Towards a greater understanding of the presence, fate and ecological effects of microplastics in the freshwater environment

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Towards a greater understanding of the presence, fate and ecological effects of microplastics in the freshwater environment

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SUMMARY

Plastics within the environment are becoming increasingly recognised as one of today's major environmental issues. Production and disposal of plastics continues to increase every year, with much of this being single-use items. Due to mismanagement of plastic waste globally, millions of tonnes of plastic ends up within the environment every year. Images of organisms entangled in plastic litter and discarded fishing gear are commonplace across the global media, often utilising images of charismatic marine megafauna such as whales and turtles, and as such, public awareness is now at an all-time high. This has translated to significant efforts to address this problem, primarily in the marine and coastal environment. This includes large-scale industry action including The Ocean Cleanup and Sky Ocean Rescue, alongside community-led action such as local litter clean-ups led by charities, and initiatives such as 'Plastic Free Communities' (linked to UK charity Surfers Against Sewage).

Despite efforts by many to reduce plastic waste entering the environment, it is not feasible to remove the majority of plastic that already resides within the environment and much of this will remain for tens, if not hundreds, of years. This is in part due to the fact that plastics will degrade over time, fragmenting and abrading into numerous small particles known as microplastics. As a result of this widespread plastic presence and subsequent degradation of large items, microplastics are now understood to be a pervasive environmental pollutant, ubiquitous across the globe. They have been found in every location that has been studied for this purpose, from remote mountain tops to the deep oceans. While it is understood that the majority of microplastics will derive from items produced and used on land, little attention has been paid to freshwaters as a receiving environment for microplastics, and the environmental and ecological implications of this. The key knowledge gaps in this area were explored in **Chapter 2**.

The sources, presence and abundance of microplastics within freshwater sediments in the River Thames Basin (UK) were investigated within **Chapter 3**. Four sites were selected to represent a range of influences, including those heavily influenced by sewage effluent, and those with little sewage input. Microplastic particles (1 mm–4 mm) were extracted from sediments using an optimised stepwise approach based on the most current literature recommendations, to include flotation, visual extraction and identification using Raman spectroscopy. Microplastics were found at all four sites. One site had significantly higher numbers of microplastics than

other sites, average 66 particles 100 g^{-1}, 91% of which were fragments. Many of the fragments at this site were determined to be derived of thermoplastic road-surface marking paints. This site was not the site most highly influenced by sewage effluent inputs, however it was directly downstream from a storm drain and therefore received urban runoff directly to the watercourse. This study therefore highlighted that the factors influencing microplastic concentration can be highly location-dependent, and that there may be a number of different routes of input for microplastics.

Due to the widespread presence of microplastics in habitats worldwide, it is recognised that microplastics are widely ingested by organisms spanning a range of trophic guilds. Despite this, prior to the research presented in this thesis, there was no evidence for ingestion of microplastics by any freshwater organisms within the UK. Following the identification of high concentrations of microplastics within sediments of the River Thames Basin (**Chapter 3**), **Chapter 4** aimed to investigate the ingestion of microplastics by a freshwater fish species within this river system, the common roach (*Rutilus rutilus*). This research also aimed to understand the factors affecting ingestion, including characteristics of the fish (size, gender) and location-specific factors based on the distance of the sampling site from the source of the river. The distance that the sampled fish could travel within the river was determined by the location of locks which would impede fish passage, and therefore each represented a known stretch of river. Microplastics were found within the gut contents of roach from six out of seven sampling sites. Of sampled fish, 33% contained at least one microplastic particle, with a maximum of six particles in one fish. Both fish size, gender and distance from the source of the river influenced the maximum number of particles a fish was likely to ingest. This study therefore provided valuable new insights into the factors influencing ingestion within riverine environments.

It is understood that plastics within the environment will associate with hydrophobic organic chemicals (HOCs), with the potential to transport these and influence their availability to organisms. These interactions were explored firstly within two separate studies. In the study presented in **Chapter 5**, polystyrene microplastics were used in combination with two different pesticides, deltamethrin and dimethoate, to investigate how microplastics may alter the toxicity of these pesticides to the model organism *Daphnia magna*. While these pesticides led to expected reductions in survival and mobility, these responses were not influenced by the presence of microplastics. Microplastics alone led to no observable responses. The research presented in **Chapter 6** further examined microplastic-HOC interactions, exposing the pond

snail *Lymnaea stagnalis* to flame-retardant chemicals polybrominated diphenyl ethers (PBDEs), in the presence and absence of nylon microplastics, to determine whether the presence of microplastics would influence PBDE accumulation and the microbiome of the snail. Only subtle effects were seen: BDE 47 accumulation was reduced while the uptake of all other congeners was not significantly affected. No effect of microplastics, PBDEs or co-exposure was observed on the microbiome diversity or community composition. Only a few operational taxonomic units were affected by PBDEs, in the absence of microplastics only.

Based on these results it was therefore concluded that microplastics were a negligible factor in influencing bioavailability, bioaccumulation and toxicity of hydrophobic organic chemicals (HOCs) under the conditions tested. This is an important observation as many studies have previously stated that microplastics will enhance the bioavailability and bioaccumulation of HOCs. These results therefore show that this is highly variable between studies and likely extremely dependent on experimental conditions and the organisms studied. It would have been expected that effects would have been seen under the highly controlled conditions used here. Given the complexity and range of possible interactions between microplastics, chemicals, organic particles and inorganic matter within the environment, it can therefore be inferred that that microplastics are not likely to significantly influence HOC bioavailability or toxicity to organisms under natural environmental conditions.

Until recently, our knowledge of microplastic in freshwater systems has been limited. This PhD research therefore aimed to take a rounded approach to the issue and as such, a range of field and laboratory studies were conducted to develop a greater understanding of the sources, environmental concentrations and ecological effects of microplastics in freshwaters. The research presented here enhances our knowledge of microplastics in freshwater systems, and explores the challenges for further microplastic research.

SAMENVATTING

De aanwezigheid van plastics in onze leefomgeving wordt in toenemende mate gezien als één van de grootste milieuproblemen van deze tijd. De productie van plastics stijgt elk jaar opnieuw en daarmee ook het afval van plastics, omdat veel plastic slechts één keer wordt gebruikt. Miljoenen tonnen plastic eindigen elk jaar in het milieu, omdat de recycling van plastics op mondiaal niveau slecht is geregeld. Iedereen kent de plaatjes van dieren die vastzitten in plasticafval of afgedankt visgerei, waarbij vaak charismatische zeedieren betrokken zijn, zoals walvissen of zeeschildpadden. Dankzij deze media-aandacht is het publieke bewustzijn momenteel op een hoogtepunt. Dit is vertaald in diverse initiatieven om het probleem te beteugelen, vooral in kust- en mariene ecosystemen. Een bekende actie vanuit de grootschalige industrie is The Ocean Cleanup and Sky Ocean Rescue, maar er zijn ook diverse initiatieven vanuit lokale gemeenschappen om het plastic afval op te ruimen en initiatieven zoals 'Plastic Free Communities' (wat gelieerd is aan de Britse liefdadigheidsorganisatie Surfers Against Sewage).

Ondanks de diverse inspanningen om de hoeveelheid plastic dat in onze leefomgeving komt terug te dringen, blijkt het merendeel van het plastic dat al in ons milieu is niet te verwijderen en zal het daar de komende tientallen tot honderden jaren blijven. Gedurende die periode zal het plastic afbreken en in kleine stukjes breken die we microplastics noemen. Als gevolg van enerzijds de wijdverspreide aanwezigheid van plastics en anderzijds de afbraak daarvan in kleine stukjes, worden microplastics tegenwoordig gezien als een prominente milieuvervuilende stof die over het hele wereld aanwezig is. Microplastics zijn op elke plek gevonden waar ernaar gezocht is, van afgelegen bergtoppen tot in de diepe oceaan. Omdat de meeste microplastics afgeleid zijn van producten die op het land geproduceerd en gebruikt zijn, is er tot nu toe weinig aandacht geweest voor zoetwatersystemen als ontvanger van microplastics, en van de ecologische en milieugevolgen daarvan. De cruciale onderzoekvragen op dit gebied zijn geëxploreerd in **Hoofdstuk 2**. In dit review artikel is de algemene stand van kennis met betrekking tot microplastics als verontreiniging in zoetwater- en terrestrische systemen beschreven, waarbij eveneens gekeken wordt hoe microplastics een ecologisch gevaar kunnen vormen in deze omgevingen. Deze beoordeling werd uitgevoerd met behulp van de beschikbare academische literatuur en diende als leidraad voor de volgende onderzoeksvragen die in dit proefschrift en in het wereldwijde onderzoeksveld.

In **Hoofdstuk 3** zijn de bronnen, aanwezigheid en de dichtheid van microplastics in zoetwatersedimenten van de rivier de Theems (Verenigd koninkrijk) onderzocht. Vier locaties werden geselecteerd om een gradiënt van milieu-invloeden te representeren van locaties die sterk beïnvloed waren door rioolafvoer tot locaties waar weinig afvalwater de rivier instroomt. De microplastic deeltjes (1-4 mm) werden geëxtraheerd met een stapsgewijze geoptimaliseerde benadering die gebaseerd was op aanbevelingen uit de meest recente literatuur en bestond uit een combinatie van flotatie, visuele extractie en identificatie met Raman spectroscopie. Op alle vier de locaties werden microplastics gevonden. Eén locatie had significant hogere waarden met een gemiddelde van 66 deeltjes per 100g, waarvan 91% bestond uit fragmenten. Veel van deze fragmenten bleken afkomstig van markeringsverf gebruikt op wegoppervlakten. Deze locatie was weliswaar niet de locatie die het meest beïnvloed was door rioolafvoer, maar was wel direct benedenstrooms van een storm drainagekanaal en kreeg daardoor direct de stedelijke afvoer. Hoofdstuk 3 benadrukt dat de factoren die de concentraties aan microplastics bepalen erg locatie-afhankelijk zijn en dat er verschillende routes zijn die voor de aanvoer van microplastics kunnen zorgen.

Vanwege de wijdverspreide aanwezigheid van microplastics, is er ook een toenemende aandacht voor de opname van microplastics door organismen. Ondanks deze aandacht, was er -voorafgaande aan het onderzoek gepresenteerd in dit proefschrift- geen bewijs voor de opname van microplastics door zoetwaterorganismen. Gebruikmakend van de resultaten uit Hoofdstuk 3 voor de rivier de Theems, had **Hoofdstuk 4** tot doel om de opname van microplastics door de vissoort Blankvoorn (*Rutilus rutilus*) in de rivier de Theems te onderzoeken. Bovendien wilden we de factoren die de opname beïnvloeden, zoals de eigenschappen van de vis (grootte en geslacht) en de effecten van de afstand tot de bron van de rivier (als maat voor menselijke beïnvloeding) onderzoeken. De afstand die een bemonsterde vis had kunnen afleggen werd vastgesteld door de locatie van sluizen die de migratie van vis tegengaan, en daardoor een stuk rivier afbakenen. Op zes van de zeven locaties werden microplastics in het darmkanaal van de voorn gevonden. Zo'n 33% van de vissen bevatte op zijn minst 1 microplastic deeltje met een maximum van 6 deeltjes per vis. Zowel de grootte van de vis, als geslacht en afstand tot de bron van de rivier beïnvloedden het maximale aantal deeltjes dat een vis kon inslikken. Deze studie bracht daarom waardevolle inzichten in de factoren die de opname van microplastics in riviersystemen bepalen.

Het is bekend dat plastics zullen binden aan hydrofobe organische verbindingen, die op hun beurt de plastics kunnen verplaatsen en zo hun beschikbaarheid voor organismen beïnvloeden.

Deze interacties werden geëxploreerd in twee afzonderlijke studies. In Hoofstuk 5 wordt een studie beschreven waarin polystyreen microplastics werden gecombineerd met twee verschillende bestrijdingsmiddelen; deltametrin en dimethoaat. Er werd onderzocht hoe microplastics de toxiciteit van deze bestrijdingsmiddelen voor het modelorganisme *Daphnia magna* veranderden. De aanwezigheid van de bestrijdingsmiddelen leidden inderdaad tot de verwachte daling in overleving en mobiliteit, maar dit bleek onafhankelijk van de aanwezigheid van microplastics. De aanwezigheid van alleen microplastics leidde tot geen respons. De relatie tussen microplastics en hydrofobe organische verbindingen werd verder onderzocht in het onderzoek beschreven in **Hoofdstuk 6**. De poelslak *Lymnaea stagnalis* werd blootgesteld aan de brandwerende chemicaliën polybrominaat difenyl ethers (PBDEs), in de aan- en afwezigheid van nylon microplastics om te bepalen of de aanwezigheid van microplastics de accumulatie van PBDEs en het microbioom van de slak zou beïnvloeden. Alleen subtiele effecten werden gevonden: De accumulatie van BDE47 was lager, terwijl de opname van de overige PBDEs onveranderd bleef. Er was geen effect van microplastics, PBDEs of de combinatie daarvan op de diversiteit of samenstelling van het microbioom. Alleen bepaalde nauwverwante bacteriën werden beïnvloed door PBDEs, maar alleen in afwezigheid van microplastics.

Op basis van deze resultaten werd geconcludeerd dat microplastics een verwaarloosbare invloed hebben op de biologische beschikbaarheid, accumulatie en toxiciteit van hydrofobe organische verbindingen. Dit is een belangrijk gegeven omdat veel eerdere studies suggereerden dat de aanwezigheid van microplastics deze processen zouden versterken. Het lijkt er dus op dat de interacties erg afhankelijk zijn van de experimentele omstandigheden en het organisme dat onderzocht wordt. Echter, juist onder de zeer gecontroleerde omstandigheden van onze proefopzet, hadden we verwacht effecten te zien. Onder natuurlijke omstandigheden zijn er nog diverse andere interacties mogelijk tussen microplastics, overige organische en anorganische verbindingen. Het ligt voor de hand dat, onder die complexiteit, de effecten van microplastics op de beschikbaarheid en toxiciteit van hydrofobe organische verbindingen onbelangrijk zullen zijn.

Dit proefschrift heeft onze kennis van microplastics in zoetwatersystemen verbeterd dankzij een diversiteit aan benaderingen, variërend van veldonderzoeken tot laboratoriumstudies. Een beter begrip van de bronnen, milieuconcentraties en de ecologische effecten van microplastics in zoetwater is verkregen. Met deze inzichten kunnen nieuwe uitdagingen voor onderzoek naar microplastics opgepakt worden.

CHAPTER 1
Introduction

1. Plastics as an environmental pollutant

In today's society, people would struggle to live without plastics. Plastics are strong, waterproof, durable and cheap, making it the material of choice for manufacturers of many everyday items including packaging, electrical items and clothing, among others. However, these features of plastics also mean they now represent a significant proportion of our waste. Despite measures to reduce plastic consumption and disposal, or to recycle plastic items, the amount discarded as plastic waste is increasing year-on-year, with the potential for much of this waste to be mismanaged and enter the environment (Jambeck et al., 2015; PlasticsEurope, 2015). The longevity of plastics implies that plastic litter that ends up in the environment will persist to leave a legacy of our 'throw-away society' for hundreds, if not thousands of years to come. With fears that the mass of plastic in the oceans could equal or exceed the weight of fish in the sea by 2050 (World Economic Forum, 2016), the general public are becoming increasingly concerned about the effects of plastics on the environment. Within the last two to three years, plastics and microplastics have begun to attract significant academic and media attention, reflecting societal concerns about the issue of waste and environmental pollution.

While plastics are durable, they invariably degrade with age, with large items fragmenting to form multiple smaller pieces, with those < 5 mm in size defined as 'microplastics' (Arthur and Baker, 2009; Moore, 2008). Despite this degradation, the resulting fragments are estimated to last for hundreds or even thousands of years within the environment (Barnes et al., 2009). Microplastics fall within two categories: primary microplastics (manufactured specifically to be smaller than 5 mm, including cosmetic microbeads, glitter and nurdles) and secondary microplastics (derived from the breakdown and weathering of large-scale plastics or plastic-containing products, such as fragments of degraded litter or microfibers from synthetic textiles) (Hartmann et al., 2019). Microplastics are of particular concern as an environmental contaminant due to their potential for ingestion by organisms, with evidence to suggest they can cause harm to organisms and ecosystems. In addition to microplastics being a particulate pollutant, microplastics may act as a source of organic chemicals to the environment in the form of plasticisers leached from plastics as they degrade (Lohmann, 2017).

2. Importance of studying microplastics

Awareness of microplastics as a potential environmental contaminant first arose in the early 1970s, with the incidental discovery of small plastic particles in marine environmental samples (Buchanan, 1971; Carpenter and Smith, 1972). This led researchers to realise that plastic pollution consisted not just of the large-scale litter that is widely visible within the environment, but that plastics were also present at a much smaller scale. Since these first observations, many studies have since used environmental sampling as a means of assessing microplastic distribution and abundance across a wide range of environments. Due to the prevalence and widespread use of plastics in all aspects of daily life, sources and emissions of microplastics to the environment as a result of product use and degradation are varied and diverse. It is recognized that the majority of microplastic waste will originate on land as this is where plastics are primarily used and discarded. However, microplastics have the capability to become widely distributed from their original source by wind, water or human actions (Lebreton et al., 2017; Nizzetto et al., 2016; Zylstra, 2013).

The marine environment is, to date, the most widely studied environment with respect to microplastic pollution, with comparatively much less understood about the contamination of freshwater systems. This is despite the understanding that rivers represent the main link between the terrestrial and the marine environment, facilitating the movement of plastics from land-based sources to the sea (Jambeck et al., 2015; Lebreton et al., 2017). However, it is highly unlikely that all particles will pass through freshwater systems unimpeded; on their journey from land to sea, microplastics will encounter a wide range of complex interactions that will influence their behaviour, transport and fate. Thus not all microplastics will reach the ocean (Castañeda et al., 2014; Dris et al., 2015; Wagner et al., 2014). Whether accumulated within sediments or passing through the water column, microplastics within rivers can become bioavailable to organisms across a range of trophic levels (Sanchez et al., 2014; Windsor et al., 2019b). A huge variety of factors will influence the potential ecological effects of microplastics including (but not limited to) environmental conditions, type of polymer, associated chemicals and size and shape of particles (Windsor et al., 2019a; Wright et al., 2013b).

The regulatory trend with microplastics is increasingly moving towards the precautionary principle of banning products without full evidence of harm (e.g. banning of microbeads in personal care products in various countries globally). However, while microbeads are relatively easy to regulate as they are usually an additional, rather than a core ingredient in products, many other applications of (micro)plastic will be far less easy to eliminate. While the public

are increasingly calling for bans or restrictions on certain plastic products, we must be certain to provide evidence of environmental release and harm in instances where banning specific plastic products may lead to a regrettable substitution, where products are replaced by potentially more harmful, and less well understood, products. This thesis aims to address the significant gaps remaining in our knowledge surrounding the sources, fate and ecological effects of microplastics in the context of these complex environmental factors.

3. Microplastics in the freshwater environment

Worldwide, humans rely heavily on freshwater systems for drinking water resources, in addition to food sources (fish and shellfish), irrigation and leisure activities. Clean water is essential for maintaining life, both aquatic and terrestrial. Contamination of freshwater systems by particulate or chemical contaminants can have significant implications for water quality, ecosystem health and function, and human health. It is therefore essential to understand how rivers may act as not only a transport pathway, but as a sink of microplastics, and the implications this may have on freshwater ecosystems and water quality.

Despite the comparative lack of research on microplastics in freshwater systems compared to the marine environment, the studies carried out to date imply that freshwaters may be equally, if not more, contaminated with microplastics than the oceans, with the highest ever concentrations of microplastics found recently in a UK river, and with flooding seen to significantly reduce sediment concentrations (Hurley et al., 2018). It is therefore critical that the scientific community works towards a greater understanding of the factors influencing microplastic accumulation and transport in freshwater environments, in addition to understanding the ecological effects, to better inform policy, industry and public decision-making.

4. Ecological impacts of microplastics

It has been observed in many studies that organisms across various trophic guilds will ingest microplastics. Microplastic ingestion may be either intentional (ingesting particles that resemble food) or unintentional (particles eaten incidentally in association with other food). It has been observed that many higher trophic organisms, including sea turtles, birds, marine mammals and fish contain (micro)plastics within their guts, likely as a result of food-chain

transfer (Campbell et al., 2017; Eriksson and Burton, 2003; Lusher, 2015). Trophic transfer is therefore likely to lead higher trophic organisms to become exposed to microplastics when otherwise they may not have done (Eriksson and Burton, 2003; Nelms et al., 2018). Ingestion by lower trophic organisms could lead to a bioaccumulation within the predators, and even (size-dependent) translocation to body tissues (Mattsson et al., 2017; Moore, 2008; Watts et al., 2014).

While microplastics have been found widespread throughout the environment, including within organisms, there is still insufficient understanding of the ecological and toxicological implications of this exposure. Physical harm may include blockage of the gut following ingestion, internal or external abrasion or inflammation, or blockage of gills leading to suffocation (Moore, 2008; von Moos et al., 2012; Wright et al., 2013b). The potential for a particle to cause harm depends on a huge variety of factors including the size and shape of the particle, concentration of plastic particles or associated chemicals (discussed in section 5), environmental conditions and also particle behaviour within the environment, determining whether an organism is likely to encounter it. Different traits of organisms will also influence their susceptibility to harm resulting from microplastic exposure. Therefore, it is also highly likely that different species will be affected in different ways by exposure to microplastics, depending on feeding behavior, metabolism, life-history and physiological characteristics (Galloway et al., 2017; Setälä et al., 2016; Wright et al., 2013b).

Microplastic exposure, in some instances, has been shown to have detrimental effects on health, metabolism, reproduction and immunity (Besseling et al., 2014; Wright et al., 2013a). However, these studies often represent very highly polluted or unrealistic scenarios and are therefore not necessarily representative of the likely exposure conditions that these organisms will encounter in the environment. Lower (more realistic) concentrations tend not to induce significant effects on commonly observed endpoints such as survival, behavior and reproduction in the short term (Lenz et al., 2016). There is not yet sufficient evidence to accurately determine the long-term impacts of microplastic contamination on organisms and ecosystems, although recent research suggests that chronic sublethal effects on the less-frequently investigated traits such as gene expression, metabolism or hormone production may have protracted but potentially significant long-term impacts on populations and the ecosystems that depend on them (Galloway et al., 2017; Jaikumar et al., 2019).

It is important to note that even at high concentrations plastics may not always be harmful; some studies suggest that microplastics may be ingested and egested without consequence (Beiras et al., 2018; Jovanović et al., 2018; Kaposi et al., 2014; Weber et al., 2018), while others show that some organisms can eat and metabolise plastic. For example, waxworms have been found to digest polyethylene, specifically due to the polymer-degrading bacteria *Enterobacter asburiae* YT1 and *Bacillus* sp. YP1 within the gut (Yang et al., 2014). A similar study was carried out which discovered that mealworms can digest and depolymerise polystyrene foam due to the gut bacterium *Exiguobacterium* sp. strain YT2, remaining as healthy over a one month test as mealworms that were fed a normal diet (Yang et al., 2015a, b). In addition to acting as a food source, plastics have also been shown to act as a microbial habitat, with the potential to acquire a distinct microbial community that is different in composition and less diverse than the surrounding environment (McCormick et al., 2014; Oberbeckmann et al., 2018; Zettler et al., 2013). While this novel substrate can be beneficial to the microbial communities which associate with plastic, the presence of plastics may also detrimentally alter the bacterial community structure within specific environments, changing the ecosystem structure by leading to the dominance of certain species. It is recognised that in order to ascertain any likely consequences of the widespread microplastic presence under realistic environmental conditions, it is important to understand the ecological impacts of microplastics not only at concentrations that are representative of those found within the environment, but also under representative timescales of exposure and with the heterogeneous mix of particles (and chemicals) to which organisms will be exposed (Lenz et al., 2016; Rist and Hartmann, 2018).

While there is a wide gap between our knowledge of the presence and abundance of microplastics in the marine environment compared to freshwaters, including rivers and lakes, our comparative understanding of organism interactions between these two systems is yet more unbalanced. While many ecological studies have focused on the presence of microplastics with wild-caught marine fish and invertebrates, far fewer address freshwater organism exposure or interactions. Further, considering our knowledge of rivers as carriers of microplastics, receiving and transporting microplastics from diverse sources and inputs, little emphasis has been put on research investigating the environmental factors influencing freshwater organism exposure, for example proximity to sources or differential exposure as a result of life history traits. This thesis therefore aims to investigate how specific sources and inputs such as wastewater effluent can be linked to organism exposure, in addition to how intraspecific

differences might influence ingestion. This will significantly increase our understanding of the factors influencing organism exposure and thus the potential for harm.

5. Plastics as a carrier of toxic chemicals

In addition to causing physical harm, there are two ways in which microplastics may impose a chemical hazard to organisms, either as a result of incorporated plasticiser chemicals, or the sorption of organic chemicals from the environment. Plastics are manufactured containing a variety of different plasticiser chemicals (e.g. phthalates, bisphenol A, dyes) which are added to plastics during manufacture, including plasticisers, flame retardants and dyes to give them different properties, for example to improve flexibility and durability (Lithner et al., 2009; Lithner et al., 2012). These chemicals are not chemically bound to the polymer structure and thus can leach out of plastic as the product ages, a process which can be accelerated by environmental conditions such as high temperatures or UV exposure (Bandow et al., 2017). This release of plasticisers allows these (potentially harmful) chemicals to become freely available within the environment and to organisms (Huang et al., 2013; Lithner et al., 2009). It has also been suggested that gut surfactants and an increased temperature within the stomach (compared to within the external environment) can facilitate plasticiser leaching from particles following ingestion (Bakir et al., 2014).

Microplastics are hydrophobic, with a large surface area to volume ratio, and so will associate with hydrophobic organic chemicals (HOCs, e.g. pesticides, polychlorinated biphenyls, polybrominated diphenyl ethers) within the environment (Ašmonaitė et al., 2018; Mato et al., 2001; Rochman et al., 2013b). This may lead to the alteration of these chemicals' toxicity and bioavailability to organisms (Rochman et al., 2013a; Teuten et al., 2009). There is widespread scientific debate as to whether plastics facilitate the uptake and bioaccumulation of these chemicals within organisms, or whether binding to plastics makes the chemicals less available, thereby reducing uptake (Bakir et al., 2016; Koelmans et al., 2016). Some studies have shown that plastics can increase bioaccumulation of HOCs within organisms. For example, PCBs have been observed to significantly accumulate within marine worms exposed to PCBs in the presence of polystyrene (Besseling et al., 2013) and fish exposed to plastics with sorbed contaminants have been seen to suffer increased hepatic stress compared to exposure to virgin uncontaminated plastics (Rochman et al., 2013a). Conversely, other studies have shown that microplastics do not change the toxicity of HOCs (Beiras and Tato, 2019) or that microplastics

may in fact reduce the bioavailability of HOCs due to strong chemical binding (Beckingham and Ghosh, 2016; Zhu et al., 2019). There is even the suggestion of ingested microplastics binding and removing HOCs that had previously been accumulated, although there is insufficient evidence to support this hypothesis (Gouin et al., 2011; Rummel et al., 2016). Recent studies have suggested that while microplastics may have an influence on bioavailability of HOCs, within a realistic environmental scenario, plastics will likely be a negligible route of transport for uptake of these chemicals compared to other modes of uptake, including ingestion of organic matter and dermal uptake directly from the water (Bakir et al., 2016; Grigorakis and Drouillard, 2018; Koelmans et al., 2016). This contrasting evidence highlights the importance of further research in this field to better understanding these microplastic-chemical associations and dynamics. An important factor to note is that the majority of these results are based on modelling exercises; therefore further experimental studies are required to verify these results (Bakir et al., 2016; Gouin et al., 2011; Koelmans et al., 2016). This need to provide comprehensive and relevant ecotoxicological data to inform and feed into models is discussed in section 7.

6. The value of field studies to inform our understanding of ecosystem exposure

Given the discrepancies between concentrations found within the field and those used within ecotoxicological tests, further field studies are essential in order to understand not only the types and concentrations of microplastics present within the environment, and temporal changes in these, but where microplastics derive from and where they accumulate. It is also important to understand how the concentrations of microplastics at different sites are affected by environmental factors, for example weather or water currents and anthropogenic factors such as urbanisation, sewage or litter input, so that we can better understand the environments that are most susceptible to microplastic accumulation and organism exposure. It is essential that we understand the presence and sources of microplastic pollution across a variety of locations and environments worldwide, in addition to presence within biota as a result of ingestion and inhalation. Without this knowledge we would be unable to determine the extent and likely effects of microplastic pollution at current or predicted future levels of environmental contamination (Adam et al., 2019; de Souza Machado et al., 2018). This information will allow for better prediction and understanding of likely interactions between

plastics and organisms, and the possible impacts of these interactions, in addition to understanding which regions and ecosystems are most at risk.

Despite a growing number of studies in this area over the last few years, robust and consistent methodologies are only now starting to emerge. This lack of consistency extends even as far as the definition of microplastics, with most studies defining these as plastic particles < 5 mm, while others use < 1 mm as a working definition (Claessens et al., 2013; Frias and Nash, 2019; Hartmann et al., 2019). It is therefore recognised that there is a need for standardisation, or at least harmonisation, of methods used for microplastic analysis across studies, to allow for accurate comparison of data (Besley et al., 2016; Rochman et al., 2017). This is especially important given the growing requirements of industries and governments for reliable and reproducible data, with the ultimate aim of using these data to inform policies, regulations and business strategies. With the understanding that all researchers will continue to use different techniques based on the samples, the research question(s) being asked and the resources available to them, it is essential to come to a consensus that data should be presented and reported in such a way that is repeatable by others, also allowing them to be interpreted correctly and compared to other relevant studies. This should include information such as (but not limited to): mesh size of sampling nets, depth and/or volume sampled, sample storage, density of separation solutions, temperature and pH for digestion protocols and polymer analysis technique (Helm, 2017; Mai et al., 2018; Rochman et al., 2017).

7. The need for realistic conditions in ecotoxicological assessments of microplastics

While field studies provide valuable information on the levels of environmental contamination, this information is not useful in itself, unless it can be put into context of environmental or ecological implications: the question of ‘so what?’. Laboratory experiments are therefore a vital tool for helping us understand the toxicological mechanisms, and biological and chemical associations, which cannot be observed purely by environmental sampling or field observations. Spatial and temporal variability in the environment are such that it can be impossible to tease apart cause and effect across biotic and abiotic variables. Many questions around the factors influencing fate, bioavailability and toxicity of microplastics (and other chemicals) cannot be answered without running specific and targeted studies under controlled conditions (Rist and Hartmann, 2018). Such controlled testing allows for small adjustments of

variables to determine the impacts of subtle changes within the system, for example different types, sizes and concentrations of plastic particles (Rist and Hartmann, 2018).

As with other pollutants, the fundamentals of environmental risk assessment can also be applied to microplastics. This requires evaluating the likelihood of exposure combined with the potential hazard (Rand, 1995; Suter, 1995). Microplastics are much more complex to risk assess compared to many chemical contaminants, as they are composites of multiple chemicals in association with a polymer (Rochman et al., 2019). Despite the importance of understanding the impacts of these chemical mixtures, assessing the impacts of individual compounds and polymers is essential first and foremost. Our understanding of the physical and chemical harm posed by microplastics of varying polymer types, sizes, and shapes, is still limited. Therefore the common approach of toxicity testing using single particle types (or simple mixtures) at high concentrations is valuable for understanding mechanisms of hazard, thresholds and modes of toxicity for microplastics with differing characteristics, in addition to informing predictive models of mixture toxicity (Au et al., 2017; Backhaus and Faust, 2012; Faust et al., 2003). While studies carried out at high concentrations exceeding the concentrations to which the organisms would currently be exposed are often met with criticism, it must be noted that environmental concentrations will inevitably increase as a combined result of increased usage and disposal of plastics, alongside degradation of existing plastic debris (Geyer et al., 2017; Thompson, 2015). Once within the environment, microplastics are difficult if not impossible to remove (Brandon et al., 2016; Lusher et al., 2014), therefore exposures at high concentrations are valuable to determine possible ‘worst-case’ future scenarios which may occur as a result of increasing environmental contamination (Huvet et al., 2016; SAPEA, 2019). These data are especially useful when combined with process-based models to determine large-scale or long-term ecological impacts of microplastics and their chemical associations (Ashauer et al., 2006; Jager et al., 2006; Kimball and Levin, 1985). Developing this knowledge on the ecotoxicological effects of different types and concentrations of microplastics to organisms of different sensitivities, under different environmental conditions, is essential for informing environmental risk assessment and regulation of microplastics (Backhaus and Faust, 2012; Huvet et al., 2016).

The majority of microplastic studies to date have used concentrations of microplastics that far exceed those found in environmental samples (Koelmans et al., 2015; Lenz et al., 2016). It is therefore often impossible to determine whether the effects seen are representative of likely consequences within real-world scenarios without considering these data in line with exposure

data. A recent review by Adam et al. (2019) assessed the likelihood of environmental risk by carrying out a meta-analysis of existing microplastic exposure and hazard data. They compared measured environmental concentrations (and therefore probability distributions of exposure) with predicted no effect concentrations (PNEC). While their analysis showed that the majority of PNECs are lower than the likely exposure, leading to little likelihood of hazard, there were a few incidences where organisms may be exposed to concentrations of microplastics above the PNEC and therefore hazard may occur (Adam et al., 2019). This applies, for example, to sensitive species in highly polluted regions. Such an assessment cannot be carried out without sufficient data on environmental concentrations and toxicity to organisms. An earlier review paper published when slightly fewer data were available did not find any likelihood of hazard when comparing exposure to toxicity (Burns and Boxall, 2018), thus highlighting the need for further research to determine where and to what extent these overlaps may occur.

This thesis aims to tackle some of the challenges in ecotoxicological microplastic research, considering that the term 'microplastics' covers a complex heterogeneous range of materials and particle types that do not exist in isolation from other environmental contaminants (Rochman, 2015; Rochman et al., 2019). Specifically, the ecotoxicological chapters of this thesis (chapters 5 and 6) address the ongoing uncertainties surrounding the interactions of microplastics with hydrophobic organic chemicals, and how these interactions may impact on different biological endpoints including mortality, chemical bioaccumulation and microbiome change. Chapter 5 also addresses the pressing need to incorporate data into models, using microplastic and associated chemical toxicology data to run a process-based survival model (Chapter 5). Using different organisms, polymers and chemicals across multiple studies provides a greater understanding of how microplastics, alone and in combination with other chemical stressors, can affect freshwater invertebrates.

8. Model freshwater organisms

In order to answer a variety of ecologically-relevant questions within this thesis, a range of organisms have been selected to study the interactions and impact of microplastics in the freshwater environment: the common roach *Rutilus rutilus*, the water flea *Daphnia magna* and the great pond snail *Lymnaea stagnalis*. These organisms are all very different in terms of morphology, life history, habitat (e.g. water column or benthic) and feeding behaviour. The species have been selected as representative of prolific freshwater families within Europe, with

a wealth of available data and/or experimental protocols available including OECD-recommended guidelines on culturing and toxicity testing (OECD, 2004, 2012, 2016). These species span different functional feeding groups and trophic levels, including lower trophic level species daphnia and pond snails, and a tertiary consumer (roach). This difference in feeding habits between species could affect their susceptibility to ingest microplastics. For example, omnivorous roach will have an additional route of microplastic exposure due to the potential for trophic transfer from both plants and invertebrates (Vasek and Kubecka, 2004), while generalist pond snails may be more likely to ingest microplastics (especially those associated with organic matter) than the more selective roach and daphnia (Elger and Lemoine, 2005; Hartmann and Kunkel, 1991; Lammens and Hoogenboezem, 1991). There are also likely intraspecific differences which will affect individual susceptibility to ingestion and possible harm, such as age, size and gender (based on possible behavioural differences). Additionally, sediment concentrations are likely higher than pelagic microplastics concentrations as microplastics sink and accumulate, leading benthic species to be more highly exposed (Leslie et al., 2017; Rodrigues et al., 2018). The type (and thus density) of polymers will also affect their availability to different organisms. For example, snails will only ingest particles that are dense enough to sink (or whose density is affected by the particle's interaction or aggregation with organic material), whereas fish and daphnia may also ingest buoyant particles that float or reside within the water column. *Daphnia magna* are the mostly widely studied species with respect to microplastic ingestion and effects (Besseling et al., 2014; Jemec et al., 2016; Ogonowski et al., 2016; Rehse et al., 2016; Rosenkranz et al., 2009), whereas no data are available for the other species.

9. Aim of the thesis

The results of field and laboratory studies can be used to help direct future research, develop our understanding of environmental and ecological processes and variation, and ultimately inform environmental policy and risk assessment. With this in mind, this thesis combines field and laboratory studies to address some of the most pressing questions in the field of microplastic research. Given the comparative lack of research on microplastics in freshwater systems, especially in the UK, this thesis therefore has the following overarching aims: to identify abundance, types and sources of microplastics in freshwater systems in the UK, and to investigate how organisms and chemicals interact with microplastics and the potential

ecological effects on a range of freshwater organisms from different functional feeding groups and trophic levels.

These aims can be fulfilled within sub-objectives:

1. To identify the gaps within the state-of-the-art on the sources, distribution, fate and behaviour of microplastics and their effects on species and ecosystems;
2. To determine the presence, abundance and types of microplastics, as well as their sources, within tributaries of the River Thames (UK);
3. To establish whether fish ingest microplastics in their natural environment, focussing on the River Thames (UK);
4. To experimentally determine whether high versus low Kow (a measure of hydrophobicity based on octanol-water partition coefficient) compounds interact differently with microplastics, potentially altering toxicological effects to *Daphnia magna*;
5. To experimentally assess whether the presence of microplastics reduces uptake of flame retardant chemicals (polybrominated diphenyl ethers, PBDEs) and alters the microbiome in the pond snail *Lymnaea stagnalis*

10. Outline of the thesis

Based on the above objectives, this thesis consists of the following chapters:

Chapter 1: Introduction to the topic and thesis aims (this chapter)

Chapter 2: A literature review to examine the state of the scientific knowledge on microplastics within freshwater and terrestrial environments, and to identify research gaps that should be addressed by subsequent chapters in this thesis.

Chapter 3: An environmental study to establish the extent of microplastic pollution within sediments of tributaries of the River Thames, to quantify and identify particles and to determine the sources of environmental particles.

Chapter 4: An environmental study to quantify microplastics from the guts of fish (*Rutilus rutilus*) within the non-tidal (freshwater) River Thames and to determine whether presence and

quantity of plastic particles can be linked to environmental factors: exposure to microplastics based on distance from the source of the river, and biological factors: size and gender of fish.

Chapter 5: A laboratory study to experimentally determine whether the presence of microplastics (1 μm polystyrene beads) affects toxicity and sublethal effects of pesticides (based on hydrophobicity and therefore binding to plastics) to *Daphnia magna* using pesticides with high and low log Kows.

Chapter 6: A laboratory study to assess how the presence or absence of microplastics (nylon fragments) may alter the accumulation of PBDEs at various concentrations within the great pond snail *Lymnaea stagnalis* and whether any effect of PBDEs (with or without microplastics) can be observed on the microbiome.

Chapter 7: A discussion to bring together the findings across all chapters of the thesis, and the scientific implications of these. This chapter includes recommendations for future research and concluding remarks.

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CHAPTER 2

Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities

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CHAPTER 2

Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities

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Abstract

Plastic debris is an environmentally persistent and complex contaminant of increasing concern. Understanding the sources, abundance and composition of microplastics present in the environment is a huge challenge due to the fact that hundreds of millions of tonnes of plastic material is manufactured for societal use annually, some of which is released to the environment. The majority of microplastics research to date has focussed on the marine environment. Although freshwater and terrestrial environments are recognised as origins and transport pathways of plastics to the oceans, there is still a comparative lack of knowledge about these environmental compartments. It is highly likely that microplastics will accumulate within continental environments, especially in areas of high anthropogenic influence such as agricultural or urban areas. This review critically evaluates the current literature on the presence, behaviour and fate of microplastics in freshwater and terrestrial environments and, where appropriate, also draws on relevant studies from other fields including nanotechnology, agriculture and waste management. Furthermore, we evaluate the relevant biological and chemical information from the substantial body of marine microplastic literature, determining the applicability and comparability of this data to freshwater and terrestrial systems. With the evidence presented, the authors have set out the current state of the knowledge, and identified the key gaps. These include the volume and composition of microplastics entering the environment, behaviour and fate of microplastics under a variety of environmental conditions and how characteristics of microplastics influence their toxicity. Given the technical challenges surrounding microplastics research, it is especially important that future studies develop standardised techniques to allow for comparability of data. The identification of these research needs will help inform the design of future studies, to determine both the extent and potential ecological impacts of microplastic pollution in freshwater and terrestrial environments.

1. Introduction

Research on microplastics as an environmental contaminant is rapidly advancing. Although marine microplastics research remains at the forefront, in recent years researchers recognising the comparative lack of studies on microplastics in freshwater environments have begun to address this field as a matter of priority, quantifying microplastics in lake and river systems and assessing exposure to, and uptake by, organisms (Dris et al., 2015b; Wagner et al., 2014). Despite the knowledge that microplastics (and indeed plastics of all sizes) are also widespread within terrestrial environments as a result of human activities, there is a dearth of studies that have quantified microplastics in terrestrial environments. In fact, much of the existing information about the environmental presence of microplastics considers terrestrial and freshwater environments only as sources and transport pathways of microplastics to the oceans. However, given that the majority of all plastics will be used and disposed of on land, both terrestrial and adjacent freshwater environments will themselves be subject to extensive pollution by plastics of all sizes, based on large amounts of anthropogenic litter from both point (e.g. wastewater treatment discharge, sewage sludge application) and diffuse (e.g. general littering) sources. As such it is highly likely that soils will act as long-term sinks for microplastic debris (Rillig, 2012; Zubris and Richards, 2005). Hence it is important to understand release rates, fate and transport of microplastics entering terrestrial systems as well as freshwater systems in order to allow for the assessment of hazards and risks posed by microplastics, and indeed plastics in general, to ecosystems.

The aim of this review is to synthesise available information relevant to understanding microplastics behaviour, fate and ecological effects within freshwater environments and soils. The review draws primarily on the published literature available from freshwater and the relatively few terrestrial microplastic studies published to date, setting out the key factors that will influence microplastic distribution, fate and exposure. One important consideration is that the processes governing distribution and exposure to plastics are not necessarily exclusive to a specific environmental ‘compartment’ (e.g. plastics within a shallow freshwater system may be exposed to similar levels of UV radiation as a particle in coastal marine systems) and plastics can be transported between compartments (e.g. from land to rivers and the sea, and from rivers and sea to land during flooding, storm events or tidal surges). Therefore it is not realistic to consider such studies in isolation from the body of marine work. Thus, where appropriate, we also include key studies from the extensive body of marine literature that will inform knowledge of the processes likely to occur in freshwaters and soils.

Microplastics as a term has quite a broad definition and can refer to a wide range of polymers, particle sizes and densities (see section 2). In this review we will predominantly focus on microplastics defined as being any polymer within the size range 1 μm to 5 mm as this is the size range which has been the major focus of reported microplastics research to date. Where information is available, we have in places included relevant information from reported studies for nanoplastics (< 100nm) as contaminants that are also likely to occur in soils and water. For the purposes of this review, microplastics and nanoplastics have been defined as per the study in which they were used/discussed and parallels drawn between the two where appropriate. However, we do not intend to carry out a complete review of nanoplastics or compare them with other nanomaterials as this topic has been previously addressed (Hüffer et al., 2017; Syberg et al., 2015). Finally, in places throughout the text, we also use the term "plastics" to refer to plastics as a whole class (macro-, micro- and nano-sized plastics). This is in order to capture the relevant influence of processes such as wind or water flow, exposure to UV, temperature fluctuations and associations with organic matter that can, alone or together, commonly affect the fate and behaviour with different sized plastic materials. The reality is that there are likely to be significant similarities between the effects and behaviours of plastics of different size classifications, for example when comparing 'large nanoplastics' to 'small microplastics'. As the size and state of plastics within the environment can change with time, we believe it is necessary to include information that extends beyond plastics in the micron size range to fully understand the drivers of microplastic and indeed all plastic transport, fate and resulting bioavailability.

Available information on plastic usage and presence on land is used in order to make informed estimations about the likely presence and effects of microplastics within terrestrial environments. This includes considering relevant data on plastic sources and transport through different environmental compartments, and therefore the organisms that may encounter and be affected by these plastics. We evaluate the available literature on ecological effects of microplastics to freshwater species (using both studies with freshwater species and any studies in comparable marine species) that can be directly related to organisms occupying the same ecological niche within aquatic and terrestrial environments. Finally, we review chemical associations and plasticiser leaching, including examples from microplastics and also large plastic products ('macroplastics') that may have implications for the toxicity of microplastics within freshwater and terrestrial environments. If we are to fully understand or predict the effects of microplastic pollution within the environment as a whole, a multidisciplinary

approach will be needed to integrate knowledge on presence and behaviour of plastic waste, particles and associated chemical pollution in the environment. Our review sets out to reflect this by drawing together knowledge from all relevant fields including waste management, nanotechnology, agriculture and toxicology. By using all available knowledge we are able to establish how previous studies can inform our knowledge of presence and effects of microplastics in terrestrial and freshwater environments and, thus, make recommendations for further research.

2. Plastic as an environmental contaminant

2.1. Plastic pollution in the environment

In 2014, annual plastic production exceeded 311 million tonnes, an increase of nearly 84 million tonnes since 2004 (PlasticsEurope, 2015; Thompson et al., 2005). By 2050 it is estimated that this may increase to a colossal 33 billion tonnes (Rochman et al., 2013a). Of anthropogenic waste materials released to the environment, plastic can constitute up to 54% by mass (Hoellein et al., 2014). Established widespread uses of plastic include packaging materials (39.5% total plastic production), building materials (20.1%), automotive components (8.6%), electronic appliances (5.7%) and agricultural materials (3.4%), with the remainder including products such as household appliances and sporting equipment (PlasticsEurope, 2015). There are approximately 30,000 different polymer materials registered for use in the European Union. A ‘polymer’ is difficult to characterise as definitions will vary between manufacturers, with much information commercially confidential. However, the European Commission report states that 84% of this 30,000 are represented by thermoplastics (Postle et al., 2012). Although they share similar characteristics, each polymer has different physical properties with respect to their plasticity and density. The density of the material in particular will be important for determining environmental fate. For example, density will influence how particles partition in the aquatic environment including whether they float on water surfaces or settle to sediment and the ease with which they will be transported by wind action across land (Zylstra, 2013). However, even when properties are known, it can be difficult to predict the fate of polymers. For example, it has been observed that supposedly buoyant particles such as polyethylene and polypropylene can be retained within sediments (Horton et al., 2017a). This could be due to biofouling or agglomeration with organic materials. These differences highlight polymers to be complex environmental pollutants.

For many plastic products their useful lifetimes are often relatively short. This is especially the case for single-use packaging materials. However, the qualities which make plastic a good material for consumer products: waterproof, durable and resistant to wear and biodegradation, can also make plastic extremely persistent (Barnes et al., 2009; Imhof et al., 2012). Many commonly-used polymers are extremely resistant to biodegradation, for example polyethylene and polystyrene (Gautam et al., 2007). Common characteristics of plastics that can impede biodegradation are high molecular weight, hydrophobicity and cross-linked chemical structure (Gautam et al., 2007; Shah et al., 2008). There is evidence that biodegradation of polymers by some organisms can occur, for example bacteria, fungi and mealworms (due to gut bacteria) (Gu, 2003; Yang et al., 2015a; Yang et al., 2015b). However, when biodegradation does occur, it is reliant on exposure of polymers to these and other specific degrading organisms that have the ability to degrade these specific polymers – conditions that may not necessarily be encountered in the environment. Indeed it has been proposed that no polymers can be efficiently biodegraded in landfill sites (Shah et al., 2008). Therefore, apart from incineration, it is understood that the vast majority of plastic ever made is still present in the environment in some form (Barnes et al., 2009; Thompson et al., 2005). It is this persistence that makes plastic pervasive as an environmental pollutant and is a main driver underpinning current concerns about the possible ecological impacts of the growing burden of plastic materials present in ecosystems. Plastic litter is present in terrestrial, freshwater, estuarine, coastal and marine environments, particularly in urbanised regions (Cole et al., 2011; Free et al., 2014; Zylstra, 2013). Plastics have been observed even in remote areas of the world including deep-sea sediments (Van Cauwenberghe et al., 2013; Woodall et al., 2014), submarine canyons (Pham et al., 2014) and encapsulated in Arctic sea ice (Obbard et al., 2014), far from any potential land-based source. It has even been observed in some locations that plastic debris can fuse together, becoming associated with volcanic rocks, sediment and organic materials forming ‘plastiglomerates’, solid rock-like substances, that have the potential to become preserved in the fossil record. As human influence begins to dominate even the most fundamental processes on earth, the potential for this evidence of human impact to last far into geological records has prompted the suggestion that we are moving into a new geological epoch from the Holocene to the ‘Anthropocene’ (Corcoran et al., 2014).

