The Ecotoxicological Impact of Microplastics on Freshwater Invertebrates

A thesis submitted for the degree of Doctor of Philosophy

School of Biological Sciences

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For my parents

who funded my study

DECLARATION

This dissertation is the result of my own work and includes nothing, which is the outcome of work done in collaboration except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

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SUMMARY / ABSTRACT

Microplastics (MPs) are small plastic particles released directly from the use of cosmetic products, or indirectly through the degradation of large plastic items under environmental conditions. Microplastics have been found in marine and freshwater environments around the world, raising concerns about the long term impact on animals and ecosystems in addition to recent discoveries of MPs entering the human food chain. The impacts of MP pollution on ecosystems and their functioning remain poorly quantified and there is a paucity of information on the impacts of MPs in freshwater ecosystems, despite the broad range of pathways through which MPs can proliferate and the extensive range of species which actively ingest MPs in these systems. This thesis aims to obtain key data on the uptake, fate and ecotoxicological impact of MPs on freshwater invertebrates. Initially, MP uptake and chronic toxicity tests were gathered by exposing the crustacean water flea *Daphnia magna* Straus 1820 (Cladocera) to polystyrene MPs of sizes 2 and 15 μm. The endpoints were mortality, growth and number of offspring. The results indicate that *D. magna* selectively uptake food particles over MPs, and that the toxicity was mainly linked to the availability of food. Moreover, a significant size dependent increase of toxicity was observed, with exposures to 2 μm sized particles being more toxic than 15 μm sized particles. Uptake, fate and toxicity of MPs were also studied in a holometabolous insect by exposing the common house mosquito *Culex pipiens* Linnaeus 1758 (Diptera) to polystyrene MPs of sizes 2 and 15 μm. Results showed both particle sizes were readily taken up by larval mosquitoes then transferred to the adults via pupae. There were more transfer of MPs size 2 μm compare it to 15 μm without any effecting on mortality and weight of adults. This work is the first to demonstrate that MPs can be transferred ontogenically through organisms with complex life histories, presenting a potential pathway for dispersal of MPs into terrestrial environments. Laboratory studies were followed up with a field study exposing a small freshwater pond community to 15 μm polystyrene MPs. The results show that a high proportion of MPs accumulated in the sediment while only a small amount remained in the water column, with a significant correlation between the number of MPs in the water and the freshwater invertebrate. The presence of MPs had no real impact on the freshwater community, with season being a more important variable. Finally, the predatory ability of non-biting midge larvae, *Chaoborus flavicans,* towards larvae of *C. pipiens* mosquitoes loaded with 2 μm was quantitatively examined by linking MP trophic transfer with predation rates in a functional response framework.

Results demonstrated a lack of effect of MPs on predation rates and correlation number of MPs transferred through predation.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS AND ACRONYMS

MP	**Microplastic**
MPs	Microplastics
PS	Polystyrene
PE	Polyethylene
PET	Polyethylene terephthalate
PP	Polypropylene
PVC	Polyvinyl chloride
WWTP	Wastewater treatment plants
PCBs	polychlorinated biphenyls
POPs	persistent organic pollutants
PAHs	polycyclic aromatic hydrocarbons
DDTs	Dichlorodiphenyltrichloroethane
D. magna	*Daphnia magna*
C. pipiens	*Culex pipiens*
PA	polyamides
LDPE	low-density PE
OECD	Organization for Economic Cooperation and Development
μm	Micrometre
Nm	Nanometre
L	Litter
mg	Milgram
ml	Millitter
g	Gram
Km	Kilometre

M^3	Cubic meter
UNIANOVA	Univariate Analysis of Variance
GLM	generalized linear model
SE	standard error
rpm	round per minuets
NMDS	non-metric multidimensional scaling
D. pulex	*Daphnia pulex*
FR	functional response
C. flavicans	*Chaoborus flavicans*

Chapter 1 GENERAL INTRODUCTION

Whilst we may consider plastic to be a modern word and substance, in fact plastics have been used for over 200 years (Meikle, 1995). The word *plastic* originally referred to any substance that was easily moulded and shaped (from the Greek adjective *plastikos*). Plastics were originally developed well before the twentieth century using natural materials such as the insect secretion shellac, latex from tree sap, rubber and celluloids (Meikle, 1995). However today when referring to plastics, we tend to mean synthetic long chain polymers originating from fossils fuels.

The first fully synthetic plastic, Bakelite (a thermosetting phenol formaldehyde resin), was invented in 1907 (Meikle, 1995). Bakelite could be shaped or moulded into almost anything and was marketed as the "material of a thousand uses". The success of Bakelite spurred chemical companies into a search for new polymers such as nylon and the onset of World War II provided the catalyst for plastic research and development which continued to grow and thrive post-war. The new plastics were seen as inexpensive, safe and clean, and product after product was replaced with plastic alternatives (Meikle, 1995). To date, an estimated 8.3 billion tons of plastic have been produced (Geyer et al., 2017). With global populations projected to rise to 9.2 billion in 2050 and as developing nations become wealthier, demand for plastics will undoubtedly increase (Bongaarts, 2009).

