Freshwater fishes and microplastics in an era of multiple stressors

Ben Parker

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Bournemouth University

Supervisors: Dr Demetra Andreou, Dr Iain D. Green, Prof J. Robert Britton & Dr Katsiaryna Pabortsava

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Abstract

Microplastics, plastics < 5 mm in size, are environmental contaminants typically arising from the breakdown of larger plastics that may cause negative impacts when consumed by freshwater fishes. However, microplastic exposure occurs in combination with other interacting multiple stressors, for example urbanisation and parasite infection, and may potentially cause a range of synergistic negative impacts. Understanding the patterns of microplastic ingestion and the interactive impacts in relation to additional stressors is thus crucial in the management of freshwater fishes to implement effective mitigation measures.

Here, a literature review, field studies, an experiment and a metanalysis were carried out to examine how microplastic loads and their effects relate to stressors and the ecological features of freshwater fishes. More specifically, the thesis aimed to determine: 1) if microplastic loads are predictable from biological and environmental features; 2) the impacts of microplastic exposure on host-parasite dynamics and feeding; and 3) the interactive effects of microplastic exposure and additional stressors.

A literature review was first undertaken to understand the ingestion and effects of microplastics in freshwater fishes and to identify knowledge gaps and testable hypotheses for further study (Chapter 2). The literature suggested microplastic loads and effects are somewhat predictable based on the ecological traits of fishes while knowledge gaps remained around the ecological impacts of microplastic exposure and the potential interactive effects with other stressors.

Field studies established baseline microplastic loadings in relation to the environmental loads and species traits across a spatiotemporal gradient in sediments, macroinvertebrates and fishes from a small urban river (Chapter 3) as well as macroinvertebrates and fishes from a larger river system (Chapter 4). Both studies revealed a low incidence of particles and that loadings were unrelated to abiotic levels and biological features.

Data from Chapters 3 and 4 then informed chronic environmentally relevant microplastic exposures for an interaction experiment with parasite exposure looking at the combined effects on fish parasite load, morphometrics and feeding (Chapter 5). Parasite exposure reduced feeding and growth, however microplastic exposure had no single or interactive effect.

Chapter 6 examined microplastic loads within juvenile eels to understand the potential impacts of microplastic contamination within several south west England eel populations. Microplastic incidence was very low and unrelated to eel length or location, suggesting little threat of microplastic contamination on juvenile eels.

Finally, a metanalysis determined the combined effects of microplastics and additional stressors in freshwater fishes and whether the type and magnitude of effects varied with the interacting stressor, response, exposure or fish features (Chapter 7). Interactions were mostly additive across different stressor and response categories, except for ecological responses for the exclusive dataset which were antagonistic, and were unrelated to microplastic or fish features.

Overall, the results suggest microplastic contamination is consistent across space, time and different taxa while the experimental and metanalysis data demonstrate largely additive interactive effects. Other stressors may currently have greater importance in the management of freshwater fishes therefore it is recommended that managers target these known stressors but continue to monitor the levels and impacts of microplastics.

Table of contents

List of figures

(version 10.3) and the World Ocean Base map-Sources: Esri, GEBCO, NOAA, National Geographic, DeLorme, HERE, Geonames.org, and other contributors.

Figure 4.2. Macroinvertebrate and fish suspected microplastic particle features. The proportion of microplastics with different morphology (A), colour (B), size (C) and polymer (D) classes, respectively are presented for particles from macroinvertebrates and fish. Panels A, B and C are for all suspected microplastic particles (macroinvertebrates: n = 61 and fish: n = 260 particles). Panel D is for a subset of suspected microplastics subjected to FTIR (macroinvertebrates: n =, fish: n = particles).

Figure 4.3. Model parameter estimates for the presence of microplastics in macroinvertebrate batches. Parameter estimates are presented for each of the taxa as well as the batch number, the number of organisms within each batch. The span around each variable represents the confidence interval with significant variables not crossing the dashed line. The taxon “Amphipoda” is absent as it is used in the model intercept to compare with other taxa.

Figure 4.4. Model parameter estimates for the presence of microplastics in fish. Parameter estimates are presented for the different species and reaches as well as for standard length and Pomphorhynchus tereticollis count. The span around each variable represents the confidence interval with significant variables not crossing the dashed line. “Bleak” and “Reach 1” are both absent from the figure as the model combines the first levels of these categorical variables in the intercept as a reference for the other model parameters.

Figure 5.1. Experimental design with timings and replication overview. Replication numbers (n = x) are given for all combinations of diet: E = mean environmental and 2E = twice mean environmental microplastic exposure; parasite exposure: I = infected and U = unexposed gammarids; prey density = 4/8/16/32/64 uninfected prey. Within diet parasite exposure conditions, fish were randomly exposed to 4/8/12/16 or 20 infected or uninfected gammarids.

Figure 5.2. The relationship between parasite load and the number of infected gammarids consumed. End parasite loads and the number of parasites ingested (via infected gammarid intermediate hosts) are given for the 32 infected fish. The model fitted line is plotted along with the standard error border margins.

Figure 5.3. Specific growth rates between the different parasite exposure categories. Boxplots show the distribution of specific growth rate values for all fishes aggregated within the Unexposed (n = 61), Exposed (n = 33) and Infected (n = 32) categories.

Figure 5.4. Functional response curves by parasite exposure category. Curves with confidence intervals are produced using data aggregated by parasite exposure status, excluding fish where no gammarids were consumed. Parasite exposure categories: Unexposed (dashed line, light grey) = fish exposed to but not infected by the parasite, Infected (solid line, dark grey) = fish exposed to and infected by the parasite and Exposed (dotted line, light blue) = individuals exposed to but not infected by the parasite.

Figure 7.1 Figure 7.1 Categorised interaction types for the inclusive and exclusive datasets. The percentage of interactions classified as additive, antagonistic and synergistic are presented for both the inclusive dataset as well as an exclusive dataset where the microplastic exposure was deemed environmentally relevant. I = refers to the number of interactions and n = the number of studies respectively.

Figure 7.2 Figure 7.2 Interaction types by interacting stressor. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Interaction types: white = antagonistic, light grey = additive, dark grey = synergistic. The number of papers is given n = as well as the number of interactions I =. Only interacting stressors with I ≥ 8 are presented as per Jackson et al. (2016a).

Figure 7.3 Interaction types by response category. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Interaction types: white = antagonistic, light grey = additive, dark grey = synergistic. The number of papers is given n = as well as the number of interactions I =. Only response categories with I ≥ 8 are presented as per Jackson et al. (2016a).

Figure 7.4 Effect sizes by interacting stressor. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Confidence intervals overlapping 0 are non-significant and indistinguishable from additive effects, those significantly above show synergistic and those below demonstrate antagonistic effects. The number of papers is given n = as well as the number of interactions I =. Only interacting stressors with I ≥ 8 are presented as per Jackson et al. (2016a).

Figure 7.5 Effect sizes by response category. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Confidence intervals overlapping 0 are non-significant and indistinguishable from additive effects, those significantly above show synergistic and those below demonstrate antagonistic effects.

List of tables

for the colour, morphology, maximum size and polymer type (confirmed via FTIR) of each of the 10 particles are presented.

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“He who works like a slave eats like a king”- Robert F. Diggs

Author’s declaration

I (BP) confirm that the research presented within this thesis is my own. The following research papers were, however, published or submitted for publication in collaboration with Demetra Andreou (DA), Iain D. Green (IDG), J. Robert Britton (JRB), Katsiaryna Pabortsava (KP), Magdalena Barrow (MB), Victoria Dominguez Almela (VDA) Fátima Amat-Trigo (FAT), Adrian C. Pinder (ACP), Rosalind M. Wright (RMW), Rose M Boardman (RMB) and Michelle C. Jackson (MCJ), with their contributions to each publication outlined below. This thesis includes reformatted versions of these publications.

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Parker, B., Andreou, D., Green, I. D., & Britton, J. R. (2021). Microplastics in freshwater fishes: Occurrence, impacts and future perspectives. *Fish and Fisheries*, *22*(3), 467-488. https://doi.org/10.1111/faf.12528 **BP, DA, IDG and JRB conceived the ideas; BP analysed the data; BP led the writing with assistance from DA, IDG and JRB (Chapter 2).**

Parker, B., Britton, J. R., Pabortsava, K., Barrow, M., Green, I. D., Almela, V. D., & Andreou, D. (2022a). Distinct microplastic patterns in the sediment and biota of an urban stream. *Science of the Total Environment*, 156477. https://doi.org/10.1016/j.scitotenv.2022.156477 **BP, JRB, KP, IDG and DA conceived the ideas; BP, JRB, IDG and DA collected the samples; BP, KP and MB processed the samples; VDA produced the land use map; BP analysed the data; BP led the writing with assistance from JRB, KP, IDG, VDA and DA (Chapter 3).** The related study data are available from the following doi: 10.18746/bmth.data.00000214.

Parker, B., Andreou, D., Pabortsava, K., Barrow, M., Green, I. D., & Britton, J. R. (2022b). Microplastic loads within riverine fishes and macroinvertebrates are not predictable from ecological or morphological characteristics. *Science of the Total Environment*, 156321. https://doi.org/10.1016/j.scitotenv.2022.156321 **BP, JRB, KP, IDG and DA conceived the ideas; BP and JRB collected the samples; BP, KP and MB processed the samples; BP analysed the data; BP led the writing with assistance from JRB, KP, IDG and DA (Chapter 4).** The related study data are available from the following doi: https://doi.org/10.18746/bmth.data.00000212.

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1 Introduction

1.1 Overview

This introductory chapter introduces several important concepts and sets out the rationale and aims of the thesis. As an integrated thesis, Chapters 2-7 are reformatted from already published or submitted chapters. Chapter 2 is a systematic review of the literature that expands upon the introductory chapter and identifies knowledge gaps for further study, Chapters 3-6 are original research chapters from field and laboratory studies, Chapter 7 is a metanalysis of the interactive effects of microplastics and Chapter 8 provides a general discussion on the research aims and implications to conclude the thesis. A general references section is provided at the end due to the different referencing styles used in the different publications and to avoid duplication of references within different chapters.

1.2 Plastics and microplastic contamination

Microplastics (plastic particles > 1 µm but < 5 mm in size) are a relatively recent type of environmental contaminant, first discovered in the marine environment (Carpenter et al., 1972; Carpenter & Smith, 1972; Thompson et al., 2004) but since then ubiquitously found in both terrestrial and aquatic environments (Andrady, 2011; Cera et al., 2020; Mai et al., 2018). The incidence of microplastics in the environment has increased with the global use, production and disposal of plastics over the past century (Pietrelli et al., 2018; Rochman, 2020; Rodrigues et al., 2019). Microplastics form from the breakdown of larger plastics by physical, chemical and biological processes (Glaser, 2019; Kundungal et al., 2019; K. Zhang et al., 2021) or can be released within the size range from industry and/or cosmetic products (Godoy et al., 2019; Guerranti et al., 2019; Napper et al., 2015). Microplastics can then be further broken down by the same processes to eventually produce nanoplastics < 1 µm in size (K. Boyle & Örmeci, 2020; Gigault et al., 2018).

Most plastic waste, and therefore microplastics, originate from the terrestrial environment (Andrady, 2011; Galloway et al., 2017) where they may accumulate and be washed into aquatic systems by wind and rain (Bondelind et al., 2020; Y. Li et al., 2020; Pramanik et al., 2020). The transport times and fate of microplastics may be impacted through features of the particles, such as shape, size, polymer density etc., as well as the environment, for example the presence of barriers/accumulation zones (Christensen et al., 2020; Drummond et al., 2020, 2022), the interactions with biota and the proximity to microplastic sources (Christensen et al., 2020; Rummel et al., 2017; Wu et al., 2019). Aquatic sediments and water samples may therefore have different microplastic profiles that differ in both space and time according to microplastic sources, transport mechanisms and environmental factors (Drummond et al., 2020, 2022; Krause et al., 2020). Freshwaters may be especially susceptible to microplastic contamination due to their proximity to human activity and as pathways connecting terestrial and marine environments (Besseling et al., 2017; Galloway et al., 2017; Siegfried et al., 2017).

Aquatic biota can interact with and ingest environmental microplastics (Collard et al., 2019; Gouin, 2020; Windsor et al., 2019) either directly due to a similarity in sight or smell to prey (de Sá et al., 2015; Procter et al., 2019; Roch et al., 2020; Savoca et al., 2017) or indirectly by consuming contaminated resources (da Costa Araújo et al., 2020; Farrell & Nelson, 2013). The consumption of microplastics by biota may be related to the environmental loadings as well as the ecology of the organism with bioaccumulation where organisms accumulate particle loads over time(Horton et al., 2018; Jâms et al., 2020; Peters & Bratton, 2016) and that particles will biomagnify and produce higher loadings in organisms occupying higher trophic levels (Campbell et al., 2017; Garcia et al., 2021; Hurt et al., 2020). Microplastic loadings within biota are therefore suggested to be predictable from the environmental levels as well as information about the ecology and habitat of a species.

Consumed microplastics might result in a wide spectrum of effects from no effect through to negative consequences on ecology, physiology and survival across a range of taxa, life stages and ecological niches (Anbumani & Kakkar, 2018; Foley et al., 2018; Prokić et al., 2019). Microplastics are thought to negatively impact organisms through a number of mechanisms such as by damaging feeding structures during ingestion, obstructing digestive structures, causing pseudosatiation, releasing harmful internalised chemicals or vectoring bound pathogens or chemicals. Exposure may generally result in increased stress and therefore investment in immune activity or physiology away from reproduction and growth (Collard et al., 2019; Limonta et al., 2019; Zwollo et al., 2021). The impacts of microplastic exposure may be impacted by both the aspects of the affected organism and the exposure, for example the size, type and especially the environmental relevance of the microplastics used (Foley et al., 2018; Franzellitti et al., 2019; Strungaru et al., 2019). Understanding the impacts of microplastic exposure and features impacting particle ingestion is essential for the management of ecosystems and biota to use information about the particular threats faced by the organisms and the effects of each to implement effective management steps and ensure that the steps taken maximise output based on the resources used.

1.3 Multiple stressors and freshwater fishes

The recent increases in the human population and their associated activities have resulted in an increased threat to ecosystems globally in what has been proposed as the Anthropocene (Crutzen, 2006; Crutzen & Stoermer, 2000; Zalasiewicz et al., 2011). This period is defined by unprecedented anthropogenic changes to ecosystems and the rapid approach of planetary boundaries and tipping points which once passed may cause ecological cascades and catastrophes that cannot be rectified (Dakos et al., 2019; Rockström et al., 2009; Steffen et al., 2015). These threats to ecosystems, often termed multiple, ecosystem or anthropogenic stressors, can be specifically traced back to human actions and include climate warming, pathogens and environmental contaminants as well as a number of novel emerging threats such as light and noise pollution (Crutzen & Stoermer, 2000; Reid et al., 2019; Zalasiewicz et al., 2011).

The impacts of ecosystem stressors may degrade ecosystem provisioning, structure and function through disrupting regulatory processes and altering foodweb structure by impacting particular organisms and/or their interactions (Cardinale et al., 2012; Naeem et al., 2012; Rapport et al., 1985). Furthermore, ecosystems do not experience stressors in isolation therefore systems may be subject to different stressor combinations and levels in both space and time (Binzer et al., 2016; Jackson et al., 2021). Combinations of stressors may result in cumulative stress to organisms with the interactions of two or more stressors typically categorised into particular types, for example additive where the combined effect is equal to the sum of the individual effects, dominance where one stressor is driving most of the overall combined effect or synergistic where the combined effect is larger than the sum of the independent effects of the stressors (Côté et al., 2016; Jackson et al., 2016a). The type of interactive effect can be highly variable even for the same combination of stressors, for example environmental pollutants and/or warming may alter host-parasite interactions to increase parasite load by increasing the infectivity of parasites or by decreasing host immunity and therefore the susceptibility to parasites (Khan & Thulin, 1991; Lafferty & Kuris, 1999; Marcogliese & Pietrock, 2011).

The consequences of multiple stressors can manifest at different levels of organisation as well as through different effects depending on the niche of the organism and its level of stress, for example warming may increase the metabolism of an organism and perhaps the feeding whereas even higher temperatures might trigger physiological responses and eventually mortality (Comte et al., 2013; Jesus et al., 2018; Marshall et al., 2010). Organisms may vary individually in their capacity to tolerate stressors within certain thresholds but may generally respond to conditions outside of their particular niche by moving to more favourable environments, adaptation or if not may go extinct (Comte et al., 2013; Jesus et al., 2018; Marshall et al., 2010). Freshwater systems, and consequently their biota, may be especially susceptible to the impacts of ecosystem stressors due to the small landcover, the distribution of these areas and their general proximity to anthropogenic activities (Dodds et al., 2013; Jackson et al., 2016b). Freshwater ecosystems are typically threatened by stressors such as climate warming, pollution through the runoff of metals, agricultural nutrients, chemicals and contaminants, pathogens as well as the presence of invasive species (Jackson et al., 2016a; Ormerod et al., 2010; Reid et al., 2019). Biota in freshwater systems may be particularly vulnerable where life history traits and physical barriers (both natural and anthropogenic) may limit the relocation to different environments (Grill et al., 2019; Ormerod et al., 2010; Stendera et al., 2012). Finally, many freshwater systems are in close proximity to human settlements and activities that may depend on them for services such as drinking water, waste disposal and recreational activities (Dodds et al., 2013; Jackson et al., 2016b;

Stendera et al., 2012). From a management perspective, it is essential to understand the threats facing ecosystems in order to effectively preserve ecosystem structure and function as well as the resulting diversity and services for both conservation and human gain (Cardinale et al., 2012; Naeem et al., 2012; Stendera et al., 2012).

1.4 Freshwater fishes as a model system

Freshwater fishes are an ideal model system in which to examine the incidence, impacts and interactions of environmental contaminants as they are a highly diverse group with various life history traits (Craig, 2016; Froese & Pauly, 2019; Matthews, 2012). Furthermore, they are found globally in a range of different environments where they may be subject to varying levels of anthropogenic stressors such as warming, barriers, pathogens and contaminants whose single impacts are often already well understood (Lange et al., 2018; Maitland, 1995). Ecologically, freshwater fishes act as important prey and predators for a range of different organisms, including humans and terrestrial animals (Craig, 2016; Maitland, 1995; Matthews, 2012) and additionally act as intermediate and final hosts for countless pathogens (Barber et al., 2000; Bauer & Al., 1962; Iyaji & Eyo, 2009). Freshwater fishes adopt various feeding habits and feed at different trophic levels, often varying with ontogeny (Davies & Britton, 2015; Froese & Pauly, 2019; Mann, 1976), therefore they are excellent communities in which to examine how species traits impact microplastic ingestion as well as the single and combined effects of microplastic contamination.

Experimentally, freshwater fishes include several useful model organisms in mesocosm and laboratory experiments due to their tolerance and adaptability to different experimental conditions as well as the ease of lab breeding (Barber, 2013; Dai et al., 2014; Powers, 1989). A number of species can be easily and ethically maintained throughout experiments in factorial experiments looking at a broad spectrum of areas such as ecology, parasitology, physiology and immunology and collecting a variety of different measures (Barber, 2013; Dai et al., 2014; Powers, 1989). Since freshwater fishes will experience increasing cumulative stress from anthropogenic pressures in the future, it is essential that experiments utilise current and predicted exposure levels for different stressors and interactions to understand the actual and future expected responses to the different stressors in order to better conserve freshwater fishes and their environments (Lange et al., 2018; Maitland, 1995; Ormerod et al., 2010). Fishes may be exposed to precise levels of environmental contaminants presented through the water medium, alongside food or within food items while the use of earlier life stages can be essential for examining the fate of contaminants and the effects on development.

1.5 Thesis aims and objectives

Microplastic contamination is an important stressor facing freshwater fishes, however there is inconsistent data on the factors that impact loadings within wild organisms and thereby whether the levels and impacts might be predicted without lethal sampling/experimentation. Additionally, freshwater fishes simultaneously experience microplastic contamination alongside other stressors such as urbanisation and parasite infection whose relative impacts and interactions are poorly understood, particularly at environmentally relevant exposure levels. Consequently, the overarching thesis aim was to determine the predictability of microplastic loadings and interactive effects in relation to interacting multiple stressors. This aim was subdivided into objectives each with particular hypotheses:

1. Determine the environmental and biological factors impacting microplastic loadings within freshwater fishes (Chapters 2, 3, 4 and 6).

   It was expected that microplastic loadings would correlate within and between the biota and sediment, that loadings would vary temporally across the season and spatially with land use and that levels would be predictable from morphological and ecological characteristics of fishes.

2. Understand the effects of microplastic and parasite exposure on biometric indices and feeding within a freshwater fish final host (Chapter 5).

   It was hypothesised that microplastic and parasite exposure would have negative single and synergistic effects on feeding, condition, specific growth rate and splenosomatic index and that fish exposed to microplastics would experience higher parasite loads.

3. Examine the interactive effects of microplastics and additional stressors on freshwater fishes and the factors affecting the impacts (Chapter 7).

   It was hypothesised that microplastics would have predominantly multiplicative/synergistic negative effects with other stressors that were consistent across stressors, response categories and life stages. It was also hypothesised that studies using environmentally irrelevant exposures would overestimate the incidence of multiplicative interactions.

Each chapter had individual aims within the three objectives:

Chapter 2: Review the factors impacting microplastic loadings and impacts in freshwater fishes and identify knowledge gaps for further study.

Chapter 3: Examine the relationship between microplastic loads in the abiotic and biotic environment and how patterns might vary between compartments in space and time.

Chapter 4: Examine if microplastic loads within macroinvertebrates and fish are predictable from their ecological and morphological traits and if loadings vary with parasite load and trophic position.

Chapter 5: Determine if environmentally relevant chronic microplastic and parasite exposure have interactive effects on the parasite load and feeding rate in a freshwater fish.

Chapter 6: Quantify the level of microplastic contamination within an endangered fish species to understand the potential contribution to its conservation status.

Chapter 7: Determine the effect sizes and interaction types between microplastics and interacting stressors.

2 Microplastics in freshwater fishes: Occurrence, impacts and future perspectives

2.1 Abstract

Microplastics (MPs) are small, plastic particles of various shapes, sizes and polymers. Although well studied in marine systems, their roles and importance in freshwater environments remain uncertain. Nevertheless, the restricted ranges and variable traits of freshwater fishes result in their communities being important receptors and strong bioindicators of MP pollution. Here, the current knowledge on MPs in freshwater fishes is synthesized, along with the development of recommendations for future research and sample processing. MPs are commonly ingested and passively taken up by numerous freshwater fishes, with ingestion patterns often related to individual traits (e.g. body size, trophic level) and environmental factors (e.g. local urbanization, habitat features). Controlled MP exposure studies highlight various effects on fish physiology, biochemistry and behaviour that are often complex, unpredictable, species-specific and nonlinear in respect of dose–response relationships. Egestion is typically rapid and effective, although particles of a particular shape and/or size may remain, or translocate across the intestinal wall to other organs via the blood. Regarding future studies, there is a need to understand the interactions of MP pollution with other anthropogenic stressors (e.g. warming, eutrophication), with a concomitant requirement to increase the complexity of studies to enable impact assessment at population, community and ecosystem levels, and to determine whether there are consequences for processes, such as parasite transmission, where MPs could vector parasites or increase infection susceptibility. This knowledge will determine the extent to which MP pollution can be considered a major anthropogenic stressor of freshwaters in this era of global environmental change.

2.2 Introduction

2.2.1 Microplastics in the environment

The ubiquity of microplastics (MPs), small plastics particles < 5 mm in diameter (Barnes et al., 2009), has recently developed into an environmental issue of high societal concern, especially as MP pollution is intricately linked to the use of plastics in everyday life (Rodrigues et al., 2019). Primary MPs are deliberately manufactured within this general size range for use in industry or various cosmetic products (Godoy et al., 2019; Guerranti et al., 2019; Yurtsever, 2019), whereas secondary MPs form from the breakdown of larger plastics through physical, chemical and biological degradation (Kundungal et al., 2019; Raddadi & Fava, 2019; Sánchez, 2019; Winkler et al., 2019).

Microplastics are highly diverse and vary in size, shape, colour, polymer type and their constituent chemicals that all affect how they behave in the environment (e.g. their transport, degradation, adsorption capacity and ultimate fate). Nevertheless, variants of polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS) and polyvinyl chloride (PVC) account for 90% of all plastic polymers used (Andrady & Neal, 2009) and, therefore, most MPs also. All MPs can be considered as dynamic, being continually modified and degraded over time to produce ever smaller particles, eventually forming nanoplastics (NPs) < 1 µm (Gigault et al., 2018).

Microplastics were identified within the marine environment in the 1970s (Carpenter et al., 1972; Carpenter & Smith, 1972), though the term was introduced later by Thompson et al. (2004), with many studies subsequently identifying MPs in freshwater and terrestrial systems, where both water (Akdogan & Guven, 2019; Bank & Hansson, 2019; Wu et al., 2019) and wind (G. Chen et al., 2020; Y. Huang et al., 2020; Y. Zhang et al., 2019) are major transport mechanisms. Several studies have also highlighted that waste plastics, including MPs, in aquatic systems typically originate from the land, demonstrating the interconnectedness of aquatic and terrestrial systems (de Souza Machado et al., 2018; Malizia & Monmany-Garzia, 2019).

Plastic particles move throughout aquatic systems and float, sink or settle depending on particle properties (density, shape etc.), environmental features (water density, salinity, flow rate etc.) and aquatic processes (e.g. water currents and storm events) (Bondelind et al., 2020; Y. Li et al., 2020). The ultimate fate of MPs is to accumulate in sinks, such as river sediments (de Villiers, 2019; Simon-Sánchez et al., 2019). MPs may also become temporarily or permanently trapped within algal mats (Feng et al., 2020) or by physical barriers such as dams (Watkins et al., 2019). Environmental perturbations, such as flooding, weather events and habitat alterations, can then free previously trapped or sunk MPs into the environment, which then gradually pass through aquatic systems and biota (Ockelford et al., 2020; O'Connor et al., 2019; von Friesen et al., 2020).

Studies on ingestion reveal that environmental MPs are consumed by a range of different taxa across varying trophic positions, feeding types and habitats (Gouin, 2020; Ribeiro et al., 2019). Many of these studies have focused on marine organisms, especially taxa of ecological, economic or conservation interest (Casabianca et al., 2019; Katyal et al., 2020; Setälä et al., 2014; Ward et al., 2019). A secondary goal is often trying to understand the potential human exposure via ingestion of contaminated fauna (Oliveira et al., 2019; Prata et al., 2020; Rainieri & Barranco, 2019; Walkinshaw et al., 2020). Complementary studies have focused on developing understandings of how MP exposure affects animal physiology, population dynamics, ecology and behaviour (Anbumani & Kakkar, 2018; Franzellitti et al., 2019; Guzzetti et al., 2018; Prokić et al., 2019). Although the main research focus has been on marine species and systems, there is increasing knowledge on how microplastics behave and their consequent effects in freshwater (J. Li et al., 2018; Strungaru et al., 2019; Triebskorn et al., 2019).

2.2.2 Issues of MPs in freshwaters and freshwater fishes

Though covering only a relatively small proportion of the surface of the earth (< 0.01%), freshwaters are highly biodiverse and support a wide range of key ecosystem services (Dodds et al., 2013). They are also already at high risk from multiple anthropogenic stressors, including nutrient pollution, habitat loss, biological invasions and climate change (Jackson et al., 2016a; Ormerod et al., 2010; Reid et al., 2019). Consequently, freshwater MPs potentially represent an additional stressor, with freshwater environments also representing a critical target habitat for future MP remediation and mitigation strategies (Karbalaei et al., 2018; J. Wong et al., 2020). As much as 80% of aquatic plastic waste originates from terrestrial sources (Andrady, 2011) and often reaches marine environments via connecting freshwaters (Galloway et al., 2017).

Freshwater fishes comprise a highly diverse taxonomic group, covering a range of trophic positions, ecological guilds and life history strategies (Noble et al., 2007). With the exception of diadromous fishes, they spend their lives within a limited area, where the presence of anthropogenic barriers may further limit their range (Grill et al., 2019). Thus, freshwater fish populations and communities may be continuously exposed to a range of MPs throughout their lives and must adapt to, or tolerate all changes within their local environment, particularly where their movement is restricted. Freshwater fish are, therefore, a key receptor and bioindicator of MP pollution and so represent strong model taxa for developing knowledge on how MPs affect the ecology and behaviour of animals, from individuals through to community levels. Consequently, in this review, we synthesise the issues and knowledge gaps relating to MPs in freshwater fishes, and suggest future research directions and approaches. The objectives of this knowledge synthesis are to: i) summarise the major sources of and transport of MPs into freshwaters; ii) outline the major ingestion-egestion and processing pathways within freshwater fish; iii) detail the principal impacts of MPs on freshwater fish; and iv) outline a series of future perspectives on research priorities and approaches. Figure 2.1, below, summarises the sources, transport, processes and pathways relating to microplastics in freshwater fish discussed in this review.

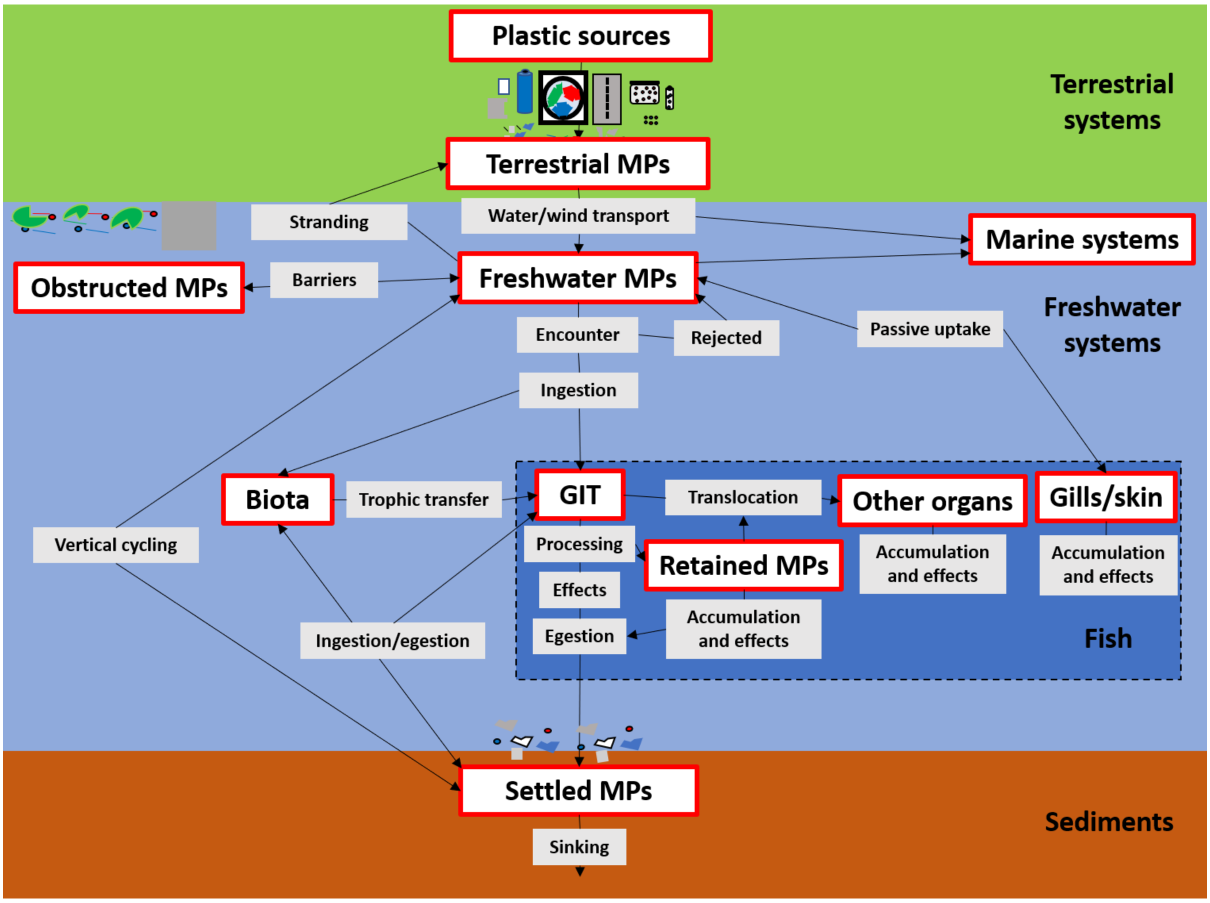


Figure 2.1. Generalised overview of microplastics in freshwater fishes including sources, transport and transfer between different biota and systems. Grey boxes indicate processes, red outlined boxes indicate abiotic and biotic compartments and arrows indicate the directionality of microplastic transfer. ‘MPs’ is used as an abbreviation for microplastics and ‘GIT’ for the gastrointestinal tract.

2.3 Occurrence of MPs in freshwater fishes: from sources to egestion

2.3.1 Sources of freshwater MPs

Most freshwater MPs originate from terrestrial systems (Andrady, 2011), with the extent of local urbanisation being a strong predictor of MP loadings in nearby water bodies, which are dependent on, and also a proxy of, local plastic usage and disposal (Kataoka et al., 2019; Tibbetts et al., 2018). The breakdown of larger plastic materials (including paints, tyres and litter) by physical, chemical and biological processes is an important source of secondary MPs to freshwaters (Fadare et al., 2020; Horton et al., 2017; Karbalaei et al., 2018; Knight et al., 2020). Recreational freshwater activities such as boating and angling may also contribute MPs or larger plastic waste directly into rivers and lakes through the degradation of plastic lines, nets, boats, waders etc., however these potential sources have yet to be investigated and quantified. MPs, along with larger litter, accumulate on impermeable surfaces or in dusts, and are washed into storm drains then water courses after sufficient rain (C. Liu et al., 2019; Roychand & Pramanik, 2020; Yukioka et al., 2019). Some MPs and smaller litter may also be transported by the wind and deposited in various urbanised or remote environments, where dispersal tends to be higher for smaller and lighter particles, particularly fibres (G. Chen et al., 2020; Y. Huang et al., 2020; Y. Zhang et al., 2020a).

The textile industry is another source of MP pollution as synthetic polymers such as polyester are commonly used to make clothing. The different synthetic materials used as well as the particular manufacturing process may dictate the sustainability and lifespan of the item of clothing (Janaina et al., 2020) and how many secondary MPs, typically fibres, are shed when the item is worn or washed (Belzagui et al., 2019; Henry et al., 2019; Hernandez et al., 2017; Napper & Thompson, 2016). Aspects of the wash cycle, the machine and the washed load may also impact both the number and type of fibres released (Cai et al., 2020; de Falco et al., 2019; L. Yang et al., 2019). Synthetic fibres from clothes washing, together with rinsed cosmetics and other flushable plastics (Guerranti et al., 2019; Morritt et al., 2014), then navigate the sewage system within wastewater.

This wastewater eventually passes through the sewage system to wastewater treatment plants (WWTPs), which treat domestic and industrial waste before release into the natural environment (often rivers and estuaries) (Ngo et al., 2019). Waste may undergo biological, chemical and physical processing to remove large debris, neutralise harmful chemicals and degrade biological materials, with these processes simultaneously degrading and modifying MPs throughout treatment (Enfrin et al., 2019; X. Li et al., 2019). Furthermore, there is evidence that MPs may reduce the efficiency of the treatment processes if they contain harmful internal or bound chemicals that can inhibit biological processing stages (Z. Zhang & Chen, 2020). While WWTPs vary in their treatment processes, even highly efficient WWTPs that approach 98% MP removal/exclusion still allow the daily discharge of substantial numbers of MPs in treated sewage effluents (Conley et al., 2019; Hidayaturrahman & Lee, 2019; Lee

& Kim, 2018). Waste sludge may accumulate up to 98% of MPs from the treated water (Gies et al., 2018; Nizzetto et al., 2016), which can account for up to 3% of biowaste by weight (Mohajerani & Karabatak, 2020), and is often modified for use as fertiliser if it is not incinerated or disposed at landfill (Edo et al., 2020; Rolsky et al., 2020). Agricultural application thereby provides a secondary opportunity for these MPs to directly reach water courses through runoff, wind dispersal and deposition, together with any MP-bound or internalised chemicals.

2.3.2 Transport of MPs in freshwater

MPs in freshwaters tend to move and behave according to plastic particle properties such as size, shape or polymer density as well as features and processes within the system (Bondelind et al., 2020; Figure 2.1). MPs may be transported floating, in the water column or carried along the bottom sediments and may have variable residence times in the environment, depending on whether MPs are ingested, impeded or sink and settle onto or into bottom sediments (Daily & Hoffman, 2020; Hoellein et al., 2019; Song et al., 2020a). Flow conditions and sediment type can often favour MP accumulation within the sediments, even for particles with relatively low polymer densities, resulting in concentrations often exceeding those of the overlaying surface waters (de Villiers, 2019; Frei et al., 2019; Simon-Sánchez et al., 2019).

MPs trapped within sediments may eventually permeate into groundwaters or aquifers before re-joining the water cycle (O’Connor et al., 2019; Re, 2019), or may be freed by storm and rain events that may resuspend trapped MPs and introduce them back into aquatic systems (Bondelind et al., 2020; de Jesus Piñon-Colin et al., 2020; Ockelford et al., 2020). Since freshwater and climatic factors may vary seasonally, there can also be some temporal variation, with environmental MP loadings typically being higher during wet seasons (Campanale et al., 2019; Eo et al., 2019; Kurniawan & Imron, 2019; Weideman et al., 2019; Yuan et al., 2019). Meandering and differences in flow and sediment profiles within systems can also produce spatial differences in MP concentrations between the littoral and mid-channel areas of rivers (G. Wong et al., 2020). The same processes also largely affect lakes and ponds, though MPs may persist in static water bodies for longer (Daily & Hoffman, 2020).

The high surface area to volume ratio, the degree of hydrophobicity and the surface structure of MPs may promote the colonisation of plastic particles by various microorganisms within natural aquatic systems, altering particle density and interactions with biota and other surfaces (Caruso, 2019; Shen et al., 2019). Degradation and modification of MPs in aquatic systems may also favour the release of internal chemicals (e.g. additives, plasticisers) and/ or the binding of various organic and inorganic chemicals present in the environment (e.g. metals, pharmaceuticals, fungicides) depending on environmental conditions, local concentration and MP properties (Caruso, 2019; Godoy et al., 2020; G.

Liu et al., 2019; Magadini et al., 2020; F. Wang et al., 2019). The modification of and binding by MPs in turn impact their transport, density and effects.

2.3.3 MP encounter rates in freshwater fishes

As ectotherms, fish activities and feeding rates are intrinsically linked to the environmental temperature, but will also vary according to the size, sex and metabolic activity of individuals, as well as the abundance, nutritional quality and processing time of their prey resources (Jobling, 1981). Consequently, these same factors may also govern the encounter and ingestion rates of MPs by fish. Although MP encounter by fish is assumed to occur mainly during active feeding, there is increasing evidence of MPs being encountered via the gills and/ or epidermis of wild freshwater fish (Abbasi et al., 2018; Hurt et al., 2020; Park et al., 2020a). Experimental studies have also demonstrated MP accumulation on the gills (Mak et al., 2019; Roch et al., 2020). Passive uptake of MPs is thus an additional route of MPs following environmental exposure during swimming and respiration. Collard et al. (2017) quantified the ingestion of anthropogenic particles in several marine fishes and found that ingestion was highest in the species with the most efficient filtration apparatus (high filtration area and small gill raker spacing). In a similar way, fish features such as gill surface area, gill structure, habitat etc. may correlate with numbers of MPs on the gills suggesting passive accumulation.

The foraging habitats of freshwater fish should also impact MP encounter rates, given MP distributions and loadings differ, with typically higher loadings in sediments compared to overlaying surface waters (Bondelind et al., 2020; Boucher et al., 2019; L. Li et al., 2019). Consequently, within a given location, pelagic species ought to encounter fewer floating MPs (e.g. less dense fibres and beads) than benthivores, with the latter then potentially encountering higher concentrations of sunk and settled MPs (e.g. denser fragments and films), provided there is also a relatively higher benthic MP concentration. Correspondingly, the trophic level of a fish and feeding guild can also affect MP exposure levels, with obligate piscivores potentially only encountering MPs passively or indirectly via ingested prey, whereas species in other feeding guilds are more likely to directly encounter MPs associated with vegetation or detritus (Hoang & Felix-Kim, 2020; Kalčíková, 2020; Ribeiro et al., 2019).