2.2. Microplastics: a brief background

Plastic debris is broadly classified by size: mega-debris (> 100 mm), macro-debris (> 20 mm), meso-debris (20-5 mm) and micro-debris (< 5 mm) (Barnes et al., 2009). Although microscale plastic particles were first observed in the marine environment in the early 1970s (Buchanan, 1971; Carpenter and Smith, 1972), it was not until 2004 that the term "microplastic" became commonly used as the result of a study by Thompson et al. (2004). Microplastics are now commonly defined as particles with the largest dimension smaller than 5 mm, although no lower size limit has been specifically defined (Arthur and Baker, 2009; Duis and Coors, 2016; Faure et al., 2012). It is understood that plastic particles in the environment will continue to degrade and become steadily smaller, eventually forming 'nanoplastics' (Koelmans et al., 2015; Mattsson et al., 2015). Microplastics in environmental samples can currently be detected down to a size of 1 μm, however few environmental studies identify particles <50 μm due to methodological limitations (Hidalgo-Ruz et al., 2012; Imhof et al., 2016).

Microplastics fall within two categories: primary and secondary. Primary microplastics are specifically manufactured in the micrometre size range, for example those used in industrial abrasives for sandblasting, either acrylic or polyester beads (von Moos et al., 2012; Zitko and Hanlon, 1991), plastic pre-production pellets ('nurdles') or in personal care products such as exfoliating agents in creams and cleansers containing polyethylene 'microbeads' (Napper et al., 2015). Primary microplastic particles are likely to be washed down industrial or domestic drainage systems and into wastewater treatment streams (Fendall and Sewell, 2009; Lechner and Ramler, 2015). Despite the capability of some sewage treatment works to remove up to 99.9% microplastic particles from wastewater (dependent on the processes employed by the treatment plant), the sheer number of particles entering the system may still allow a significant number to bypass filtration systems and be released into the freshwater environment with effluent (Carr et al., 2016; Murphy et al., 2016).

Secondary microplastics are formed as a result of meso and macroplastic litter fragmentation. Plastics are susceptible to the effects of UV radiation and high temperatures which can cause chemical changes making plastics brittle and thus more susceptible to fragmentation (Andrady, 2011; Barnes et al., 2009; Hidalgo-Ruz et al., 2012; Ivar do Sul and Costa, 2014; Rillig, 2012; Shah et al., 2008). Fragmentation increases surface area and number of particles per unit of mass. Both exposure to sunlight and wave action are primary causes of fragmentation in marine waters. On land, especially at the soil surface, fragmentation of plastics is thought to occur readily as a result of direct exposure to UV radiation from sunlight, aided also by temperature

fluctuations which will generally be greater than those in sea water (Andrady, 2011). Similarly, exposure to UV may be higher in small shallow aquatic systems such as ponds and rivers than in large lakes or the open ocean. However, many freshwater environments may lack the fragmentation potential that is offered by turbulence and wave action in coastal waters, especially in rocky tidal areas (Barnes et al., 2009). An additional source of secondary microplastics is derived from synthetic fabrics, which can shed up to 1900 fibres per garment during washing (Browne et al., 2011). Although microfibres are secondary particles they will be released to the environment along with primary microplastics through wastewater effluents and sludge application. Hence in this respect the fate and transport of these fibres may be more closely aligned with that of primary microplastics, based on similar release routes.

3. Sources, environmental presence and transport of microplastics

3.1. Sources of microplastics to freshwater and terrestrial environments

A significant direct input of primary microplastics to terrestrial environments has been identified as being through the application of sewage sludge containing synthetic fibres or sedimented microplastics from personal care or household products to land (Habib et al., 1996; Zubris and Richards, 2005). Polymers used in synthetic textiles include polyester and nylon, while polyethylene or polypropylene are commonly used as microbeads or glitter in cosmetics. As sewage treatment works are efficient in removing the majority of microplastic particles from wastewater, many of the particles that are removed will be retained within the sludge (Magnusson and Norén, 2014; Mintenig et al., 2017). This suggests that the major routes of release for secondary microfibres and primary microplastics are the same. In Europe it is common practice to compost and pasteurise sewage sludge for use as agricultural fertiliser as well as dispose of large quantities of sludge produced by wastewater treatment to land (DEFRA, 2012). Between four and five million tons dry weight of sewage sludge are applied to arable land every year in the European Union (Cieślik et al., 2015; Willén et al., 2016), although application rates are highly variable between countries (Nizzetto et al., 2016b). Despite regulations on harmful substances within sludge applied to land, microplastics are not yet considered by these and thus the mass of microplastics inadvertently applied to land annually may exceed 400,000 tonnes – higher than the mass currently estimated to be present in oceanic surface waters worldwide (Nizzetto et al., 2016b). Zubris & Richards (2005) found that soils with a known history of sewage sludge application contained significantly higher

concentrations of synthetic microfibres than soils which had not received sewage sludge. In some field sites, synthetic microfibres were found 15 years after the last sludge application (Zubris and Richards, 2005). This suggests that microplastics and synthetic fibres are likely to accumulate in soils after repeated sludge applications.

Those particles that are not retained within the sewage sludge, or removed by skimming during the treatment process, will enter the environment via effluent input to rivers. For primary microplastics and secondary microfibres, effluent from sewage treatment is thought to be a major source of microplastics to freshwater bodies. Synthetic microfibres have been identified by many studies as the most abundant microplastic particle type found throughout freshwater, terrestrial and marine environments (Browne et al., 2011; Dubaish and Liebezeit, 2013; Free et al., 2014; Zubris and Richards, 2005), with primary microbeads from personal care products also likely to be a significant contributor to microplastic pollution (Castañeda et al., 2014; Murphy et al., 2016; Napper et al., 2015). However, it must be noted that the sampling equipment and methodology will influence the size of particles observed, and therefore may determine the dominant particle type observed. For example, because fibres have at least one very small dimension, they may not always be retained on a mesh even if the length of the fibre exceeds the mesh size. This variation in sampling methodology could lead to fragments or pellets being erroneously identified as the most abundant particle type and may make comparison of particle types and abundances between studies difficult (Dris et al., 2015b; Ivleva et al., 2016).

Due to the small size of primary microplastics they are unlikely to be removed by existing screening of debris, with coarse screens retaining particles >10 mm and even the finest screens retaining particles >1.5 mm (Fendall and Sewell, 2009). An important predictor of microplastic partitioning in sewage treatment will be particle density, with dense particles settling to sludge and buoyant particles floating in effluents (Fig. 1). The extent to which this occurs will also depend on a number of relevant processes that may affect the characteristics of the microplastics. For example, the aggregation of microplastic particles, either with themselves or more likely with other (organic) particulate materials can increase size and density leading to an increase in sedimentation rate (Long et al., 2015). The growth of bacterial biofilms on microplastic surface may again increase particle weight and density, resulting in settling (Cozar et al., 2014; Kowalski et al., 2016; Moret-Ferguson et al., 2010).

Figure 1 shows a schematic diagram of waste water treatment processes and how particle partitioning is likely to occur through processing. Removal of coarse debris with physical

screens, primary settling lagoons and aerobic oxidation are common across many treatment plants, additional settling lagoons and tertiary treatments may also be present. Plastic materials will generally not be degraded at any point throughout the process and as a consequence, any plastic not removed for disposal during the initial filtering steps will remain in the solids or the effluent after processing. Many microplastics from sewage treatment works will therefore ultimately be directly released to the environment in effluents or through sludge application to land. Other methods of sludge disposal include landfilling, incineration and even in production of cement for use in construction. In these cases, plastic particles are likely to be well-contained and so unlikely to leach into the surrounding environment (Browne et al., 2011; Cieślik et al., 2015; Dubaish and Liebezeit, 2013; Rillig, 2012; Zubris and Richards, 2005).

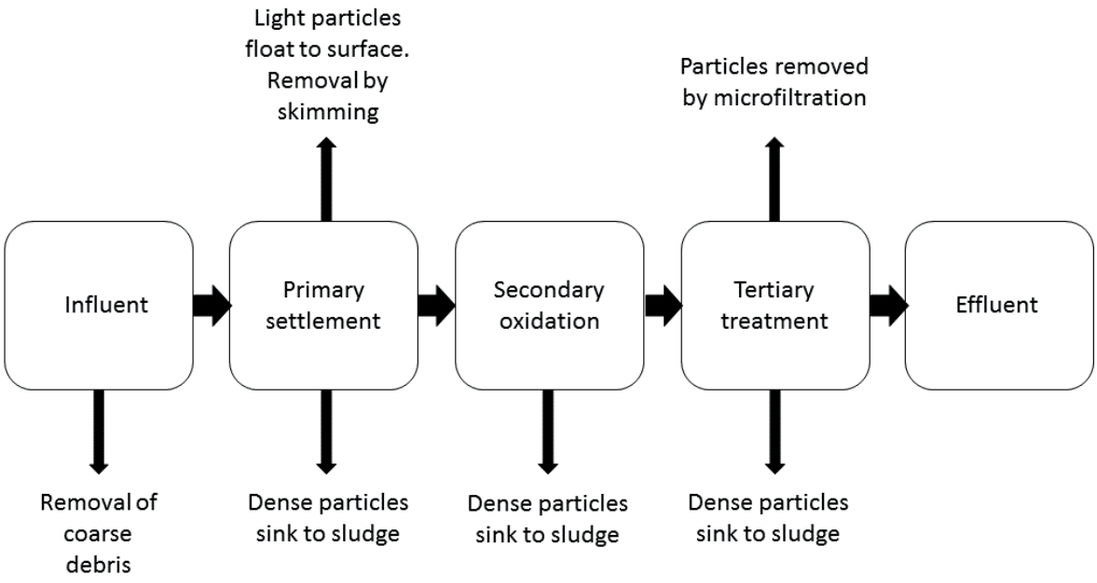


Figure 1. Schematic diagram of standard wastewater treatment processes and particle behaviour influenced by density at each stage of treatment. Adapted from Baird and Cann (2012).

A recent study observed microbeads originating from cosmetic products in wastewater treatment influents and effluents at seven wastewater reclamation plants in California, in which waste waters were treated for reuse with tertiary treatment. The treatment processes at these plants resulted in the complete removal of microparticles (45–400 μm) from water outputs, as a result of tertiary treatment including surface skimming, sludge settling and microfiltration processes (Carr et al., 2016). After secondary treatment only (elimination microfiltration), effluents contained on average one plastic particle per 1140 litres of effluent, compared to an

estimated one particle per litre in the influent (Carr et al., 2016). No fibres were found despite these being the most frequently reported kind of microplastics found in environmental samples, however as previously highlighted, this may be a result of the sampling technique used. Murphy et al. (2016) similarly found that microplastics were significantly reduced in effluent following a secondary treatment process. In this study, plastic flakes and fibres were the two most abundant microplastic types (67.3% and 18.5% respectively), with microbeads only contributing to 3% of total particles. For this mixture of materials, average microplastic concentrations reduced from 15.7 particles litre^{-1} (± 5.23) in sewage treatment influents to 0.25 particles litre^{-1} (± 0.04) in final effluents, which represents a 98% reduction in microplastic concentrations (Murphy et al., 2016). Other recent studies have reported similar high removal rates: 95% (Talvitie et al., 2017), 97% (Mintenig et al., 2017) and 99% (Magnusson and Norén, 2014). Notably, these proportions of partitioning between solid waste and effluent are similar to estimates that have been provided for nanomaterials: 90% removal of titanium (Ti) associated with titanium dioxide (TiO_2) nanoparticles (Johnson et al., 2011), 96% removal of Ti (Westerhoff et al., 2011), 94% removal of surfactant-coated silicon dioxide (SiO_2) nanoparticles (Jarvie et al., 2009). This suggests that similar processes may affect the fate of microplastics as they do poorly soluble and potentially inert nanomaterials such as gold and titanium dioxide during waste water treatment (e.g. heteroaggregation), and highlights the importance of interdisciplinary research for understanding the fates and behaviours of microplastics and nanoparticles and the parallels that can be drawn between them (Bouwmeester et al., 2015; Syberg et al., 2015). Despite the significant removal of particles from treated wastewater, given the large volumes passing through wastewater treatment plants the remaining 5%, or less, of the microplastics that are not filtered out will likely represent a large number and mass entering the freshwater environment in effluent (Murphy et al., 2016; Ziajahromi et al., 2016). It is also important to note that these results are based on efficient current-generation wastewater treatment processes that may not be widely available or utilised worldwide. In many countries, untreated sewage is input directly to watercourses without treatment (Duis and Coors, 2016; Hammer et al., 2012). Where the most modern facilities are not available, these estimates could fall short by up to 100-fold in places.

Sources of secondary microplastics derived from plastic litter are both numerous and diverse, ranging from releases during municipal solid waste collection, processing and land-filling, release from transportation and disposal systems to individuals creating litter either accidentally or intentionally (Fig. 2). This includes large plastic items and sanitary waste input

to rivers via combined sewage overflows (CSOs). Runoff via drainage ditches from agricultural land, or storm drains from roads containing plastics such as tyre wear particles, vehicle-derived debris or fragments of road-marking paints is another significant source of riverine microplastic loads (Browne et al., 2010; Eriksen et al., 2013; Galgani et al., 2015; Horton et al., 2017a; Tibbetts, 2015). Additionally, wind action may also transport lighter plastic items into water bodies or across land (Zylstra, 2013) and there is evidence to suggest that anthropogenic fibres can be transported and deposited by atmospheric fallout. This appears to be especially significant in urban areas, with deposition increasing during periods of rain (Dris et al., 2016). Although the fibres found in atmospheric studies were not exclusively synthetic (<33% fibres were pure polymers), with an estimated deposition of between 3-10 tonnes of fibres deposited annually in an area approximately 2500 km^2 (based on the Paris region), this may therefore still represent a significant pathway of microplastics from consumer products to the environment (Dris et al., 2017; Dris et al., 2016). Airborne particles are determined to originate from a variety of sources including construction materials, artificial turf and household dust (Magnusson et al., 2016).

Another direct source of secondary microplastics to land is the use and fragmentation of agricultural plastics. For example, plastic mulches and polytunnels are used to control temperature and moisture, and retard weed growth in agricultural and horticultural applications (Kasirajan and Ngouajio, 2012; Kyrikou and Briassoulis, 2007; Rillig, 2012; Steinmetz et al., 2016). Polymer seed coatings can also be used to control germination (Clayton et al., 2004). These may consist of various polymers and often contain incorporated pesticides and fertilisers. Commonly used polymers for seed coatings are non-biodegradable and therefore following germination, will remain in the soil (Schultz et al., 2014; Turnblad and Chen, 1998). Additional products used in agriculture include bale twines and wraps, containers, packaging and netting, all of which have the potential for dispersal within the environment (Scarascia-Mugnozza et al., 2012). Exposure of these materials to sunlight and high temperatures may lead to their relatively rapid fragmentation after which they are difficult to completely remove from soils. Dense polymers are more likely to remain in soil and ultimately to be transported into deeper soil layers, whereas lighter polymers will be more likely to be transported by wind and water action either to other terrestrial locations or to surface waters. To our knowledge, to date there are no studies which quantify microplastic presence at terrestrial field sites. Based on the above evidence, however, it is highly likely that microplastics will be present within terrestrial

environments and, if investigated in detail, may be found to be as equally pervasive as they are in freshwater and marine environments (Nizzetto et al., 2016a).

3.2. Presence of microplastics in the freshwater environment

Studies of microplastics in freshwater environments are rapidly advancing, with microplastic particles found across a range of freshwater environments worldwide, including lakes and rivers. Area of water surface, depth, wind, currents and density of particles are all factors determining transport and fate of particles within these aquatic systems (Eriksen et al., 2014; Eriksen et al., 2013; Fischer et al., 2016; Free et al., 2014). Given the lack of terrestrial studies to date, it is necessary to use our knowledge of microplastics in the freshwater environment, notably sediments, to infer the presence and behaviour of microplastics in soils and to inform future sampling efforts.

A study carried out on lake beaches by Imhof et al (2013) measured microplastics found in sediments of two beaches on the north and south shores of Lake Garda (Italy). Particle numbers between these sites were significantly different, with these differences attributed to the prevailing southerly wind direction transporting plastics either directly or by surface water movement to the opposite shore (Imhof et al., 2013). The number of local sources, together with factors including water surface area, depth, wind, currents and density of particles are all factors determining transport and fate of particles within these aquatic systems and can lead to large variation, even within a relatively small area (Castañeda et al., 2014; Eriksen et al., 2014; Eriksen et al., 2013; Fischer et al., 2016; Free et al., 2014). Another significant factor influencing particle presence and abundance is urbanisation of the area surrounding and influencing the waterbody. Eriksen et al. (2013) conducted a study in the Great Lakes (USA) and found that downstream of highly populated Detroit and Cleveland metropolitan areas, particle concentrations ranged from 280,947-466,305 particles km^{-2}. In Lake Huron, where the shorelines are less influenced by the presence of major urban centres, particle concentrations estimated from sampling were generally orders of magnitude lower, ranging from 456-6541 particles km^{-2}, with one trawl finding no particles (Eriksen et al., 2013). A similar study of the remote lake Hovsgol (Mongolia) also found microplastics present in all samples at concentrations comparable to those found in the Great Lakes (Table 1). Although the area surrounding Lake Hovsgol has a low population density, poor local waste management and inputs of wastewater are blamed for the presence of microplastic particles in the lake (Free et

al., 2014). Additionally, the smaller volume of Lake Hovsgol, compared to the Great Lakes of the USA, may be an important reason for microplastic concentrations being comparable between these two studies.

Urbanisation has also been observed to be a significant factor influencing presence of microplastics in riverine environments, with plastics being introduced from a variety of sources including effluent, road runoff, littering and atmospheric deposition (discussed further in Section 3.1). Mani et al. (2015) and Yonkos et al. (2014) are among those who have found microplastics in higher abundances at sites in close proximity to urban areas than at more remote sites. However, although particle numbers are regularly found to be high near urban areas, this is not the only factor influencing presence of microplastic particles. For example, Horton et al. (2017a), in addition to finding high numbers of particles downstream of urban discharge points, also found particles in rural areas where few human-associated inputs would be expected.

Given the growing need to make comparative assessments in order to identify regional, national and global trends in microplastic distribution, it would be desirable to be able to collate the available data to conduct meta-analyses. However, a major challenge to this is that no standard protocol for collecting particles from environmental samples exists, with different authors using different approaches. While many studies use broadly similar techniques to extract microplastics from environmental samples, including size fractionation, digestion of organic matter and density separation, the specific parameters of methods differ between studies regarding volume of sample studied, upper and lower particle size limits, density separation media and particle identification criteria (Besley et al., 2016; Hidalgo-Ruz et al., 2012). Given that many methods currently rely on visual identification, there are also many opportunities for the introduction of sampling error, bias or omission of particles of certain size or density, leading many results to be qualitative rather than quantitative (Ivleva et al., 2016). Although many studies have established ‘standard methods’ for particle extraction in an effort to introduce consistency across studies, these methods are in fact quite disparate. Moreover, studies are still identifying new and reportedly more effective criteria. Thus no standardised methods have yet been agreed (Rochman et al., 2017; Stock et al., 2019). An additional issue is the use of non-standard units of measurement for reporting microplastic concentrations. In order to compare studies where units are not consistent, units must be transformed to units per volume, either as particles per litre of sampled water or as particles per kilogram of sediment (see Table 1). It is therefore of utmost importance that authors detail results in all units, or

provide sufficient detail on the sampling methodology to do so (Phuong et al., 2016; Van Cauwenberghe et al., 2015). These differences between studies highlight the need for continued efforts to standardise methods for microplastic extraction and quantification, as has been recognised in environmental nanomaterial research (Delay et al., 2010).

Table 1. Summary of selected freshwater microplastic environmental sampling studies, covering a range of freshwater environments (water, plus benthic and shore sediments of lakes and rivers). Selected studies were those which quantified specifically microplastics and provided sufficient methodological detail to allow for conversion of units, to standardise by volume or mass for comparability. Converted units for water and sediment were calculated by multiplying area sampled by sampling depth to estimate total volume, then converting this volume into litres or kg (dry weight). For sediment this calculation is based on typical dry sediment bulk density of 1.3 g cm^{-3} (Sekellick et al., 2013) Conversion was not required where the study already reports results as particles L^{-1} or kg^{-1}. For details of additional freshwater studies, refer to (Dris et al., 2015b).

Water body type	Sample type	Sample location and description	Study findings (reported units)	Study findings (converted units)	Study
Lake	Water	Great Lakes (USA) 16 cm sampling depth	Average particle concentration 43,000 km^{-2}	Average 0.00027 particles L^{-1}	Eriksen et al. (2013)
Lake	Water	Lake Hovsgol (Mongolia), sampling depth 16 cm	Average particle concentration 20,264 km^{-2}	Average 0.00012 particles L^{-1}	Free et al. (2014)
Lake	Benthic sediment	Lake Ontario (Canada) sampling depth 8 cm	26 particles in 42.2 g (station 403) 9 particles in 103.2 g (station 208)	616.1 particles kg^{-1} (station 403) 87 particles kg^{-1} (station 208)	Corcoran et al. (2015)
Lake	Shore sediment	Lake Garda (Italy), sampling depth 5 cm	Average particle abundance 1108 and 108 m^{-2} (north and south shores respectively)	Average 17 particles kg^{-1} (north) 1.7 particles kg^{-1} (south)	Imhof et al. (2013)
Lake	Shore sediment	Lake Garda (Italy), sampling depth 5 cm	Average particle abundance 75 m^{-2}	Average 1.2 particles kg^{-1}	Imhof et al. (2016)

Table 1 (continued)

Water body type	Sample type	Sample location and description	Study findings (reported units)	Study findings (converted units)	Study
Lake	Water and shore sediment	Lake Chiusi (Italy)	Average particle abundance 234 kg^{-1} sediment, 3.02 m^{-3} surface water	Average 0.03 particles L^{-1} surface water	Fischer et al. (2016)
		Lake Bolsena (Italy)	Average particle abundance 112 kg^{-1} sediment, 2.51 m^{-3} surface water	Average 0.025 particles L^{-1} surface water	
Lake	Water and benthic sediment	Taihu Lake (China)	Particle abundance range: 3.4 – 25.8 L^{-1} surface water 11 – 234.6 kg^{-1} benthic sediment	-	Su et al. (2016)
Lake	Benthic and shore sediments	Lake Ontario (Canada)	Average particle abundance 980 kg^{-1} lake benthic 140 kg^{-1} lake beach	-	Ballent et al. (2016)
River	Water	Great Lakes tributaries (USA)	Particle abundance range: 0.05 – 32 m^{-3}	0.00005 – 0.032 particles L^{-1}	Baldwin et al. (2016)
River	Water	River Seine, urban area (Paris, France)	Average particle abundance 30 m^{-3} (plankton trawl)	Average 0.03 particles L^{-1}	Dris et al. (2015a)
			Average particle abundance 0.35 m^{-3} (manta trawl)	Average 0.00035 particles L^{-1}	
River	Water	Various rivers (Switzerland)	Average particle abundance 7 m^{-3}	Average particles 0.007 L^{-1}	Faure et al. (2015)
River	Water	River Danube (Austria)	Average particle abundance 0.32 m^{-3}	Average 0.00032 particles L^{-1}	Lechner et al. (2014)
River	Water	River Rhine (various), sampling depth 18 cm	Average particle abundance 892,777 km^{-2}	Average particles 0.005 L^{-1}	Mani et al. (2015)

Table 1 (continued)

Water body type	Sample type	Sample location and description	Study findings (reported units)	Study findings (converted units)	Study
River	Water	Nine different rivers, Chicago area (USA)	Average particle abundance 2.4 m^{-3}, upstream sewage treatment works (STW) Average particle abundance 5.7 m^{-3}, downstream STW	Average particles 0.002 L^{-1} Average particles 0.006 L^{-1}	McCormick et al. (2014)
River	Water	Rivers: Papatsco Corsica Rhode Magothy Sampling depth 15 cm	Average particle abundance 155,374 km^{-2} 40,852 km^{-2} 67,469 km^{-2} 112,590 km^{-2}	Average particles 0.001 L^{-1} 0.00027 L^{-1} 0.00045 L^{-1} 0.00075 L^{-1}	Yonkos et al. (2014)
River	Shore sediment	Rivers Rhine and Main (Germany)	Particle abundance range: 228 - 3763 kg^{-1}	-	Klein et al. (2015)
River	Benthic sediment	Lake Ontario tributaries (Canada)	Average particle abundance 610 kg^{-1}	-	Ballent et al. (2016)
River	Benthic sediment	St Lawrence river sediments, sampling depth 10-15 cm (Canada).	Average particle abundance 13,759 m^{-2}	Average approx. 70.6-105.8 particles kg^{-1} (depending on depth sampled)	Castañeda et al. (2014)
River	Benthic sediment	River Thames Basin (UK), sampling depth approx. 10cm	Average particle abundance range: 185 kg^{-1} to 660 kg^{-1} depending on site.	-	Horton et al. (2017a)
River	Benthic sediment	Beijiang River (China)	Particle abundance range: 178 - 554 particles kg^{-1}	-	Wang et al. (2016)

The numbers of particles reported in marine and freshwater surface waters are extremely variable. Concentrations of microplastics in marine surface waters have been reported from 0.0005 particles L^{-1} (Carson et al., 2013) (calculated as per Table 1) to 16 particles L^{-1} (Song et al., 2014) with a range of intermediate concentrations reported (Lusher et al., 2014; Zhao et al., 2014). Studies of freshwater surface samples generally show concentrations comparable to the lower end of the reported marine surface concentrations such as those seen by Carson et al. (2013) (see Table 1). Dris et al. (2015a) highlight the consequence of using different mesh sizes when determining the number of particles observed. When sampling with a plankton net (80 µm mesh), up to 100-fold more particles can be collected compared to use of a manta net (330 µm mesh). This effect of mesh size is an important consideration when comparing surface water studies, as differences in sampling method and equipment may lead to inconsistencies that prohibit the comparability of datasets (Cole et al., 2011). However, despite this variation, it remains possible that freshwater concentrations comparable to the higher marine concentrations will be found, likely within urban areas.

Studies in river sediments consistently report abundances of microplastics in the tens to hundreds of particles kg^{-1} (Table 1), values that are broadly comparable to those reported in marine sediment studies. For example, Dekiff et al. (2014) and Nor and Obbard (2014) reported marine microplastic concentrations in the range from individual particles to tens of particles per kilogram of dry sediment, consistent with a study of the sediments of the St Lawrence River (Castañeda et al., 2014). Hundreds of particles per kilogram of dry sediment were reported by Horton et al. (2017a) in UK river sediments, values also reflected by Laglbauer et al. (2014) in coastal sediments in Slovenia. At the highest concentrations, thousands of particles kg^{-1} of dry sediment have been reported in river sediments in Germany (Klein et al., 2015), comparable to the 2000-8000 particles kg^{-1} reported by Mathalon and Hill (2014) in coastal sediments in Canada.

Efforts in colloid science and nanotoxicology have shown the value of working towards standard methods for key measurements of colloid and nanomaterial characteristics, such as size, stability and surface properties (Hassellov et al., 2008; Montes-Burgos et al., 2009). Similar efforts seem warranted in the microplastic community with respect to environmental sampling and qualification. Currently in the field of microplastics research, there are two widely accepted methods of polymer identification – Fourier transform infra-red (FTIR) spectroscopy and Raman spectroscopy, although both have drawbacks. Alternative identification methods such as differential scanning calorimetery (DSC) and thermo-

gravimetric analysis (TGA) have been tested but not been widely applied (Dumichen et al., 2015). Of the sampling configurations available for FTIR, there are two that are most common: attenuated total reflectance (ATR) and or transmission (or absorbance). ATR is not effective for analysing very small particles due to the fact that the sample needs to be large enough to cover an 'ATR window' in order for a satisfactory spectrum to be obtained (typically > 1 mm). Additionally, while in transmission mode refractive or scattering artefacts can occur, most notably for particles with irregular surfaces (Harrison et al., 2012). Raman spectroscopy can be overridden by fluorescence from some polymer particles, while other interferences may occur if particles are dirty or contain larger amounts of filler, such as dyes or plasticisers (Löder and Gerdts 2015). These limitations reduce the possibility of determining probable sources, fate and potential short and long-term environmental impacts of these microplastics as well as advising policy makers on how to regulate microplastic pollutants. It could be that in order to effectively identify environmental polymers, a combined and complementary approach is required, for example using both spectroscopy and thermal analysis (Gigault et al., 2016; Majewsky et al., 2016; Sgier et al., 2016). It will be important to use the experience of working with microplastics in aquatic environments, especially sediments, to inform methods for terrestrial studies.

3.3. Transport of microplastics within the environment

Estimating the quantity of plastic litter which is released to the environment is difficult due to a lack of data and international variations between plastic waste generation and disposal. These disparities arise as a result of international differences in societal attitudes, education and investment in waste management infrastructure. For example, in China in 2010, 76% of plastic waste (8.82 million metric tonnes) was considered to be mismanaged, compared with 2% (0.28 million metric tonnes) in the United States (Jambeck et al., 2015). Mismanaged waste accounts for plastic released to land by littering and wind-blown debris. The best available estimates for managed and mismanaged plastic waste worldwide are from Jambeck et al. (2015), who modelled how much plastic waste was emitted globally to the oceans from land-based sources during 2010. Our estimates presented in Table 2 focus on Europe and assume that the proportion of waste that is mismanaged in the European Union (EU) is equivalent to that of the United States (2%). This is a reasonable assumption based on similarities in national income and development of waste management infrastructure, evidenced by the application of EU wide policies governing waste management, such as the 1999 EC landfill directive (1999/31/EC)

(European Council, 1999). Based on this assumption we estimate how much of this mismanaged waste, plus the additional source of microplastics from sewage sludge application, is likely to remain on land annually within Europe (Table 2).

Table 2. Waste management data and estimates of plastic waste released to terrestrial and freshwater (continental) environments, based on figures for the European Union. Rows highlighted in grey are those directly related to plastic within continental environments. ¤Values for specific waste management practises do not account for mismanaged waste. *Managed and mismanaged waste figures are calculated based on the proportion of waste categorised as managed or mismanaged in the United States: 2% (Jambeck et al., 2015). ¥Values are calculated based on mismanaged waste to include plastics within sewage sludge, minus plastic that is transported to the oceans. Some sources, such as atmospheric fallout have not been considered due to the limited data available. [1]PlasticsEurope (2015) [2]Jambeck et al. (2015) [3]Nizzetto et al. (2016b)

Plastic handling/disposal	Plastic million metric tonnes/year
Plastic production (EU total, 2014)[1]	59
Plastic waste (EU total, 2014)[1]	25.8
Managed plastic waste (-2% mismanaged waste)*	25.28
Landfill (EU total)[1¤]	8
Recycling (EU total)[1¤]	7.6
Energy recovery (EU total)[1¤]	10.2
Mismanaged plastic waste (2% of plastic waste in the EU)*	0.52
Plastic in sewage sludge (EU total)[3]	0.063 - 0.43
Ocean input (EU total)[2]	0.04 - 0.11
Total mismanaged plastic waste remaining in continental environments (EU) ¥	0.47 - 0.91

Plastic materials used in consumer, domestic and agricultural products in Europe amounted to 59 million metric tonnes in 2014 (PlasticsEurope, 2015). Mismanaged plastic waste within the EU is calculated at 520,000 metric tonnes (plastic waste – managed waste).In addition to this, it is estimated that between 63,000 and 430,000 metric tonnes of microplastics in sewage sludge are deposited on land annually (Nizzetto et al., 2016b). As a result, we calculate that in

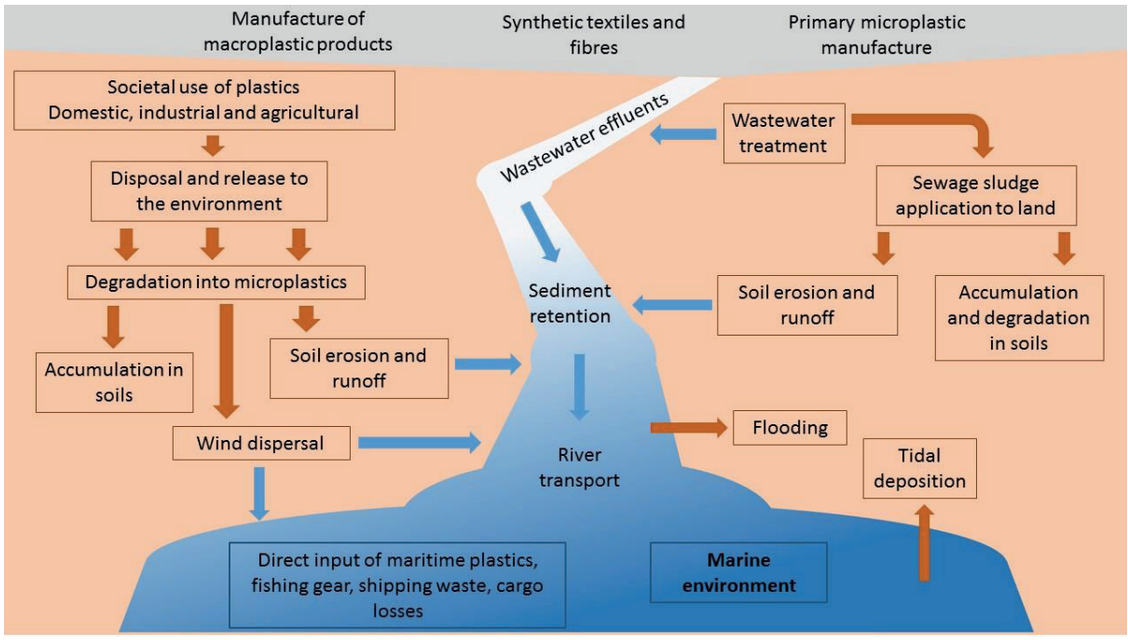
the EU between 473,000 and 910,000 metric tonnes of plastic waste is released and retained annually within continental environments, between 4 and 23 times the amount estimated to be released to oceans (Table 2). With the current lack of data on microplastics in soils, it is not possible to distinguish between particles that are retained within terrestrial environments and those retained within freshwater systems. As plastic production and thus environmental deposition increases, this will also result in greater accumulation, and larger amounts being ultimately transferred to the marine environment. However, for a considerable time into the future it remains likely that the amount of plastic deposited and retained within continental environments will exceed that entering the oceans. It is important to note that the study by Jambeck et al. (2015) considers all waste within the US to be well-managed, with the exception of litter (2% of all waste). However, it is possible that some fraction of the waste that is considered to be well-managed could enter the environment during waste processing (e.g. as wind-blown debris or mechanical or human error). Therefore it remains plausible that the figures for mismanaged waste may be higher than the stated value. When it is also considered that there may be additional pathways of release that are poorly known, such as atmospheric deposition, then it may be the case that the calculations presented here may be an underestimation of plastic releases.

Freshwater and soil systems are subject to both point and diffuse inputs of plastics and so great research effort is warranted to understand transport, exposure and ecological effects of microplastics in these systems. This knowledge will also inform our understanding of rivers and freshwater bodies as transport pathways for plastics from land to oceans (Jambeck et al., 2015; Lechner et al., 2014; Rillig, 2012). It has been estimated that between 70-80% of marine plastics are transported to the sea through the conduits provided by rivers (Bowmer and Kershaw, 2010). Recognising this need, freshwater environments have received more attention than terrestrial environments thus far as they are seen as a direct link between land-based plastic waste and the open oceans, as well as interest in the toxicological impact of microplastics on freshwater ecosystems (see Table 1). Studies of microplastics in soil ecosystems are, however, notably lacking (Lwanga et al., 2016; Zubris and Richards, 2005).

Figure 2 shows a conceptual diagram of the main flows of microplastics within and between three environmental compartments: terrestrial, freshwater and marine. A key concept of the diagram is partitioning of plastic particles between aquatic and terrestrial environments, highlighting that plastic debris will not only be transported by rivers from land to sea, but that even once in the aquatic environment, may also return to land during high tide or flooding

events (Fig. 2). The extent of overall deposition, retention and transport of microplastics will depend on many factors including human behaviours, such as littering or recycling, particle characteristics such as density, shape and size, weather, including wind, rainfall and flooding, and environmental topography and hydrology. This variation can make predicting the spread of litter difficult (Zylstra, 2013). Transport of plastic particles within river systems will be largely affected by the same factors affecting sediment transport, such as hydrological characteristics and environmental conditions (Nizzetto et al., 2016a). Conditions such as low flows and change in river depth or velocity (for example, on a bend) may lead to deposition of particulate matter, whereas high velocity flood conditions and erosion could lead to mobilisation of previously sedimented particles, in addition to the introduction of particles via runoff (Milliman et al., 1985; Naden et al., 2016; Walling, 2009). Surrounding land-use can also affect the dynamics of sediment and particulate transport within a river due to erosion, use of soils, irrigation and runoff (Chakrapani, 2005). Plastic residing in river systems may also be subject to in-situ degradation, either by photodegradation or mechanical fragmentation (Williams and Simmons, 1999).

Figure 2. Conceptual diagram of microplastic sources and flows throughout and between anthropogenic, terrestrial, freshwater and marine environmental compartments.



To date only scant attention has been paid to investigating sources, fate and transport of microplastics in terrestrial environments. However, it not unreasonable to suggest that microplastics are widely present across land. Litter has been widely reported as a common observation, with many studies commenting on land based (macro)plastic debris (Derraik, 2002; Hoellein et al., 2014; Jambeck et al., 2015; Townsend and Barker, 2014; Williams and Simmons, 1999; Zylstra, 2013).

4. Microplastics as an environmental hazard

4.1. Ecological impacts of microplastics

4.1.1. Organism interaction and ingestion of microplastics

Based on the evidence of widespread presence of plastics, it is highly likely that organisms in terrestrial and freshwater ecosystems will encounter microplastic particles. Depending on the particle size and the physiological and behavioural traits of the organism, there is an opportunity for the ingestion of these particles by invertebrates and vertebrates. Indeed such consumption has been widely observed in many marine species. Although plastic is largely excreted following ingestion, there is evidence to suggest that microplastics can be retained in the gut over timescales beyond those expected for other ingested matter (Browne et al., 2008). Further, there is evidence that particles may even cross the gut wall and be translocated to other body tissues, with unknown consequences (Browne et al., 2008; Farrell and Nelson, 2013; von Moos et al., 2012). Given the similarity of some phyla that are commonly found in freshwater and marine ecosystems (e.g. nematodes, annelids, molluscs, arthropods) and indeed in soils, similar findings of ingestion in species in these ecosystems are almost inevitable. Since many of these species, likely to take up microplastics, are important to ecosystems (Lavelle, 1997; Sampedro et al., 2006) ecosystem processes such as decomposition and nutrient cycling may be affected by microplastic exposure. Further, there is the potential for food web effects either through effects on keystone species or possibly through the trophic transfer of microplastics themselves.

Research to date, predominantly on marine species, has shown the ingestion of microplastic particles in a wide range of species at many organisational levels and with different feeding strategies, including detritivores, filter feeders and predators. In addition to accumulation of particles in organisms at lower trophic levels (Browne et al., 2008), there is also evidence for the trophic transfer of microplastic particles between marine species, especially bivalves and

crustaceans (Farrell and Nelson, 2013; Van Cauwenberghe and Janssen, 2014; Watts et al., 2014). This is also likely to occur in terrestrial ecosystems in a similar manner to that of observed trophic transfer and accumulation of gold nanoparticles between earthworms *Eisenia fetida* and bullfrogs *Rana catesbeina* (Unrine et al., 2012). Gold nanoparticles are comparable to (nano)plastic particles in that are they are similarly poorly soluble (Bouwmeester et al., 2015). There is also evidence that exposure to inert anthropogenic particles can cause physical damage to body tissues (Lahive et al., 2014; Van Der Ploeg et al., 2013).

As far as we are aware, to date only three terrestrial species, the earthworms *Lumbricus terrestris* (Lwanga et al., 2016) and *Eisenia andrei* (Rodriguez-Seijo et al., 2017) and the nematode *Caenorhabditis elegans* (Kiyama et al., 2012), have been studied in the literature exposed to microplastic particles under laboratory conditions and with ingestion being observed. Among freshwater organisms, the filter feeder *Daphnia magna* has been observed to ingest microplastics (Besseling et al., 2014; Casado et al., 2013; Rehse et al., 2016). Synthetic fibres have also been observed in the digestive systems of freshwater fish collected from the wild, indicating consumption either directly or in association with consumed prey items (Sanchez et al., 2014). Through such consumption, mobile organisms such as fish, mammals and birds may also contribute to the dispersal of microplastics over long distances following the ingestion and subsequent egestion of consumed microplastics (Eerkes-Medrano et al., 2015). A major factor that is known to influence particle ingestion by organisms is particle to mouth size ratio, with smaller particles having greater potential to be ingested by a greater range of organisms. If ingested by lower tropic level organisms, this may support further transfer and accumulation along food chains (Cole et al., 2013; Farrell and Nelson, 2013; Setälä et al., 2014).

4.1.2. Observed toxicological effects of microplastics

Ingestion of microplastic particles by marine invertebrates has been linked with a wide range of sub-lethal effects including reduced reproduction, reduced growth of individuals and reduced fitness. These are generally the result of the physical effects of ingested microplastics including internal damage such as lacerations, inflammatory responses and plastic particles replacing digestible food, causing individuals to reduce feeding hence resulting in lower energy intake, although effects vary between species and plastic types (Moore, 2008; von Moos et al., 2012; Wright et al., 2013a; Wright et al., 2013b). While there are fewer studies conducted to date with soil and freshwater species, the studies that have been conducted generally confirm

the potential for microplastics to have detrimental effects on the physiology of species across many ecological niches.

In a recent soil study, Lwanga et al. (2016) observed mortality in *Lumbricus terrestris* earthworms exposed to polyethylene particles; mortality was increased by 8% at a concentration of 450 g kg^{-1} polyethylene (in overlying leaf litter) and 25% mortality at 600 g kg^{-1}. Reduced growth and negative effects on burrow construction were also observed. As the concentrations of plastic litter micro-fragments found on soil surfaces are currently unknown, it is difficult to place the concentrations that are used in this study within the range of possible microplastic concentrations that may occurs in soils. The exposure concentrations would certainly seem high compared to expected microplastic levels resulting from diffuse pollution. However, it remains possible that they may be consistent with exposure around some point sources, especially following *in situ* degradation. This finding that annelid worms can be affected by microplastics is consistent with a number of studies conducted for marine species. For example, in a study of *Arenicola marina* exposed to uPVC (unplasticised PVC) particles experienced weight loss and reduced lipid reserves were observed. A uPVC treatment of 10 g kg^{-1} dry sediment reduced energy reserves by 30% while at a uPVC concentration of 50 g kg^{-1} dry sediment, energy reserves were reduced by 50%. This effect overall suggests that exposure to UPVC causes metabolic stress to marine benthic sediment worms (Wright et al., 2013a). Due to the close relatedness of worm species in terms of morphology and how they feed in sediment it is likely that similar effects would be observed in freshwater and terrestrial worm species (Rillig, 2012). In the marine copepod, *Tigriopus japonicas*, Lee et al. (2013) found that although acute exposure (96 hours) to three different particle sizes (0.05, 0.5 and 6 µm) of polystyrene microbeads, had no impact on the survival rate of adults, in a two generation chronic exposure experiment mortality was observed at concentrations above 12.5 µg ml^{-1}, with the second generation observed to be much more sensitive than the first generation, especially when exposed to the nano-scale particles (0.05 µm). Larger particles in contrast (6 µm) had no effect on survival even over two generations, although fecundity was affected at concentrations above 25 µg ml^{-1}. Although the species of copepod used in this study were marine, they are directly comparable to freshwater copepod species and other planktonic filter feeding organisms like *Daphnia* sp. This implies that toxic effects of microplastics may be size-dependent either as a result of particle ability to permeate body tissues or to cause greater inflammatory response. Studies conducted with nanoplastics also highlight possible size dependent influences on toxicity for both acute survival effects (Besseling et al., 2014; Nasser

and Lynch, 2016) and different reproductive effects observed in response to smaller particle fractions (Lee et al., 2013).

It is also important to consider how alteration of particle characteristics over different environmental timescales may affect toxicity. Exposure to artificially aged (nano)polystyrene has been found to cause mortality, growth and reproduction effects to the standard test species *Daphnia magna* over a 21-day period, whereas pristine nano-polystyrene particles caused no significant effects on mortality. Mixtures of nano-polystyrene and fish kairomones (known to cause stress in *D. magna*) produced an additive effect on body size and reproductive endpoints, indicating that exposure to plastic particles can exacerbate existing environmental stress responses (Besseling et al., 2014). Many studies investigating the toxicological impacts of microplastics have used virgin plastic particles. However, if aged and contaminated, particles can have the potential for greater chemical transfer than virgin particles (see section 4.2.2.). This use of pristine particles could thus lead to a potential underestimation of the toxicological impacts of microplastic exposure under more realistic environmental exposure scenarios. Recently the nanotoxicology research community have recognised the need to conduct experiments with environmentally 'aged' nanomaterial forms (Judy et al., 2015; Lahive et al., 2017). Common nanomaterial transformations, such as hetero- and homo-aggregation, changes in surface charge and in particular the development of a surface 'corona' of associated macromolecules and chemicals may all occur for both nanoparticles and microplastics (Syberg et al., 2015). Hence future studies with these 'aged' particle forms may be needed to more accurately identify the possible effects of anthropogenic materials in real environments (Schultz et al., 2015).

When considering microplastics and chemical co-transport, principles used in mixture toxicology may be useful to assess these multifaceted stresses in the environment. Given that most environmental microplastic studies quantify microplastics by number of particles rather than by weight (as is more common for bioassays), and none to our knowledge have yet detected nanoplastics in environmental samples, it is not yet possible to determine whether the concentrations used in these studies are environmentally relevant. This is a similarly common criticism of microplastic studies in that the concentrations of particles used are likely not environmentally realistic. Even though the relationship between environmental concentrations and those used in toxicity bioassays is not fully established, it is likely that the concentrations used in laboratory tests are comparable to only the highest levels of environmental contamination. However, it is still valuable to understand the potential ecological implications

of microplastic pollution at these high concentrations as a contribution to understanding of hazard and developing risk assessments. Further, given that environmental concentrations of microplastics are likely to increase with input and fragmentation of plastics already present in the environment, the future presence of higher concentrations can be expected (Phuong et al., 2016).

4.2. Microplastics as a chemical hazard

4.2.1. Leaching of plasticiser chemicals in freshwater and terrestrial environments

Plastic materials often contain a wide range of plasticiser chemicals to give them specific physical properties such as elasticity, rigidity, UV stability, flame retardants and colourings (Browne et al., 2013; Lithner et al., 2009; Moore, 2008; Teuten et al., 2009). Many of the chemicals associated with plastics have been identified as either toxic or endocrine disruptors including bisphenol-A, phthalates such as di-n-butyl phthalate and di-(2-ethylhexyl) phthalate, polybrominated diphenyl ethers (PBDEs) and metals used as colourings (Hua et al., 2005; Kim et al., 2006; Lithner et al., 2009; Oehlmann et al., 2009; Rochman et al., 2013c; Teuten et al., 2009). Additive chemicals like these are weakly bound, or not bound at all to the polymer molecule and as such these chemicals will leach out of the plastic over time. Such releases can be facilitated in environments where particle dispersal is limited and where plastics will experience UV degradation and high temperatures (Andrady, 2011). The locations where microplastics may accumulate in soil and surface waters are therefore likely to be subject to the possible release of these chemicals from plastics and their subsequent transfer to water, sediment and organisms. Lithner et al. (2009) showed that different plastic items can leach toxic chemicals into water that can cause varying effects on *Daphnia magna*. Different items made of the same polymer may have varying toxicity effects following leaching, based on the type and amount of plasticisers added during manufacture. This demonstrates that plastic materials can act as a source of complex leachate mixtures to the environment.