As a result of poor waste management and improper plastic disposal, plastic waste has dramatically accumulated in the environment (Derraik 2002; Thompson, et al. 2009). As early as the 1960s, plastic debris was recorded in the oceans and a growing awareness of environmental issues was developing (Ryan et al., 2009). Although it is easy to focus on the larger and more evident plastic debris such as the Great Pacific Garbage Patch (Howell et al., 2012) most plastic waste is microscopic. Under environmental influences such as ultraviolet light and physical abrasion the degradation of larger plastic particles leads to the production of microplastics (MPs) (Wagner et al., 2014). As a consequence, much of the plastic pollution found on the ocean surface is dominated by particles smaller than 1 cm in diameter (Hidalgo-ruz et al., 2012). However not all MPs are the result of degradation of larger particles. Many are released into the environment in this form, particularly from domestic wastewater. Although most of the research to date has focussed on the pollution of the world's oceans, plastic debris has been found in numerous aquatic systems, including European rivers (Lechner et al., 2014; Morritt et al., 2014), lakes (Horton et al., 2017) and mangrove swamps in India (Kukulka et al., 2012). Waste emanating from domestic sewage is often released into freshwaters prior to reaching the ocean. Here a multitude of organisms are exposed to the pollution

and yet at the beginning of this work, very little research had focussed specifically on freshwater organisms and MPs. This thesis is therefore a branch of a developing science to determine whether the effects of MPs measured in marine environments are also found in freshwater systems.

1.1 Literature Review

Plastics have been manufactured and exported globally since 1959 (Free et al., 2014), as they are cheap, easy to manufacture and have properties that allow them to replace natural products, including wood, stone and glass (Cole et al., 2011). These special plastic characteristics that allow them to be moulded, cast and spun or used as coatings have resulted in their extensive use (Thompson et al., 2009a). Due to the resilient properties of plastic (lightweight, strong, corrosion-resistant and high thermal properties), the demand continues to increase (Barnes et al., 2009). Plastic synthesis involves the primary polymers in addition to some other chemicals that are added during the plastics production to improve plastics performance (Cole et al., 2011). These additives include inorganic material such as carbon and silica which are used to increase plastic stabilization, reinforce and enhance flexibility (Thompson et al., 2009). Plastic production can vary from one product to another, for example plastics that are used in packaging material can be classified into Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polyethylene terephthalate (PET); and Polyvinyl chloride (PVC) (Andrady, 2011). Each of these plastics have the potential to break-down into smaller particles in the environment (Moore, 2008). Approximately 8300 million metric tons of virgin plastic have been produced worldwide, most of which ends up in the landfill or littering the natural environment (Geyer et al., 2017).

Plastic pollution has detrimental impacts on marine fauna through ingestion and entanglement (Gregory, 2009). Plastic pellets were first detected in the guts of sea birds in the 1960s (Ryan et al., 2009). By the late 1970s/early 1980s wide concern was developing over the sheer volume of cylindrical, virgin plastic pellets that were being ingested by pelagic seabirds e.g. (Shomura and Yoshida, 1985). In the decades that have followed there have been numerous accounts of marine debris ingestion by a great variety of seabirds (Gregory, 2009). Over 100 species of seabirds such as albatrosses, petrels, penguins and gulls are known to ingest plastic artefacts and/or become entangled with them (Laist, 1997). For birds such as albatrosses, anything floating on the ocean's surface was likely to be edible throughout their entire evolutionary history and they are unable to differentiate between food and rubbish.

In addition to the growing awareness of the issues associated with plastic litter and marine vertebrates, the 1970s saw the first records of plastic debris on the sea bed (Ryan, 1988). Fishermen trawling in the North Sea were catching plastic sheets 80-400m deep. They had sunk because of colonisation with calcareous bryozoan and algae which made them heavy enough to sink to the sea bed (Galgani et al., 2000).

There have been many studies on the physical impact of macroplastic ingestion on vertebrates (Wright et al., 2013). Impacts include: external or/internal abrasions and ulcers; and blockages of the digestive tract, which could be result in starvation, satiation and physical decline (Ryan et al., 2009). Any physical impact on the organisms can lead to reduced fitness, productivity, drowning, damage of feeding ability, the ability of damaging toxicants from seawater and death (Gregory, 2009). In addition to the possibility of adverse effects from ingestion of plastic themselves, toxic responses could be result from the leaching or adhering of other contaminants on plastic pellets (Cole et al., 2011). Entanglement is a major issue which can lead to both external and internal wounds, impairment of natural behaviour and drowning (Laist, 1997). As well as sea birds, plastics impact turtles, whales and dolphins; sharks, seals, fish and marine invertebrates (Gregory, 2009). Marine turtles that feed on jellyfish are often full of plastic bags which look extremely similar to their normal food. Three quarters of turtles examined from Mediterranean areas were found to have ingested plastic bags (Tomas et al., 2002). Legislation on the use of plastic bags in a number of European countries will hopefully reduce the extent of littering but much of the plastic waste comes from the fishing industry. Prior to the 1950s, marine nets and ropes were made of natural fibres which rotted relatively quickly if lost at sea, unlike the modern plastic replacements. Large marine vertebrates are often entangled in plastic ropes and nets by mistake or get trapped by plastic containers and strapping bands (Galgani et al., 2014).

1.2 What are Microplastics?

The result of degradation of large plastic particles causes the production of secondary microplastics (MPs) which can accumulate in aquatic environment (Cole et al., 2011). Microplastics are defined as *"the barely visible plastic particles that can pass through a 500µm sieve "* (Andrady, 2011). The working group on Good Environmental Status (WG-GES) had redefined the plastic particles to include the range of small particles that can be readily ingested by organisms to 'macroplastics' as >5 mm, 'mesoplastic' for particles between $\leq$ 5 to >1 mm, 'microplastics' items $\leq$1 mm to > 0.1 µm and 'nanoplastics' $\leq$ 0.1µm (Gigault et al. 2018; Fendall et al., 2009; Charles et al., 2008).