2.3.4 MP ingestion by freshwater fish

The ingestion patterns of MPs in freshwater fishes have been well documented (Table 2.1) and may vary depending on the encounter rate, MP characteristics and whether the particle is externally identified as a non-resource item (Collard et al., 2019; Markic et al., 2020). Gape size constrains the prey and MP sizes a fish can ingest, with larger fish generally having larger maximum particle sizes and/ or MP loadings (Dantas et al., 2012; Pegado et al., 2018; Ramos et al., 2012; Ryan et al., 2019), although there are exceptions (McNeish et al., 2018; Slootmaekers et al., 2019; Vendel et al., 2017). A general review of plastic ingestion in animals suggests that body size alone can explain as much as 42% of variations in plastics consumed by animals and that the ratio of the maximum ingestible plastic size for an animal relative to its body size is approximately 1:20 (Jâms et al., 2020). Sex has also been implicated as a possible explanation for differences in freshwater fish MP patterns (Horton et al., 2018; Su et al., 2019b), although this might be an artefact of body size, given patterns of sexual dimorphism in fish (G. A. Parker, 1992).

The issue of body-size is, however, complicated at the individual level, given that ontogenetic dietary shifts facilitate changes in prey items and sizes, which then result in changes in trophic position (TP) (Campbell et al., 2017; Hurt et al., 2020; F. R. Khan et al., 2020; McNeish et al., 2018; Roch et al., 2019). With their increased gape, body size, and trophic position, MPs are potentially accumulated in the gastrointestinal tract (GIT) of apex predators, as demonstrated in populations of largemouth bass (*Micropterus salmoides*, Centrarchidae) (Hurt et al., 2020) and northern pike (*Esox lucius*, Esocidae) (Campbell et al., 2017), which both had higher GIT MP levels compared to fishes of lower TP from the same system. However, pike have also been found to have fewer MPs than other fishes (Roch et al., 2019), suggesting some complexity in the transfer of MPs within food chains and the limitations of current ‘snapshot’ MP screening methods. Moreover, a recent general review of plastic ingestion in animals suggested no evidence of bioaccumulation or biomagnification (Gouin, 2020) where MPs cross the intestinal barrier then translocate into and accumulate within tissues, respectively. It should however be noted that ingestion research to date is heavily biased towards MP accumulation in the gastrointestinal tract which, while inside the body, is considered external as it is continuous with the environment at the mouth and anus, though several studies have identified MP bioaccumulation within the liver, brain and muscle of freshwater fish (Abbasi et al., 2018; Batel et al., 2016; J. Ding et al., 2020; Su et al., 2019b). The reported mean MP incidence of 20%, a mean of four particles per individual and a general range of up to 10 pieces per individual (Gouin, 2020) is largely consistent with ingestion studies in freshwater fishes (Table 2.1). The trophic transfer of MPs has been demonstrated experimentally from water fleas (*Daphnia magna*, Daphniidae) to fathead minnows (*Pimephales promelas*, Cyprinidae) (Elizalde-Velázquez et al., 2020), brine shrimp (*Artemia* sp., Artemiidae) to zebrafish (*Danio rerio*, Cyprinidae) (Batel et al., 2016) and from tintinnid (*Favella* sp., Ptychocyclidae) to inland silversides (*Menidia beryllina*, Atherinopsidae) (Athey et al., 2020). Thus, ingestion of MPs via resource items is a viable transfer mechanism in freshwater fish.

The relationship between fish consumption rates and MP exposure concentration followed a Type II functional response curve in banded tilapia (*Tilapia sparrmanii*, Cichlidae), with consumption of MPs even at low concentrations (Mbedzi et al., 2019). Experiments have also suggested that the probability of ingestion is elevated where MPs have similar characteristics (appearance, smell and/ or taste) to common prey resources (de Sá et al., 2015; Roch et al., 2020). The ingestion of a red MP fibre by a sight-feeding fish may occur, perhaps due to its similarity to chironomid larvae, whereas the binding or leaching of info-chemicals, such as dimethyl sulphide, can induce ingestion by taste-feeding marine copepods (Procter et al., 2019) and fish (Savoca et al., 2017). While dimethyl sulphide is not present in freshwaters, most likely through an absence of the microorganisms that produce it (Zink & Pyle, 2019), similar freshwater info-chemicals might be discovered.

The increased ingestion rates of MPs when coupled with a feeding cue is supported by experimental evidence that many fish will readily reject MPs except when they are presented in combination with food items (de Sá et al., 2015; S. W. Kim et al., 2019; Xiong et al., 2019). It could also explain why older MPs with altered structures and chemistry might be more likely to be consumed than virgin MPs, given that older MPs tend to degrade, develop biofilms or bind other chemicals over time (X. Chen et al., 2019; Song et al., 2020b). The adsorption and uptake of MPs by aquatic plants is another understudied concentrating mechanism that has the potential to increase the ingestion probability of associated MPs by herbivorous fish (Kalčíková, 2020). Nevertheless, debate remains over important questions such as whether fish are able to distinguish MPs from prey resources and to assess the suitability of MPs as a food source prior to ingestion, if fish learn to avoid or ingest MPs, whether ingestion is deliberate and under which conditions does MP ingestion increase (Huuskonen et al., 2020; B. Li et al., 2020; Peters & Bratton, 2016; Ramos et al., 2012; Ryan et al., 2019).

Table 2.1. Overview of studies accessible before the 15th May 2020 investigating microplastic ingestion in at least one wild freshwater fish (including estuarine/temporarily-freshwater fish). Studies are presented in chronological order. ‘Species’ denotes the number of species studied, ‘N’ the sample size, ‘FO’ the percentage frequency of microplastic occurrence, ‘Mean’ is the mean number of microplastics per individual and ‘Polymer’ lists the main polymers confirmed through chemical techniques.

Species	Country	N	FO	Mean	Polymer(s) †	References
3	Brazil	182	23	-	(PA) ‡	(Possatto et al., 2011)
2	Brazil	569	7.9	-	(PA) ‡	(Dantas et al., 2012)
3	Brazil	425	13.4	-	(PA) ‡	(Ramos et al., 2012)
1	France	186	12	-	-	(Sanchez et al., 2014)
4	Switzerland	40	7.5	-	-	(Faure et al., 2015)
44	USA	419	8.2	-	PP, PES, AC, PS	(Phillips & Bonner, 2015)
2	Tanzania	40	20	-	PE, PU, PET	(Biginagwa et al., 2016)
2	USA	436	45	0.8	-	(Peters & Bratton, 2016)
1	South Africa	70	73	3.8	-	(Naidoo et al., 2016)
1	Brazil	530	64.2	-	-	(Ferreira et al., 2016)
2	UK	76	66	0.5	PES, PA, AC, PET	(McGoran et al., 2017)
6	China	-	95.7	2.4	CE, PET, PES	(Jabeen et al., 2017)

1	Brazil	48	83	3.6	-	(Silva-Cavalcanti et al., 2017)
2	Switzerland	25	24	1.15	-	(Roch & Brinker, 2017)
69	Brazil	2233	9	1.06	-	(Vendel et al., 2017)
13	China	35	25.7	0.86	PE, PA	(K. Zhang et al., 2017)
5	Canada	181	73.5	3.28	-	(Campbell et al., 2017)
11	Argentina	87	100	19.2	-	(Pazos et al., 2017)
4	South Africa	36	100	-	-	(Naidoo et al., 2017)
3	Portugal	120	38	1.67	PE, PP, PET, PA, RAY	(Bessa et al., 2018)
3	Australia	93	-	1.37	PET, RAY	(Halstead et al., 2018)
1	China	30	60	4.3	PP, PE	(Cheung et al., 2018)
1	UK	64	32.8	0.69	PE, PP, PET	(Horton et al., 2018)
11	USA	74	85	-	-	(McNeish et al., 2018)
46	Brazil	189	13.7	1.2	PA, RAY, PE	(Pegado et al., 2018)
2	Brazil	125	-	-	-	(Silva et al., 2018)
1	France	60	15	0.15	PET, PP, PAN, PEVA	(Collard et al., 2018)
21	UK	876	32	-	PET, PA, PP	(McGoran et al., 2018)

16	Brazil	172	26.7	0.56	PE, PVC, PP, PA, PMMA	(M. C. Andrade et al., 2019)
1	Canada	74	59	1.15	-	(Collicutt et al., 2019)
1	Belgium	78	9	0.1	PET, EVA, PVC, PP, PVA, PA, CE	(Slootmaekers et al., 2019)
2	China	-	-	1.7	PE, PP	(Lv et al., 2019)
13	China	217	-	-	PET, PP, PE	(Su et al., 2019a)
2	Brazil	529	> 50	1.4/1.5	-	(Ferreira 2019a)
9	China	279	50	7	PE, PP, PET	(K. Zheng et al., 2019)
1	China	11	91	7.64	PE, PP	(Yuan et al., 2019)
1	Australia	180	19.4	0.6	PET, RAY, PA, PP	(Su et al., 2019b)
3	Brazil	529	58	1.46	-	(Ferreira et al., 2019b)
1	USA	44	100	9	-	(Ryan et al., 2019)
22	Germany	1167	18.8	0.2	-	(Roch et al., 2019)
1	Argentina	21	100	9.9	-	(Blettler et al., 2019)
2	USA	96	100	-	-	(Hurt et al., 2020)
2	Poland	389	54.5	1.16	-	(Kuśmierek & Popiołek, 2020)
4	South Africa	174	52	0.79	RAY, PET, PA, PVC	(Naidoo et al., 2020)

8	Thailand	107	72.9	1.76	-	(Kasamesiri & Thaimuangphol, 2020)
32	China	120	30/47	2.83	PE, PET, PP, PS	(J. S. Huang et al., 2020)
6	South Korea	6	100	22	PFTE, PE, RAY	(Park et al., 2020a)
2	Egypt	43	76.7	4.91	PE, PET, PP	(F. R. Khan et al., 2020)
22	Colombia	302	7	0.23	PA, EVA, Latex	(Garcés-Ordóñez et al., 2020)
4	China	126	-	-	-	(B. Li et al., 2020)

† Alphabetised polymer abbreviations: ‘AC’, acrylate; ‘CE’, cellophane; ‘EVA’, ethylene vinyl acetate; ‘PA’, polyamide; ‘PAN’, polyacrylonitrile; ‘PE’, polyethylene (high or low density); ‘PES’, polyethersulphone; ‘PET’, polyethylene terephthalate/polyester; ‘PEVA’, polyethylene vinyl acetate; ‘PTFE’, polytetrafluoroethylene; ‘PMMA’, polymethyl methacrylate; ‘PP’, polypropylene; ‘PS’, polystyrene; ‘PU’, polyurethane; ‘PVA’, polyvinyl alcohol; ‘PVC’, polyvinyl chloride; ‘RAY’, rayon.

‡ Denotes studies in which the polymer was reported without chemical confirmation.

2.3.5 MP processing and egestion

Following ingestion, the morphology of both the GIT and ingested MPs may affect their passage through the fish (Jabeen et al., 2017) and whether they temporarily or permanently lodge on to GIT structures, such as coils or projections. Differences in GIT structure have been suggested as a driver of species-specific differences in MP levels (Jabeen et al., 2017), although uncertainty remains over how particular MP morphometrics and physicochemical properties affect their passage rates through fishes and whether there are systematic biases in the MP screening of fish GITs as a consequence.

Internal environments within fish will differ in temperature and pH which may modify MPs and promote the release of certain harmful MP-associated chemicals (Coffin et al., 2019; F. R. Khan et al., 2017; Lee et al., 2019; Wu et al., 2020). These in vitro studies suggest that a variety of chemical and physiological cues in different species and sections of the GIT may change the absorption profile of MPs to leach out chemicals internalised within the particle as well as those bound to the external surface of the particle. Chemicals within MPs often include additives such as flame retardants and bisphenols which are added to plastics to achieve certain properties (Q. Chen et al., 2019; Gunaalan et al., 2020; B. Sun et al., 2019), while externally bound chemicals may include a variety of pharmaceuticals, fertilisers, pesticides and heavy metals encountered and bound in the freshwater environment (Atugoda et al., 2020; Bradney et al., 2019; Caruso, 2019; Guan et al., 2020). MPs may therefore vector or leach a range of different chemicals into fish and other biota that then produce effects according to factors such as the type of chemical, concentration, where the chemical is released within the GIT and whether the chemicals are taken up across the intestinal barrier (Bradney et al., 2019; Gunaalan et al., 2020).

MP size and shape are also important features affecting the processing of ingested MPs, particularly as small particles, typically < 10 µm, may translocate across the intestinal barrier, reaching the blood and eventually the rest of the body (Ribeiro et al., 2019). The range of particles that may cross the intestinal barrier does, however, vary with species and only particles of a particular shape, size and chemistry may pass (Ribeiro et al., 2019). The MPs ingested by fish may already be capable of translocation, however MPs may also be modified and degraded internally throughout the GIT by processes such as maceration, digestion etc. which may produce particles capable of translocation within fish, though this has yet to be investigated.

Within fish, translocated MPs, NPs and their associated contaminants have been recovered from regions such as the liver, muscle and brain (e.g. Abbasi et al., 2018; Batel et al., 2016; Ding et al., 2020; Su et al., 2019b), suggesting some risk of MP trophic transfer to piscivorous fauna. The MPs present in a fish at the point of capture should thus be considered a snapshot representing those currently trapped, as well as those yet to be egested or translocated. The varying individual diets, rates of internal physiological processes and time since egestion once caught/ euthanised will, therefore, provide varying GIT MP loadings, even if the fish are of the same species, size and sex, and from the same local environment. Additionally, processing tissues of the same fish may provide a greater level of data on fish MP exposure over a slightly longer time period. Tissue MP levels could be correlated to both gut concentrations and factors such as body condition which may depend more on translocated tissue MP concentrations than those accumulated, temporarily or permanently, in the gut.

Laboratory experiments have demonstrated that goldfish (*Carassius auratus*, Cyprinidae) may clear 50% of MPs within 10 hours and 90% within 33 hours of ingestion (Grigorakis et al., 2017), although egestion rates vary within and between species, depending on the MP and GIT structure, food availability and stomach fullness (Elizalde-Velázquez et al., 2020; Gouin, 2020; Hoang & Felix-Kim, 2020; Xiong et al., 2019). For example, fibres tend to accumulate at higher levels relative to fragments and pellets, as they are harder to egest (Qiao et al., 2019b). Faecal pellets may act as a concentrated source of MPs and organic material which may be utilised by a range of pelagic or benthic organisms as it sinks and settles, transferring MPs and material between biota (Hoang & Felix-Kim, 2020; Ribeiro et al., 2019).

2.4 Impacts of ingested MPs on freshwater fishes

Following ingestion, MPs can affect fish via three broad, non-mutually exclusive ways: (i) through physical effects of the MP itself (e.g. blocking the GIT or causing false satiation); (ii) the leaching of plasticisers, additives and other harmful chemicals from within the MPs; and (iii) by the desorption of harmful pollutants bound to the MPs (Strungaru et al., 2019). The effects of MP exposure on freshwater fish are thus highly variable (Table 2.2, Figure 2.2), depending on the interaction of the MP exposure (e.g. concentration, size, polymer and shape) and the ecology of the species. Effects vary from no effect to measurable changes in feeding rates, movement, gene expression, physiology, development, and/ or survival (Jovanović, 2017; W. Wang et al., 2019).

In general, much of the experimental MP literature for freshwater fish has focussed on using various life stages of cyprinids (Table 2.2), especially zebrafish, as the model species to test the effects of MP exposure, most likely due to their extensive use in toxicology research (Dai et al., 2014). The broad diet and different feeding behaviours of zebrafish (Froese & Pauly, 2019) allow several MP exposure methods, including presented paired with food items (Batel et al., 2018; Lei et al., 2018; Lu et al., 2016), within feed (Mak et al., 2019) and/ or via contaminated prey resources (Batel et al., 2016, 2020).

Within controlled experimental studies, freshwater fish MP exposure levels have been highly variable, based on the type, size and shape of MPs, and, taking weight alone, span at least five orders of magnitude from 10 µg l^{-1} (Qiao et al., 2019a) up to 6 g l^{-1} (LaPlaca & van den Hurk, 2020). While these exposure levels are largely within the variation of recorded freshwater environmental loads (e.g. C. Li et al., 2020; J. Li et al., 2018; Tibbetts et al., 2018), some higher exposure levels can be considered ecologically irrelevant based on these current freshwater data (e.g. LaPlaca & van den Hurk, 2020; Mazurais et al., 2015). Several studies have replicated specific environmental MP loadings appropriate to the life stage and habitat of the model fish (e.g. Mazurais et al., 2015; Rochman et al., 2014, 2017), with Naidoo & Glassom (2019) additionally also replicating the types and shapes of MPs in the exposure regime.

Several studies have demonstrated dose-dependent impacts of MP exposure on freshwater fish, though these effects may only occur at a particular MP concentration, suggesting MP thresholds for impact, with the relationship between exposure and impact thus being more complex than a simple linear dose-effect relationship (Lei et al., 2018; Mazurais et al., 2015; Qu et al., 2019; Y. Zhao et al., 2020). MP impacts are often exaggerated when the fish is co-exposed to an additional chemical (Banaee et al., 2019; Qiao et al., 2019b; Roda et al., 2020), although antagonistic interactions can also occur where MPs modulate the harmful effects of another chemical in the fish, or may also have no interaction at all (Hatami et al., 2019; Oliveira et al., 2013; Wen et al., 2018a). MP effects may also be life stage-specific, and are occasionally more detrimental to larval than adult fish, especially when MP exposure affects development (Pannetier et al., 2020).

Table 2.2. Overview of studies accessible before the 15th May 2020 investigating impacts of microplastics on freshwater fish (including estuarine/migratory-freshwater fish), grouped by taxa. Bracketed letters refer to the life-stage(s) of taxa: ‘A’, adult; ‘E’, embryo; ‘J’, juvenile; ‘L’, larvae.

Taxa	Impact(s)	Source
Acipenser transmontanus, Acipenseridae	Protein levels and feeding behaviour altered (J)	(Rochman et al., 2017)†
Ambassis dussumieri, Ambassidae	Growth, survival and body condition reduced (J)	(Naidoo & Glassom, 2019)
Barbodes gonionotus, Cyprinidae	Increased protein levels; epithelial thickening (J)	(Romano et al., 2018)
Carassius auratus, Cyprinidae	Reduced body weight; mouth and GIT damage GIT damage; oxidative stress; altered growth/activity (L)	(Jabeen et al., 2018) (Yang et al., 2020b)
Clarias gariepinus, Clariidae	GIT damage; protein levels/blood chemistry altered (J) Altered protein levels; liver damage; oxidative stress (J)	(Karami et al., 2016)† (Iheanacho & Odo, 2020)
Cyprinus carpio, Cyprinidae	Oxidative stress; protein levels/blood chemistry altered (J) Blood chemistry, protein and immune activity altered (J) Reduced growth; GIT damage; protein levels altered (L)	(Hatami et al., 2019)† (Banaee et al., 2019)† (Xia et al., 2020)
Danio rerio, Cyprinidae	No impact on protein levels or GIT structure (A) GIT damage; metabolism and protein levels altered (A)	(Batel et al., 2016)† (Lu et al., 2016)

	Altered protein levels in adults only (A + E)	(Batel et al., 2018)†
	GIT damage leading to some mortality (A)	(Lei et al., 2018)
	GIT damage; protein levels and gut biota altered (A)	(Jin et al., 2018)
	Altered protein levels in larvae only (E + L)	(LeMoine et al., 2018)
	Metabolism, protein levels and gut biota altered (L)	(Wan et al., 2019)
	Protein activity and swimming behaviour altered (L)	(Qiang & Cheng, 2019)
	Altered protein levels (E)	(Cormier et al., 2019)†
	Altered protein levels	(Qiao et al., 2019b)†
	GIT damage; protein levels and behaviour altered (A)	(Limonta et al., 2019)
	GIT damage; protein levels and behaviour altered (A)	(Mak et al., 2019)
	GIT damage; metabolism and gut biota altered	(Qiao et al., 2019a)
	Condition, protein levels and metabolism altered (A)	(Y. Zhao et al., 2020)
	No adverse effects (A)	(Batel et al., 2020)†
	Oxidative stress; protein levels altered (A)	(Yu et al., 2020)†
	Protein levels and metabolism altered; delayed hatching (E)	(Duan et al., 2020)
	Protein activity altered due to lead leaching (L)	(D. Boyle et al., 2020)

Dicentrarchus labrax, Moronidae	Protein levels altered and some mortality (L)	(Mazurais et al., 2015)
Fundulus heteroclitus, Fundulidae	Protein levels altered and some mortality (A)	(LaPlaca & van den Hurk, 2020)
Lates calcarifer, Latidae	Co-exposure impaired feeding and swimming (J)	(Guven et al., 2018)†
Menidia beryllina, Atherinopsidae	Reduced growth rate (L)	(Athey et al., 2020)†
Misgurnus anguillicaudatus, Cobitidae	Co-exposure induced oxidative stress (A)	(Qu et al., 2019)†
Oreochromis niloticus, Cichlidae	Blood and body chemistry altered; anaemia induced (J) Altered brain activity and metabolism (A)	(Hamed et al., 2019) (J. Ding et al., 2020)
Oryzias latipes, Adrianicthyidae	Protein levels, signalling and germ lines altered (A) Morphology, behaviour and protein levels altered (E + L)	(Rochman et al., 2014)† (Pannetier et al., 2020)
Pimephales promelas, Cyprinidae	No adverse effects (L) Protein levels altered and some mortality (A)	(Malinich et al., 2018) (LaPlaca & van den Hurk, 2020)
Pomatoschistus microps, Gobiidae	Metabolism and neurotransmission altered (J) Predatory efficiency and performance reduced (J) Protein levels and neurotransmission altered (J)	(Oliveira et al., 2013)† (de Sá et al., 2015) (Luís et al., 2015)†
Prochilodus lineatus, Prochilodontidae	DNA damage; blood, brain and protein activity altered (J)	(Roda et al., 2020)†
Symphysodon aequifasciatus, Cichlidae	Predatory behaviour, metabolism and signalling altered (J)	(Wen et al., 2018b)

	Oxidative stress; protein levels altered (J)	(Wen et al., 2018a)†

† Indicates studies investigating an interaction between MPs and an additional chemical

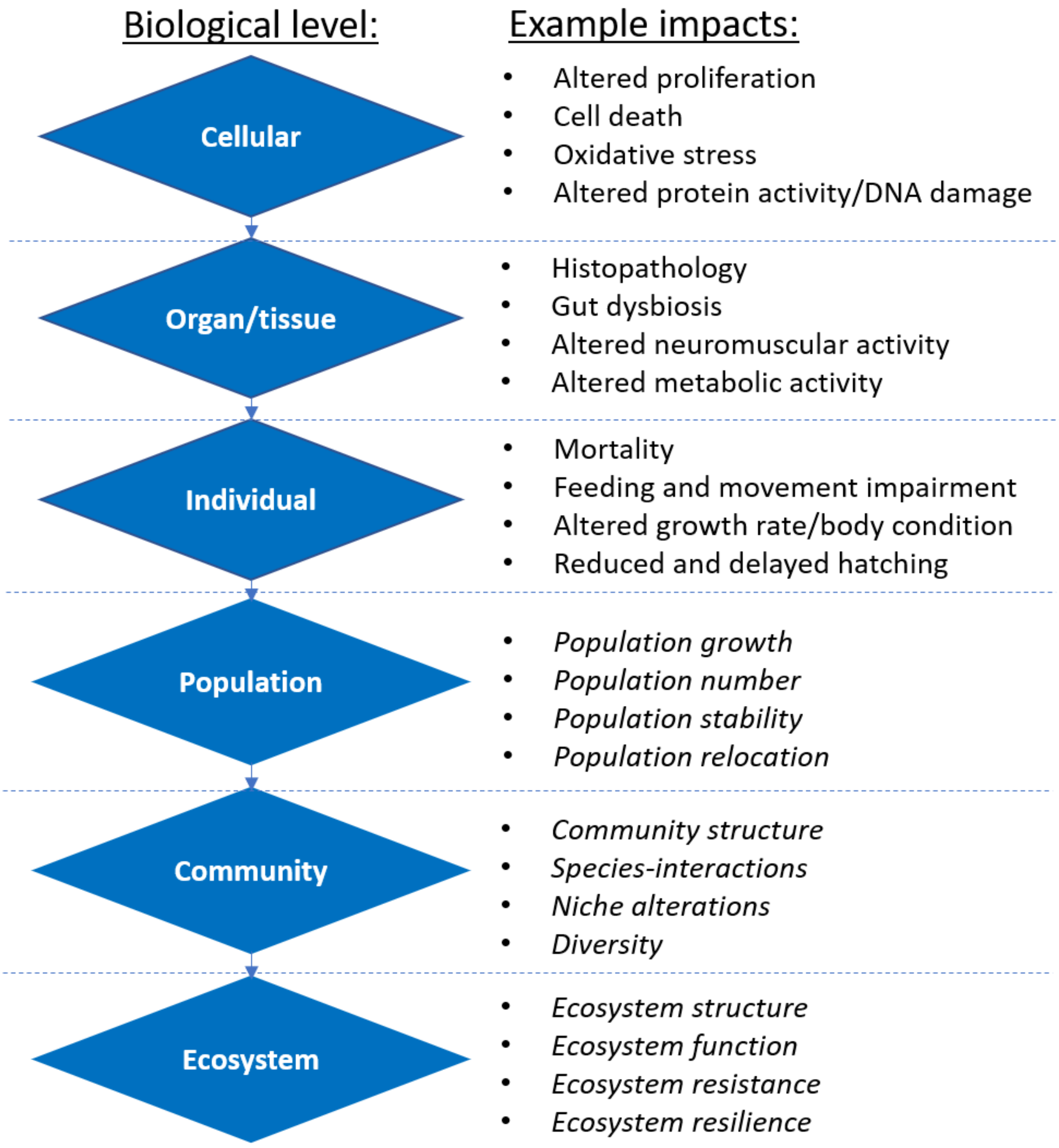


Figure 2.2. Overview of microplastic impacts on freshwater fish at different levels of biological complexity and how impacts can scale up to effects at higher levels of organisation. Several common impacts are given for each biological level. Population, community and ecosystem level impacts resulting from freshwater fish exposure are thus far unknown, and so suggested potential impacts are italicised.

2.4.1 Physiological impacts

The most common impacts of MP exposure have been recorded at the level of the individual fish, or lower, with impacts most typically including modified patterns of expression and/ or protein activity but also no effect (Figure 2.2, Table 2.2). Chemicals within or bound to the MP may also be released within the fish, causing a range of impacts including altered immune activity, expression or blood biochemistry in response to the foreign MP and any associated chemicals (Table 2.2). GIT oxidative stress and histological damage are common impacts at the organ/tissue level (Figure 2.2), where the processing of MPs and/ or any associated chemicals induce immune response, resulting in localised cell damage and altered morphology of physiological structures (Lu et al., 2016; Yu et al., 2020). Changes in GIT morphology may also alter the types and activity of symbiotic microorganisms, resulting in gut dysbiosis and altered metabolism (J. Ding et al., 2020; Jabeen et al., 2018; Qiao et al., 2019a; Xia et al., 2020; Y. Zhao et al., 2020). Typically, sufficient cellular level effects scale-up to tissue/organ then individual level impacts etc. as different numbers and types of cells are affected by MP exposure (Figure 2.2).

Blood biochemistry and immune biomarkers may indicate the absorption and translocation of MPs or associated chemicals across the gut, and are useful indicators for identifying dysfunction and damage in various non-GIT organs and tissues (e.g. Banaee et al., 2019; Hamed et al., 2019; Karami et al., 2016; Qiao et al., 2019b; Roda et al., 2020). Changes to blood composition may result in anaemia, altered immune function and nutrient supply throughout the body (e.g. Hatami et al., 2019; Karami et al., 2016; Roda et al., 2020). MPs in the brain may interfere with the endocrine or central nervous system, which can impact individual growth, body condition, behaviour and/ or survival (e.g. Athey et al., 2020; LaPlaca & van den Hurk, 2020; Lei et al., 2018; Xia et al., 2020). Functional disruption of key organs, such as the kidneys, liver and brain, can then impact body fluid composition, neurotransmitter and endocrine pathways (Walpitagama et al., 2019; X. Wang et al., 2019), with survival consequences for the individual (Figure 2.2).

2.4.2 Biological consequences

Behavioural alterations resulting from MP exposure often occur as MPs and/ or their associated chemicals impact cells in the brain or central nervous system which may negatively affect swimming activity and/ or survival in freshwater fishes (Limonta et al., 2019; Mak et al., 2019; Qiang & Cheng, 2019; Yang et al., 2020b). Impairments to swimming behaviour might be temporary, however some studies also suggest more damaging effects if MP exposure impacts early development (Duan et al., 2020; Pannetier et al., 2020). Fish eggs can also externally bind MPs and/ or uptake smaller NPs that can alter gaseous exchange and delay hatching times (Batel et al., 2018; Duan et al., 2020).

MP-induced reductions in predatory behaviour and efficiency have been demonstrated in juvenile common goby (*Pomatoschistus microps*, Gobiidae), most likely through affecting the discrimination of co-presented prey and MP items (de Sá et al., 2015). Changes to predator-prey dynamics may modify food webs and communities through altering interaction strengths (Figure 2.2), and is particularly relevant since higher trophic levels and predators appear to be more vulnerable to MPs than their prey (Q. Huang et al., 2020). While goldfish experimentally rejected MPs that could not be chewed and swallowed, this processing damaged the mouth (Jabeen et al., 2018), potentially affecting their subsequent foraging behaviours and consumption rates.

2.4.3 MPs as biological vectors

MPs are often implicated to aid in the binding and transport of various pathogens and invasive species (Caruso, 2019; Shen et al., 2019), however the potential role of MPs in macro-parasite transport remains unexplored. As MP uptake in fish occurs predominately through feeding (Gouin, 2020; Ribeiro et al., 2019), then the association of parasites to MPs may potentially benefit trophically transmitted parasites through increasing their transmission probabilities to fish hosts. MPs might also indirectly increase transmission rates and parasite virulence by suppressing the immune response and/ or the general condition of the impacted individual (Limonta et al., 2019; Luís et al., 2015). The trophic transmission of parasites can often involve parasite manipulation of the behaviour of the intermediate host to promote their ingestion by a final host (Thomas et al., 2010). For example, infection of three-spined stickleback (*Gasterosteus aculeatus*, Gasterosteidae) by the cestode *Schistocephalus solidus* (Schistocephalidae) results in infected fish foraging on smaller prey items (Barber & Huntingford, 1995) and altering habitat utilisation to increase predation encounter and subsequent parasite transfer to a final bird host (Barber et al., 2004). These parasite-mediated behavioural modifications can result in trophic differences between infected and uninfected fish within populations (Britton & Andreou, 2016), potentially also altering their exposure to MPs.

Nevertheless, the consequences of MPs for freshwater fish host-parasite relationships remain uncertain. In marine environments, anecdotal evidence suggests that MPs and parasites accumulate in the same part of the gut in seals (Hernandez-Milian et al., 2019), and fish closer to urban areas have both higher MP and parasite loads (Alves et al., 2016). Microplastics and trophic (ingested) parasites are also most likely accumulated in the same way, with typically more of both in larger and/ or predatory individuals (Lester & McVinish, 2016). Several exposure studies in freshwater fish have also suggested that MP exposure might increase susceptibility to parasites or disease (Lester & McVinish, 2016; Luís et al., 2015), though this was not studied. In addition, environmental plastics do attract and harbour distinct bacterial and fungal assemblages compared to natural particles, many of which can be pathogenic to fish, and may provide similar mechanisms for transporting parasites (M. Gong et al., 2019; Munier & Bendell, 2018; Vethaak & Leslie, 2016).

2.5 Future perspectives

2.5.1 Experimental approaches

MP exposure studies have revealed a wide range of effects in freshwater fishes (Table 2.2), but there remains considerable uncertainty in how these translate into measurable impacts in wild populations, particularly above the individual level (Figure 2.2). Understanding the impacts of MPs on freshwater fish requires, at least in part, controlled studies that enable the decoupling of the impacts of MPs from other stressors and that use appropriate exposure conditions and model species. Therefore, it is recommended that exposure studies initially test the effects of current (or predicted) ecologically relevant MP concentrations and ensure that the concentrations, sizes, shapes and polymers of the MPs reflect the natural exposure levels of that species and life-stage. In controlled studies to date, there has been a bias towards using spherical beads during exposure (e.g. de Sá et al., 2015; Mazurais et al., 2015; Oliveira et al., 2013), so there is also a need to further investigate the impacts on freshwater fish arising from exposure to fragments, fibres and films, especially as these are already known to have different egestion rates in freshwater fish (Qiao et al., 2019b). The actual exposure mechanism should also aim to simulate how a particular fish might encounter MPs in the wild, such as sprinkling floating MPs and food for surface filter feeders, but spiking resource items with MPs for species at higher trophic levels.

Most studies have exposed fish to MPs via the water when feeding (e.g. Batel et al., 2018; Lei et al., 2018; Lu et al., 2016), with this an appropriate exposure route for most of the investigated species and life stages to date, typically larvae/juveniles of cyprinid zooplanktivores. However, there have been fewer impact studies using benthic-feeding or piscivorous fishes (e.g. de Sá et al., 2015; Iheanacho & Odo, 2020; Karami et al., 2016; Oliveira et al., 2013), as well as a lack of studies exposing fish via contaminated resources (e.g. Batel et al., 2016, 2020; Rochman et al., 2017), despite this often being a more effective MP transmission route than coupled with commercial fish food (Athey et al., 2020). Consequently, there remains a lack of knowledge over whether benthic fishes, which often tend to ingest more MPs than pelagic feeders within the same environment (McGoran et al., 2017, 2018; McNeish et al., 2018), are similarly disproportionally impacted by MP exposure. Trophic transfer studies could additionally determine if MP transfer efficiencies are similar from prey to predatory fish as between invertebrates and fish, which might explain different MP ingestion patterns in wild fish occupying different niches and trophic levels (Campbell et al., 2017; Hurt et al., 2020; McNeish et al., 2018; Roch et al., 2019).

For scaling up from individual MP impacts to higher levels of biological organisation (Figure 2.2), the controlled conditions provided within mesocosm studies provide a strong experimental framework using fully-factorial designs that enable the effects of MPs on community structure and function to be quantified. Such studies enable complexity to be developed and investigated, including how MP impacts are affected by other stressors, such as warming and nutrient enrichment. For example, studies utilising mesocosms have experimentally demonstrated the trophic transfer of NPs across several trophic levels into top-predator fish (Chae et al., 2018; Mattsson et al., 2015), where quantified impacts included histological damage and alterations to feeding, shoaling behaviours and metabolism.

Alternative experimental approaches, such as exposure studies translocating organisms within mesh cages, also provide opportunities to identify how different species respond to different MP levels in the environment, as well as whether previous exposure to MPs may impact susceptibility to future exposure. Similar studies have already been completed in marine systems for blue mussel (*Mytilus edulis*, Mytilidae) (Kazour & Amara, 2020) and European flounder (*Platicthys flesus*, Pleuronectidae) (Kazour et al., 2018) and are another promising experimental framework for future MP research in freshwater fishes.

2.5.2 Nonfatal field sampling

Current field sampling methods tend to result in fish being euthanised for analyses in the laboratory that provide limited short-term data (Ferreira et al., 2016; Possatto et al., 2011). Correspondingly, future studies should consider using non-destructive sampling methods where feasible. For example, stomach flushing has been used to recover up to 95% of spiked MP samples from juvenile Mexican crocodiles (*Crocodylus moreletii*, Crocodilidae) (Gonzalez-Jauregui et al., 2019), with flushing techniques already used in fish dietary analyses (e.g. Correa & Anderson, 2016; Kamler & Pope, 2001). It should, however, be noted that stomach lavage is only suitable for larger fish and can potentially cause damage, and sometimes fatality, in smaller and/ or agastric individuals. The application of non-fatal MP recovery techniques can then be complemented by the ecological application of stable isotope analysis (SIA) that can provide temporal and spatial information on fish diet composition (Grey, 2006). While SIA would be unable to quantify MP loads in individual fish, it does enable information to be developed for populations in relation to their trophic (isotopic) niches (Jackson et al., 2012) and the extent of individual dietary specialisation (Araújo et al., 2011), that can be tested against the numbers of MPs recovered from the GIT. This complementary approach could also identify whether differences in MP loads between individuals are a consequence or driver of dietary specialisation (Britton & Andreou, 2016). For MPs encountered passively, such as those on the gills (Hurt et al., 2020) and epidermis (Abbasi et al., 2018), then the use of ‘skin scrapes’ and ‘gill swabs’ (e.g. Roberts et al., 2009; Young et al., 2008) could prove suitable and non-invasive methods to quantify this mechanism of MP uptake.

Research investigating the bioaccumulation of MPs has so far focused almost exclusively on those found in the GIT (Gouin, 2020), despite being continuous with the external environment and smaller MPs being known to translocate to other parts of the body (Ribeiro et al., 2019), including the liver, muscle and brain of freshwater fish (e.g. Abbasi et al., 2018; Batel et al., 2016; Ding et al., 2020; Su et al., 2019b). The development of non-destructive techniques for tracing MPs in fish organs can potentially utilise MP carbon isotopes, as these have been used experimentally to demonstrate the incorporation of MP materials by microbes and to track the trophic transfer of these particles into animals (Taipale et al., 2019), as fluorescent dyes are often problematic and less reliable (Schür et al., 2019).

2.5.3 Laboratory analyses

Reviews of current laboratory processing techniques highlight a range of different methods of digestion and MP analyses (Collard et al., 2019), with particular techniques working better for different species (Bianchi et al., 2020), but that might under- or over-estimate counts for particular polymers (Karami et al., 2017). A single, standardised procedure for all MP processing is, therefore, not possible, although attempts should be made to reduce the signal-noise ratio in samples and to comprehensively outline and critique the processing steps. In a review of plastic ingestion in wild freshwater fish, Collard et al. (2019) made numerous recommendations to standardise fish MP processing protocols, including on sample sizes, MP target size and visualisation methods. In addition to these recommendations, where additional density separation of samples is employed, floatation reagents should have a minimum relative density of 1.5 g cm^{-3} to maximise the number of MPs recovered from samples and to reduce systematic underrepresentation of denser plastics (Coppock et al., 2017; Quinn et al., 2017).

Contamination is a recurring problem in MP research and it is important that all reasonable attempts are made to eliminate contamination and to determine the efficacy of implemented contamination control steps by quantifying remaining sources of contamination. Best practice reviews suggest the wearing of non-plastic personal protection equipment, the use of laminar flow cabinets to minimise atmospheric sample exposure, carrying out procedural blanks, and ensuring reagents and equipment are filtered and cleaned prior to use are all effective ways to reduce contamination to acceptable levels (e.g. < 10% of sample counts) (e.g. Collard et al., 2019; Dehaut et al., 2019; J. Gong & Xie, 2020; Lusher et al., 2017; Scopetani et al., 2020). Studies should additionally outline the steps taken to reduce contamination, as well as declare any residual contamination and/ or subsequent data correction.

MP visual screening varies with individual experience and the particular shapes, sizes and colours of MP particles (Cadiou et al., 2020). MP screenings under microscopy should aim to be conservative and carried out blind, in a randomised order, with a pre-determined and standardised search time and search criteria to reduce bias and variation in counts. The processing of samples by multiple observers is another effective way of reducing individual bias by assessing inter-observer reliability.

Visual screening should also be supplemented by chemical confirmation, through spectroscopy or other techniques, on a subset of samples (e.g. 10%) to confirm the proportion of suspected MPs that are actually plastics and to determine polymer types. Automated techniques, such as image processing software, should also be favoured preferentially to more subjective manual visual processing (J. M. Andrade et al., 2020; Dehaut et al., 2019; Renner et al., 2019). Many studies on MPs in freshwater fish have not utilised chemical techniques, indicated by those studies in Table 2.1 without polymer data, and so for these studies estimates of MP incidence are likely to be unreliable and to include various other particles of non-plastic origin. As an example, Collard et al., (2018) identified approximately a quarter of anthropogenic particles as plastics while Slootmaekers et al., (2019) found only half of suspected MPs were actually plastic. The chemical analysis of suspected MPs is therefore critical to determine reliable MP counts and to assess the actual risks of MP pollution faced by freshwater fish.

