton et al. 2018; Tibbetts et al. 2018; Townsend et al. 2019; Khan et al. 2020; Kuśmierek and Popiołek 2020; Mao et al. 2020a; Uurasjärvi et al. 2020; Woodward et al. 2021.

1) Particles have no cellular or organic structure.
2) Particles have an unnatural shape.
3) Fibres are equally thick throughout their length, are not segmented or twisted flat ribbons, and have 3D bending (i.e., not entirely straight).
4) Particles are not shiny, have clear and homogenous colour and if transparent or white, must be examined under high magnification and fluorescence to exclude organic origin.
5) Particles have a homogenous texture.
6) Particles maintain structural integrity when compressed, without being brittle.

Supplementary Information B2 Criteria used to classify suspected microplastic particles by shape, summarised from Castañeda et al. 2014; Free et al. 2014; Wagner et al. 2014; Dris et al. 2015; Phillips and Bonner 2015; McCormick et al. 2016; Su et al. 2016; Anderson et al. 2017; Cable et al. 2017; Jabeen et al. 2017; McGoran et al. 2017; Sruthy and Ramasamy 2017; Vaughan et al. 2017; Di and Wang 2018; Hendrickson et al. 2018; Jiang et al. 2018; Pivokonsky et al. 2018; Rodrigues et al. 2018a; Su et al. 2018; Wen et al. 2018; Akindele et al. 2019; Andrade et al. 2019; Ding et al. 2019; Townsend et al. 2019; Watkins et al. 2019b; Watkins et al. 2019a; Yin et al. 2019; Campanale et al. 2020; Constant et al. 2020; Egessa et al. 2020; Hwi et al. 2020; Mani and Burkhardt-Holm 2020; Mao et al. 2020a; Mao et al. 2020b; Martínez Silva and Nanny 2020; Pariatamby et al. 2020; Scircle et al. 2020; Wang et al. 2020a; Wang et al. 2020b; Wardlaw and Prosser 2020; Zobkov et al. 2020; Bertoldi et al. 2021; Bujaczek et al. 2021; Felismino et al. 2021; Garcia et al. 2021; Huang et al. 2021a; Lin et al. 2021; Parvin et al. 2021

1) Fragment – Hard, irregular shaped cube with at least one smooth plane, angular, jagged, incomplete, and 3D.
2) Bead/Pellet – Hard, round, spherical, ovoid discs, cylinders, and 3D.
3) Foam – Lightweight, sponge or bubble-like, and surface is not smooth.
4) Fibre – Thin, fibrous, thread-like, slender, elongated, cylindrical, equally thick throughout (not tapered at ends), not entirely straight, 3D bending, and length is >3 times width.
5) Film – Thin with two smooth planes, 2D, flat, irregular in shape soft, and flexible.
6) Other.

Supplementary Information B3 Limit of Detection (LOD)/Limit of Quantification (LOQ) calculations per shape for procedural blank adjustment (Bråte et al. 2018).

LOD was calculated as the average of total procedural blank particles for each individual shape plus 3x standard deviation, i.e., $LOD_{Sn} = \overline{X_{ySn}} + \left(3 \times SD_{ySn}\right)$ and **LOQ** was calculated as the average of total procedural blank particles for each individual shape plus 10x standard deviation, i.e., $LOQ_{Sn} = \overline{X_{ySn}} + \left(10 \times SD_{ySn}\right)$, where X = sample, Y = procedural blank, S = shape, $\bar{X}$ = mean, and SD = standard deviation of the mean. Sample counts were reported if their mean exceeds the LOQ. Sample counts were excluded if their mean is < LOQ and > LOD where microplastics are present but unquantifiable, or <LOD where microplastics are indistinguishable from the background.

Table B4 List of gauging stations on the River Taff from which average, minimum, and maximum flow rate (m3/s) were calculated.

ID	Name	Grid Reference
57005	Taff at Pontypridd	ST0792489715
57006	Rhondda at Trehafod	ST0528390946
57017	Rhondda Fawr at Tynewydd	SS9325998687
57004	Cynon at Abercynon	ST0794095652
57007	Taff at Fiddlers Elbow	ST0892095153
57015	Taff at Merthyr Tydfil	SO0430806814
57001	Taf Fechan at Taf Fechan Reservoir	SO060117
57002	Taf Fawr at Llwynon Reservoir	SO0118611166

Appendix C

Table C1 Percentage of macroinvertebrate individuals per mesocosm that belong to each family, grouped by their Functional Feeding Guild (FFG). Only taxa with contributions higher than 2% in any group, are reported. Mesocosms: H = Hanwell, S = Sidaway, C = Carpenter, and D = Davies.

		Acidic				Circumneutral			
		Benthic		Leaf-dwelling		Benthic		Leaf-dwelling	
FFG	**Family**	**H**	**S**	**H**	**S**	**C**	**D**	**C**	**D**
Shredder	Leuctridae	45.5	74.1	37.6	18.8	19.4	27.2	33.9	55.7
	Thaumaleidae	8.9	12.5	0	0	3.9	18.9	0	0
	Nemouridae	0	0.3	5	13.9	0	0	16.5	23.6
	Limnephilidae	1.4	0.8	7.2	0.6	0	0.6	0	0
	Lepidostomatidae	0	0	8.8	0.6	0	0	0.3	0
Filtering Collector	Simuliidae	34.9	0.3	5	3.6	7.7	2.4	0.8	0.7
	Oligochaeta	2.6	2.4	3.3	3.6	1.9	1.2	6.2	2.1
	Scirtidae larvae	0	0	0.6	8.5	0	0	5.4	0
	Hydropsychidae	0	0.8	0	3	7.7	0	0	1.4
Gathering collector	Chironomidae	0	0.3	30.4	26.1	2.6	1.2	23	5.7
	Baetidae	1.7	3.2	0	0	16.8	8.9	0	0
Predator	Rhyacophilidae	0	1.8	0	9.7	0.6	0.6	2.4	5
	Dytiscidae adult	2.9	0	0.6	3.6	0	0.6	0.5	1.4
Scraper/ Grazer	Heptageniidae	0	0	0	1.2	34.2	36.7	0.8	0
	Lymnaeidae	0	0	0	0	0	0	8.4	0