Degradation of polymers by environmental conditions takes place through a number of routes; Photo-degradation results from the exposure to sunlight over time, initiating photo-oxidative degradation in which large polymer molecular weight is decreased and oxygen-rich functional groups are released (Andrady, 2011; Browne et al., 2007). Another significant source of breakdown is biodegradation, this process works by living organisms such as microorganisms converting the polymers to CO_2 this process called complete mineralisation, although for most polymers only partial

breakdown occurs (Andrady, 2011). Additionally, there is also thermooxidative degradation which is slow oxidative degradation under moderate temperatures (Andrady, 2011). All these processes can affect the plastic materials and cause continuous break-down to polymers over the time period until they become tiny in size (Ryan et al., 2009).

Not all MPs are the result of secondary breakdown of larger plastics. Primary MPs are manufactured to add to consumer care products such as toothpaste or facial scrubs which will pass through filtration systems of the wastewater treatment plants and enter aquatic environments (Browne et al., 2007; Horton et al., 2017; Napper et al., 2015). Approximately 70% to 80% of MPs in marine environments are thought to originate from inland sources and are carried out from rivers to the oceans (Andrady, 2011). Wastewater treatment plants (WWTP) effluents, fishery activities, cargo shipping and direct shoreline disposal are considered to be potential sources of contamination (Birch et al., 2015; Claessens et al., 2011; Dubaish et al., 2013; Horton et al., 2017; Peng et al., 2017; Zubris and Richards, 2005) (Figure 1.1).
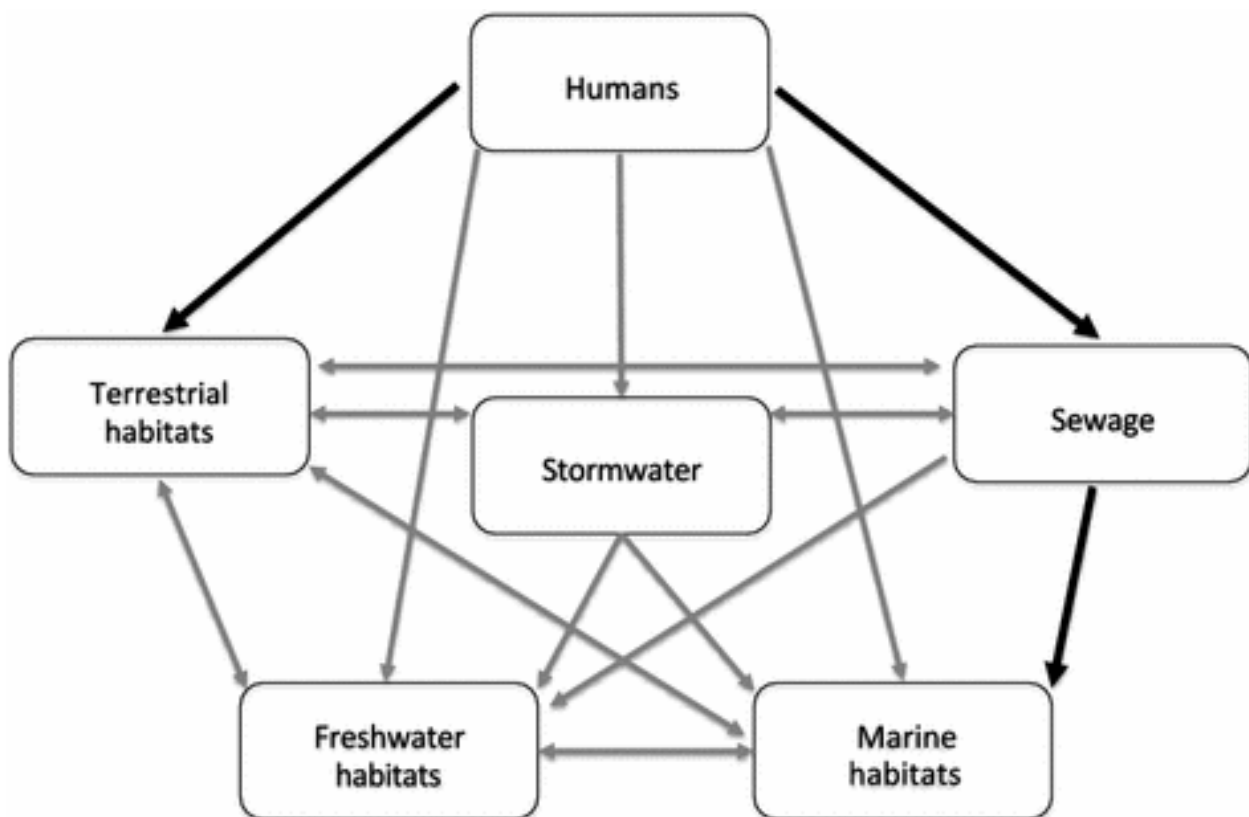
Figure 1-1 Hypothesized (grey) and known (black) sources and pathways of microplastic into habitats. (Browne, 2015)