2.5.4 Scaling up complexity

Much MP research in freshwater fish has been on single species (Table 2.1) and has focussed on individual level effects and below (Table 2.2, Figure 2.2). Consequently, as knowledge develops, there is a need to fill in the gaps to understand MP impacts at population, community and ecosystem levels (Figure 2.2), as well as continuing to investigate the relative impacts of body size, sex, trophic level and other traits that affect MP ingestion patterns. Field studies should aim to collect representative communities with sufficient numbers of fish per trophic level and functional group (excluding those of conservation concern), with consideration of sampling across different seasons and under different river flows and levels.

2.6 Conclusions

Both field and laboratory approaches have, to date, contributed knowledge on how particular characteristics of freshwater fish affect the encounter, ingestion, effects and egestion of MPs. Studies highlight considerable variability in ingestion patterns and effects, but that these differences can generally be explained through a combination of traits of the fish (e.g. size, trophic level, habitat), its environment (e.g. the extent of local urbanisation) and the MP characteristics (e.g. size, shape, polymer, internal/external chemicals). Future field research should identify MPs in novel fish species and locations, and develop population level and community-wide assessment approaches using standardised methods that maximise MP recovery while minimising contamination. Experimental studies should ensure that exposure regimes and routes are environmentally relevant and investigate uptake and effects in a wider range of species, including piscivorous and benthic fishes. The interactive impacts of MPs with other anthropogenic stressors are also required, given that MP pollution is likely acting as a further stressor in environments already exposed to multiple stressors. Data generated through field- and laboratory-based studies can then move towards a more quantitative assessment of the risks faced by particular freshwater fish, with criteria on freshwater MPs able to be incorporated into freshwater monitoring programmes and species action plans.

3 Distinct microplastic patterns in the sediment and biota of an urban stream

3.1 Abstract

Urban freshwaters, their sediments and resident biota are often highly susceptible to microplastic contamination from catchment-specific sources. Water velocity and spatiotemporal dynamics within the system can impact microplastic loads, while biological features may additionally impact levels within freshwater biota. Here, we investigated the spatiotemporal variations in microplastic loads collected from sediment, macroinvertebrate and fish samples from an urban watercourse (Bourne Stream) in Dorset, southwest England. Sediment particles were mostly fragments of colours (especially orange and purple) whereas microplastics in both macroinvertebrates and fishes were blue/green and fibres. Across all sample types, the dominant particle size class was ≤ 100 µm. Median (M) and range (R) of microplastic loads within each sample type were sediment: M = 0.06, R = 0-0.36 particles g^{-1}; macroinvertebrates: M = 0, R = 0-4 particles per batch; and fishes: M = 1, R = 0-6 particles per individual. Sediment loads varied spatially, with the highest load in the most upstream site, whereas biotic loads did not vary across space and time. Macroinvertebrate batch loadings varied between taxa and feeding guild, with counts significantly higher in annelids but lower in herbivores. Fish counts were higher in species with true, differentiated stomachs, but with the effects of species, feeding guild and body size being non-significant. Within sites, mean microplastic loads did not correlate between sediment, macroinvertebrate and fish samples. These results suggest that sediment freshwater microplastic loadings may vary spatially but that these trends are not reflected by, or correlated to, those in the biota where ingestion varies with biological traits. Assessments of freshwater microplastic contamination must therefore consider sampling spatiotemporally and across different biotic communities to fully understand the scale of contamination, and to subsequently undertake effective mitigation steps.

3.2 Introduction

Microplastics (plastics < 5 mm in maximum size) are a form of environmental contaminant whose prevalence throughout freshwaters has been revealed in recent decades and that are thought to negatively impact a range of organisms (Barnes et al., 2009; Campbell et al., 2017; Eerkes-Medrano et al., 2015). Most aquatic microplastics originate from the terrestrial environment (Andrady, 2011) and, in freshwaters, typically originate from secondary particles produced through the washing of synthetic clothes, the breakdown of larger plastics and from tyre wear particles (Siegfried et al., 2017; van Wijnen et al., 2019). Particles may be washed or deposited into freshwaters through rain and wind respectively (Dris et al., 2016; Eerkes-Medrano et al., 2015). Particle features (e.g. shape and polymer density) and hydro-morphological conditions (e.g. water velocity and river morphology) influence the riverine movement of microplastics (Daily & Hoffman, 2020; Hoellein et al., 2019).

Riverine particles can have very short residence times, travelling several kilometres within hours, but can also have prolonged residence times under lower flow conditions where increased particle settling and/or obstruction may occur (Drummond et al., 2020, 2022). Trapped microplastics, including buoyant particles, often accumulate within sediments (de Villiers, 2019; Frei et al., 2019; Simon-Sánchez et al., 2019), and may be aided by the formation of biofilms on the particle that aid sinking (Besseling et al., 2017). Flooding events can remobilise trapped particles and may export 70 % of microplastics from riverine sediments (R. Hurley et al., 2018). Spatiotemporal variations in local conditions, microplastic sources and transport may influence the fate and interactions of microplastics, resulting in differences in sample loads over time and space (de Carvalho et al., 2021a; Park et al., 2020b; Rodrigues et al., 2018). For example, one study found 74 % of plastic emissions from riverine into marine systems occurred between May and October (Lebreton et al., 2017).

The ingestion of freshwater microplastics by macroinvertebrates and fishes is now well documented (Collard et al., 2019; B. Parker et al., 2021; Windsor et al., 2019), where microplastic loads within the biota are often related to the prevailing environmental conditions and the biological traits of the focal species (Garcia et al., 2021; Horton et al., 2018; Park et al., 2020b). Microplastic loads within macroinvertebrates and/or fishes have been shown to be higher in organisms with higher trophic positions (Garcia et al., 2021), larger body sizes (Garcia et al., 2021; Horton et al., 2018; Park et al., 2020b) and in demersal- (bottom) relative to column-feeding fishes (Merga et al., 2020). Biological traits, such as the structure of the gastrointestinal tract, are also thought to impact the processing and/or egestion of microplastics (Bosshart et al., 2020; Jabeen et al., 2017; Roch et al., 2021). For example, the lack of a complete stomach in some fishes (agastric condition) may impact particle egestion and therefore microplastic loads. Collectively, these findings suggest microplastic loads within individuals should be predictable according to their biological traits.

Urban rivers typically connect terrestrial to marine systems and are often areas of concentrated human population density that may experience frequent urban runoff and effluent discharge, exposing these systems and their biota to the effects of microplastic contamination (Lebreton et al., 2017; Siegfried et al., 2017; van Wijnen et al., 2019). Furthermore, as urban freshwaters tend to be highly modified, with dams, weirs and channels all present to aid flood relief and urban drainage (Grill et al., 2019), these may trap and accumulate particles (Lebreton et al., 2017; Mani et al., 2015; K. Zhang et al., 2015). Differences in waste sources and river hydrology across space and time may influence particle loads and therefore corresponding levels within the environment and biota of urban freshwaters.

Studies have previously demonstrated the occurrence of microplastics in the environment and biota of large urban freshwaters (de Carvalho et al., 2021a; Garcia et al., 2021; Park et al 2020a, 2020b).

However, the complexity of these systems due to the variety of different microplastic sources within a large catchment area potentially makes it difficult to understand spatiotemporal variations and the relationship between abiotic and biotic particle loads. Consequently, the present study quantified microplastic loads within the sediment, and within the macroinvertebrate and fish communities of a small urban watercourse, where microplastic inputs are primarily through runoff. The relationships in microplastic loads were tested between the different sample types, and according to seasonal and site differences, as well as the biological traits of the sampled biota. We hypothesised that 1) microplastic loads significantly increase with distance downstream towards an urban centre (Bournemouth), 2) microplastic loads are highest in winter months due to higher rates of runoff, and 3) microplastic loads are higher in organisms occupying higher trophic levels (omnivores and carnivores) as well as in fishes that are larger, demersal-feeding and gastric (with complete stomachs and differentiated gastrointestinal tracts).

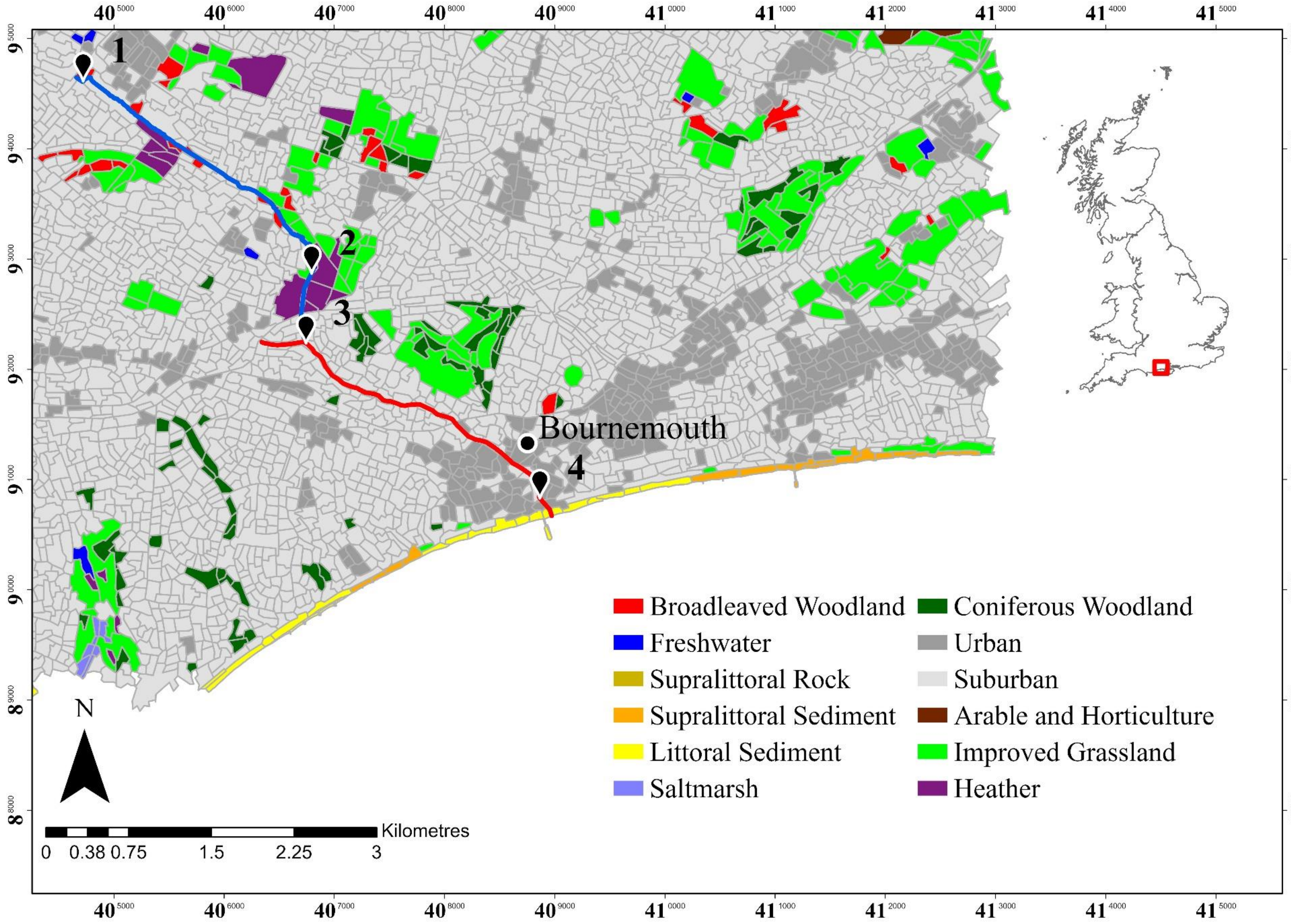
3.3 Materials and methods

3.3.1 Study site and sampling

The River Bourne or Bourne Stream (hereafter 'Bourne') is an urban watercourse in southwest England that is 7 km long, with two narrowly separated tributaries totalling 13 km of waterway, and with a catchment size of approximately 14 km² (Figure 3.1). The Bourne is entirely within the highly suburban Bournemouth-Christchurch-Poole conurbation and passes through areas of Poole and Bournemouth, including Bournemouth town centre, before its confluence with the sea. The upper tributary starts below a major road and passes through suburban areas as well as heathland areas whereas the lower tributary passes through busier public parks and gardens then through the town centre (Figure 3.1). Under normal conditions, the Bourne is < 5 m in maximum width and < 1.5 m in maximum depth,and includes various physical modifications such as weirs and grates, with the lower section also being stone-channelled. The gradient, size and general land use of the catchments means that although the water velocity and level tend to respond relatively rapidly to heavy rainfall, flooding frequently year-round, the Bourne typically returns to normal levels within several days. The water sources of and to the Bourne are poorly documented, although the stream is believed to receive most of its water from the nearby Bournemouth Water output (originally sourced from the local Hampshire Avon and/or Dorset Stour rivers), surface runoff (there are approximately 60 documented surface water discharges, although the locations and exact contributions are not known) and as drainage from the heath area (Bourne Stream Partnership, 2000).

Four sites were selected for sampling along the entire length of the stream that were representative of the general land cover and stream features (Figure 3.1, Table 3.1). Site 1, just downstream of the source, was close to a suburban area and major road, site 2 was within a heath area, mostly accessible by foot, site 3 was within a suburban public park and garden in the lower tributary, and site 4 was within Bournemouth town centre. All sites were sampled on five occasions between April 2019 and January 2020 (24th April 2019 and 1st May 2019; 8th July 2019; 1st October 2019; 4th December 2019 and 21st January 2020), outside of flooded periods to exclude the impacts of flood events. Sediment samples were collected on all occasions for all sites except for site 4, within the lower stone-channelled section, as no fine sediment accumulations were present. Sediment samples were collected using a customised soft sediment suction corer made of metal (10 cm diameter × 15 cm height, Figure S1.1). Three samples were collected from the middle of the watercourse within straight sections at 1 metre intervals (replicates 1, 2 and 3, respectively) for the three sites on each sampling occasion ($3 \times 3 \times 5 = 45$ samples). Samples were transferred into clean glass jars with the aid of metal spoons before thoroughly rinsing the equipment between samples with river water to prevent any carryover. The layering of each core sample was not preserved and wet samples were kept at room temperature until processing.

Macroinvertebrate samples were collected on all sampling occasions by kick sampling with a standard 1 mm mesh hand net. Care was taken to sample all microhabitats (e.g. gravel beds, vegetation, deeper pools, and riffles) within each site and continued until > 50 organisms had been collected. Organisms were transferred into containers and frozen at -4 °C until later processing. In contrast, fish samples were collected on two separate occasions only (27th September 2019 and 30th January 2020), as lethal sampling on more occasions was considered inappropriate due to the absence of prior data and the assumed limited abundance of the fish assemblage. A total of 160 fishes were sampled, with collection of 20 fish per site per sampling occasion that were representative of the local fish community and size ranges. Five fish species were sampled for microplastic analyses: stone loach *Barbatula barbatula*, three-spined stickleback *Gasterosteus aculeatus*, minnow *Phoxinus phoxinus*, roach *Rutilus rutilus* and chub *Squalius cephalus*. Sampling was carried out using a combination of electric fishing (Smith Root LR24) and dip netting until sufficient fish were collected. Fishes were euthanised in the field in line with a Schedule 1 Method of Humane Killing under the 1986 UK Animals (Scientific Procedures) Act (concussion then destruction of the brain) before freezing at -4 °C until later processing.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2
3
4
Bournemouth
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0.75
1.5
2.25
3
Kilometres
Broadleaved Woodland
Freshwater
Supralittoral Rock
Supralittoral Sediment
Littoral Sediment
Saltmarsh
Coniferous Woodland
Urban
Suburban
Arable and Horticulture
Improved Grassland
Heather

Figure 3.1. Location of the Bourne Stream and land cover within the catchment area. The upper (blue) and lower tributary (red) are coloured and the four sampling sites are numbered. Land cover categories are based upon Land Cover Map 2020 © UKCEH 2021. The initial river layers were extracted as shapefiles from Ordnance Survey data (OS Open Rivers, 2021) followed by removal of catchments and drainage outside the Bourne scope using ArcGIS Pro (version 2.7.1). Contains Ordnance Survey data © Crown Copyright 2007 (100017572) and 2021 (Open data).

Table 3.1. Bourne Stream site information. For each site: Dist is the distance downstream, WV the mean water velocity (± SE) collected during the sampling period, D the depth range and Sediment gives the sediment structure.

Site	Dist (km)	WV (m s^{-1})	D (cm)	Sediment
1	0.07	0.12 ± 0.02	5-30	90 % silt, clay and fine sand 10 % gravel
2	2.9	0.19 ± 0.04	10-40	80 % gravel 15 % silt, clay and fine sand 5 % cobbles
3	3.6	0.30 ± 0.05	5-50	70 % gravel 20 % cobbles 10 % silt, clay and fine sand
4	6.4	0.31 ± 0.03	5-20	95 % cobbles (stone channelled) 5 % silt, clay and fine sand

3.3.2 Sediment sample processing

Due to considerable variations in sediment types and volumes, wet sediment samples were first filtered using a lidded stainless-steel sieve stack and filtered water to remove materials ≥ 5 mm (therefore not microplastics) and very small particles < 38 µm. Jars and sieves were thoroughly rinsed through several times and the filtered material dried overnight at 50 °C within metal containers. The dried sediments were then thoroughly mixed using glass-/metalware and a 50 g subset (44 g for a single sample) removed. Subsamples were subject to density separation, adapted from Rodrigues et al., (2020), by mixing for 2 min in a 100 ml solution of zinc chloride (1.5 g cm^{-3}) within a glass beaker, allowing the covered beakers to stand for 30 min and then drawing up the supernatant using a widened fresh glass pipette. The supernatant was rinsed through several times with filtered water then vacuum filtered through a 13 mm diameter 26 µm mesh stainless steel circular filter (The Mesh Company, Warrington, UK), which was kept within a foil capped container and allowed to dry. The zinc chloride was recycled and reused following a standard method (Rodrigues et al., 2020).

3.3.3 Biotic sample processing

In the laboratory, the macroinvertebrate samples were defrosted, rinsed in filtered water to exclude any external particles, and grouped into batches of up to five of the same taxa within samples, as per Garcia et al. (2021). Batches were transferred into glassware and the number of organisms was recorded. Individual fish samples were defrosted and identified to species before recording the standard length (nearest mm). Samples were then carefully dissected to remove the entire gastrointestinal tract which was transferred into a glass container. Whole macroinvertebrate batches and fish gastrointestinal tracts, including their contents, were digested through submersion in 30 % hydrogen peroxide (3:1 reagent:sample volume) at 60°C under gentle rotation (30 rpm) for 48 h until clear (excluding shells). The resulting material was then vacuum filtered through a 13 mm diameter 26 µm mesh stainless steel circular filter (The Mesh Company, Warrington, UK), thoroughly rinsed through twice with filtered water, and was allowed to dry within a foil capped container.

3.3.4 Microplastic microscopy and spectroscopy

Entire filters were screened for suspected microplastics using microscopy (Leica M165C) at up to 120× magnification for a set 5 min search period in an attempt to standardise search effort. All suspected microplastics were identified that met standard criteria, such as unnatural colours, consistent shapes and lacking biological features (Nor & Obbard, 2014). Individual particles were allocated a shape morphology (bead: near-perfect spherical; fibre: long, thin and flexible or fragment: irregular 3D shape), colour category (blue/green, grey/black, pink/red, other) and a size class ((26-100, 101-200, 201-300, 301-400, 401-500, 501-600, 601-700, 701-800, 801-900, 901-1000 and 1001-5000 µm) based on the longest particle dimension, measured at 120× magnification using the eye-piece graticule.

To assess both the quality of microplastic identification and identify the particular polymers for a subset of particles, 200 suspected microplastics ≥ 100 µm in size were selected for polymer analysis using a micro-Attenuated Total Reflectance (micro-ATR) accessory attached to a Spotlight™ 400 FTIR Imaging System coupled to a Frontier™ IR Spectrometer (PerkinElmer, Llantrisant, UK). Due to COVID-19 pandemic related constraints, no additional particles < 100 µm were analysed. Particles were scanned from 650-4000 cm^{-1} (mid-IR region) at 8 cm^{-1} spectral resolution and 10 accumulations (co-added spectra) per scan, using a background collected in air using the same settings, though with additional co-added spectra (n = 120). Sample spectra were compared to a reference polymer library (18,711 polymer types; spectra database from S.T. Japan-Europe GmbH, Germany/Japan) using PerkinElmer Spectrum™ 10 software to identify the top 5 highest scoring matches. An arbitrary match score of ≥ 70% was considered a successful match and each particle was assigned to a successful matching hit. As particles were already suspected to be microplastics, special priority was given to successful plastic then additive hits when assigning particles to one of the top scoring matches. Individual polymer hit types were later grouped into broader categories: polyolefin, polyester, polyamide, other-plastic, additive, and non-plastic.

3.3.5 Quality assurance and control

The environmental exposure time of the samples was minimised both in the field, through careful storage and rinsing. Within the laboratory, samples were only uncovered when adding reagents and vacuum filtering (both stages performed within a pre-cleaned flow cabinet) and under the microscope when screening for suspected microplastics and selecting particles for FTIR analysis. All equipment was cleaned prior to use through rinsing with filtered water (1.2 µm, Whatman glass microfibre filters) and/or furnacing. Reagents were also filtered prior to use (1.2 µm, Whatman glass microfibre filters). Previous studies indicate that hydrogen peroxide can damage and discolour common polymers, producing white/clear materials and leading to underestimates (Nuelle et al., 2014). Since hydrogen peroxide digestion of biotic samples occasionally produced white and/or clear remains and white/clear equipment was used throughout sample processing (e.g. glass vials, Whatman filters, squeezy bottles, white cotton lab coat), all white/clear materials were excluded during screening.

Additionally, 5 sediment and 61 biotic hydrogen peroxide procedural blanks (> 10 % of each sample type) were carried out and processed as above to determine background contamination levels. The biotic blanks were collected for two sets of samples with identical methods and processed simultaneously. Although 7 suspected fibre contaminants were recovered from biotic blanks, no corrections were applied as their colours were highly variable and inconsistent. By contrast, black fibres were recovered from 60 % of the sediment blanks, therefore all black fibres were excluded from sediment screens. Early sample processing revealed some turquoise fragment contaminants which were traced to a broken pump valve which was immediately replaced and all resulting contaminants excluded.

3.3.6 Statistical analyses

All analyses were performed in R version 3.5.1 (R Core Team, 2018). Due to observed overdispersion in the data, Akaike Information Criterion (AIC) values were used to compare the fit of a saturated Poisson family generalised linear model (GLM), with an identical saturated negative binomial variant (NBGLM), excluding interactions, of each model. The NBGLM variant was selected where its AIC value was at least two points lower than the competing standard Poisson model and all AIC values for each pair of models are given in the results. Sediment sample counts were first standardised by dry sub-sample mass (typically 50 g) and were then related to the replicate number (indicative of distance downstream within the site), sampling site and month using a GLM. For the macroinvertebrates, batch microplastic counts were tested using a GLM, with taxa, number of organisms within the batch, site and sampling month as fixed factors. A separate GLM tested for differences in loads between macroinvertebrate feeding guilds (detritivore, herbivore, omnivore and predator). For testing differences in microplastic counts within individual fishes, a NBGLM was performed using species, standard length (pre-scaled) and site as fixed factors. Separate independent NBGLMs were additionally carried out to determine any differences in counts between fishes with and without distinct stomachs (agastric fishes have a continuous and undifferentiated gastrointestinal tract) and feeding guild (benthopelagic or demersal), assigned using species data from FishBase (www.fishbase.org; Froese & Pauly, 2021). Finally, Spearman's rank correlations tested mean microplastic loads within sites between different sample type pairs. Where error is expressed around the mean, it is the standard error unless otherwise stated.

3.4 Results

3.4.1 Sediment data

In total, 169 particles were recovered from 44 out of 45 sediment samples (98 % incidence). Sediment loads ranged from 0 to 0.36 particles g^{-1} with a mean of 0.08 ± 0.01 particles g^{-1}. The GLM best fitted these data (GLM: AIC = -116, NBGLM: AIC = 38) and revealed sediment counts were significantly lower in site 2 and site 3 relative to site 1 (site 2: $t = -4.65$, $p < 0.001$; site 3: $t = -3.41$, $p < 0.01$), although loads did not vary with month and replicate number ($p > 0.05$; Figure 3.2A, Table S3.1). Sediment samples were dominated by fragments, various ‘other’ colours (mostly orange, yellow and purple), and particles ≤ 100 µm (Figure 3.3).

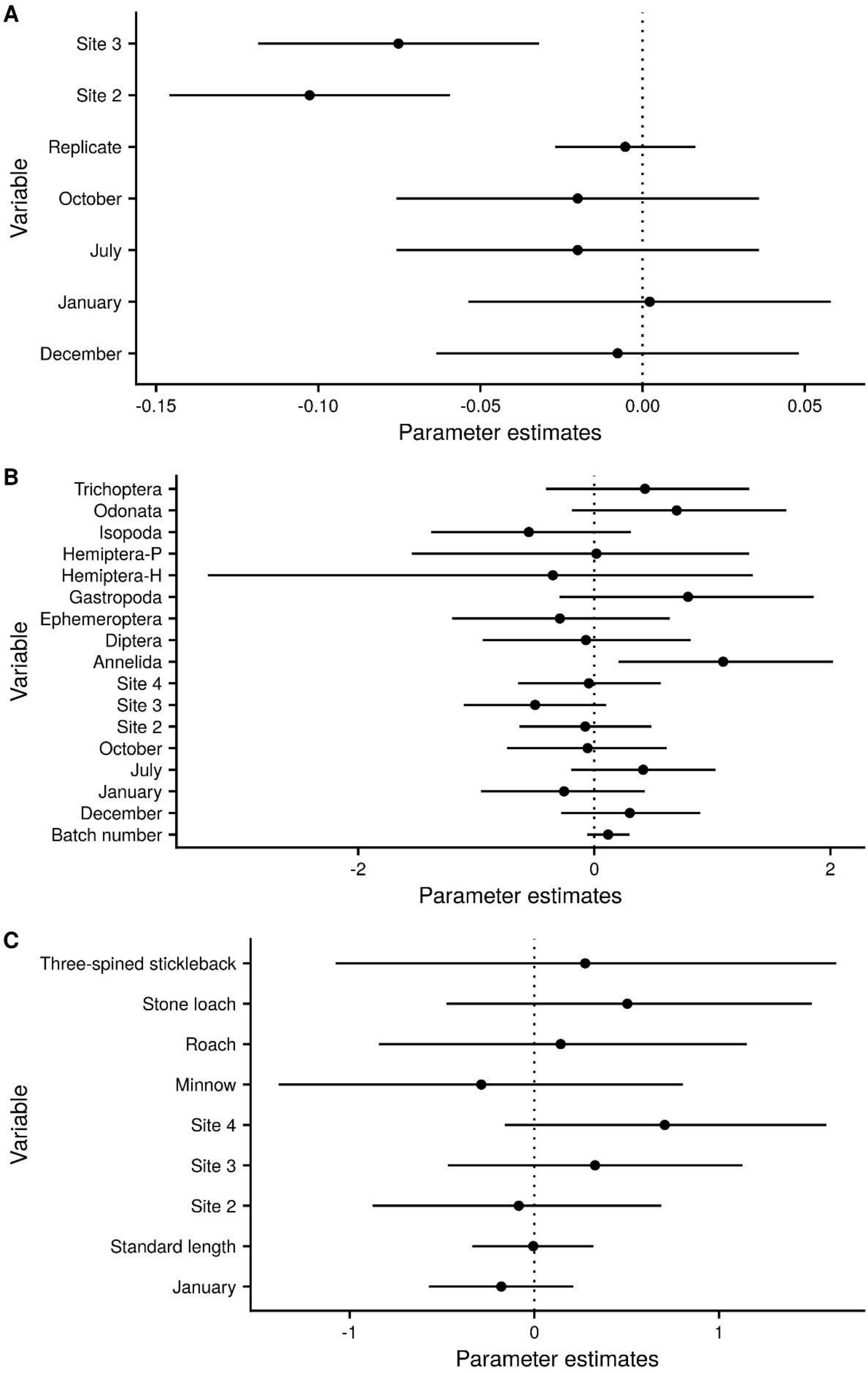

Figure 3.2. Model parameter estimates for the presence of microplastics in different sample types. Parameter estimates are presented for separate saturated models on microplastic counts in sediments (A), macroinvertebrate batches (B) and fishes (C). Variables vary for the different

sample types. The span around each variable represents the confidence interval with significant variables not crossing the dashed line. Certain variable estimates are absent that are combined in each model intercept, against which the other factors are compared.

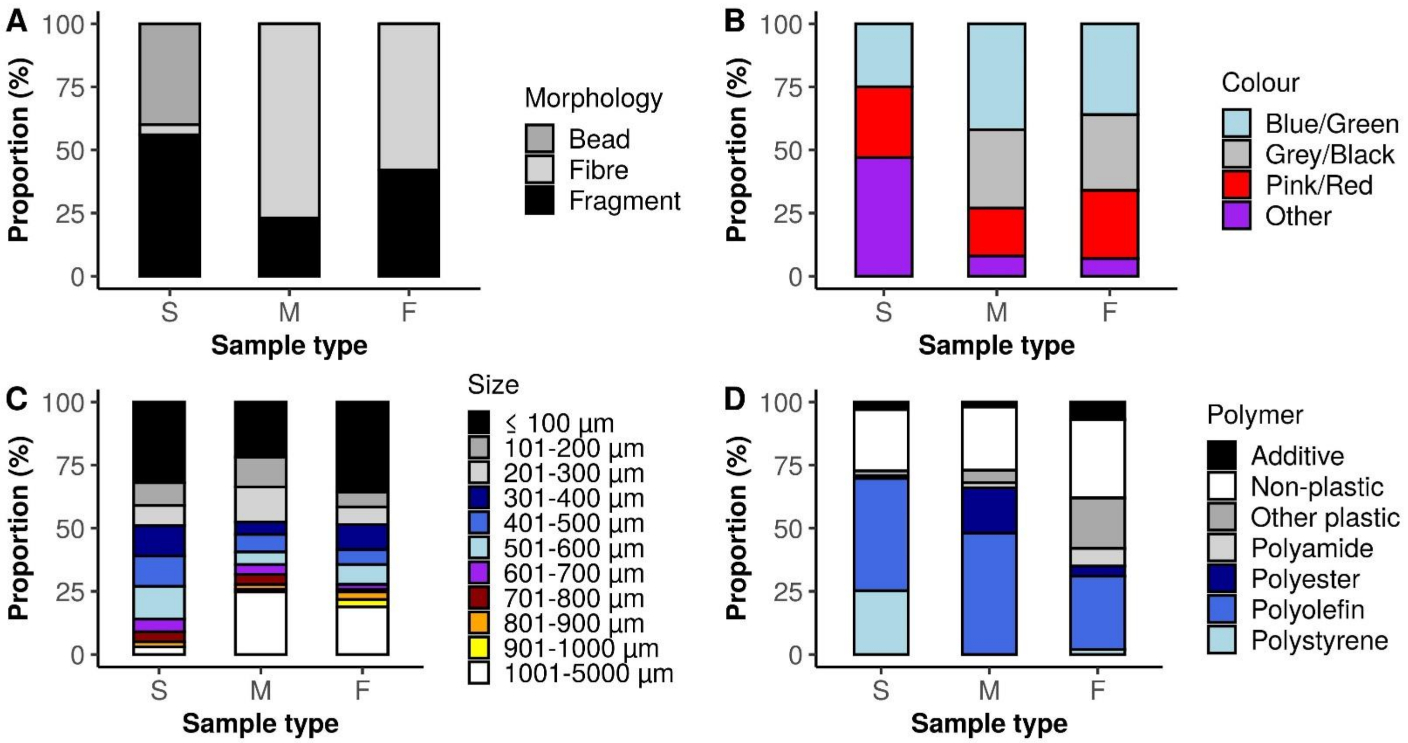

Figure 3.3. Suspected microplastic particle features for all sample types. The proportion of microplastics with different morphology (A), colour (B), size (C) and polymer (D) classes, respectively are presented for particles from sediments (S), macroinvertebrates (M) and fishes (F). Panels A, B and C are for all suspected microplastic particles (sediments: n = 169; macroinvertebrates: n = 111 and fishes: n = 157 particles). Panel D is for a subset of suspected microplastics subjected to FTIR (sediments: n = 88; macroinvertebrates: n = 44 and fishes: n = 55 particles).

3.4.2 Macroinvertebrate and fish counts

A total of 806 macroinvertebrates were processed as 220 pooled batches of up to 5 organisms. There were 111 particles recovered from 80 of the 220 batches (36 % incidence), with a mean of 0.50 ± 0.05 particles per batch. Incidences within taxa ranged from 24 % in Diptera and Isopoda to 67 % in Annelida, and mean counts per organism ranged from 0.06 in Isopoda to 0.56 in Annelida (Table 3.2). The GLM variant was selected (GLM: AIC = 430, NBGLM: AIC = 431) and revealed significantly higher counts in Annelida ($t = 2.37$, $p < 0.05$), but counts did not vary with batch number, sampling sites or months ($p > 0.05$, Figure 3.2B, Table S3.2). The particles were mostly fibres, blue/green and 1001-5000 µm in size (Figure 3.3). An independent GLM (GLM: AIC = 415, NBGLM: AIC = 415) testing for differences between feeding guilds indicated that microplastic loads were significantly lower in herbivores relative to detritivores ($z = -4.21$, $p < 0.001$, Table S3.3).

Table 3.2. Macroinvertebrate summary data. For each taxon: G denotes the guild: D; detritivore, H; herbivore, O; omnivore, PR; predator, B; number of batches, N; number of organisms, MPs; number of microplastics recovered, B (%); incidence within batches; M (B); mean for batches and M (N); mean for individual macroinvertebrates.

Taxa	**G**	**B**	**N**	**MPs**	**B (%)**	**M (B)**	**M (N)**
Amphipoda	O	21	89	10	33	0.48	0.11
Annelida	D	18	34	19	67	1.06	0.56
Diptera	H	25	88	11	24	0.44	0.13
Ephemeroptera	H	33	160	12	33	0.36	0.08
Gastropoda	D	10	24	8	50	0.80	0.33
Hemiptera (Herbivorous)	H	3	11	1	33	0.33	0.09
Hemiptera (Predatory)	PR	6	10	3	33	0.50	0.30
Isopoda	H	55	266	15	24	0.27	0.06
Odonata	PR	23	52	15	48	0.65	0.29
Trichoptera	O	26	71	17	46	0.65	0.24

For fishes, 157 particles were recovered from 86 of the 160 individuals (54 % incidence). The mean number of particles per fish was 0.98 ± 0.10. Incidences within species ranged from 42 % in *S. cephalus* up to 69 % in *B. barbatula*, and means ranged from 0.63 ± 0.22 items per fish in *S. cephalus* up to 1.46 ± 0.35 in *G. aculeatus* (Table 3.3). In the NBGLM (GLM: AIC = 453, NBGLM: AIC = 446), fish counts did not vary between species, sites, months or with standard length ($p > 0.05$; Figure 3.2C, Table S3.4). The particles within fish gastrointestinal tracts were mostly fibres, blue and ≤ 100 µm in maximum length (Figure 3.3). Independent gastrointestinal tract (AIC = 455, NBGLM: AIC = 443) and feeding guild (AIC = 455, NBGLM: AIC = 442) NBGLMs revealed that microplastic counts were higher in gastric than agastric fishes ($z = 2.33$, $p < 0.05$, Table S3.5), but did not differ between feeding guilds ($p > 0.05$, Table S3.6).

Table 3.3. Fish species summary data. For each species: F denotes the primary feeding guild: D; demersal, BP; benthopelagic, GIT indicates the structure of the gastrointestinal tract: A; agastric (undifferentiated stomach) and G; gastric (differentiated stomach, N indicates the total number of each species sampled, SL the mean standard length ± standard deviation, MPs the total number of microplastics recovered, FO the frequency of occurrence, M the mean and R the range.

Species (Family)	F	GIT	N	SL (mm)	MPs	FO (%)	M	R
Barbatula barbatula (Nemacheilidae)	D	G	26	61.3 ± 16.6	31	69	1.19	3
Gasterosteus aculeatus (Gasterosteidae)	BP	G	24	37.3 ± 8.9	35	63	1.46	6
Phoxinus phoxinus (Cyprinidae)	D	A	56	58.1 ± 10.5	48	48	0.86	5
Rutilus rutilus (Cyprinidae)	BP	A	35	103.0 ± 38.4	31	51	0.89	4
Squalius cephalus (Cyprinidae)	BP	A	19	101.1 ± 53.2	12	42	0.63	3

3.4.3 Correlations between sample types and polymer information

Within sites, mean microplastic loadings for sediments, macroinvertebrates and fishes were not significantly correlated with those of other sample types (Spearman's rank correlations: sediment-macroinvertebrates $r = 0.5$, $S = 2$, $p > 0.05$; sediment-fish $r = 0.5$, $S = 2$, $p > 0.05$; macroinvertebrates-fish $r = -0.5$, $S = 6$, $p > 0.05$, Table S3.7). Of the 200 analysed particles, 187 suspected microplastics were identified (match 1 score ≥ 70 %), of which 83 % of 88 sediment, 78 % of 44 macroinvertebrate and 60 % of 55 analysed fish particles were identified as microplastics (Figure 3.3D). The dominant microplastic class was polyolefin in all sample types (Figure 3.3D).

3.5 Discussion

The level of microplastic contamination within the urban Bourne Stream was assessed within sediment, macroinvertebrate and fish samples to identify the significance of spatiotemporal variation, and the sample type and traits of the analysed species. It was expected that loads would vary over space and time, with increased loads with distance downstream and in winter months (November, December, January), while it was also expected that biotic loads would vary between taxa/species and be higher for predatory organisms, larger, demersal-feeding and gastric fishes. Sediment loads were found to vary with site only, however spatiotemporal trends were absent in macroinvertebrate batches and individual fish. Counts varied with macroinvertebrate taxa and guild whereas fish loads were higher in gastric individuals with a distinct gastrointestinal tract.

3.5.1 Microplastics in sediments

Loads within Bourne sediments (maximum of 0.36 particles g^{-1}) were comparable to those of other UK urban freshwater sediments in both rivers (Blair et al., 2019: 0.16-0.43 particles g^{-1} dry weight; Horton et al., 2017: averages of 0.19-0.66 particles g^{-1} within sites) and lakes (Vaughan et al., 2017: 0.25-0.3 particles g^{-1} dried sediment), when scaling by weight. It is important to note that this study likely underestimates the number of particles due to the exclusion of white/clear particles, black fibres, particles below the examined size range, as well as microplastics with a particle density ≥ 1.5 cm^{-3}, including colonised particles. Additionally, the subsampling of sediments and the degree of dissipation may also under- or over-estimate sediment loadings. In contrast to other studies in urban river (e.g. Blair et al., 2019; Horton et al., 2017) and lake (Vaughan et al., 2017) sediments, the present study identified fragments, not fibres as the dominant plastic morphology. Horton et al. (2017) additionally identified a dominance of synthetic dyes, with very few polyolefins, in contrast to the present study.

The sources of the fragments in the present study were likely to include the degradation of paints and other plastics (Horton et al., 2017; Siegfried et al., 2017), while the beads were of a comparable shape and polymer type to those recovered from cosmetic products (Napper et al., 2015), although Napper et al. (2015) identified beads as predominately polyethylene in comparison to polystyrene in the present study. Within the Bourne Stream, counts varied between sites, with significantly lower levels in sites 2 and 3 than for site 1, where the water velocity was lowest, and in contrast to our hypothesis but supporting the notion of freshwater sediments acting as sinks for microplastics (de Villiers, 2019; Frei et al., 2019; Simon-Sánchez et al., 2019). Due to its low water velocity, site 1 likely represents an accumulation zone since both buoyant and denser polymers were recovered, the samples were 15 cm deep and no beads were recovered from the biota. It is, however, important to note that water velocity and volumetric flow likely varies seasonally, particularly in response to rainfall events, during which sampling did not take place. As such, the observed spatial differences may also result from seasonal hydrological variations not captured in the study. Some beads were characterised as organic materials by FTIR (e.g. yeast, data not presented), most likely due to the formation of biofilms (Besseling et al., 2017). The highly colonised and degraded nature of these particles and with beads being more prevalent at site 1 (with lowest water velocity, nearer the start of the stream) would support this being a plastic legacy, in line with data suggesting particles may exist within riverine sediments for several years under lower flow conditions (Drummond et al., 2022).