As a major environmental sink for all types of plastic waste, landfill material and the leachates arising from landfill sites are highly likely to contain high concentrations of plasticiser chemicals (do Nascimento Filho et al., 2003; Slack et al., 2005; Yamamoto et al., 2001). Within a landfill site chemical conditions change over time with regards to temperature fluctuation, oxygen presence, acid/alkaline conditions and dissolved organic carbon all of which have the potential to change plasticiser leaching (Teuten et al., 2009; Xu et al., 2011). Large scale

chemical monitoring studies have identified the presence of phthalate esters (plasticiser chemicals) in a wide range of agricultural and peri-urban soils in various regions of China. Zeng et al. (2008) analysed soil samples from a range of field sites around Guangzhou city, China. The study identified 16 phthalate compounds with concentrations for individual phthalate found ranged from 0.195–33.5 mg kg^{-1} dry weight soil. The highest concentration of phthalates were found in an agricultural soil, in close proximity to a water course into which wastewater was discharged from nearby industrial activities including manufacture and disposal of plastics and this was identified as the key source of phthalates in soil. Similarly, Kong et al. (2012) analysed soil samples from farmland finding concentrations of phthalates ranging from 0.05–10.4 mg kg^{-1} dry weight. The highest concentrations were found in vegetable plots close to domestic rubbish sites, from which phthalates could be expected to leach. High concentrations were found at sites close to busy roads and at wasteland sites where plastic debris abundance was high. Further to these studies, Wang et al. (2013) sampled soils used for vegetable production near Nanjing (east China). Measured concentrations of phthalates ranged between 0.15–9.68 mg kg^{-1} dry weight; the highest concentrations were found at sites where plastic mulches and polytunnels were in use. Proximity to municipal solid waste sites and application of sewage sludge were also identified as major sources of phthalates, indicating leaching of plasticiser chemicals from plastic particles deposited on land. Taken together, the results suggest that plastic materials release chemicals to soil via a number of the pathways and are a potential source of plasticisers to soils. This may have significant implications for terrestrial locations where microplastic concentrations are high, although further studies are needed to confirm this early evidence.

4.2.2. Microplastic associations with organic pollutants

Microplastics themselves are widely understood to bind to a range of different hydrophobic organic chemicals (HOCs) within the environment, such as organochlorine pesticides, PAHs, PCBs, PBDEs, dioxins and metals (Besseling et al., 2013; Mato et al., 2001; Rochman et al., 2013d). This may be especially significant in continental freshwater and terrestrial environments, where concentrations of these chemicals are expected to be higher than in marine systems, due to proximity to the use of these chemicals (Dris et al., 2015b). HOCs are recognised as having high lipophilicity (i.e. high octanol/water partition coefficient, Kow), determining whether a chemical will dissolve in water and remain in solution). Chemicals with such a high Kow will typically have a strong affinity for adsorption to organic and particulate

matter within water, soil and sediment. These same characteristics, in addition to factors including hydrophobicity of polymer, large or abraded surface properties and biofouling, mean that HOCs also have the potential for sorption to plastic materials (Karapanagioti and Klontza, 2008; Teuten et al., 2007). Microplastics and representative chemicals from many POP classes may become associated in waste streams (e.g. sewage effluent and sludge, landfill waste and leachate) or in anthropogenically influenced environments. Hence, the interactions between microplastics and organic pollutants are particularly pertinent in freshwaters inland, especially those in close proximity to industrialised and populated areas with a high discharge of industrial and domestic wastewater, where small dispersal areas can lead to high pollutant concentrations (Eerkes-Medrano et al., 2015; Free et al., 2014). This will be especially relevant in agricultural areas where plastic products are used in close proximity or in association with the application of hydrophobic chemicals such as some pesticides.

Changes to environmental conditions will influence equilibrium dynamics between chemicals and plastics, impacting on chemical accumulation and bioavailability (Bakir et al., 2016; Bakir et al., 2014; Karapanagioti and Klontza, 2008; Koelmans et al., 2016). Additionally, particle size and texture will affect the capacity of microplastics to either adsorb or leach contaminants and indeed plasticiser additives. The greater surface area per unit of mass as particles decrease in size increases the potential for surface chemical interactions and thus binding with hydrophobic chemicals. Physically weathered particles are expected to have a larger surface area as a result of cracking and abrasion which increases overall surface area (Ivar do Sul and Costa, 2014; Teuten et al., 2009). Such environmentally-induced changes may be particularly relevant for terrestrial microplastics, which may be exposed to high levels of UV radiation and wind. The ecological impacts of plastic-chemical associations are difficult to predict due to the many interactions between polymers, plastic additives, adsorbent characteristics and environmental conditions which will impact on bioavailability (Bakir et al., 2014; Koelmans et al., 2016; Velzeboer et al., 2014).

5. Future research recommendations

As this review highlights, the largest gaps in current knowledge are in our understanding of microplastic pollution in terrestrial ecosystems, especially environmental concentrations, sources and ecological impacts. In freshwater systems, knowledge of concentrations of microplastics is rapidly growing. However, in most instances this knowledge has yet to be

related to ecological effects. Due to the lack of quantitative data, it is difficult to assess quantitatively the exact nature of the microplastic hazard in these systems and how the consequences of microplastic presence in these ecosystems will manifest themselves. Indeed this is true of microplastics research as a whole, where the long term implications of microplastics are still unclear compared to better-studied chemical pollutants.

There is a large degree of uncertainty around the volume, composition and diversity of microplastic particles entering the environment. Information on the scale of production is available as is some data on plastic entry into major waste management systems, however current release rates from these streams either by deliberate or accidental release of refuse or wind action is not quantified. This route from accidental release and littering is, hence, one of the greatest uncertainties for emission predictions. This review highlights the complex challenge of understanding the dynamics and impacts of microplastics as an environmental pollutant, especially understanding microplastics in a freshwater and terrestrial context, but also demonstrates how information from marine studies can be used to infer or predict what may occur in these less studied systems. In a similar way, nanomaterial research can also provide insights into particulate behaviour and fate.

To progress the field of research, it is of utmost importance in the first place to define 'microplastics' clearly as an environmental contaminant, and thereafter to develop standardised methods for collecting, processing and analysing environmental samples. Such standardisation has the potential to reduce ambiguity and thus allow direct comparison between studies with a view to understanding sources and transport pathways. Spectroscopy methods have already been used to identify freshwater and terrestrial nanoparticles and the continued development of such methods, as well as alternatives such as differential scanning calorimetery (DSC) and thermo-gravimetric analysis (TGA), is important to provide additional information on the polymers present in terrestrial and freshwater ecosystems.

While an ideal scenario would be to reduce the amount of plastic entering the environment, the challenges of reduction from changes in manufacturer and consumer behaviour mean that releases can be expected to continue for some time. Given the volume of plastic currently present in the environment, and the likely increase of microplastics due to fragmentation, it therefore remains important to understand the potential effects of this ever-accumulating pollution (Nizzetto et al., 2016a; Phuong et al., 2016).

Based on the evidence presented in this review, it is clear that our understanding of microplastics in the environment is rapidly developing. However, there are still fundamental gaps in the knowledge and many questions still remain. In summary, the most important questions remaining are:

1) What is the current extent of microplastic pollution in terrestrial environments, and how does this compare to known contamination in aquatic environments? Which polymers are most abundant and does this vary across habitats and regions?
2) To what extent do environmental conditions and properties of different plastic materials affect microplastic behaviour and bioavailability under the conditions that are found in freshwater and terrestrial environments?
3) Are adverse effects primarily due to physical impacts of the particle itself, chemical toxicity or mixture effects, and does this vary between polymers and species? Are there parallels that can be drawn with what is known concerning mechanisms of action for some nanoparticles?
4) What are the likely ecological implications of plastics under realistic exposure conditions (i.e. microplastics of the type and concentrations likely to be encountered by organisms)?

6. Conclusions

The available literature reporting information on plastic use and release suggests that primary and certainly secondary microplastics are likely to be found ubiquitously across terrestrial and freshwater environmental compartments due to their proximity to most point and diffuse sources. Both primary and secondary microplastics entering the environment will persist and continue to fragment to smaller particles. These smaller fragments are likely to pose a greater risk to organism health due to their increased likelihood of uptake, increased surface area for interactions with chemicals and greater number of particles per unit of bulk mass (Jeong et al., 2016; Lee et al., 2013). The focus on nanoparticle hazards has recently generated a greater understanding of the behaviour of particulate pollutants, as well as methods for their detection and hazard assessment. Clear parallels exist from this work to future studies with nanoparticles, with collaboration between the disciplines likely to improve understanding (Bouwmeester et al., 2015; Syberg et al., 2015). This takes the more environmentally relevant approach that it is necessary to understand the fate, behaviour and impacts of microplastics as an environmental

pollutant and, therefore, their potential implications for keys ecosystem components and processes.

As microplastics can act as both a direct (particulate) hazard and an indirect (chemical) hazard, unravelling ecological effects may call for the application of approaches for mixture toxicity may be beneficial for the analysis of combined plastic-chemical effects. Despite land being the least studied environmental compartment, many of the ecological risks of microplastics identified in aquatic species will also apply to terrestrial ecosystems due to the many ecological and taxonomic parallels that exist between resident species. Studies on the dynamic interactions between plastic particles, plasticiser additives and environmental contaminants is also a field that needs to be expanded to understand how organic chemical partition coefficients to plastics are altered in the presence of sediment and soil. Studies of chemical dynamics within the gut of organisms are also needed in order to better understand the processes that govern bioaccumulation of plasticisers and co-transported chemicals. Ultimately, studies are needed to link the finding in the field studies to laboratory results to better understand both environmentally relevant scenarios of real-world risks posed by microplastics and the underlying mechanisms.

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CHAPTER 3

Large microplastic particles in sediments of tributaries of the River Thames, UK – abundance, sources and methods for effective quantification

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CHAPTER 3

Large microplastic particles in sediments of tributaries of the River Thames, UK – abundance, sources and methods for effective quantification

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Abstract

Sewage effluent input and population were chosen as predictors of microplastic presence in sediments at four sites in the River Thames basin (UK). Large microplastic particles (1 mm – 4 mm) were extracted using a stepwise approach to include visual extraction, flotation and identification using Raman spectroscopy. Microplastics were found at all four sites. One site had significantly higher numbers of microplastics than other sites, average 66 particles 100 g^{-1}, 91% of which were fragments. This site was downstream of a storm drain outfall receiving urban runoff; many of the fragments at this site were determined to be derived of thermoplastic road-surface marking paints. At the remaining three sites, fibres were the dominant particle type. The most common polymers identified included polypropylene, polyester and polyarylsulphone. This study describes two major new findings: presence of microplastic particles in a UK freshwater system and identification of road marking paints as a source of microplastics.

1. Introduction

Since the 1960s plastics have become widely manufactured and used, with global production of plastics reaching 311 million tonnes in 2014, 59 million tonnes of which were produced in Europe (PlasticsEurope, 2015). However, only 17.9 million tonnes were recycled or used in energy recovery processes in Europe in 2014 (PlasticsEurope, 2015). Their inherent durability and longevity which make plastics such a favourable commercial material are also the characteristics that allow them to persist in the environment (Barnes et al., 2009). Degradation of large plastic items can be a very slow process therefore plastics may persist in the environment over long timescales (Andrady, 2011; Hidalgo-Ruz et al., 2012), even in the range of hundreds of years (Barnes et al., 2009). However, despite the wide-ranging use and disposal of plastic products and the recognised abundance of plastic litter worldwide, the importance of understanding the fate and impacts of these plastics within the environment has only recently started to be addressed.

Microplastics, plastic particles <5mm in size, are a specific concern given their small scale and potential for widespread environmental dispersal. The first reports of synthetic fibres and pellets as marine environmental contaminants emerged in the early 1970s (Buchanan, 1971; Carpenter and Smith, 1972), however direct research into this field was not pursued until the last decade (Thompson et al., 2004). Since 2004, many studies have investigated the presence and effects of marine microplastic debris (Arthur and Baker, 2011; Faure et al., 2012; Law et al., 2014; Lusher et al., 2015; Van Cauwenberghe and Janssen, 2014). The majority of plastic debris found in the marine environment (70-80%) has land-based sources and rivers are considered an important medium for transfer of this debris (Arthur and Baker, 2011; Bowmer and Kershaw, 2010; Hirai et al., 2011; Jambeck et al., 2015; Sadri and Thompson, 2014; Wagner et al., 2014; Zbyszewski and Corcoran, 2011; Zbyszewski et al., 2014). Comparatively few studies have actually been published on microplastics in freshwater or terrestrial environments, although this field of research is growing with a number of papers recently published on microplastics in freshwater systems (Corcoran et al., 2015; Klein et al., 2015; Lechner et al., 2014; Sanchez et al., 2014; Zbyszewski and Corcoran, 2011; Zbyszewski et al., 2014), with the greatest proportion of microplastic debris in freshwater environments being observed near to industrialised areas (Dubaish and Liebezeit, 2013; Eriksen et al., 2013; Sadri and Thompson, 2014; Zbyszewski and Corcoran, 2011).

Microplastics fall into 2 categories: primary and secondary. Primary microplastics are those which were manufactured with the intention of them being of a micro scale, for example those

used in cosmetics or exfoliating scrubs (such as glitter and 'microbeads') or virgin pellets used in the plastic production industry. Secondary microplastics are those that have formed as a result of macroplastic degradation, for example breakdown of *in situ* litter (Andrady, 2011; Barnes et al., 2009; Rillig, 2012; Shah et al., 2008) or the washing of artificial fabrics in the laundry, which can lead to the loss of up to 1900 fibres into wastewater per wash (Browne et al., 2011). Within these categories, microplastics are categorised into 2 size brackets: 'large microplastic particles' (LMPP, 1 mm-5 mm) and 'small microplastic particles' (SMPP, < 1 mm). Over time, LMPPs may become SMPPs or even nanoplastics, due to degradation within the environment (Andrady, 2011; Koelmans et al., 2015; Lambert and Wagner, 2016).

Sources of microplastic particles to the environment are numerous and varied. Sewage treatment works (STWs) are a critical link in the microplastic transport and distribution web given that many plastic particles including microbeads and synthetic fibres will enter these STWs. If not physically filtered out within the plant itself then they will be discharged to rivers via effluent or incorporated into sludge (Habib et al., 1996; Zubris and Richards, 2005). Sludge may in turn be applied to agricultural land (DEFRA, 2012), leading to direct terrestrial implications, in addition to potential for runoff into watercourses. STW outfalls discharge directly into rivers representing a point source discharge of particles to freshwater environments. Thus, sewage outfalls have been recognised as a likely significant source of microplastic pollution to the oceans (Arthur and Baker, 2011; Browne et al., 2011). Additional sources include degradation of macroplastic debris such as sanitary waste from sewage treatment overflows, plastic packaging, particle runoff from roads in the form of tyre wear particles or parts of vehicles and runoff from land containing degraded litter (Andrady, 2011; Eriksen et al., 2013; Galgani et al., 2015; Hidalgo-Ruz et al., 2012). Another source was recently recognised in the form of polymer composite paints. Due to the low polymer composition of paints, these are likely to be more brittle than pure polymers and therefore break down quickly into smaller particles in the environment (Imhof et al., 2016; Song et al., 2014; Takahashi et al., 2012).

The aim of this study was to investigate the presence, abundance and types of microplastics within tributaries of the River Thames basin (UK). This study investigated the link between two expected and related drivers of microplastic input, sewage effluent input and population density, with the presence of microplastics in river sediments. The River Thames catchment in the UK was selected as the location for our survey as it is the UK's second longest river and the river basin supports many large urban areas, receiving effluent from a population of over

13 million (Bengtson Nash et al., 2006; National Statistics, 2002). Although likely acting as a source of microplastics to the marine environment, the Thames also has the capability to act as a sink for some plastic particles due to flow dynamics: in the Thames estuary (and other estuaries), water near the riverbed has a tendency to flow landward, meaning that some of the debris entering the river may be retained within estuarine sediments (Board, 1973). Sediment was our selected medium for analysis given that microplastics can accumulate in sediments at an order of magnitude higher than in the water column (Hoellein et al., 2016). This indicates the potential for rivers to act as a sink for environmental microplastics. Studies of macroplastic in the Thames have shown there to be an abundance of litter being transported down the Thames (Morritt et al., 2014). To our knowledge, however, with the exception of estuaries this is the first study investigating microplastics in the Thames catchment or indeed any freshwater system in the UK.

2. Materials and methods

2.1. Sampling site selection and sample collection

Sampling sites within the Thames river basin were selected based on two variables; average % effluent present in the river as estimated using the Low Flows 2000 (LF2000) WQX (Water Quality eXtension) model (Williams et al., 2009) and population equivalent density as calculated using population within the catchment area (of known area) served by the upstream sewage treatment works (Pottinger et al., 2013; Williams et al., 2009). Selected sites comprised three tributaries of the Thames: the River Leach, the River Lambourn and The Cut (two sites). These rivers are regularly monitored for a range of water quality and biological characteristics as part of the ongoing Thames Initiative project and are therefore well characterised (Bowes et al., 2014). Four sampling sites were selected to represent scenarios ranging from low sewage input and population equivalent density, Leach (SU228996) and Lambourn (SU429721) through an intermediate site, The Cut site 1 (SU859704, upstream of an effluent outfall) to a site with high sewage input and population equivalent density, The Cut site 2 (SU855732, downstream of an effluent outfall) (Figures 1 and 2). Samples were collected between 28th August and 3rd September 2014 to correspond with seasonal low flow conditions. At each site four sediment samples were collected at 1 m intervals along a 3 m transect running parallel to the bank at 1 m distance, therefore giving four replicate samples per site. The sediment surface

was sampled in all cases to approximately 10 cm depth using a stainless steel scoop, collected to fill a 1L glass Kilner jar, ensuring that minimal excess water was retained.

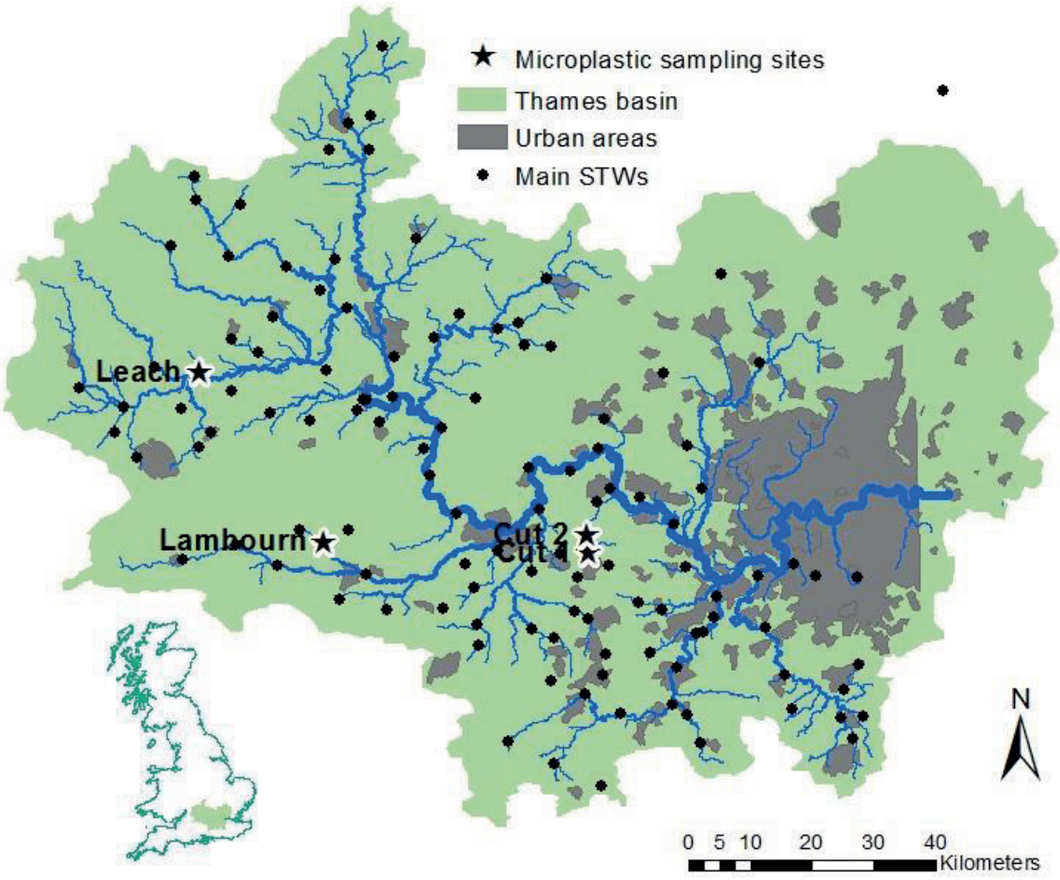

Fig. 1. Map to show locations of sampling sites within the Thames basin and the UK.

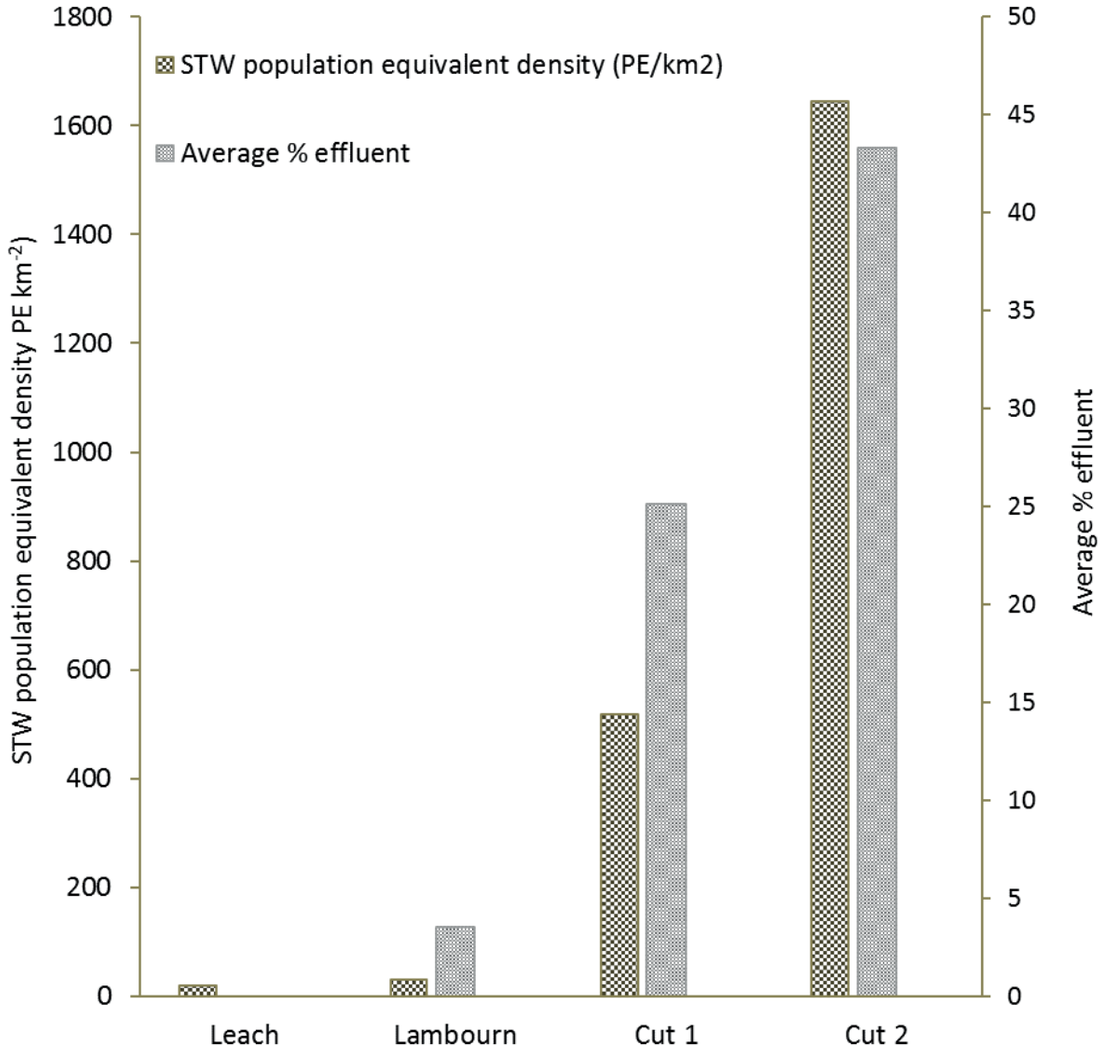

Fig. 2. Site characteristics including average percentage effluent in the river at the sampling sites and population equivalent density of upstream sewage treatment works.

2.2. Sample processing

The sediments were processed in three steps in order to find and separate microplastic particles: 1) visual inspection of whole sample, 2) flotation and 3) further visual inspection of unfloated material. This three-step process was designed to remove microplastic particles with maximum thoroughness and efficiency, without the need for custom-made equipment (Claessens et al., 2013; Imhof et al., 2012), based on the assumption that each step would not in itself be sufficient to recover all microplastics. To determine whether any of the three steps could be eliminated from future analyses to further streamline the process, the effectiveness of each step

for microplastic removal was compared, based on percentage removal of total microplastic particles. As methodological limitations prevent accurate determination of small microplastic particles <1 mm, before undertaking the steps to extract microplastics particles the 1 L sediment samples were each wet-sieved to retain two size fractions, 1-2mm and 2-4mm. These sizes were selected for analysis as indicators of the types and likely sources of microplastics present in this environment while remaining visible and easily quantifiable. Two fractions were specified in order to differentiate between abundances of microplastics of different sizes. Both size fractions from each site were carefully rinsed into individual clean containers and oven-dried at 80°C. This temperature is below the melting point of all common polymers and wouldn't be expected to alter the inherent particle shape considered for the analysis (Kalpakjian and Schmid, 2008). Once dry, samples were weighed and total dry weight calculated, then covered to prevent airborne contamination and stored for sorting and analysis.

2.2.2. Extraction step 1: Visual inspection of sieved sediments

The first sorting step was a visual inspection of the entire sample using a binocular light microscope at 6x magnification (Wild Heerbrugg, Switzerland, with Photonic PL2000 cold light source), in order to determine to what extent this step could remove all microplastics and potentially eliminate the necessity for flotation in future analyses. For each sample, all sediment from the 2-4mm fraction was inspected for 15 mins and the 1-2mm fraction for 25 mins (subsample of 40 g where the total 1-2 mm size fraction exceeded this). These time frames were found to be sufficient based on the time taken to manually skim through sediment of this size and remove visible microplastic particles from surrounding organic and inorganic matter. In order to be selected, all particles sorted from sediment were required to conform to the following criteria as outlined by Nor and Obbard (2014): no visible cellular or organic structures, particles/fibres are not segmented and if fibres, were equally thick throughout their entire length and should not be tapered at the end. Two additional criteria were specified by Nor and Obbard, however these were considered unsuitable as they would have led to dismissal of likely plastics (homogenously coloured and not shiny) (Nor and Obbard, 2014). Furthermore, based on initial observations these criteria alone were deemed insufficient for identifying all potential microplastic particles and eliminating non-plastics, as many particles in the sample appeared to be anthropogenic in origin while not conforming to the above conditions. In order to avoid missing/misidentifying particles we therefore employed additional

measures whereby particles were only identified as microplastics if they also met at least two of the following criteria: 1) unnaturally coloured compared to the majority of other particles in the sample (e.g. bright blue, yellow etc.) and appear to be a homogenous material or texture, 2) unnaturally brightly coloured coating on another particle, 3) unnatural shape e.g. perfectly spherical, 4) fibre that remained intact with a firm tug/poke with tweezers, 5) shiny/glassy, 6) flexible/can be compressed without being brittle. All particles identified as microplastics according to the above criteria were removed and stored for subsequent analysis using Raman spectroscopy.

Particles were identified and quantified as fragments (angular and solid, likely derived of larger items broken down), fibres (likely derived of synthetic textiles) or films (flexible and very thin, likely derived of large packing materials).

2.2.3. Extraction steps 2 and 3: Flotation and visual inspection of sediments post-flotation

Following the initial visual sorting, the remaining material from each sample was transferred to 250ml glass beakers, each filled to approximately 75 ml volume, using a sufficient number of beakers to accommodate the whole sample to allow for separation by flotation. A concentrated $ZnCl_2$ solution (Bonnymans, UK) was prepared to a concentration of 1.7-1.8 kg L^{-1}, for use in the flotation. This solution is denser than the plastic particles with the highest expected density in the sediments and should therefore float all plastic particles (e.g. PVC density is $\leq$ 1.58 g cm^{-3} (Nuelle et al., 2014), lower than the density of the $ZnCl_2$ solution, > 1.7 g cm^{-3}). The concentrated $ZnCl_2$ solution was poured on top of the sediment in the beaker leaving an approximately 1cm gap to the brim of the beaker. The beaker was then covered with Parafilm® to make a watertight seal, and shaken vigorously for 30 seconds. After settling for 2 hours, the beaker was placed into a larger vessel and the Parafilm® removed and any attached particles rinsed back into the beaker. Additional $ZnCl_2$ solution was gently poured into the beaker allowing the floating particles to overflow into the larger vessel. The outside of the smaller beaker was then rinsed into the overflow container to remove any adhered particles. These shaking and overflow steps were then repeated twice more to maximise the retrieval of the buoyant particles (Claessens et al., 2013). The remaining sediment was stored for further visual inspection. The overflow liquid was vacuum filtered through 1.2 µm Whatman GF/C glass microfibre filter papers (GE Healthcare Life Sciences, UK) to collect floated particles and the filter was then flushed thoroughly with deionised water to remove all traces of $ZnCl_2$. Given that particles were already size-sieved and > 1 mm, the pore size of these filters allowed

for the retention of particles > 1 mm. The filtered particles were then oven-dried on the filter paper at 60°C before analysis. These filtered particles were initially inspected using the binocular light microscope varying between 6-40x magnification (Wild Heerbrugg, Switzerland, with Photonic PL2000 cold light source) to distinguish plastic from non-plastic using the selection criteria outlined above.

The third and final step of the process was to visually inspect the material that remained sedimented following the flotation step. This step was included as a precaution to investigate whether dense particles such as polymer-based composites had not been originally observed or floated in the density separation step. The remaining unfloated sediments were rinsed with deionised water and vacuum filtered through 1.2 µm Whatman GF/C glass microfibre filter papers (GE Healthcare Life Sciences, UK) to remove $ZnCl_2$ residues and visually inspected for 25 mins per sample. Microplastic particles were identified and removed according to the same criteria as before. This final step, allowed the effectiveness of the previous two steps to be assessed for microplastics recovery from sediments.

In order to account for potential handling and airborne contamination three control samples were also run by passing approximately 400-500 ml of the $ZnCl_2$ solution through the vacuum filter (an equivalent volume to that filtered per field sample) onto 1.2 µm Whatman GF/C glass microfibre filter papers and analysing under the binocular light microscope for contamination.

2.3. Sample analysis: Raman spectroscopy

Given the large number of particles extracted overall, 20% of particles were subsampled for chemical characterisation using Raman spectroscopy (HR800UV, Jobin Yvon Horiba, France, with integrated Olympus BX41 microscope). To prevent bias in particle selection, all the particles from each sample were tipped onto a 40 mm by 40 mm grid and a random number generator used to determine the x and y coordinates from which to take each particle (20% total from each sample).

Spectra were acquired at 50x magnification using a near infra-red laser (785 nm) to limit fluorescence and the filter adjusted accordingly with each particle based on colour (to prevent burning or melting of dark coloured particles). Acquisition time was 30 seconds, accumulation 2, grating 600 with the range set to 600-3200 cm^{-1} to ensure the entire fingerprint region was accounted for. Spectra were analysed using BioRad KnowItAll® Informatics System - Raman

ID Expert (2015) software using single and multiple component and functional group analyses to compare spectra to a database of known compounds. This software carries out optimised corrections for spectral matching including interdependent corrections of the baseline, intensity distortion and axis shift with further manual correction possible for noise and baseline correction. The software matches each sample spectrum to several potential reference spectra. Sample spectra were compared to matched reference spectra and the most appropriate match was selected based on matching peak wavenumber positions.

2.4 Data analysis

Particle numbers across all the sites were first checked for normal variance structure using a Kolmogorov-Smirnov test. Where non-normal variance structure was found data were log transformed and normality confirmed prior to further analysis. Post-normalisation, analysis of particle numbers, types and sizes across all four sites were carried out using two way analysis of variance (ANOVAs) using site, size fraction and the interaction term as fixed factors. For comparing particle numbers between sites, one way analysis of variance (ANOVA) was used. Where significant differences were found across sites or particle fraction size, a post-hoc Tukey test was used to identify significant differences between conditions.

3. Results

3.1. Sorting method

The three control filters analysed to assess contamination during processing, contained an average of two fibres per filter paper. These may arise from aerial deposition and from clothing. Compared to the number of fibres found across all field samples (578 total, with even the least polluted site, the Leach, containing 69 fibres), this contamination was deemed to be negligible.

In order to determine the effectiveness of the different sorting methods the proportion of particles recovered in each step were compared. The most effective method of particle removal was flotation, which extracted between 51% (The Cut site 1) and 82% (Lambourn) of the total particles removed combining all three steps. In comparison, number of particles removed in the initial timed search by eye was between 16% (The Cut site 1 and Lambourn) and 37% (The Cut site 2) of the total particles. However following steps one (timed search by eye) and two

(flotation), 97% of the total number of particles extracted were found for three out of four sites (excluding The Cut site 1). The final step which was a search of sediment post-flotation found less than 3% of the total particles recovered for these three sites. However, for the most polluted site (The Cut site 1) even after these two steps of the combined method, 34% particles (of total removed overall) remained in the sediment (determined by the third step of a search through sediment post-flotation). Overall an average 75% of the total recovered particles were extracted by initial sorting and flotation, this being 98% Cut site 1 was excluded.

3.2. Particle presence, abundance and size

Microplastic particles were found at all of the sampling sites. There were clear and significant differences in both the number and types found between the four sites (both ANOVA, p <0.001). However the mass of sediment in the 1-4mm size range varied between sites with total dry weights of sediment in the 1-4 mm size fractions being significantly lower in the Leach, Lambourn and Cut 2 samples than those from The Cut site 1 (ANOVA, $p < 0.01$, Tukey, $p <$ 0.05). This was due to variation in sediment composition and grain size. For example, total dry weights of sediment between 1-4 mm from the total 1 L sample from the Leach (average 128 g) were less than the Cut site 1 (429 g) due a greater proportion of sediment particles <1 mm at the Leach (table 1). To standardise between sites for comparability, particle numbers were therefore expressed as a number of microplastic particles per 100 g dry weight of sediment in the 1-4mm size range for both microplastic size fractions and all particle types, and all statistical analysis carried out on these corrected data. Total and corrected numbers are reported in table 1, with significant differences found between sites for both number and types of microplastics following correction for sediment weight (ANOVA, p <0.001, Tukey, p <0.05).

Site was a highly significant factor determining the total number of microplastics particles per 100 g sediment (ANOVA, p <0.005). The highest number of particles was recovered from The Cut site 1, the second most sewage-impacted site, with an average of 66 ± 7.7 particles per 100 g across the four replicates (table 1) found following the three-step extraction method. The high number of particles recovered here was, however, not significantly different from the most sewage effluent impacted site (The Cut site 2) (average 33.2 ± 16.1 particles per 100 g, Tukey, p >0.05). The lowest numbers of microplastic particles were found at the Leach and Lambourn sites, which had the lowest sewage effluent input and population equivalent density (average 18.5 ± 4.2 particles and 22.1 ± 9.5 particles respectively). Comparisons indicated that total

counts from both these sites were significantly lower than the more polluted Cut 1 site (Tukey, $p < 0.05$), but not significantly different from each other (Tukey, $p > 0.05$).

There was a significant difference between the two microplastic particle size fractions found across all sites (ANOVA, $p < 0.005$), with the number of particles in the 1-2 mm fraction consistently being higher on average than in the 2-4 mm fraction (table 1). This difference was consistent across all the sites (ANOVA, $p = 0.142$).

3.3. Type of particles

There was a significant difference between the types of particles found across sites (ANOVA $p < 0.001$). This was due to the significantly lower numbers of films which comprised only 3.3% of particles (average 2.2 particles per 100 g) compared to the other two particle forms (Tukey, $p < 0.05$, table 1). The difference between fragments and fibres was not significant (average 17.2 particles per 100 g, 49.3% overall and average 16.5 particles per 100 g, 47.4% overall respectively, Tukey, $p > 0.05$).

Site significantly influenced the types of particles found (ANOVA $p < 0.001$). The Cut site 1 was significantly different from all other sites in that the dominant type of particles (Tukey, $p < 0.05$) found at this location were fragments, comprising 80.8% of particles (corrected for sediment weight, Fig. 3). Fibres were the most abundant particle type at all other sites, although there were no significant differences between the numbers of fibres found between sites (Tukey, $p > 0.05$, table 1). Films were the least abundant particle type and showed no significant differences in abundance between sites (Tukey, $p > 0.05$, table 1).

Analysis of fragments from The Cut site 1 identified a specific particle form that was not observed at any of the other sites. These unique fragments were red and yellow and were often found to incorporate glass beads (which themselves were also found independently). As non-plastics these glass beads were not included in overall site microplastic counts, however their presence was observed in all samples from this site. Shards of glass were also easily identifiable due to their brittle nature and were not counted.

Table 1. The average (± St Dev) sediment weight (g in 1-4 mm size range from 1 L samples, *n*=4), total number of microplastic particles for four 1 L sediment samples and average (± St Dev) number of microplastic particles per sample (*n*=4) reported both as total particles extracted per 1 L sediment and weight-corrected to particles per 100 g sediment (1-4 mm fraction) from sediment samples from four sites in the Thames basin, namely two rural sites Leach and Lambourn (with low population equivalent densities and low sewage input) and the urban sites Cut 1 and Cut 2 (with high population equivalent density and high sewage input). Average (± St Dev) numbers of microplastic particles per site (*n*=4) are reported for both totals and within the categories of particle size distribution split into two size ranges (1-2mm & 2-4mm) and three particle types ("fragments", "fibres" and "films"); averages that do not share a common letter are significantly different (Tukey $p < 0.05$).

					Size distribution (particles per 100g)		**Particle types (per 100g)**		
Site	**Average dry weight of sediment 1-4 mm (g) from 1 L total sample**	**Total actual number of particles 1-4 mm at site (total of 4 replicates)**	**Average actual number of particles (1-4 mm)**	**Average number of particles per 100g (1-4 mm)**	**Average number of particles (1-2 mm) (A)**	**Average number of particles (2-4 mm) (B)**	**Average number of fragments (A)**	**Average number of fibres (A)**	**Average number of films (B)**
Leach	128.1 ± 50.8 (B)	88	22 ± 5.2 (A)	18.5 ± 4.2 (A)	10.2 ± 3.1	8.3 ± 1.9	3.2 ± 0.6 (CDE)	14.7 ± 3.8 (BCD)	0.6 ± 1 (E)
Lambourn	191.1 ± 62.7 (B)	149	37.3 ± 5.8 (A)	22.1 ± 9.5 (A)	14.1 ± 5.4	8.1 ± 5.3	3.1 ± 3 (CDE)	16.9 ± 5.7 (BC)	1.7 ± 1.6 (DE)
The Cut site 1	459 ± 148.4 (A)	1190	297.5 ± 85.5 (B)	66 ± 7.7 (B)	41.9 ± 3.4	24.1 ± 5	53.3 ± 7.8 (A)	12.1 ± 4.5 (BCDE)	0.6 ± 0.3 (E)
The Cut site 2	225.8 ± 88.6 (B)	252	63 ± 25.4 (A)	33.2 ± 16.1 (AB)	20.5 ± 12	12.7 ± 4.6	9.1 ± 9.6 (BCDE)	22.3 ± 7.1 (B)	1.7 ± 1.3 (DE)

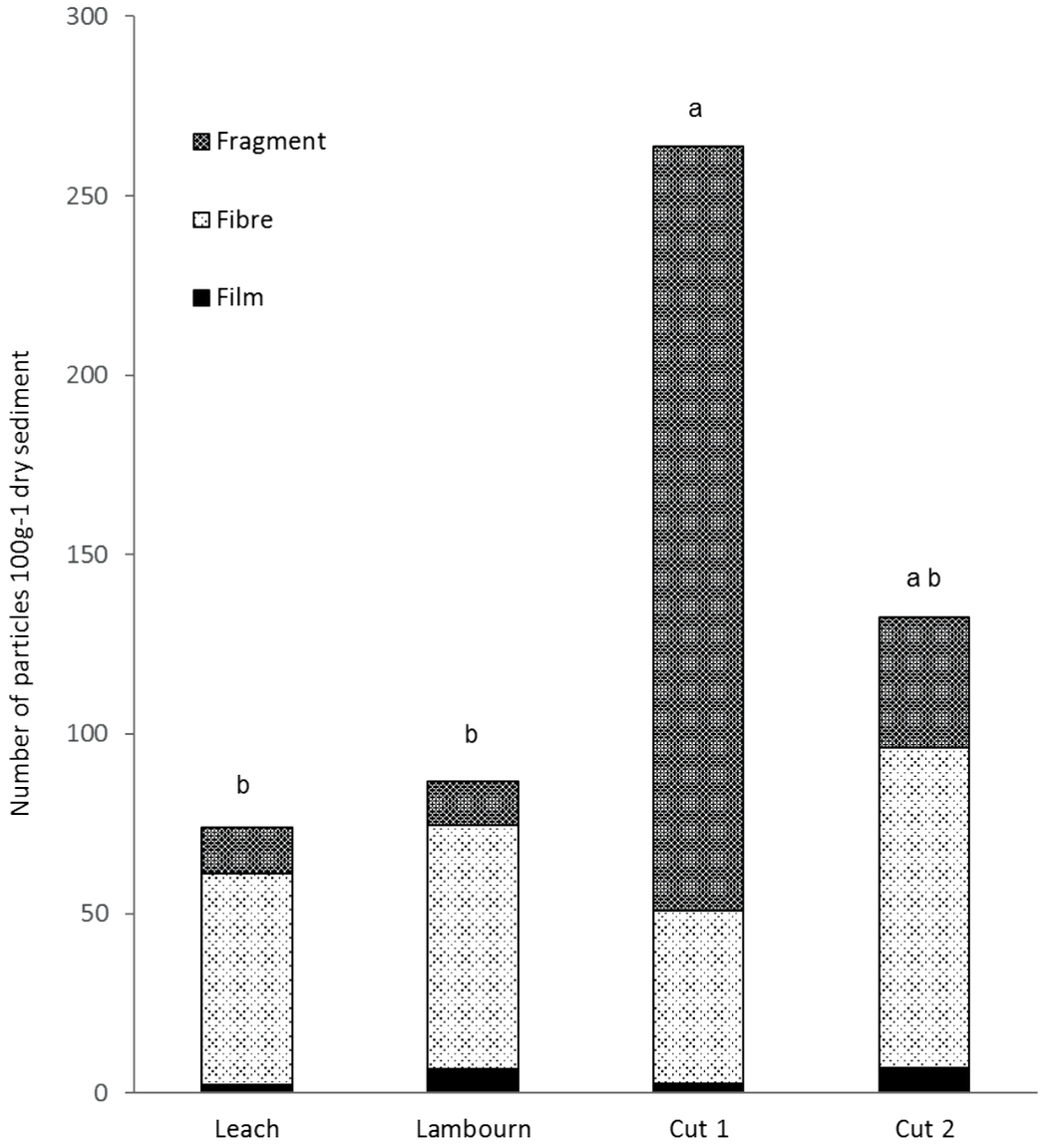

Fig. 3. Total number of microplastic particles at each site per 100 g dry weight of sediment, and number of different types of particles (fragment, fibre and film) within this total. Different letters indicate significant differences between the number of microplastic particles per 100 g at each site.

3.4. Plastic types

A total of 336 particles (20% total) were analysed using Raman spectroscopy with BioRad KnowItAll® Informatics System - Raman ID Expert (2015) software to determine their chemical composition. The particles chosen were evenly distributed across all samples and size fractions. Of the particles analysed, many could not be identified due to poor quality spectra

(due to fluorescence/lack of identifiable peaks), or a spectrum was present but was not recognised either using the KnowItAll software or by eye. Therefore 111 out of 335 (33%) particles could be identified to chemical composition.

Of these 111 identifiable particles, eight (7%) were found to be natural substances such as shell or organic matter, while the other 103 (93%) were of anthropogenic origin. The majority of these spectra (62%) related to dyes, as opposed to the plastic materials in which they are impregnated (Fig. 4). Dyes detected included those commonly added to plastics and plastic composites, including copper phthalocyanine, mortoperm blue, hostasol green and chrome yellow (Clariant International Ltd, 2011; Imhof et al., 2016; Lewis, 2005; Okazaki and Suzuki, 1976; Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe et al., 2013). A total of 34 analysed particles could be identified specifically to their polymer composition. The types of polymer identified were polyester/polyethylene terephthalate (PET, 14 particles) polypropylene (PP, five particles), polyarylsulphone thermoplastic (five particles), polyethylene (PE, two particles), polystyrene (PS, one particle), and poly vinylchloride (PVC, one particle). Additional polymers found include polycarbonate and composites such as acrylonitrile/PMMA thermoplastic blend and polyurethane/resin composite; these were all grouped under 'other polymers' (Fig. 4).

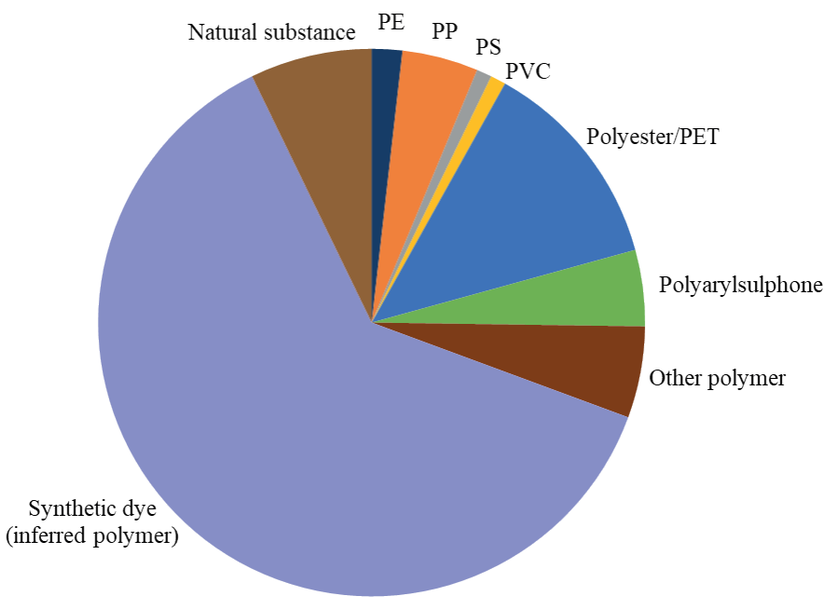

Fig. 4. Proportional compositions of 111 identifiable particles characterised by Raman analysis (of an original 336 analysed particles) across all sites including polymers, dyes (inferred to be polymers) and particles misidentified as plastics (natural substances)

4. Discussion

In terms of quantification method evaluation, the initial sorting and flotation steps combined successfully removed 75% of microplastic particles with the other 25% remaining in the residual sediment. Recovery would have been at 98% if the particles at The Cut site 1 were excluded, as 34% of these could not be floated due to their dense nature. However an initial manual sort by hand and microscope through an amount of dry sediment alone appears to be ineffective, as a maximum of 37% particles were removed in this sorting step. Many of the microplastics manually sorted would also be expected to float, therefore this suggests that flotation is the most effective method for removing microplastics from river sediments, with a subsequent sort through the remaining sediment post-flotation to remove dense particles. Given the thorough stepwise process of particle extraction, it was considered that these steps carried out in succession were successful in removing all microplastic particles from the sediment. However, for efficiency, the initial pre-flotation search cannot be considered fully effective on its own and may be eliminated as it can be assumed that all particles removed in this step would be extracted in the following two steps. The presence of these dense microplastics present in the unfloated fraction highlights the complexity of microplastics as an environmental contaminant; these will often likely be polymer-based composites and therefore will not behave as the pure polymer would be expected to. This stepwise methodology works to extract particles even from complex sediment samples in a cost-effective manner. It is necessary to carry out multiple steps of particle extraction to account for dense particles therefore the suggested protocol for future samples would be to carry out a flotation using a concentrated $ZnCl_2$ solution, followed by a timed manual sort of the remaining sediment to remove any unfloated plastic particles.

This study shows for the first time in the UK that microplastics are present in river sediments, with microplastic particles observed at all sites including both urban and rural locations. Despite being the second most anthropogenically influenced in terms of effluent input and population equivalent density, the highest sediment microplastic burden was found at The Cut site 1 (although not significantly different from the more highly effluent polluted Cut site 2, Fig. 3). The dominant type of particle at this site was fragments, as opposed to fibres at the other three sites. Hence at this site there is the indication of a source of fragment additional to the sources at the other three sites. The characteristics and chemical nature of particles found (e.g. predominantly coloured, angular fragments) suggest that many of these particles found were locally-derived secondary microplastics rather than primary microplastics from consumer

products or secondary artificial fibres introduced by sewage effluent. Factors contributing to the relatively high plastic fragment input at The Cut site 1 are likely to be the presence of a storm drain immediately upstream from the sampling location carrying local urban runoff to the watercourse and the urban nature of the site, on the outskirts of a large town. This implies that, at this site, runoff rather than sewage effluent is the dominant input. The high sewage-based input at The Cut site 2 may be reflected in it having the highest number of fibres when calculated per 100 g (Fig. 3).