The effluent of plastic industries is a likely source of primary MPs through the discharge of plastic resin pellets (Wagner et al., 2014). Resin pellets come in different colours (white, old white, off-white, orange, brown), size (mesoplastics or microplastics) (Karkanorachaki et al., 2018) and materials (polyethylene and polystyrene) (Wagner and Lambert, 2018). Sewage effluents and sludge are another likely source of contamination although sludge contain higher concentrations of MPs than in effluents (de Sá et al., 2018). Sewage sludge is used for landfilling and as fertilizer in agriculture that may increase the possibility to transfer the MPs to the rivers, lakes and seas through surface runoff (Nizzetto et al., 2016; Wagner et al., 2014). Microplastic particles are known to enter the wastewater treatment plants through domestic products; clothes washing (Browne et al., 2011; Napper & Thompson, 2016), personal care products (Duis and Coors, 2016; Fendall and Sewell, 2009; Kalčíková et al., 2017) and toothpaste (Leslie, 2014), and have recently been found in human stools (Schwabl et al., 2018).

1.3 Presence of microplastics in marine environment

Microplastics are ubiquitous and abundant in marine systems from the Arctic to Antarctic and from the surface to the deep sea as well as in terrestrial ecosystems worldwide (Lusher et al., 2017; Wagner and Lambert, 2018). Even with standardized monitoring approaches, which are rare, the abundance and distribution of marine plastic litter show considerable spatial variability (Table 1.1).

Studies have estimated that at least 5.25 trillion plastic particles weighing 268,940 tons ranging from ≤4.75 mm to ≥ 4.75 mm in diameter are currently floating in seas around the world (Eriksen et al., 2014). Distribution may be ubiquitous, but the rates at which plastics accumulate vary widely depending on proximity to large cities, anthropogenic uses of the sea shore and maritime activities (Galgani et al., 2000). As a general pattern, accumulation rates appear to be lower in the southern than in the northern hemisphere with some of the highest amounts of floating particles found in the Mediterranean (Eriksen et al., 2014). The distribution of MPs show huge differences in concentrations depending on the ocean current (Yu et al., 2018) with an average of 4600 particles m^{-3} in the North-eastern Pacific ocean (Desforges et al., 2014). Under the action of tides and ocean current MPs in the marine environment often stay in the coastal zone from where they spread from the polluted to unpolluted shores (Yu et al., 2018). In some instances there was no relation to MPs found in the beach and the tourism activities (Laglbauer et al., 2014). However another study on tourist beaches located in Huatulco Bay, USA found evidence that tourism-based activities and effluents discharged from the hotels and restaurants are the main source of MPs (Retama et al., 2016).

Some MPs tend to sink into the sediment which means that they are likely to contaminate deep sea beds.

The methodologies used to collect the water samples in the environment are not standardized and the founding from the reported studies varied significantly which made it difficult to estimate the total number of microplastics in the aquatic environment (Koelmans et al., 2017) as summarised in (Table 1.1).

Also the sizes of MPs are differ in the studies as most of the researchers used 330 µm mesh to collect the MPs from the surface water while only one study used smaller mesh size 80 µm and found that number of MPs was 100,000 times higher (Li et al., 2018). As a results we cannot establish environmental concentrations of small MPs such as 2µm since this has never been measured (Rivers et al., 2019).

During their production, many plastics have additives such as plasticizers and the antibacterial chemical triclosan added. Microplastics are considered to be vectors for chemical contaminants such as polychlorinated biphenyls (PCBs) which are used as an additive during plastic production to increase elasticity and flexibility (Graham and Thompson, 2009a). More chemicals can be adsorb onto their large surface area (Ivar do Sul et al., 2014). A study conducted to examine the pre-production and post-consumer fragments found in the marine environment, found that most of the plastic pellets contained different concentrations of persistent organic pollutants (POPs) such as PCBs, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides such as Dichlorodiphenyltrichloroethane (DDTs), and aliphatic hydrocarbons (Rios et al., 2007). Persistent organic pollutants are considered among the most persistent anthropogenic organic compounds released into the environment, some of these organic compounds are highly toxic and have comprehensive long-term effects on the organisms, such as endocrine disruption, mutagenicity and carcinogenicity (Sultan et al., 2001). In addition to that POPs are chemically stable and hard to degrade in the environment and could be accumulated in the food chain (Gouin et al., 2011).

Table 1-1 Summary of selected marine microplastic environmental sampling studies, covering a range of marine environments (water, plus benthic and shore sediments in oceans and seas). Units provided as published in the studies.

Location	Maximum observed concentration	Reference
Coastal Water, Sweden	102,000 particles m^{-3}	(Norén and Naustvoll, 2010)
Coastal beach, Slovenia	69 particles/ 30 g of sediment	(Retama et al., 2016)
Harbour sediment, Sweden	50 particles L^{-1}	(Noren, 2007a)
Industrial harbour sediment, Sweden	3320 particles L^{-1}	(Noren, 2007b)
Industrial coast sediment, Sweden	340 particles L^{-1}	(Noren, 2007b)
Open ocean, New England	3 particles m^{-3}	(Wright et al., 2013)
Open Ocean, North Waste Atlantic	67,000 particles Km^{-2}	(Colton et al., 1974)
Turkish territorial waters of the Mediterranean Sea	16,339 - 520,213 particles km^{-2}	(Güven et al., 2017)
Beach, UK	8 particles kg^{-1}	(Thompson et al., 2004)
Estuarine sediment, UK	31 particles kg^{-1}	(Thompson et al., 2004)
Subtidal sediment, UK	86 particles kg^{-1}	(Thompson et al., 2004)
Coastal Water, California	3 particles m^{-3}	(Doyle et al., 2011)
Subtidal sediment, Florida	214 particles L^{-1}	(Graham and Thompson, 2009a)
Subtidal sediment, Maine	105 particles L^{-1}	(Graham and Thompson, 2009b)
Ship-breaking yard sediment, India	89 mg kg^{-1}	(Reddy et al., 2006)
Harbour sediment, Belgium	7 mg kg^{-1}	(Claessens et al., 2011)
Continental shelf sediments, Belgium	1 mg kg^{-1}	(Claessens et al., 2011)
Beach, Belgium	93 items kg^{-1} (dry weight)	(Claessens et al., 2011)