3.5.2 Macroinvertebrate microplastic loads

The individual incidence and mean numbers of suspected microplastics within macroinvertebrates are largely comparable to studies investigating loads within comparable taxa (Bertoli et al., 2022; Garcia et al., 2021; Pastorino et al., 2021). That fibres were dominant in this study is consistent with other studies (Pastorino et al., 2021), however the present study identified higher loadings within annelids only. It was expected that microplastic loads would be higher in macroinvertberates of higher trophic positions, as suggested by previous studies (e.g. Garcia et al., 2021). However, within the Bourne, lower loads were found in herbivorous relative to detritivorous macroinvertebrates only and were therefore not higher in predatory organisms. The higher incidence of microplastics in annelids may suggest a higher encounter rate and/or that microplastics are retained for longer, as suggested for *Tubifex tubifex* (Annelida) from an urban waterbody in the UK (R. R. Hurley et al., 2017). That annelids often live in and feed on the subsurface sediment and detritus may explain the increased particle loads that were likely ingested from these environments. Detritivores, to which annelids were designated in this study, did have significantly higher loadings relative to herbivores but no other feeding guilds and may require further investigation to understand this trend.

3.5.3 Fish microplastic loads

The incidence of suspected microplastics within fishes (54 %) was within the published range for European freshwater fishes (Collard et al., 2019; B. Parker et al., 2021). The particle counts, incidences and features were also comparable to other studies using the same species (Atamanalp et al., 2021; Garcia et al., 2021; Roch et al., 2019). Counts did not vary between sites or sampling months, as well as biological traits such as feeding guild and body size, despite our prediction based on trends observed in other freshwater fishes (Garcia et al., 2021; Horton et al., 2018; Park et al., 2020b). These data thus do not support biomagnification (higher microplastic loads within fishes at higher trophic levels) or bioaccumulation within the gastrointestinal tract (higher loads in larger organisms), as indicated in some previous studies (Garcia et al., 2021; Horton et al., 2018; Park et al., 2020a). However, microplastic loads were higher in gastric fishes with complete stomachs (three-spined stickleback and stone loach), as detected elsewhere (Bosshart et al., 2020; Jabeen et al., 2017; Roch et al., 2021). This result is potentially important as it can help identify those species at particular risk from microplastic contamination that are also of high conservation concern (B. Parker et al., 2021). The fish community within the Bourne Stream was fairly depauperate, with European eel (*Anguilla anguilla*) the only piscivorous fish present, but samples were not taken from this species due to their critically endangered status (IUCN, 2020) and low abundance in samples. Consequently, analysing more complex fish communities may better determine the impacts of biological traits on their microplastic loads. Finally, while only the gastrointestinal tract was processed here, microplastics are known to accumulate in other regions such as the gills, skin and organs (Park et al., 2020a). This study, therefore, could have systematically underestimated the total number of microplastics in fishes and discounted the possibility of any variations in these tissue loadings relating to the same examined biological features.

3.5.4 Spatiotemporal variation and comparisons between compartments

Although spatiotemporal variations in microplastic loadings have previously been demonstrated in abiotic and biotic samples (de Carvalho et al., 2021a; Rodrigues et al., 2018; Skalska et al., 2020) spatial trends were only observed in sediment loadings. It was expected that microplastic loads would increase with distance downstream as the Bourne approaches the town centre, as supported for other study systems (e.g. Horton et al., 2018; Park et al., 2020b), but sediment loadings were highest in the first, low-velocity site and did not vary spatially in the biotic samples. Furthermore, it was also expected that the ‘flashy’ nature of the stream would result in higher loads within winter due to increased surface runoff, however studies have also demonstrated lower loadings within winter months due to export via flooding (R. Hurley et al., 2018). The present study found no differences between sampling occasions within any sample types. Overall, these trends demonstrate an accumulation of microplastics within the sediments of sites with low water velocity but that these variations are not mirrored in the biota, suggesting biota in areas with high sediment microplastic contamination are not necessarily at greater risk of particle ingestion. While organisms and particles may be mobile within the system, potentially obscuring spatial trends, there was limited evidence for differences within sites, despite the distinct areas of the stream sampled. The absence of any temporal variations may suggest a consistent level of contamination across the year or perhaps that any variations occur at a much different scale, for example, immediately after flooding events or over a number of years. Future studies could investigate loads within paired samples collected directly before and after heavy rainfall events as well as upstream and downstream of various barriers, such as weirs and locks, to better examine the impacts of local spatiotemporal dynamics as well as flooding and barriers respectively. Longer-term time series monitoring may also examine how microplastic loads vary with natural or engineered changes to the hydrology of urban freshwaters, which could additionally explore how these changes impact microplastic profiles in the environment and biota.

Sediment microplastic samples had different features but were of comparable polymer classes to biotic microplastics, as also detected in other studies (e.g. de Carvalho et al., 2021a). The Bourne sediments were dominated by fragments and were unique in containing beads that were mostly identified as polystyrenes, while biotic samples included mostly blue fibres and a larger diversity of microplastics including more polyesters. Due to the depth of the sediment samples and absence of beads within the biota, it is likely that these particles had been trapped in the sediment for some time (Drummond et al., 2022; Frei et al., 2019; Simon-Sánchez et al., 2019), were unavailable to the biota and may have originated from cosmetic products due to their similar shape (Napper et al., 2015). The differences in dominant particle features between the different compartments suggest biota may actively ingest/interact with and expel/egest particular particles based on their characteristics (e.g. size, shape, colour), as supported by field (Garcia et al., 2021) and experimental data (Roch et al., 2021) on freshwater biota. Furthermore, the lack of significant relationships in microplastic counts between fish species, as well as the absence of a relationship with fish body size, would suggest that the studied species were generally able to egest microplastics to prevent their accumulation, although, as previously noted, fishes with distinct gastrointestinal tracts were found to have higher levels, and may therefore be at greater risk from microplastic contamination. Finally, the distinct patterns in microplastic loadings relating to the spatiotemporal dynamics and biological features, as well as the lack of correlation between sample types, suggests that the ingestion of microplastics is more dependent on biological traits than environmental loads, with important implications for management and microplastic mitigation.

3.6 Conclusions

The present study simultaneously examined microplastic levels in sediments, macroinvertebrates and fishes from an urban stream and related these levels to spatiotemporal dynamics, the biological features of biota, as well as loads within other sample types. Limited spatial (sediment only) but no temporal dynamics were observed, loadings were not correlated between sample types and counts did vary with some biotic traits such as macroinvertebrate taxa and guild as well as fish gastrointestinal tract structure. These data suggest that sediments in low-velocity areas may accumulate high numbers of microplastics, although the ingestion of particles by biota is independent of sediment loadings and may depend more on biological traits. In conclusion, biotic and sediment loadings in urban freshwaters were not significantly correlated and varied with different factors, therefore assessments spanning multiple sample types are essential for understanding the variations in microplastic loads within the ecosystem to better manage urban freshwaters and mitigate microplastic contamination.

4 Microplastic loads within riverine fishes and macroinvertebrates are not predictable from ecological or morphological characteristics

4.1 Abstract

Microplastics are a relatively new but important form of freshwater contamination that can be ingested by a range of different species, with particle counts thought to be predictable from species ecology and morphology. Here, we report levels of microplastics in a 26 μm-5 mm size range within the macroinvertebrate and fish community of a lowland river (Dorset Stour, SW England), and test the hypothesis that counts are predictable from characteristics such as feeding guild, body length and trophic position. Macroinvertebrates (n = 257, 12 taxa) and fish (n = 418, 9 species) were collected from distinct river reaches by kick sampling and rod and line angling, respectively. Batches of whole macroinvertebrates and individual fish gastrointestinal tracts were digested with 30 % hydrogen peroxide before microplastic screening and FTIR polymer confirmation on a particle subset. Particles were found in 40 % of pooled macroinvertebrate batches (taxa incidences: 14-75 %) and 39 % of fishes (species incidences: 29-47 %). Dominant particle feature categories were ≤ 100 μm, blue/green, fragments and fibres identified as various polyolefins. Although particle counts in macroinvertebrates were highest in Ephemeroptera (mean of 0.74 particles per individual), the relationships between particle loads, batch number and guild were all non-significant. In fishes, particle counts were not significantly related to species, stomach structure, feeding guild or body length, with spatial differences also not apparent across the catchment. Individual fish particle counts were similarly not significantly associated with their trophic positions (calculated from bulk $\delta^{15}N$ values for a subset of fishes) and parasite load of *Pomphorhynchus tereticollis*. Correlations between fish and macroinvertebrate particle counts within specific river reaches were also not significant. In entirety, these results indicated although loadings of microplastic particles were relatively consistent within the two communities, they were not predictable from any of their ecological or morphological characteristics.

4.2 Introduction

Microplastics (MPs), defined as plastic particles with length < 5 mm, are a relatively recent but pervasive form of contamination within aquatic systems (Cera et al., 2020; Eerkes-Medrano et al., 2015; J. Li et al., 2018). Common sources of freshwater MPs include the degradation of synthetic clothing during washing, tyre wear particles and the gradual breakdown of other larger plastics (Siegfried et al., 2017). Accumulated MPs within the catchment are then washed and/or deposited into water bodies via water and wind (Cera et al., 2020; Eerkes-Medrano et al., 2015). MPs vary widely in shape, specific gravity, size and chemistry and, depending on system and particle properties, may pass through or accumulate in regions such as bottom sediments (Besseling et al., 2017; Horton & Dixon, 2018).

Riverine MP contamination may often vary spatially and temporally depending on land use, particularly the degree of urbanisation, as well as due to meteorological variations that alter hydrological conditions (de Carvalho et al., 2021a; Skalska et al., 2020; Stanton et al., 2020).

MPs in freshwater systems may be ingested by a range of organisms, including macroinvertebrates and fish, with studies often finding biotic ingestion levels proportional to MP levels in the environment (Horton et al., 2018; Peters & Bratton, 2016). Various studies suggest that the encounter, ingestion and egestion of MPs differs between taxa and that counts within the gastrointestinal tract can be predicted from the biological characteristics of the species (Bertoli et al., 2022; Garcia et al., 2021; McNeish et al., 2018). Studies often reveal increased particle loadings at higher trophic levels (Campbell et al., 2017; Garcia et al., 2021; McNeish et al., 2018) and in demersal-feeding fishes (Merga et al., 2020; L. Zhang et al., 2021). Furthermore, larger organisms can be particularly susceptible to ingesting MPs (Garcia et al., 2021; Horton et al., 2018; McNeish et al., 2018) and can accumulate particles through bioaccumulation (particles accumulate within the organism over time), although this is not always apparent. Finally, the structure of the gastrointestinal tract can impact the ability of individuals to egest particles (Bosshart et al., 2020; Jabeen et al., 2017; Roch et al., 2021).

Ingested MPs and associated chemicals, for example plasticisers (e.g. adipates and terephthalates) and additives (e.g. butadiene rubber and crosslinked acrylics), can cause a range of negative impacts on the feeding and physiology of freshwater biota, and can result in mortality (Collard et al., 2019; Naqash et al., 2020; B. Parker et al., 2021). Particles can even translocate into other organs if sufficiently small (J. Ding et al., 2018; J. Kim et al., 2020; Lu et al., 2016). Moreover, freshwater organisms are often additionally and simultaneously exposed to other stressors, such as climate change and urbanisation, which might also negatively impact organisms and potentially interact with the effects of MPs (Jenny et al., 2020; Reid et al., 2019; Y. Zhang et al., 2020b). Parasite infection has also been tentatively linked to MP contamination (Alves et al., 2016; Banihashemi et al., 2021; Limonta et al., 2019; Luís et al., 2015), with suggestions that higher parasite loads increase the susceptibility of individuals to having higher MP loads or parasite infection might influence ingestion of MPs (B. Parker et al., 2021).

If the MP loads within organisms differ predictably according to their biological characteristics then those species and/ or life-stages can be more easily identified, their impacts assessed more specifically, and management strategies designed and implemented should the impacts be considered as too damaging (B. Parker et al., 2021). However, this requires community-level data examining MP loads within taxa across multiple trophic levels and feeding guilds, and an understanding of how other anthropogenic stressors may impact the individual and population response to MPs (B. Parker et al., 2021). Correspondingly, the aim here was to determine the particle loads of MPs in freshwater communities and test these against their ecological and/ or morphological characteristics. Through analyses of MP loads of fish and macroinvertebrate communities across a lowland river in SW England

(Dorset Stour) spanning a gradient of urbanisation, we posit that: (1) within specific reaches of river, there will be a positive relationship in MP loads between macroinvertebrates and fish; (2) MP loads in macroinvertebrates will be highest in predatory and omnivorous guilds; (3) fish MP loads will be higher in larger organisms, those infected with parasites, and demersal fishes, as well as in those with differentiated gastrointestinal tracts; and (4) biotic MP loads will increase with distance downstream (as a proxy of the extent of urbanisation in the study catchment), and correlate with trophic position and parasite number within individual fishes.

4.3 Materials and methods

4.3.1 Study river and reaches

The study river was the Dorset Stour (hereafter "Stour") in Southern England (Figure 4.1), which has a main channel length of approximately 100 km, drains a catchment area of 1240 km^2 and has a human population size of approximately 400,000 people, most of whom live in the lower catchment (Environment Agency, 2012). Along its length, the Stour passes several settlements and sewage treatment works before emptying into Christchurch Harbour (Figure 4.1). The river has a gradient of land use along its course from principally agricultural in upper sections to increasingly urbanised in lower sections, especially near Bournemouth, Christchurch and Poole (Figure 4.1).

For the purposes of sample collection and subsequent analyses, the river was split into four distinct sections reflecting the changes in surrounding land use and different physical and hydrological characteristics along the length of river (Figure 4.1). Reach 1 (distance from source approximately 0 to 40 km) was the most upstream section, characterised by a narrow channel (typically < 10 m), with relatively deep sections (over 3 m depth) and low levels of urbanisation. Reach 2 (approximately 40 to 58 km) has a wider channel and sits between the settlements of Sturminster Newton (population approximately 5,000) and Blandford Forum (population approximately 9,000). Reach 3 (approximately 58 to 88 km) includes wider (> 20 m) sections, more variable depths including pools, riffles, impoundments and two sewage treatment works discharging into the river. Wimborne is the largest settlement in the reach (population approximately 16,000). Reach 4 was the furthest downstream (approximately 88 to 98 km) with wide sections of variable depths, a single sewage treatment works and much of the surrounding land use being urban, where the population size of the Poole-Bournemouth-Christchurch conurbation is over 500,000, although not all of this area is within the Stour catchment (Figure 4.1).

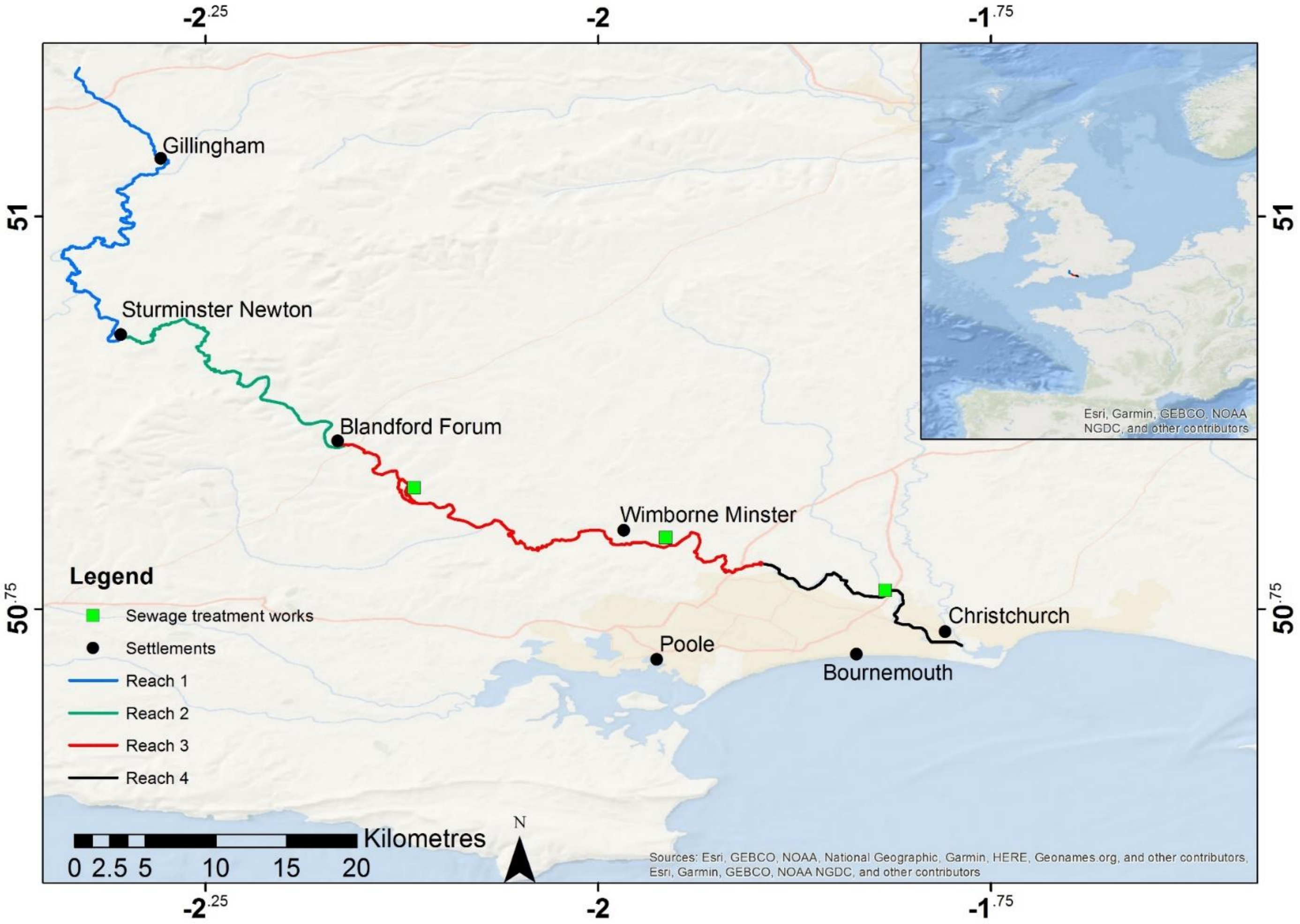

Figure 4.1. Map of the Dorset Stour water body. The four reaches of the river are colour coded with settlements and sewage treatment locations shown. Map produced using ArcMap (version 10.3) and the World Ocean Base map-Sources: Esri, GEBCO, NOAA, National Geographic, DeLorme, HERE, Geonames.org, and other contributors.

4.3.2 Sample collection and laboratory processing

Macroinvertebrate samples were collected in April and August 2019 only within each reach in shallower riffle sections by kick sampling with a standard 1 mm mesh kick net and sweeping the margins in deeper sections. Approximately 40 organisms were collected for each reach, euthanised in the field and frozen at -4 °C until processing. Where feasible, all microhabitats within locations were sampled, ensuring that the sample was representative of the local community (e.g. all major feeding guilds were present). In the laboratory, macroinvertebrate samples were defrosted and assigned to taxa, typically to order level. Within samples and taxa, up to five individuals were grouped together in batches based on incidence and size, as per similar studies (Garcia et al., 2021). Batches were placed into foil-capped glass vials with the number of organisms recorded.

A total of 418 freshwater fish representative of the local community within each reach were collected across 9 separate months (2018: July, August, September; 2019: January, February, August, September, November and 2020: March), mainly through rod and line, with smaller species captured using sweep nets. Nine common species of fish were sampled; bleak *Alburnus alburnus*, stone loach *Barbatula barbatula*, bullhead *Cottus gobio*, three-spined stickleback *Gasterosteus aculeatus*, dace *Leuciscus leuciscus*, perch *Perca fluviatilis*, minnow *Phoxinus phoxinus*, roach *Rutilus rutilus* and chub *Squalius cephalus*. Larval samples were not collected to prevent taking fish too small for dissection. Larger chub, roach, dace and perch were generally not taken to avoid removing fish of angling importance (as the river is heavily used for catch-and-release angling). Kept individuals were euthanised in the field using a Schedule 1 Method of Humane Killing under the UK legislation Animals (Scientific Procedures) Act by concussion and destruction of the brain and transporting on ice before freezing at -4 °C until processing.

In the laboratory, the fish were then defrosted, identified to species, the standard length recorded and a small section of dorsal muscle taken for stable isotope analyses. The entire gastrointestinal tract, including the gastrointestinal contents, was then dissected out and transferred into a foil-capped glass. The entire gastrointestinal tract was additionally pressed within a glass compressorium and subject to a brief (< 2 min) parasite screen under a microscope (LEICA M165C) at up to 80x magnification to record the number of acanthocephalan *Pomphorhynchus tereticollis* parasites before carefully returning gastrointestinal tracts into their containers. This particular parasite was selected specifically as the focal parasite to use in analyses as it is easily and accurately identified within freshwater fish final hosts and also as the parasite is ingested by, and later trophically transported via, a macroinvertebrate (*Gammarus* spp.) intermediate host (Kennedy, 2006). While the parasite is capable of infecting several fish species year-round, its definitive final host in the study system is *S. cephalus*, where it is often found in relatively high prevalence (Hine & Kennedy, 1974a, 1974b).

Batches of whole macroinvertebrates and individual extracted fish gastrointestinal tracts were processed using a methodology adapted from Avio et al. (2015). Samples were digested within glassware by submersion (3:1 reagent-sample volume) in 30 % hydrogen peroxide and incubating at 60 °C within a shaker set at 30 rpm for 48 h. The resultant solution was then vacuum filtered through a sterile 13 mm, 26 µm mesh stainless-steel filter (The Mesh Company, Warrington, UK). Containers and funnels were thoroughly rinsed through several times with filtered water (1.2 µm, Whatman glass microfibre filters) and the filters were stored and left to dry in clear polypropylene caps with foil lids.

Entire filters were screened under a stereo microscope (LEICA M165C) at up to 120 x magnification for 5 min each. Suspected MPs were identified based on previous defined criteria, such as distinct and consistent colours and shapes, as well as their lack of internal biological features (Nor & Obbard, 2014). For every suspected MP, the morphology (fibre; long, thin and flexible shape or fragment; irregular shape) and colour category (blue/green; grey/black; pink/red; other) were recorded. Particles were also assigned size classes, typically corresponding to 100 µm increments, by measuring the maximum dimension size of each particle using the eye piece graticule at 120 x magnification (26-100, 101-200, 201-300, 301-400, 401-500, 501-600, 601-700, 701-800, 801-900, 901-1000 and 1001-5000 µm).

4.3.3 Stable isotope data

Samples of macroinvertebrates (principally *Gammarus* spp. to represent fish putative prey resources) and fish dorsal muscle (n = maximum 10 per species per reach) were dried to constant mass at 60 ˚C and analysed at the Cornell University Stable Isotope Laboratory (New York, USA) for bulk $\delta^{15}N$ in a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, USA) interfaced to a NC2500 elemental analyser (CE Elantach Inc., USA). Analytical precision of the $\delta^{15}N$ sample runs was estimated against an internal standard sample of animal (deer) material every 10 samples, with the overall standard deviation estimated at 0.08 and 0.04 % respectively. To then determine the trophic position (TP) of each individual fish, their data were applied to the following equation at the reach level:

$$TP = \left(\frac{\delta^{15}N_{fish} - \delta^{15}N_{macroinvertebrates}}{3.4}\right) + 2$$

Where $\delta^{15}N_{fish}$ is the nitrogen ratio for each fish, $\delta^{15}N_{macroinvertebrates}$ is the mean nitrogen ratio of the macroinvertebrate prey within each reach (data not presented), 2 is the trophic position of primary consumers and 3.4 the fractionation between trophic levels (Post, 2002).

4.3.4 Polymer identification with vibrational spectroscopy (Attenuated Total Reflectance)

The polymer identity/type of 98 suspected MPs ≥ 100 µm in maximum length was determined using micro-Attenuated Total Reflectance (micro-ATR) accessory attached to Spotlight™ 400 FTIR Imaging System coupled to Frontier™ IR Spectrometer (PerkinElmer, Llantrisant, UK). The number of particles analysed represented approximately 30 % of all suspected MPs, however due to logistical and equipment-related constraints, no particles < 100 µm were identified. For each suspected plastic particle, ATR spectra were collected over the mid-IR spectral region (650-4000 cm^{-1}) at 8 cm^{-1} spectral resolution and 10 accumulations (co-added spectra) per scan. The IR background was collected in air under the same spectral settings but with an increased number of co-added spectra ($n = 120$). The collected individual spectra were then compared to the spectra in the reference polymer library (18,711 polymer types; spectra database from S.T. Japan-Europe GmbH, Germany/Japan) using PerkinElmer Spectrum™ 10 software. The comparison generated 5 top matches to the library spectra. A match score of ≥ 70 % was considered a successful hit and particles were assigned to the highest scoring successful polymer hit that was a plastic polymer, plastic additive or non-plastic, as appropriate. As the particles were already suspected to be plastic, special preference was given to successful plastic hits, for example, a particle with “yeast” as the highest matching hit, but with “polyethylene” as the next matching hit would be assigned “polyethylene”. Individual polymer hit types were later grouped into broader categories: polyolefin, polyester, polyamide, other-plastic, additives, and non-plastic.

4.3.5 Quality and contamination control

To reduce the potential for contamination, the time samples were exposed to the environment was minimised and, wherever possible (except when using large external equipment), processing was performed within a pre-cleaned flow cabinet. Plasticware was avoided wherever possible, sampling equipment was cleaned before use and between samples by furnacing or rinsing several times with filtered water (1.2 µm, Whatman glass microfibre filters) and all reagents were pre-filtered (1.2 µm, Whatman glass microfibre filters). As the hydrogen peroxide digestion of organic material often resulted in white/clear samples, white and clear materials were assumed organic and deliberately ignored throughout screening. Studies have shown that hydrogen peroxide may damage and/or discolour common polymers such as polyethylene, polypropylene and polyamide (e.g. Nuelle et al., 2014), therefore leading to potential underestimation.

Additionally, 61 procedural blanks containing filtered hydrogen peroxide only were carried out and processed as above alongside the samples to determine any contamination in the reagents or introduced during processing, recording both the morphology and colour of contaminants. These procedural blanks assessed the level of contamination for these samples as well as those of another project which used similar processing methods and that were processed alongside these samples. Seven fibre contaminants were detected within blanks (maximum of 1 fibre per blank) and since the colour of fibre contaminants was highly variable and inconsistent, no corrections were applied.

4.3.6 Statistical analyses

All analyses used suspected particle counts identified through visual microscopy and uncorrected by the FTIR results. Statistical analyses were performed in R version 3.5.1 (R Core Team 2018), using the glmer.nb function from the package MASS (Venables & Ripley, 2002), to perform a negative binomial linear mixed effect model (NBLME) for the fish count data.

Due to overdispersion in the data, Akaike Information Criterion (AIC) values were used to compare the fit of Poisson (GLM/GLME) and negative binomial variants (NBGLM/NBGLME) of identical saturated general linear models (without interactions). The negative binomial variant was selected where this model had an AIC value two points lower than the competing Poisson model. The macroinvertebrate data best fitted a negative binomial family general linear model (GLM: AIC = 217, NBGLM: AIC = 214.6) and the fish count data a negative binomial family general linear mixed effects model (GLME: AIC = 941.6, NBLME: AIC = 916.8).

The macroinvertebrate NBGLM tested for differences in pooled MP counts using taxon and the number of organisms as fixed factors. An additional NBGLM then tested for differences in macroinvertebrate counts between batches based on their different feeding guilds. To examine relationships between MP loadings and fish characteristics, the NBLME used fixed effects of river reach, standard length (after scaling), species and *P. tereticollis* number, with sampling date used as a random effect. Additional, separate NBGLMs were also performed on the fish count data to identify any differences between feeding guild (demersal and benthopelagic) and also gastrointestinal tract structure (agastric and gastric), determined from FishBase species data (www.fishbase.org;Froese & Pauly, 2021). Finally, trophic position was tested by correlation (Pearson’s) versus MP load for a subset of fish.

4.4 Results

4.4.1 General incidence of microplastics and particle features

There were 61 suspected MPs recovered from 40 % of 85 pooled macroinvertebrate batches (totalling 257 organisms). The taxa incidences within batches ranged from 14 % for Hemiptera (Predatory) to 75 % in Annelida (Table 4.1), whereas mean counts per individual ranged from 0.06 in Diptera up to 0.74 in Ephemeroptera (Table 4.1). There were 260 suspected MPs recovered from the gastrointestinal tracts of 418 fish, with particles found in 39 % of individuals. MP counts ranged from 0 to 6 per fish (mean ± standard error = 0.62 ± 0.05), with species incidence ranging between 29 % (perch *Perca fluviatilis*) and 47 % (stone loach *Barbatula barbatula* and minnow *Phoxinus phoxinus*) (Table 4.2). *P. tereticollis* was identified in 22 fishes (5 % prevalence); in infected fishes, median abundance was 1 parasite, maximum 42.

Table 4.1. Macroinvertebrate summary data.

Taxa	Guild	Batches	Number of organisms	Number of microplastics	Batch incidence (%)	Mean for batches	Mean for organisms
Amphipoda	Omnivore	12	56	4	25	0.33	0.07
Annelida	Detritivore	4	9	5	75	1.25	0.56
Coleoptera	Predator	3	7	2	33	0.67	0.29
Diptera	Herbivore	4	16	1	25	0.25	0.06
Ephemeroptera	Herbivore	10	19	14	50	1.40	0.74
Gastropoda	Detritivore	9	21	6	67	0.67	0.29
Hemiptera (Herbivorous)	Herbivore	5	17	2	20	0.40	0.12
Hemiptera (Predatory)	Predator	7	17	2	14	0.29	0.12
Isopoda	Herbivore	9	36	6	44	0.67	0.17
Megaloptera	Predator	4	4	1	25	0.25	0.25
Odonata	Predator	11	42	10	36	0.91	0.24
Trichoptera	Omnivore	7	13	8	57	1.14	0.62

Table 4.2. Fish summary data.

Species (Family)	Feeding type	Stomach structure	Sample number	Standard length (mm)	Microplastic number	Frequency of occurrence (%)	Microplastic mean	Microplastic range
Alburnus alburnus (Cyprinidae)	Benthopelagic	Agastric	22	90.36 ± 18.54	10	32	0.45	3
Barbatula barbatula (Nemacheilidae)	Demersal	Gastric	19	39.63 ± 11.38	17	47	0.89	4
Cottus gobio (Cottidae)	Demersal	Gastric	14	30.21 ± 6.18	10	43	0.71	4
Gasterosteus aculeatus (Gasterosteidae)	Benthopelagic	Gastric	27	29.92 ± 4.16	15	41	0.56	3
Leuciscus leuciscus (Cyprinidae)	Benthopelagic	Agastric	74	130.72 ± 34.84	42	38	0.57	5
Perca fluviatilis (Percidae)	Demersal	Gastric	31	153.52 ± 28.59	11	29	0.35	2
Phoxinus phoxinus (Cyprinidae)	Demersal	Agastric	93	55.55 ± 11.66	71	47	0.76	4

Rutilus rutilus (Cyprinidae)	Benthopelagic	Agastric	96	114.82 ± 34.40	55	35	0.57	6
Squalius cephalus (Cyprinidae)	Benthopelagic	Agastric	42	130.67 ± 45.96	29	38	0.69	5

Suspected MPs in the macroinvertebrate samples were dominated by fragments and in fish samples by fibres (Figure 4.2A); particles from all samples were mostly blue/green (Figure 4.2B) and ≤ 100 µm (Figure 4.2C). FTIR indicated that 59 % of suspected particles from whole macroinvertebrates and 63 % from fish gastrointestinal tracts were MPs (Figure 4.2D). In macroinvertebrates, polyolefins (e.g. polyethylene, polyheptene and polypropylene) were the most common polymer type identified (47 %; n = 8 particles, Figure 4.2D), with this also the case for fishes (37 %; n = 30 particles, Figure 4.2D).

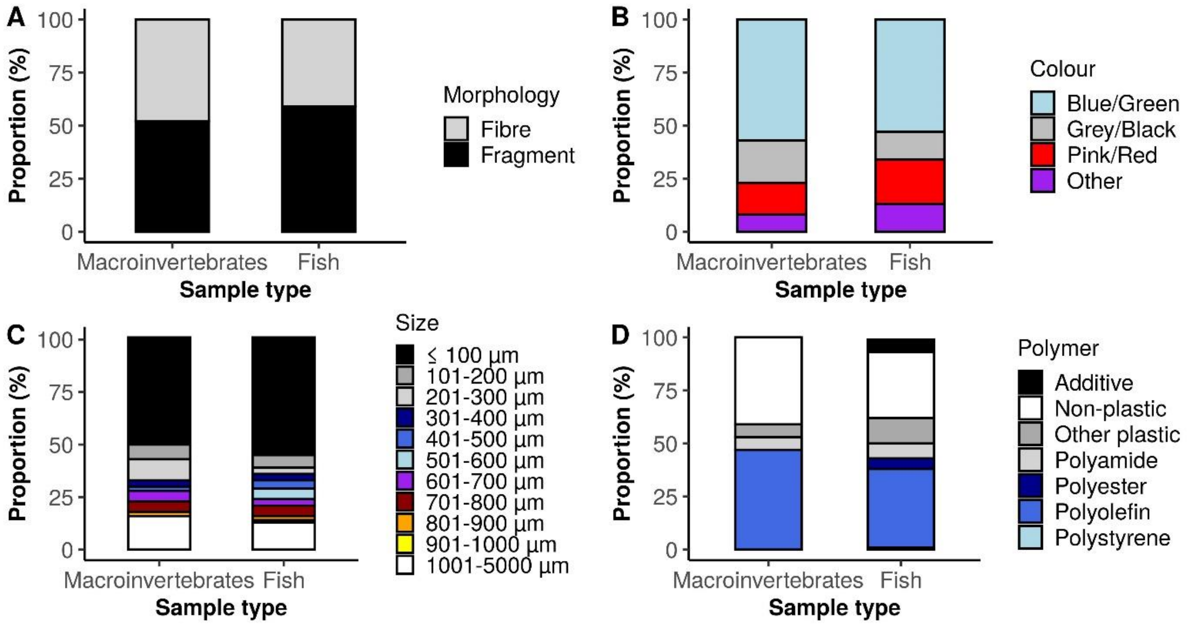

Figure 4.2. Macroinvertebrate and fish suspected microplastic particle features. The proportion of microplastics with different morphology (A), colour (B), size (C) and polymer (D) classes, respectively are presented for particles from macroinvertebrates and fish. Panels A, B and C are for all suspected microplastic particles (macroinvertebrates: n = 61 and fish: n = 260 particles). Panel D is for a subset of suspected microplastics subjected to FTIR (macroinvertebrates: n = 17, fish: n = 81 particles).

4.4.2 Relationships between MP loads and biological characteristics

In the macroinvertebrates, MP number was significantly higher in Ephemeroptera batches than in other groups (NBGLM; $p = 0.03$, Table S4.1), but with differences between the other groups and the number of organisms being non-significant ($p > 0.05$, Figure 4.3). There were no significant differences in MP counts between macroinvertebrate guilds (NBGLM; $df = 84$, $p > 0.05$, Table S4.2).

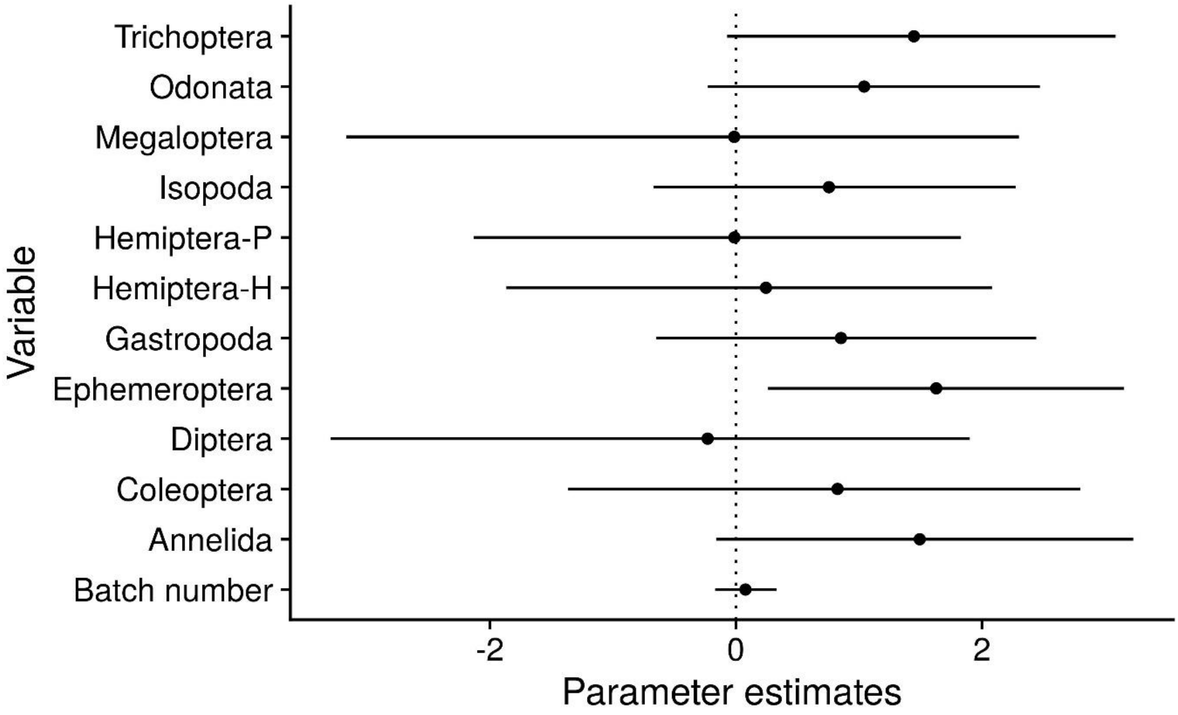

Figure 4.3. Model parameter estimates for the presence of microplastics in macroinvertebrate batches. Parameter estimates are presented for each of the taxa as well as the batch number, the number of organisms within each batch. The span around each variable represents the confidence interval with significant variables not crossing the dashed line. The taxon "Amphipoda" is absent as it is used in the model intercept to compare with other taxa.

In fish, differences in MP loads were not significantly related to any of the fixed effects and their factor levels: species, standard length and reach (NBLME; $p > 0.05$, Table S4.3, Figure 4.4). There were also no significant differences in fish MP loads between both the primary feeding type (as demersal/benthopelagic; NBGLM: df = 417, $p > 0.05$, Table S4.4), and gastrointestinal tract structure (agastric/gastric; NBGLM: df = 417, $p > 0.05$, Table S4.5). The relationship between trophic position and microplastic load was also non-significant (Pearson's correlation: $r = -0.06$, df = 224, $p > 0.05$). Finally, the correlation between mean MP loads in macroinvertebrates and fish within river reaches was also not significant (Pearson's correlation: $r = -0.71$, df = 2, $p > 0.05$).