Fragments and fibres were both found in significant numbers, with fragments dominating the particles found at the Cut site 1 and fibres being the dominant particle type at the other three sites. Films were found only at low numbers. The abundance of fibres at all sites suggests the influence of sewage effluent, even for the Leach where there is only one upstream STW and negligible effluent input (Fig. 2). Given that there were still a considerable number of particles found at this site there may be an alternative anthropogenic influence, for example airborne contamination (Peters and Bratton, 2016) or agricultural runoff (e.g. from plastic mulching) (Rillig, 2012). With UK policy of significant amounts of sewage sludge applied to land in the UK (80% of all sludge) (DEFRA, 2012), it is possible that such fibres may be derived from sludge applied to surrounding arable land entering the watercourses via runoff. Runoff from septic tank systems may also be a source (Butler and Payne, 1995).

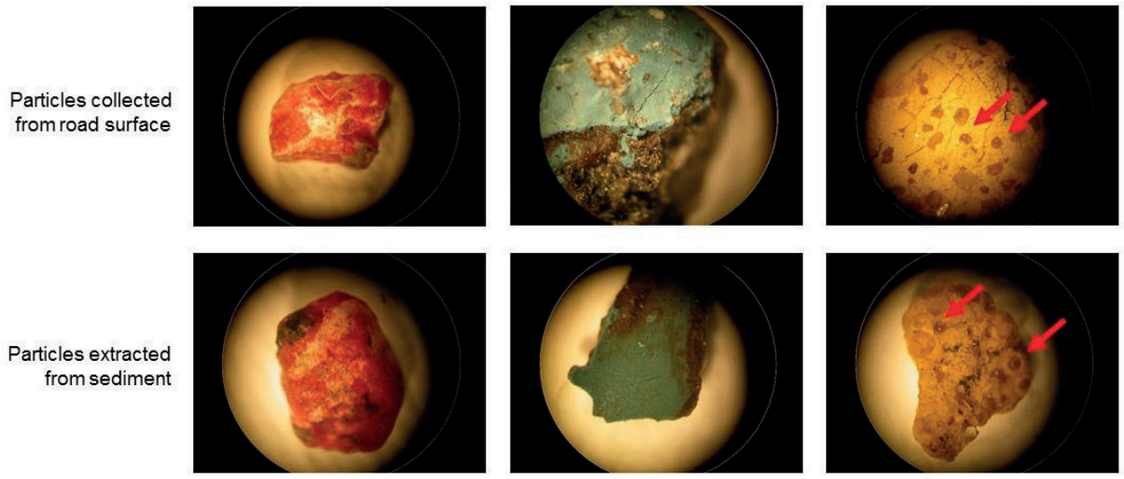

Fig. 5. Photographs comparing particles collected directly from coloured road surfaces/road marking paints (top row) to particles extracted from sediment samples at The Cut site 1 (bottom row). These particles all fit within the 2-4 mm size range. Photos were taken using a Nikon Coolpix 4500 camera with a Nikon Coolpix MDC lens attachment on a Nikon SMZ800 stereo microscope with Photonic PL2000 cold light source at varying magnifications. Arrows highlight incorporated glass beads, present both in particles taken from road marking paints and in environmental samples.

Of the particles remaining unfloated throughout the sorting process, a number were identified to be dense composites of road-marking paints, aggregates, a painted coating on a dense particle or high density mineral-polymer mixtures (Corcoran et al., 2015). These materials are composites of polymer resin, thermoplastic, bitumen and pigment (often with incorporated glass beads for reflectivity) (Conserva and Dupont, 2011; National Association of City Transportation Officials, 2014). In addition, key features of the particles identifying road markings as a source include their colour (predominantly red and yellow), incorporated glass beads and site location downstream of the storm drain input. Raman analysis showed many of these particles to contain dyes, for example many yellow particles contained chrome yellow, a yellow pigment commonly added to thermoplastic road marking paints (Okazaki and Suzuki, 1976). To confirm this identification as road-derived particles, particles were collected from road surfaces upstream of the Cut site 1 storm drain. Visual inspection and Raman analysis showed that particles collected directly from road-based coatings and paints matched those extracted from the sediment samples (Fig. 5). Some of these particles appeared to be partially coated in paint indicating that some of the coating had degraded and highlighting the potential for small particles to degrade further. The incorporated glass beads observed, which are lost to the environment with wear and were also observed independently in samples, do not fit the definition of microplastics (Kemsley, 2010).

Polymers give a weak Raman scatter and therefore an incorporated dye is likely to override the polymer spectrum (Imhof et al., 2016; Smith and Dent, 2005). Given the strong dye spectra observed in many of the coloured particles, and lack of other peaks, it can therefore be inferred that the particles identified as pigments are all dyed polymers or polymer composites (Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe et al., 2013). Unidentifiable particles were also inferred to be plastics as fluorescence is a common problem when analysing polymer particles using Raman spectroscopy (Löder and Gerdts, 2015). For the purpose of this study, paints, pure polymers and composites were all considered as microplastics as per Song et al (2014), although some authors will distinguish ‘micropaints’ and microplastics separately due to varying polymer composition (Imhof et al., 2016). However as all polymers are composites to some extent (containing fillers, pigments and plasticisers) very few environmental plastics will be ‘pure’ polymers; there is currently no threshold of polymer content to distinguish between pigmented polymer and polymer incorporated within a paint.

Previous marine studies have identified plastic pellets associated with tarry residues or attached to tar-based substances (Gregory, 1983). The observations of road-derived particles here

indicate that materials similar in nature are also entering river systems and may add another aspect to microplastic presence and behaviour in this environment. It has previously been noted that microplastics can be transported via road surface runoff originating from degraded litter, pieces of car-related debris such as bumpers or hubcaps, tyre wear particles (Browne et al., 2010; Eriksen et al., 2013; Galgani et al., 2015; Tibbetts, 2015), however to our knowledge this is the first study to note the presence of microplastics derived directly from the road surface and associated markings. Paint particles have previously been found in UK estuarine sediments, however these were not linked to road surface degradation (Takahashi et al., 2012). Little is known about the long term fate and behaviours of these materials in rivers. Such releases are likely to be widespread and difficult to avoid; efforts in infrastructure and civil engineering management would be needed to limit such emissions.

Using Raman spectroscopy, polymer types including polypropylene, polyethylene, polyvinyl chloride, polyester and polystyrene were found at the sites. These were all expected as these are among the most widely used plastics in consumer products (PlasticsEurope, 2015). Another polymer found at three out of four sites in relatively high numbers was polyarylsulphone thermoplastic (Fig. 4). This was not expected as it is not one of the most commonly used polymers. This polymer has high thermal resistance and is used to replaces ceramics and glass in a variety of applications including household goods and electrical equipment (Rosato and Rosato, 2004). One expected polymer, nylon was not observed here, although this does not necessarily indicate its absence at these sites.

These findings highlight the ubiquitous nature of plastic as an environmental pollutant, even in rural areas with no expected significant inputs. The results presented here can be taken as an indicator of microplastic pollution in the Thames Basin. Despite the combination of different sorting methods these are not guaranteed to be without error, given that 7% of particles analysed by Raman were found to be of natural origin. However, this error is far lower than the 70% predicted by Hidalgo-Ruz et al. (2012). To some extent, predictive estimates of microplastic abundances can be made based on known site characteristics (including effluent input and population served by upstream STWs). However, alternative factors are important to take into account when trying to predict microplastic pollution in this size range; other sources such as terrestrial run-off and inputs from storm drains cannot be disregarded. Additional factors to take into consideration include surrounding land use, population density in the area surrounding the sampling site (as opposed to population equivalent served by upstream STWs) and alternative inputs to the watercourse (such as storm drains and drainage ditches).

These results provide evidence of rivers as a source of microplastics to the sea, however the factors influencing presence, abundance and behaviour of microplastics in a riverine environment are complex and difficult to predict. Within a river, sediment transport and dynamics including flow speed and channel depth can control the flow of particles, both natural and artificially produced, *en route* from land to ocean (Phillips et al., 2000; Smith et al., 2003). An accurate assessment of microplastics in any environment needs first and foremost knowledge of the range of potential sources, behaviour of particles in the environment and an understanding of the factors that mediate the inputs. Further research needs to be done at these locations to include the small scale particles (<1 mm) and also particles within the water column and on the surface. The density of polymers is an important consideration given that the particles observed in sediment are likely to be of denser polymers; in flowing waters buoyant particles may have been transported downstream before they could become biofouled and dense enough to sink (Andrady, 2011; Van Cauwenberghe et al., 2013). Additional studies and modelling of fate and transport of these particles within river systems need to be carried out in an attempt to better predict where they will end up. It is also highly likely that seasonal changes in river flow will affect the presence and transport of microplastics within riverine systems. Therefore sampling in different weather and seasonal conditions would help develop understanding of the degree to which rivers act as a sink of microplastics and a source to the marine environment.

5. Conclusions

This study is the first to report relative amounts and types of microplastics present across different locations both in the Thames basin, and also in any low-lying river catchment in the UK. Despite the uncertainties and complexities with predicting and analysing microplastic pollution, microplastics were observed at all sites and inference can be made as to sources. While it is clear that the number and types of microplastics observed in this study are not the entirety of microplastic pollution at these sites, these results give a representative indication of the proportions of plastics between these sites and the factors that influence their presence and abundance, specifically sewage and road-derived input, plus in situ degradation of litter, especially in urban areas. The majority of microplastics from this study were deemed to be secondary microplastics i.e. broken down from larger items. To some extent, different types of particles could be attributed to different sources i.e. road surface markings made of

thermoplastic composite paints, fibres derived from synthetic textiles and fragments of large litter items such as plastic bottles (polypropylene) and packaging materials (polystyrene). Sewage and effluent input is also a likely significant source given that many of the particles found were fibres, especially in the most polluted sites that receive a high volume of effluent such as those at The Cut sites 1 and 2. However these results indicate that despite the evidence for sewage influences at these sites, in certain locations sewage effluent may be a less significant source of large microplastic particles than direct runoff from land. This study highlights the importance of rivers as a source of microplastics and other anthropogenic litter to the ocean, but also as a sink for dense plastics and anthropogenic particles with potential for environmental and ecological impacts.

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CHAPTER 4

The influence of exposure and physiology on microplastic ingestion by the freshwater fish *Rutilus rutilus* (roach) in the River Thames, UK

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The influence of exposure and physiology on microplastic ingestion by the freshwater fish *Rutilus rutilus* (roach) in the River Thames, UK

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Abstract

Microplastics are widespread throughout aquatic environments. However, there is currently insufficient understanding of the factors influencing ingestion of microplastics by organisms, especially higher predators such as fish. In this study we link ingestion of microplastics by the roach *Rutilus rutilus,* within the non-tidal part of the River Thames, to exposure and physiological factors. Microplastics were found within the gut contents of roach from six out of seven sampling sites. Of sampled fish, 33% contained at least one microplastic particle. The majority of particles were fibres (75%), with fragments and films also seen (22.7% and 2.3% respectively). Polymers identified were polyethylene, polypropylene and polyester, in addition to a synthetic dye. The maximum number of ingested microplastic particles for individual fish was strongly correlated to exposure (based on distance from the source of the river). Additionally, at a given exposure, the size of fish correlated with the actual quantity of microplastics in the gut. Larger (mainly female) fish were more likely to ingest the maximum possible number of particles than smaller (mainly male) fish. This study is the first to show microplastic ingestion within freshwater fish in the UK and provides valuable new evidence of the factors influencing ingestion that can be used to inform future studies on exposure and hazard of microplastics to fish.

1. Introduction

Microplastics (plastic particles <5 mm) are an emerging environmental contaminant of growing concern due to their abundance and persistence throughout the environment. Microplastics can enter rivers via runoff and drainage systems, effluent input and breakdown of in situ litter. Once in the aquatic environment, it is highly likely that these will be encountered and ingested by pelagic or benthic organisms. In the case of higher trophic organisms such as fish, ingestion may be direct (from the water column or sediment) or indirect (ingestion of organisms that have previously ingested microplastics) (Campbell et al., 2017; Desforges et al., 2015; Setälä et al., 2014). There is a growing body of evidence for microplastic ingestion by freshwater fish (Biginagwa et al., 2016; Peters and Bratton, 2016; Sanchez et al., 2014; Silva-Cavalcanti et al., 2017) with studies finding up to 100% contamination within sampled fish in some areas (Pazos et al., 2017). However, based on a lack of evidence, we are currently unable to determine the extent to which freshwater fish are ingesting microplastics, the complex variety of factors that may influence ingestion and any implications this may have for ecosystems.

Rivers are highly dynamic environments and along its course, a river will be subject to an accumulation of land-derived inputs, for example road runoff, agricultural runoff, wastewater inputs and litter, all of which can contribute to the burden of microplastics within the watercourse (Horton et al., 2017a; Lechner et al., 2014; Morritt et al., 2014; Nizzetto et al., 2016a). The majority of microplastic particles entering the freshwater environment are likely to be derived from the breakdown of larger items, for example single-use packaging items, tyre and road paint particles, or fibres from synthetic fabrics (Boucher and Friot, 2017; Browne et al., 2011; Horton et al., 2017a). It is assumed that a proportion of microplastics (although not all) entering a river will be buoyant and easily transported downstream. Since the sources of (micro)plastic particles are anthropogenic, a site downstream of populated or industrial areas is likely to contain more microplastics than sites that have been subject to little anthropogenic input (Dris et al., 2015b; Horton et al., 2017a; McCormick et al., 2014). As such, sites further from the river source would be expected to be subject to a greater variety of inputs (Mani et al., 2015).

Assuming there is exposure, physiological traits of fish, such as size, may determine whether an individual will ingest microplastics, and the number of particles the fish may ingest. For example, larger roach will consume more in general due to increased energy demands (Hölker

and Breckling, 2001), which increases their potential for ingestion of microplastic particles. Therefore, susceptibility to ingestion and volume of uptake, given exposure, will be determined by physiological characteristics. Combined, these two factors (exposure and likelihood of ingestion) are expected to determine the number of particles that an individual fish can ingest. Microplastics present within the guts of fish may be considered a representation of microplastic pollution within the river, as a proportion of microplastics within the environment are likely to be contained within biota (van Sebille et al., 2015). The higher the number of microplastics an individual ingests, the more likely the particles are to have an adverse health effect, such as reduced capacity for food ingestion and reduced scope for growth (Murray and Cowie, 2011; Watts et al., 2015). Indeed, dose-dependent effects are commonly seen with the most significant effects on organisms following ingestion at the highest exposure concentrations of microplastics (Au et al., 2015; Besseling et al., 2014; Ziajahromi et al., 2017). However, there is a recognised discrepancy between the concentrations within the environment and those used within laboratory exposures, therefore more data is needed from field studies on actual ingestion to inform future laboratory tests (Lenz et al., 2016).

In this study we investigated microplastic ingestion by roach *Rutilus rutilus* (Linnaeus 1758) in the River Thames; the second longest river in the UK. Studies have shown the Thames to be contaminated with both microplastic (Horton et al., 2017a) and macroplastic litter (Morritt et al., 2014), in addition to evidence of microplastic ingestion by marine fish living within the tidal Thames estuary (McGoran et al., 2016). However, no studies to date have yet investigated microplastic ingestion by freshwater fish within the non-tidal Thames. Roach are an indicator species (Havelková et al., 2008; Hellawell, 1972) and abundant throughout the UK in rivers, lakes and ponds. They are omnivorous, eating a wide variety of food from a range of sources including plant matter, benthic invertebrates and zooplankton (Elliott et al., 2015; Wintle, 2011). They are an important component of the aquatic food chain, supporting a number of predatory fish such as pike, and mammals including otters (Bean and Winfield, 1995; Hansson et al., 1998; Webb, 1975).

The aim of this study was to investigate whether wild-caught roach ingest microplastics within the non-tidal part of the River Thames, and how this relates to the location of the sampling site (which may influence exposure to microplastics) and physiological traits of the fish (determining likelihood and volume of ingestion). We hypothesised that exposure of fish to plastic particles will be determined by the distance from the source of the river. Further, we

hypothesised that the number of microplastic particles in the fish will reflect their feeding habits based on energy requirements and will therefore be influenced by size and gender.

2. Materials and methods

2.1. Sampling sites and fish collection

Rutilus rutilus (roach) were collected from the River Thames between July and October 2013 (following the spawning season) by Environment Agency staff in connection with regular fish population surveys, using electrofishing techniques. Fish were collected from seven sites along the main body of the River Thames, spanning a distance of 203 km, between 36 km and 239 km from the source of the river (Table 1 and Fig. 1). In this study, the source of the river relates to the source of the longest tributary (River Churn). Sampling was conducted between locks, except at the two sites furthest upstream, Cricklade and Castle Eaton, where no locks were present.

A minimum of six roach, which had a minimum fork length (size from the tip of the nose to the middle of the caudal fin rays) of 100 mm each, were collected per site. Caught fish were sacrificed with an overdose of an anaesthetic (0.4 ml/L 2-phenoxyethanol) and their weights and fork lengths recorded. They were then frozen on site by placing them in a liquid nitrogen-cooled container and stored at -80°C until further processing. In order to process the fish, individuals were allowed to warm up to a semi-frozen state and dissected, during which the entire digestive tracts were removed and the gender of the individuals was recorded. Digestive tracts were placed in 15ml centrifuge tubes and stored at -80°C until analysis.

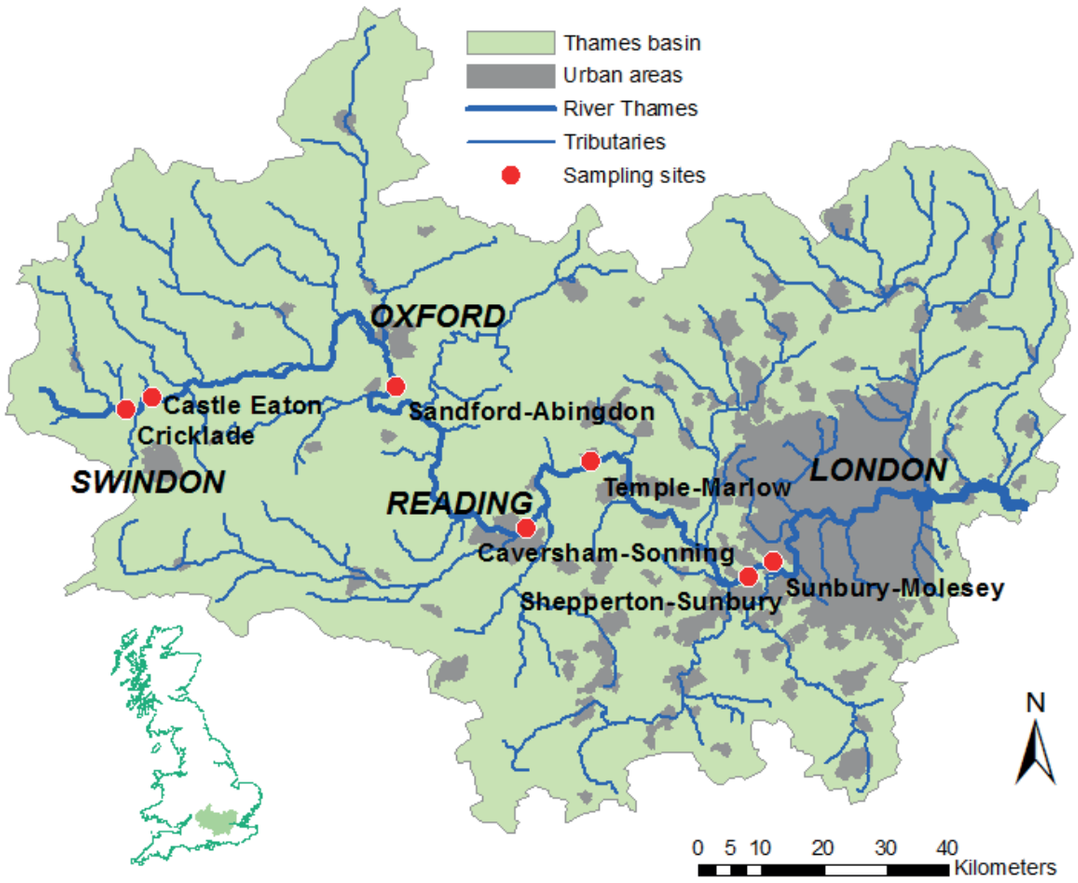

Fig. 1. Map showing locations of sampling sites on the River Thames. Sampling was undertaken in the stretch between locks (detailed by the site name) and therefore markers are placed approximately between the two locks, except for Cricklade and Castle Eaton where there are no locks and the markers denote the exact sampling location. See table 1 and table S2 for further details on sampling sites. The main urban centres are also marked.

2.2. Gut dissection and microplastic extraction

Fish tissues were removed from the freezer one fish at a time, and dissected as the tissue thawed. The entire digestive tract of fish (buccal cavity to anus) was cut open and all contents scraped out with a stainless steel spatula (hereafter referred to as 'gut content' for simplicity). Contents were spread on a Whatman GF/C glass microfibre filter paper (47 mm diameter, 1.2 µm mesh, GE Healthcare Life Sciences, UK) and immediately analysed. To eliminate possible contamination, all filters and tools were examined for particles before gut content analysis. Due to the small amount of gut content in each fish, it was possible to manually and thoroughly sort

through the content and therefore it was not necessary to digest the organic matter. Gut contents were searched under a binocular microscope (Wild Heerbrugg, Switzerland, with Photonic PL2000 cold light source) using a 6x magnification for a maximum of 15 minutes (this time frame based on the amount of time required to thoroughly search the largest volume of gut content), using a stainless steel spatula and forceps to move contents around as necessary. Forceps were used to remove microplastic particles to a clean filter paper. Gut contents were only exposed to the air during this 15 minute period. Following removal of contents, the inside of the gut itself was also examined to check that no particles had been missed. All particles were visibly incorporated into gut content when they were removed and were therefore believed not to be derived from airborne contamination. Between fish, all dissection tools were rinsed thoroughly with deionised water, wiped with ethanol and a lint-free tissue (Kimwipes, Kimtech Science, USA) and observed under the microscope before use to eliminate the possibility of cross-contamination.

Particles were removed as per Horton et al. (2017a) and were required to meet all of the following selection criteria, originally set out by Nor and Obbard (2014): 1) no visible cellular or organic structures, 2) unsegmented, 3) fibres of homogenous width (not tapered) and at least two of the additional criteria: 1) unnaturally coloured or with a brightly coloured coating (e.g. bright orange, blue etc.), 2) appear to be of homogenous texture/material, 3) abnormal (un-natural) shape e.g. perfectly spherical, 4) fibre that remained unbroken if tugged with tweezers, 4) reflective/glassy, 5) flexible without being brittle.

2.3. Polymer identification

Particles removed were quantified and half of the total number of particles (22/44) were analysed by Raman spectroscopy (HR800UV, Jobin Yvon Horiba, France, with integrated Olympus BX41 microscope) using Horiba LabSpec 6 software to give a qualitative representation of chemical composition of the microplastic particles as per Horton et al. (2017a). It was not possible to analyse all particles as some were lost following quantification due to their small size. Acquired spectra were compared to matched reference spectra using BioRad KnowItAll® Informatics System - Raman ID Expert (2015) software and the most appropriate match was selected based on matching peak wavenumber positions and a minimum 80% correlation between unknown and matched spectra (Horton et al., 2017a).

2.4. Data analysis

In this study, we first analysed the maximum likely ingestion for individual fish as a function of distance from the source of the river, as a measure of exposure. Subsequently, we analysed how physiological characteristics influence the actual ingestion compared to the maximum likely ingestion at the location. By dividing the analysis into these steps, we believe to stay close to the true mechanisms of microplastic ingestion and obtain a good understanding of the ingestion by individual fish. Determining an average ingestion at each site would not have provided these insights and would have given a population estimate only.

Firstly to test our hypothesis that the maximum likely ingestion of microplastics was related to the distance downstream from the river source, a quantile regression on the 95% quantile was carried out based on all the raw ingestion data for each fish compared to distance downstream (using the upstream point of the 0-7 km sampling stretch). A quantile regression draws a linear function of an independent variable (here, distance downstream from the river source) such that a given proportion of the observations (in this case, ingestion by individual fish) are below the line. In this instance the upper 95% (τ) was chosen as representing the maximum likely ingestion (Cade et al., 1999). For robustness, the quantile regression was resampled by bootstrapping (999 iterations), a recognised method for testing hypotheses regarding quantile regression models. The significance of the regression coefficients of the quantile regression indicate the significance of the relationship between the fitted line (maximum likely ingestion) and distance from the source. Bootstrapping makes no assumptions and so is particularly suitable when sample sizes are small and/or data are not normally distributed (Fox, 2015).

Second, we tested the hypothesis that the deviation in the actual uptake by an individual from the maximum likely uptake (at a given distance from the river source, based on the 95% quantile regression) is based on physiological traits. This gives a measure of whether fish with certain physiological characteristics are more or less likely to achieve the maximum ingestion at a given exposure. The physiological traits measured were fork length and gender (Fig. 2.). A two-way ANOVA was used to identify whether fork length, gender or their interaction were significantly influencing the deviation in uptake.

Given that sewage is often identified as a significant contributor of microplastics to the freshwater environment, we also carried out ANOVAs to determine whether maximum ingestion (based on resampled data) or average ingestion (based on raw data) were influenced by modelled sewage input. Statistical tests were all carried out using R statistical software.

3. Results and discussion

3.1 Microplastic ingestion

A total of 64 fish, 30 females and 32 males (the genders of two individuals were not identified), were caught at seven sites. The minimum number of fish was six (Sunbury-Molesey) and the maximum was 13 (Temple-Marlow) (Table 1). Caught fish measured between 100 mm and 184 mm and therefore likely represented both adults and juveniles (Table S1). From all sampled fish (64), 32.8% of roach (21) ingested a total of 44 microplastic particles giving a mean ingestion value of 0.69 particles ± 1.25 (SD) per fish (Table 1). Microplastics were observed in the guts of fish from six out of seven sites, whereas at one site (Sandford-Abingdon, 106 km from the source of the river) none of the sampled fish contained plastics.

The majority of particles were fibres (75%), followed by fragments (22.7%) and pellets (2.3%) (Fig. S1 shows a representation of types of particles found). Although particles were not individually measured, all were less than 5 mm and as such considered microplastics. A lower size limit was not set or measured, however all particles observed were of a size that could be removed by hand using forceps. There was limited ability to analyse these particles using Raman spectroscopy. Fifteen out of the 22 analysed particles were unidentifiable due to fluorescence or insufficient spectrum intensity, which are common problems when analysing environmental polymers using Raman spectroscopy (Horton et al., 2017a; Löder and Gerdts, 2015). Of the remaining seven particles, all were of anthropogenic origin and included polyethylene, polypropylene and polyester and a synthetic dye, neolan green (Fig. S2 and table S2). This data can therefore only be considered qualitative, showing the presence of commonly-used polymers. Although it cannot be completely ruled out that some of the unidentified particles may have been organic, or non-polymeric anthropogenic materials, those identified in the study met the criteria from previously successful criteria for microplastic identification (Horton et al., 2017a).

The results presented here complement the results of a recent study by McGoran et al. (2017) who found microplastics within the guts of two different species of marine fish within the estuarine River Thames, also consisting predominantly of fibres. Based on high microplastic inputs to rivers (Horton et al., 2017a; Lechner and Ramler, 2015; Murphy et al., 2016), it is therefore likely that ingestion by freshwater fish is occurring worldwide, especially those in close proximity to, or downstream of, urbanised areas (Dris et al., 2015b; Peters and Bratton, 2016; Sanchez et al., 2014; Silva-Cavalcanti et al., 2017).

Table 1. Site characteristics, sampling undertaken at each site and the numbers of microplastics found. ^Where fish were taken from a stretch between two locks, this distance relates to the upstream end of the stretch. *as calculated using the Low Flows 2000 (LF2000) WQX (Water Quality eXtension) model (Williams et al., 2009). Raw data for each site and individual fish are available in tables S1 and S2.

Site	Distance from source of river (km)^	Average percentage sewage within the watercourse*	Number of fish	Fork length range (mm)	Gender ratio (M:F)	Number of fish containing microplastics	Percentage of fish containing microplastics	Maximum number of ingested microplastic particles by any individual
Cricklade	36	13.3%	8	147-184	2:6	5	62.5	2
Castle Eaton	43	22.4%	11	106-181	1:10	1	9.1	1
Sandford-Abingdon	106-113	12.9%	7	144-164	4:2 (NA = 1)	0	0	0
Caversham-Sonning	162-166	12.8%	9	123-178	8:1	5	55. 6	3
Temple-Marlow	187-190	14.9%	13	100-153	9:4	3	23.1	3
Shepperton-Sunbury	234-239	15.9%	10	105-161	4:5 (NA = 1)	4	40	3
Sunbury-Molesey	239-243	16.2%	6	122-150	4:2	3	50	6

3.2. Microplastics in fish in relation to environmental factors

Analysis of the quantile regression (the fitted line for maximum ingestion) showed a significant relationship: the maximum ingestion of microplastics by individual roach increased with increasing distance from the source of the River Thames (p <0.005 significance of quantile regression, based on bootstrapped coefficients, Fig. 2). This likely reflects the fact that the number of inputs of microplastics to the river are increasing with distance from the river source, due to increasing urbanisation as the Thames flows towards London. However, given that the abundance of microplastics in surface waters of the River Thames has not yet been determined, it is not possible to directly relate the results of plastic ingestion here to the riverine concentrations of these plastics. A trend of increasing microplastic concentration with increasing distance from the source of the river has previously been observed in the River Danube (Lechner et al., 2014) and the river Rhine (Mani et al., 2015). When looking simply at the size of fish in relation to distance from the source, the size of fish did not significantly change with distance downriver (p=0.85, t test). This implies therefore that the difference in ingestion with distance was independent of any size-related differences. The 'maximum likely ingestion' approach allows for comparison of individual fish and therefore better insights into the factors that may influence ingestion.

The finding that the majority of plastic particles in this study were fibres, in addition to the identification of polyester (derived from synthetic textiles), suggests sewage to be a significant contributor to this contamination. Although sewage inputs can give an indication of population pressures, with greater concentrations of microplastics often found within the environment downstream of effluent outfalls (Estahbanati and Fahrenfeld, 2016; McCormick et al., 2014), these values cannot be used to infer the extent of microplastic pollution as they do not necessarily correlate with environmental concentrations due to inputs from other sources (Boucher and Friot, 2017; Horton et al., 2017a). Indeed this study found no relationship between sewage inputs and microplastic ingestion by fish (P > 0.05, ANOVAs for average and maximum ingestion). However, the range of sewage inputs between these sites is not large (average sewage content of the river flow between 12.8-22.4% depending on the sampling site), therefore if analysing sites with a greater range sewage inputs, this result may be different.

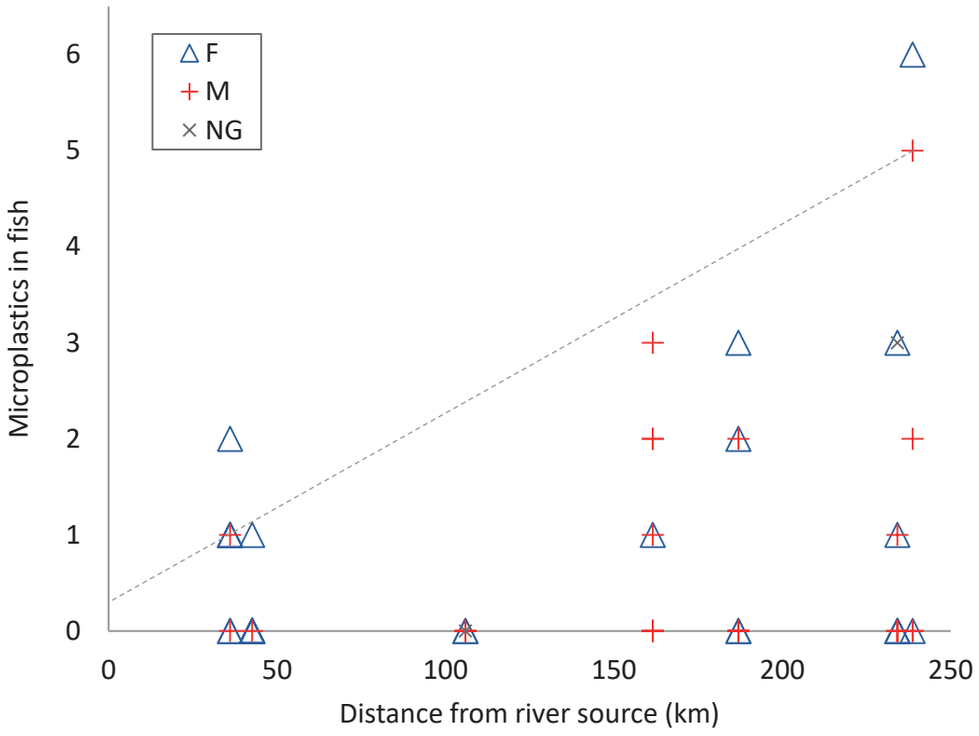

Fig. 2. Number of ingested microplastics in relation to distance from the source of the river. Each data point represents an individual fish, F = female, M = male and NG = no gender (gender not recorded). Some data points overlap therefore there are fewer visible points than fish. The predicted maximum number of microplastics that could be ingested by individual fish at a given distance downstream of the source is shown by the fitted line, which is based on 95% quantile regression. This line therefore represents maximum likely microplastic ingestion based on 95% of fish.

3.3. Microplastics in fish in relation to life history

Although exposure (based on distance from the source of the river) is an important factor determining whether, and to what extent, fish will have the potential to come into contact with and ingest microplastics, ingestion cannot be fully explained by location alone. This is evident in the variability between individuals at each site and the fact that at Sandford-Abingdon (106-113 km from the source of the river) no fish contained microplastics. At a given exposure, physiological characteristics will also influence the likelihood of roach ingesting microplastics, and the number they may consume.

When considering simply presence or absence of microplastics within the gut, there was no significant difference between males and females ($p > 0.05$, Wilcox test). However, the

deviation in actual uptake from the predicted maximum exposure was significantly dependent on gender ($p < 0.05$, ANOVA; Fig. 2). On average, male fish had three particles fewer than the maximum whereas female fish had 1.8 fewer particles on average. Female ingestion was therefore higher (based on less deviation from the maximum). The main effect of fork length was significant ($p < 0.05$, ANOVA): as fork length increases, deviation decreases, therefore larger fish are more likely to attain the maximum ingestion (fig. 3). Although females in this study were significantly bigger than males, with an average size of 148 mm (± 23.3 mm, SD) compared to a male average size of 136 mm (± 19.5 mm, SD) ($p < 0.05$, t-test), gender and fork length effects were not related ($p > 0.05$, interaction effect of the two-way ANOVA) indicating that both gender and fork length influenced ingestion independently.

The increase in ingestion of microplastics with increased fish size correlates with an increased volume of food required to meet the higher energy demands of larger fish (Hölker and Breckling, 2001) leading to a greater chance of direct or indirect ingestion of microplastics. This also suggests that smaller fish are far less likely to reach the maximum ingestion than larger fish at the same exposure. Other studies relating fish size to microplastic ingestion show varying results (Foekema et al., 2013; Peters and Bratton, 2016). This implies that life stage may also influence particle ingestion due to feeding habits.

It is not fully understood why gender would influence microplastic ingestion; this difference could not be explained simply by the larger female size. It could be that gender-specific differences due to the previous spawning event led to greater energy requirements by females (Foltz and Norden, 1977; Lambert and Dutil, 2000) and therefore a greater volume of food consumed (and thus incidental microplastic ingestion). Studies have shown that even water quality can lead to gender-specific differences in fish feeding (Horppila et al., 2011). This is a more complex matter than can be addressed within this study, so this should be another subject for future investigation.

In the current study, in addition to filamentous algae and plant matter, shells were also observed in the guts of some roach indicating the ingestion of molluscs such as bivalves and gastropods. Given the potential for filter-feeding molluscs to ingest microplastic fibres (Farrell and Nelson, 2013; Van Cauwenberghe and Janssen, 2014), there is the possibility that observed particles were ingested by means of food-chain transfer rather than direct ingestion. A recent study on a range of freshwater fish species found that gut microplastic burden varied significantly between species depending on feeding habits and trophic transfer, with apex predators containing the

highest numbers of microplastics, presumably due to ingestion via trophic transfer (Campbell et al., 2017). The presence of filamentous algae indicates that fibrous material will be ingested and that plastic fibres may therefore be unintentionally ingested along with visually similar filamentous algae.

As far as we are aware, this study is the first to relate ingestion of microplastics in a freshwater fish species to gender, fish size and distance from the source of the river. These results suggest that physiological characteristics may be equally as important as environmental characteristics for influencing ingestion of microplastics by fish. Ingestion (likelihood and volume) is therefore a result of a complex combination of factors.

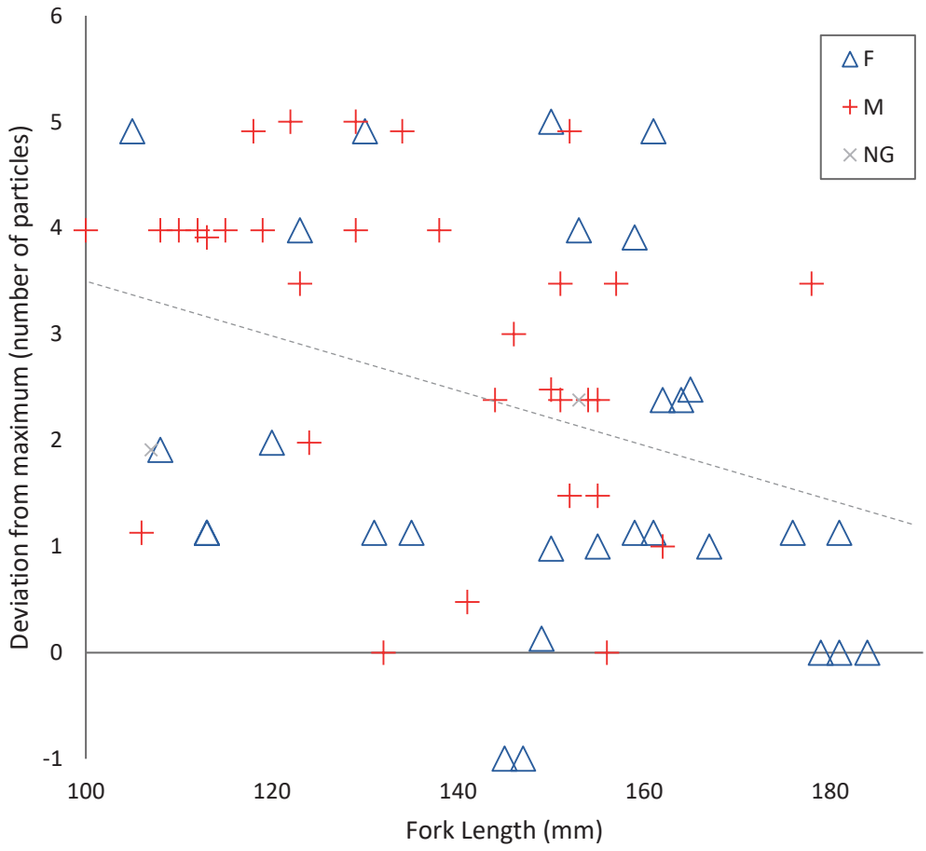

Fig. 3. Deviation from the predicted maximum ingestion (based on 95% quantile regression) compared to fish fork length. Each data point represents an individual fish, F = female, M = male and NG = no gender (gender not recorded). The fitted line was derived from the intercept and slope calculated by ANOVA.

3.4. Implications of microplastic ingestion

Recent studies highlight the potential for damaging effects of microplastics on fish health and fitness. These include changes to immunity (Greven et al., 2016), metabolism (Mattsson et al., 2014), neurotransmission (Oliveira et al., 2013), endocrine function and reproduction (Rochman et al., 2014), and behaviour (Espinosa et al., 2016; Mattsson et al., 2014). Lu et al. (2016) found particles less than 5 μm led to oxidative stress and inflammation within the liver. If plastic particles become nano-sized, they have the potential to cross the blood-brain barrier leading to brain damage and changes in behaviour (Mattsson et al., 2017). Individually or combined, these effects could have severe consequences on fish populations long-term, with significant implications for ecosystems.

5. Conclusions

Microplastics are being ingested by roach, and it is therefore likely that many other species of freshwater fish in the River Thames will also ingest microplastics. The number of microplastic particles in the guts of individuals is understood to be the result of two processes, exposure (which is likely to increase with distance downstream) and physiological characteristics of the fish. In this study, larger, female fish were more likely to reach a maximum ingestion at a given exposure, believed to be a result of increased energy requirements and thus feeding. This understanding gained from this study will help in interpreting findings from future studies data on the occurrence of microplastics in guts of fish worldwide, as well as identifying which fish are most likely to consume microplastics.

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CHAPTER 4
SUPPLEMENTARY INFORMATION

Table S1. Raw data showing site details and physiological characteristics of fish in relation to number and types of microplastics found within individuals. Where fish were sampled between two locks, the distance from the source of the river is given as the range between these two locations. For analysis and presentation of data, the upstream distance was used. *'NG' refers to 'no gender' *i.e.* the gender of the fish was not recorded.

Location	Distance from source (km)	Fishing date	Fork length (mm)	Gender	Fragments	Fibres	Pellets	Total particles in fish
Cricklade	36	11th Oct 2013	147	F	0	2	0	2
			155	F	0	0	0	0
			156	M	0	1	0	1
			162	M	0	0	0	0
			167	F	0	0	0	0
			179	F	1	0	0	1
			181	F	0	1	0	1
			184	F	0	1	0	1
Castle Eaton	43	11th Oct 2013	106	M	0	0	0	0
			113	F	0	0	0	0
			113	F	0	0	0	0
			113	F	0	0	0	0
			131	F	0	0	0	0
			135	F	0	0	0	0
			149	F	0	1	0	1
			159	F	0	0	0	0
			161	F	0	0	0	0
			176	F	0	0	0	0
			181	F	0	0	0	0
Sandford-Abingdon	106-113	2nd Jul 2013	144	M	0	0	0	0
			151	M	0	0	0	0
			153	NG*	0	0	0	0
			154	M	0	0	0	0
			155	M	0	0	0	0
			162	F	0	0	0	0
			164	F	0	0	0	0
Caversham-Sonning	162-166	11th Jul 2013	123	M	0	0	0	0
			141	M	0	3	0	3
			150	M	0	1	0	1
			151	M	0	0	0	0

Location	Distance from source (km)	Fishing date	Fork length (mm)	Gender	Fragments	Fibres	Pellets	Total particles in fish
			152	M	1	1	0	2
			155	M	0	2	0	2
			157	M	0	0	0	0
			165	F	0	0	1	1
			178	M	0	0	0	0
Temple-Marlow	187-190	2nd Sep 2013	100	M	0	0	0	0
			108	M	0	0	0	0
			110	M	0	0	0	0
			112	M	0	0	0	0
			115	M	0	0	0	0
			119	M	0	0	0	0
			120	F	0	2	0	2
			123	F	0	0	0	0
			124	M	0	2	0	2
			129	M	0	0	0	0
			138	M	0	0	0	0
			150	F	3	0	0	3
			153	F	0	0	0	0
Shepperton-Sunbury	234-239	9th Sep 2013	105	F	0	0	0	0
			107	NG*	1	2	0	3
			108	F	3	0	0	3
			113	M	0	1	0	1
			118	M	0	0	0	0
			130	F	0	0	0	0
			134	M	0	0	0	0
			152	M	0	0	0	0
			159	F	0	1	0	1
			161	F	0	0	0	0
Sunbury-Molesey	239-243	10th Sep 2013	122	M	0	0	0	0
			129	M	0	0	0	0
			132	M	1	4	0	5
			145	F	0	6	0	6
			146	M	0	2	0	2
			150	F	0	0	0	0

Table S2. Locations of relevant locks on the River Thames

Lock name	Latitude (degrees, minutes, seconds)	Longitude (degrees, minutes, seconds)
Cricklade	51° -21' -20.448"	-1° 9' - 9.988"
Castle Eaton	51° -20' -16.953"	-1° 13' -28.063"
Sandford	51° -18' 29.719"	-1° -14' 1.748"
Abingdon	51° -20' 13.856"	-1° -16' - 8.556"
Caversham	51° 28' -21.500"	-0° 2' 9.170"
Sonning	51° 28' 22.516"	-0° 5' - 4.951"
Temple	51° -27' 7.424"	-0° 12' 21.804"
Marlow	51° -26' 2.096"	-0° 14' - 7.670"
Shepperton	51° 23' - 4.762"	-0° -28' 27.451"
Sunbury	51° 24' 18.344"	-0° -24' -21.768"
Molesey	51° 24' 17.322"	-0° -21' 14.498"

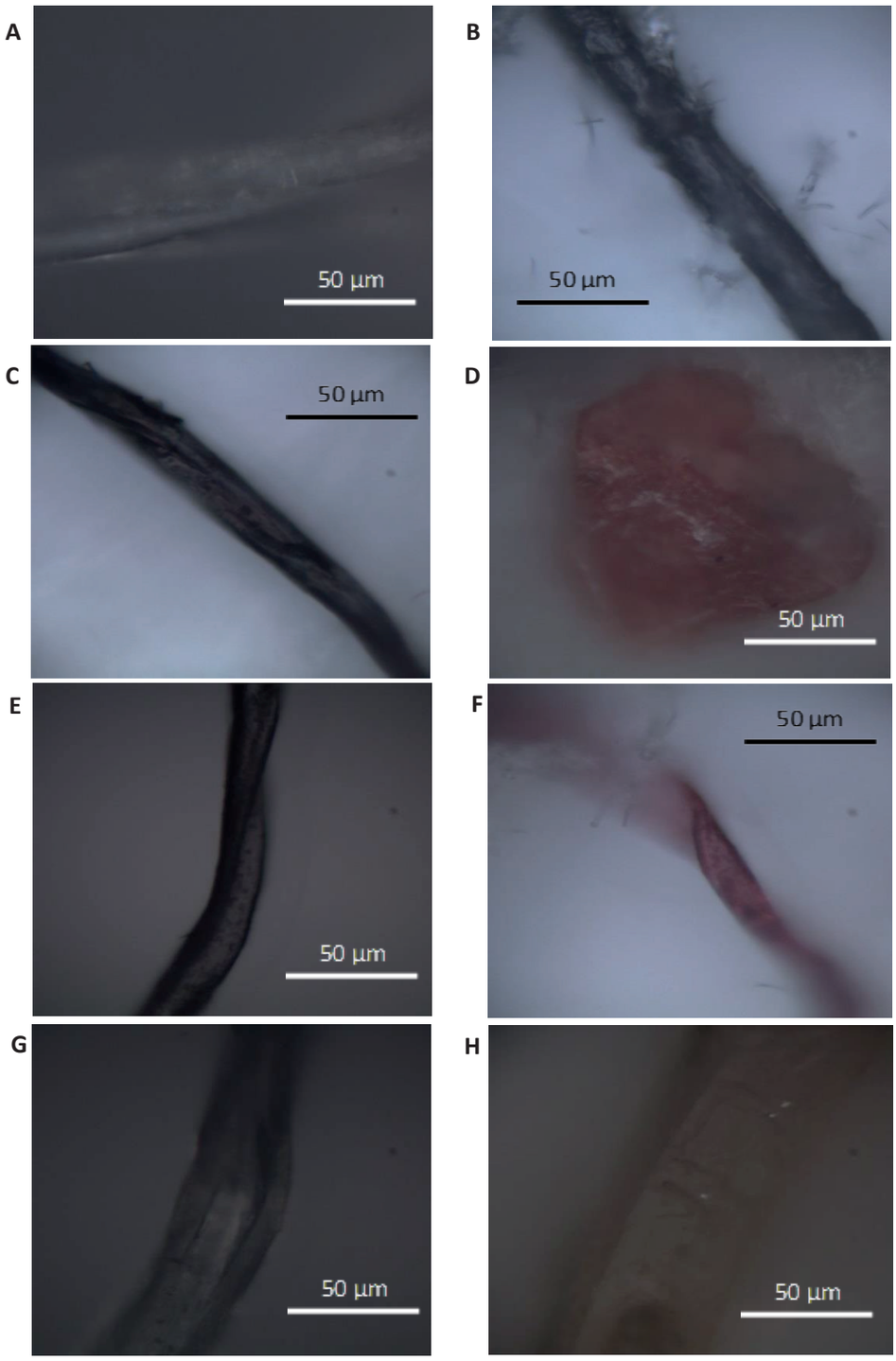
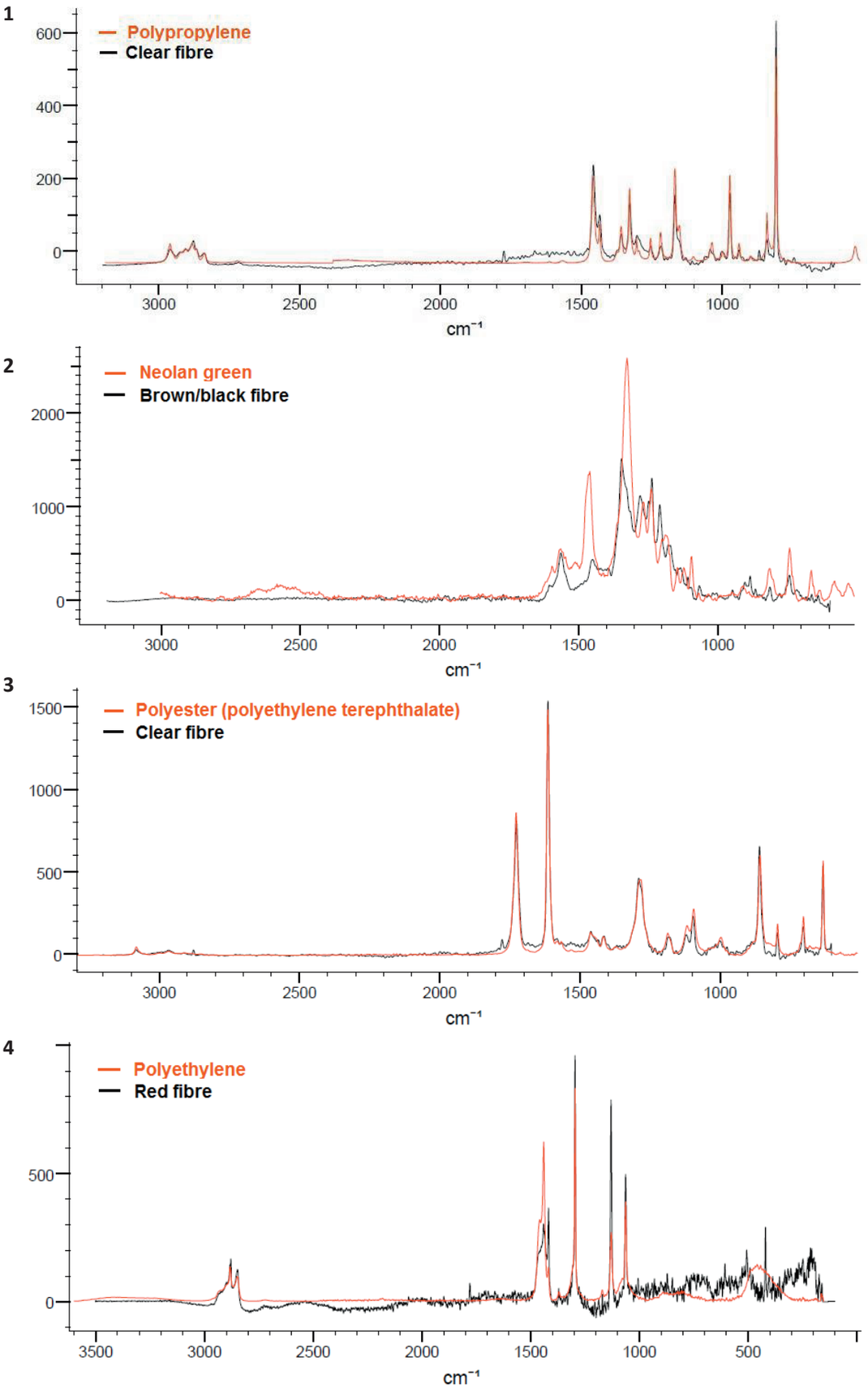


Fig S1. Images of a selection of representative particles at 50x magnification. Images A-C represent particles from fish collected between Caversham-Sonning. Images D-F represent particles from Temple-Marlow. Image G represents a particle from Sunbury-Molesey. Image H represents a particle from Castle Eaton. These images therefore show particles found within fish throughout the length of the non-tidal River Thames. Particles A, C G and H could be accurately identified and correspond to spectra 1, 2, 3 and 5 below (Fig. S2 and table S2).