Beach, Portugal	6 particles m^{-2}	(Martins and Sobral, 2011)
Beach, East Frisian Islands, Germany	621 particles 10g^{-1}	(Liebezeit and Dubaish, 2012)
Subsurface waters of the Atlantic Ocean	1.15 particles m^{-3}	(Kanhai et al., 2017)
Northeast Atlantic Ocean	2.46 particles m^{-3}	(Lusher et al., 2014).
South Atlantic	Total: 29.7 X 10^{10} Pieces	(Eriksen et al., 2014)
North Atlantic	Total: 93X10^{10} Pieces	(Eriksen et al., 2014)
North Pacific	Total: 199x10^{10} Pieces	(Eriksen et al., 2014)
South Pacific	Total: 49.1 X 10^{10} Pieces	(Eriksen et al., 2014)
Indian Ocean	Total: 130 X 10^{10} Pieces	(Eriksen et al., 2014)
North-eastern Pacific and coastal British Columbia	4600 particles m^{-3}	(Desforges et al., 2014)
Beach, Malta	>1000 particles m^{-2}	(Turner and Holmes, 2011)
Chilean continental coast	27 items m^{-2}	(Hidalgo-ruz et al., 2012)
Beaches on the Canary Island Fuerteventura	Sediment: 1–30 g L^{-1}	(Baztan et al., 2014)
Beaches on the Canary Island Lanzarote	Sediment: <1–109 g L^{-1}	
Beaches on the Canary Island La Graciosa	Sediment: <1–90 g L^{-1}	
Gulf of Finland, northern Baltic Sea	< 10 particles m^{-3}	(Setälä et al., 2016)
Baltic sea	10^{2}–10^{4} particles m^{-3}	(Gorokhova, 2015)
Ross Sea (Antarctica)	1.18 particle m^{-3}	(Setälä et al., 2016)

1.3.1 Ingestion and ecotoxicological impacts of microplastics on marine organisms

Since the beginning of the 21st century, the number of studies looking at the interaction of MPs with the biota has risen dramatically and is rising exponentially at present (Figure 1.2)
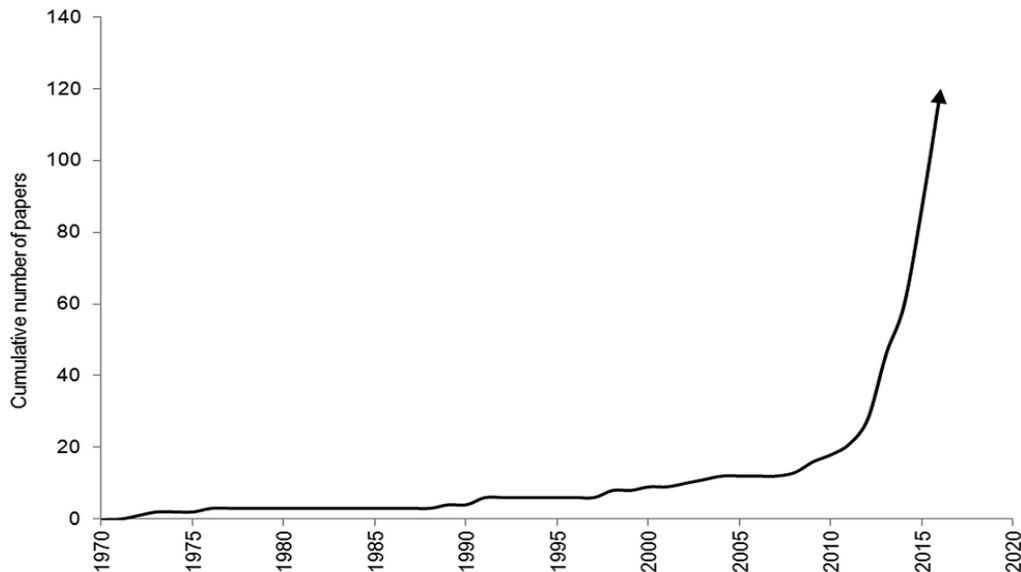
Figure 1-2 Cumulative number of papers on biota interactions with microplastics (Lusher et al., 2017).