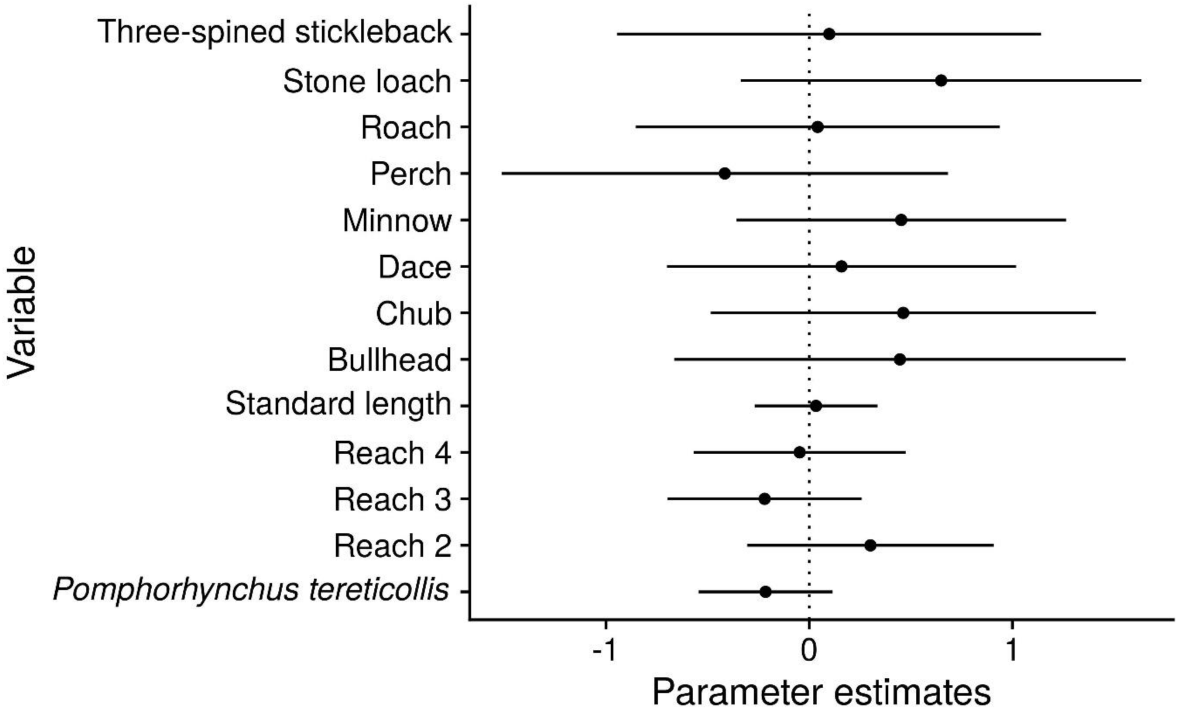

Figure 4.4. Model parameter estimates for the presence of microplastics in fish. Parameter estimates are presented for the different species and reaches as well as for standard length and *Pomphorhynchus tereticollis* count. The span around each variable represents the confidence interval with significant variables not crossing the dashed line. “Bleak” and “Reach 1” are both absent from the figure as the model combines the first levels of these categorical variables in the intercept as a reference for the other model parameters.

4.5 Discussion

An understanding of how species traits may impact the ingestion of microplastics is an important tool in conservation and ecosystem management to identify those organisms that are particularly susceptible to microplastic contamination. If microplastic levels are highly predictable from traits, at-risk organisms may already be identified without prior lethal sampling and may, in some circumstances, allow the better prioritisation of management resources, for example if a particular species of conservation interest is unlikely to be susceptible to microplastic contamination those resources may be focussed elsewhere. Conversely, if traits have a low predictive value then data for the system and organisms may first be needed (including lethal sampling) to identify organisms at risk and then to allocate resources based on these data. The present study found suspected counts were mostly unpredictable from traits with only pooled macroinvertebrate counts varying between taxa whereas counts were unrelated to all other features.

4.5.1 Macroinvertebrate MP counts and features

The general results revealed a low incidence of suspected MPs in freshwater macroinvertebrates in the study river (0.07-0.89 particles per organism), of which 59 % of suspected particles ≥ 100 µm were confirmed to be MPs through FTIR, and that particles were predominately blue/green, fragments ≤ 100 µm. Their irregular shape suggested these particles originated from the degradation of larger plastics, although their precise source is hard to trace. The mean MP counts per individual recorded are largely comparable to those of recent studies of MPs in macrobenthic invertebrates from an Italian and French river, respectively (Bertoli et al., 2022; Garcia et al., 2021). However, despite many similar taxa being assessed, these other studies identified MPs dominated by black fibres and polyesters in contrast to the blue/green polyolefin fragments found in the present study. In a remote high-mountain lake, no MPs were detected in macroinvertebrates, fish and sediments, and only polyesters in snow (Pastorino et al., 2021), and so the differences in particle counts and features likely reflects the distinct sources, levels and transport of plastic pollution within each catchment. Differences in processing methods may also impact the recovered particles, for example the use of a 26 µm minimum particle size and hydrogen peroxide reagent may systematically underestimate particle loads whereas the FTIR results simultaneously suggest count inflation within both sample types.

The batch MP counts were unrelated to the number of organisms in each batch, however there were some differences between taxa with higher MP loads in mayfly larvae (Ephemeroptera) relative to other taxa. This result was contrary to expectation, since these organisms are primarily collector gatherers and scrapers, feeding on plants, algae or organic debris - although organic material within freshwaters may potentially capture and accumulate MPs (Nel et al., 2018). By contrast, higher MP loads within macroinvertebrates of higher trophic positions were detected by Garcia et al. (2021). It is possible that the particle features are comparable to blue-green algae resources and were therefore ingested by mayfly larvae, as supported by higher loads within other scraper macroinvertebrates (Bertoli et al., 2022), although no differences were identified between guilds. Alternatively, or in addition, macroinvertebrates such as *Gammarus duebeni* are capable of fragmenting particles (Mateos-Cárdenas et al., 2020), so this might also be possible within Ephemeroptera, but requires further investigation.

4.5.2 Fish MP incidences and features

Both the incidence range (29-47 %) and mean number of MPs (mean ± standard error = 0.62 ± 0.05) within the studied species are very similar to those of roach from the River Thames, UK (Horton et al., 2018), but are higher than several other studies examining many of the same species within Europe (Collard et al., 2018; Faure et al., 2015; Garcia et al., 2021; Roch et al., 2019; Sainio et al., 2021; Uurasjärvi et al., 2021). However, the findings of the present study are also lower than for several other systems investigating the same species (Atamanalp et al., 2021; Galafassi, Sighicelli, et al., 2021; Kuśmierek & Popiołek, 2020). Taken together, these results show that MPs are frequently found within biota, but that actual values have some context dependency arising from, for example, the catchment characteristics and the manner of sample processing. In addition to the previously discussed sample processing limitations, the present study did not investigate microplastic loads within other parts of fishes, for example the gills and liver, and therefore likely underestimates absolute counts for the individual.

The present study recovered small (≤ 100 µm), blue/green fibres of various polyolefins, particularly polyethylene and polypropylene, in general agreement with the particles recovered from other freshwater fishes (Collard et al., 2018; Galafassi, Sighicelli, et al., 2021; Horton et al., 2018; Uurasjärvi et al., 2021). The blue/green fibres here may have originated from sources such as ropes, commonly used in agriculture, boating and nets, and often made of polyethylene, polypropylene and other polyolefins. Hydrogen peroxide digestion is known to discolour certain particles (Nuelle et al., 2014) and so some white/clear MPs may have been excluded, as well as those particles smaller than the mesh size, and so the estimates from this study can be considered conservative. It should also be stressed that no chemical confirmation was carried out for particles < 100 µm, which were most abundant, and so the correct identification of smaller particles as suspected MPs may be less reliable and size-dependent.

In contrast to our predictions, there were no differences in macroinvertebrate or fish MP counts between the different reaches. It was expected that MPs would increase with distance downstream from the source, given the increasing level of urbanisation which has been linked to higher MP loads in both the biota and environment of various other freshwater systems (e.g. de Carvalho et al., 2021a; Horton et al., 2018; Park et al., 2020b). No such relationship was detected, despite the differences in hydrology and land use that were used to designate the four distinct river reaches. The absence of spatial differences here may reflect consistent environmental loadings within the system, that the egestion rate of particles was sufficient to prevent accumulation, and/or that spatial variations in MP loadings may occur at a finer (non-reach) resolution, for example if higher loadings are present in organisms immediately downstream of urban settlements. Since we did not collect accompanying water and/or sediment samples, we cannot speculate on the potential spatial differences in MP loads within the abiotic environment. However, the relationship between freshwater urbanisation and MP loadings in the environment and biota is well supported (e.g. de Carvalho et al., 2021a; Horton et al., 2018; Park et al., 2020b).

Despite finding no significant correlations between macroinvertebrate and fish MP counts within reaches, the most dominant particle features (morphology, colour and size) were the same for both sets of biota. This would suggest that the particles within the system are largely the same, there was no evidence for trophic transfer and biomagnification, given MP counts were unrelated to ecological or physiological characteristics. The polymers recovered from the biota are amongst the most common types found (Andrady & Neal, 2009) and, while it is difficult to describe the exact source of MP particles, possible sources in the system likely include common freshwater sources such as secondary particles from the breakdown of paints, plastic containers etc. (Siegfried et al., 2017). The popularity of the Stour for catch and release angling may also introduce some MPs through the use of angling baits (de Carvalho et al., 2021b) or through the degradation of plastic items.

4.5.3 Fish ecology, morphology and MP loads

It was expected that larger fish would have higher MP loads, as these individuals may require increased volumes or different foods that impact their direct and indirect encounter of MPs, with some studies identifying such correlations in some freshwater fishes (Garcia et al., 2021; Horton et al., 2018; McNeish et al., 2018). It is possible that the use of baited rod and line fishing as well as the exclusion of very small and large individuals may have narrowed the size ranges of fish within this study and skewed the trophic data in favour of larger, predatory individuals. However, no differences were found between taxa either, which vary largely in size and ecological characteristics. The lack of a relationship with body size suggests that the studied freshwater fishes had a similar encounter and/or turnover rate of MPs (D. Sun et al., 2021). This latter point is supported by the lack of variation between fish with different gastrointestinal tract structures, which were hypothesised to impact the egestion of particles (Bosshart et al., 2020; Jabeen et al., 2017; Roch et al., 2021). Since body size (indicative of age) was not correlated with MP counts, we also found no evidence of particle bioaccumulation (the accumulation of particles in older/larger individuals) within the gastrointestinal tract, as suggested in laboratory studies of the freshwater cyprinid goldfish *Carassius auratus* (Grigorakis et al., 2017). While particles may potentially accumulate elsewhere in the brain and/or liver (J. Ding et al., 2018), the processing method used in this study would have excluded any particles 1-25 µm in size (through filtering with a 26 µm mesh filter) that may best be able to translocate the gastrointestinal tract of the studied organisms (J. Kim et al., 2020; Lu et al., 2016). Laboratory experiments have demonstrated particle egestion in various freshwater fishes (e.g. Hoang & Felix-Kim, 2020; Roch et al., 2021), with egestion times typically rapid (< 24 h), size-dependent and influenced by fish body shape. The absence of relationships between fish MP counts and biological features in the present study may reflect a high egestion relative to ingestion rate while the present study also found a dominance of ≤ 100 µm that have been shown to have a longer retention time within similar species (Roch et al., 2021).

No differences in MPs were found between species, despite them representing a range of feeding guilds and trophic positions, and other studies suggesting some differences between feeding guilds (Campbell et al., 2017; McNeish et al., 2018; Roch et al., 2019), with Garcia et al. (2021) also finding significant correlations between fish MP counts and trophic position (Garcia et al., 2021). While we found no such relationship, we echo the conclusions of Garcia et al. (2021) that stable isotopes are invaluable in MP research and should be used wherever possible instead of assigning guilds at the species level, since the ecology of freshwater fishes is often highly variable in space and time and individuals may be dietary specialists (Araújo et al., 2011). Several species from this study, such as smaller shoaling *P. fluviatilis* (Davies & Britton, 2015) and *S. cephalus* (Mann, 1976), may switch to solitary ambush, piscivorous feeding at larger sizes and target larger prey, likely influencing their encounters with MPs, and so stable isotope analyses better encapsulate the individual feeding ecology than species-level allocation or dietary analysis. Predatory salmonids, eels (*Anguilla anguilla*) and pike (*Esox lucius*) are also present in the study system and tend to act as apex predators together with perch, but were excluded due to conservation interest, marine life stages and popularity for catch and release angling. The inclusion of these species may better evaluate the role of trophic position and feeding guild on MP loads within the study system.

Feeding guild has been suggested to impact MP loads, with higher levels suggested in demersal feeding fish (Merga et al., 2020; L. Zhang et al., 2021). However, some of the lowest MP detection rates have been found within various demersal feeding freshwater fishes (Bosshart et al., 2020; Pastorino et al., 2021; Sanchez et al., 2014; Slootmaekers et al., 2019). The results of the present study found no differences in MP loads between feeding types (demersal and benthopelagic), though it should be noted that several additional demersal species (gudgeon; *Gobio gobio* and rudd; *Scardinius erythrophthalmus*) were excluded as their incidence was so low. Since the demersal species from this system are often smaller than benthopelagic species (Table 4.2) it is difficult to disentangle the interactions of size, trophic position and feeding type, though this study found no variation in loads between any of these features.

4.5.4 Fish Pomphorhynchus tereticollis counts and MP loads

Contrary to our hypotheses, there was no relationship between fish *P. tereticollis* load and MP counts. We expected that the ingestion of both MPs and trophically transmitted particles might correlate, given studies in both the wild (Alves et al., 2016) and laboratory (Banihashemi et al., 2021; Limonta et al., 2019; Luís et al., 2015) have suggested a potential interaction. A potential positive feedback mechanism was hypothesised in which stressors such as MP exposure and/or parasite infection might increase feeding to compensate for any negative impacts and therefore the encounter of additional particles and/or parasites (Lafferty & Kuris, 1999; Lester & McVinish, 2016; B. Parker et al., 2021), though this was not demonstrated. The present study investigated only a single generalist freshwater fish parasite, *P. tereticollis,* which was considered a good model to investigate this potential interaction with MPs as a trophic parasite, though further studies should continue to investigate this potential relationship. It is also possible that the sample size was too small to include suitable numbers of fish with different infection and contamination status combinations to detect any interaction. However, these findings do support the independence of parasite infection and MP contamination. Future work exclusively sampling and processing a number of *S. cephalus*, the preferred host of the acanthocephalan parasite used in the present study, within the system (Hine & Kennedy, 1974a, 1974b), across all size classes throughout the year would be the best way to examine this dynamic.

4.6 Conclusions

Here we assessed the baseline MP loads from the macroinvertebrate and fish communities of a lowland river and tested if particle counts could be predicted from biological characteristics. The particles recovered were mostly ≤ 100 µm, blue/green fragments and fibres of various polyolefins and, while loads were higher within Ephemeroptera macroinvertebrate batches, counts were otherwise unrelated to all other biological features studied. The consistency of particle loads and features within the macroinvertebrate and fish community suggests that the encounter, ingestion and egestion of MPs may be uniform within the system and largely unpredictable from species ecology and morphology. The initial processing of freshwater biota is therefore still crucial to identify organisms within a system that are particularly susceptible to microplastic contamination in order to select appropriate and effective mitigation steps.

5 Parasite infection but not chronic microplastic exposure reduces the feeding rate in a freshwater fish

5.1 Abstract

Microplastics (plastics <5 mm) are an environmental contaminant that can negatively impact the behaviour and physiology of aquatic biota. Although parasite infection can also alter the behaviour and physiology of their hosts, few studies have investigated how microplastic and parasite exposure interact to affect hosts. Accordingly, an interaction experiment tested how exposure to environmentally relevant microplastic concentrations and the trophically transmitted parasite *Pomphorhynchus tereticollis* affected the parasite load, condition metrics and feeding rate of the freshwater fish final host chub *Squalius cephalus*. Microplastic exposure was predicted to increase infection susceptibility, resulting in increased parasite loads, whereas parasite and microplastic exposure were expected to synergistically and negatively impact condition indices and feeding rates. Following chronic (≈170 day) dietary microplastic exposure, fish were exposed to a given number of gammarids (4/8/12/16/20), with half of the fish presented with parasite infected individuals, before a comparative functional response experiment tested differences in feeding rates on different live prey densities. Contrary to predictions, dietary microplastic exposure did not affect parasite abundance at different levels of parasite exposure, specific growth rate was the only condition index that was lower for exposed but uninfected fish, with no single or interactive effects of microplastic exposure detected. However, parasite infected fish had significantly lower feeding rates than unexposed fish in the functional response experiment, with exposed but uninfected fish also showing an intermediate decrease in feeding rates. Thus, the effects of parasitism on individuals were considerably stronger than microplastic exposure, with no evidence of interactive effects. Impacts of environmentally relevant microplastic levels might thus be relatively minor versus other stressors, with their interactive effects difficult to predict based on their single effects.

5.2 Introduction

Aquatic ecosystems are simultaneously threatened by increasing levels of stressors such as environmental contaminants, climate change, parasites and infectious diseases (Crutzen & Stoermer, 2000; Zalasiewicz et al., 2011). Microplastic (plastics <5 mm in size) contamination is a topical stressor within freshwater systems that can induce a range of lethal and sublethal effects in exposed animal populations, alter food web structure, and cause direct and indirect effects on ecosystem structure, function and services (Eerkes-Medrano et al., 2015; C. Li et al., 2020a; J. Li et al., 2018). While typically produced on land from the degradation of larger plastics, microplastics are then dispersed into aquatic ecosystems via water and wind, with particles then ingested by resident biota (Collard et al., 2019; Eerkes-Medrano et al., 2015; Windsor et al., 2019).

Microplastics have been ubiquitously recovered from the gastrointestinal tract, skin and gills of wild freshwater fishes (Collard et al., 2019; B. Parker et al., 2021). Additionally, the negative behavioural, physiological and ecological effects of microplastic and parasite exposure singly raise major concerns, especially if exposure increases the susceptibility to additional stressors, such as parasite infection (Collard et al., 2019; B. Parker et al., 2021). Microplastic exposure has been shown to reduce feeding and morphometrics in fishes through the increased stress, immune and metabolic costs (Foley et al., 2018; B. Parker et al., 2021; Salerno et al., 2021) with ecological consequences (B. Parker et al., 2021; Wootton et al., 2021). While the single effects of microplastic and parasite exposure are better known, the potential interactive effects of chronic plastic exposure (e.g. > 90 days) with additional stressors remains poorly understood.

Parasite infections can act as considerable stressors to animal populations through their substantial physiological and ecological host consequences (Barber et al., 2000; Lafferty, 2008; Slavík et al., 2017). Parasite infections can negatively impact fitness and population dynamics of hosts, alter the symmetry of competition between infected and uninfected individuals, and modify host phenotypes through differences in the expression of life history traits, behaviours and habitat utilisation (Barber et al., 2000; Hatcher et al., 2006, 2012). Individual host responses to infections include altering their life-history traits prior to maturity when individuals allocate more resources to gonadal development than growth and survival to ensure reproduction before resource depletion and/or castration (Agnew et al., 2000; Michalakis & Hochberg, 1994). Where the parasite has a complex lifecycle involving trophic transmission then the behavioural modification of infected intermediate hosts can increase the probability of their consumption by final hosts (Barber et al., 2004; Barber & Huntingford, 1995; Lagrue et al., 2007). Microplastic exposure has been posited to alter investment in the host immune system, with the increased immune cost and any subsequent compensatory changes to foraging likely to impact both the encounter and susceptibility to parasites and therefore patterns of trophic transmission (B. Parker et al., 2021).

Increased parasite transmission and abundance has often resulted from other environmental contaminants, for example trace metals and oils, where exposure can suppress host immune responses and/or alter parasite pathogenicity (Khan & Thulin, 1991; Lafferty & Kuris, 1999; Tort, 2011). In zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), microplastic exposure has altered the regulation of gene expression and immune cells (Limonta et al., 2019; Zwollo et al., 2021), although studies assessing the relationships between microplastic loads and parasite infection levels in wild populations remain inconclusive (Alves et al., 2016; B. Parker et al., 2022a). While the exposure to both microplastics and pathogenic microorganisms in controlled conditions resulted in synergistic effects in the clinical parameters of rainbow trout (Banihashemi et al., 2021), the exposure of zebrafish to microplastics did not significantly alter their bacterial infections or mortality rates (N. Ding et al., 2022).

Although microplastic and parasite exposure can thus individually elicit considerable physiological and immunological responses in fish, the extent to which this exposure alters the outcomes of parasite exposure, and how the interaction of parasite and microplastic exposure affects the performance of individual fishes (e.g. in foraging) remains highly uncertain. The use of morphometric indices such as condition factor, and the relative spleen, liver and gonad weights, could provide useful information about the differential impacts of microplastic exposure on the general health, immune response, metabolic function and reproductive investment of fishes (Chenet et al., 2021; Mancia et al., 2020). The relative size of the spleen as a proxy of immune activity, as well as the general body condition, might be particularly responsive to microplastic and parasite exposure if impacted hosts increase investment in the immune system relative to feeding and growth (B. Parker et al., 2021), although this mechanism has yet to be demonstrated experimentally. To overcome this knowledge gap, the interactive effects of chronic microplastic contamination and exposure to different numbers of a trophically transmitted parasite were tested experimentally to assess the consequences for parasite loadings, fish morphometric indices and feeding rates. We test the hypotheses that, relative to the control diet: (1) feeding on microplastics increases fish parasite loads across a range of different parasite exposure levels (2) microplastic exposure increases spleen size while reducing fish growth and condition, and (3) the interaction of exposure to microplastics and parasites has negative synergistic effects on fish feeding rates (indicated by altered comparative functional response metrics).

5.3 Materials and methods

5.3.1 Experimental animals and husbandry

A total of 150 juvenile chub (*Squalius cephalus* L.) were used as the model host species. To minimise variability in their starting lengths and mass, and to use fish that had not been exposed previously to the acanthocephalan parasite, the fish were sourced from a local hatchery (Sheath et al., 2018). These fish had been pond-reared with diets that were only partially supplemented by formulated feeds and thus had experience of feeding on natural prey. Their mean starting standard length and wet weight (±SE) was 6.43 ± 0.02 cm and 4.26 ± 0.05 g. To acclimatise fish to the laboratory environment, they were held in relatively large groups (≈30) for 10 days in 100 L aquaria at 17 °C under a 16:8 h light-dark regime, with water quality maintained on a flow-through system. Concomitantly, 15 of the fish were selected at random and tested for the presence of both microplastics and intestinal parasites. This involved their euthanasia (overdose of tricaine methanesulfonate, MS222), followed by dissection of the intestinal tract and its screening using a glass compressorium (Hauptner) under a stereomicroscope (BMDZ, Brunel Microscopes Ltd.). No parasites or microplastics were detected in these fish. At the end of the acclimation period, the fish were measured, weighed and transferred into individual experimental tanks (Exo Terra Standard Faunarium Medium: PT2260, L x W x H: 30 × 19.3 × 20.6 cm, Supplementary material: Figure S5.1), with each fitted with a small corner filter with filter medium (Xin You XY-2008) and a plastic PVC pipe tunnel (D x L:7 × 9 cm). All fish were fed a control diet for 7 days before changing to their experimental diet.

5.3.2 Experimental procedure

The experimental design involved a three-step process: (1) chronic exposure to microplastics; (2) exposure to the acanthocephalan parasite; and (3) the functional response experiment (Figure 5.1).

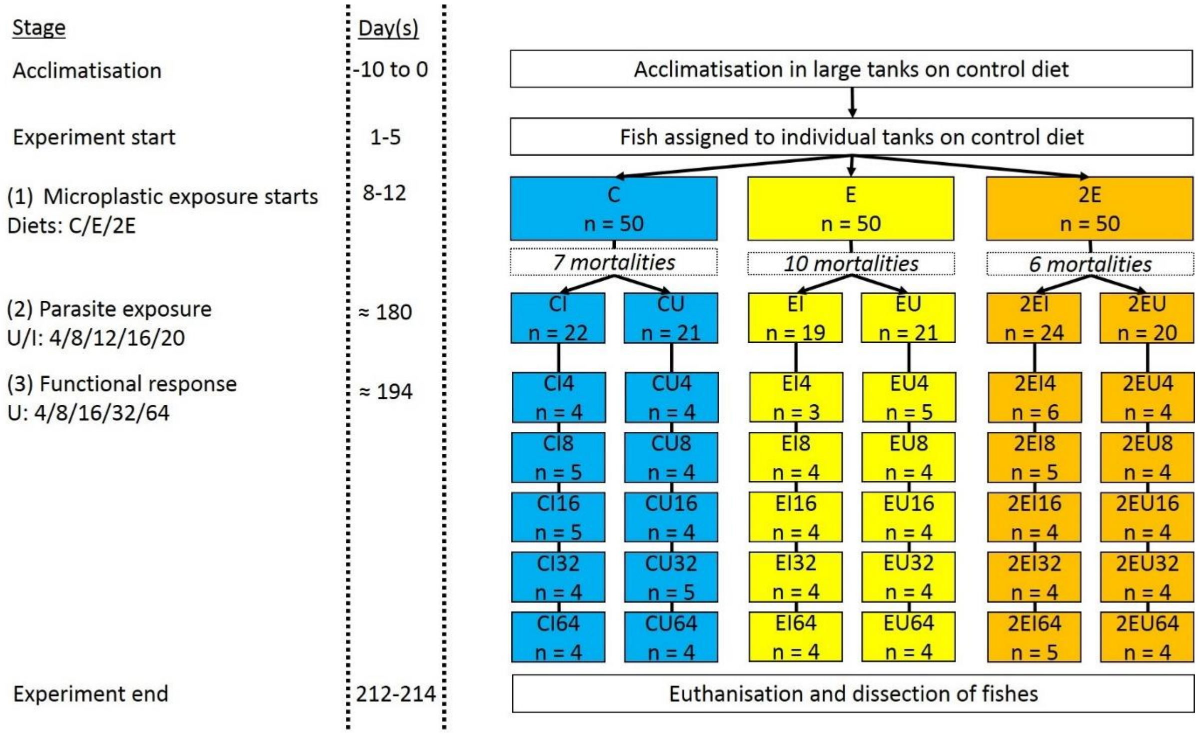

Figure 5.1. Experimental design with timings and replication overview. Replication numbers (n = x) are given for all combinations of diet: E = mean environmental and 2E = twice mean environmental microplastic exposure; parasite exposure: I = infected and U = unexposed gammarids; prey density = 4/8/16/32/64 uninfected prey. Within diet parasite exposure conditions, fish were randomly exposed to 4/8/12/16 or 20 infected or uninfected gammarids.

5.3.2.1 Chronic exposure to microplastics

Following their random allocation to the tanks, fish chronic microplastic exposure was mediated through their diet, where three diet conditions were used ($n = 50$ per diet condition): (1) control (C; no microplastic exposure) (2) environmental exposure (E; 0.5 microplastic particles d^{-1}) and (3) twice the environmental exposure (2E, 1 microplastic particle d^{-1} based on E) through feeding with control and microplastic-spiked pellets. These exposure levels were largely based on the mean loadings and features of microplastic particles recovered from the gastrointestinal tracts of wild chub in two water courses within southern England (B. Parker et al., 2022a, 2022b): the Bourne Stream 0.63 ± 0.22 and Dorset Stour 0.69 ± 0.19 particles, where ≈ 70% of all recovered particles were <1 mm in size and predominately polyolefins, such as polyethylene. Wild chub from both study systems were assumed to trophically ingest the microplastics directly and/or indirectly via contaminated prey items (B. Parker et al., 2022a, 2022b), thus spiked food pellets were considered as the most appropriate microplastic exposure method, based on Coppens' 2 mm diameter Premium Select Carp Pellets. As the feed pellets potentially already contained microplastic particles (de Carvalho et al., 2021b), 100 pellets were randomly selected and processed to confirm that no microplastics were present.

Control fish received 4 normal feed pellets every day, corresponding to 1% of the starting mean body mass, where E fish received 1 spiked pellet and 3 normal feed pellets or the control diet on alternating days (for a mean exposure of 0.5 microplastic particles d^{-1}) and 2E fish received 2 spiked pellets and 2 normal feed pellets or the control diet on alternating days (for a mean exposure of 1 microplastic particle d^{-1}). Irregular shaped microplastics were produced from blue polyethylene sheets (PE8, Lows of Dundee) through the repeated cutting and sieving of particles 0.1–1 mm in size. Spiked pellets were made by individually embedding single microplastics into wetted pellets, reforming them and allowing them to dry overnight at 50 °C. Pre-experiment trials using non-experimental fish indicated that the spiked pellets sank and retained their shape in the water, and were then consumed whole rapidly by fish. Approximately 50% of the microplastic particles were then recoverable from the gastrointestinal tracts of the fish the day after feeding, suggesting the likelihood of plastic accumulation was low (data not presented). The size range of selected particles (0.1–1 mm) was also deliberately selected to exceed those that may translocate the gastrointestinal barrier and reach other parts of the body such as the liver, brain and muscle (B. Parker et al., 2021).

The fish were initially exposed to the microplastics for ≈170 days, during which water chemistry was monitored to ensure it remained within safe limits (NH_3: <0.2, NO_2: <0.5, NO_3: <70 mg L^{-1}) for the species with 50% water changes used where necessary. In the initial 50 days of the experiment, 23 of the fish died, but mortality was not significantly related to the experimental diet (C: 7, E: 10, 2E: 6; Pearson's Chi-squared test: $\chi^2 = 6$, $df = 4$, $p = 0.20$). These fish were removed from all subsequent data analyses (Figure 5.1) and no further mortality was observed after this initial period.

5.3.2.2 Parasite exposure

The acanthocephalan parasite used in the experiment was *Pomphorhynchus tereticollis* (Rudolphi, 1809), which has a complex lifecycle involving a freshwater amphipod (*Gammarus* spp.) intermediate host and a fish final host, usually chub in Southwest England (Andreou et al., 2020; Hine & Kennedy, 1974a, 1974b; Kennedy, 2006). Parasite infected gammarids are easily identifiable by the presence of an orange spot observable through the body (Hine & Kennedy, 1974a, 1974b; Kennedy, 2006). To test how different levels of parasite exposure interacted with microplastic exposure, at the end of the microplastic exposure period, all fish were randomly assigned within diets and exposed to one of a pre-determined gammarid abundance groups (n = 4, 8, 12, 16 or 20). Half of the surviving fish were exposed to infected gammarids and the remainder to uninfected ones. All gammarids were collected from the River Avon, Hampshire (50.8864, −1.788279), by kick sampling with a 1 mm mesh net. Exposure was always on the day of gammarid collection. Initial screening of 50 gammarids revealed no microplastics were present and a further 40 were examined to confirm that the parasite was at the particular life stage and size infective to fish. Prior to gammarid exposure, the fish were starved for 24 h and the PVC tunnel and corner filter temporarily removed and replaced with an air stone to continue aeration but prevent gammarids seeking refuge behind the corner filters. Following the addition of the pre-determined number and infection status of gammarids, fish were left to consume them for 24 h. At the end of this period, any remaining gammarids were siphoned out and counted, the corner filters and tunnels were then added back to tanks, and fish resumed their experimental diet of pelleted food the following day. Surviving gammarids were not reused.

5.3.2.3 Functional response experiment

Fourteen days after parasite exposure (a time sufficient for attachment and infection within the gastrointestinal tract (Hine & Kennedy, 1974a, 1974b; Kennedy, 2006)), all fish were used in a comparative functional response experiment, where the prey were all live uninfected gammarids (collected from the field site). Fish were subject to a 24 h starvation period prior to the trial to standardise hunger levels. Tunnels and corner filters were then removed from tanks and a specific number (4, 8, 16, 32, 64) of gammarids randomly assigned within diet-parasite exposure combinations (Figure 5.1). Fish were allowed to feed without disturbance for 1 h before the remaining gammarids were recovered and counted by siphoning through a sieve. Corner filters and tunnels were then added back to the tank and the fish returned to their experimental diet. Individual fish were exposed to a single prey density and surviving gammarids were not reused.

5.3.3 Experiment conclusion and data collection

Following the functional response experiment, the fish were fed their experimental diet for six more days before being euthanised (MS222 overdose), re-measured, weighed and then dissected, with removal of the gastrointestinal tract and spleen. Gastrointestinal tracts were then pressed to 1 mm thickness using a glass compressorium (Hauptner) and screened under stereomicroscope (BMDZ, Brunel Microscopes Ltd.) for counting the number of microplastics and parasites present. Screenings were performed blind to the microplastic and parasite exposure, and any parasites were removed and weighed to more accurately determine the total end fish body weight.

Several morphometric indices relating to body condition, growth rate and immune activity were calculated for all individuals surviving until the experiment end:

$$\textit{Fulton's condition factor (K)} = 100 \times \frac{W}{SL^3}$$

$$\textit{Specific growth rate (SGR)} = \frac{100 \times (\ln(W_E) - \ln(W_S))}{\Delta t}$$

$$\textit{Splenosomatic index (SSI)} = 100 \times \frac{SW}{W}$$

where W is total body weight (excluding the weight of all parasites), SL standard length, W_E and W_S are the end and start weights, respectively, Δt the change in time (days) between measurements, and SW the end spleen weight.

To test the effects of parasite exposure in the morphometric and functional response analyses, fish were assigned to three different parasite exposure categories depending on the status of the presented gammarids and the end parasite load (identified during dissection): (i) unexposed (fish fed with uninfected gammarids that were thus uninfected; (ii) exposed fish (fish presented with infected gammarids but were uninfected on dissection), and (iii) infected (fish presented with infected gammarids and that had parasites within the gastrointestinal tract). The data for 12 individuals (4 for each diet) were excluded from the analyses as no prey items were consumed which was assumed to be an unnatural behaviour. A single parasite was recovered from an unexposed fish, assumed to have resulted through the accidental addition of an infected gammarid, and was subsequently excluded from the analyses. After assigning to parasite exposure categories, 61 fish were unexposed, 33 were exposed, and 32 were infected (n = 67 parasites recovered, mean ± SE: 2.09 ± 0.25 parasites per infected fish).

5.3.4 Statistical analyses

All data analyses were carried out in RStudio version 3.5.1 (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com). Within each analysis, the model selection process first compared between a pair of beyond optimal models with all fixed effects and their interactions: a linear mixed effects model (LMEM) containing batch and rack as random effects, with a simpler general linear model (GLM) on the basis of Akaike Information Criterion (AIC). The simpler model was selected where the AIC value was two points lower. The progressing model was then subject to a top-down approach, as outlined by Zuur et al. (2009), working backwards and sequentially removing the least significant term in each iteration until an optimal model was reached (where all remaining variables were significant or all remaining variables were non-significant). The optimal model was then checked for overdispersion using the ratio between the residual variance and degrees of freedom (Zuur et al., 2009) where ratios <1 indicate no overdispersion. Overdispersion was not identified in any of the analyses.

For infected fish, a Poisson model examined if parasite loads were related to the interaction of microplastic exposure and the number of infected gammarids consumed (indicating parasite exposure) to examine if infection was higher for fish exposed to microplastics. Separate Gaussian models were then performed to test the interaction of microplastic exposure and parasite exposure categories on change in Fulton's condition factor (ΔK), SGR and SSI as indices of general health, growth throughout the experimental period and immune investment, respectively.

For the number of uninfected gammarids consumed within the functional response experiment, first a Poisson GLM, Poisson LMEM and a negative binomial LMEM were compared on the basis of AIC to determine the best model fit. The progressing model tested the number of uninfected prey items consumed based on the interaction of the parasite and microplastic exposure categories. Comparative functional response curves were then determined for significant effects only using functions from the package “FRAIR” (Pritchard et al., 2017). Attack rate (a) and handling rate (h) were calculated for data aggregated by each of the above parasite exposure categories (excluding those individuals where 0 gammarids were consumed) using “frair_fit” (based on a Type II response, $a = 1.2$, $h = 0.015$) and “frair_boot” (200 iterations), before carrying out pairwise comparisons between parasite exposure categories using the “frair_compare” function. Attack rate is the rate at which an organism encounters prey items at a particular density, whereas the handling rate defines the time taken to process a prey item.

5.4 Results

5.4.1 Microplastic and parasite exposure impacts on parasite load and fish morphometrics

No fish accumulated microplastics during the experiment, with dissections at its conclusion revealing no remaining plastics in the gastrointestinal tracts, either from the experimental treatments or other sources. The best fitting model for the parasite load data was the general linear model structure (Poisson GLM AIC = 113, Poisson LMEM AIC = 117). The resulting optimal parasite load model indicated that parasite load increased with the number of parasites ingested only (Poisson GLM: $\chi^2 = 4.06$, df = 1, $p < 0.05$, Figure 5.2, S5.1 models), with microplastic exposure having non-significant single and interactive effects ($p > 0.05$, S5.1 models).

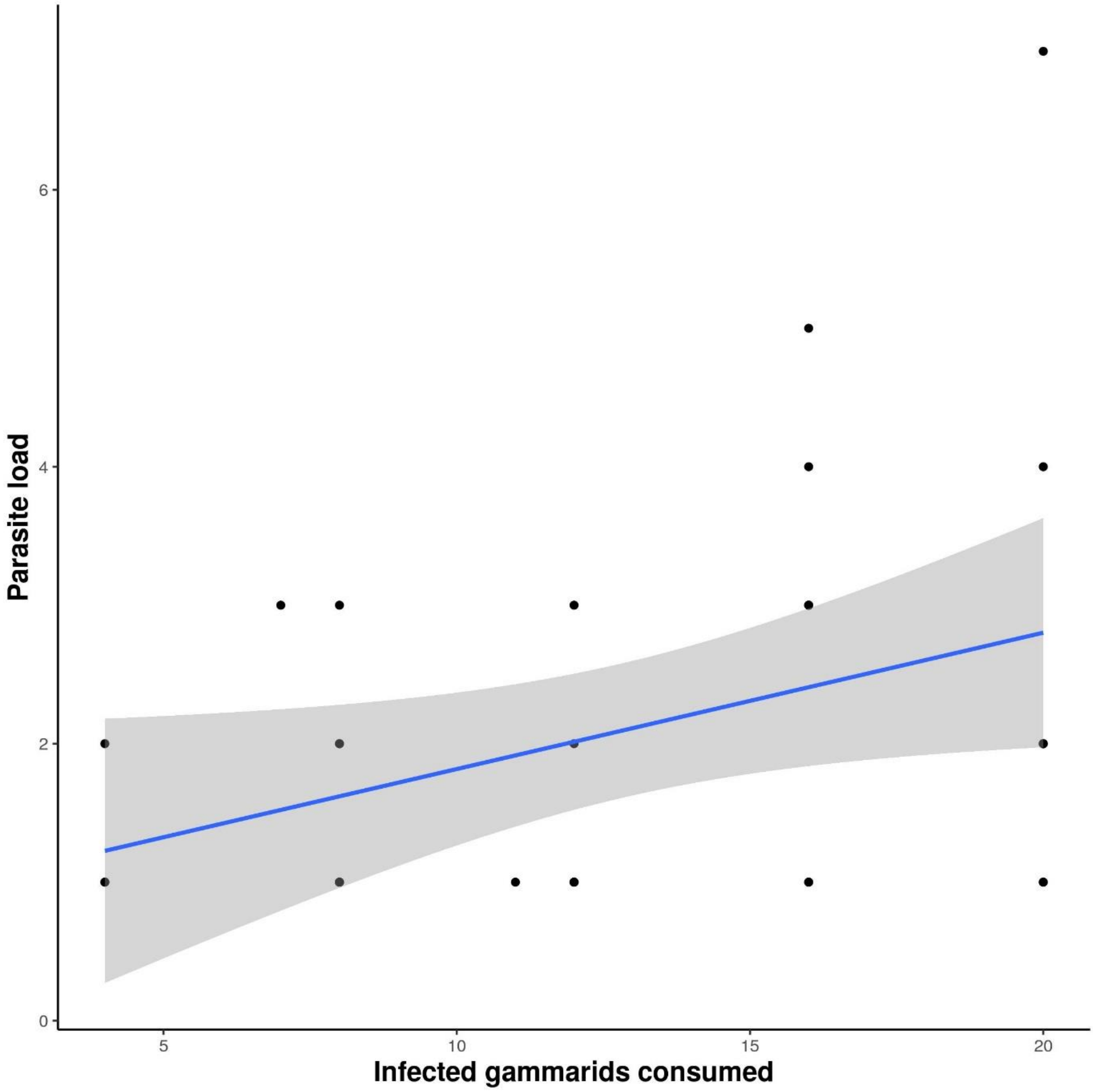

Figure 5.2. The relationship between parasite load and the number of infected gammarids consumed. End parasite loads and the number of parasites ingested (via infected gammarid intermediate hosts) are given for the 32 infected fish. The model fitted line is plotted along with the standard error border margins.