1
Polypropylene
Clear fibre
600
400
200
0
3000
2500
2000
1500
1000
cm⁻¹
2
Neolan green
Brown/black fibre
2000
1000
0
3000
2500
2000
1500
1000
cm⁻¹
3
Polyester (polyethylene terephthalate)
Clear fibre
1500
1000
500
0
3000
2500
2000
1500
1000
cm⁻¹
4
Polyethylene
Red fibre
500
0
3500
3000
2500
2000
1500
1000
500
cm⁻¹

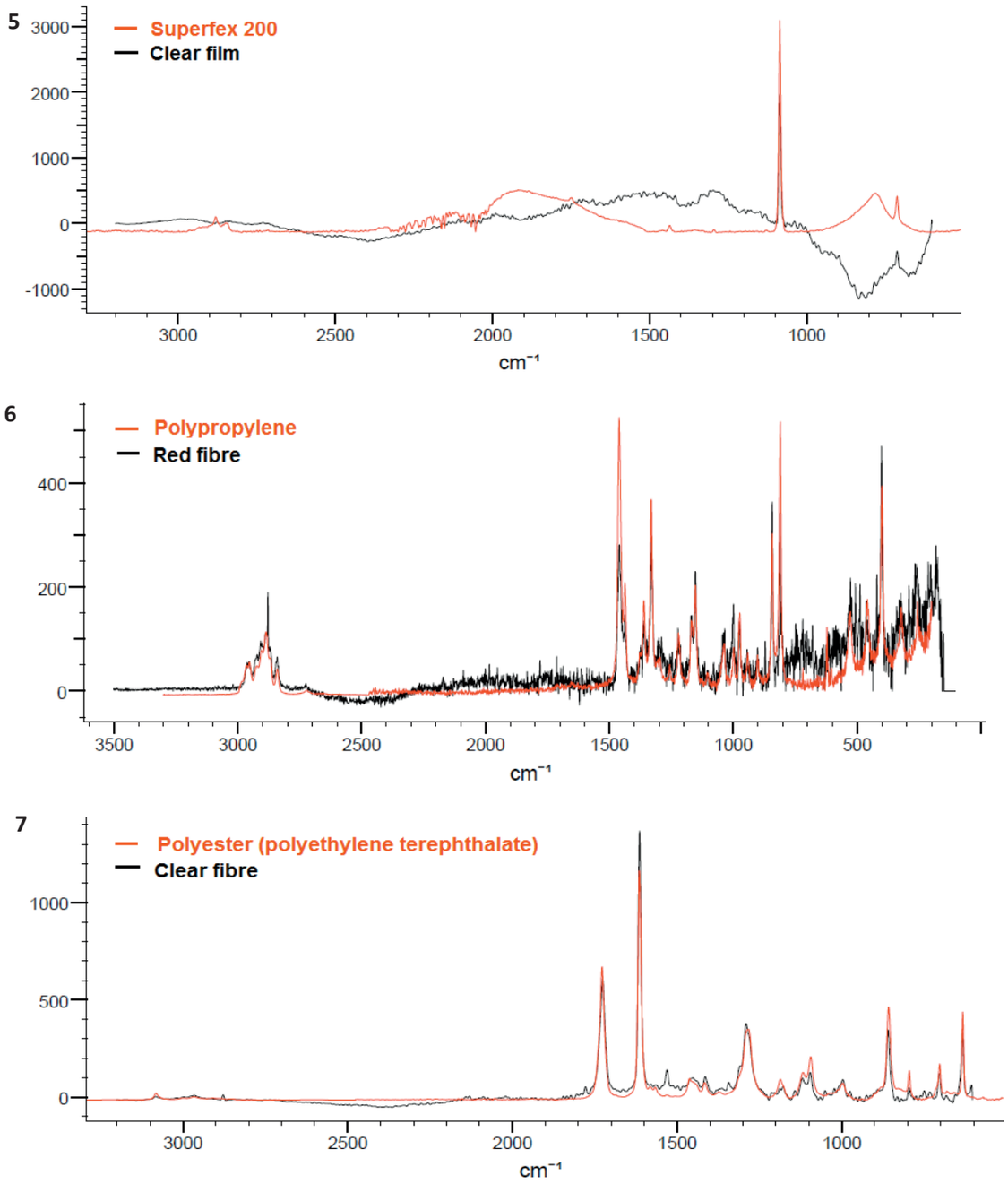


Fig S2. Spectra of identifiable particles and matched compounds using BioRad KnowItAll® Informatics System - Raman ID Expert (2015) software. Further particle information is available in table S3.

Table S3. Information associated with particles identifiable using Raman spectroscopy

Spectrum	Particle type	Particle colour	From (location)	% match	Substance name	Classification
1	Fibre	Clear	Caversham-Sonning	92	Polypropylene	Polymer
2	Fibre	Brown/black	Caversham-Sonning	87	Neolan Green	Dyestuff
3	Fibre	Clear	Sunbury-Molesey	97	Polyester	Polymer
4	Film	Clear	Shepperton-Sunbury	88	Superfex 200	Fluoropolymer
5	Fibre	Red	Shepperton-Sunbury	82	Polyethylene	Polymer
6	Fibre	Red	Castle Eaton	90	Polypropylene	Polymer
7	Fibre	Clear	Cricklade	95	Polyester	Polymer

CHAPTER 5

Acute toxicity of organic pesticides to *Daphnia magna* is unchanged by co-exposure to polystyrene microplastics

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CHAPTER 5

Acute toxicity of organic pesticides to *Daphnia magna* is unchanged by co-exposure to polystyrene microplastics

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Abstract

Daphnia magna were exposed to two pesticides in the presence or absence of microplastics (300 000 particles ml^{-1} 1 μm polystyrene spheres) and to microplastics alone. The pesticides were dimethoate, an organophosphate insecticide with a low log Kow, and deltamethrin, a pyrethroid insecticide with a high log Kow. *Daphnia* were exposed to a nominal concentration range of 0.15, 0.31, 0.63, 1.25, 2.5, 5 mg l^{-1} dimethoate and 0.016, 0.08, 0.4, 2, 5 and 10 μg l^{-1} deltamethrin. Exposure to polystyrene microplastics alone showed no effects on *Daphnia magna* survival and mobility over a 72 hour exposure. In the dimethoate exposures, mobility and survival were both affected from a concentration of 1.25 mg l^{-1}, with effects were seen on mobility from 28 hours and survival from 48 hours, with greater effects seen with increasing concentration and exposure time. In deltamethrin exposures, survival was affected from a concentration of 0.4 μg l^{-1} and mobility from a concentration of 0.08 μg l^{-1}. Effects of deltamethrin on mobility were seen from 5 hours and on survival from 28 hours, with greater effects on survival and mobility seen with increasing concentration and exposure time. Contrary to expectations, pesticide toxicity to *Daphnia magna* was not affected by the presence of microplastics, regardless of chemical binding affinity (log Kow). This therefore suggests that polystyrene microplastics are unlikely to act as a significant sink, nor as a vector for increased uptake of pesticides by aquatic organisms.

1. Introduction

Microplastics are a pollutant of increasing environmental concern based on their ubiquitous and persistent nature. It is widely recognised that microplastics will form biological and chemical associations within the environment. For example microplastics may become associated with algae or bacteria (biofilms) (Hoellein et al., 2016; McCormick et al., 2014) or may sorb organic chemicals due to their hydrophobic nature (Bakir et al., 2012; Koelmans et al., 2016; Mato et al., 2001). The potential for association of hydrophobic organic chemicals (HOCs) with microplastics has been recognised and has prompted studies on whether this association will affect the bioavailability of HOCs, and thus their toxicity to organisms. Studies have shown that microplastics can make HOCs either more bioavailable, by acting as a vector for uptake following ingestion (Avio et al., 2015a; Chen et al., 2017; Rochman et al., 2013c), or less bioavailable due to strong irreversible binding of HOCs to microplastics, removing HOCs from solution and remaining bound even if ingested (Beckingham and Ghosh, 2016). It has even been suggested that microplastics may lead to the removal of HOCs from body tissues following the ingestion of clean plastics by a previously contaminated organism (Koelmans et al., 2013). The majority of studies on microplastics and chemical associations to date have focussed on the marine environment. However, concentrations of HOCs and microplastics in continental terrestrial and freshwater environments are expected to be higher than marine environments due to their proximity to the sources combined with limited dispersal and dilution, thus highlighting the importance of studying terrestrial and freshwater systems (Dris et al., 2015b; Horton et al., 2017b).

The capacity for a chemical to bind to microplastics is, among other factors, determined by its hydrophobicity, usually expressed as the log Kow value. Kow represents the partition coefficient between octanol and water (Brooke, 2014). A chemical with a high log Kow will have a lower water solubility than less hydrophobic substances (with a lower log Kow), meaning that it will preferentially bind to organic particulate matter within the system rather than remaining within solution (Lee et al., 2014; Mackay et al., 1980). It is therefore expected that a chemical with a high log Kow (high hydrophobicity) will also have a higher affinity for binding to microplastics in an aqueous system than a chemical with a lower log Kow (higher hydrophilicity) (Wang et al., 2018b). Such interactions can potentially remove the chemical from solution and concentrate it on the surface of the plastic, thereby changing bioavailability (Gouin et al., 2011; Lee et al., 2014; Velzeboer et al., 2014). The aim of this study was therefore to investigate how the presence of microplastics would affect the toxicity of high and low log

Kow organic pesticides to a relevant freshwater organism, the cladoceran *Daphnia magna*. Pesticides were chosen as their toxicity is well-documented. The starting hypothesis was that the presence of microplastics within an aquatic solution would reduce the toxicity of a pesticide with a high log Kow, due to its high binding capacity to the microplastics making it less bioavailable (Beckingham and Ghosh, 2016; Koelmans et al., 2013), whereas the toxicity of a low log Kow pesticide would be less affected by the presence of microplastics.

2. Materials and methods

2.1. The test chemicals

We chose two pesticides to represent chemicals with high and low log Kow, both with known toxicity to *Daphnia magna*. Dimethoate and deltamethrin were chosen both for their differing chemical properties (specifically log Kow) and because they are environmentally relevant, being representative of two widely used classes of insecticides. Both pesticides target receptors associated with nervous system function to cause neurotoxicity. Dimethoate is an organophosphate insecticide with a low log Kow (0.704) (Pesticide Properties Database, 2017b). It is relatively soluble in water (between 23.5-39.8 g l^{-1} at 25°C) (Pesticide Properties Database, 2017b; Sigma-Aldrich, 2017). It was first registered for use in 1962 and is still widely applied to agricultural land worldwide (Van Scoy et al., 2016). Deltamethrin is a pyrethroid insecticide also widely used in agriculture (Ren et al. 2009) and aquaculture (Ernst et al. 2014). Deltamethrin is very poorly soluble in water, with a solubility between 0.2-2 µg l^{-1} at 25°C (Mestres and Mestres, 1992; Pesticide Properties Database, 2017a). Due to this hydrophobic nature, with a log Kow reported between 4.6 (Kaneko, 2010) and 6.2 (PubChem Compound Database, 2017), deltamethrin entering a water body would be expected to adsorb readily to particulate matter such as microplastics, in addition to sediment and organic matter (Lee et al., 2014; Lee et al., 2002).

2.2. The test organism

Daphnia magna is commonly used for ecotoxicological testing and as such, toxicity data are readily available for *D. magna* for both deltamethrin and dimethoate toxicity (Andersen et al., 2006; Toumi et al., 2013), as well as information on microplastic uptake and toxicity (Besseling et al., 2014; Jemec et al., 2016; Rehse et al., 2016). This makes them an ideal species for

investigating how toxicity may be influenced by the interaction of these pesticides with microplastics.

D. magna were taken from the Leiden University culture which has been continuously maintained for over six years in the laboratory. According to the OECD guideline 202, *D. magna* were cultured in glass containers with Artificial ElendtM4 medium at a density of 1 individual/10 ml of ElendtM4 medium (OECD, 2004). The culture medium was refreshed twice a week. The test organisms were fed *ad libitum* with *Raphidocelis subcapitata* algae and maintained inside a temperature-controlled chamber (20 ± 1 °C) under a 16:8 light-dark cycle. Throughout the duration of culturing, sensitivity of the test species was checked every six months using the standardized toxicity test conducted with $K_2Cr_2O_7$ as a reference compound (OECD, 2004).

2.3. Preparation of the microplastic beads

Microplastics as fluorescent polystyrene beads were purchased from Phosphorex (USA) with a nominal size of 1 µm, as a solution containing DI water, an anti-microbial agent (sodium azide) and a surfactant (Tween 20). The size of particles was confirmed by TEM as being 1.2 ± 0.2 µm (mean ± SD) (Fig S1). Previous experimental studies have shown that microplastics within the size range 20 nm – 5 µm are commonly ingested by *D. magna*, as they represent a similar size range as their common algal food sources (Besseling et al., 2014; Ogonowski et al., 2016; Rehse et al., 2016; Rist et al., 2017; Rosenkranz et al., 2009). Both sodium azide and Tween 20 may act as toxicants and so the beads were washed in order to remove these from the solution used for microplastic spiking. For washing, the supplied stock of beads (1 ml) was diluted to approximately 12 ml with Milli-Q water, vortexed to mix and then centrifuged at 5180 g (5000 rpm) (Beckman Coulter Avanti J-E centrifuge, USA) for 5 minutes. The supernatant was then carefully pipetted leaving approximately 1 ml of solution containing the particles at the bottom. These cleaning steps of dilution and centrifuging were then repeated twice more to ensure maximum removal of the sodium azide and Tween20. Following the final cleaning step the solution was diluted with Milli-Q water to give a total stock solution volume of 10 ml. The number of beads per ml of this new bead stock was measured using a flow cytometer (BD Accuri C6, BD Biosciences, USA). This bead stock was used for spiking the test medium to a nominal concentration of 300 000 particles ml^{-1}. This concentration is roughly equivalent to the number of algal cells that daphnids would be exposed to in an excess food

situation (*i.e.* under culture conditions) and equates to approximately 0.29 µg ml^{-1} (287.7 µg l^{-1}, calculations in SI).

2.4. Preparation of the test solutions

A dimethoate (PESTANAL®, analytical standard, Sigma Aldrich Ltd, UK) stock solution of 1 g l^{-1} was prepared directly in Elendt artificial freshwater. In order to produce the required concentrations, the appropriate amount of stock solution was made up to 250 ml with Elendt artificial freshwater. Based on toxicity values of dimethoate to *D. magna*, with 48 h LC_{50} ranging from 0.86-2 mg l^{-1} (Beusen and Neven, 1989; Syberg et al., 2008), exposure concentrations were made in the range 0.156, 0.313, 0.625, 1.25, 2.5, 5 mg l^{-1} (0.68, 1.36, 2.73, 5.45, 10.9, 21.8 µM) .

To spike the test medium with deltamethrin it was necessary to dissolve it in a solvent carrier due to its low solubility in water. Deltamethrin (certified reference material, Sigma-Aldrich Ltd, UK) was dissolved in acetone to prepare a stock solution of 10 000 µg l^{-1}. A serial dilution of this stock, was made by further dilution in acetone to create a deltamethrin concentration series for spiking into artificial freshwater. A volume of 375 µl of the relevant stock was added to 250 ml Elendt artificial freshwater (giving an acetone concentration of 0.15 % within the exposure solution) in order to give the required exposure concentration range: 0.016, 0.08, 0.4, 2, 5 and 10 µg l^{-1} (0.03, 0.16, 0.79, 3.96, 9.9, 19.79 nM). These exposure concentrations were based on literature toxicity data for *D. magna* with 48 h LC_{50}s ranging from 0.038-0.45 µg l^{-1} (Ren et al., 2009; Xiu et al., 1989) and 24 h LC_{50}s ranging from 0.113-9.4 µg l^{-1} (Toumi et al., 2013; Xiu et al., 1989).

For both pesticides, treatments were prepared with and without microplastics. For the microplastic treatments, the polystyrene bead stock solution was added to the exposure solutions after the artificial freshwater had been spiked with the chemicals. The appropriate volume of stock solution (as determined using the flow cytometer) was added to a volume of 250 ml of spiked solution to give a nominal concentration of 300 000 particles ml^{-1}. Four replicates of 40 ml exposure solution held in 50 ml glass jars were prepared for each treatment. With an average particle size of 1.2 µm ± 0.2 µm, the average surface area of the microplastics within 40 ml was calculated as approx. 38-74 cm^2 dependent on variation in particle size (surface area calculations are in SI). This concentration of particles provides a comparable surface area to that of the glass vessel (40 ml water was calculated to cover approx. 63 cm^2 of

the internal surface area). Thus introduction of microplastics at this concentration effectively doubles the surface area available for chemical binding. Each jar was allowed to equilibrate for 24 hours before introduction of the organisms (Lee et al., 2002).

Control treatments consisted of artificial freshwater only (further referred to as 'control'), artificial freshwater with microplastics only (equal to microplastic concentrations in pesticide exposures: 300 000 particles ml^{-1}, further referred to as 'microplastic control'), artificial freshwater with acetone (0.1 %, further referred to as 'acetone control'), and artificial freshwater with both microplastics (300 000 particles ml^{-1}) and acetone (0.1%) (further referred to as 'microplastic and acetone control'). These solutions were made and distributed to glass jars 24 hours prior to introduction of daphnids as per pesticide treatments.

2.5. Acute Toxicity Tests

Following the equilibration period, five neonates (< 24 hours old) were added to each jar. Errors were made in some vessels with 4 neonates added to a vessel (4 vessels overall) or 6 neonates added to a vessel (3 vessels overall). This was taken into account during the data analysis. Jars were completely randomised throughout the exposure to avoid systematic bias. *Daphnia* were observed at 5, 8, 21, 28, 48 and 72 hours. To enable resuspension of any settled particles, each test jar was gently mixed at each observation point by drawing approx. 1-2 ml of exposure media in and out of a glass pipette three times. Aqueous pH was measured in one jar from each concentration at the beginning and the end of the test. The organisms were not fed for the duration of the experiment. Mortality was recorded as per OECD protocol 202 (OECD, 2004). Impaired mobility was also recorded at each time point. This was defined as an individual that was alive, as seen by the clear movement of limbs, but was not able to swim effectively *i.e.* swimming erratically or not swimming effectively in a forward direction, and additionally showing no response to gentle agitation with a glass pipette tip. Sub-lethal behavioural effects are commonly seen in organisms when testing pesticides with a neurotoxic mode of action (Desneux et al., 2007; Haynes, 1988; Sørensen et al., 1995).

2.6. Chemical analysis

Water samples for chemical analysis were taken (1 ml dimethoate, 2 ml deltamethrin) at 0, 24 and 72 hours after preparation of the solutions for deltamethrin treatments and 0 and 72 hours

for dimethoate treatments. Fewer dimethoate measurements were taken than for deltamethrin, as dimethoate was expected to be less complex in terms of chemistry, with concentrations not expected to change over time (Eichelberger and Lichtenberg, 1971; Roast et al., 1999). Samples were spun in 1ml glass tubes (2 tubes per sample) in a centrifuge at approx. centrifugation 6000 G (8000 rpm) for 5 minutes (Eppendorf 24-place Fixed-angle rotor, FA-45-24-11-HS) to remove microplastics and samples were subsequently stored in a fridge at 5°C in the dark prior to analysis. Three replicate samples were taken from a medium and a high nominal concentration for each chemical (0.625 and 5 mg l^{-1} dimethoate, 0.4 and 10 µg l^{-1} deltamethrin) at each of the above specified time points. Chemical analysis was carried out by Wageningen Environmental Research (Alterra), and full details of chemical sampling and analytical procedures are available in the Supplementary Information (SI).

2.7. Data analysis

To determine differences between treatments with and without microplastics at different time points for each chemical, survival frequency data for each chemical were analysed using a Chi-squared (χ^2) test (Microsoft Excel), where treatments without microplastics were the 'expected' and those with microplastics were the 'observed'. Mobility frequency data were analysed using Fisher's exact test (R statistical software) due to a number of zero values (no daphnids swimming normally) which would not be accurately represented using the χ^2. Both tests accounted for any odd numbers where too few or too many neonates had been added initially. Effects on survival and mobility with respect to chemical concentrations and time were evaluated using ANOVA for each endpoint and each chemical, with time points and concentrations considered as factors (R statistical software). A *post-hoc* Tukey HSD test was carried out to determine pairwise differences with time and concentration (R statistical software). Chemical data were analysed using ANOVA with time considered as a factor. A *post-hoc* Tukey HSD test was carried out to determine pairwise differences with time and nominal concentration (R statistical software).

Further analyses of the survival data over time were carried out using a process-based survival model. The model assumes that the toxicant must be first taken up in the organism before it can exert an effect. The kinetics are described with a one-compartment model and the effects is described with the 'stochastic death' model. The model is extensively described in Jager et al. (2006a) and Kooijman and Bedaux (1996). This model is accepted by the OECD (OECD,

2006), where an additional elaborate (mathematical) description can be found with examples of the use of the model. The model links exposure concentrations to a survival probability using three parameters for the whole time-course of the exposure (the No Effect Concentration (NEC): a threshold for toxic effects, the killing rate (k_r): a measure for the toxic potency of the compound, and the elimination rate (k_e) as a kinetic parameter).

Parameter values for dimethoate were calculated using the known (measured) chemical exposure concentrations and the survival data. The parameter values were subsequently compared to independent values obtained from literature for verification. For deltamethrin, the uncertainties related to the actual exposure concentrations prompted a 'reverse modelling' approach. Literature toxicity values for deltamethrin to *D. magna* (Xiu et al., 1989) were used to derive the model parameters, which were subsequently used to fit the model output to the survival data, allowing back-calculation of actual exposure concentrations (further details on this approach are available in the SI). The benefits of including the modelling are threefold: 1) to validate the results of the traditional statistical analysis, 2) to calculate the actual concentrations of pesticides that the *Daphnia* are exposed to and 3) to determine toxicity effects over time, allowing for extrapolation of toxicity estimates beyond the timeframe of the experiments. Together, these benefits allowed us to better understand the dynamics of toxicity within the experiment.

3. Results

3.1. Daphnia survival

Daphnia survival in the controls without microplastics or chemicals, and in the acetone controls, was 100%. This high control survival validates the criteria of the toxicity test according to OECD guidelines for *Daphnia magna* acute toxicity testing (OECD, 2004). Microplastics alone did not affect survival over the 72 hour test period with only one mortality in the microplastic control treatment (5%) after the 72 hour exposure period and 100% survival in the microplastics and acetone control treatments. While it may be the case that some particles could have aggregated and therefore were not within an edible size range for *D. magna*, without the use of a microscope, microplastics were clearly visible within the guts of daphnids as a white mass indicating that ingestion did occur during the exposure.

There was a significant effect of pesticide exposure concentration on survival ($p < 0.01$ for both pesticides, ANOVA). There were also a significant effect of exposure time on survival (p

< 0.01 for both pesticides, ANOVA) and a significant interaction between concentration and time also occurred ($p < 0.01$ for both pesticides, ANOVA). Over the 72 h exposure, significant effects were seen on survival at exposure concentrations above 1.25 mg l^{-1} for dimethoate ($p < 0.01$, ANOVA + Tukey HSD) and above 0.4 µg l^{-1} for deltamethrin ($p < 0.05$, ANOVA + Tukey HSD). When considering time, significant effects on survival were seen from 48 hours in dimethoate treatments above 2.5 mg l^{-1} ($p < 0.01$, ANOVA + Tukey HSD, Table 1a) and from 28 hours in deltamethrin treatments above 2 µg l^{-1} ($p < 0.01$, ANOVA + Tukey HSD, Table 2a). For both pesticides there was no significant difference in the survival of organisms based on the presence or absence of microplastics ($p > 0.05$ at every time point, χ^2) To give a visual representation of this similarity, the survival and mobility probability was calculated and the deviance between treatments with and without microplastics depicted (Figs. 1a and 2a). Deviance was calculated as the difference in survival (or mobility) probabilities for treatments without MPs (– MP) vs. those with MPs (+ MP) at given concentrations.

3.2. Daphnid mobility

There were also concentration-dependent effects on daphnid mobility. There was a significant effect of pesticide exposure concentration on mobility ($p < 0.01$ for both pesticides, ANOVA). There were also a significant effect of exposure time on mobility for both chemicals ($p < 0.01$ for both pesticides, ANOVA) and a significant interaction between concentration and time also occurred for both chemicals (ANOVA, $p < 0.01$ for both chemicals). Over the 72 h exposure, significant mobility impairment was observed in *Daphnia* exposed to dimethoate at concentrations of 1.25 mg l^{-1} and above ($p < 0.01$, ANOVA + Tukey HSD). Similarly, *Daphnia* exposed to 0.08 µg l^{-1} deltamethrin and above suffered significant mobility impairment ($p < 0.05$, ANOVA + Tukey HSD). When considering time, significant effects on mobility were seen from 21 hours for dimethoate at 5 mg l^{-1} ($p < 0.01$, ANOVA + Tukey HSD, Table 1b) and from 5 hours for deltamethrin at 10 µg l^{-1} ($p < 0.01$, ANOVA + Tukey HSD, Table 2b). The presence of microplastics resulted in no significant difference in the number of daphnids suffering impaired mobility for either chemical at any time point ($p > 0.05$, Fisher's exact test). As for survival, plots for deviance were created to give a visual representation of this similarity using deviance in probability of normal mobility of treatments with vs. without microplastics (Figs 1b and 2b). Effects on mobility were seen at earlier time points than effects on survival, as would be expected given that sublethal behavioural effects are a precursor to mortality.

Table 1. Survival probabilities (Table 1a) and probabilities of normal mobility (Table 1b) for *D. magna* exposed to dimethoate at each concentration and time point, calculated by dividing the remaining surviving neonates by the original 20 to give a probability between 0-1.

Table 1a)

Dimethoate exposure concentration (mg l^{-1})		Time (h) 0	5	8	21	28	48	72
0	**Without MP**	1	1	1	1	1	1	1
0	**With MP**	1	1	1	1	1	1	1
0.156	**Without MP**	1	1	1	1	1	1	1
0.156	**With MP**	1	1	1	1	1	0.9	0.8
0.313	**Without MP**	1	1	1	1	1	1	1
0.313	**With MP**	1	1	1	1	1	0.6	0.5
0.625	**Without MP**	1	1	1	1	1	1	1
0.625	**With MP**	1	1	1	1	1	0.9	0.6
1.25	**Without MP**	1	1	1	1	1	1	0.6
1.25	**With MP**	1	1	1	1	1	0.9	0.7
2.5	**Without MP**	1	1	1	0.8	0.8	0.4	0
2.5	**With MP**	1	1	1	1	1	0.6	0
5	**Without MP**	1	1	1	0.7	0.7	0.2	0.1
5	**With MP**	1	1	1	0.9	0.8	0.2	0

Table 1b)

Dimethoate exposure concentration (mg l^{-1})		Time (h) 0	5	8	21	28	48	72
0	**Without MP**	1	1	1	1	1	1	1
0	**With MP**	1	1	1	1	1	1	1
0.156	**Without MP**	1	1	1	1	1	1	1
0.156	**With MP**	1	1	1	1	1	1	0.8
0.313	**Without MP**	1	1	1	1	1	1	1
0.313	**With MP**	1	1	1	1	1	0.8	0.4
0.625	**Without MP**	1	1	1	1	1	1	1
0.625	**With MP**	1	1	1	1	1	0.9	0.5
1.25	**Without MP**	1	1	1	1	1	0.9	0.4
1.25	**With MP**	1	1	1	1	1	0.7	0.6
2.5	**Without MP**	1	1	1	1	0.5	0	0
2.5	**With MP**	1	1	1	0.9	0.7	0.2	0
5	**Without MP**	1	1	1	0.7	0.3	0	0
5	**With MP**	1	1	1	0.4	0.4	0	0

Table 2. Survival probabilities (Table 2a) and probabilities of normal mobility (Table 2b) for *D. magna* exposed to deltamethrin at each concentration and time point, calculated by dividing the remaining surviving neonates by the original 20 to give a probability between 0-1.

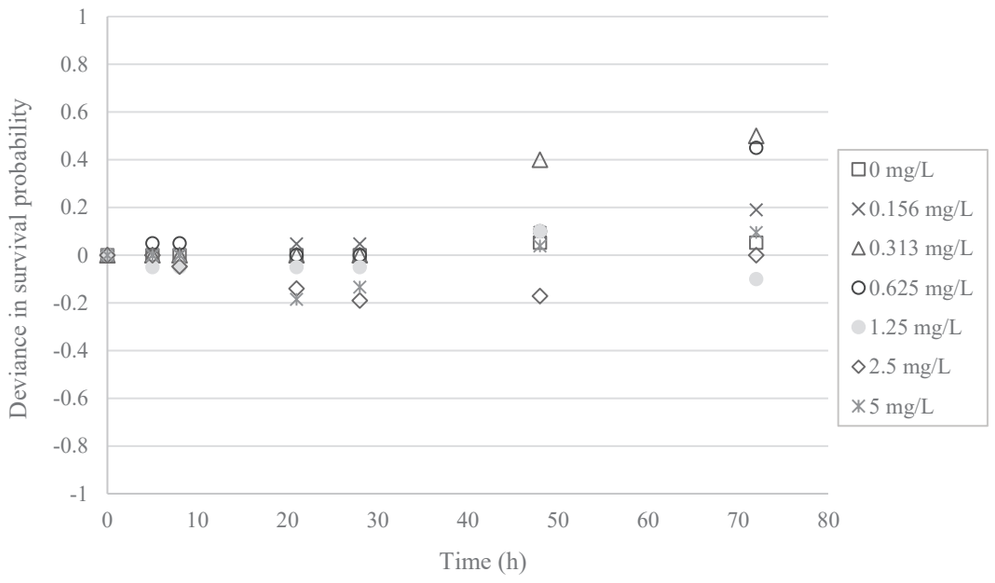
Table 2a)

Deltamethrin exposure concentration (µg l^{-1})		Time (h) 0	5	8	21	28	48	72
0	**Without MP**	1	1	1	1	1	1	1
0	**With MP**	1	1	1	1	1	0.9	1
0.016	**Without MP**	1	1	1	1	1	1	1
0.016	**With MP**	1	1	1	1	1	1	1
0.08	**Without MP**	1	1	1	1	1	1	0
0.08	**With MP**	1	1	1	1	1	0.9	0
0.4	**Without MP**	1	1	1	0.9	1	0.9	0
0.4	**With MP**	1	1	1	1	0.9	0.7	0
2	**Without MP**	1	1	1	0.9	0.7	0.5	0
2	**With MP**	1	1	1	1	0.7	0.6	0
5	**Without MP**	1	1	1	0.7	0.7	0.2	0
5	**With MP**	1	1	1	0.8	0.8	0.5	0
10	**Without MP**	1	1	1	1	0.7	0.3	0
10	**With MP**	1	1	1	1	0.6	0.2	0

Table 2b)

Deltamethrin exposure concentration (µg l^{-1})		Time (h) 0	5	8	21	28	48	72
0	**Without MP**	1	1	1	1	1	1	1
0	**With MP**	1	1	1	1	1	0.9	1
0.016	**Without MP**	1	1	1	1	1	1	1
0.016	**With MP**	1	1	1	1	1	1	1
0.08	**Without MP**	1	1	1	0.9	1	0.7	0
0.08	**With MP**	1	1	1	1	1	0.6	0
0.4	**Without MP**	1	1	1	0.7	0.4	0.2	0
0.4	**With MP**	1	1	1	0.7	0.3	0	0
2	**Without MP**	1	0.9	0.8	0.1	0.1	0	0
2	**With MP**	1	1	0.8	0.1	0	0	0
5	**Without MP**	1	0.9	0.7	0.1	0.1	0	0
5	**With MP**	1	1	0.6	0.1	0.2	0.1	0
10	**Without MP**	1	0.4	0.6	0.1	0	0	0
10	**With MP**	1	0.4	0.6	0.2	0	0	0

1a



1b

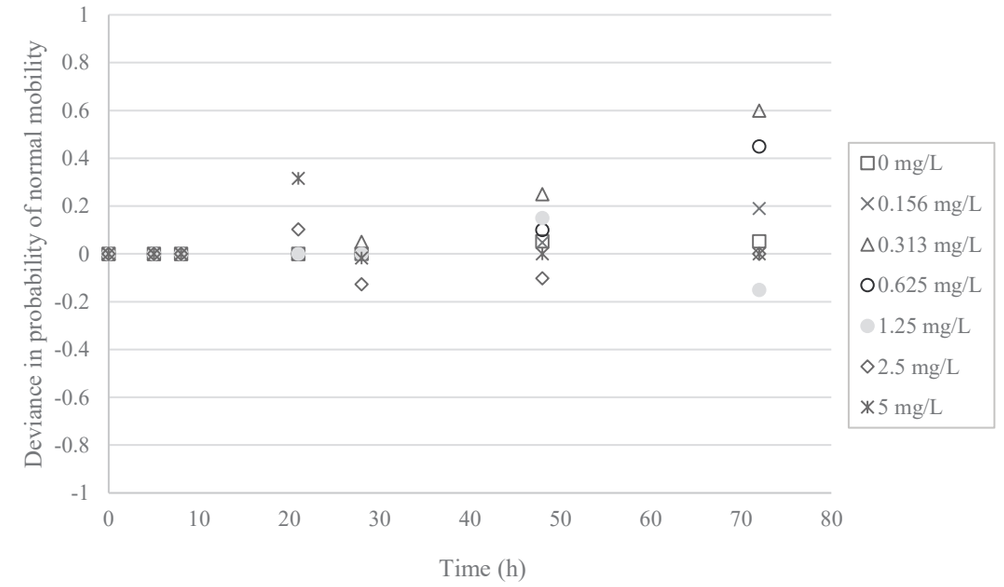
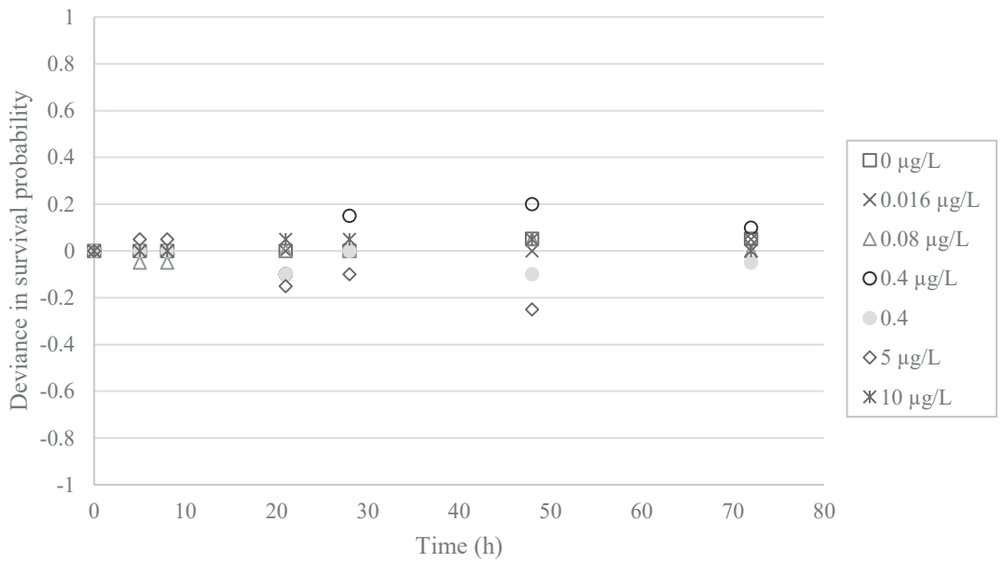


Fig. 1. Data for dimethoate showing 1a) a comparison of survival probabilities (the deviance in survival probability based on a ratio of survival probability without microplastics and with microplastics) and 1b) a comparison of normal mobility probabilities (calculated as for 1a). Deviations from 0 indicate the extent of the difference when microplastics were present. The closer to 0, the more similar the data. Full survival and mobility probability values for dimethoate are presented in Tables 1a and 1b respectively.

2a



2b

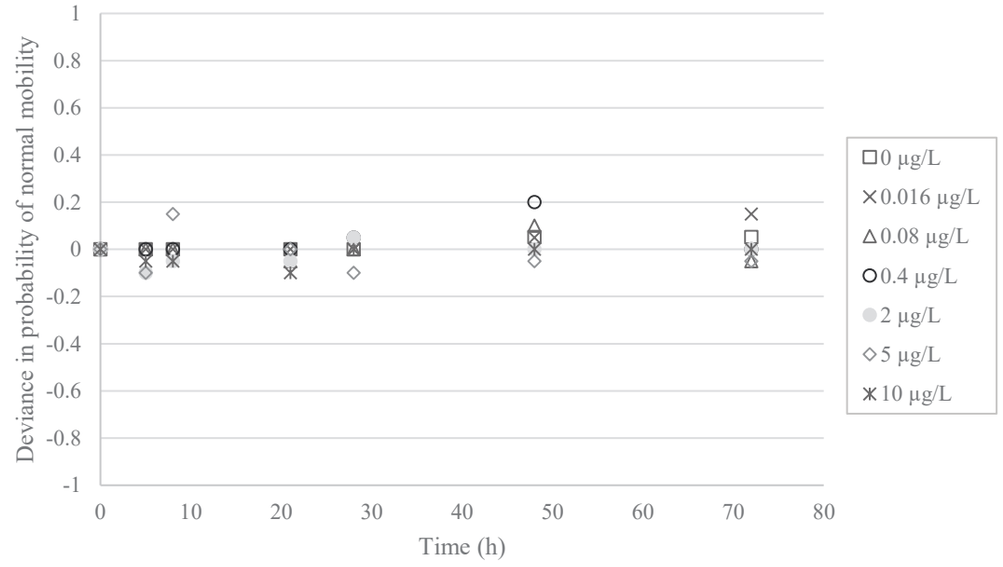


Fig. 2. Data for deltamethrin showing 2a) a comparison of survival probabilities (the deviance in survival probability based on a ratio of survival probability without microplastics and with microplastics) 2b) a comparison of normal mobility probabilities (calculated as for 2a). Deviations from 0 indicate the extent of the difference when microplastics were present. The closer to 0, the more similar the data. Full survival and mobility probability values for deltamethrin are presented in Tables 2a and 2b respectively.

3.3. Chemical concentrations

The pH remained consistent throughout the test with a mean pH of 7.81 (± 0.17 SD) across treatments at 0 hrs and 7.9 (± 0.05 SD) at 72 hours.

All measured dimethoate concentrations were lower than the nominal concentrations, ranging from (average) 59-63% of nominal values, although this difference was not significant ($p > 0.05$, t-test, Table S1). Measured concentrations of dimethoate did not vary significantly over time ($p > 0.05$, ANOVA) and there was no effect of microplastics on the measured concentrations of dimethoate ($p > 0.05$, ANOVA) (Figs. 3a and 3b). There was no significant effect of microplastics on concentration over time (interaction $p > 0.05$, ANOVA).

There was a significant difference between nominal and measured deltamethrin concentrations ($p < 0.01$, t-test), with average measured concentrations ranging from 3.7-20.5% of the nominal concentrations (Table S2). Due to an apparent difference in trend between the low and high nominal concentrations measured (Figs. 4a and 4b), these were analysed separately to tease apart concentration-dependent effects. At the low nominal concentration (0.4 $\mu g\ l^{-1}$), there was no effect of microplastics or time on the measured concentrations (both $p > 0.05$, ANOVA), nor an interaction of time and microplastics ($p > 0.05$, ANOVA). At the highest nominal concentration (10 $\mu g\ l^{-1}$), both microplastics and time significantly influenced the measured concentrations, with concentrations lower when microplastics were present (both microplastics and time $p < 0.01$, ANOVA), and with an initial significant decrease in concentration up to 24 hours (0-24 h, $p < 0.01$, ANOVA + Tukey HSD, 24-72 h, $p > 0.05$, ANOVA + Tukey HSD). There was no significant effect of microplastics on concentration over time (interaction $p > 0.05$, ANOVA).

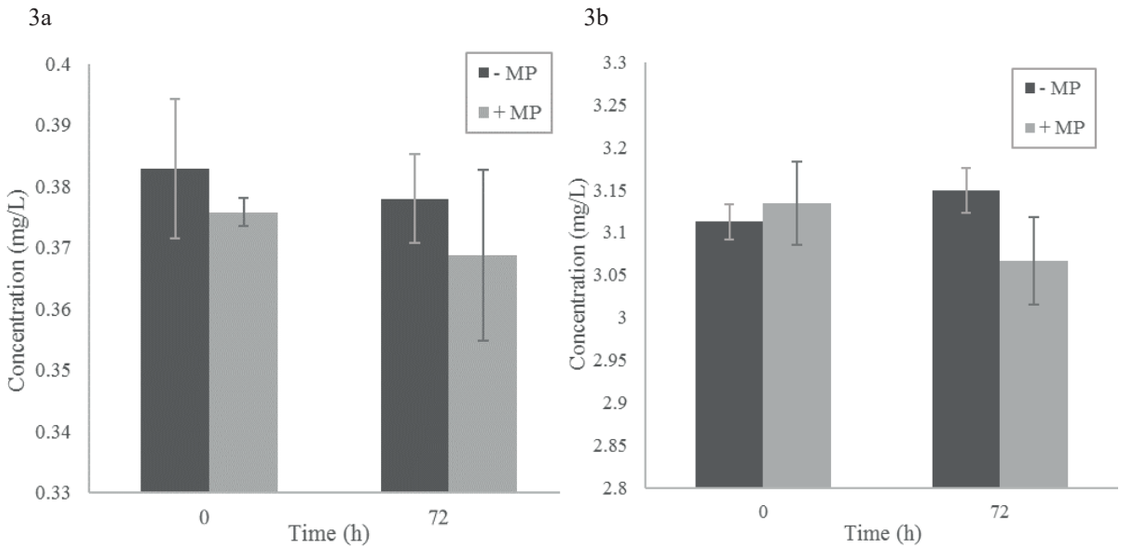


Fig. 3. Average measured concentrations based on three replicate samples of dimethoate (± SD) at different time points taken from treatments with nominal concentrations (a) 0.625 mg l^{-1} and (b) 5 mg l^{-1}, with or without microplastics, at each time point. '- MP' = no microplastics, '+ MP' = with microplastics.

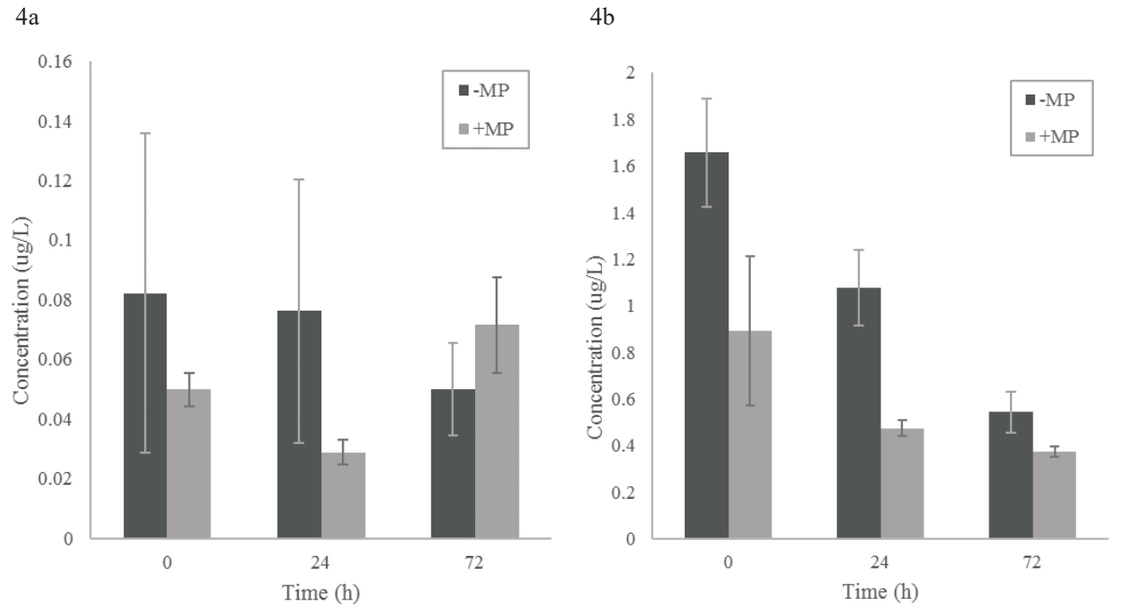


Fig. 4. Average measured concentrations based on three replicate samples of deltamethrin (± SD) at different time points taken from treatments with nominal concentrations (a) 0.4 µg l^{-1} and (b) 10 µg l^{-1} b, with or without microplastics, at each time point. '- MP' = no microplastics, '+ MP' = with microplastics.