Due to their small size and their presence in both benthic and pelagic ecosystems, MPs have the potential to be ingested by a wide range of marine biota (Thompson et al., 2009a; Yu et al., 2018). A growing body of research is showing that in general, marine organisms are unable to differentiate between food and plastic particles (Moore, 2008; Murray & Cowie 2011). The ingestion of MPs sized 20-200 μm has been observed in the gut of collected samples of the omnivore amphipod (*detritivores*), lugworms (deposit feeders), and barnacles (filter feeders) (Thompson et al., 2004). MPs ingestion has also been reported in the benthic holothurians (sea cucumbers) *Thyonella gemmate, Holothuria floridana, H. grisea* and *Cucumaria frondosa* (Graham and Thompson, 2009a). Also laboratory studies have confirmed the accumulation of 2 μm MPs in the digestive cavity and tubules of *Mytilus edulis* after 12 hours of exposure (Browne et al., 2007) and in the gills, stomach and lysosome system after 3 hours of exposure (Moos et al., 2012). Moreover, MPs were present in the circulation system for up to 48 days after exposure (Browne et al., 2007). The marine polychaete *Arenicola marina* (lugworm) is capable of selecting the plastic size (Wright et al., 2013), the exposure to PS MPs size 10, 30 and 90 μm results to higher accumulation on the tissue for 10 μm rather than 30 μm while larger particles are rejected (Phuong et al., 2016). Which shows small MPs

could be hazardous to marine organisms as they may cause blockage in the passage of food through the intestinal tract (Murray & Cowie 2011; Tourinho et al., 2010). However, some studies have demonstrated that some marine organisms have the ability to remove unwanted materials such as sediments and particles from their body (Thompson, Swan, et al. 2009; Andrady & Neal, 2009).

Ecotoxicological work conducted to examine the impact of different concentrations and sizes of MPs on marine organisms have found detrimental effects; *Sparus aurata* (gilthead seabream) exposed to PVC and PE particles of size 40-150 µm (1, 10 and 100 mg ml^{-1}) displayed decreased phagocytosis and increased respiratory burst activities and upregulated expression of nrf2 gene (Espinosa et al., 2018); whilst *Tigriopus japonicus* exposed to PS size 0.5 µm and 6 µm in concentrations 2.2×10^{10} - 1.1×10^{12} MPs L^{-1} and 1.3×10^{7} - 6.6×10^{8} MPs L^{-1} respectively showed a reduction in fecundity (Lee et al., 2013). The feeding capacity of the pelagic copepods (*Calanus helgolandicus)* exposed to 7.5×10^{4} MPs L^{-1} PS 20 µm MPs had mixed effects with a reduction in the consumption of algae and a decreased reproductive output, but no effect on egg production or survive (Cole et al., 2015).

The chronic exposure of *Nephrops norvegicus* (lobster) to PP microfibers 1.5 mg L^{-1} resulted in a reduction in feeding rate, body mass and metabolic rate as well as catabolism of stored lipids (Welden and Cowie, 2016). Moreover, the exposure of PS microplastic size 2 and 6 µm 32 mg L^{-1} to *Mytilus galloprovincialis* led to direct toxic effects at tissue, cellular and molecular levels (Paul-Pont et al., 2016). *Crassostrea gigas* (Pacific Oyster) exposed to PS MPs for 2 months led to a 38% decrease in oocyte number 23% reduction in sperm velocity and a significant energy shift from reproduction to growth (Sussarellu et al., 2016). However there are many studies that show no impact on the parameters chosen. Sea urchins (*Tripneustes gratilla)* exposed to different concentrations of fluorescent PE MPs size 10–45 µm experience no effect on their survival rate (Kaposi *et al.*, 2014); Isopods (*Idotea emarginata)* exposed to fluorescent MPs size 10 µm at concentration 12 MPs mg^{-1} and PS fragments size 1–100 µm at concentrations 20 fragments mg^{-1} and fluorescent acrylic fibres 20 µm–2.5 mm at concentrations of 0.3 mg g^{-1} show no significant effect on growth and survival *(Hämer* et al.*,* 2014).

1.3.2 Trophic transference of microplastics

Many organisms that eat MPs are themselves eaten by predators, which has led to a number of studies investigating the accumulation of MPs through the food chain (Anbumani & Kakkar 2018; Wagner & Lambert 2018; de Sá *et al*. 2018; Browne *et al.*, 2007; Setala et al 2014) (Figure 1.3). If MPs are eaten by zooplankton, a relatively modest number of MPs in the environment (just a few

MPs per individual) could lead to high levels at higher tropic levels if a large number of contaminated zooplankton are eaten (Desforges et al., 2014).
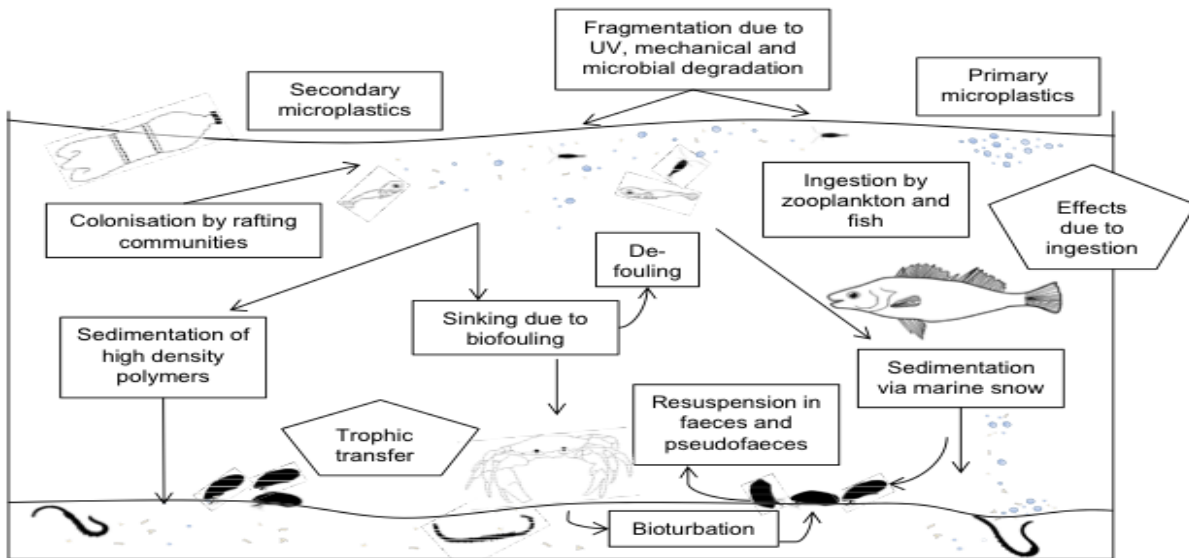
Figure 1-3 Source and accumulation of microplastics in the marine environment (Wright *et al*., 2013).