The best fitting models for all morphometric data were GLM rather than LMEM variants (Change in condition: Gaussian GLM AIC = 5, Gaussian LMEM AIC = 36; Specific growth rate: Gaussian GLM AIC = −366, Gaussian LMEM AIC = −315; Splenosomatic index: Gaussian GLM AIC = −183, Gaussian LMEM AIC = −136). Microplastic exposure, parasite exposure and their interaction had no effect on change in condition (all factors $p > 0.05$, S5.2 models). Specific growth rate varied between parasite exposure categories (Gaussian GLM: $\chi^2 = 8.14$, df = 2, $p < 0.05$) and was lower for exposed relative to unexposed fish (Figure 5.3, S5.3 models, Table S5.1). Splenosomatic index did not vary between microplastic, parasite exposure categories, or their interaction (all factors $p > 0.05$, S5.4 models).

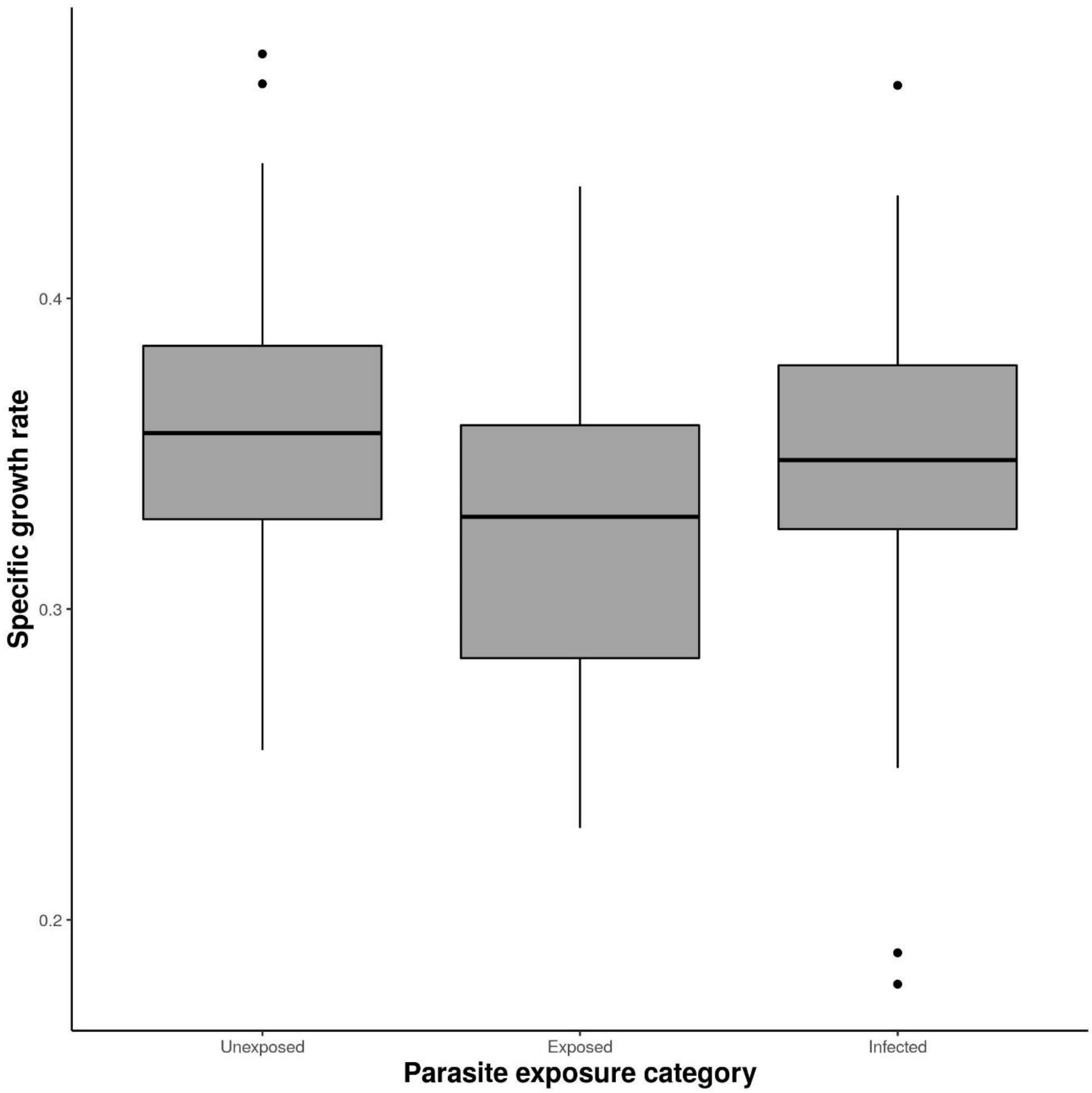

Figure 5.3. Specific growth rates between the different parasite exposure categories. Boxplots show the distribution of specific growth rate values for all fishes aggregated within the Unexposed (n = 61), Exposed (n = 33) and Infected (n = 32) categories.

Comparative functional responses

The best fitting model for the number of gammarids consumed was a negative binomial LMEM variant (Poisson GLM AIC = 1572, Poisson LMEM AIC = 1541, Negative binomial LMEM AIC = 837). The optimal model indicated that the number of gammarids consumed differed between parasite exposure categories (Negative binomial LMEM: $\chi^2 = 20.14$, df = 2, $p < 0.001$, S5.5 models). Correspondingly, the functional response data were grouped by the parasite exposure categories, revealing that fish infected with parasites had significantly lower attack rates but higher handling times than unexposed fishes (Table 5.1, Figure 5.4). Additionally, infected fish had significantly lower attack rates than exposed fish, whereas unexposed individuals had lower handling rates compared to fish exposed to, but not infected by, the parasite (Table 5.1, Figure 5.4).

Table 5.1. (A) Functional response coefficient estimates for aggregated parasite exposure categories. Attack (a) and handling rates (h) for all parasite exposure categories are reported after excluding fish where no gammarids were consumed. (B) Outputs of pairwise functional response coefficient tests. Tests compare differences in attack (Da) and handling rate (Dh) between all parasite exposure categories, excluding fish where no gammarids were consumed. SE refers to standard error and significance levels are denoted by “*”.

(A)

Factor level	Attack rate (a)	Handling rate (h)
Unexposed	3.27	0.02
Exposed	3.11	0.06
Infected	1.67	0.07

(B)

Comparison	Coefficient	Estimate ± SE	z value	p value
Unexposed-Exposed	Da	0.16 ± 0.55	0.29	0.77
	Dh	-0.04 ± 0.00	-8.25	< 0.001 ***
Unexposed-Infected	Da	1.60 ± 0.39	4.06	< 0.001 ***
	Dh	-0.05 ± 0.01	-6.67	< 0.001 ***
Exposed-Infected	Da	1.44 ± 0.56	2.59	< 0.01 **
	Dh	-0.01 ± 0.01	-1.64	0.10

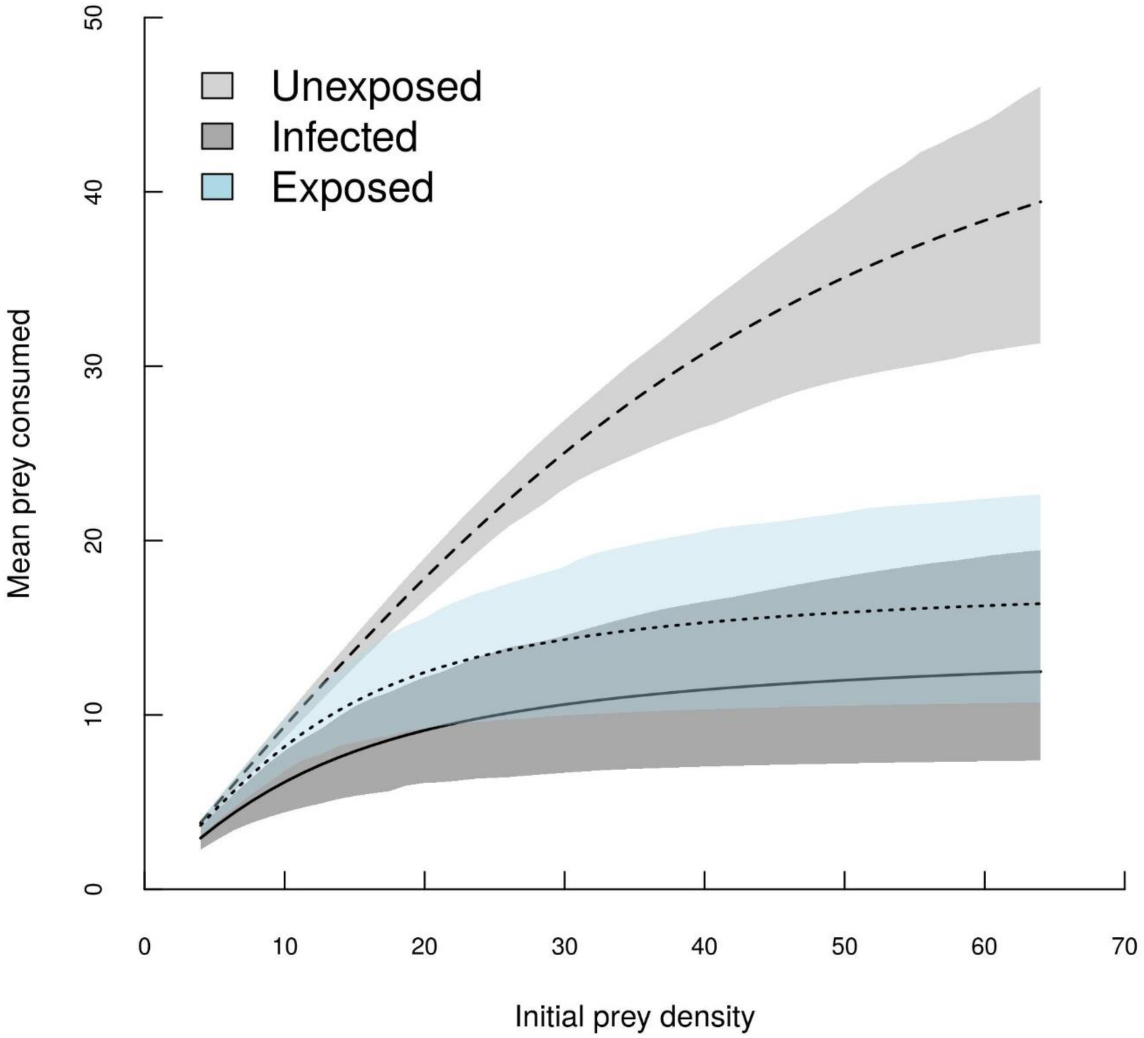

Figure 5.4. Functional response curves by parasite exposure category. Curves with confidence intervals are produced using data aggregated by parasite exposure status, excluding fish where no gammarids were consumed. Parasite exposure categories: Unexposed (dashed line, light grey) = fish exposed to but not infected by the parasite, Infected (solid line, dark grey) = fish exposed to and infected by the parasite and Exposed (dotted line, light blue) = individuals exposed to but not infected by the parasite.

5.5 Discussion

This study is the first to investigate the potential interactive effects of environmentally relevant microplastic and parasite exposures on the parasite load, morphometrics and feeding of a freshwater fish. The results revealed that the effects of parasite exposure and infection on functional response parameters were substantially stronger than chronic microplastic exposure, with no significant interactive effects. Within infected fish, diet did not impact parasite load and microplastic exposure had no effect on fish morphometrics; however, specific growth rate was lower in exposed relative to unexposed fish. Significantly lower feeding rates were observed in infected relative to unexposed fish. Additionally, infected fish had a lower attack rate and unexposed fish a lower handling rate relative to exposed individuals. This is a highly important result since the fish were chronically exposed to environmentally relevant microplastic exposure levels to determine if their effects on host-parasite dynamics are similar to those of other environmental contaminants (Khan & Thulin, 1991; Lafferty & Kuris, 1999; Tort, 2011).

5.5.1 Parasite load and morphometric indices

Contrary to our hypotheses, no interactive effects were observed, with diet not impacting parasite load and load only increasing with the number of ingested parasites. This result of microplastic exposure not interactively impacting the susceptibility of organisms to parasite infection is also contrary to assumptions that microplastic exposure may impair immune function (Limonta et al., 2019; Masud et al., 2022; B. Parker et al., 2021) and additionally suggests that any correlation between microplastic and parasite loads in wild and experimental fish may be coincidental (Alves et al., 2016). Parasite loads were positively related to the number of parasites consumed and high parasite exposures were required to achieve infection, as also found elsewhere (Sheath et al., 2016, 2018). However, some fish in the present study did not become infected, even after consuming >10 parasites, perhaps due to differences in the experimental conditions, host response and/or perhaps differences in parasite infectivity (Hine & Kennedy, 1974a, 1974b; Kennedy, 2006). Fish in the present study were sourced from a local hatchery to reduce variations in size, age and genetic variation, as well as to ensure that fish had no previous exposure to the parasite and microplastic exposure used. As such, a population of wild *S. cephalus* would likely be more resistant to the microplastic and parasite treatments due to prior exposure, reduced stress and/or a higher resistance to parasites (Oliva-Teles, 2012; Wysocki et al., 2007), however individual variation in responses would likely also be much greater due to greater genetic and individual variation (Kohlmann et al., 2007; Norris et al., 1999).

Microplastic exposure had no effect on fish morphometric indices, while parasite exposure only impacted the specific growth rate for exposed but not infected fish. These results were contrary to the hypothesis that condition and specific growth rate will be lower in fish that experienced chronic microplastic and shorter term parasite exposure, since polyethylene ingestion (Hu et al., 2022; Jabeen et al., 2018; Ottová et al., 2005; Šimková et al., 2008; Tarasco et al., 2022) and *P. tereticollis* exposure (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015) can both negatively affect body condition (and other morphometrics) in *S. cephalus* and other cyprinid fishes. Similarly, the splenosomatic index, a proxy of spleen size and immune function, was also predicted to be higher in fish exposed to microplastics as well as parasites, given the metabolic, immunological and pathological costs to microplastic (Chenet et al., 2021; M. Zhu et al., 2020) and parasite exposure singly (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015). The absence of these relationships could result from the use of environmentally realistic microplastic exposures in the present study, based on two rivers in southern England (B. Parker et al., 2022a, 2022b), as other studies have demonstrated effects at higher and/or environmentally unrealistic exposures (Hu et al., 2022; Jabeen et al., 2018; Tarasco et al., 2022). Alternatively, it is possible that the experimental exposure was impacting other organs such as the liver and gastrointestinal tract, not investigated here but previously demonstrated to be impacted by microplastic exposure (Foley et al., 2018; B. Parker et al., 2021; Salerno et al., 2021). Additionally, the period of parasite exposure was relatively short compared to other studies (Sheath et al., 2016, 2018) and may thus exert effects on other morphometrics over longer timescales.

5.5.2 Comparative functional responses

Microplastic and parasite exposure had no interactive effect on the number of gammarids consumed, with the aggregated functional response curves only identifying reduced feeding in infected - and to a lesser degree exposed - fish versus unexposed fish. This result partially supported the hypothesis that parasite exposure reduces feeding and is consistent with previous work that identified significantly lower attack rates and higher handling times for fish exposed to this parasite (Sheath et al., 2018). The ingestion of *P. tereticollis* parasites via infected intermediate hosts may result in reduced feeding due to, for example, pseudo-satiation, blockage of the gastrointestinal tract or altered behaviour (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015). *Pomphorhynchus* spp. infection in wild *S. cephalus* has been shown to be associated with several histopathologies of the small intestines, locally to the attachment sites of parasites, as well as increased levels of immune cells (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015), supporting a negative single effect of infection that might also impair feeding. Parasite attachment may depend on factors such as parasite fitness affecting their ability to attach, the immune response mounted by the fish, the condition of the organ surface structure and the remaining space available which likely impacts the efficiency of trophic transfer and the subsequent handling of food items if digestive structures are damaged (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015). The handling rate for exposed fish was significantly higher than for unexposed individuals and was no different than for infected individuals, suggesting that even exposure to the parasite and/or infected prey items may have negatively impacted feeding, perhaps through a short-term physiological or immune response and/or cost (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015). Paired with the lower specific growth rate observed, parasite exposure thus resulted in both a behavioural and physiological change within fish exposed to parasites.

In contrast to the hypothesis, microplastic exposure did not impact the number of infected parasites consumed. It was posited that microplastic exposure would reduce the feeding of fish through mechanisms such as increased metabolic stress, immune investment and physiological damage to feeding apparatus, as identified in other freshwater cyprinids (Hu et al., 2022; Jabeen et al., 2018; Tarasco et al., 2022), which might induce behavioural and feeding changes. Discrepancies may arise from the different exposure conditions, especially the level and type of microplastics used, as well as the particular organism whereby environmentally irrelevant exposures may produce artificial effects not seen in and relevant to nature. While few studies have directly investigated the impact of microplastic exposure on comparative functional responses, no impact of different types and concentrations of microplastic were detected in European green crab *Carcinus maenas* feeding on blue mussels *Mytilus edulis* (Cunningham et al., 2021). Finally, the greater retention time for smaller particles and/or fibres may mean that the impacts of microplastic exposure may depend as much on the particle features as the concentration and might lead to the systematic over- or under-estimation of the negative impacts of microplastic exposure depending on the particular particles used (Hoang & Felix-Kim, 2020; S. W. Kim et al., 2019; Xiong et al., 2019).

5.5.3 Microplastic exposure

No microplastics, from the experimental diets or introduced throughout, were identified in any of the fish during blind dissections at the end of the experiment, suggesting no contamination and that the desired microplastic exposure levels were achieved in the experiment comparable to the loadings seen in wild *S. cephalus* (B. Parker et al., 2022a, 2022b). Further, pre-experiment assessments of pellets, source fish and gammarids ensured that the only exposure of the experimental fish to microplastics was through the spiked pellets. The pilot studies indicated approximately 50% of microplastics were retained the day after feeding, which is comparable to levels detected in other cyprinids under controlled conditions (Hoang & Felix-Kim, 2020; S. W. Kim et al., 2019; Xiong et al., 2019). However, these studies highlight that particle features, fish body size and feeding rates will impact egestion, therefore pilot egestion studies are crucial to determine particle turnover (Hoang & Felix-Kim, 2020; S. W. Kim et al., 2019; Xiong et al., 2019). Additionally, we emphasise that the exact particle retention times at different exposure levels were not determined and thus fish may have differed in their egestion times and therefore actual loadings at any particular time (Hoang & Felix-Kim, 2020; S. W. Kim et al., 2019; Xiong et al., 2019). The recovery of microplastics from wild *S. cephalus* and other freshwater fishes (Collard et al., 2018; B. Parker et al., 2022a, 2022b) demonstrates not all particles are immediately egested, and that the encounter and ingestion rate might often exceed egestion. While studies have found that polyethylene microplastic ingestion can impact the feeding of freshwater cyprinids by altering the buccal cavity (Jabeen et al., 2018), no such effects were detected here.

5.6 Conclusions

This experiment represents, to our knowledge, the first interaction experiment to investigate how environmentally relevant microplastic loadings and acanthocephalan parasite exposure affects the host-parasite relationships, morphometrics and feeding ecology in a freshwater fish. Although microplastics are considered an environmental contaminant of high concern, detrimental effects on fish hosts were not evident in the behavioural functional response metrics and morphometric indices, perhaps due to the use of environmentally derived exposure levels. In contrast, both exposure to and infection by parasites increased the handling but decreased the attack rate of foraging fish, whereas the specific growth rate was lower in exposed fish only, indicating a cost of both exposure and infection. It is important to emphasise that reductions in feeding rate and the reduced specific growth rate for exposed fish were detectable only two weeks after parasite exposure, yet no alterations to feeding or morphometrics were observed even after several months of microplastic exposure. The absence of interactive effects between environmentally relevant microplastic and parasite exposures suggests microplastics have minor effects when compared with other stressors, although we suggest additional interaction studies are needed to understand the conditions under which more severe impacts could manifest. Finally, future studies should investigate the potential interactive effects of microplastics with other parasites spanning different costs of infection and mechanisms of action.

6 Low microplastic loads in riverine European eel (*Anguilla anguilla*) from SW England during their marine-freshwater transition

6.1 Abstract

The microplastic loads in elvers of the critically endangered European eel *Anguilla anguilla*, sampled in the lower reaches of three English rivers, were very low (incidence: 3.3 %, mean ± SD: 0.03 ± 0.18 particles) and did not vary with body length or between rivers. Particles were mostly black, polyolefins, fibres and fragments of size 101-200 µm. Current levels indicate a low contamination pressure locally and, consequently, management efforts might prioritise mitigating the effects of other stressors affecting the species.

6.2 Introduction

Microplastics (plastics < 5 mm in maximum size), are typically produced from the environmental degradation of larger plastic items and then transported into freshwater systems by wind and rain (Andrady, 2011; Galloway et al., 2017). Microplastics are ingested by a wide range of freshwater fishes either through direct feeding or indirectly by the ingestion of contaminated resources (Azizi et al., 2021; Collard et al., 2019; B. Parker et al., 2021). Experimentally, the ingestion of plastic particles has induced a range of detrimental effects on survival, physiology, behaviour and reproduction depending on the extent of exposure and the affected species, but with some studies also indicating no effect (Collard et al., 2019; B. Parker et al., 2021). Fish ingestion rates of microplastics has been associated with their biological traits with, for example, higher loadings expected in species at higher trophic levels through biomagnification, while larger/older individuals are expected to accumulate microplastics over time through bioaccumulation (Garcia et al., 2021; McNeish et al., 2018; Munno et al., 2022). However, the evidence supporting the relationships of fish biological traits and microplastic loadings is often equivocal (Covernton et al., 2021; B. Parker et al., 2022a, 2022b).

The European eel *Anguilla anguilla* (Linnaeus) (‘eel’ hereafter) is a critically endangered catadromous fish and important predatory fish found throughout Europe (Pike et al., 2020) whose panmictic population has undergone significant declines in recent decades, where causal factors include exploitation, riverine barriers to migration, altered ocean currents and environmental pollution (Baltazar-Soares et al., 2014; Geeraerts & Belpaire, 2009). Eel trophic ecology is intimately linked to their body size, with smaller eels generally feeding on macro-invertebrate prey but with increasing proportions of fish in their diet with increasing body size and head width (Cucherousset et al., 2011; Pegg et al., 2015), which has also been related to the accumulation of mercury and several organic pollutants (De Meyer et al., 2018). The eel lifecycle includes their transition from marine to freshwater environments at their glass-eel (non-pigmented) and elver (pigmented) stages when there is potential for individuals to transport marine plastics into the freshwater environment (Menéndez et al., 2022). Studies on the incidences of microplastics in eels are conflicting, with no particles in the River Garonne, France (Garcia et al., 2021), while incidences in three rivers discharging into the Bay of Biscay reported 2.74 microplastics per gram of glass eel (Menéndez et al., 2022), with loadings related to concentrations in the adjacent freshwater and marine environment (Garcia et al., 2021). Given these discrepancies in loadings, the aim here was to investigate the incidence of microplastic contamination within European eel elvers from three rivers in SW England, with testing of the effects of location and body size. It is posited that larger eels would have higher microplastic counts. Due to their conservation and ecological importance it is thus important to understand the incidence of microplastics in different eel populations to identify the impacts and sites of microplastic exposure within their critical marine-freshwater transition stage.

6.3 Materials and methods

Elver samples (n = 300, mean total length ± SD = 81.1 ± 24.2, minimum = 64, maximum = 170 mm) were collected from the lower reaches of the Rivers Frome, Piddle and Huntspill in SW England between the 1st June 2021 and the 24th August 2022. For the Piddle, samples were collected at an elver pass located on the most downstream weir of the river (50.68809, -2.12414), approximately 3 km upstream of its confluence with Poole Harbour. Elvers were captured by attaching a net on the upstream egress of the pass, where the period between setting and lifting the net was < 18 h. Elvers were sampled from the River Frome using electric fishing (Smith Root LR24) on a side channel located approximately 8 km from its confluence with Poole Harbour (50.67954, -2.18150). The Hunstpill was sampled as per the Piddle, where the elver pass was located at the tidal barrier (51.22025; -2.98444). All samples were euthanised by anaesthetic overdose (MS-222) and frozen. The collection of samples was for the purposes of another study where destructive sampling was required and the relevant ethical and legislative approvals were in place (UK Home Office Project Licence P47216841; Environment Agency permit reference EP/EW027-C-042/19919/01). The project about microplastics in eels was approved by the ethics panel for the UK Home Office Project Licence PA2C7C4E6. In the study areas, elvers and larger eels are considered as highly abundant and thus the sampling was not impacting the sustainability of the local eel population.

Within the laboratory, the elvers were defrosted and total length (nearest mm) recorded, before the gastrointestinal tracts were removed and stored in individual glass vials. Subsequent processing of the gastrointestinal tracts were as per previous works (B. Parker et al., 2022a, 2022b) but a potassium hydroxide digestion (10 ml, 15 %, incubation at 60 °C for 48 h at 30 rpm) was used to reduce the amount of organic residue remaining. After digestion, the resulting samples were then vacuum filtered through a steel filter (13 mm diameter, 48 µm mesh; The Mesh Company Warrington). Dried filters were screened using microscopy (Leica M165C; up to ×120 magnification), with suspected microplastics identified using criteria such as size, shape and colour (Nor & Obbard, 2014). The colour, morphology and maximum size (measured at ×120 magnification; eyepiece graticule and later converted to µm) of suspected particles were recorded within a set 5 min search period which was sufficient to cover the entire filter several times.

Regarding the reduction of sample microplastic contamination in the laboratory, all elver samples were measured, dissected, potassium hydroxide added and vacuum filtered within a pre-cleaned flow cabinet. After adding the digestion reagent, sealed samples were transferred into an incubator with the sealed filtered steel discs later opened under the microscope to screen them. Glass and metal ware was used wherever possible instead of plastics and the glass vials and filter discs were sterilised prior to use by furnacing at 500 °C. The potassium hydroxide and water used to rinse through the containers was first filtered through a glass microfibre filter (1.2 µm, Whatman glass microfibre filters) with the vacuum filtering equipment rinsed through twice with the same filtered water prior to use. Procedural blanks for the digestion reagent were also carried out, as per previous studies (B. Parker et al., 2022a, 2022b), alongside the actual samples with 1 blank sample carried out for every 10 samples processed within the batch. Procedural blanks were then processed as the actual samples and subject to the same microplastic screen under microscope. Blue fibres were recovered from 4 of 32 blank samples and were therefore excluded as suspected contaminants from all eel samples (n = 15 blue fibres) and subsequent analyses.

Suspected microplastics recovered from the elvers were then subject to polymer analysis using a micro-Attenuated Total Reflectance (micro-ATR) accessory coupled to a Spotlight^{TM} 400 FTIR Imaging System coupled to a Frontier^{TM} IR Spectrometer (PerkinElmer, Llantrisant, UK). Spectra were collected from 650-4000 cm^{-1} and ran against reference databases using an arbitrary match score of 0.7 as a “hit” (B. Parker et al., 2022a, 2022b). The hits were later compiled into broader polymer categories giving special preference to plastic over organic matches (only those ≥ 0.7) as per Parker et al. (2022a, 2022b). Suspected microplastic counts (determined from screening) were then corrected based on this FTIR information data, excluding undeterminable and non-plastic particles from the count data to provide absolute microplastic counts. Then a Poisson linear mixed effects model testing the microplastic counts against the interaction of body length and river as a fixed effect, with sampling date as a random effect, was completed and compared against a comparable Poisson general linear model (lacking the random effect) and a general linear model using a negative binomial distribution. A reduction of two points in Akaike Information Criterion (AIC) was considered as a significant difference between models, with the retained model having the lowest AIC value. This model was then subjected to backward selection (Zuur et al., 2009), involving removal of the least significant term at each iteration (based on the p value) until an optimal model was reached where all remaining terms were either all significant or non-significant. Analyses were performed using RStudio version 3.5.1 (R Development Core Team, 2021).

6.4 Results

Elver sizes were largest in the River Frome and smallest in the Huntspill (Table 6.1A), where many of the analysed Huntspill individuals were still non-pigmented. Of 27 suspected microplastic particles, 10 were confirmed by FTIR as being microplastics (3.3 % incidence; Table 6.1B). Confirmed microplastics were equal proportions of fibres and fragments, with black, 101-200 µm and polyolefins being the dominant microplastic categories of colour, size and polymer, respectively (Table 6.1B). The best fitting model was the Poisson general linear model (Poisson general linear model: AIC = 97.3, Poisson linear mixed effects model: AIC = 99.3, Negative binomial general linear model: AIC = 99.3). This model indicated that microplastic counts did not vary with the main or interactive effect of river and body length (reverse order of removal: length: Total length $\chi^2 = 0.19$, df = 1, $p > 0.05$; River*Total length $\chi^2 = 1.24$, df = 2, $p > 0.05$; River $\chi^2 = 1.29$, df = 2, $p > 0.05$, S6.1 models).

Table 6.1. A) Descriptive statistics for the 300 juvenile *Anguilla anguilla* recovered from the 3 study rivers. B) Features of the confirmed microplastic particle. Details for the colour, morphology, maximum size and polymer type (confirmed via FTIR) of each of the 10 particles are presented.

A)

Descriptive statistic	River Frome	River Huntspill	River Piddle
Sample number	72	105	123
Mean total length ± standard error (mm)	108 ± 19	71 ± 3	82 ± 10
Microplastic number	4	3	3
Frequency of occurrence (%)	5.5	2.9	2.4

B)

River	Colour	Morphology	Size (µm)	Polymer
Frome	Blue	Fragment	110	Additive
	Blue	Fragment	144	Polyolefin
	Yellow	Fibre	274	Polyolefin
	Pink	Fibre	309	Polyamide
Huntspill	Black	Fibre	439	Polyolefin
	Black	Fibre	466	Polyolefin
	Black	Fibre	974	Polyolefin
Piddle	Clear	Fragment	110	Polyolefin
	Red	Fragment	117	Polyester
	Red	Fragment	130	Polyolefin

6.5 Discussion

The presented incidence and number of confirmed microplastics was much lower than a comparable study finding several particles per gram of juvenile eel (Menéndez et al. 2022). The average load and frequency of occurrence (0.03 particles per eel and 3.3 % respectively) are additionally amongst the lowest reported within freshwater fishes (B. Parker et al., 2021, 2022a, 2022b). However, no plastic particles were recovered from 40 *Cottus gobio* from an alpine lake (Pastorino et al., 2021) and from 11 *A. anguilla* from the Garonne, France (Garcia et al., 2021). Such variability in plastic loadings might arise from the different microplastic pressures/local urbanisation levels, dietary differences between lifestages and the processing methods used in the present study, for example Menéndez et al. (2022) used a seven-day hydrogen peroxide digestion and a much smaller filter pore size (0.45 µm) which may affect the type and number of recovered particles based on their susceptibility to different digestion methods (Avio et al., 2015; Nuelle et al., 2014). Structures such as dams and weirs have been shown to impact the accumulation of microplastics (Mani et al., 2015; Watkins et al., 2019) and there is additionally a knowledge gap on the time taken for these eel life-stages to by-pass such structures and use elver passes. It is also possible that the duration between capture and elver removal from the net may have allowed the excretion of any ingested particles or the ingestion of particles within the traps, potentially under- or overestimating microplastic loads and the frequency of occurrence within the Piddle and Huntspill. Nevertheless, if many of the plastic particles were ingested/egested by elvers prior to their collection then this would indicate that microplastic turnover in freshwaters is relatively short.

The microplastics recovered from within the eel samples likely originate either from pelagic feeding by the leptocephalus stage in or around the Sargasso sea, known to contain floating plastics (Carpenter & Smith, 1972) or by freshwater feeding as glass eel and elvers. However, a small proportion of the intermediate glass eel stage do feed within estuarine environments, providing opportunities for plastic ingestion there (Bardonnet & Riera, 2005; Van Wichelen et al., 2022). Notwithstanding, as the Frome eels were collected some distance from the tidal limit and were considered to have been in the river for some time, then it is likely their plastic items were all of freshwater origin. The application of stable isotope analysis to migratory fishes used in microplastic loading studies could thus link the trophic ecology of individuals with levels of environmental contaminants (Garcia et al., 2021; B. Parker et al., 2022b). As Menéndez et al. (2022) found that river and sea water microplastic contamination predicted contamination levels of individual eels from rivers draining into the Bay of Biscay, the differences in plastic loadings with our study might reflect differences in abiotic microplastic loads between the study locations, however no differences were identified between sites. The use of samples here that had already been collected for the purposes of a separate study meant that there was no opportunity to concomitantly collect and analyse abiotic water and/or sediment samples to investigate this further.

Contrary to predictions, microplastic loadings were unrelated to eel body length. It was assumed that larger individuals would have a larger gape size to access to a greater size range of microplastic particles as well as prey items from which to ingest microplastics, as suggested by several studies on freshwater fishes (Garcia et al., 2021; Park et al., 2020b). The findings of the present study mirror those of previous studies suggesting no relationship between fish size and microplastic load (B. Parker et al., 2022a, 2022b). The size of the recovered microplastics (< 1 mm) may approach the diameter of the gastrointestinal tract of some individuals (in the mm range, data not presented), however the present study did not record any metrics or biomarkers to assess the potential impact of microplastic ingestion.

While it is possible that juvenile eels in the system are highly susceptible to microplastic contamination and so survivorship bias prevented the observation of higher incidences and loadings above a single particle, the data presented by Menéndez et al. (2022) for eels in the Bay of Biscay suggest eels can have much higher plastic loadings than we detected and still survive. This further emphasises that the lower frequency of occurrence and loadings in our eels was probably more reflective of a lower microplastic contamination in the sampled freshwaters.

6.6 Conclusions

In summary, the results here indicate a low incidence of microplastics in glass eel and elvers immigrating into freshwaters in southwest England, with microplastic loads not varying with body length (as a proxy of age and trophic position) or river. It is thus recommended that eel conservation efforts in these areas continue their focus on other stressors that potentially impact their recruitment into freshwater, including exploitation and barrier passage, while further monitoring the incidence and impacts of microplastics on these populations.

7 Additive effects explain the interactions between microplastics and interacting stressors in freshwater fishes

7.1 Abstract

Exposure to microplastics can cause a range of negative single effects on the ecology, physiology and immunology of freshwater fishes. However, fishes are typically exposed to a range of additional multiple stressors simultaneously for which the interactive effects with microplastics are poorly understood. Here, using a meta-analysis, we tested whether microplastic and secondary stressor interactions are mostly additive, antagonistic or synergistic and whether the effect size magnitudes and directions could be related to the interacting stressor, response category, microplastic and fish features. Analyses were repeated for a subset of data for which the microplastic exposure was considered environmentally relevant. Across the final dataset of 150 interactions from 28 studies, most interactions were classified as additive or antagonistic, and the dominant type was consistent across datasets, interacting stressors and response categories. Additionally, response effect size estimates were antagonistic (significantly lower than null predictions) for ecological responses within the exclusive data subset but were otherwise within the range of null additive model predictions when grouped by stressor and response category. Finally, effect sizes did not differ with microplastic exposure features, fish life-stage and family. Overall, these results suggest that additive effects best explain the interactions of microplastics and interacting stressors for freshwater fishes.

7.2 Introduction

Microplastics, plastics < 5 mm in size, are a type of environmental contaminant ubiquitously found within freshwater systems globally (C. Li et al., 2020b; J. Li et al., 2018; Talbot & Chang, 2022). Particles may be ingested by aquatic biota (Collard et al., 2019; B. Parker et al., 2021; Wootton et al., 2021) and may cause a range of detrimental effects on the ecology, immunology and physiology of affected organisms (Foley et al., 2018; Salerno et al., 2021; W. Wang et al., 2019). The exact consequences of microplastic contamination may be highly variable depending on the features of the exposure and species with a spectrum of effects ranging from cellular to ecological (Cera et al., 2020; B. Parker et al., 2021). Meta-analyses on the impacts of microplastics provide contrasting findings, with one study finding mostly neutral and few negative impacts of microplastic exposure (Foley et al., 2018), while another suggested a range of negative impacts on feeding, for juvenile and benthopelagic fishes (Salerno et al., 2021). The consequences of current and future microplastic contamination levels may thus be highly context dependent due to the variations in experimental exposure used, the lifestage and the limited number of species utilised (B. Parker et al., 2021).

Freshwater organisms are simultaneously exposed to other stressors in addition to microplastic contamination, including pathogen infection, and inorganic and organic pollutants, which may also be having concomitant physiological and ecological consequences (Lange et al., 2018; Reid et al., 2019; Stendera et al., 2012). Freshwater biota may be particularly susceptible to stressors due to their proximity to anthropogenic activities from which most stressors originate (Ormerod et al., 2010). The impacts of combined stressors are especially concerning as multiple stressors may result in interactive effects where the actions of one stressor may alter those of another resulting in different interaction types with consequences for biota (Khan & Thulin, 1991; Lange et al., 2018; Morgan et al., 2001). Interaction types include additive effects (combined effect is equal to the sum of the single effects), synergistic effects (combined effect is greater than the sum of the single exposures) and antagonistic effects (combined effect is lower than the sum of the single effects) (Jackson et al., 2016a; Morris et al., 2022). Understanding the exposure of particular stressors, and their relative and interactive effects, is thus an important step in implementing effective remediation and/ or mitigation steps to appropriately target the most disruptive stressor(s).

Microplastic exposure with a second interacting stressor is often predicted to have negative effects because of reallocation of resources into immune functions and away from reproduction or growth (Naqash et al., 2020; B. Parker et al., 2021). However, the independent effects of microplastic exposure may be highly variable depending on the environmental relevance of the microplastic exposure, the lifestage and the ecology of organisms (Foley et al., 2018; B. Parker et al., 2021; W. Wang et al., 2020). While synergistic interactions between microplastics and other stressors are often assumed, to our knowledge no study has yet to analyse the combined experimental effects of microplastics and interacting secondary stressors on freshwater fishes. Consequently, using a meta-analysis approach, the present study determined how the types of interaction, effect directions and magnitudes relate to the combinations of interacting stressors, response categories and features of the microplastic exposure and fish exposed. We hypothesised that: 1) most interactions would be synergistic in nature and that studies with environmentally irrelevant exposures above natural levels would overestimate synergistic interactions; 2) the predominant type of interaction will be the same across different interacting stressors and categories of measured response; 3) the direction and magnitude of mean effect sizes will be consistent across interacting stressors and responses, but would also be overestimated for studies using environmentally irrelevant exposures, and 4) the response effect size is related to features of the microplastic exposure and the traits of the exposed fish.

7.3 Materials and methods

7.3.1 Data search and extraction

Research papers, accessible before the 2nd September 2022, were identified from research databases (Web of Science, JSTOR, SCOPUS and EBSCO, Table S7.1) using several different searches corresponding to microplastics ("Stressor 1") and another interacting stressor ("Stressor 2") including climate change, pollutants and pathogens (see Table S1 for full search terms and details). The initial 2986 hits were screened for suitability so that they only included articles that: 1) exposed fish to microplastic particles (hereby defined as ≥ 1 µm but < 5 mm); 2) experimentally tested the interactive effects of microplastics and another anthropogenic stressor in a freshwater fish, as identified by FishBase (Froese & Pauly, 2021); 3) used animals rather than tissues or cells; and 4) manipulated both stressors using a factorial design that included a control. Studies that only looked at the accumulation of microplastics and/or another contaminant were not included. This process generated 36 original research articles for further consideration (Table S7.2).

Data was provided by the paper authors, available within the manuscript, or digitally extracted from plots using GetData Graph Digitizer (http://www.getdata-graph-digitizer.com/). Extracted data included all the responses measured, mean responses with standard deviation, sample numbers, interacting stressor, exposure conditions, and exposed fish features. Interacting stressors were categorised into different groups: warming, inorganic pollutants, organic pollutants, pathogens and plastic additives. Defined responses were additionally allocated into different response categories of biochemical (levels of chemicals within the fish), ecological (measures of movement, interactions, mortality and condition), immunological (measures of immune cells and associated chemicals), metabolism (the activity and expression of molecules involved with the breakdown and formation of other molecules but also morphological measures such as muscle thickness), and stress response (chemicals and molecules associated with the response and reaction to oxidative and toxic stress). Studies were categorised as environmentally relevant or not (Y/N), based on the inclusion of a text justification of the exposures used in relation to environmental levels. Reference to other studies with the same exposure levels did not satisfy this condition unless those studies included a justification of the exposure levels. Where a response was measured over a time series, data for the final timepoint only was used. This step recorded 858 interactions initially from the 36 studies.