3.4. Model analysis

Fitting of separate stochastic death models for both dimethoate and deltamethrin gave an estimation of toxicity over time at the experimental exposure concentrations and provided a consistent fit with the survival data (Figs. S2 and S3). For dimethoate, the model-derived LC_{50} was 0.5 mg l^{-1} (the full range of model-derived LC_x values for dimethoate available in Table S6). For deltamethrin, the model-derived LC_{50} was 0.023 µg l^{-1} (the full range of model-derived LC_x values for deltamethrin are available in Table S7). For both pesticides, the model shows no difference in pesticide exposure, or survival, with or without microplastics. For deltamethrin, using the reverse modelling approach, the survival data were used to determine the actual exposure concentrations as an indirect and complementary assessment of the measured concentrations (Table 3).

Table 3. Nominal concentration range of deltamethrin compared to modelled exposure concentrations and measured concentrations.

Nominal Concentration (µg l^{-1})	Nominal Concentration (nM)	Modelled Concentration (µg l^{-1})	Modelled Concentration (nM)	Measured Concentration (µg l^{-1})
0.016	0.03	0.012	0.024	-
0.08	0.16	0.03	0.06	-
0.4	0.79	0.04	0.079	0.05
2	3.96	0.08	0.16	-
5	9.9	0.08	0.16	-
10	19.79	0.09	0.18	0.40

The reverse modelling to predict actual exposure concentrations indicated that the concentrations in the three highest test treatments are more or less equal. This is likely governed by the solubility limit, which would therefore be around 0.08-0.09 µg l^{-1} (close to the reported value of 0.2 µg l^{-1} (Mestres and Mestres, 1992; Pesticide Properties Database, 2017a). The reported 48 h LC_{50} taken from literature that informed the parameters used for this model estimation was at the lower end of the scale: 0.038 µg l^{-1} (Xiu et al., 1989), compared to 0.32-0.63 µg l^{-1} reported by (Toumi et al., 2013), although is comparable to that reported in other studies (0.05-0.6 µg l^{-1} reported by (Day and Maguire, 1990). With higher input values the calculated exposure concentrations may have been higher.

4. Discussion

4.1. Biological effects

Although microplastics are commonly implicated in causing physiological damage to organisms, leading to reduced fitness and mortality (Lee et al., 2013; Rehse et al., 2016; Wright et al., 2013a), no microplastic-specific effects on mobility or survival were seen in this acute test, despite the high concentration of microplastics used and visual confirmation of ingestion. This result is in accordance with a number of other studies where high concentrations of microplastics were shown to cause no observable detrimental effects (Hämer et al., 2014; Kaposi et al., 2014; Weber et al., 2018b). Although other acute studies have measured subtle effects of exposure to microplastics that may have occurred, for example immune responses, gut blockage, reduced assimilation efficiency or reduced scope for growth (Blarer and Burkhardt-Holm, 2016; Cole et al., 2015; Jeong et al., 2016; Lo and Chan, 2018), these were beyond the scope of this study which was not planned to determine the effects of microplastics alone, but to determine whether the presence of microplastics influenced the toxic effects of pesticides.

Contrary to the hypothesis that microplastics would lead to a reduction in toxic effect of the high log Kow pesticide deltamethrin, the results showed no alteration in the acute toxicity of either deltamethrin or dimethoate to *D. magna*, regardless of the chemical binding capacity (log Kow) (Figs. 1 and 2, Tables 1 and 2). Mortality and mobility impairment increased with concentration and time for both pesticides, as expected, however the concentrations at which detrimental effects occurred were not influenced by the presence of microplastics. This is also highlighted by the results of the stochastic death modelling.

4.2. Linking biological effects to chemical exposure

The measured concentrations for deltamethrin were significantly lower than expected across all treatments, on average between 3.7-20.5 % the nominal concentration, depending on the time the sample was taken and the presence of microplastics (Fig. 3). Measured concentrations were highly variable, especially at the lower measured concentrations when microplastics were present (Fig. 4a). Additional replicate samples would have helped to reduce this variability and may have helped to clarify whether the lack of significance was simply due to high variability. However, regardless of the significant differences found in measured deltamethrin

concentrations between treatments with and without microplastics at higher concentrations (Fig. 4b), no differences in toxicity were observed. This highlights that the chemical dynamics within the system were complex and that while some binding of pesticides to microplastics may have occurred, this did not reduce the bioavailability of the two pesticides enough to lower the resulting observed toxicity. As predicted, there was no significant difference in water concentration with or without microplastics for dimethoate, supporting the lack of difference in the survival and mobility data, and no significant change in concentrations over time (Fig. 3). This difference between deltamethrin and dimethoate highlights that hydrophobicity of chemicals can influence binding and removal from solution, influencing different chemicals in different ways, however toxicity is more complex to predict.

Due to the high hydrophobicity of deltamethrin, it is likely that this pesticide bound strongly to both the glass vessel and the microplastic particles (where present) (Lee et al., 2002; Sethi et al., 2014; Wheelock et al., 2005). To overcome this, we introduced a 24 h equilibrium period following the suggestion made by Lee et al. (2002). Nonetheless it turned out extremely difficult to make accurate quantifications of the deltamethrin concentrations in water, as deltamethrin is also likely bind to organic matter including the *Daphnia* and any associated organic detritus or excreta. This means that, despite the 24 h equilibration phase, the equilibrium likely shifted when the *Daphnia* were introduced to the solution, highlighted by the significant reduction in concentration within the aqueous solution within the first 24 hours. This is a highly dynamic system and the equilibrium is likely to continue to shift over time leading the chemical to be associated with different substrates at different times. This highlights the complexity of working with deltamethrin, with binding, availability and ease of chemical extraction dependent on substrates available and methods used.

Due to the discrepancy between measured and nominal concentrations for deltamethrin, we were not able to directly relate toxicity to nominal or measured chemical concentrations. It was for these reasons that we carried out the reverse modelling approach to determine the likely exposure concentrations the *Daphnia* were exposed to (Table 3) and thus enable us to determine the toxicity of deltamethrin (SI). The model showed that, probably as a result of the limit of solubility of the hydrophobic insecticide, the top three concentrations of deltamethrin (nominal concentrations 2, 5, and 10 $\mu g\ l^{-1}$) were in fact likely to have been almost identical at 0.08-0.09 $\mu g\ l^{-1}$ (Table 3). This was reflected in the survival and mobility matrices showing survival and mobility to be comparable across the top three concentrations (comparing top three concentrations across survival and mobility, all $p > 0.05$ ANOVA + Tukey HSD, Table

2). This highest calculated exposure concentration was below the expected lower limit of solubility (0.2 µg l^{-1} at 25°C). This could be due to the combined effects of a lower temperature than stated for maximum solubility (experiments were run at 20°C ± 1°C) and additional dissolved constituents in the Elendt artificial freshwater, both of which may have led to a decreased capacity for dissolution.

Although the highest concentrations of deltamethrin used in this study were above solubility, the actual value for solubility is uncertain, reported between 0.2-2 µg l^{-1} (Mestres and Mestres, 1992). EC_{50} values for deltamethrin for effects on mortality and immobilisation in *D. magna* reported in the literature are highly variable, ranging from 0.11 to 9.4 µg l^{-1} at 24 h and 0.03 to 0.63 µg l^{-1} at 48 h (Toumi et al., 2013; Xiu et al., 1989). The highest of these values, particularly for the 24 h exposure time, hence are well above stated solubility. In this study, the modelled 96 h LC_{50} of 0.023 µg l^{-1} is in the same order of magnitude as the literature value of 0.01 µg l^{-1} calculated by Xiu et al. (1989), although it should be noted that their calculation was based on nominal concentrations. Many studies focus solely on nominal concentrations, not taking into account solubility or binding issues, while studies that do seek to determine concentrations find measured concentrations to be vastly reduced from nominal values (Lee et al., 2002; Toumi et al., 2013; Wheelock et al., 2005).

The modelling allowed us to compare the toxicity observed in this study to literature data (SI) and enabled us to develop a better understanding of the biological effects seen under given chemical and microplastics exposures. For dimethoate, measured concentrations were much closer to stated nominal concentrations, and were consistent over time. Model estimations for toxicity of dimethoate in this study based on the measured chemical data showed exposures to be comparable with or without microplastics, with our LC_{50} results shown to be comparable to literature values (SI).

4.3. Binding of pesticides to microplastics

Different polymers have different affinities for chemical binding and therefore may have differing propensities for altering the toxicity of associated chemicals. For example, it has been reported that polyethylene and polypropylene will have greater affinities for chemical sorption than polyvinyl chloride (PVC) or polyethylene terephthalate (PET) (Rochman et al., 2013b). Polystyrene has been suggested as having a lower affinity for hydrophobic chemical sorption than polyethylene, but higher than PVC (Wang and Wang, 2018). It is nonetheless recognised

that polystyrene will associate with hydrophobic organic chemicals within the environment (Liu et al., 2015; Rochman et al., 2013d). The concentration of polystyrene particles used in this experiment (300 000 particles ml^{-1}) is far above the concentrations that will likely be found within the freshwater environment (see Horton et al. (2017b) for an overview of freshwater microplastic studies), although this exposure level is within the range of other experimental studies using microplastics (Lu et al., 2016; Ogonowski et al., 2016; Rehse et al., 2016; Setälä et al., 2014). This study was therefore intended to give a representation of the possible effects of interactions between microplastics, pesticides and freshwater organisms in a scenario where microplastics were highly abundant.

The presence of microplastics would have provided an increased surface area available for chemical binding (in this instance the surface area of the microplastics was calculated to be approximately equivalent to that of the vessel, effectively doubling the surface area). Therefore a lower concentration of deltamethrin would have been expected in the water when microplastics were present. The chemical measurement results confirm this effect, as at the highest exposure concentration of deltamethrin (nominal concentration of 10 µg/l), water concentrations were significantly lower when microplastics were present (Fig. 4b). This implies that deltamethrin was binding to microplastics (inferred by a reduced concentration in water when compared to an equivalent nominal concentration without microplastics). However, it is important to note that despite the difference with and without microplastics at the highest concentration of deltamethrin (nominal concentration 10 µg l^{-1}), the reduced concentration in the presence of microplastics was not observed at the lower concentration measured (nominal concentration 0.4 µg l^{-1}) (Fig. 4a). In the higher nominal exposure levels (10 µg l^{-1}), the decline in measured concentration continues after the 24 h equilibration period highlighting the complex chemical dynamics within the solution, with the introduction of daphnia likely to alter the equilibrium. Questions remain surrounding the dynamics and kinetics of chemical behaviour and toxicity in relation to the presence of microplastics. However, as there were no significant effects on survival and mobility between microplastic and non-microplastic treatments in this study, these complex dynamics do not appear to affect the overall bioavailability, and as a result, acute toxicity of the chemicals.

4.5. Outlook

If effects are to be seen with respect to chemicals in association with microplastics, especially their facilitation of chemical uptake and toxicity, it is most likely that these would be seen under controlled laboratory conditions where uncontaminated organisms are exposed to contaminated plastics (of a size that enables ingestion), as opposed to in the environment where organisms will already have been exposed to a variety of different chemicals (Koelmans et al., 2016). This study was designed to enable optimum chemical binding and ingestion of microplastics by *D. magna*. Given the high concentration of microplastics in this study and, thus, the high surface area available for binding, an alteration in the bioavailability and toxicity of hydrophobic deltamethrin (high log Kow) would have been expected, whereas dimethoate (low log Kow) would be expected to be consistently bioavailable and toxic regardless of the presence of microplastics (Cole et al., 2011; Teuten et al., 2009). In contrast, our results show that there was no effect of microplastics on the response of daphnids to either of the two pesticides, despite the very different chemical characteristics. The vector effects, or so-called 'Trojan Horse' effects, as ascribed to microplastics (Rochman et al., 2014; Rochman et al., 2013d) were not observed. It is therefore unlikely that microplastics will exert short-term effects on pesticide toxicity under real field conditions where sediment and organic matter would compete with microplastics for binding of chemicals. Additionally, in areas highly polluted with pesticides or other organic chemicals, the presence of microplastics is unlikely to alter the availability of these pollutants (Tanaka et al., 2018). In terms of chemical toxicity associated with microplastics, it is feasible that plasticisers will pose a greater chemical risk to organisms than sorbed hydrophobic chemicals (Devriese et al., 2017; Lohmann, 2017). Although polymer, particle and chemical-specific, these data are a valuable contribution to the wider understanding of microplastic and chemical associations, and the complexities underlying these mechanisms.

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CHAPTER 5

SUPPLEMENTARY INFORMATION

S1. Area and mass calculations

S1.1. Surface area calculations

Particles were calculated using TEM as being 1.2 µm ± 0.2 µm in diameter (fig S1). Surface area was therefore calculated for particles of 1 µm and 1.4 µm to account for variation, using the equation:

$A = 4\pi r^2$ (equation 1)

Calculated surface area ranged from 3.14 μm^2 for a 1 µm particle and 6.15 μm^2 for a 1.4 µm particle (median 1.2 µm ± 0.2 µm). Given a concentration of 300 000 particles ml^{-1}, the number in 40 ml solution was approximately 12 000 000. This therefore gave a total particle surface area per vessel of between 37.7 cm and 73.9 cm.

The surface area of the inside of the vessel was calculated to be approximately 62.8 cm^2 based on a depth of 3.8 cm and a diameter of 4.2 cm when filled with 40 ml water.

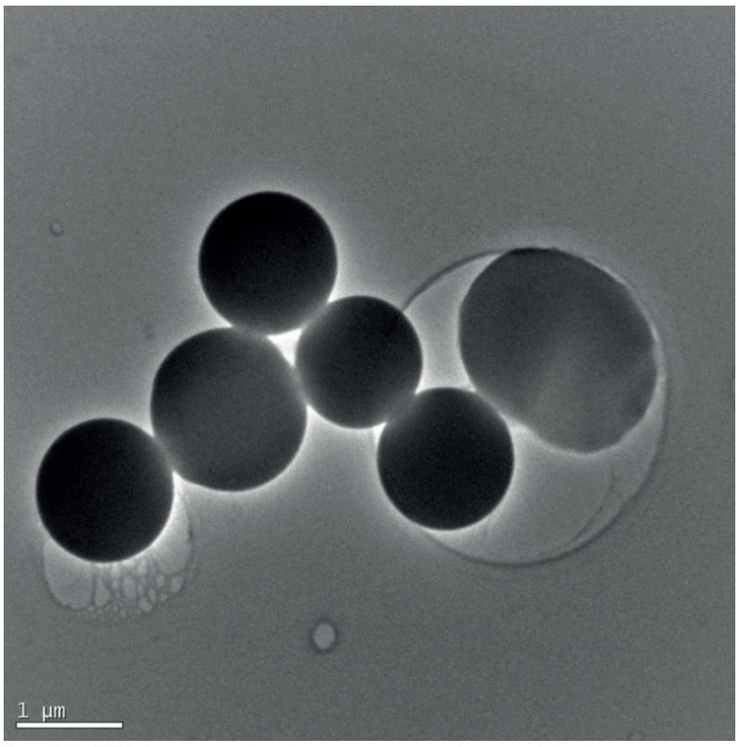


Fig. S1. TEM image of polystyrene particles used in the exposures.

S1.2. Particle mass calculations

Particle mass was calculated by taking the known particle density: 1.06 g cm^{-3}, and the mean particle radius: 0.6 µm (0.00006 cm). The volume of an individual sphere was calculated using the equation:

$V=4/3\ \pi r^3$ *(equation 2)*

This gave a particle volume of 9.05 x 10^{-13} cm^3. Volume was then multiplied by density to give the mass of one particle: 9.59 x 10^{-13} g (9.59 x 10^{-7} µg). This could then be multiplied by 300 000 to give the mass of particles per ml: 2.88 x 10^{-7} g ml^{-1} (0.29 µg ml^{-1}) and then by 1000 to give the mass of particles per l: 0.00029 g l^{-1} (287.7 µg l^{-1}).

S2. Chemical analysis methods

For the dimethoate treatments, 1 ml samples were taken from three replicate vessels of two different nominal concentrations (5 mg l^{-1} and 0.625 mg l^{-1}) at 0 and 72 hours and the microplastic treatments centrifuged as before. From the centrifuged microplastic samples, 800 µl was carefully pipetted into a glass vial to avoid resuspending the particles and 400 µl methanol added. The non-microplastic samples were not centrifuged and 500 µl methanol was added to the 1 ml sample. Vials were tightly sealed with a cap (phenolic cap with aluminium liner) and were then shaken well to mix.

For the deltamethrin treatments, 2 ml samples were taken from three replicate vessels of two different nominal concentrations (10 µg l^{-1} and 0.04 µg l^{-1}) at 0, 24 and 72 hours (based on times of daphnia exposure). Following removal, the microplastic samples were immediately spun in 1ml glass tubes (2 tubes per sample) in a centrifuge at approx. 6000 G (8000 rpm) for 5 minutes (Eppendorf 24-place Fixed-angle rotor, FA-45-24-11-HS) and the 1.6 ml (800 µl per tube) supernatant carefully pipetted off to avoid resuspending the particles. This was transferred to a glass vial and 1.6 ml hexane added. The non-microplastic samples were not centrifuged and 2 ml hexane was added to the 2 ml sample. The microplastic and non-microplastics samples were then treated the same by shaking the sample with the hexane vigorously for 1 minute in a glass vial tightly sealed with aluminium foil and parafilm and then pipetting 1.2 ml of the hexane fraction into a 2ml brown glass vial (Sigma Aldrich). Vials were tightly sealed with a cap (phenolic cap with aluminium liner, Sigma Aldrich).

All chemical samples were analysed at Wageningen Environmental Research (Alterra). The analytical method was developed at the laboratory of the Environmental Risk Assessment team.

Dimethoate samples were diluted 100 times with acetonitrile-ultrapure water by using a Dilutor Hamilton 600 series. The diluted samples were analysed using an Agilent LC-MS×MS suite (Agilent 6460 Triple Quad LC/MS) equipped with autosampler (Agilent G1329B), pump (Agilent G1311B (Quat. pump)), an ESI (+Agilent Jet Stream) source and a column thermostat (Agilent G1316A). The separation was performed in reverse phase LC (Column: Agilent Zorbax Eclipse XDB C18; 4.6 mm x 150 mm, 5 micron) under gradient elution of Eluents C (Milli-Q water (Advantage A10) + 0.1 % v/v formic acid) and Eluent D (Acetonitrile + 0.1 % formic acid). The initial composition of the mobile phase (40%:60%, C:D) was first held for 2 mins, then changed in 1 min to 20%:80% (C:D) (between 2 and 3 minutes run time), held for 3 minutes (between 3 and 6 minutes run time), changed back to the initial composition over 1 minute (between 6 and 7 minutes) and held there 1 more minute (between 7 and 8 minutes). The flow rate and column temperature were fixed at 0.7 $mL.min^{-1}$ and 35°C, respectively. Dimethoate retention time was ca. 2.5 minutes and was detected by monitoring the 230 m/z – 198.9 m/z transition (quantifier), qualified with additional peaks at m/z = 171 and 125. Injected samples were quantified by peak area using the calibration curve constructed from calibration standards included in the same sample sequence.

Deltamethrin was measured in the hexane extract by using an Agilent 6890 gas chromatograph equipped with an electron capture detector (ECD). Three microliters of the extract was injected via split injection and analysed in a wall-coated open tubular (WCOT) fused silica column (Varian CP Sil5) using He gas as the mobile phase. The oven temperature was programmed so that the initial temperature of 50°C was held for 7 minutes after which, the temperature was ramped at a rate of 50°C min^{-1} to a final temperature of 300°C minutes and held for 15:30 minutes. Retention time for deltamethrin was approximately 25.3 minutes. Injected samples were quantified by peak area using the calibration curve constructed from calibration standards included in the same sample sequence.

Table S1. Nominal and average measured concentrations (three replicate samples) for dimethoate treatments

Nominal concentration (mg l^{-1})	Microplastic treatment	Time point	Average measured concentration (mg l^{-1})	Standard deviation
0.625	NO	0	0.383	0.011
0.625	NO	72	0.378	0.007
0.625	YES	0	0.376	0.002
0.625	YES	72	0.369	0.014
5	NO	0	3.112	0.021
5	NO	72	3.149	0.027
5	YES	0	3.134	0.049
5	YES	72	3.067	0.051

Table S2. Nominal and average measured concentrations (three replicate samples) for deltamethrin treatments

Nominal concentration (µg l^{-1})	Microplastic treatment	Time point	Average measured concentration (µg l^{-1})	Standard deviation
0.4	NO	0	0.082	0.054
0.4	NO	24	0.076	0.044
0.4	NO	72	0.050	0.015
0.4	YES	0	0.050	0.006
0.4	YES	24	0.029	0.004
0.4	YES	72	0.072	0.016
10	NO	0	1.657	0.234
10	NO	24	1.077	0.161
10	NO	72	0.544	0.089
10	YES	0	0.892	0.322
10	YES	24	0.475	0.035
10	YES	72	0.375	0.021

S3. DEB modelling methods

S3.1. Modelling approach

The Stochastic Death model was used to model the data. This model is extensively described in the original paper by Kooijman and Bedaux (1996) and is accepted by the OECD (OECD, 2006). In addition, see Jager et al. (2011) for an extensive review on the different survival models.

The model needs three parameters to describe the whole time course of toxic effects:

1) No Effect Concentration (NEC): a toxicological threshold for effects
2) Killing rate (k_r): a measure for the toxicity of the compound
3) Elimination rate (k_e): a kinetic parameter determining the kinetics of the compound

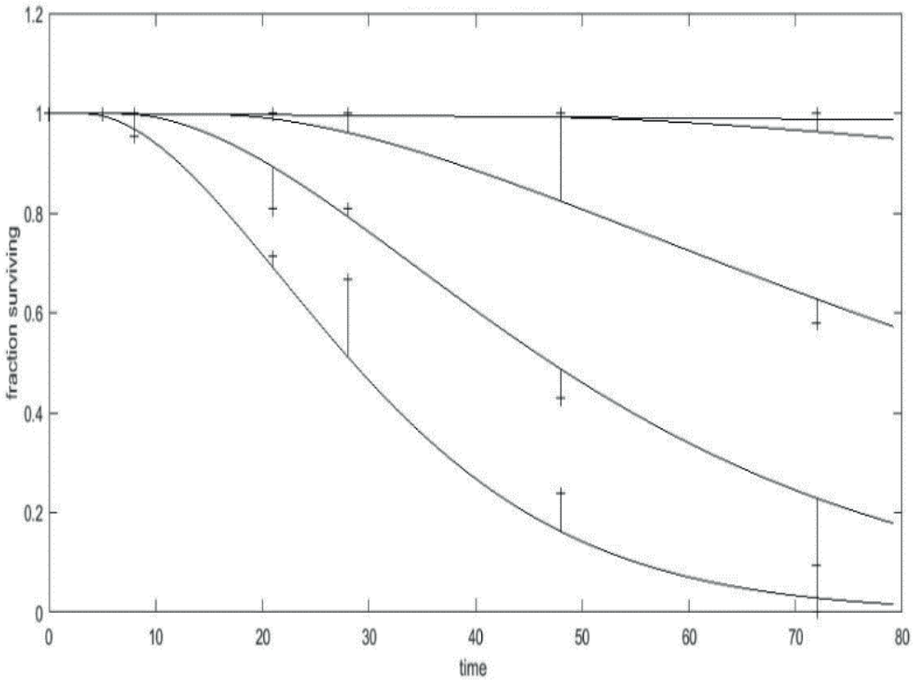
There is an additional parameter (the blank killing rate (BKR)) to take control mortality into account. The NEC is the most important parameter as this reflects the inherent sensitivity of the species for a toxicant. Usually this parameter is also the parameter value with the smallest confidence interval.

Parameter values can be estimated from the raw data of a survival experiment (e.g. Hesketh et al. (2016)), given multiple points in time, as the approach is basically a TK-TD approach. The model can also be used, if the parameter values are known, to back-estimate the exposure concentrations if the survival probabilities are taken from the experiments.

S3.1.1. Dimethoate

Actual concentrations were measured for two nominal concentrations (5 and 0.625 mg/L nominal) at the start of the exposure and at the end of the exposure (24 hrs and 96 hrs after preparing the exposure solutions). Concentrations were stable over the measurement period and there is a constant fraction of the nominal concentrations for the two measured concentrations (0.625 and 5 mg l^{-1}), this fraction equals 61% of the nominal concentrations both for treatments with and without microplastics. The exposure concentrations calculated based on measured values therefore gave a range of 0, 0.08, 0.15, 0.3, 0.6, 1.2, 2.4 mg l^{-1}. There appears to be no effect of the microplastics on the actual concentrations. This was the starting point for the parameter estimates. The results of the parameter estimates are summarised in Table S3 (all expressed in µ moles).

a



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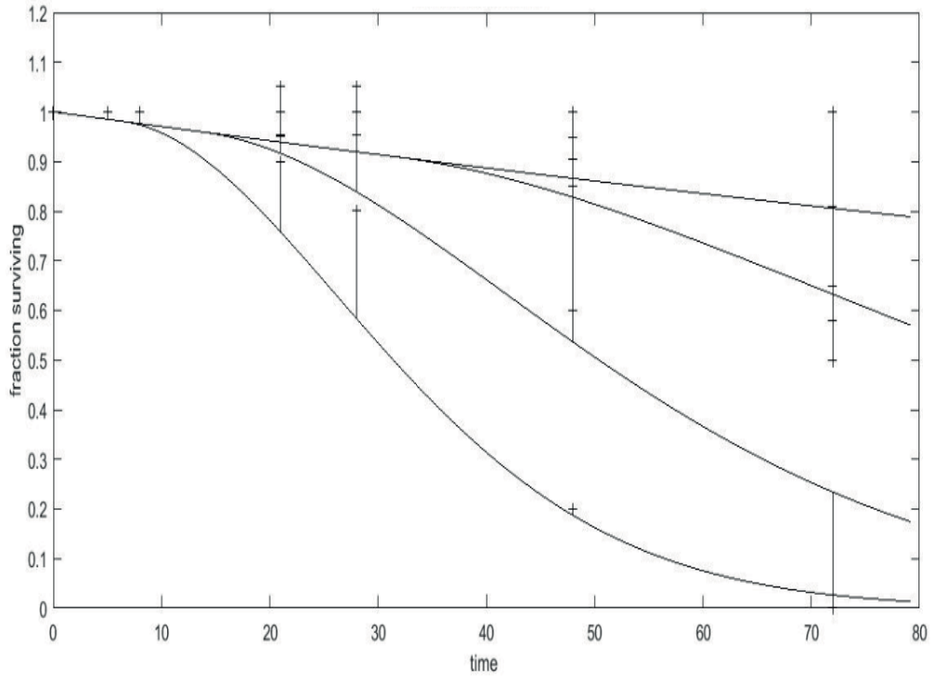


Figure S2. Model fit to dimethoate survival data (+ symbols). Each line represents a different concentration, although for visual clarity, some concentrations have been removed. Fig. S2a shows the model fit to the data without microplastics, fig. S2b shows the model fit to the data with microplastics.

The estimated parameter values are identical with and without microplastics (as could be expected as there are no differences in the survival matrices (see the results section of the main text). In addition, the value found for the No Effect Concentration in this research is in perfect agreement with an earlier estimate of 0.63 µM (Baas et al., 2016). LC_x values were calculated (Table S6) and compared to literature values (section S3.1.).

Table S3. Estimated parameter values for dimethoate with and without microplastics. Where present, numbers in brackets represent 95% confidence intervals.

Experiment	**BKR (hr^{-1})**	**NEC (mg l^{-1})**	**NEC (µM)**	**k_r (mg l^{-1} hr^{-1})**	**k_r (µM hr^{-1})**	**k_e (hr^{-1})**
Dimethoate without microplastics	1.7E-04	0.147 (0.101)	0.64 (0.44)	0.0053 (0.0039)	0.023 (0.017)	0.011 (0.009)
Dimethoate with microplastics	2.7E-03	0.105 (0.039)	0.46 (0.17)	0.023*	0.1*	0.004 (0.001)

* fixed in model

S3.1.2. Deltamethrin

As there was a large discrepancy between nominal and actual exposure concentrations for deltamethrin, the nominal chemical exposure concentrations cannot be used to inform the parameters of the model and obtain a reliable estimate of deltamethrin toxicity. We therefore needed to carry out reverse modelling based on known toxicity data, to allow us to estimate actual exposure concentrations and toxicity within our experiment. An independent estimate of the parameter values can be carried out if we have at least three LC_{50} values at different points in time that can be taken from the available literature. In the US-EPA ECOTOX database (US EPA, 2017) we can find 24, 48 and 96 hr LC_{50} values for *Daphnia magna* exposed to deltamethrin (most of the reported data contain only one point in time and are therefore of no use for a TK-TD approach). There is a significant range in the 48 hr LC_{50} values in different publications (Toumi et al., 2013; Xiu et al., 1989), but the numbers presented here (Table S4) are in line with the general picture that emerges from the database. With these values a NEC, killing rate and elimination rate could be derived (Table S5). From these parameters, a model was fit using survival over time (including 96 h, beyond the scope of the test) and thus

extrapolating to a realistic exposure concentration range (table 1). LC_x values were calculated (table S7) and compared to literature values as validation of the concentration measurements (section S3.2.).

Table S4. Toxicity data for daphnia exposed to deltamethrin over a 96 hour time period (Xiu et al., 1989)

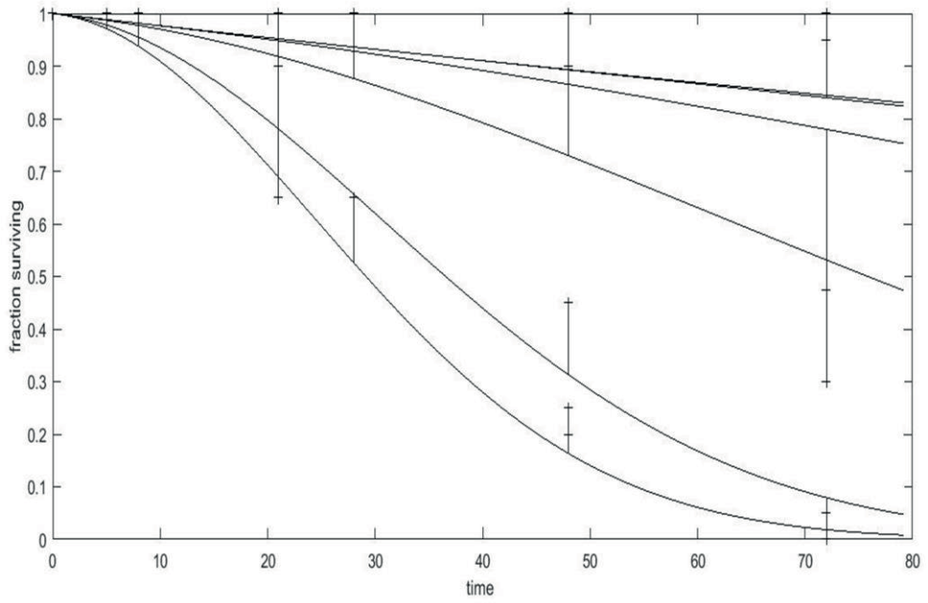
hr	**LC_{50} (ug l^{-1})**
24	0.13
48	0.038
96	0.01

Table S5. Estimated parameter values for deltamethrin.

Experiment	**BKR (hr^{-1})**	**NEC (ug l^{-1})**	**NEC (nM)**	**k_r (ug l^{-1} hr^{-1})**	**k_r (nM hr^{-1})**	**k_e (hr^{-1})**
Deltamethrin	1.7E-04	0.004	0.008	0.56	1.1	0.32

For the purposes of comparison to, and extrapolation from, other studies, for deltamethrin we can only focus on the data without microplastics. As the survival data shows no significant difference whether microplastics are present or not it is therefore reasonable to assume these are the same and therefore only one set of parameter values are presented (Table S5).

a



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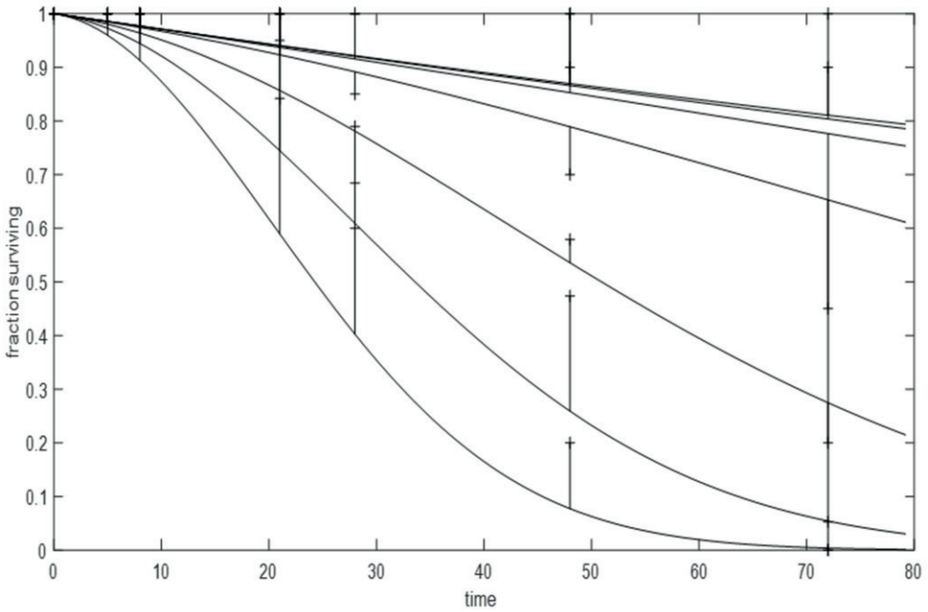

Fig. S3. Model fit to deltamethrin survival data (+ symbols). Each line represents a different concentration although for visual clarity, some concentrations have been removed. Fig. S3a shows the model fit to the data without microplastics, fig. S3b shows the model fit to the data with microplastics.

S4. Model-based LC_{50} values

S4.1. Dimethoate

The 48 h LC_{50} for dimethoate based on measured values was 1.22 mg l^{-1} which very closely resembles the 48 h LC_{50} value of 1.1 mg l^{-1} reported by Andersen et al. (2006). Beusen and Neven (1989) reported LC_{50} values of 1.7 and 2 mg l^{-1} for open and closed experimental systems respectively, values which are also very similar to our 48 h LC_{50}. Although all reported literature values are based on nominal concentrations, the limited difference between nominal and actual concentrations means these can be accurately compared.

Table S6. Modelled LC_x values for dimethoate at different time points based on calculated exposure concentrations.

LC_x (mg l^{-1})	Time (hr)			
	24	48	72	96
1	0.8	0.41	0.3	0.25
5	1.05	0.5	0.34	0.28
10	1.31	0.57	0.39	0.3
50	3.48	1.22	0.71	0.5
90	9.08	2.77	1.47	0.99

S4.2. Deltamethrin

The 48 h LC_{50} value of 0.046 µg l^{-1} as calculated by the model is comparable to the 48 h LC50 value of 0.12 µg l^{-1} reported on the deltamethrin safety data sheet (Sigma-Aldrich, 2017). The result is also within a similar range to that reported by Toumi et al. (2013) who calculated 48 h LC_{50} values of 0.32 µg l^{-1} and 0.63 µg l^{-1} based on measured concentrations, with variation dependent on the strain of *D. magna*. The modelled value for 96 h LC_{50} is 0.023 µg l^{-1}, which is in the same order of magnitude as the literature value of 0.01 µg l^{-1} calculated by Xiu et al. (1989). However these values should be treated with caution as these concentrations are approaching/exceeding the solubility limit of deltamethrin, and are often based on nominal concentrations.

Table S7. Modelled LC_x values for deltamethrin at different time points based on calculated exposure concentrations.

LC_x (µg l^{-1})	Time (hr)			
	24	48	72	96
1	0.024	0.015	0.012	0.011
5	0.032	0.018	0.014	0.012
10	0.040	0.021	0.016	0.013
50	0.118	0.046	0.029	0.023
90	0.321	0.109	0.064	0.046

Although 48 and 96 hour LC_{50}s for deltamethrin can be broadly compared to those of other studies, there is huge variability within the literature which suggests that determining LC_{50}s for deltamethrin is complicated, as solubility and LC_{50} can both be influenced by factors such as temperature, pH and vessel material.

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CHAPTER 6

Accumulation of polybrominated diphenyl ethers and microbiome response in the great pond snail *Lymnaea stagnalis* with exposure to nylon (polyamide) microplastics

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CHAPTER 6

Accumulation of polybrominated diphenyl ethers and microbiome response in the great pond snail *Lymnaea stagnalis* with exposure to nylon (polyamide) microplastics

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Abstract

Microplastics attract widespread attention, including for their potential to transport toxic chemicals in the form of plasticisers and associated hydrophobic organic chemicals, such as polybrominated diphenyl ethers (PBDEs). The aims of this study were to investigate how nylon (polyamide) microplastics may affect PBDE accumulation in snails, and the acute effects of nylon particles and PBDEs on survival, weight change and inherent microbiome diversity and community composition of the pond snail *Lymnaea stagnalis*. Snails were exposed for 96 hours to BDEs-47, 99, 100 and 153 in the presence and absence of 1% w/w nylon microplastics in quartz sand sediment. No mortality was observed over the exposure period. Snails not exposed to microplastics lost significantly more weight compared to those exposed to microplastics. Increasing PBDE concentration in the sediment resulted in an increased PBDE body burden in the snails, however microplastics did not significantly influence total PBDE uptake. Based on individual congeners, uptake of BDE 47 by snails was significantly reduced in the presence of microplastics. The diversity and composition of the snail microbiome was not significantly altered by the presence of PBDEs nor by the microplastics, singly or combined. Significant effects on a few individual operational taxonomic units (OTUs) occurred when comparing the highest PBDE concentration with the control treatment, but in the absence of microplastics only. Overall within these acute experiments, only subtle effects on weight loss and slight microbiome alterations occurred. These results therefore highlight that *L. stagnalis* are resilient to acute exposures to microplastics and PBDEs, and that microplastics are unlikely to influence HOC accumulation or the microbiome of this species over short timescales.

1. Introduction

Microplastics are a widely-recognised pollutant. The impacts of microplastics on biota and ecosystems, and their interactions with other environmental pollutants under various environmental conditions, are highly uncertain and existing studies have produced contradictory results (see discussions of expert committee summarised in the report published by SAPEA (2019)). Due to the high affinity of microplastic surfaces for hydrophobic organic chemicals (HOCs), there is potential for particles to sorb HOCs (Hirai et al., 2011; Karapanagioti et al., 2011; Rochman et al., 2013d), which may lead to elevated or reduced bioaccumulation of HOCs by organisms that ingest these microplastics (Bakir et al., 2016; Besseling et al., 2013; Rochman et al., 2013c). However, other studies have not found clear evidence for microplastics altering bioaccumulation or toxicity of HOCs (Ašmonaitė et al., 2018; Beiras and Tato, 2019; Besseling et al., 2017; Horton et al., 2018). The question therefore remains as to whether microplastics will significantly alter the impacts of HOCs on organisms.

Within the group of HOCs, polybrominated diphenyl ethers (PBDEs), brominated hydrocarbons commonly used as flame-retardants, are one of the priority pollutant groups. They are found widely throughout the environment (Guan et al., 2007; Hassanin et al., 2004), including in riverine sediments (up to 16088 ng g^{-1} dry weight total PBDEs in riverbank sediment in China (Luo et al., 2007)). As with other persistent organic pollutants, due to their relatively high log Kow, PBDEs sorb to particulate and organic matter within the environment, and to fatty tissues of organisms where they can bioaccumulate (Rahman et al., 2001). Where microplastics and PBDEs occur together, there is the likelihood of interactions. One environmental study found microplastics had surface concentrations of PBDEs up to 9900 ng g^{-1} (Hirai et al., 2011), suggesting the potential for such interactions to influence organism exposure. Chua et al. (2014) and Rochman et al. (2013c) have shown that the presence of microplastics within experimental systems can lead to increased body burdens of PBDEs in amphipods and fish, with the type and concentration of microplastics affecting the dynamics of bioaccumulation. Microplastics can also change the way in which different PBDE congeners are accumulated, with higher brominated congeners more likely to be accumulated when microplastics are present (Chua et al., 2014).

The gut microbiome is important for nutrition, metabolic function and immunity, with perturbations to the microbial community understood to have implications for organism health and fitness (Licht and Bahl, 2018; Zhu et al., 2018a). A number of studies have been carried

out to determine the effects of PBDEs on the gut microbiome of various organisms. Chen et al. (2018) investigated the effects of BDE-71 on the gut microbiome of zebrafish, finding that, in the presence of BDE-71, bacterial diversity was significantly reduced, and bacterial metabolic functioning was altered in a 7-day exposure. Li et al. (2018) showed BDEs-47 and 99 to significantly affect the gut microbial diversity of mice, leading to up- and down-regulation of 45 bacterial OTUs (5-day exposure), while Wang et al. (2018a) also found BDE-47 to also lead to a significant reduction in mouse gut microbial diversity and an alteration in the community structure (21-day exposure). Studies have shown that microplastics can similarly alter the gut microbiome of both vertebrates (Jin et al., 2018; Lu et al., 2018) and invertebrates (Zhu et al., 2018a; Zhu et al., 2018b). These studies clearly show that microbiome alterations, expressed as species richness and diversity, are a sensitive endpoint responding to HOC and microplastic exposure, even over short timescales. Therefore, microbiome analysis together with host fitness could provide a fast screening tool for assessing the effects of combined HOCs and microplastics during acute exposures.

The aim of this study was to investigate the effects of microplastics and PBDEs, individually and in combination, on the accumulation, physiology and microbiome of the great pond snail *Lymnaea stagnalis* (Linnaeus 1758). Molluscs have been shown to bioaccumulate organic chemicals (and metals) as they lack the oxidase systems to metabolise xenobiotic substances (Geyer et al., 1982). These traits make them well suited as test organism for investigating organic pollutant accumulation (Amorim et al., 2019). Although microplastics and PBDEs have been shown to individually alter the gut microbiome of organisms once ingested, no studies to date have investigated the effects of co-exposure to these pollutants with respect to microbiome responses. We hypothesise that increasing PBDE sediment concentrations will lead to significant changes in the microbiome community (diversity and composition) and that the presence of microplastics will reduce this effect through strong binding of PBDEs, making them less bioavailable to microbiota within the gut. We also hypothesise that the presence of microplastics will reduce PBDE accumulation in the snail.

2. Materials and methods

2.1. Organisms

Adult *Lymnaea stagnalis* were obtained from Blades Biological, UK, and were acclimatised for one week under laboratory conditions prior to the exposure. Cultures were maintained and

exposure studies carried out using ISO artificial freshwater as recommended by the OECD for *L. stagnalis* (OECD, 2016). An air pump with an air stone was provided for system oxygenation. Stock cultures and exposures were maintained at 20°C with a 16:8 h light:dark cycle. Snails in culture were fed well-washed iceberg lettuce *ad libitum*. No food was provided during test exposures. Preliminary experiments showed *L. stagnalis* to ingest and egest the nylon microplastics used for this study (personal observation).

2.2. Microplastic particles

Nylon 6 powder (mono-constituent substance, density 1.13 g cm^{-3}) was purchased from Goodfellow (Huntingdon, UK). This powder consisted of heterogeneous fragments <50 µm, with a mean size of 13-19 µm, measured using a Coulter Counter (Multisizer 3, Beckman, USA) and had been previously stained with Nile Red dye.

2.3. PBDEs

Method 527 PBDE Mixture was purchased from LGC Standards (Teddington, UK). This mixture contained BDE- 47, 99, 100, 153 and PBB- 153 (PBB-153 was not considered or measured throughout this study), each at a concentration of 500 µg ml^{-1} in ethyl acetate. With respectively log Kows of 6.81, 7.32, 7.24, and 7.9 these BDEs were all highly hydrophobic. These congeners are commonly detected within aquatic organisms and have a high propensity for bioaccumulation (Hirai et al., 2011; Shanmuganathan et al., 2011). A serial dilution was carried out in ethyl acetate in order to provide the ultimate concentrations of each BDE congener in sediment of 3000, 1500, 750, 375, 188 and 94 ng g^{-1}. These concentrations were chosen to reflect concentrations found within freshwater sediments (Luo et al., 2007; Sellström et al., 1998; Yin et al., 2017).

2.4. Experimental setup

Experimental treatments consisted of either microplastics (1% nylon powder by sediment mass) or sediment without added microplastics. Microplastic treatments were prepared by weighing 0.8 g nylon powder and mixing with white quartz sand (SiO_2, particle size 210-300 µm, Sigma-Aldrich, Poole, UK) to make up to 80 g. For each treatment, 1 ml of each diluted

PBDE stock was added to the 80 g quartz sand substrate (with or without microplastics, hereafter referred to as 'sediment') and stirred for 2 minutes 30 seconds using a glass rod. This bulk mixture was divided between six replicate 100 ml glass exposure vessels (13 g per vessel). As a solvent carrier was used for spiking the PBDEs into the sediment, an ethyl acetate solvent control was also set up (1.25 % ethyl acetate in sediment) by carrying out this procedure with ethyl acetate only. Following dosing, the vessels were left under a fume hood for two days with occasional agitation to ensure complete evaporation of the solvent. Blank control treatments were made by mixing nylon powder and quartz sand using the same procedure, but without the need for solvent evaporation.

To prevent suspension of nylon particles due to water surface tension, a small spray bottle of ISO test water was used to spray eight times onto the surface of the dry sediment. 100 ml of ISO test water was then gently introduced to the vessel and the water surface sprayed another seven times to break the water surface tension and allow any floating nylon particles to sink (15 sprays total). Vessels were left to equilibrate for 48 hours prior to introducing the organisms.

Before being added to the test vessels, each snail was rinsed in ISO test water and the shell gently rubbed with a gloved finger to remove any faeces/algae present and patted dry with a tissue. Each snail was weighed and length of shell measured; only snails > 25mm were used in the bioassays at which size all individuals can be expected to be mature (Coeurdassier et al., 2004; Zonneveld and Kooijman, 1989).

During exposures, jars were covered with Parafilm® to prevent escape of snails, pierced 10 times to allow for oxygenation. Exposures ran for 96 hours. Snails were observed daily to check for mortality. At the end of the exposure, snails were removed from the water, washed in DI water, patted dry with tissue and weighed. Snails were euthanised and preserved: of the six replicate snails for each treatment, three were preserved for microbiome analysis (directly placed into ethanol) and three for tissue PBDE concentration analysis (immediately frozen at -80°C). Snails were not depurated before weighing or preservation as it was decided that analysing organisms with a full gut would give a more natural representation of environmental exposure and associated internal concentration. The overlying water from the exposure vessels was poured away and sediments were dried in a temperature-controlled chamber at 25°C until dry (approx. 2 days). Sediment PBDE concentrations were measured in the dried samples at the end of the experiment.

2.5. Chemical analysis

Half of a snail was thawed, removed from the shell and dissected lengthways to obtain a representative sample of the whole body. This tissue was then weighed, ground with sand and dried with anhydrous sodium sulphate. Each sample (snail/sediment) was spiked with labelled recovery standards (^{13}C BDE 47, ^{13}C BDE 126 and ^{13}C BDE 153; Cambridge Isotope Laboratories) and soxhlet extracted in dichloromethane (DCM) for 16 h. A small portion of the extract was evaporated to zero volume and the lipid content was determined gravimetrically. The remaining of the extract was cleaned using automated size exclusion chromatography followed by deactivated (5% deionised water; w/w) alumina column.

The clean extract, was then spiked with labelled internal standards (BDE 77and ^{13}C BDE 138; Cambridge Isotope Laboratories) and 100 µl of sample was injected into a GC-MS (Agilent) with programmable temperature vaporization (PTV) inlet. The PTV injector was kept at 55°C for 0.45 min, and heated to 325°C at a rate of 700°C min^{-1} and kept at 325°C for 5 min. Then the temperature was reduced to 315°C min^{-1} at a rate of 10°C min^{-1}.The GC-MS had a 25 m HT8 column (0.22 mm internal diameter and 0.25 µm film thickness, SGE Milton Keynes, UK) and the carrier gas was helium (2.0 ml min^{-1}). The temperature programme was: isothermal at 80°C for 2.4 min, 25°C min^{-1} to 200°C, 5°C min^{-1} to 315°C and was held at 315°C for 9.8 min. Residues were quantified using internal standard method and also calibration curves of the standard PBDEs (Cambridge Isotope Laboratories) and were recovery corrected. The mean recoveries were: ^{13}C BDE 47- 85%, ^{13}C BDE 126 – 105% and ^{13}C BDE 153- 96% and the LOD was 0.109 ng g^{-1} wet weight.