Studies have fed predators organisms previously exposed to MPs. Small fish, which had eaten plastic fibres, were fed to the lobster *Nephrops norvegicus* which subsequently had plastic fibres in their guts (Murray and Cowie, 2011). Likewise crabs (*Carcinus maenas*) fed on *M. edulis* which had been exposed to 0.5 μm fluorescent PS MPs, accumulated the MPs in their haemolympth, guts, ovaries and gills (Farrell and Nelson, 2013). In this study MPs were found up to 21 days after crabs had been provided with contaminated mussels. Therefore residence time of MPs inside the organism will also impact the transfer of MPs through trophic levels. This has implications for the health of marine organisms, the wider food web and humans.

Concerns have been voiced not just about trophic transference of the MPs but also any chemicals they contain. In a study where zebrafish were fed Artemia contaminated with MPs on which benzo[a]pyrene had been adsorbed, desorption was measured in zebrafish gut and was associated with an induction of the stress enzyme cyptochrome P450 (Batel et al., 2016), It is thought that the uptake of contaminants from MPs will be greater for organisms at a higher trophic level since the release of these chemicals has been shown to be more rapid under conditions that simulate the guts of warm blooded organisms (Bakir et al., 2014).

Many studies have been conducted under laboratory conditions but the limited field evidence from higher trophic level organisms in a variety of habitats suggests that trophic transfer of MPs may be a common phenomenon (Au et al., 2017). Most MPs are of a size whereby aquatic invertebrates, the food source of many at higher tropic levels, will normally ingest food. Microplastics have been found in the faeces of many higher trophic organisms including mammals, birds and fish which implies bioaccumulation (Lusher et al., 2012).

1.4 Presence of microplastics in freshwater environment

Microplastic pollution in freshwater environments has begun to attract attention worldwide (Wagner and Lambert, 2018). Studies suggested that the wastewater treatment plants and industrial effluent are the source of MP pollution in freshwater ecosystem (Horton et al., 2017; McCormick et al., 2014; Windsor et al., 2019). These studies imply that MPs are not only marine pollutants but also significant freshwater contaminants (Yu et al., 2018). The past ten years has seen a rapid increase in research looking at the presence of MPs in freshwater environment including; rivers (riverine shoreline, shallow water and sediments), lakes, ponds, and reservoirs (Wagner and Lambert, 2018). Results have demonstrated alarming quantities of MPs in these ecosystems (Table 1.2). Even a remote mountain lake in Mongolia was contaminated with MPs (Free et al., 2014). Similarly, microplastic particles have been detected in the surface water of Lake Erie, Lake St. Clair, and the Laurentian Great lakes, USA (Eriksen et al., 2013), Lake Huron, Canada (Zbyszewski and Corcoran, 2011) Table 1.2 Almost 99.9% of the microbeads found in the studies were particles less than 2mm in diameter, which is close to what is used in the consumer facial products (Castañeda et al., 2014). However the concentrations of particles reported in freshwater studies lack of consistence specially they used different sampling methods and units for quantifications (Horton et al., 2017).

Table 1-2 Summary of selected freshwater microplastic environmental sampling studies, covering a range of freshwater environments rivers (riverine shoreline, shallow water and sediments) and lakes. Some unites were converted as published in (Horton *et al*., 2017).

Location	Maximum observed concentration	Reference
Europe		
River Thames, UK	Sediment: 66 Particles $100g^{-1}$	(Horton et al., 2016)
River seine, France	Average 0.03 particles L^{-1}	(Dris et al., 2015)
River Danube, Austria	2- 4.2 ton	(Law, 2010)
River Danube, Austria	0.32 particles L^{-1}	(Lechner et al., 2014)
Rhine-main area, Germany	Sediment: 4000 particles kg^{-1}	(Klein et al., 2015)
Rhine-main area, Germany	Water: 0.005 particles L^{-1}	(Mani et al., 2015)
Rhine, Elbe, Mosel, and Neckar, Germany	Sediment: 34-64 particles kg^{-1}	(Wagner et al., 2014)
Various rivers, Switzerland	0.007 particles L^{-1}	(Faure et al., 2015)
Lake Iseo, Italy	Sediment: 40,000 particles km^{-2}	(Sighicelli et al., 2018)
Lake Maggiore, Italy	Sediment: 39,000 particles km^{-2}	(Sighicelli et al., 2018)
Lake Garda, Italy	Sediment: 25,000 MPs km^{-2}	(Sighicelli et al., 2018)
Lake Garda, Italy	South shore: 1.7 particles kg^{-1} North shore: 17 particles kg^{-1}	(Hannes K. Imhof et al., 2013; Imhof et al., 2016)
Lake Chiusi, Italy	Water:0.03 particles L^{-1}	(Faure et al., 2015)
Lake Bolsena, Italy	Water:0.025 particles L^{-1}	(Faure et al., 2015)
Various lake, Switzerland	Sediment: 20 particles kg^{-1}	(Faure et al., 2015)
USA and Canada		
Californian rivers, USA	30-109 particles m^{-3}	(Moore et al., 2005)