Due to a lack of replication within response variables, the number of correlated variables measured within studies and differences in the number of responses measured, the top 3 most frequent independent response measures were identified within each response category (Table S7.3) using a simple vote counting method after checking for instances where the same response was given different names. Only the responses from the 3 most frequent responses (up to 1 response per response category within each study) in ranked frequency order were retained in a reduced dataset. After excluding interactions for which the interactive effect could not be calculated or classified, and after removal of any interactions with an abnormally low or high effect size (< -30, > 30) (as defined by Morris et al. (2022)), this produced a final dataset of 28 studies with 150 interactions (hereafter the "inclusive dataset"). A subset of the inclusive dataset was then also produced that included only studies where the microplastic exposure was environmentally relevant (hereafter the "exclusive dataset") as described above.

7.3.2 Effect sizes and interaction types

To assess interaction type for each single response, an additive null model was used (Folt et al., 1999) as a baseline for comparison, and was calculated as per Morris et al. (2022):

$$Simple\ additive\ model = S_1 + S_2 - C$$

Where S_1 is the response to the microplastic singly, S_2 the response to the stressor 2 singly and C the control.

Hedges' d was calculated as:

$$Hedges'd = J\frac{X_O - X_P}{s}$$

$$Correction\ factor\ (J) = 1 - \frac{3}{4(n_o + n_p - 2) - 1}$$

$$Pooled\ standard\ deviation\ (s) = \sqrt{\frac{(n_o - 1)(s_o)^2 + (n_p - 1)(s_p)^2}{(n_o + n_p) - 2}}$$

Where X_o is the observed combined response to stressors 1 and 2, X_p the predicted combined response (from the best fitting null model), n_o and n_p the sample sizes for the observed and predicted responses respectively, J is a weighting factor to correct for small sample bias, and s is the pooled standard deviation.

The variances used to provide inverse weights of Hedges' d (d) were calculated as:

$$Inverse\ weight\ of\ Hedges'd\ (Vd) = \frac{n_o + n_p}{n_o n_p} + \frac{d^2}{2(n_o + n_p)}$$

Where n and SD are the sample sizes and standard deviations for the treatment (T) and control (C) conditions respectively.

For each interaction, the pooled sample size, its associated t-value and standard error were then used to calculate 95 % confidence limits. The interaction type was defined as additive where the confidence intervals crossed 0, antagonistic where both confidence limits were below 0 and synergistic where both confidence limits were above 0 following Jackson et al. (2016a).

7.3.3 Statistical analyses

Analyses were carried out in RStudio version 3.5.1 (R Development Core Team, 2021). The mean interaction effect sizes grouped by the interacting stressor and response category for both datasets were estimated using the "metafor" package in R (Viechtbauer, 2010) and also to evaluate the fit of an additive null model from weighted meta-analyses. Hedges' d (Hedges, 1981) was selected as a standardised mean difference (with bias correction) between the best fitting model and the observed responses for different second stressor and response categories. Effect size directions were inverted to compare the strength of effects on an absolute scale (Jackson et al., 2016a).

Separate mixed effects linear models for the inclusive and exclusive dataset with fixed effects of microplastic morphology and polymer, fish lifestage and family, and fish life-stage only, respectively, were performed with "ID" nested within "Study" as random effects. This was to account for non-independence of observations from the same study and to allow the true effect sizes to vary across observations.

7.4 Results

7.4.1 Interaction types across datasets, stressors and response categories

Within the inclusive dataset (150 interactions, 28 studies), interactions were mostly classified as additive, whereas those in the exclusive dataset (62 interactions, 13 studies) were mostly antagonistic (Figure 7.1). Synergistic interactions were the least frequent for both datasets. Classifying by interacting stressor, interactions with organic pollutants were mostly antagonistic, organic pollutants were additive, and pathogens had an equal proportion for additive and antagonistic (Figure 7.2). For the exclusive dataset, antagonistic interactions were most common for both inorganic and organic pollutant interacting stressors.

Regarding response category, antagonistic interactions were most frequent for biochemical, ecological and immunological response parameters in the inclusive dataset, whereas metabolic and stress response measures were mostly additive (Figure 3). For the exclusive subset, interactions were predominately antagonistic for ecological and metabolic parameters, and additive for stress response categories.

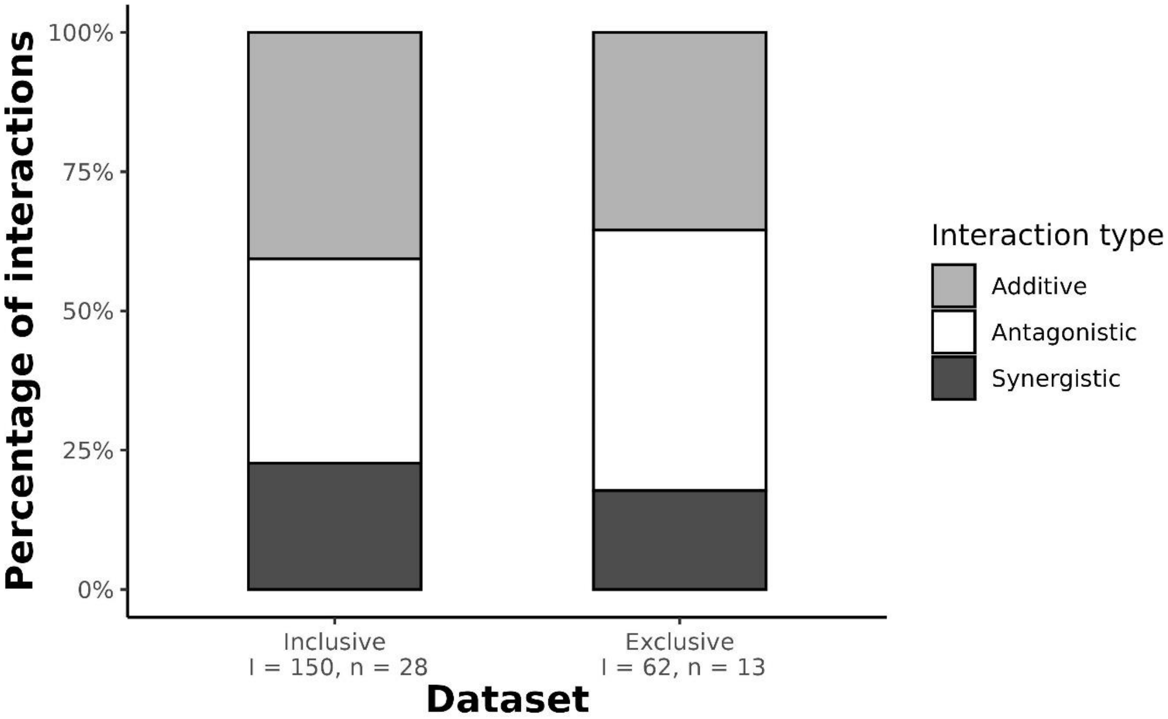

Figure 7.3 Categorised interaction types for the inclusive and exclusive datasets. The percentage of interactions classified as additive, antagonistic and synergistic are presented for both the inclusive dataset as well as an exclusive dataset where the microplastic exposure was deemed environmentally relevant. I = refers to the number of interactions and n = the number of studies respectively.

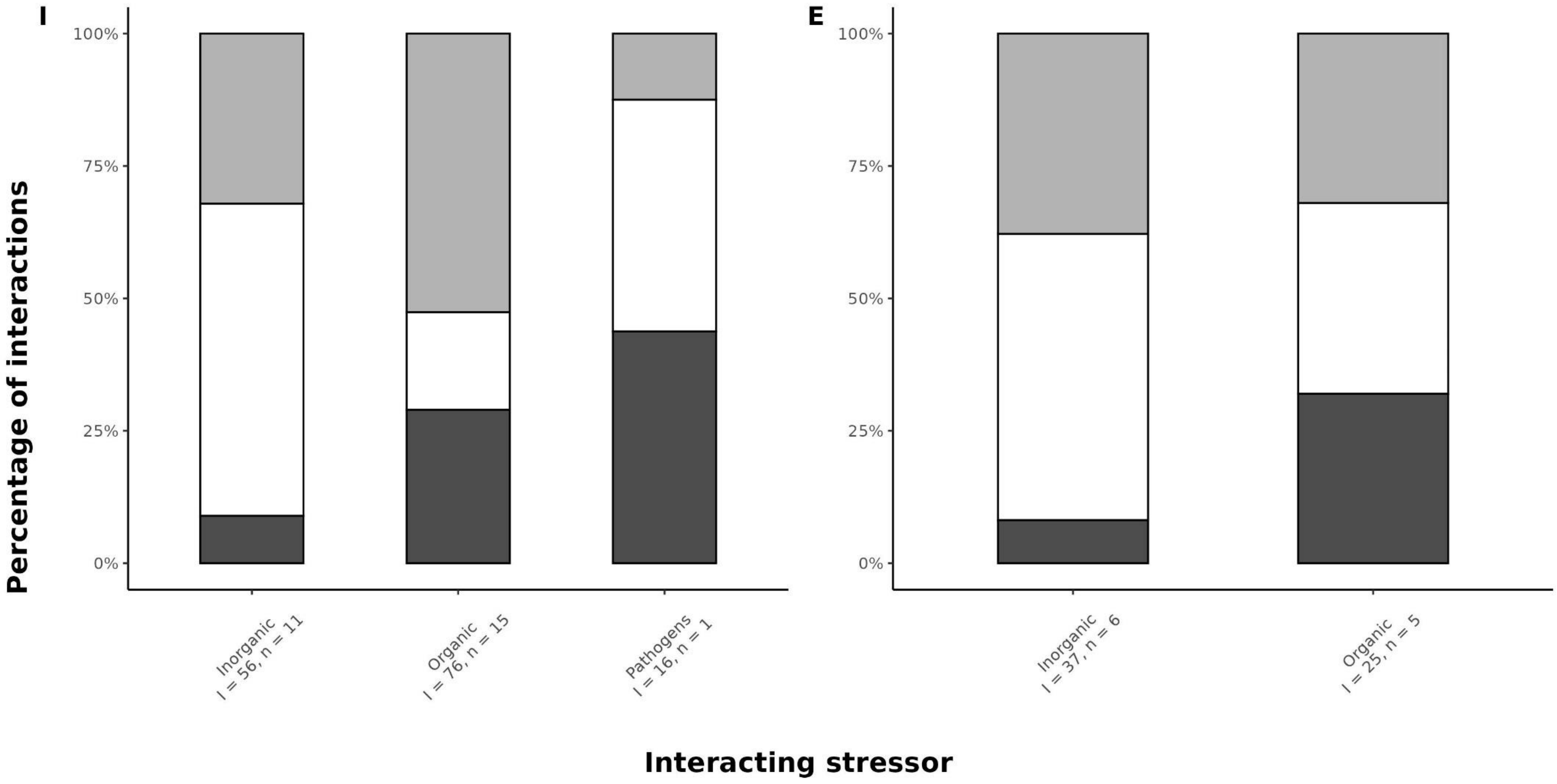

Figure 7.4 Interaction types by interacting stressor. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Interaction types: white = antagonistic, light grey = additive, dark grey = synergistic. The number of papers is given n = as well as the number of interactions I =. Only interacting stressors with I ≥ 8 are presented as per Jackson et al. (2016a).

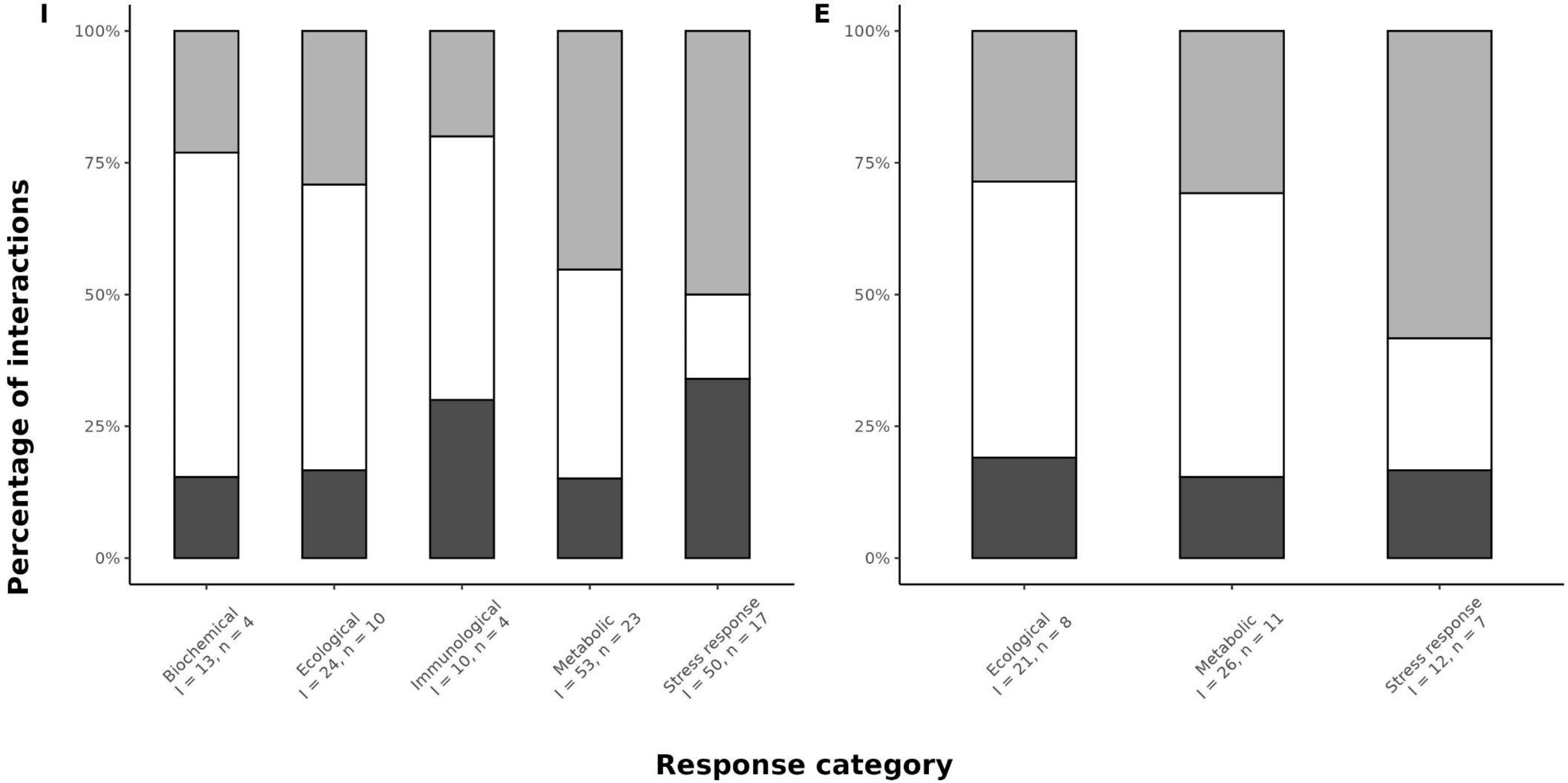

Figure 7.3 Interaction types by response category. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Interaction types: white = antagonistic, light grey = additive, dark grey = synergistic. The number of papers is given n = as well as the number of interactions I =. Only response categories with I ≥ 8 are presented as per Jackson et al. (2016a).

7.4.2 Effect size data

Mixed effects models indicated that the effect size confidence intervals for all interacting stressors demonstrated additive effects (confidence intervals overlapped 0) for both the inclusive and exclusive datasets (Figure 7.4). Grouping by response category, the models revealed that effect sizes were antagonistic (significantly lower than the additive model predictions) for ecological responses in the exclusive data subset but were otherwise no different than additive models (Figure 7.5). Separate linear mixed effects models then indicated that effect size did not differ with microplastic morphology, polymer type, fish life-stage and family for the inclusive or life-stage in the exclusive dataset (all factors $p > 0.05$, Figure 7.6, Table S7.4).

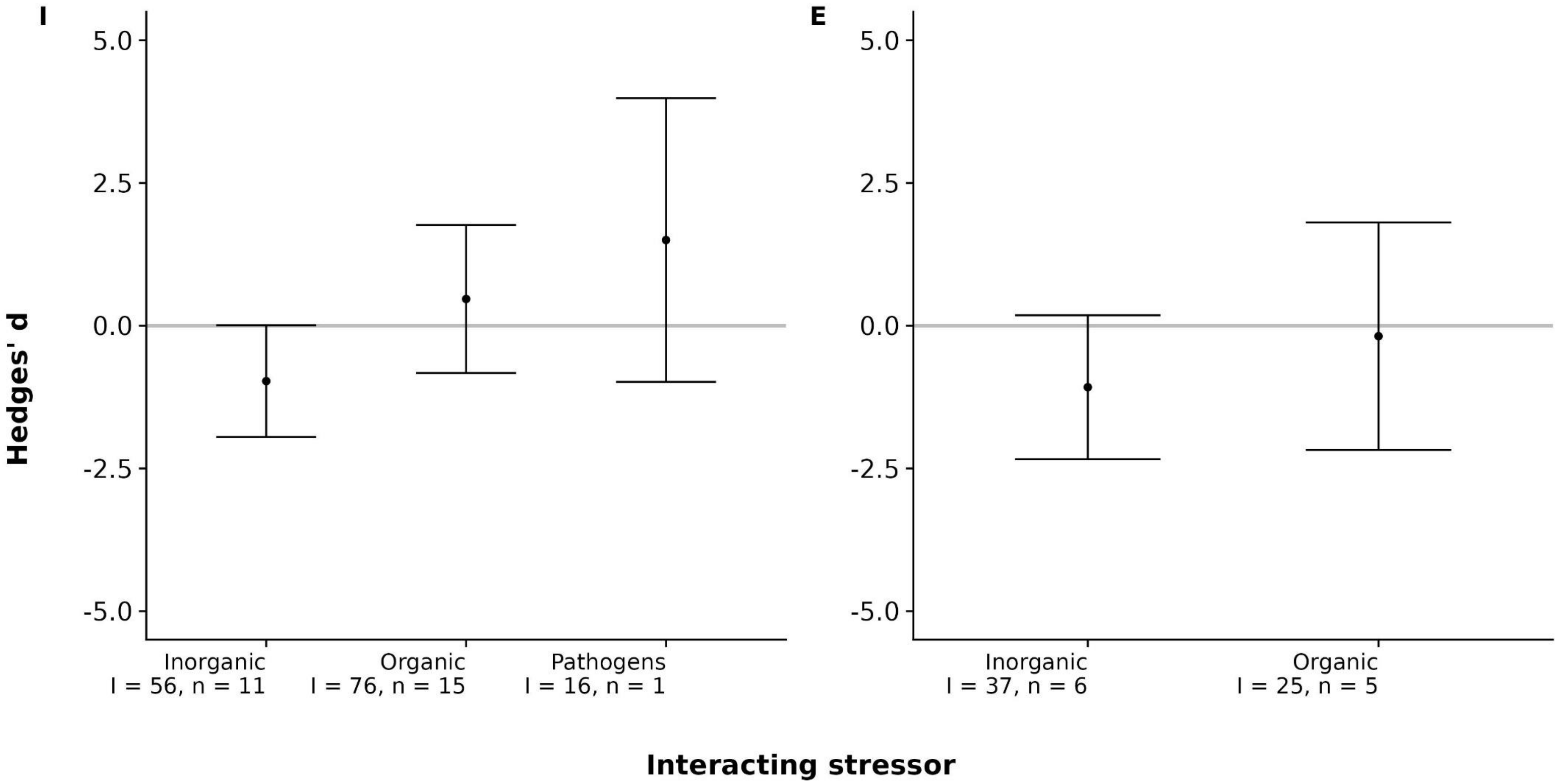

Figure 7.4 Effect sizes by interacting stressor. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Confidence intervals overlapping 0 are non-significant and indistinguishable from additive effects, those significantly above show synergistic and those below demonstrate antagonistic effects. The number of papers is given n = as well as the number of interactions I =. Only interacting stressors with I ≥ 8 are presented as per Jackson et al. (2016a).

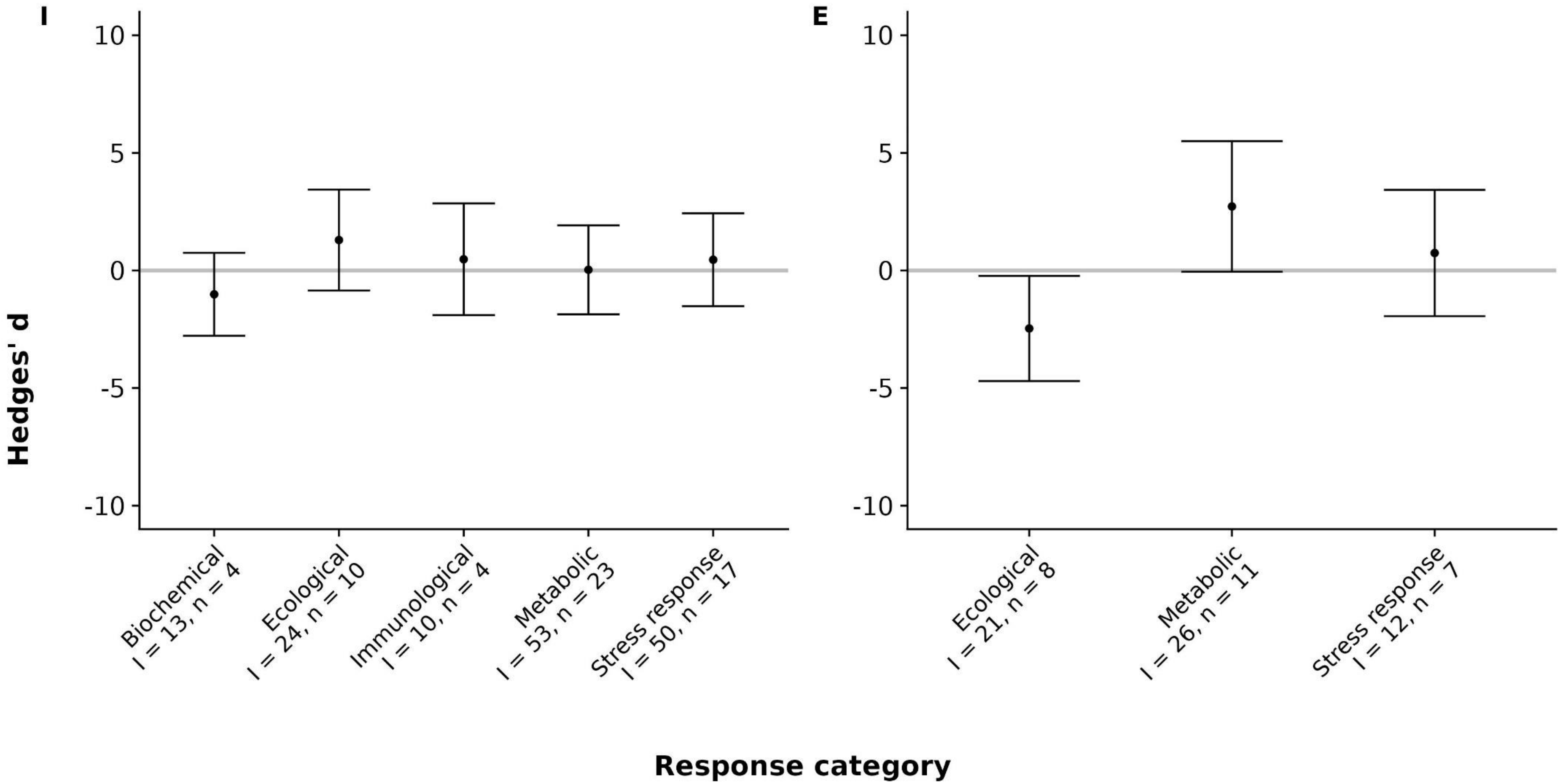

Figure 7.5 Effect sizes by response category. I is the inclusive dataset and E the exclusive dataset where microplastic exposure is deemed environmentally relevant. Confidence intervals overlapping 0 are non-significant and indistinguishable from additive effects, those significantly above show synergistic and those below demonstrate antagonistic effects. The number of papers is given n = as well as the number of interactions I =. Only response categories with I ≥ 8 are presented as per Jackson et al. (2016a).

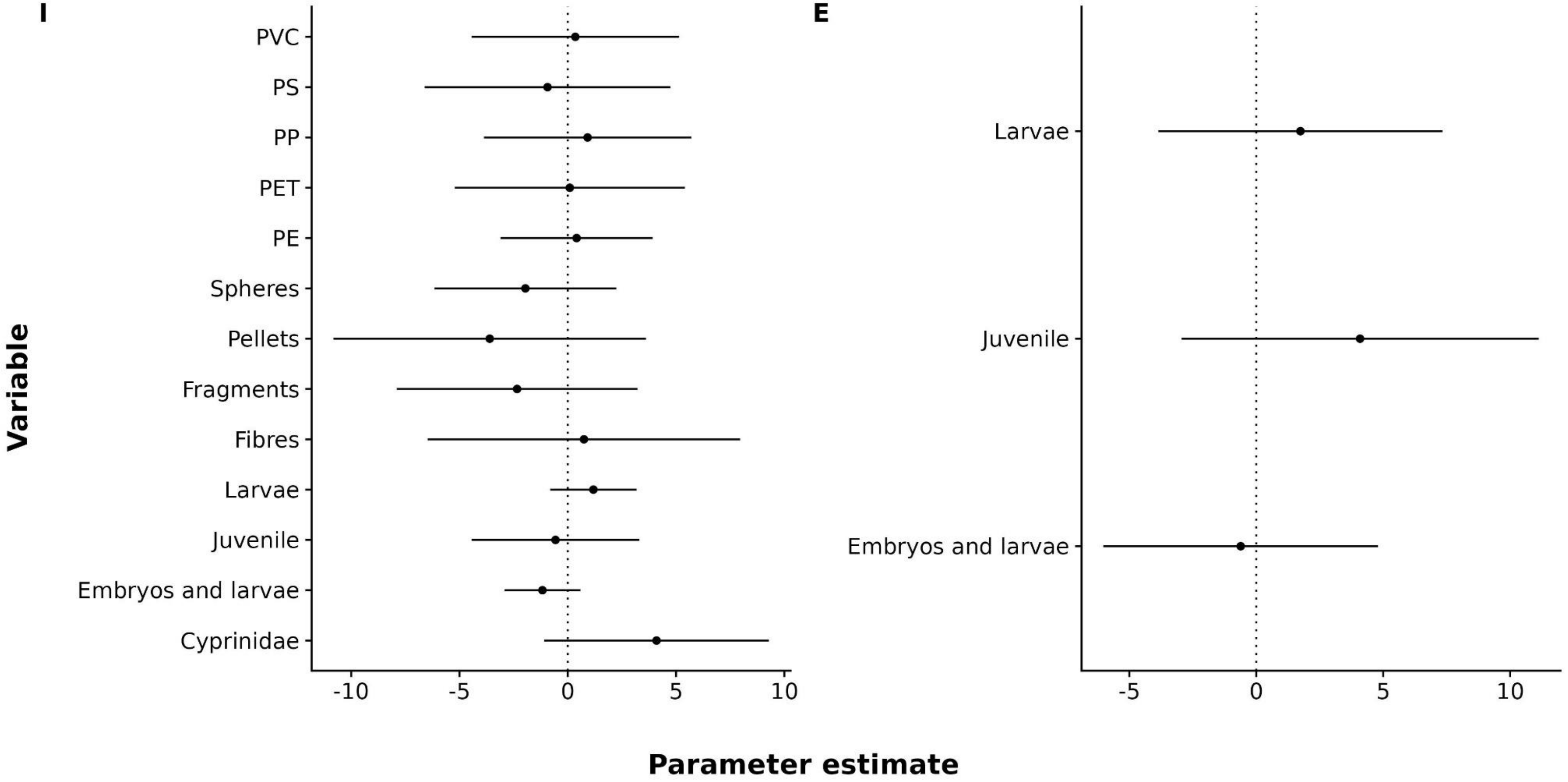

Figure 7.6 Model parameter estimates for effect size by microplastic morphology, polymer, fish life-stage and family. Parameter estimates are presented for the inclusive (I) and exclusive (E) dataset where microplastic exposure was considered environmentally relevant. Variables vary for the different datasets with the span around each variable represents the confidence interval (significant variables do not cross the dashed line). Variable estimates are absent where combined in each model intercept, against which the other factors are compared.

7.5 Discussion

7.5.1 Dominant interaction types

In contrast to the hypotheses, most stressor interactions here were classified as additive or antagonistic. Grouping by both the interacting stressor and the response category, this was also the case with synergistic effects being less frequent than predicted. Again, this was contrary to the hypotheses, but supported other meta-analyses investigating multiple stressors in freshwaters (Jackson et al., 2016a; Morris et al., 2022). Additionally, there was no evidence of synergistic interactions being overestimated for the inclusive dataset relative to the exclusive dataset of studies with environmentally relevant microplastic exposures. We recognise that the reported ranges of microplastic loadings in freshwater environments and biota span several orders of magnitude (C. Li et al., 2020b; B. Parker et al., 2021). Thus, the categorisation of studies as environmentally relevant or not may be contentious, though the analyses of the separate datasets are still informative. Nevertheless, these results suggest that, in most cases, microplastic exposure does not amplify the negative effects of the other stressors discussed. However, stressor interactions can vary both spatially and temporally (Jackson et al., 2021), and dominant interactions also exist whereby a single stressor may drive the overall interactive effect (Jackson et al., 2016a; Morris et al., 2022).

The low number of interactions classified as synergistic can be considered as a positive result for ecosystem management, as it suggests that - in most cases at least - microplastics may exert no or even a compensatory effect on the interacting stressor. Such a result might be useful as managers can target the greatest perceived stressor within their system without concern for synergism. The lack of synergism is especially important given Foley et al. (2018) demonstrate some publication bias towards studies reporting negative effects for growth, reproduction, consumption and survival when looking at the single effects of microplastic exposure. The present study classified similar responses within the "ecological" category and observed an antagonistic effect for ecological responses within the exclusive dataset only. Assuming some similar publication bias within the dataset in the present study, and additionally any bias resulting from the use of non-environmentally relevant microplastic exposures, the present study might have been likely to overestimate the negative effects of microplastic interactions. Despite this, no negative impacts were found. Future interaction studies and subsequent metanalyses will help to clarify the interactive effects and to examine these potential biases.

7.5.2 Effect sizes

The effect size analyses also indicated that the responses to microplastics and interacting stressors were no different to null additive predictions in most cases except for ecological responses within the exclusive dataset only. This was in contrast to the hypotheses but once again in agreement with the results of other multiple stressor metanalyses (Jackson et al., 2016a; Morris et al., 2022). In support of one of the study hypotheses, the size and direction of the effect sizes were largely consistent when grouped by both interacting stressor and response category, finding no significant impacts of multiple exposure. Once again, no differences were found between the inclusive and exclusive dataset. In partial support of the study hypotheses, overall ecological responses were antagonistic for the exclusive dataset but additive for the inclusive dataset and therefore the effects were somewhat greater in the inclusive dataset for this response only since in antagonistic interactions the combined effect is lower than the sum of the single effects.

Our findings suggest that microplastic exposure and interacting stressors have comparable impacts on the biota while the response data suggest the different categories of response may be equally susceptible to exposure with the only exception for ecological responses in the exclusive dataset. Meta-analyses on the single effects of microplastics provide conflicting data, with an early study suggesting mostly neutral and a few negative impacts in fish and invertebrates (Foley et al., 2018) whereas a later study found negative impacts on the feeding, behaviour and growth responses of fish (Salerno et al., 2021), hereby classified within “Ecological” responses. Our results better support those of the former study, with microplastics likely having a neutral or even an antagonistic effect on the interacting stressor, though these studies used a broader range of taxa, included all responses (the present study used only repeated responses) and did not take into account the environmental relevance of microplastic exposure (Foley et al., 2018; Salerno et al., 2021). Despite this, both studies demonstrated negative impacts of microplastic exposure in at least one response category, typically ecological measures, whereas the present study found effect sizes were largely additive (most confidence intervals spanned the null additive model predictions) but antagonistic for ecological responses in the exclusive dataset. Both Foley et al. (2018) and Salerno et al. (2021) demonstrate some significantly lower effect sizes (indicating larger negative effects) for fish consumption and behaviour, respectively, however the present study found interactions were antagonistic (less negative) for comparable ecological measures in the exclusive dataset.

Mixed effects models found that effect sizes did not vary with microplastic morphology, polymer, fish life-stage or family or just fish life-stage for the inclusive and exclusive datasets, respectively. It was assumed that the magnitude and direction of the responses would vary with aspects of the microplastic exposure (morphology and polymer type) as well as the fish (family and life-stage), as suggested by other studies (Salerno et al., 2021). Experiments suggest microplastic features, such as shape, can impact microplastic ingestion and therefore their effects (Xiong et al., 2019). However, there is no evidence that features impacted the effects of the multiple exposure. Studies suggest that particle size can also impact microplastic uptake and impacts (Hoang & Felix-Kim, 2020; Xiong et al., 2019), though the role of particle size was not investigated within the present study.

7.5.3 Recommendations for further research

The sample sizes for the number of studies and interactions in the present study may be considered low compared to other analyses (Jackson et al., 2016a; Morris et al., 2022), but reflect the available literature and the rigorous screening process undertaken here. Certain stressors and responses were excluded based on small sample sizes or as suspected outliers to provide a conservative estimate of the interactive effects. As such, more interaction studies are required to increase the level of replication, especially for interacting stressors such as warming and plastic additives, as well as for responses such as biochemical and immunological responses that were removed from analyses due to the low sample sizes. Additionally, there are also some knowledge gaps around the potential interactions of known stressors such as eutrophication and especially warming where interactions have already been investigated in freshwater biota such as *Daphnia* sp. (Hiltunen et al., 2021; Sadler et al., 2019). Habitat degradation has also been demonstrated to have an interactive effect with microplastic exposure within marine fishes (McCormick et al., 2020), though more studies on habitat degradation are needed. Understandably, most of the interaction studies in the present study were for inorganic and organic contaminants, since microplastics are assumed to sorb these chemicals and/or increase their toxicity (Naqash et al., 2020; B. Parker et al., 2021), but further studies might focus on other stressors that could result in indirect effects through cumulative stress (B. Parker et al., 2021). Similarly, the use of different microplastic morphologies (beads were most common) and study organisms (*Danio rerio* were most commonly used), such as *Squalius cephalus* (B. Parker et al., 2023), will improve the generalisation of the study findings, particularly as Salerno et al. (2021) indicate negative overall single effects of microplastic exposure for two freshwater fishes; *Cyprinus carpio* and *Perca fluviatilis*. These species are thus excellent alternative model species to study the interactive effects of microplastics and additional stressors if single negative effects have already been demonstrated.

A number of studies initially considered were excluded on the basis of methodology, with many studies lacking a factorial study design that included blank controls and all combinations of single and combined treatments. Furthermore, pseudo-replication was fairly common in studies that were both included and excluded in the analyses, and the data were often digitised from figures where these were not available in the article or from the author. It is, therefore, recommended that editors, reviewers and authors are wary of pseudo-replication and non-factorial study designs, while also ensuring raw data are submitted together with the associated article. Finally, it is important that the microplastic exposure used is justified by the author and ideally uses current or predicted microplastic exposure conditions justified in the text to allow evaluation by reviewers and the reader. However, the present study recognises that the span of freshwater microplastic levels globally can justify most levels of microplastic exposure (C. Li et al., 2020b). In several studies, the microplastic exposure information (morphology, polymer etc) was absent, resulting in the removal of these studies from mixed effects models using microplastic features and so authors should provide as much information as possible to allow analyses such as the present study.

7.6 Conclusions

The present study found that additive and antagonistic interactions dominated the interactive effects of microplastics and interacting stressors in freshwater fishes. This was true across stressors and response categories and was largely unrelated to the environmental relevance of microplastic exposure. Similarly, effect size magnitude and direction were consistently indistinguishable from additive null model predictions, suggesting a comparable sensitivity to the interacting stressors and for the different response categories. Taken together, the results suggest that most interactions between microplastics and interacting stressors are not synergistic within freshwater fishes and therefore management options can separately remediate or mitigate these stressors. However, knowledge gaps remain around the interactions with known stressors, such as warming and eutrophication, and more care should be taken in the future to ensure the design of factorial interaction studies and that the data generated may be used in additional analyses. Finally, there is no evidence that the environmental relevance of microplastic exposure impacted the results, except for ecological responses where interactions were antagonistic, though future research should carefully consider the details of the exposure and utilise study organisms and exposure combinations not previously investigated.

8 General discussion

Microplastic contamination remains a significant topic facing freshwater fishes globally (Collard et al., 2019; Wootton et al., 2021) and can be found within most freshwaters investigated (C. Li et al., 2020a; Talbot & Chang, 2022). The continued improper production, use and disposal of plastics by humans has resulted in large and ever-increasing amounts of plastics and microplastics within the environment (Lebreton & Andrady, 2019) as particles degrade and fragment over time with loadings in some areas expected to double by 2030 (Hale et al., 2020). Freshwater microplastics may cause a range of negative ecological, physiological and morphological effects upon ingestion by freshwater fishes (Foley et al., 2018; W. Wang et al., 2020) and can additionally exacerbate the impacts of additional stressors such as chemical pollutants and viruses through cumulative stress (Naqash et al., 2020; Seeley et al., 2023), however this was not supported by this thesis. Understanding the predictability of particle ingestion and their impacts as well as identifying the interactive effects of microplastics with additional stressors is thus a crucial step in the management of freshwater fishes and mitigation of microplastic contamination.

The aim of the thesis was to utilise field studies, experimental results and data from the literature to assess the risk posed by microplastics to freshwater fishes and, in particular, how the impacts of additional stressors may further modify the impacts of microplastics. A literature review first identified the factors believed to impact microplastic ingestion (Chapter 2) before the testing of these hypotheses using abiotic and biotic samples collected from the Bourne Stream (Chapter 3) and Dorset Stour (Chapter 4). Baseline microplastic data from the field studies were then used to inform the microplastic exposure in a factorial interaction experiment investigating the interactive effects of microplastic and parasite exposure on fish parasite load, biomarkers and feeding (Chapter 5). Next, the microplastic loadings within juvenile *Anguilla anguilla* were determined to examine if microplastics are likely impacting the survival of eels during their marine-freshwater transition and whether body size may predict their loadings (Chapter 6). Finally, a metanalysis collated data on the effects of microplastics and additional stressors from the wider literature to examine if the interactive effects are largely additive or multiplicative and whether the sign and size of the response is related to details of the exposure and biological features (Chapter 7)

8.1 The predictability of microplastic loadings in wild freshwater fishes

The findings of the initial global literature review (Chapter 2) suggested that microplastic loadings within freshwater fishes and their effects would be highly predictable based on features such as the size of an individual, its ecology as well as its proximity to potential sources of contamination, however the results of the subsequent field studies (Chapter 3, 4 and 6) did not substantiate these predictions. In contrast to the predictions, while sediment microplastic loadings did vary between sites the loads within macroinvertebrates and fishes did not vary with land use and did not correlate between sample types or with seasons (Chapter 3). Microplastic loads are assumed to vary both in space depending on hydrological parameters such as flow as well as the proximity to urban microplastic sources and also in time due to seasonal variations in microplastic production, degradation and transport after precipitation events (Stanton et al., 2020; Talbot & Chang, 2022). By contrast, the thesis findings reveal a relatively consistent and low microplastic loading within different communities across multiple rivers within SW England (Chapter 3, Chapter 4, Chapter 6). The loadings of microplastics recovered from freshwater environments and biota within SW England are amongst some of the lowest recorded (Collard et al., 2019; C. Li et al., 2020a) and may therefore be relatively unaffected by spatiotemporal variations for this reason.

Similarly, microplastic loadings varied only with invertebrate taxa, guild and stomach morphology within the Bourne Stream (Chapter 3), invertebrate taxa within the Dorset Stour (Chapter 4) and were unrelated to all features for juvenile *A. anguilla* (Chapter 6). While some variations were observed, trends were inconsistent across the different study systems and therefore loadings were not predictable from the biological traits investigated either, in contrast to the findings of other studies which suggest high predictability (Garcia et al., 2021; McNeish et al., 2018; Salerno et al., 2021). The thesis additionally found no empirical support for the processes of bioaccumulation, the increase in microplastic loads within larger/older individuals, or biomagnification, the increase in microplastic loadings within higher trophic levels, both assumed and often supported by the literature (Horton et al., 2018; McNeish et al., 2018). The absence of these relationships does support those of other studies (McIlwraith et al., 2021; Wootton et al., 2021) and again supports a low and consistent baseline microplastic load within the communities and may also suggest a suitable rate of egestion, depending on the shape and size of particles that prevents particle accumulation (Hoang & Felix-Kim, 2020; McIlwraith et al., 2021). Furthermore, particles are known to accumulate within other regions such as the gills, muscles, liver (Galafassi, Campanale, et al., 2021) and would be expected to be highest within the oldest individuals of apex fish predators (Campbell et al., 2017; Garcia et al., 2021) which were explicitly avoided within the field studies described in this thesis, likely underestimating these processes.