2.6. Ingestion of microplastics

The snail tissue remaining following the chemical analysis was analysed using a fluorescence microscope (Olympus BX41 microscope with an Olympus U-LH100HG 100W mercury lamp using the green filter of the Cy3 (Olympus U-M39004) filter cube, with Olympus analySIS software) to verify ingestion of microplastics by the snails.

2.7. Microbiome analysis

2.7.1. DNA extraction and sequencing

DNA was extracted from three snails per treatment (whole snail excluding shell) following the protocol described in the SI. Sample DNA required an additional cleaning step through the application of Genomic DNA Clean & Concentrator kit (Zymo research, USA) under the manufacturer's recommended protocol. Resultant DNA was quantified using the nanodrop 8000 UV-Vis spectrophotometer (ThermoFisher scientific, USA).

Approximately 40 ng of template DNA was amplified using Q5 high-fidelity DNA polymerase (New England Biolabs, Hitchin, UK) each with a unique dual-index barcode primer combination (Kozich et al., 2013). Individual PCR reactions employed 25 cycles of an initial 30 s, 98°C denaturation step, followed by an annealing phase for 30 s at 53°C, and a final extension step lasting 90 s at 72°C. Primers were based upon the universal primer sequence 341F and 806R (Takahashi et al., 2014). An amplicon library consisting of ~550 bp amplicons spanning the V3-V4 hypervariable regions of encoding for the 16S small subunit ribosomal RNA gene (16S rRNA), was sequenced at a concentration of 6 pM with a 10% addition of control phiX DNA, on an Illumina MiSeq platform using V3 chemistry (Illumina Inc., San Diego, CA, USA).

2.7.2. Bioinformatics analysis

Sequenced paired-end reads were joined using VSEARCH (Rognes et al., 2016), quality filtered using FASTX tools (hannonlab.cshl.edu), length filtered with the minimum length of 300 bp, presence of PhiX and adapters were checked and removed with BBTools (jgi.doe.gov/data-and-tools/bbtools/), and chimeras were identified and removed with VSEARCH_UCHIME_REF (Rognes et al., 2016) using Greengenes Release 13_5 (at 97%) (DeSantis et al., 2006). Singletons were removed and the resulting sequences were clustered into operational taxonomic units (OTUs) with VSEARCH_CLUSTER (Rognes et al., 2016) at 97% sequence identity (Tindall et al., 2010). Representative sequences for each OTU were taxonomically assigned by RDP Classifier with the bootstrap threshold of 0.8 or greater (Wang et al., 2007) using the Greengenes Release 13_5 (full) (DeSantis et al., 2006) as the reference. Unless stated otherwise, default parameters were used for the steps listed. The raw sequence

data reported in this study have been deposited in the European Nucleotide Archive under study accession number PRJEB27672 (ERP109787).

2.8. Statistical analysis

2.8.1. Chemistry data

Sediment concentration and snail body concentration data were log transformed for normality. As only one sediment concentration was measured per treatment, it was assumed that each of the three snails analysed per treatment was exposed to this measured concentration. To compare the concentrations of PBDEs in sediment and organisms with and without microplastics, only treatments with added PBDEs were included in the analyses of chemical data (i.e. no control treatments) as the control treatments showed very low or non-detected values which could not be log-transformed. Two-way ANOVAs were carried out for each BDE congener, and the total PBDEs, to determine the relationship between snail tissue concentration, the concentration of PBDEs in the sediment and the presence of microplastics (R statistical software).

2.8.2. Snail weight data

A two-way ANOVA was conducted considering the effects on snail weight change of PBDE concentration and presence of microplastics as factors, and also their interaction.

2.8.3. Microbiome data

After quality filtering, a total of 2626755 sequences remained. One sample was removed from the analysis due to low sequencing efficiency (<6000 sequences). Rarefaction curves were used to ensure the sample depth represented the full community. To account for uneven sequencing depth (inherent in NGS platforms) samples were normalized to lowest sequence depth using the rarefy_even_depth function in the R package 'Phyloseq V 1.22.3' (McMurdie and Holmes, 2013). For simplicity, for microbiome analysis with respect to PBDE concentration, nominal PBDE concentrations were used. In order to assess any subtle changes, communities were subdivided into 'core' OTUs (occurring in >50% of samples, at an abundance of >2%) and 'non-core' (all other community members), using the function 'prevalence' in the R package

'microbiome' (McMurdie and Holmes, 2013). Analyses were firstly carried out on the whole community and subsequently on the subdivided core and non-core communities.

To visualise the relationship between 16 rRNA sequence-based community profiles from different treatments, nonmetric multidimensional scaling (NMDS) was performed using the 'metaMDS' function, based on dissimilarities calculated using the Bray–Curtis index. Additionally, bacterial diversity were assessed using Fishers log series [alpha], as this is largely unaffected by sample sizes > 1000 (Magurran, 2004). Differences in bacterial diversity for each PBDE compound and nominal PBDE concentration were tested through the multiple Kruskal-Wallis (H) test, a test which does not assume data normality, using the function 'kruskalmc' in R package 'Pgirmess' version 1.6.9 (Giraudoux et al., 2018). An additional Kruskal-Wallis test was run to determine whether there were differences in microbiome diversity between control and solvent control treatments (Fig. 3). Similarity percentages breakdown procedure (SIMPER) was used to infer the importance of community members within treatments (Clarke, 1993) and again Kruskal-Wallis was used to test significance. Finally, the effect of PBDE concentration, presence of microplastics and their interaction upon community dissimilarity was assessed using the Bray–Curtis index through Permutational Multivariate Analysis of Variance (PERMANOVA, using the 'ADONIS' function in R package 'Vegan' v2.0-10 (Anderson, 2001; Oksanen et al., 2013)). Taxonomic composition was plotted using the R package 'ggplot2' (Wickham, 2016). For each treatment, relative abundances per treatment were calculated to account for unequal sampling, taking into account the combined data of the three replicates (Figs. 5, S4 and S5).

3. Results

3.1. Concentration of PBDEs in the presence and absence of microplastics

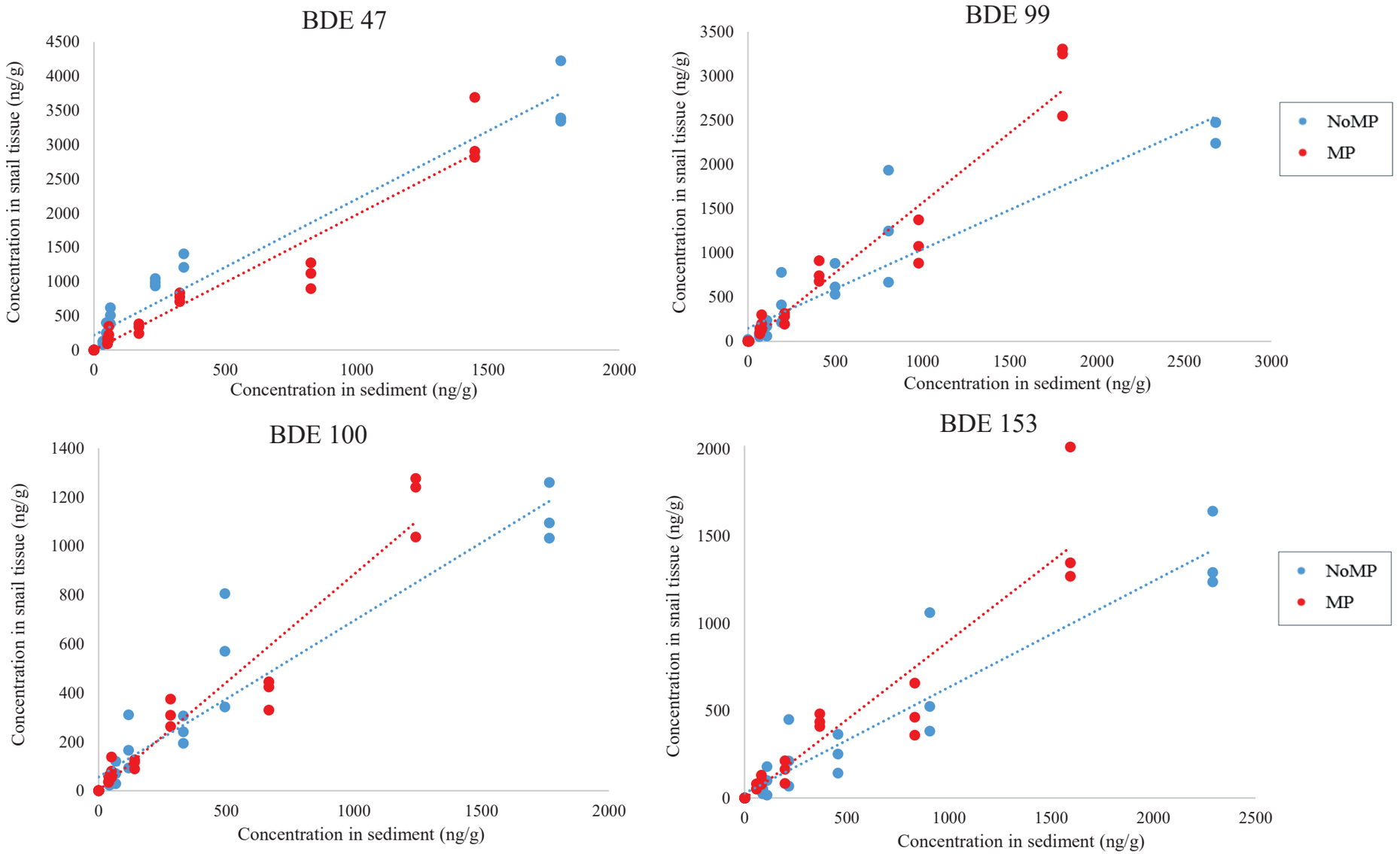
The control treatments (no PBDEs, with and without microplastics) contained trace concentrations of PBDEs in some instances, although most (overall 73%) were below the detection limit of 0.108 ng g^{-1}. The concentrations of different PBDE congeners in relation to its nominal concentrations varied between 41% and 74% (Table 1). When considering all congeners and concentrations both with and without microplastics, measured sediment concentrations overall were 54% of the nominal concentration. PBDEs were therefore present within sediment at statistically significantly comparable concentrations regardless of the presence or absence of microplastics ($p > 0.05$, ANOVA).

The (measured) sediment PBDE concentrations significantly affected PBDE uptake by snails, for all PBDEs independently and combined, higher sediment concentrations resulted in a significantly higher snail body burden ($p < 0.01$, ANOVA, Fig. 1). BDE 47 was the only PBDE congener that showed a significant effect of microplastics on the uptake of PBDEs by snails, with microplastics leading to a significantly lower body burden ($p < 0.01$, ANOVA, Fig. 1). There were no significant interactions between the concentration of PBDEs within the sediment and the presence of microplastics for any of the congeners ($p > 0.05$, two-way ANOVA).

Table 1. Nominal and measured sediment concentrations for each BDE congener, for all PBDE treatments with and without microplastics. Sediment PBDE concentrations were measured at the end of the experiment (one replicate per treatment).

		Measured sediment concentration (ng g^{-1})				
	Nominal concentration (ng g^{-1}, per BDE)	**BDE 47**	**BDE 100**	**BDE 99**	**BDE 153**	**Total BDEs**
Without microplastics	0	0	0	0	0	0
	0 (solvent control)	0	0	0	0	0
	94	34.42	42.67	66.63	88.41	232.12
	188	46.80	67.44	108.19	109.85	332.27
	375	61.92	117.09	191.70	215.99	586.70
	750	233.24	332.20	499.45	456.24	1521.14
	1500	341.34	494.64	805.90	906.48	2548.36
	3000	1776.98	1765.79	2681.38	2290.35	8514.49
With microplastics	0	0	0	0	0.41	0.41
	0 (solvent control)	0	0	6.93	0.54	7.47
	94	50.77	39.42	68.30	58.05	216.54
	188	56.24	50.58	78.46	81.65	266.92
	375	170.47	141.11	209.81	197.16	718.55
	750	326.44	281.85	408.44	367.71	1384.44
	1500	825.13	667.41	978.23	832.01	3302.77
	3000	1449.14	1242.28	1804.11	1593.52	6089.05

Fig. 1. Measured PBDE concentrations in sediment, compared to the concentration within snails, for each BDE congener, with and without microplastics. 'No MP' = without microplastics, 'MP' = with microplastics.



3.2. Survival and weight change

There was 100% survival throughout the exposure. A significant difference was observed in snail wet weight change between microplastic and non-microplastic treatments, with non-microplastic treatments losing significantly more weight on average (0.11 ± 0.13 g) than microplastic treatments (0.03 ± 0.12 g) (two-way ANOVA, $p < 0.01$, Fig. 2). Concentration of PBDE had no effect on weight change (two-way ANOVA, $p > 0.05$) and there was no interaction between PBDEs and microplastics (two-way ANOVA, $p > 0.05$).

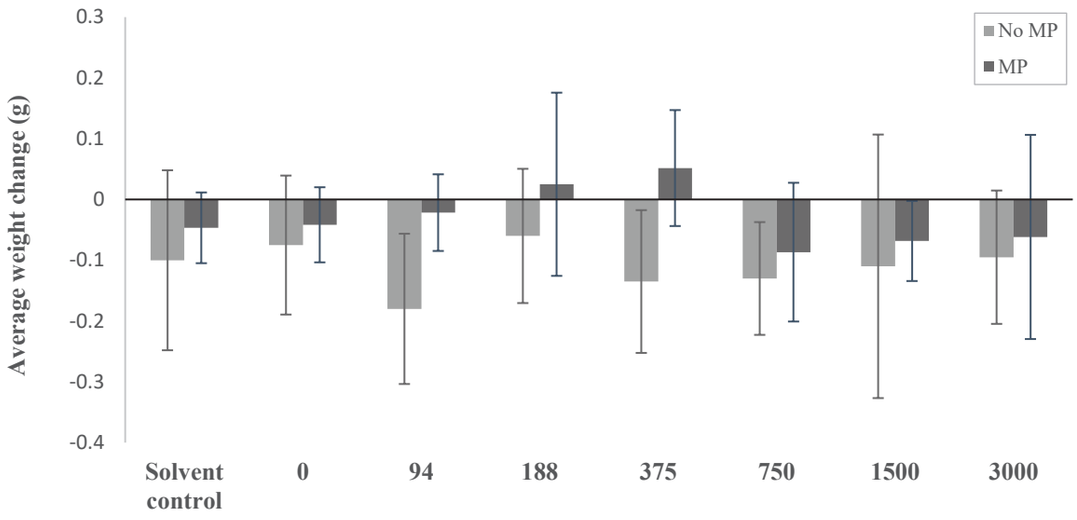


Fig. 2. Average snail wet weight change for all PBDE treatments (nominal concentration in ng g^{-1} within sediment) with and without microplastics, based on weight difference between 0 and 96 hours exposure. 'No MP' = without microplastics, 'MP' = with microplastics.

3.3. Ingestion of microplastics

Ingestion of microplastics was qualitatively confirmed using fluorescence microscopy (Fig. S1). Microplastics are clearly visible within the tissues of the snails, both on the surface of the sample (Fig. S1, G and I) and behind membranes (i.e. within organs, Figs S1. D, F and H). Based on the way the samples were prepared and analysed, it is not possible to quantitatively analyse ingestion, nor to identify the specific locations where microplastics were found or accumulated. However, microplastics were visibly present within all snails exposed to microplastics (Fig. S1 D-I).

3.3. Microbiome data

3.3.1. Control treatments

Using a multiple-comparison Kruskal-Wallis test, there were no significant differences in microbial diversity (Fisher's Log alpha) between blank controls and solvent controls, nor between control treatments with and without microplastics (Fig. 3. $p > 0.05$, Kruskal-Wallis). This highlights that there was no effect of the solvent control, or of microplastics alone (in the absence of PBDEs), on snail microbiome structure.

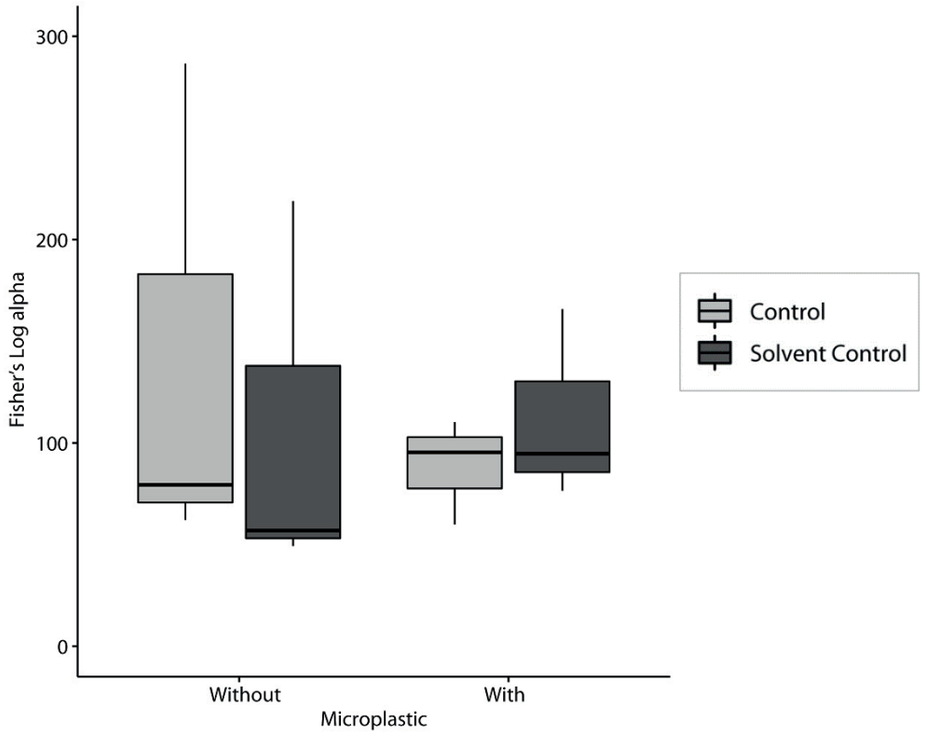


Fig. 3: Comparison of changes in microbial community diversity of the snails (Fisher's Log alpha) in the blank and solvent controls, with and without microplastics.

3.3.2. Community composition and diversity

Community diversity assessed by Fisher's log alpha (Fig. 4) showed no significant differences between different PBDE concentrations (all $p < 0.05$, multiple Kruskal-Wallis, H). However, although not significant, it should be noted that diversity does appear to be lower at higher PBDE concentrations when microplastics are absent, while the diversity of communities in treatments with microplastics appear largely unaffected by PBDE concentration (Fig. 4). This

decline in diversity pattern could be related to a loss in richness (count) of non-core OTUs in treatment with high concentrations of PBDE, in the absence of microplastics (Fig. 4 and Fig S2).

The visualisation of the community composition in the NMDS suggested some clustering at high PBDE concentrations in the absence of microplastics (Fig. S3). However, the Permutational Multivariate Analysis of Variance showed that none of this clustering was significant ($p > 0.05$ for all comparisons). Replicate variability was high and possibly rendered this non-significance. Attempting to introduce more stringent criteria to determine core OTUs did not change the results.

3.3.3. Taxonomic microbiome composition

The greatest number of 16S rRNA gene sequences within the *L. stagnalis* microbiome were found to be from the Gammaproteobacteria, Betaproteobacteria, Alphaproteobacteria, Flavobacteria and Bacilli (Fig. S4) irrespective of treatment. The most dominant order across all treatments are the Enterobacterales (Fig. 5), and within that order the genus *Klebsiella* (Fig. S5). When comparing individual OTUs in the controls vs the highest PBDE concentration, similarity of percentage (SIMPER) analysis shows that in the absence of microplastics there was a significantly higher relative abundance of OTUs 5512 and 4432 (both identified as belonging to the Enterobacteriaceae) in the highest PBDE concentration treatment (Table S1). There was also a significant reduction in OTU 8733 (identified as belonging to the Flavobacteriaceae), in the highest dose treatment compared to the control (Kruskal-Wallis test $P < 0.05$, df =1). In contrast, no significant differences were observed in individual relative OTU abundance when microplastics were present (Table S1). Some orders are present only in PBDE treatments, notably sulfate-reducing bacteria (Desulfobacterales and Syntrophobacterales).

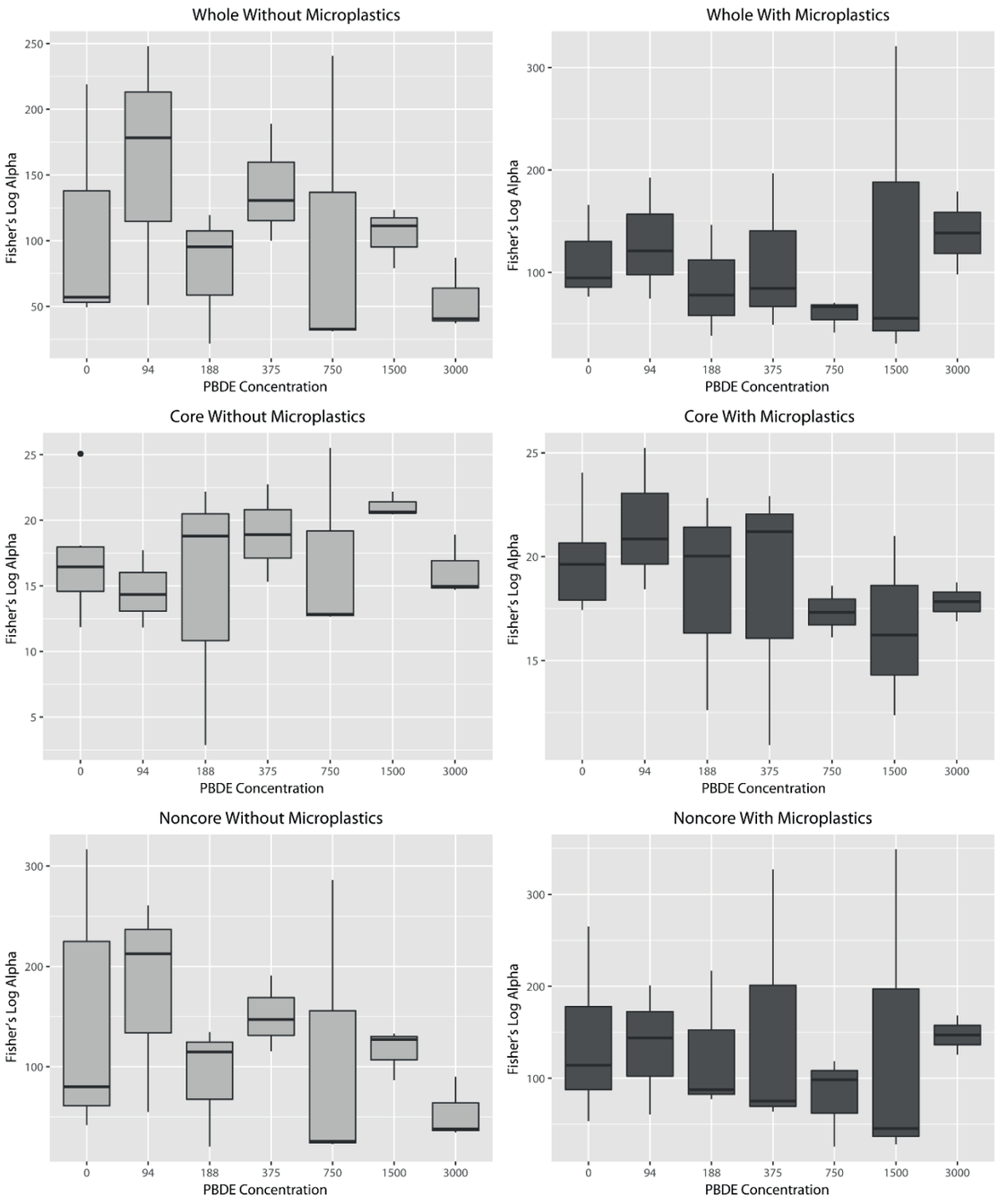


Fig. 4. Boxplots to show differences in microbial diversity in snails exposed to PBDEs in the absence and presence of microplastics, showing whole data for the whole microbial community, then subsequent separation into 'core' and 'non-core' community.

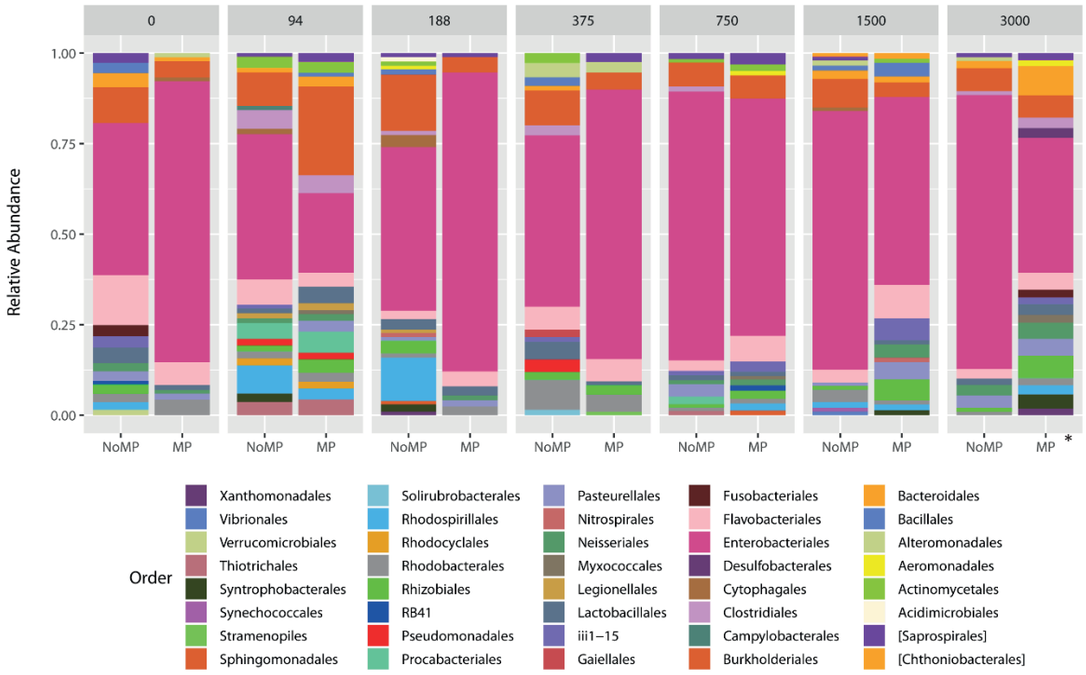


Fig. 5. Order composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with and without microplastics (present/absent). Relative abundance was calculated as rarefied number of sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3) are plotted on Y axis. For ease of representation taxa of an abundance of <0.02 (2%) from an individual sample were excluded. *Note one sample was removed from this treatment due to inefficient sequencing, therefore *N*=2.

4. Discussion

4.1. The snail microbiome

The individuals used in this test were taken from the field and acclimated for this test, therefore the microbial data acquired here is likely representative of biological variability within wild *L. stagnalis.* While the microbiome analysis in this study considered the whole microbiome of the snail, it is expected that the majority of OTUs derive from the gut bacterial community. This is highlighted in the communities across all treatments being dominated by Enterobacterales (Fig. 5), a common order comprising gut bacteria (Hu et al., 2018). For example, *Klebsiella*, the most dominant genus observed here within the family Enterobacteriacae (Fig. S5), is a polysaccharide degrader linked to the presence of cellulase (Imran et al., 2016). The *L. stagnalis* core microbiome in this study appears to be similar to other freshwater snails and

associated habitats including Enterobacterales, Flavobacterales and Bacillales corresponding to lactic acid production (food fermentation) and cellulose degradation (Béguin, 1990; Dar et al., 2017; Hu et al., 2018).

4.2. PBDE accumulation and effects on microbiome

All sediment PBDE concentrations were lower than the nominal concentrations when measured at the end of the exposures. The half-lives of BDEs-47, 99, 100 and 153 are all estimated to be approximately 14,400 hours in sediment (Wania and Dugani, 2003), therefore degradation over the experimental time period is not likely to have been a significant factor leading to the discrepancies between nominal and measured concentrations observed here (estimated 0.3% loss due to degradation over 96 hours based on a half-life of 14,400 hours). Some loss of PBDEs may have occurred as a result of volatilisation during the solvent evaporation step, and some may also have bound to the walls of the glass exposure vessels.

BDE 47 has the lowest log Kow, which would indicate a greater (although still low) partitioning into the water phase than for the other more hydrophobic PDBEs. In a marine study, Mizukawa et al. (2009) found that proportionally, higher brominated BDE congeners (BDE 209) associated most strongly with sediments, while the composition within overlying seawater was dominated by lower brominated congeners (predominantly BDE 47, but also including BDEs 99 and 100). In our study, BDE 47 followed by BDE 99 accumulated most in the snails, with higher internal concentrations compared to the other congeners (Fig. 1). This corresponds with evidence which shows that BDEs 47 and 99 are the most bioavailable PBDE congeners, due to a lower molecular weight and smaller molecules than higher brominated congeners (Liang et al., 2010; Mizukawa et al., 2009; Watanabe and Sakai, 2003; Zhang et al., 2016).

There were a number of sulfate reducing bacteria observed within snails exposed to PBDEs (most notably Desulfobacterales and Syntrophobacterales, Fig. 5), bacteria also recognised to be associated with the debromination of PBDEs (Zhao et al., 2018). These bacteria have not commonly been described in relation to other freshwater snail species (Hu et al., 2018) and were not present within the controls. Burkholderiales, one of the dominant orders found within these snails across all treatments, are also associated with PBDE degradation, especially lower brominated congeners (Robrock et al., 2009).

PBDE concentration had no significant influence on the microbiome, a result which is in contrast to other studies which found that PBDEs affected bacterial community composition and diversity in sediments and within guts, with changes being congener-dependent (Li et al., 2018; Wang et al., 2018a; Yen et al., 2009). This difference is likely to be because these studies represent different exposure scenarios (via food or water) and also generally used much higher PBDE concentrations (e.g. $\mu g\ g^{-1}$ concentrations in food), although Chen et al. (2018) found significant microbiome community shift in zebrafish exposed to just 5 ng L^{-1} in water. We therefore reject the starting hypothesis that increasing PBDE sediment concentrations lead to significant structural changes in the microbiome community over an acute timescale.

4.3. Effects of microplastics on snail physiology and microbiome

There was no effect of any exposure condition on survival. Microplastics did subtly affect the wet weight of the snails. In general, the weight of all snails declined throughout the experiment, likely due to the lack of food within the exposure. However, this decline was less pronounced in snails exposed to microplastics (average 0.03 g weight decline in microplastic-exposed snails, compared to average 0.11 g decline in non microplastic-exposed snails). The reasons for this difference are not clear; most microplastic exposure studies observe a more pronounced weight decline in exposed organisms (Besseling et al., 2013; Zhu et al., 2018a).

The lack of significant influence of microplastics on the microbiome (Fig. 3) is in contrast to other studies on the microbiome response in invertebrates (Zhu et al., 2018a; Zhu et al., 2018b). For example, Zhu et al. (2018b) found a significant increase in the family Bacillaceae within collembolan guts following exposure to microplastics, while our analysis found the order Bacillales to be present in both the microplastic and non-microplastic treatments (Fig 5). Many gut bacteria are derived from, and influenced by, ingested material, therefore feeding behaviour is likely to have a significant influence on the gut microbiome (Turnbaugh et al., 2009; Zhu et al., 2018b). It was chosen not to feed the snails during the acute exposure, and hence any alterations within the microbiome community could be ascribed solely to the microplastic, PBDEs and their interaction. Despite the lack of significance of microplastics alone, the microbiome analysis suggests that microplastics can subtly influence PBDE impacts on the microbiome. For example, while not significant, there appears to be a tendency for the diversity of non-core bacteria to be lower at higher PBDE concentrations in the absence of microplastics, a trend which is not evident when microplastics were present (Fig. 4, Fig S2). Microplastics

also appear to slightly reduce variability between individuals within the microplastic controls compared to non-microplastic controls i.e. 'reference' gut conditions (Figs. 3 and 4). Within natural conditions, a higher microbial diversity between individuals may be beneficial for populations, increasing resilience to perturbation (Heiman and Greenway, 2016; Lozupone et al., 2012).

4.4.Influence of microplastics and PBDE co-exposure on accumulation and microbiome

Microplastics did not influence sediment PBDE concentrations. This result was expected as the microplastics were not removed from the sediment samples before analysis, therefore during analysis, PBDEs were likely to have been extracted from both the sediment and microplastics simultaneously. The concentrations of PBDEs within the sediment significantly affected the amount of PBDEs taken up within the snail, in line with the expected relationship between external exposure concentration and snail body burden.

Given that snails were not depurated before chemical analysis of the whole body, this analysis took into account any chemicals present within the gut content, in addition to those in snail tissues. Microplastics did not influence the uptake of BDEs-99, 100, 153, nor PBDE uptake as a whole. Therefore these PBDEs were equally available regardless of the presence of microplastics and our hypothesis was not supported. This is in contrast to previous studies carried out on microplastic and PBDE interactions, where microplastics have been shown to enhance uptake of PBDEs into fish tissue (Rochman et al., 2013c).

Previous studies have shown that PBDEs can transfer from microplastics into body tissues (Chua et al., 2014; Rochman et al., 2013c). Hence, the concentrations measured here are indeed likely to be a combination of both gut content and tissue concentrations, especially as our preliminary studies have shown that the nylon particles are ingested by snails (personal observation.). PBDEs entering tissues are unlikely to be taken up only by ingestion of contaminated particles, as the foot of the snail will be exposed to the sediment-based PBDEs by direct contact with the sediment, and to aqueous phase PBDEs through contact with the water phase (Bakir et al., 2016). To allow uptake into tissues, desorption of the chemical from the sediment (or microplastic) surface, whether externally or within the gut, is needed as a prelude to uptake. While it is anticipated that the main route of exposure to PBDEs was via the sediment (either dermally or via ingestion) (Mizukawa et al., 2009), aqueous phase uptake may also be important and the precise nature of exposure may also vary dependent on the behaviour

of the BDE congener: BDE 47 was the only PBDE whose concentration in snails was significantly reduced in the presence of microplastics. BDE 47 is the congener with the lowest log Kow at 6.81, which would be expected to sorb the least strongly to particles (both microplastics and sediment) compared to the other congeners (although it is still highly hydrophobic). This reduced binding affinity could have led to greater BDE 47 partitioning into the water phase in the absence of microplastics, facilitating uptake. The presence of microplastics may have increased the partitioning of BDE 47 to sediment through the addition of a further surface binding phase with a high affinity for HOCs, thus reducing BDE 47 in the more bioavailable water phase, resulting in reduced bioavailability and uptake (Fig. 1).

While microplastics can sorb chemicals, other media (e.g. organic matter, sediment) may also accumulate HOCs and therefore should be also be taken into account when considering pathways for exposure and bioavailability (Bakir et al., 2016; Koelmans et al., 2016). Further, if considering trophic transfer, the interactions with the sediment also indicate the importance of measuring organisms with a full gut, as we did within this study (rather than depurated organisms as is usually the case in chemical bioaccumulation studies), given that PBDEs associated with the gut content may also be bioavailable.

No consistent significant differences were observed in snail microbiome community diversity in response to either the microplastic or PBDE treatments, although a trend for reduced diversity at high PDBE concentrations in the absence of microplastics was suggested, which warrants further investigation. Hence, our hypothesis of chemical effects on the snail microbiome, influenced by microplastics, was not supported over the short exposure timescale used. When investigating the differences in abundance of specific OTUs, significant differences were seen in the abundance of Enterobacteriaceae and Flavobacteriaceae between the control and high PBDE concentration, only when microplastics were absent (Table S1). Enterobacterales can be induced to bloom within the gut under conditions of stress, for example inflammatory responses produced by the gut immune system (Stecher et al., 2012), which may explain their increase in the presence of high PBDE concentrations. Flavobacterales have been associated with polymer degradation (Mergaert and Swings, 1996; Nogales et al., 2011) and have been commonly found associated with marine plastic debris (Bryant et al., 2016; Oberbeckmann et al., 2018) which could explain their decline in the absence of microplastics (combined with high PBDE concentrations), although it is not possible to link those characteristics directly to this study. The fact that these results were seen only in the absence

of microplastics suggests that microplastics may be buffering the effects of PBDEs on the microbiota, although only subtly.

4.5. Long term implications and outlook

Short and long-term exposure are likely to lead to very different microbial community responses, therefore acute exposures can provide information on initial responses to perturbation that would be not be observed during chronic tests (Shade et al., 2012). There is evidence to suggest that microbiomes will respond very quickly to perturbations, for example a study by Yen et al. (2009) found that BDEs 153 and 154 rapidly and irreversibly changed the bacterial community within sediment (within 24 hours). Studies which have found significant changes in organism microbiomes following invertebrate exposure to microplastics usually run for longer timescales, e.g. enchytraeids exposed for seven days (Zhu et al., 2018a) and collembolans exposed for 56 days (Zhu et al., 2018b).

The subtle variations in response of the snail microbiome to microplastic exposure, PBDE exposure and co-exposure over a 96 hour exposure indicated that these stressors do affect the structure of the gut community. However, overall response to aspects such as overall diversity were not evident to the same extent as for studies with other species conducted over longer exposure times. These results, therefore, highlight the complexity of responses of organisms to microplastics and organic chemicals, and show the importance of carrying out further studies to understand the interaction between microplastics and HOCs and their influence on organisms in a variety of exposure scenarios and time-scales.

5. Conclusions

Microplastics did not affect survival of the snails. The weight of all snails generally declined throughout the exposure period, however, this decline was lower in snails exposed to microplastics. An increased concentration of PBDE in the sediment led to an increased body burden within the snails, however microplastics did not significantly influence this uptake when considering all PBDE congeners overall. BDE 47 was the only congener influenced by the presence of microplastics, leading to a significantly reduced internal concentration in the presence of microplastics. Overall, the diversity and composition of the snail microbiome was

not significantly altered by the presence of PBDEs or microplastics, or both combined. However, when considering individual OTUs, significant effects on individual responses were found that can be functionally linked to the exposure of snails to the PBDEs added, a result only observed in the absence of microplastics. This suggests that microplastics influence how PBDEs will impact on specific OTUs. In summary, these results suggest that microplastics and PBDEs have a limited effect both individually and when combined on HOC accumulation and the microbiome of *Lymnaea stagnalis* within an acute exposure. However the subtle effects seen highlight the importance of carrying out further studies to better understand the mechanisms causing the interaction between microplastics and HOCs given that these relationships may become more pronounced over extended time-scales.

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CHAPTER 6

SUPPLEMENTARY INFORMATION

DNA extraction procedure

Three snails were analysed per treatment. Frozen snails were defrosted, removed from the shells, then rinsed in phosphate buffered saline prior to DNA extraction. In order to capture the entire snail microbiome, DNA was extracted from a whole snail through the application of both chemical and enzymatic lysis. Per 250 mg of snail tissue 100 µl of lysis buffer (10 ppmL 1 M Tris pH7.5, 1 ppmL 0.5 M EDTA, 2 ppmL 10% SDS and 4 ppmL 5 M NaCl, made up in molecular grade water) and 20 µl proteinase K solution (20 mg ml^{-1}) was added. Tissue was ground and mixed with a disposable polypropylene tissue pestle and handheld tissue grinder. To ensure complete cell lysis, samples were incubated at 37°C overnight. When samples were fully lysed, proteins were removed through the addition of 600 µl 5M NaCl (per 250 mg of snail weight), mixed well through vortexing and allowed to precipitate for 10 mins at room temperature. 700 µl of lysate was moved to a clean tube and centrifuged at 20000 x g for 10 minutes. Supernatant was transferred to a new tube, mixed by inversion and DNA precipitated through the addition of 650 µl absolute ethanol. DNA was pelleted through centrifugation at 20000 x g for 10 minutes. Ethanol was removed and pelleted DNA was cleaned using 400 µl 70% ethanol. Pellet was centrifuged again at 20000 x g for 2 minutes and ethanol aspirated. Pelleted DNA was air dried to remove residual ethanol and resuspended in 500 µl molecular grade water. Sample DNA required an additional cleaning step performed through the application of Genomic DNA Clean & Concentrator kit (Zymo research) under the manufacturer's recommended protocol. Resultant DNA was quantified using the nanodrop 8000 UV-Vis spectrophotometer (ThermoFisher scientific).

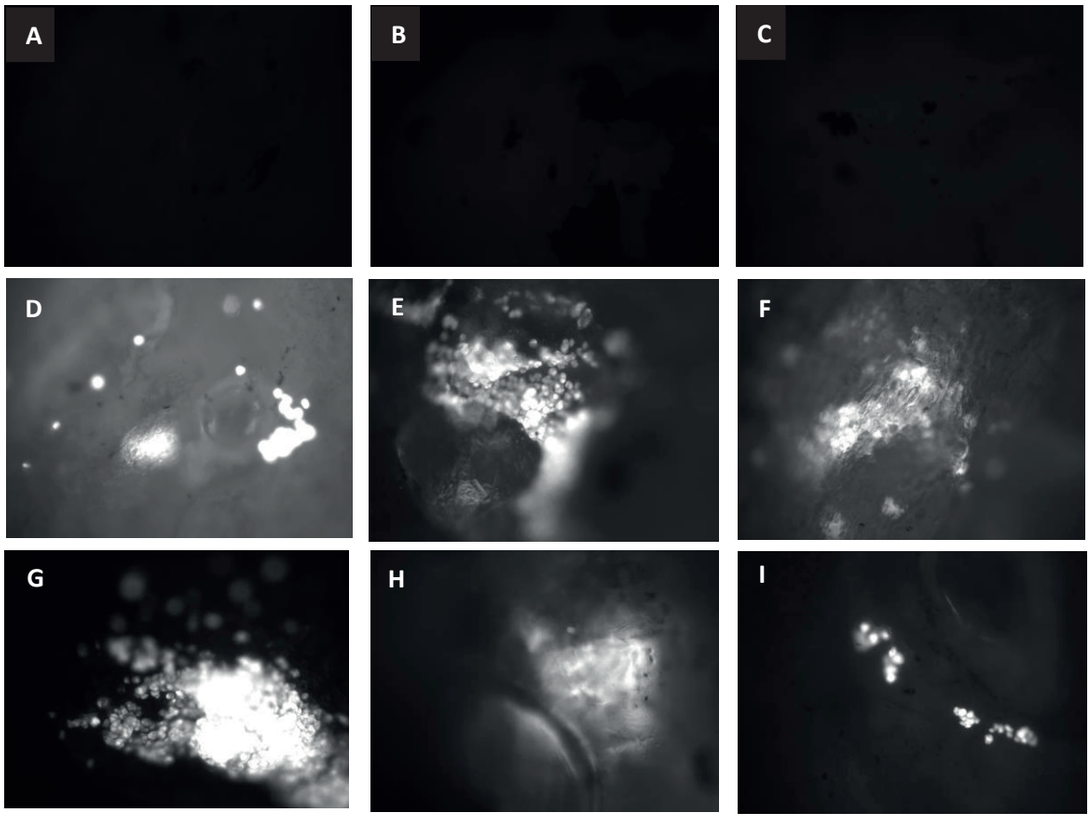

Fig S1. Microscope images showing fluorescently labelled nylon particles within the guts of snails: blank controls (no microplastics) = A-C, microplastic controls = D-F and 3000 ng g^{-1} PBDEs with microplastics = G-I.

Table S1. The OTUs which show the most change in abundance between the highest nominal PBDE concentration (3000) and control treatment, without microplastics (a) or with microplastics (b). Significance at the P > 0.05 level was determined through the application of a Kruskal Wallis test. Significant OTUs are highlighted with an asterisk. Taxonomic identity is given at the highest resolution, using a 97% threshold. ^abundance based on rarefied values

(a) Without Microplastics

	Average^	Standard deviation	ratio	Average^ in 3000 ng g^{-1}	Average^ in solvent control	Cumulative sum	Kruskal-Wallis chi-squared	df	P value	Taxonomic Identity
OTU5512*	0.02	0.02	1.48	357.33	74.67	0.28	3.86	1	0.0495*	Enterobacteriaceae
OTU4432*	0.02	0.01	1.48	355.67	81	0.32	3.86	1	0.0495*	Enterobacteriaceae
OTU32	0.10	0.06	1.79	2851.33	1621.67	0.18	2.33	1	0.1266	Enterobacteriaceae (*Klebsiella*)
OTU2245	0.01	0.01	0.96	84	35	0.42	0.05	1	0.8273	Enterobacteriaceae (*Serratia marcescens*)
OTU8733*	0.03	0.02	1.27	146.67	547	0.24	3.86	1	0.0495*	Flavobacteriaceae
OTU12263	0.01	0.02	0.72	16.33	154	0.36	1.19	1	0.2752	Leptotrichiaceae (*Streptobacillus moniliformis*)
OTU3412	0.01	0.01	1.69	170.67	172.67	0.34	0.05	1	0.8273	Pasteurellales
OTU16390	0.01	0.01	0.93	15	119.33	0.41	2.33	1	0.1266	Rhodospirillaceae
OTU10409	0.01	0.01	1.15	98.33	172	0.39	0.43	1	0.512	Bacteroidales
OTU3010	0.01	0.01	1.45	144.67	124.67	0.38	0.05	1	0.8273	Neisseriaceae

(b) With Microplastics

	Average^	Standard deviation	ratio	Average^ in 3000 ng g^{-1}	Average^ in solvent control	Cumulative sum	Kruskal-Wallis chi-squared	df	P value	Taxonomic Identity
OTU5512	0.02	0.01	1.18	140.5	252.67	0.29	0	1	1	Enterobacteriaceae
OTU4432	0.01	0.01	1.16	118	230.67	0.31	0.33	1	0.5637	Enterobacteriaceae
OTU32	0.19	0.09	2.07	892.5	3265.67	0.26	3	1	0.0833	Enterobacteriaceae (*Klebsiella*)
OTU2245	0.01	0.01	1.03	218	53.67	0.33	0	1	1	Enterobacteriaceae (*Serratia marcescens*)
OTU8733	0.01	0	3.03	172.5	140.33	0.35	0	1	1	Flavobacteriaceae
OTU12263	0.01	0	1.87	87.5	10	0.43	3	1	0.0833	Leptotrichiaceae (*Streptobacillus moniliformis*)
OTU3010	0.01	0.01	1.12	197.5	69	0.37	0.33	1	0.5637	Neisseriaceae
OTU3412	0.01	0.01	1.2	214.5	120.67	0.39	0.33	1	0.5637	Pasteurellales
OTU16395	0.01	0.01	1.07	103.5	19	0.41	0	1	1	Rhizobiaceae
OTU10409	0.01	0.01	1.5	212	69.67	0.40	3	1	0.0833	Bacteroidales
OTU999	0.02	0.01	1.18	140.5	252.67	0.29	0	1	0.2207	Desulfobacteraceae

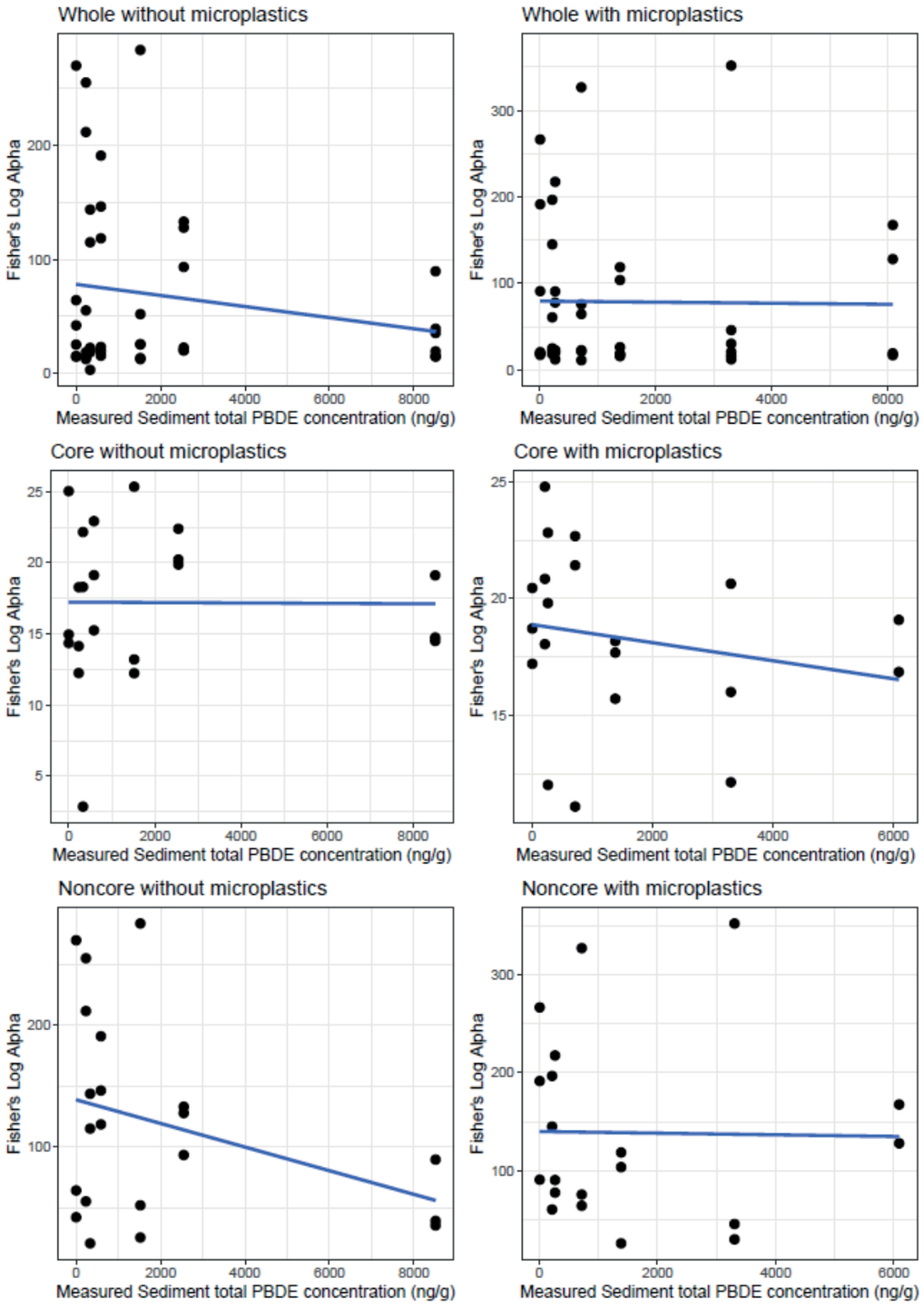

Fig S2. Linear regression of Fisher's log alpha diversity in relation to total sediment PBDE concentration (all congeners combined). Each data point represents an individual snail. Only one sediment concentration value was measured per treatment, therefore these regression lines are to provide a visual representation of the data only.

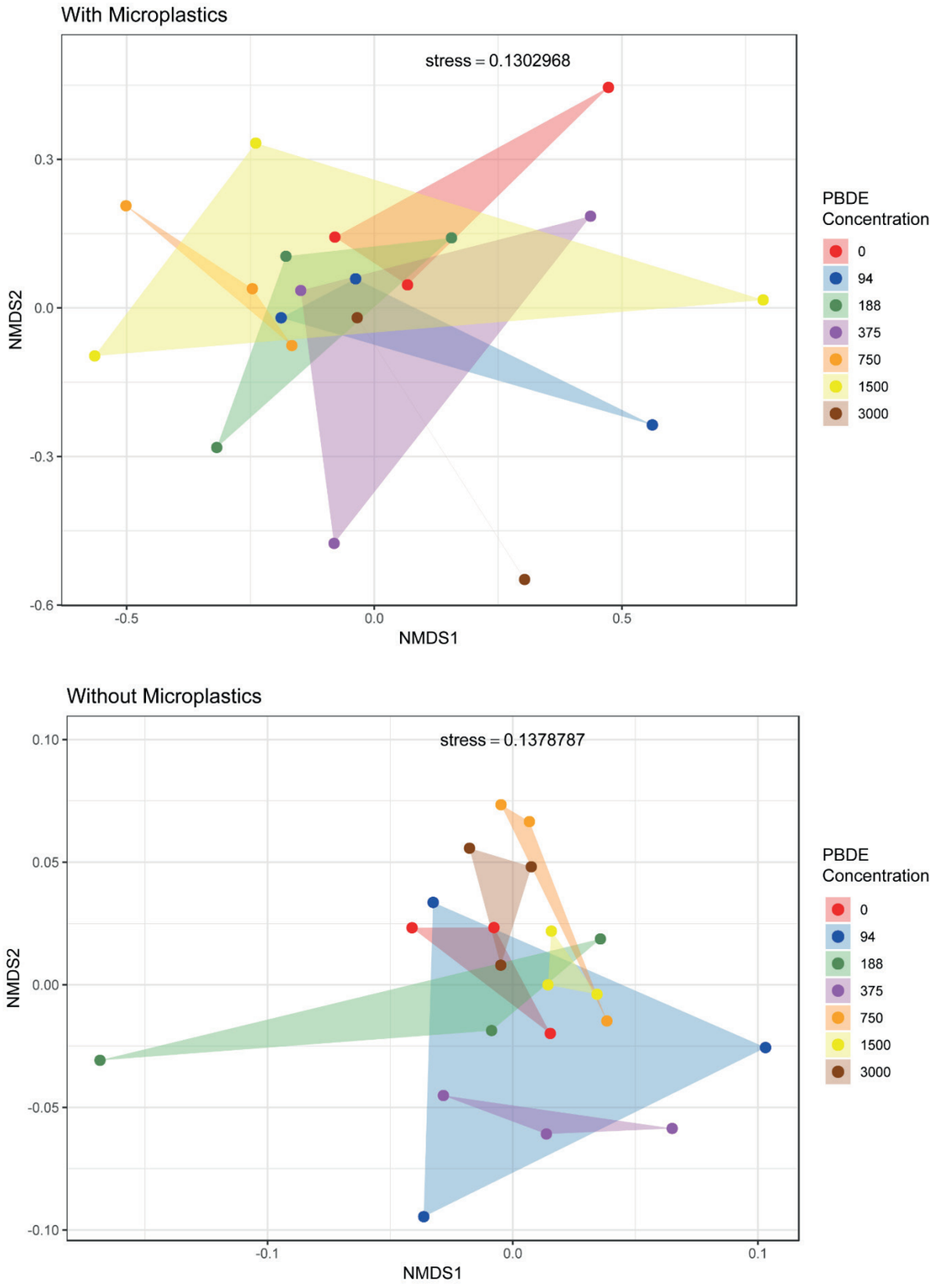

Fig S3. NMDS plots showing community dissimilarity for each PBDE treatment, with or without microplastics.

Fig. S4. Class composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with and without microplastics (present/absent). Relative abundance was calculated as rarefied number of sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3) are plotted on Y axis. For ease of representation classes of an abundance of <0.02 (2%) from an individual sample were excluded. *Note one sample was removed from this treatment due to inefficient sequencing, therefore *N*=2.

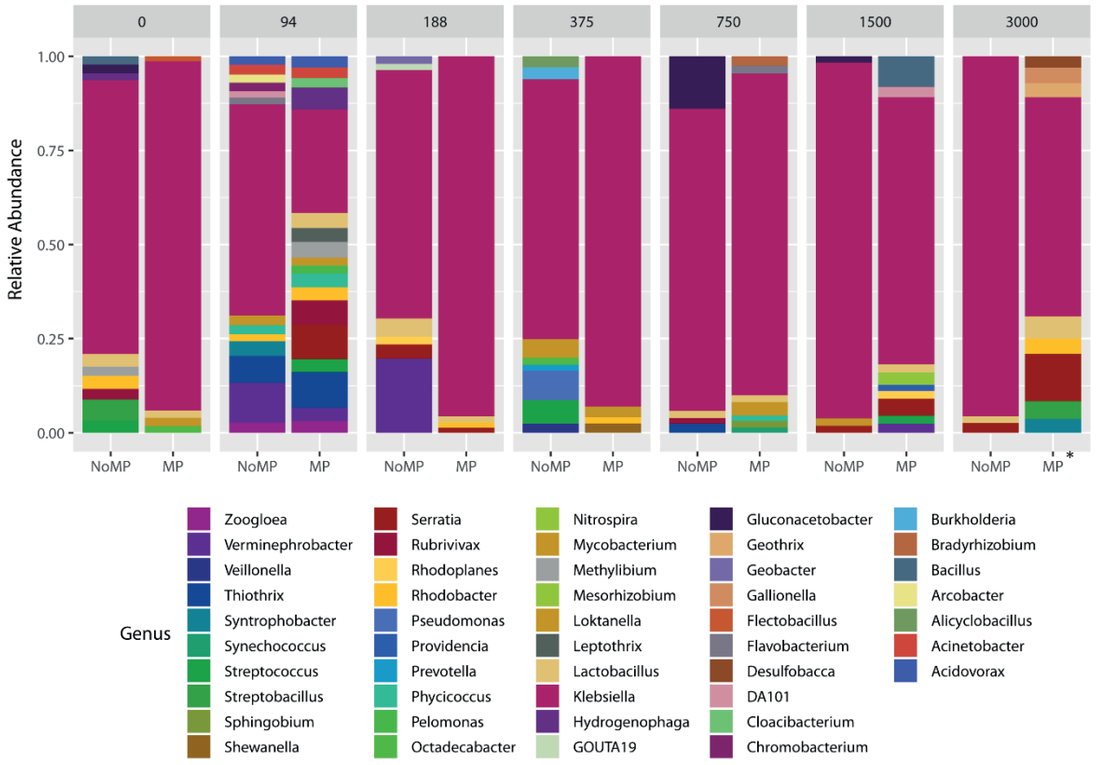

Fig S5. Genus composition of bacterial communities at each nominal PBDE concentration (ng g^{-1}), with and without microplastics (present/absent). Relative abundance was calculated as rarefied number of sequences in OTU/total sequences in each sample (= 6359), relative abundances per treatment (*N*=3) are plotted on Y axis. For ease of representation genera of an abundance of <0.03 (3%) from an individual sample were excluded. *Note one sample was removed from this treatment due to inefficient sequencing, therefore *N*=2.

CHAPTER 7

Discussion

1. Microplastics: a rapidly expanding field of research

This PhD thesis has been written during a time when the state of the knowledge of microplastics has been rapidly expanding, with multiple articles now published on the topic every week. This research effort has corresponded with an increased public awareness, often under the impression that microplastics are inherently toxic and harmful within the environment, despite the lack of clear evidence to support this view. It is unusual for such keen interest in a topic to precede scientific evidence, and as such, research has had to work fast to provide answers to the basic overarching questions posed by the public such as 'how much plastic is in the environment?' and 'is this a problem for ecosystem and human health?'. Despite this awareness of the potential problems associated with plastic pollution, the manufacture and usage of plastics continues to grow and the amount of plastic waste within the environment is predicted to increase (Geyer et al., 2017; Jambeck et al., 2015).

Chapter 2 was published at the time when this proliferation in research and understanding, especially of microplastics in freshwaters, was just beginning. This review comprehensively examined the current state of the knowledge of microplastics in freshwater and terrestrial environments, identifying the major knowledge gaps and the significant questions that needed to be addressed to develop our understanding in this area. This review highlighted the limited knowledge of microplastics in freshwater systems compared to the marine environment, and demonstrated where comparisons can be made between the two systems, to inform our understanding and help develop forward-thinking research questions. It also emphasised the lack of knowledge of microplastics in terrestrial environments, despite the widespread manufacture and use of plastics on land, including agricultural practises, in addition to industrial and domestic use. This made particularly clear the challenges involved in microplastics research, including the lack of method standardisation for environmental surveys, which hinders comparability between data and will be essential to address in the research field going forward.

There are contradictory results across studies on both the ecotoxicological effects of microplastics, and on the effects of associated chemicals (Beckingham and Ghosh, 2016;

Koelmans et al., 2016; Rochman et al., 2013c). This is partly due to the heterogeneous nature of the particles covered by the term 'microplastics' which can refer to particles of any polymer type, shape and size (below 5 mm) (Rochman et al., 2019), in addition to the variation in species' sensitivity to physical and chemical stressors (Adam et al., 2019). This makes it extremely difficult to predict the effects of microplastics on species and ecosystems. Such heterogeneity must, therefore, be taken into account when designing toxicity studies, in order to ensure that future studies will enhance our understanding of how these varied particle characteristics (e.g. size, shape, polymer) will influence their hazard. This must also be considered across a range of ecologically important species in order to determine which species and ecosystems may be most at risk from microplastic exposure.

Writing the review helped to clarify a number key questions considered in this thesis and my future research including: 1. What are the sources of microplastics to freshwater environments, how are microplastics transported and do they accumulate within freshwater sediments? 2. Do freshwater organisms interact with/ingest microplastics and can this be linked to environmental or physiological factors? 3. Does acute exposure to microplastics lead to eco(toxico)logical effects? 4. Do microplastics mediate the effects of different hydrophobic organic chemicals on toxicity, bioaccumulation and the microbiome? These questions have been addressed to an extent in the following thesis chapters, advancing our understanding of these key research challenges.

2. Microplastics in UK rivers – sources and ecological interactions

Before the publication of **Chapters 3 and 4**, no studies had been carried out to investigate the presence of microplastics within UK freshwater systems, and there was no knowledge of ingestion by UK freshwater organisms. This was a critical gap in our understanding of microplastic presence, distribution and ecological interactions, and therefore the first two studies carried out as part of this PhD thesis intended to address these two questions. As one of the most economically and commercially significant rivers in the UK, the River Thames catchment was chosen as our study system for both the environmental and the ecological studies.

It is understood that microplastics will derive from a wide range of sources and will be transported to the freshwater environment in different ways including via land run-off, drainage systems or sewage effluent input. The latter represents a variety of sources that will be

commonly released to wastewaters including microfibres released from synthetic fabrics when laundered and microbeads from cosmetic products (Ziajahromi et al., 2016). This is the easiest route of input to quantify and characterise based on known volumes of sewage treated, known populations contributing to sewage treatment systems within specific areas, and existing models to correlate these data with river flow data to estimate riverine effluent concentrations (Williams et al., 2009). For **Chapter 3**, sewage effluent input and population density were therefore chosen as predictors of microplastic presence in sediments at four sites in the River Thames basin (UK) Microplastics were found at all four sites. One site had significantly higher numbers of microplastics than other sites, average 66 particles 100 g^{-1}, 91% of which were fragments. Contrary to our hypothesis, this was not the site receiving the highest concentrations of effluent, but it was a site downstream of a storm drain outfall receiving urban runoff. Many of the fragments at this site were determined to be derived from thermoplastic road-surface marking paints, showing a clear and unimpeded pathway directly from the road surface to the river sediment. Road marking paints as a source of microplastics to the freshwater environment had not previously been described, and therefore these data were a significant contribution to the field of microplastics research. At the remaining three sites, fibres were the dominant particle type, as is the case in the majority of environmental microplastic studies (Barrows et al., 2018; Carr, 2017). These were present even at the sites with little sewage influence, further suggesting that effluent may not always be the dominant route of entry for microplastics to the riverine environment. For example, recent research has highlighted the likely high contribution of atmospheric transport and deposition of particles to regions where inputs may not otherwise have been expected to be significant (Allen et al., 2019).

A study has since been carried out which found that microbeads dominated the microplastics found within sediments at multiple rivers around Manchester, UK (Hurley et al., 2018a). This is likely due to the size range analysed, as the majority of particles observed by Hurley et al. (2018a) were < 1 mm, smaller than those analysed in our study. These results also show the extent to which the methods used for sampling and analysis may influence the result seen. With the results presented in Chapter 3, it is not possible to determine whether microbeads were present, given that the majority of these are likely to be < 1 mm, and therefore they would not been observed using our techniques. Additionally, their study showed the influence of intermittent weather events on particle concentrations, with concentrations in sediments significantly reduced following flooding (Hurley et al., 2018a). This highlights the importance of reporting the environmental conditions at the time of sampling, and undertaking time-series

sampling where possible. All rivers will experience periods of high and low flows, and therefore this is likely to be a significant factor influencing microplastic concentrations within riverine environments. Given that our study was a 'snapshot' in time, having been carried out on a single sampling occasion, it would be recommended for future studies to include temporal variation to better understand the dynamics of microplastic presence in sediments and surface waters.

While it has since been further acknowledged that road paints will be a source of microplastics to freshwater systems, few subsequent studies have shown evidence for road paints within environmental samples. Instead interest has shifted to the likely high contribution of tyre-wear particles to the number of microplastic particles in freshwaters (Abbasi et al., 2017; Boucher and Friot, 2017; Kole et al., 2017; Verschoor et al., 2016). However, paints as plastic composites remain of interest, especially within the marine environment, given their widespread use on boats and ships. Indeed paint particles have been found in marine sediments and surface waters (Chae et al., 2015; Reddy et al., 2006). It is important to note that such particles derived from maritime activities may pose the additional hazard of being derived from antifouling paints with biocidal properties, containing high levels of metals such as copper and zinc (Brennecke et al., 2016).

Following the identification of microplastics with the River Thames basin (UK), it was decided to investigate ingestion of microplastics by fish within this habitat. **Chapter 4** provided the first study of ingestion of microplastics by the freshwater fish (the common roach, *Rutilus rutilus*) within the River Thames. In combination with existing relevant literature (Andrade et al., 2019; McGoran et al., 2018; Sanchez et al., 2014), this study provided an insight into the factors influencing fish exposure and ingestion within riverine environments. This study aimed to link fish ingestion to environmental exposure (i.e. increased distance downstream from the source of the river was expected to increase exposure) and physiological factors (fish length and gender). Microplastics were found within the gut contents of roach from six out of seven sampling sites. Of the fish sampled, 33% contained at least one microplastic particle. This corresponds with a number of other studies which have found similar proportional contamination of fish with microplastics: 28% of fish sampled within the Adriatic Sea (Avio et al., 2015b), 32% within the Thames Estuary and the Clyde (McGoran et al., 2018), 36.5% in the English channel (Lusher et al., 2013). The majority of particles were fibres (75%), with fragments and films also seen (22.7% and 2.3%, respectively). This also corresponds to other

studies which found fibres to be the dominant particle type: 68% (Lusher et al., 2013), 83% (Steer et al., 2017) and 88% (McGoran et al., 2018).

Despite some similarities of our data to other studies, it must be noted that results across studies are also extremely variable in many respects. For example, while the particle types found by Steer et al. (2017) were dominated by fibres and were, therefore, proportionally related to what we found, contrary to our results they found only 2.9% of fish (larvae) had ingested microplastics. In our study, larger fish were found to be more likely to ingest a predicted maximum number of particles at a given location (based on quantile regression) than smaller fish. Female fish were more likely to ingest the predicted maximum number of particles than male fish. To our knowledge, gender-specific differences have not been highlighted in other microplastic ingestion studies. This suggests that intra- or interspecific factors such as size, gender or life stage may have a significant influence on feeding habits and thus ingestion of microplastics. Such factors are not currently well understood or described and this may explain many of the differences seen between studies. Further research on how organism physiology will influence ingestion is therefore recommended.

Where possible, ingestion studies should also seek to quantify surrounding environmental microplastic concentrations, for example within the water column, as this will enable an understanding of the link between exposure and ingestion. Such data providing evidence of direct links between exposure and ingestion are surprisingly scarce. Understanding of fish exposure to microplastics could be furthered by investigating trophic interactions to determine the likely dominant routes of microplastic uptake across different species, relationships which, to date, have been little studied within freshwater systems (Chae et al., 2018; Windsor et al., 2019b). Such knowledge will be valuable in informing future studies of microplastic impacts on fish health, and the wider ecological and economic implications of this (Lusher et al., 2017).

3. Challenges and recommendations for method development

Methods for microplastic sampling, sample processing and analysis have developed considerably in the last few years, even since the inception of this PhD. At the time of carrying out the analytical work for **Chapters 3 and 4** it was commonplace to visually identify and quantify microplastics by eye using a binocular microscope, and then verify this identification using spectroscopy, methods focussed on and optimised within these chapters. Similarly, the minimum size of particle commonly identified was in the hundreds of microns scale. More

recently, studies are increasingly moving towards more automated and technologically advanced methods, for example using fluorescence staining and image analysis, FTIR mapping, or mass quantification using thermo-analytical methods. Such methods eliminate bias and allow for the identification of much smaller particles down to tens or even 1 µm (Cabernard et al., 2018; Erni-Cassola et al., 2017; Simon et al., 2018). However, the more manual methods are still widely utilised by researchers who are using relatively recently-published literature for guidance.

With respect to the reporting of microplastics data, these may be reported in different units *i.e.* either by mass or number of particles, leading to difficulties when attempting to compare results between studies. To some extent the units reported are reliant on the methods used – manual and spectroscopic methods rely on counting and characterising individual particles, whereas chemical analysis techniques produce results by mass of polymer. This has implications for interpretation, comparison and utilisation of data across different studies. Where extrapolation is possible, for example using quantitative data on number, size and polymer type of particles to also estimate a mass, this is advisable to enable greater comparability between studies. Due to the fast rate of knowledge expansion and method development in this field, it must be acknowledged that some lag is inevitable if allowing all researchers to 'catch up'. In fact, while methods have significantly improved in recent years, it is recognised that due to the continually improving analytical capabilities for microplastic detection, quantification and polymer analysis, method development is likely to continue at pace for some time.

The utilisation of different techniques, methods and units for collection and reporting of microplastic data is a widely-recognised hindrance to comparability of data between studies. While there is a call for development of protocols (including by the British Standardisation Committee, BSI, and the International Standardisation Organisation, ISO), based on equipment accessibility and also sample heterogeneity, it may not be reasonable to suggest that all studies adhere to a strict standard protocol. Further, the technical capabilities will likely continue to progress with the development of analytical equipment that is fit for purpose, and therefore standard methods determined now could be outdated within a few years. As such, care must be taken to regularly evaluate and update any written protocols and recommendations that may be put into place. Despite this, a few key recommendations can be made now, and should be agreed across the research community. As a minimum, all methods should be reported step-by-step, including all specifications of sampling or analytical equipment used, the time/depth/volume/weight of sample taken, particle size analysed (minimum and maximum),

how subsamples were selected, any software parameters for undertaking data analysis and any calculations used to extrapolate to concentrations. It is essential to detail any uncertainty or ambiguity in microplastic analysis, for example, where reporting false positives or negatives may significantly influence results.

A critical consideration for high-quality analysis of environmental samples is that of contamination controls and blanks. Since the early microplastic studies it has been recognised that samples may become contaminated in the lab by airborne particles, although often little in the way of contamination control is implemented (Hidalgo-Ruz et al., 2012; Koelmans et al., 2019). In the past, at most this may have consisted of a petri dish left open to the air and later examined for contamination. However, recently it has been noted that more rigorous controls are necessary, including blank process controls to account for contamination throughout all sample collection and processing steps. Recent analyses have suggested that the majority of studies to date have not carried out sufficient controls to ensure the reliability of the data collected (Hermsen et al., 2018; Koelmans et al., 2019). It has also been recognised that plastic laboratory equipment in itself may shed particles and contaminate samples, therefore glassware is recommended over plastic wherever possible. It is also suggested to wear natural fabrics during sample collection and processing, although this is not always practical (especially in the case of fieldwork). Within the lab, a cotton lab coat should be worn to cover clothing (Woodall et al., 2015).

It is also highly recommended to carry out positive controls to account for any particle loss during processing, as is the case with chemical analyses, to determine what proportion of particles are actually being recovered from the matrix (Koelmans et al., 2019). It is possible that many studies provide an underestimation of particles as a result of ineffective recoveries based on the extraction procedures used. This is especially the case for very small particles which may have been missed due to filtering or sieving carried out as part of the sample collection or processing methods (Hurley et al., 2018b).

A collaborative effort across the research community to adhere to such recommendations will allow for the utilisation or comparison of these methods in future studies as appropriate for the question being asked, and thus allow for the harmonisation, rather than the standardisation of methods (Rochman et al., 2017). Therefore, while not always directly comparable to other studies, resulting data can be compared with known limitations. If repeating the studies published within this thesis, contamination controls would undoubtedly be more rigorous and

recovery standards would have been implemented. However, these studies were carried out in line with recommendations at the time.

It is especially important to bear in mind that, when considering recommendations for future microplastic research, analytical techniques may not be of interest simply to academics, but ultimately to governments who may wish in the future to regulate microplastics across a range of systems. Furthermore, the industries that will be required to monitor their contributions in line with these regulations will need to keep up to date with methodological recommendations, in anticipation of needing to take action. Without comparable and repeatable methods, reliable scientific evidence to inform regulations will be difficult. Given this, and for the inclusion of developing countries in progressing the research, it is important that simple, time-efficient and cost-effective methods remain valid for the analysis of microplastics, providing sufficient information on these methods and the limitations of the study are provided in any published reports. For non-academics, or those with limited resources, it may not always be necessary to identify every particle by polymer type, shape and size if simply quantifying microplastics is the desired outcome. However, contamination controls must be in place, quality assurance must be met (e.g. accounting for particles found in blank samples), polymer confirmation is needed to eliminate false positives, even if only on a subsample, and reporting must be clear. Based on recent methodological developments, going forward it would always be recommended to use methods which eliminate bias (rather than identification by eye), the most simple of which is fluorescent staining combined with image analysis (Erni-Cassola et al., 2017; Maes et al., 2017).

4. Microplastic toxicity and effects on chemical bioavailability

There is still a degree of uncertainty as to whether or not microplastics influence the bioavailability and toxicity of hydrophobic organic chemicals. The variability in results between studies is largely likely to be due to the complexities of comparing different polymers, chemicals, organisms and environmental matrices, all of which will influence chemical associations and dynamics. It is too crude to consider simply ‘microplastics’ and ‘hydrophobic chemicals’ as single contaminants due to the great diversity among these materials (Rochman et al., 2019).

Given that microplastics and chemicals within the environment will rarely, if ever, occur independently, it is essential that we understand how these mixtures may differently influence

species and ecosystems compared to individual pollutant exposures, as are usually carried out within the lab. For this reason, within the studies detailed in **Chapters 5 and 6**, two different polymer types were selected, with differing densities (low = polystyrene and high = nylon) alongside two particular types of chemicals with uses that could lead to their release to the environment (1. pesticides: dimethoate and deltamethrin, and 2. flame-retardants: PBDEs). As test organisms two different species were chosen, *Daphnia magna*, a keystone species within pelagic food webs and *Lymnaea stagnalis,* a benthic feeder. Despite the intentional variability of chemicals, polymers and organisms within and between these studies, the overall outcome was the same: microplastics had no influence on chemical toxicity or bioavailability. Microplastics did not influence the toxicity of chemicals (*Daphnia magna* exposed to polystyrene and pesticides, **Chapter 5**) or the accumulation of chemicals (*Lymnaea stagnalis* exposed to nylon and PBDEs, **Chapter 6**). This result is in line with a number of other recent studies which found that microplastics did not influence bioavailability or toxicity of hydrophobic organic chemicals (Ašmonaitė et al., 2018; Devriese et al., 2017). Due to the relatively low mass of microplastics within the environment, a greater proportion of hydrophobic chemicals will associate with natural organic and inorganic particulate matter. Under natural environmental conditions, microplastics are therefore likely to have a negligible effect on the bioavailability of associated chemicals (Bakir et al., 2016; Koelmans et al., 2016).

Comparing the results in **Chapters 5 and 6** to previous studies on microplastics and associated chemical toxicity in this field highlights the variability between the results of different studies, which are highly dependent on the organisms exposed, the particles used and the exposure conditions. For example, the PBDE exposure conditions in **Chapter 6** were very different in many ways to a study carried out by Rochman et al. (2013c), who found that the presence of microplastics significantly influenced the bioaccumulation of PBDEs in fish. When considering chemical accumulation, a different organism would likely have accumulated PBDEs (and different congeners) differently. For example, some organisms are more likely to bioaccumulate hydrophobic chemicals than others, based on lipid content (Gobas, 1993; Mackay and Fraser, 2000). It could be valuable to evaluate such responses to microplastics in association with other indicators of organism sensitivity where available. For example, existing toxicity data of common chemical pollutants may help to understand the underlying mechanisms and specific traits which can influence sensitivity, including inter- and intraspecific differences such as metabolism or life stage (Baas and Kooijman, 2015; Mohammed, 2013). Such understanding of differential organism responses may help in

predicting susceptibility to harm by microplastics. However, given that microplastics pose a combined chemical and particulate threat (Rochman, 2013), it should also be acknowledged that responses to microplastics may differ greatly to other stressors, and thus continued research into these effects under varying conditions is essential. In addition to the organism itself, the particle properties, including size, shape and polymer type will significantly influence the likelihood of ingestion and possible hazard. Finally, the route and duration of exposure can also significantly influence the effects seen, for example, PBDEs spiked directly into the water may have behaved differently and been differently bioavailable compared to the sediment-based exposure we carried out. It is therefore critical that we consider these factors when interpreting data, as they have implications for our understanding of the effects of microplastics and hydrophobic chemicals, both individually and in combination.

It is not feasible to experimentally test all the possible permutations of species, particles and environmental conditions, therefore it would be valuable to learn from other areas of ecotoxicology which have considered this challenge. For example, a traits-based approach to understanding sensitivity has been recommended for nanotoxicology research, based primarily on understanding how organism morphology and physiology will determine sensitivity. This involves extrapolating known interactions between organisms, particles and the environment to enable prediction of how these factors will interact across a wider range of conditions, and the subsequent likelihood of harm (Song et al., 2011). Alternatively, read-across models may take a different approach by combining knowledge of the physico-chemical properties of particles and their interactions with the environmental matrix to predict exposure (Gajewicz, 2017; Quik et al., 2018). While potentially valuable for making predictions in future microplastic research, both of these approaches rely on sufficient availability of data, which for microplastics is still lacking.

5. Microbiome response to microplastics and flame-retardants

One aspect of the potential biological effects of microplastics that has been little studied is that of the microbiome. It is known that the gut microbiome is susceptible to perturbation as a result of chemical or physical stress (Moya and Ferrer, 2016). The limited response of the microbiome to microplastics and PBDEs observed in **Chapter 6** was an unexpected finding, as most studies investigating the effects of PBDEs and microplastics (independently) have found significant effects relating to an increased availability of hydrophobic organic chemicals

(Chen et al., 2018; Zhu et al., 2018a). However, these previous studies ran for a minimum of seven days whereas ours was a 96 hour exposure, implying that exposure duration is likely to be highly significant. It is understood that acute and chronic microbiome responses will be different (Shade et al., 2012), with some communities resilient to short-term or 'pulse' perturbations (Sommer et al., 2017). Therefore, it is likely to have been the case that in this study, the pulse exposure was insufficient in duration to induce a change in the microbial diversity or community composition. Pulse exposures are highly relevant in an environmental context, as inputs and chemical concentrations within the environment will fluctuate enormously, especially within highly dynamic environments such as rivers. As such, organisms are rarely likely to be exposed to consistent concentrations of chemicals (Handy, 1994; Reinert et al., 2002). However, it must be considered that microplastics will not readily degrade and therefore can accumulate within environmental sinks (Browne et al., 2011; Corcoran et al., 2015; Turner et al., 2019). In a relatively enclosed and undisturbed environment where microplastics can accumulate, there is therefore the potential for organisms to be chronically exposed. This highlights the need to consider how chronic low-level exposures may differ from acute exposures when trying to interpret organism and ecosystem responses to microplastic pollution. Further, differences between organism microbiome responses will be species-specific, for example relating to feeding habits and gut retention time. This therefore requires linking our understanding of environmental concentrations and conditions (exposure) to the effects seen in laboratory exposures (hazard).

6. Implications and impact of this research

It is clear that microplastics are everywhere within the environment. While there is a lot of available information on microplastics in the oceans, there is still a lesser understanding of microplastics in freshwater and terrestrial systems, despite the recent increase in research. Many of the knowledge gaps and research questions outlined in **Chapter 2** still remain. Rather than focussing on simple presence and abundance studies, in future studies it will be important to investigate the factors influencing microplastic behaviour and fate, for example, biofouling, weathering and degradation, to better understand where and why microplastics accumulate. While flux and transport models are becoming increasingly more commonplace to assess the volumes and transport of microplastics within the environment, it is essential that data are available with which to parameterise and validate these models, across a variety of different

environmental scenarios. For example, it is recognised that lakes can act as sinks for microplastics. Using sediment cores, this can provide stratigraphic evidence of microplastic accumulation that can be directly related to temporal trends in microplastic deposition (Turner et al., 2019). This is in contrast to riverine environments where flow conditions can lead to the rapid mobilisation of particles (Horton and Dixon, 2018). Identifying local sources, inputs and hotspots at specific sites (for example road marking paints as identified in **Chapter 3**), in addition to the types and characteristic of particles found, will provide new information to inform models. This is especially important given that some models may have been originally developed to predict chemical concentrations and transport, while microplastics by their nature will behave very differently to the majority of chemical pollutants. Such field data will, therefore, enable an improved understanding of the importance of these localised inputs to the more widespread movement of microplastics within river systems. If inputs or accumulations are found to be significant, for example from urban drainage, this will have implications for civil engineering and town planning decisions, helping to inform the cost-benefit assessment of materials used within urban settings, in addition to the design and regulation of such systems. This evidence will therefore contribute to better-informed decision-making for policy and industrial practices based on a greater knowledge of microplastic sources, environmental inputs, and mechanisms of transport, allowing for targeted industry and location-specific mitigation measures to be implemented.

Knowing that microplastics are widespread throughout the freshwater (and wider) environment and understanding hotspots of contamination, then developing a greater knowledge of organism interactions with microplastics, and the associated hazard, is critical for determining potential ecological effects. It is clear from the findings in **Chapter 4** that variations in ingestion between individuals can be highly intra-specific. A greater understanding of the physiological factors influencing ingestion within and between species is therefore essential, alongside a sound understanding of the environmental factors influencing exposure.

The studies detailed within **Chapters 5 and 6** aimed specifically to investigate the influence of microplastic presence on chemical toxicity and accumulation, therefore we used very high concentrations of microplastics in order to maximise the likelihood of interactions between the organisms, microplastics and chemicals. Concurrently it was also possible to observe the response of organisms to such concentrations of microplastics alone. Our results suggest that even very high concentrations of the microplastics tested within these studies will not be of ecological importance over acute timescales, however from these data it is not possible to

predict how this relates to other types and sizes of microplastics. Where very high (unrealistic) concentrations of microplastics are used in future studies, this approach must be justified as being worthwhile and valuable for our understanding of microplastics as a pollutant. For example studies should seek to understand mechanisms of toxicity or chemical transfer processes, across a range of different polymers, particle sizes, ages and additive chemicals, where subtle effects may be difficult to interpret at lower concentrations (Huvet et al., 2016; Kuhn et al., 2018). It has been suggested that more realistic, chronic exposures could induce subtle, sub-lethal effects leading to longer-term ecosystem consequences (Au et al., 2015; Jaikumar et al., 2019; Redondo-Hasselerharm et al., 2018). While effects over chronic timescale are not always seen (Bruck and Ford, 2018; Weber et al., 2018a), such studies are likely to be more ecologically relevant than analysing acute responses to high concentrations of microplastics. Given that microplastics will not degrade quickly, environmental exposures are likely to be long-term (i.e. months, years or even decades). Therefore, experimental exposures in the order of weeks or even months would allow for the investigation of the potential sub-lethal or multigenerational effects of microplastics. It should be noted that even if the input of plastics to the environment were halted, environmental concentrations will continue to increase due to the degradation of existing plastics. Therefore it is reasonable to assume that organisms will be exposed to ever-increasing concentrations of microplastics and nanoplastics (Mattsson et al., 2015). This must be therefore taken into consideration when thinking about future worst-case scenarios and risk assessment.

If effects of microplastics and adsorbed chemicals on organisms are to be seen, it is expected that they would have been observed within a pristine, controlled setup, such as those used in the studies presented with **Chapters 5 and 6**. The lack of effects therefore imply that within the environment where geological, chemical and biological processes are more complex, the interactions between the organisms and pollutants investigated in these experiments are likely to be insignificant. In addition to the chemicals that may associate with microplastics once they enter the environment, many plasticiser chemicals are incorporated into plastics during manufacture, which are not chemically bound to the polymer structure and will leach out of the plastic over time (Geiss et al., 2009; Godwin, 2011). While hydrophobic organic chemicals (HOCs) are widespread within the environment, plasticisers would not be widely present in the absence of plastics. Given the negligible microplastic-facilitated toxicity of externally associated HOCs seen in the research presented within this thesis, it is likely that plasticisers will become significantly available to organisms as a result of microplastic presence, due to

leaching (Devriese et al., 2017; Lohmann, 2017). Plasticisers have been proven to leach out of common plastic products and cause toxic effects to aquatic organisms (Lithner et al., 2009; Lithner et al., 2012). It has been suggested that leaching of plasticisers from microplastic particles is likely to be size-dependent and as such has further toxicological implications when considering the degradation of particles within the environment (Coffin et al., 2019). However, research in this area is still limited and therefore in future studies it would be recommended to prioritise the investigation of plasticiser toxicity (including rates of leaching and effects of weathering) over the investigation of sorbed organic chemicals.

In order to effectively develop our understanding of the effects of microplastics within the environment, it is especially important that future research looks to better design experimental studies to make them relevant and relatable to predicted or actual environmental conditions, concentrations and particle transformations (Kuhn et al., 2018; Lenz et al., 2016). Due to methodological limitations, we are currently unable to understand fully the range of microplastics present within the environment, especially with respect to the lower size limit of particles present (Huvet et al., 2016). The lower particle size limit which can currently be simultaneously quantified and analysed to polymer type (using micro-FTIR imaging) is approximately 10-20 μm (Liu et al., 2019; Mintenig et al., 2019; Simon et al., 2018). It is assumed that plastic will undergo a continual degradation from macroplastic to microplastic, eventually degrading to nanoplastics (Mattsson et al., 2015). However, analytical techniques for detecting nanoplastics within environmental samples are in their infancy (Nguyen et al., 2019; Schwaferts et al., 2019). Therefore recommendations cannot easily be made for monitoring, and thus regulation of plastic particles < 10 μm. Within laboratory assays, this also limits the ability to trace particles, thus impeding understanding of toxicological mechanisms. Recent developments in producing novel, metal-doped nanoplastic particles could provide a solution to these detection limitations, *i.e.* plastic particles containing a rare metal component allowing for analysis of the metal as a proxy for the presence of the nanoplastics (Mitrano et al., 2019).

If, and when, environmental regulations for microplastics are implemented, it would be recommended to use the risk assessment approach of comparing predicted environmental concentrations to ‘ecologically acceptable concentrations’, as is often the case with chemical regulation (Crane and Giddings, 2004; Hommen et al., 2010; Rico et al., 2016). Efforts have been made to synthesise and interpret existing microplastic data on exposure and hazard by Adam et al. (2019) and Burns and Boxall (2018). These reviews highlight not only the

variability in responses between different species, but also within species, where responses (for example NOEC based on particle concentration) can be orders of magnitude different, even when exposed to the same shape and type of polymer. This may be due to different exposure conditions (for example different culture conditions or particle sizes) but can still occur even where all other exposure conditions appear to have been the same (Adam et al., 2019; Martins and Guilhermino, 2018; Rehse et al., 2016). Where species have been the subject of multiple studies, it is therefore possible to make better estimates of responses to microplastics, although it is still necessary to understand the full range of exposure conditions, in order to be able to determine the factors influencing any differences seen. Although substance regulations are usually based on the risk assessment framework, where concerns are high this type of review may be bypassed. For example, this year the European Chemicals Agency (ECHA) have proposed a restriction on all 'intentionally added microplastics' as a precautionary measure, despite little substantial evidence of harm, (ECHA, 2019). This restriction proposal aims specifically to restrict or regulate the use of microplastics added to products where their use will result in direct or indirect release to the environment. This will include products not previously regulated for microplastics including, for example, detergents, sunscreens, paints and seed coatings. Depending on the product, this may require a restriction on placing the product on the market (where microplastics will certainly enter the environment as a result of using the product), specific labelling requirements (to minimise releases to the environment where this may occur indirectly during use) or improved reporting requirements to improve the standard of information available to consumers. While this only applies to primary microplastics and does not cover the more substantial inputs from secondary sources, this could still prevent ~36,000 tonnes microplastics entering the environment annually (ECHA, 2019).

It is important to be aware that the pristine particles often used within laboratory exposures are not representative of those found within the environment. Plastics are complex heterogeneous mixtures of polymers and chemicals and as such, it is not feasible to predict microplastic effects based solely on limited studies of homogenous polymers and particle types. Laboratory studies must therefore be designed effectively to enable the determination of toxicological effects of plastics with well-defined characteristics, based on size, shape, polymer type, externally associated chemicals and weathering, to better determine the specific factors leading to any toxic effects seen (Kuhn et al., 2018; Rochman et al., 2019; Vroom et al., 2017). Given the potential for environmental transformation to significantly influence the toxicity of particles, this requires a greater understanding of these particle characteristics and transformations within

the environment. This is an area of research that has been extensively studied for engineered nanomaterials, but less is understood about how these processes will influence microplastic toxicity (Schultz et al., 2015; Syberg et al., 2015). Microplastic research is extremely multi-disciplinary, spanning ecotoxicology, microbiology, chemistry, geography, hydrology and more, and therefore it is essential that researchers collaborate to address these key questions.

The findings in presented this thesis have contributed to our understanding of microplastics as a pollutant within freshwater systems, having particular impact within the UK. This has enabled dialogue with UK regulators including the Department for Environment, Food and Rural Affairs (Defra) and the Environment Agency, regarding necessary future research and possible implications for policy and regulation around plastic items and microplastics. Microbeads within wash-off cosmetic products were banned in the UK from 2017 following a public consultation (Defra, 2016). The proposal for microplastic restriction by ECHA (ECHA, 2019) will apply to a far greater range of products and thus will be much more difficult to implement. While these restrictions are helping to prevent some microplastics entering the environment, it must be borne in mind that primary microplastics such as microbeads and glitter are only infrequently found within environmental samples. Instead the majority of particles in fact consisting of secondary microplastics formed by the degradation of larger items (Anderson et al., 2017; Boucher and Friot, 2017; Ryan, 2015). The degradation processes leading to this fragmentation are not well defined in the context of the many different polymer types present within the environment. Developing a sound understanding of these processes and their implications for conventional polymers, in addition to the relatively recent development of degradable and biodegradable polymers, will be a great challenge for scientists, regulators and industry alike (Napper and Thompson, 2019).

7. Outlook

When considering microplastics it is important to remember that microplastics form but one small part of the plastic issue. Given that microplastics usually derive from the breakdown of macroplastics, and will further degrade to form nanoplastics, it is not reasonable to consider microplastics as a separate entity (Blair et al., 2019; Lambert and Wagner, 2016). Rather, microplastics should be considered within the bigger picture of plastic manufacture, use and disposal, as a wide range of different materials from various sources (Rochman et al., 2019). By the time microplastics reach the environment, it is almost certainly too late for mitigation

or removal, due to their small size and their ability to be transported large distances (Allen et al., 2019; Horton and Dixon, 2018). Thus if any potential effects or implications of microplastics in the environment are to be avoided, they must be prevented from entering or forming within the environment in the first place.

In response to the growing global recognition of plastics as a widespread and persistent environmental pollutant, a number of small and large-scale initiatives are in place. These include, for example, individuals changing their plastic use habits, producers using natural or recycled packaging materials, microplastic removal devices such as filters or laundry bags design to capture fibres, and the development of bioplastics and degradable plastics. However, the benefits and disadvantages of novel products or plastic alternatives are not well understood by consumers due to the lack of specific definitions and regulations regarding these products. One example is that of biodegradable plastics: a common misconception is that biodegradable plastics can be thrown anywhere in the environment and will rapidly degrade to form harmless, natural materials. However, research has shown that the majority of products labelled degradable and biodegradable will not mineralise within the environment under any meaningful amount of time (Napper and Thompson, 2019). Further confusion arises from the labelling of such plastics, with terms including ‘oxodegradable’, ‘biodegradable’ and ‘compostable’, all inferring that the product will fully degrade but in reality, many such items have differing properties and will often degrade only under very specific conditions (Lambert and Wagner, 2017).

Many plastics are also theoretically recyclable, for example polyethylene terephthalate (PET), although whether they are in fact recycled depends on the waste management practices within the country or region (Eriksen et al., 2019). Across much of Europe, for example, the waste management infrastructure is unable to handle the amount of recyclable waste produced and much of this is shipped to Asian countries (Brooks et al., 2018). Here some of it will be recycled, but much of it will also end up being (often illegally) incinerated or landfilled (Ray, 2008). Where plastics are mechanically recycled, the majority of recycling practices lead to ‘downcycling’ whereby the product becomes less pure and is used to create lower-quality products with each cycle, ultimately becoming a product which requires landfill or incineration (Rahimi and García, 2017). Alternative options for recycling exist, for example pyrolysis (also known as thermal cracking) to break the polymer down to its constituent monomer components. However, further work is needed to assess the economic viability and large-scale feasibility of such processes (Brems et al., 2012). It has been suggested that ‘problem’ plastics,

which cannot be readily recycled and often end up as contaminants within the environment, such as PVC and expanded polystyrene packaging, should be phased out (WRAP, 2019). As such, in order to effectively design a product for that can be recycled, provisions must be made for the product's end of life during the design and manufacture stage. While the general public can influence the decisions and practises of businesses based on demand, ultimately it is the manufacturers and large organisations who can address the plastic pollution problem, based on the materials they use and the products they design.

Despite the recognition that plastic waste management and pollution is an increasing problem, the manufacture rate of plastics continues to increase and is projected to increase ~400% by the year 2050 (World Economic Forum, 2016). In order to prevent the continued accumulation of plastic within the environment, and any resulting harmful effects, global discussions and collaborations are needed to tackle the problem from all angles. This includes a better understanding of the sources and fate of plastics, the degradation and behaviour of traditional plastics and their alternatives, and the toxicity and long term ecological effects of the wide variety of different particle shapes, sizes and polymer types. With such knowledge we can inform future regulations, mitigations and solutions to what is indisputably one of the most prominent environmental issues today.

8. Conclusions

In recent years, efforts in microplastic research have increased significantly. This PhD research was carried out at a time when there was little knowledge of microplastic inputs, presence within the environment, availability or toxicity to freshwater organisms. The research presented within this thesis has enhanced our knowledge across these areas, and although many questions remain, these questions have become better refined and informed. In order to progress further our understanding of microplastics as an environmental pollutant, it will be essential to coordinate research efforts in understanding both the fate and effects of microplastics within the natural environment, combined with hazard studies determining toxicity and effects thresholds over realistic exposure scenarios and timescales.

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CURRICULUM VITAE

Alice Avory Horton was born on 3rd July 1988 in Romsey, UK. Alice completed her GCSEs at the Mountbatten School in Romsey, followed by A-levels in Human Biology, Chemistry and German at Peter Symonds College in Winchester. In 2006 Alice moved to Brighton, where she studied for her bachelor's degree in Biology at the University of Sussex, UK, graduating in 2009. After a period of work and travel, in 2011 Alice decided to further pursue her academic interests and began a master's degree in Oceanography at the University of Southampton, UK, based at the National Oceanography Centre (NOC). This opened her eyes to new aspects of science at the forefront of research and discovery, and helped her to realise her interest in the effects of environmental pollution on organisms and ecosystems. During her Master's she also volunteered on a research cruise in the North Atlantic (aboard the RRS Discovery) which gave her the unique experience of a different type of scientific working environment. These experiences encouraged her to pursue a relevant career in aquatic science.

Following a stint working as a Field Scientist for an aquatic environmental consultancy (APEM Ltd, UK) from 2013-2014, Alice took a position as a Research Associate at the Centre for Ecology & Hydrology (CEH), UK, in 2014. This was a pivotal turning point in her career, enabling her to develop an expertise in microplastic pollution in freshwater environments, in addition to providing excellent opportunities for career progression. It was during this appointment that Alice registered to study for her PhD at Leiden University, the Netherlands, alongside her employment. In 2017 Alice progressed to the role of Ecotoxicologist, and was also awarded a NERC Knowledge Exchange Fellowship to build the UK Microplastics Network, an interdisciplinary network promoting collaboration and communication surrounding microplastic-focussed research and relevant activities in the UK. Having this fellowship alongside a research-focussed job allowed Alice fantastic opportunities to share relevant knowledge with others, developing relationships, building new collaborations and travelling within the UK and overseas.

In August 2019 Alice returned to NOC in Southampton as an Anthropogenic Contaminants Scientist, to develop her research in microplastics and plastic pollution within the marine environment. Alice continually aspires to a lifetime of adventure and discovery, and is excited to see what the future holds.

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