While the lack of predictability between the biological features of biota and their microplastic loadings can be considered negative based on the assessment of microplastic contamination within environments using indicator species, this can also be considered beneficial from a management perspective as freshwater fishes seem to experience comparable microplastic loadings in the wild and can thus be treated as a single "management unit". This means that particular individuals differing in species, ecology, size etc. do not differ in their susceptibility to microplastic contamination and therefore species under commercial and/or conservation concern may not be disproportionately impacted. The current low loadings and lack of ecological and morphological relationships suggest microplastic contamination may thus play a minor role in the management of freshwater fishes. It should, however, be stressed that the numbers of microplastics reported in this thesis are exclusively from SW England and are among the lowest found (Collard et al., 2019; B. Parker et al., 2021) therefore relationships with biological and/or environmental features may be easier to detect in systems with greater numbers or variation in loadings.

8.2 The impacts of microplastics on ecological interactions

The interaction experiment (Chapter 5) aimed to determine the interactive impacts of exposure to an environmentally relevant exposure of microplastics (determined from Chapter 3 and Chapter 4) and a parasite on the parasite load, biomarkers and feeding rate within a freshwater fish final host. It was expected that microplastic exposure might increase the susceptibility of a freshwater fish host to parasite infection through a process of cumulative stress and/or immune suppression (Foley et al., 2018; Jabeen et al., 2018; Salerno et al., 2021), however this was not evident. Parasite loads have been shown to correlate with microplastic loadings (Alves et al., 2016) and to be ingested via comparable trophic routes (Lester & McVinish, 2016), though no impact was observed. Similarly, microplastic exposure had no single or interactive effect on fish biomarkers (condition, specific growth rate or splenosomatic index) as proxies of general fish health and immune investment, demonstrated to be impacted by other stressors and environmental contaminants (Kumar Verma & Prakash, 2019; Seppänen et al., 2009). Furthermore, microplastic exposure had no impact on the feeding rate through either the encounter of prey items, the attack rate, or the processing time for prey items, the handling rate, (Chapter 5). The absence of an effect of microplastic exposure suggests that environmentally relevant exposures may be insufficient to impact ecological interactions and that studies using unrealistic exposures may be overestimating the biological impacts of microplastic exposure (Foley et al., 2018; Salerno et al., 2021). Alternatively, no microplastics were recovered from within the fishes upon dissection and so it may be the case that particles were not retained within the fish long enough to induce any effects on the measured responses.

By contrast, several weeks of parasite exposure produced an impact on the specific growth rate which was reduced for exposed fish only but more importantly reduced the feeding rate in infected individuals and somewhat in exposed individuals relative to the control group (Chapter 5). Several months of microplastic exposure had no impact on any of the recorded metrics compared to parasite exposure which would suggest that the particular interactive effects are likely an additive type model or dominance by parasite exposure. Identifying asymmetry in multiple stressor interactions is an important tool in the management and mitigation of ecosystems to determine the particular stressor responsible for a negative observed effect (Folt et al., 1999; Morris et al., 2022) and to effectively improve ecosystems. Interestingly, parasite effects were observed for fishes exposed to but not infected by the parasite which suggests some cost to the individuals as the parasite passed through, potentially inducing immune responses, damaging the gastrointestinal tract etc. as observed for the parasite genus of interest (Bosi & Sayyaf Dezfuli, 2015; Dezfuli et al., 2002, 2015).

Additionally, data from the metanalyses revealed that the combined ecological impacts of microplastic exposure and an additional stressor on freshwater fishes were best fit by additive and antagonistic interactions whereby overall effects were equal to or less than the sum of the individual effects, respectively (Chapter 7). This trend was evident when only considering a data subset of environmentally relevant exposures and thus suggests a lack of synergistic microplastic effects previously assumed within the literature (Mao et al., 2022; Naqash et al., 2020). That the ecological effects within freshwater fishes are driven by additivity means that the management of freshwater fisheries need not target a particular stressor. If a particular stressor is much easier or cheaper to address, a knowledge that microplastic interactions tend to be additive or antagonistic means that management steps can more efficiently target the larger perceived problem.

8.3 The interactive effects of microplastics and additional stressors

Data from the wider literature on the interactive effects of microplastics and additional stressors on freshwater fishes were gathered to determine the types of interactions, whether interaction types vary with response categories, environmental relevance and whether the size and direction of the interactive effect is related to features of the microplastic exposure and/or the organism. Synergistic interactions whereby the effect of one stressor amplifies the negative effects of another are perhaps the most detrimental result for ecosystem management (Folt et al., 1999; Jackson et al., 2016a) and are assumed to result from microplastic interactions, however additive and antagonistic interactions were most prevalent (Chapter 7). This trend was consistent for both datasets across second stressor types, response categories and effects were additionally unrelated to microplastic exposure and fish features (Chapter 7). This result is very important in the context of understanding the interactive role of microplastic contamination and additional stressors on freshwater fishes and is in line with other metanalyses which find predominantly additive and dominant interaction types &(Jackson et al., 2016a; Morris et al., 2022).

The low frequency of synergistic effects and the overlap of data with null additive models thus show that microplastic contamination is not currently amplifying the impacts of other freshwater stressors and therefore the impacts of other stressors such as warming, disease and eutrophication with established interactive effects are likely of higher priority to mitigate against (Kratina et al., 2012; Marcos-López et al., 2010). Despite this result, microplastics are still known to have negative single effects on various biota (Foley et al., 2018; Salerno et al., 2021) but might be targeted by later management methods once other stressors are resolved. In support of the metanalysis findings, the interaction experiment additionally found no interactive effects of microplastic and parasite exposure

(Chapter 5) and only found single impacts of parasite exposure on specific growth rate and more evidently on feeding rate. These single effects may have the capacity to modify ecological interactions and therefore fish communities in the wild, however empirical mesocosm studies are currently lacking to test the interactive effects of microplastics and other stressors at the population and community levels.

8.4 Microplastic contamination "win-win" scenarios in ecosystem management

While the results of this thesis suggest current levels of microplastic contamination likely cause additive and/or minor effects, the global interest around plastic pollution provide excellent opportunities to simultaneously address multiple stressors through "win-win" scenarios. These are scenarios in which addressing microplastic contamination will also positively address another co-occurring stressor. Plastic pollution is a relatively novel stressor with great global concern and emotional appeal to the general public (Catarino et al., 2021; Males & Van Aelst, 2020) and so management steps designed to mitigate plastic pollution but also another stressor might be more attractive politically and to the taxpayers. For example, a reduction in plastic production will reduce the number of microplastics entering the environment as well as the release of carbon dioxide and other greenhouse gases during the production of plastics that contribute to climate change which is arguably a greater threat to aquatic systems (Kvale, 2022; Stafford & Jones, 2019)

Alternatively, treating stressors as a group rather than individually may also be beneficial to understand how an action may positively address multiple stressors. However, it is also recognised that other drivers such as societal health must come first as seen by the massive return to using single-use plastics during the COVID-19 pandemic and the subsequent reversal of years of plastic reduction (Benson et al., 2021; Shams et al., 2021). Potentially good candidate stressors for this type of management option include various organic and inorganic pollutants since microplastics often bind these contaminants (Caruso et al., 2019; Naqash et al., 2020) and so the manual removal of plastic waste might remove harmful pollutants from the environment too. Similarly, pathogens are also known to colonise microplastic particles (Caruso et al., 2019) thereby microplastic reduction and/or removal might reduce aquatic disease incidence. Finally, the contribution to plastic pollution should be considered and balanced in the implementation of other management strategies. For example, designing using more sustainable recycled or non-plastic building materials that do not impact biota but also reduce the environmental impact of plastics (da Silva et al., 2021; Millican & Agarwal, 2021). Many microplastics are produced from tyre wear particles and road paints (Siegfried et al., 2017; van Wijnen et al., 2019). Therefore actions that attempt to reduce traffic through car share schemes, car exclusion zones and improved public transport will have an inevitable impact on microplastic levels, climate change, air pollution and likely the quality of life.

8.5 Policy, management and industry implications for plastic pollution

An estimated 79 % of all plastics ever produced are in the environment or landfill (Geyer et al., 2017) and so potential solutions to microplastic contamination will involve mitigation steps in the terrestrial environment to both reduce plastic production and prevent its entry into the aquatic environment. It is estimated that 80 % of marine plastic pollution originates from land, travelling via freshwaters (Andrady, 2011; Galloway et al., 2017) and so this is the stage at which plastic pollution should be addressed. The issue of microplastic contamination is compounded as microplastics are continuously fragmented into smaller particles in the environment and may accumulate in sinks such as sediments (Daily & Hoffman, 2020; Hoellein et al., 2019; Song et al., 2020a). The legacy of plastic pollution is therefore difficult to address due to the number of plastics in the environment, the difficulty of understanding their distributions and the number of particles in remote regions. While individuals have a subtsnatial plastic footprint that they might work to reduce (Amadei et al., 2022; Kaur et al., 2018; Sheldon & Norton, 2020), larger scale change will require international policy-led global behavioural and societal changes to the use and disposal of plastics to produce a more circular and greener economy (Kaur et al., 2018; Sheldon & Norton, 2020).

Key sources of aquatic microplastics include tyre wear particles, synthetic clothing and the breakdown of larger plastics (Siegfried et al., 2017; van Wijnen et al., 2019) and are good targets for mitigation. Policy steps should aim to increase the recycling of plastic items so that they are viewed as resources by encouraging industries to adopt fully recyclable packaging and more sustainable manufacturing decisions but still understanding the needs of industry and consumers (Raubenheimer & Urho, 2020). Additionally, increasing the recycling efficiency, stopping the international export of waste and stopping the burning of plastic and other waste would also help close the loop on the plastic cycle (Klemeš et al., 2020; Sheldon & Norton, 2020) as well as combatting climate change, air pollution and other related issues. Policy might also promote and implement technologies using grants and tax breaks that exclude and then use the microplastics passing into waste waters by modifying clothing regulations, adding filters to washing machines and/or waste pipes (De Falco et al., 2019; Periyasamy & Tehrani-Bagha, 2022). Finally, processes and/or technologies that remove plastic wastes from the environment are also required, however the success of removal must be balanced against the potential for harm to biota (Barcelo & Pico, 2020; Parker-Jurd et al., 2022).

8.6 Future recommendations

Despite a number of advances in the field of study throughout the completion of this thesis, there still remain several important knowledge gaps/limitations that warrant recommendations for future work. Firstly, the field sampling of microplastics within freshwater fishes typically involves the lethal sampling of organisms before dissection, chemical digestion and screening (Lusher et al., 2017; Stock et al., 2019). As a method of monitoring, this current practice may be relatively counterproductive given the sacrifice of individuals to understand the threat faced by microplastic contamination given the relatively low incidence and the relatively minor impacts of exposure for certain regions and studies (Collard et al., 2019; Foley et al., 2018). There are a number of non-lethal sampling techniques that may be utilised to assess the incidence of microplastics within wild individuals for example the use of novel gill swabs, skin scrapes, mucus samples (Smith, 2002) as well as the expansion of techniques such as gastric lavage, previously utilised to examine microplastic loadings within reptiles (Gonzalez-Jauregui et al., 2019; Micheal Mackenzie et al., 2021). Furthermore, additional samples may be gathered in a non-lethal way from wild fishes for example taking scales for aging, mucus for isotope work, quantifying stress or contaminant levels, stable isotope analyses and DNA work (Cerveny et al., 2016; Winter et al., 2019). Proof of concept studies are therefore required to identify the suitability of non-lethal methods as alternatives to lethal sampling. Where lethal sampling is necessary, the data collected may be maximised through the collection of materials for microplastic quantification, isotope analyses and DNA work through the cross-collaboration of researchers with different expertise.

Secondly, studies investigating the effects of microplastic exposure must ensure that only environmentally relevant exposures or predicted future levels are carried out to avoid biasing the literature on the actual impacts of microplastics. Various metanalyses identify general effects of microplastic exposure on different response variables (Foley et al., 2018; Salerno et al., 2021), however studies make no differentiation between the results of studies using environmentally relevant exposure levels and those that do not. Researchers should ensure that they justify the exposures selected within a manuscript in terms of current levels in a particular environment or a predicted future level to test and editors and reviewers should be wary of studies that do not justify the exposure selected or appear to increase concentrations until an effect is seen. From an ethical standpoint, ethical welfare bodies must ensure that the benefits of microplastic exposure experiments are maximised and ensuring environmentally relevant exposure levels are used is one such step. The publishing of all experimental results, particularly negative results whereby microplastic exposure has no impact, is another important part of the process to progress the research area and ensure that experiments are not unnecessarily repeated. Editors and reviewers should consider these publications based on the study design and in particular the use of appropriate factorial design, experimental and contamination controls. Studies investigating the impacts of microplastic exposure at the population level and above are also needed to understand how microplastic exposure might alter population dynamics, ecological interactions, the community structure and ultimately the services provided by freshwater ecosystems. While the higher-level consequences are predicted and discussed, studies have yet to actually assess the community level impacts of microplastic exposure on freshwater fishes which is important for the management of these communities.

Finally, additional factorial studies using environmentally relevant exposures are crucial to understand the effects of microplastics and additional stressors on freshwater fishes. The effects of microplastic exposure do not occur in isolation (Naqash et al., 2020; Seeley et al., 2023) and so interaction studies are essential to understand the interplay between different stressors and under which conditions and scenarios the impacts are particularly negative. Remarkably little data is available for important interacting stressors such as warming and eutrophication which can modify the impact of other environmental contaminants (Kratina et al., 2012; Marcos-López et al., 2010) and there are a number of additional emerging and novel freshwater stressors such as noise and light pollution (Reid et al., 2019) for which there are no data at all. The management of freshwater fishes and the mitigation of microplastic contamination requires an up to date understanding of the stressors faced and the relative impacts of each and therefore the development of ecological models from empirical experiments may predict how freshwater fishes are likely to respond to different combinations and levels of stressors. The success of managing freshwater fishes in an era of global change also depends on having openly available raw experimental data that can be utilised in metanalyses to draw meaningful conclusions and correctly weight the results from a range of different studies using different exposures, study organisms and designs. To this end, researchers should openly publish raw data alongside their manuscripts at the time of publication to avoid the need for manual data extraction from the manuscript and help advance the field of research. Editors and reviewers should also encourage the transparent publication of research data to aid this process which will have the additional benefit of simultaneously reducing academic misconduct.

8.7 Concluding remarks

Overall, this thesis has contributed a number of important and novel insights into the features impacting microplastic loadings in wild freshwater fishes, the impacts of environmentally relevant microplastic exposure on ecological and morphological measures and the interactive effects of microplastics together with other stressors. Through combining field data, experimental results and information from the wider literature the results highlight that microplastic loadings within freshwater fishes are relatively low, consistent and largely unrelated to biological features. Based on data for rivers in SW England, current microplastic loadings are unlikely to be having major impacts on fishes and that the impacts of microplastic exposure are likely overshadowed by the impacts of other stressors. Taken together, these results suggest that microplastic contamination may currently be a red herring compared to the impacts of other stressors and therefore efforts to manage freshwater fishes and their environment would be more successful in targeting other stressors. The dominance of additive and antagonistic interactions within the wider literature further supports that microplastic contamination can be considered a background stressor, however the future monitoring of microplastic loadings is essential. Finally, more data is required to understand the likely microplastic loadings, single and interactive effects under varying future predicted levels of microplastic contamination and under which scenarios microplastics might amplify the negative effects of particular interacting stressors.

9 References

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10 Appendices

10.1 Chapter 3 supplementary materials

Figure S3.1. Photograph of the custom metal soft sediment suction corer. The corer has a 10 cm diameter and 15 cm height. The side nozzle expels the water and the valve is used to maintain suction when removing the core.

Table S3.1. Results of the Poisson generalised linear model testing for relationships between sediment microplastic counts (per gram), sites, seasons and replicates. Significance thresholds are indicated using “*”.

Factor	Level	Estimate	SE	t value	p value
(Intercept)	-	0.15	0.03	4.75	0.000031 ***
Site	Site 2	-0.10	0.02	-4.65	0.000042 ***
	Site 3	-0.08	0.02	-3.41	0.0016 **
Season	July	-0.02	0.03	-0.70	0.49
	October	-0.02	0.03	-0.70	0.49
	December	-0.01	0.03	-0.27	0.79
	January	0.00	0.03	0.08	0.94
Replicate	-	-0.01	0.01	-0.48	0.63

Table S3.2. Results of the generalised linear model testing for relationships between batch macroinvertebrate microplastic counts, taxa, number of organisms, sites and seasons. Significance thresholds are indicated using “*”.

Factor	Level	Estimate	SE	z value	p value
(Intercept)	-	-1.21	0.54	-2.24	0.025 *
Taxa	Annelida	1.09	0.46	2.37	0.018 *
	Diptera	-0.07	0.44	-0.16	0.87
	Ephemeroptera	-0.29	0.47	-0.63	0.53
	Gastropoda	0.79	0.54	1.46	0.14
	Hemiptera-H	-0.35	1.06	-0.33	0.74
	Hemiptera-P	0.02	0.71	0.03	0.98
	Isopoda	-0.55	0.43	-1.30	0.19
	Odonata	0.70	0.46	1.52	0.13
	Trichoptera	0.43	0.44	0.99	0.32
Batch number	-	0.12	0.09	1.28	0.20
Site	Site 2	-0.08	0.28	-0.27	0.79
	Site 3	-0.50	0.31	-1.64	0.10
	Site 4	-0.05	0.31	-0.15	0.88
Season	July	0.41	0.31	1.33	0.18
	October	-0.06	0.34	-0.17	0.87
	December	0.30	0.30	1.00	0.31
	January	-0.26	0.35	-0.73	0.47

Table S3.3. Results of the independent generalised linear model testing for relationships between batch macroinvertebrate microplastic counts and guild. Significance thresholds are indicated using “*”.

Level	Estimate	SE	z value	p value
(Intercept)	-0.04	0.19	-0.19	0.85
Herbivore	-1.05	0.25	-4.21	0.000026 ***
Omnivore	-0.52	0.27	-1.90	0.06
Predator	-0.44	0.30	-1.45	0.15

Table S3.4. Results of the negative binomial generalised linear model testing for relationships between fish microplastic counts, species, standard length, sites and seasons.

Factor	Level	Estimate	SE	z value	p value
(Intercept)	-	-0.28	0.53	-0.53	0.60
Species	Minnow	-0.29	0.56	-0.52	0.61
	Roach	0.14	0.50	0.29	0.78
	Stone loach	0.50	0.50	1.00	0.32
	Three-spined stickleback	0.28	0.69	0.40	0.69
Standard length	-	-0.01	0.16	-0.04	0.97
Site	Site 2	-0.08	0.39	-0.22	0.83
	Site 3	0.33	0.40	0.83	0.41
	Site 4	0.71	0.44	1.61	0.11
Season	January	-0.18	0.20	-0.90	0.37

Table S3.5. Results of the independent negative binomial generalised linear model testing for relationships between fish microplastic counts and stomach condition. Significance thresholds are indicated using “*”.

Level	Estimate	SE	z value	p value
(Intercept)	-0.19	0.12	-1.53	0.13
Gastric	0.47	0.20	2.33	0.02 *

Table S3.6. Results of the independent negative binomial generalised linear model testing for relationships between fish microplastic counts and feeding guild.

Level	Estimate	SE	z value	p value
(Intercept)	0.00	0.14	0.00	1.00
Demersal	-0.04	0.20	-0.19	0.85

Table S3.7. Results of the Spearman’s rank correlation tests within sites examining relationships between combinations of mean sample loads.

Sample type	Macroinvertebrate	Fishes
Sediment	r = 0.5, S = 2, p = 1	r = 0.5, S = 2, p = 1
Fishes	r = -0.5, S = 6, p = 1	

10.2 Chapter 4 supplementary materials

Table S4.1. Results of the negative binomial generalised (NBGLM) model testing for relationships between batch macroinvertebrate microplastic counts with taxa and batch number (number of organisms within the batch). Hemiptera-H and Hemiptera-P refer to herbivorous and predatory groups within Hemiptera, respectively. “*” denotes a significant factor level ($p < 0.05$).

Factor	Level	Estimate	Standard error	z value	p value
(Intercept)	-	-1.45	0.82	-1.76	0.08
Taxa	Annelida	1.49	0.86	1.73	0.08
	Coleoptera	0.83	1.05	0.79	0.43
	Diptera	-0.23	1.19	-0.19	0.85
	Ephemeroptera	1.63	0.75	2.18	0.03*
	Gastropoda	0.85	0.78	1.09	0.28
	Hemiptera (H)	0.24	0.97	0.25	0.80
	Hemiptera (P)	-0.01	0.98	-001	0.99
	Isopoda	0.76	0.73	1.04	0.30
	Megaloptera	-0.01	1.30	-0.01	0.99
	Odonata	1.04	0.68	1.54	0.12
	Trichoptera	1.45	0.80	1.81	0.07
Batch number	-	0.08	0.13	0.59	0.56

Table S4.2. Results of the independent negative binomial generalised model testing for relationships between batch macroinvertebrate microplastic counts and feeding guild.

Level	Estimate	Standard error	z value	p value
(Intercept)	-0.17	0.41	-0.40	0.69
Herbivore	-0.03	0.50	-0.06	0.95
Omnivore	-0.29	0.56	-0.53	0.60
Predator	-0.34	0.53	-0.65	0.52

Table S4.3. Results of the negative binomial generalised mixed effects model (NBGLME) testing for relationships between fish microplastic counts with species, standard length and parasite count.

Factor	Level	Estimate	Standard error	z value	p value
(Intercept)	-	-0.69	0.45	-1.55	0.12
Species	Bullhead	0.45	0.57	0.79	0.43
	Chub	0.46	0.48	0.96	0.34
	Dace	0.16	0.44	0.36	0.72
	Minnow	0.45	0.41	1.09	0.27
	Perch	-0.42	0.56	-0.74	0.46
	Roach	0.04	0.46	0.09	0.93
	Stone loach	0.65	0.50	1.29	0.20
	Three-spined stickleback	0.10	0.53	0.19	0.85
Standard length	-	0.03	0.15	0.21	0.83
Reach	Reach 2	0.30	0.31	0.97	0.33
	Reach 3	-0.22	0.24	-0.90	0.37
	Reach 4	-0.05	0.27	-0.18	0.86
Parasite count	-	-0.22	0.17	1.28	0.20

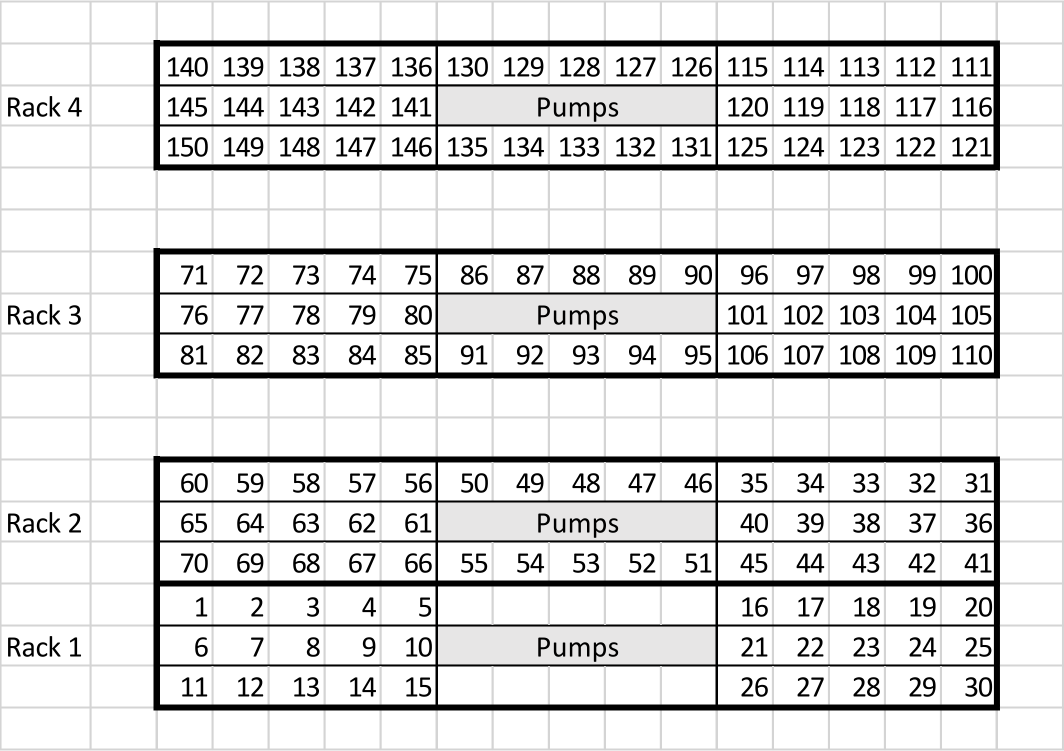
Table S4.4. Results of the independent negative binomial generalised model (NBGLM) testing for relationships between fish microplastic counts and feeding guild.

Level	Estimate	Standard error	z value	p value
(Intercept)	-0.55	0.10	-5.49	< 0.01
Demersal	0.18	0.16	1.16	0.25

Table S4.5. Results of the independent negative binomial generalised model (NBGLM) testing for relationships between fish microplastic counts and stomach condition.

Level	Estimate	Standard error	z value	p value
(Intercept)	-0.46	0.09	-5.27	< 0.01
Gastric	-0.08	0.19	-0.44	0.66

10.3 **Chapter 5 supplementary materials**



	140	139	138	137	136	130	129	128	127	126	115	114	113	112	111
Rack 4	145	144	143	142	141	Pumps					120	119	118	117	116
	150	149	148	147	146	135	134	133	132	131	125	124	123	122	121
	71	72	73	74	75	86	87	88	89	90	96	97	98	99	100
Rack 3	76	77	78	79	80	Pumps					101	102	103	104	105
	81	82	83	84	85	91	92	93	94	95	106	107	108	109	110
	60	59	58	57	56	50	49	48	47	46	35	34	33	32	31
Rack 2	65	64	63	62	61	Pumps					40	39	38	37	36
	70	69	68	67	66	55	54	53	52	51	45	44	43	42	41
	1	2	3	4	5						16	17	18	19	20
Rack 1	6	7	8	9	10	Pumps					21	22	23	24	25
	11	12	13	14	15						26	27	28	29	30

Figure S5.1. Schematic of experimental tank layout. The positions of racks in relation to each other are presented as well as the experimental tanks (5 per shelf) and air pumps.

Statistical model structure

In the following section, we give the R formula for all models considered in the selection process within each analysis. Akaike Information Criterion (AIC) was used to compare between linear and mixed effects models as well as between different mixed effects models. A backwards stepwise process was performed as per Zuur et al. (2009), using a model family based on the data and removing the least significant term each iteration to produce the optimal model where all remaining variables are either significant or non-significant. The models in each step are presented with the action(s) taken following each iteration within brackets. The output of each optimal model (bold, underlined) is then presented.

Response variables: P = end parasite load, K = change in body condition, SGR = specific growth rate, SSI = splenosomatic index, N = number of uninfected gammarids consumed within the functional response.

Fixed effects: G = the number of infected gammarids consumed (indicating parasite exposure), D = the microplastic diet exposure category, C = the parasite exposure category.

Random effects: B = the particular batch the tank was in (dictated on which days the tank received their feeding pellets), R = the particular rack the tank was in (dictated on which day the parasite exposure occurred).

S5.1 models. Parasite load model selection process and outputs for the optimal model. The models considered in the parasite load analysis using only infected fish are shown.

paraglm <- glm (P ~ G * D, family = "poisson") AIC = 113.062

paralme <- glmer (P ~ G * D + (1|B) + (1|R), family = "poisson”) AIC = 117.036

(paraglm progresses on the basis of AIC)

paramod1 <- glm (P ~ G * D, family = "poisson")

(D removed: χ^2 = 0.052, df = 2, p = 0.974, interaction term therefore also removed)

paramod2 <- glm (P ~ G, family = "poisson")

(Optimal model reached)

G: χ^2 = 4.065, df = 1, p = 0.044 *

S5.2 models. Change in condition model selection process and outputs for the optimal model. The models considered in the change in condition analysis using only surviving fish are shown.

kglm <- glm (K ~ C * D, family = "gaussian") AIC = 4.814

klme <- glmer (K ~ C * D + (1|B) + (1|R), family = "gaussian") AIC = 35.709

(kglm progresses on the basis of AIC)

kmod1<- glm (K ~ C * D, family = ”gaussian”)

(D removed: $\chi^2 = 0.210$, df = 2, p = 0.900)

kmod2 <- glm (K ~ C, family = ”gaussian”)

(Optimal model reached)

C: $\chi^2 = 2.315$, df = 2, p = 0.314

S5.3 models. Specific growth rate model selection process and outputs for the optimal model. The models considered in the specific growth rate analysis using only surviving fish are shown.

SGRglm <- glm (SGR ~ C * D, family = "gaussian") AIC = -366.014

SGRlme <- glmer (SGR ~ C * D + (1|B) + (1|R), family = "gaussian") AIC = -315.451

(SGRglm progresses on the basis of AIC)

SGRmod1<- glm (SGR ~ C * D, family = ”gaussian”)

*(C*D interaction removed: χ^2 = 0.683, df = 4, p = 0.953)*

SGRmod2<- glm (SGR ~ C + D, family = ”gaussian”)

(D removed: χ^2 = 0.150, df = 2, p = 0.928)

SGRmod3<- glm (SGR ~ C, family = ”gaussian”)

(Optimal model reached)

C: χ^2 = 8.139, df = 2, p = 0.017

S5.4 models. Splenosomatic index model selection process and outputs for the optimal model. The models considered in the splenosomatic index analysis using only surviving fish are shown.

SSIglm <- glm (SSI ~ C * D, family = "gaussian") AIC = -183.137

SSIlme <- glmer (SSI ~ C * D + (1|B) + (1|R), family = "gaussian") AIC = -135.571

(SSIglm progresses on the basis of AIC)

SSImod1<- glm (SSI ~ C * D, family = ”gaussian”)

*(C*D interaction removed: χ^2 = 0.637, df = 4, p = 0.959)*

SSImod2<- glm (SSI ~ C + D, family = ”gaussian”)

(D removed: χ^2 = 0.269, df = 2, p = 0.874)

<u>SSImod3<- glm (SSI ~ C, family = ”gaussian”)</u>

(Optimal model reached)

C: χ^2 = 5.314, df = 2, p = 0.070

S5.5 models. Number of gammarids consumed model selection process and outputs for the optimal model. The models considered in the number of gammarids consumed analysis using only fish included in the functional response analyses are shown.

FRglm <- glm (N ~ C * D, family = "poisson") AIC = 1571.705

FRpoissonlme <- glmer (N ~ C * D + (1|B) + (1|R), family = "poisson") AIC = 1540.98

(FRpoissonlme progresses on AIC)

FRnblme <- glmer (N ~ C * D + (1|B) + (1|R), family = "poisson") AIC = 837.193

(FRnblme progresses on AIC)

FRnblme <- glmer (N ~ C * D + (1|B) + (1|R), family = "poisson")

(C*D interaction removed: χ^2 = 0.583, df = 4, p = 0.965)

FRnblme1 <- glmer (N ~ C + D + (1|B) + (1|R), family = "poisson")

(D removed: χ^2 = 1.747, df = 2, p = 0.417)

FRnblme2 <- glmer (N ~ C + (1|B) + (1|R), family = "poisson")

(Optimal model reached)

C: χ^2 = 20.142, df = 2, p < 0.001 ***

Table S5.1. General linear model outputs for the optimal specific growth rate model. The results of the optimal model summary are given with significance levels. The unexposed category is included within the model intercept.

Coefficients	Estimate	Standard Error	t value	p value
(Intercept)	0.355	0.007	52.213	< 0.0001 ***
Exposed	-0.033	0.011	-2.851	0.005 **
Infected	-0.010	0.012	-0.892	0.374

10.4 Chapter 6 supplementary materials

In the following section, we give the R formula for all models considered in the selection process within the microplastic load models. Akaike Information Criterion (AIC) was used to compare between linear and mixed effects models before a backwards stepwise process was performed, as per Zuur et al. (2009), removing the least significant term each iteration to produce the optimal model where all remaining variables are either significant or non-significant. The models considered in each step are presented with the action(s) taken following each iteration within brackets. The output of each optimal model (bold, underlined) is then presented.

Variables: MP = the confirmed microplastic load (after exclusion of contaminants and FTIR confirmation), R = the river samples were collected from, L = the eel total length, D = the date the sample was collected on.

S6.1 models. Microplastic load model selection process and outputs for the optimal model. The models considered and selection process are presented using the confirmed microplastic loads.

MPGLMP <- glm (MP ~ R * L, family = "poisson") AIC = 97.3

MPGLMERP <- glmer (MP ~ R * L + (1|D), family = "poisson”) AIC = 99.3

MPGLMNB <- glm.nb (MP ~ R * L) AIC = 99.3

(MPGLMP progresses on the basis of AIC)

MPGLMP <- glm (MP ~ R * L, family = "poisson")

*(L and interaction removed: $\chi^2 = 1.24$, df = 1, p = 0.67, L*R: $\chi^2 = 0.19$, df = 1, p = 0.67)*

<u>MPGLMP1 <- glm (MP ~ R, family = "poisson")</u>

(Optimal model reached)

R: $\chi^2 = 1.29$, df = 2, p = 0.53

10.5 Chapter 7 supplementary materials

Table S7.1 Search terms and resulting hit numbers for the initial database searches. The number of hits are given from the initial searches within each of the databases: WoS (https://www.webofscience.com/wos/woscc/basic-search), JSTOR (https://www.jstor.org/), EBSCO (https://web.s.ebscohost.com/ehost/search/advanced?vid=0&sid=97dd22e6-5e1b-4a87-85f0-1be8b2bfea5c%40redis) and SCOPUS (https://www-scopus-com.bournemouth.idm.oclc.org/search/form.uri?display=basic&zone=header&origin=searchbasic#basic). All 2986 hits were accessible on the 2nd September 2022 and were screened further.

Stressor	Search	WoS	JSTOR	EBSCO	SCOPUS
Warming	"microplastic" AND "fish" AND "climate change"	38	58	19	24
	"microplastic" AND "fish" AND "warming"	6	33	10	8
	"microplastic" AND "fish" AND "temperature"	25	58	49	48
	"microplastic" AND "fish" AND "climate"	47	74	56	32
Pathogens	"microplastic" AND "fish" AND "pathogen"	6	13	7	23
	"microplastic" AND "fish" AND "parasite"	4	8	4	9
	"microplastic" AND "fish" AND "infection"	3	13	8	14
	"microplastic" AND "fish" AND "disease"	10	39	10	53
Habitat modification	"microplastic" AND "fish" AND "habitat"	57	61	92	125
	"microplastic" AND "fish" AND "urbanisation"	1	3	3	15
	"microplastic" AND "fish" AND "urbanization"	10	11	15	15
	"microplastic" AND "fish" AND "modification"	4	17	3	11
	"microplastic" AND "fish" AND "habitat degradation"	2	2	3	3
Pollutants	"microplastic" AND "fish" AND "nutrient"	6	41	9	19
	"microplastic" AND "fish" AND "contaminant"	53	32	64	179

	"microplastic" AND "fish" AND "metal"	28	46	59	94
	"microplastic" AND "fish" AND "pollutant"	70	42	149	833

Table S7.2 Considered research papers on the interaction of microplastics and an interacting stressor in freshwater fishes. For each study the assigned stressor is given, the species used, the environmental relevance of the microplastic exposure (justified within the text), how the data were extracted M = manually with the aid of a plot digitiser, P = data present in or with the paper and A = data were provided from the author. The studies are also identified where the data were included in the final dataset and therefore the analyses.

Interacting stressor	Species	Relevance	Data	Reference	Final dataset?
Warming	Symphysodon aequifasciatus	N	M	(Wen et al., 2018a)	Y
Inorganic pollutants	Danio rerio	Y	M	(R. Zhang et al., 2020)	Y
	Danio rerio	Y	M	(J. L. Zheng et al., 2022)	Y
	Danio rerio	N	M	(J. Zhu et al., 2022)	Y
	Danio rerio	N	M	(Qiao et al., 2019b)	Y
	Danio rerio	Y	M	(Santos, et al., 2022b)	N
	Danio rerio	Y	P	(Santos et al., 2022a)	Y
	Danio rerio	Y	M	(Santos, Luzio, et al., 2021)	Y
	Danio rerio	Y	M	(Santos, Félix, et al., 2021)	Y
	Danio rerio	Y	P	(Santos et al., 2020)	Y
	Danio rerio	N	M	(Cheng et al., 2021)	Y
	Danio rerio	N	M	(Qin et al., 2021)	Y
	Cyprinus carpio	N	M	(Hoseini et al., 2022a)	N
	Cyprinus carpio	N	M	(Hoseini et al., 2022b)	N
	Cyprinus carpio	N	P	(Banaee et al., 2019)	Y
Organic pollutants	Danio rerio	Y	M	(Yang et al., 2020a)	Y
	Danio rerio	Y	M	(Luo et al., 2021)	Y
	Danio rerio	N	M	(J. Zhang et al., 2021)	Y

	Cyprinus carpio	Y	M	(C. Zhang et al., 2021)	Y
	Cyprinus carpio	Y	M	(J. Chen et al., 2022)	Y
	Danio rerio	Y	M	(C. Li et al., 2022)	Y
	Danio rerio	N	M	(H. J. Zhao et al., 2020)	Y
	Danio rerio	N	M	(Sheng et al., 2021)	Y
	Clarias gariepinus	N	M	(Karami et al., 2016)	Y
	Oncorhynchus mykiss	Y	M	(Karbalaei et al., 2021)	N
	Oreochromis niloticus	N	M	(Y. Huang et al., 2021)	Y
	Misgurnus anguillicaudatus	N	M	(Qu et al., 2019)	Y
	Salmo trutta	N	P	(Schmieg et al., 2020)	Y
	Oncorhynchus mykiss	N	P	(Hanachi et al., 2021)	N
	Danio rerio	N	A	(Z. Huang et al., 2023)	Y
	Gasterosteus aculeatus	Y	A	(Masud et al., 2022)	N
	Danio rerio	N	A	(Schell et al., 2022)	Y
	Danio rerio	Y	M	(Hanslik et al., 2022)	Y
Pathogens	Oncorhynchus mykiss	N	M	(Banihashemi et al., 2021)	Y
Plastic additives	Danio rerio	Y	M	(H. Wang et al., 2022)	N
	Danio rerio	N	A	(He et al., 2021)	N

Table S7.3 Top 3 most frequent responses within each response category. The top 3 most frequently occurring responses are given within each response category gathered from the final 36 studies. Measure responses are given in ranked order and decreasing frequency. Often authors would name the same responses differently across different studies therefore terms were not explicit e.g. “Total immunoglobulin level” would be included within the response “Immunoglobulin level”.

Response category	Response
Biochemical	Albumin Cholesterol level Globulin levels
Ecological	Body length/body length growth Total distance moved Mortality
Immunological	Complement C3 level/expression Lysozyme activity Immunoglobulin level
Metabolic*	Acetylcholinesterase expression/activity Catalase activity/expression Heart rate
Stress response	Superoxide dismutase activity/expression Malondialdehyde levels Glutathione peroxidase activity/expression

*Metabolic also included morphological measures such as muscle thickness

Table S7.4 Results of the linear mixed effects models relating Hedges’ d effect sizes to microplastic and fish features. Parameter estimates are given for A) the inclusive dataset model with microplastic morphology, polymer, fish lifestage and B) the exclusive dataset model with fish lifestage.

A)

Factor	X^2	df	p value
Microplastic polymer	1.17	5	0.95
Microplastic morphology	2.76	4	0.60
Fish lifestage	5.07	3	0.17
Fish family	2.40	1	0.12

B)

Factor	X^2	df	p value
Fish lifestage	2.16	3	0.54