Chicago, Illinois, USA	Downstream: 17.93 (11.05) particles m^{-3} upstream:1.94 (0.81) particles m^{-3}	(McCormick et al., 2014)
Los Angeles river, USA	12,000 particles m^{-3}	(Moore et al., 2005)
St. Lawrence River, Canada	1.4×10^5 particles m^{-2}	(Wright et al., 2013)
Grate lake, USA	0.00027 particles L^{-1}	(Eriksen et al., 2013)
Lake Winnipeg, Canada	Sediment:193,420 (± 115,567) particles km^{-2}	(Anderson et al., 2017)
Lake Ontario, Canada	Benthic: 616.1 particles kg^{-1}Sediment: 87 particles kg^{-1}	(Ballent et al., 2016)
Asia		
Beijiang River, China	Sediment:178–554 particles kg^{-1}	(Wang et al., 2017)
Xiangxi river of Three Gorges Reservoir, China	surface water: 80-864 particles m^{-2} sediment: and 0.55×10^5 - 342×10^5 items km^{-2}	(Zhang et al., 2017)
Taihu Lake, China	Water: 3.4–25.8 particles L^{-1} Sediment: 11–234.6 particles kg^{-1}	(Su et al., 2016)
Lake Hovsgol, Mongolia	Water: 0.00012 particles L^{-1} Sediment: 20,264 particles km^{-2}	(Free et al., 2014)

1.4.1 Ingestion and ecotoxicological impact of microplastics on freshwater organisms

The first studies of MP ingestion in freshwater animals began to emerge in the mid 2014s (Wagner and Lambert, 2018). This included work on wild gudgeon (Gobio gobio) where 12% of a sample of 186 adult fish from 11 French streams were shown to contain MPs (Sanchez et al., 2014). A later study conducted to examine the European flounder (*Platichthys flesus*) and European smelt (*Osmerus eperlanus*) from the Thames river, UK, found that 75% of the European flounder and 20 % of European smelt samples contained plastic fibres in their gut system (McGoran et al., 2017). A very recent study found up to 0.14 MPs/ mg tissue in 50% of samples of macroinvertebrates (Baetidae, Heptageniidae and Hydropsychidae) collected from a riverine valley in South Wales, UK, an area which is close to the waste water treatment (WWTP) works (Windsor et al., 2019).

The majority of work with freshwater invertebrates has been in the laboratory, answering questions related to ingestion, uptake, depuration and ecotoxicological effect (Anbumani and Kakkar, 2018). A common methodology is to expose animals to fluorescent MP beads. This allows their fate to be followed easily through the use of fluorescent microscopy and to date studies has been conducted with various types of plastic of various sizes using *Lumbriculus variegatus, Daphnia magna, Gammarus pulex, Notodromas monacha, Potamopyrgus antipodarum* (Imhof et al. 2013) Table 1.3.

Most research has found that MPs uptake is concentration-dependent and time-dependent (Bruck and Ford, 2018; Canniff and Hoang, 2018; Rehse et al., 2016; Wagner and Lambert, 2018).

The ingestion of MPs is likely to have varied impacts on an organism depending on the size, shape, concentrations and exposure period, and the feeding method of the organism (Redondo-Hasselerharm et al., 2018). Very small PS MPs (20 and 1000 nm) can cross the *D. magna* gut epithelium where they are accumulated in lipid storage droplets (Rosenkranz et al., 2009). Although the majority of findings have not confirmed this observation (Wagner and Lambert, 2018), work on *Daphnia galeata* exposed to PS nanoparticles size 52 nm confirmed the transfer of particles from the external body to the internal organs, the thoracic appendices, ovaries, caudal appendices, and brood chamber, as well as the storage in lipid droplets (Cui et al., 2017).

Although PS (20-500µm) decreased growth rates in *Gammraus pulex* (Redondo-Hasselerharm et al., 2018), several studies have examined the ecotoxicological effect of MPs on mortality, reproduction and growth rate and found that any effect was more related to availability of food rather than toxicity of MPs (Table 1.3). However it could depend on the type or size of plastic used; fibres were more

toxic than spherical MPs to *D. magna*, a result which could be due to the clogging of fibres in the gut system (Jemec et al., 2016) and smaller sized MPs (1 μm) increased immobilisation to *D. magna* (Rehse et al., 2016) and decease growth rate and induced stress defence on *Daphnia pulex* (Liu et al., 2019).

Moreover, only a few studies have examined the effect of MPs combined with other chemicals or environmental stressors. The exposure of MPs sizes 50 nm and 10 μm combined with phenanthrene which is a model compound of PAHs known to have carcinogenic and mutagenic impact on aquatic organisms, to *D. magna* shows that smaller MPs tend to have higher adsorption rate of hydrophobic contaminants (Ma et al., 2016). Whereas exposure to MPs and high temperature had a negative effect on the survival rate compared to the impact of MPs at lower temperature (Jaikumar et al., 2018). In the same study the exposure of primary MPs had more toxic effect on *Ceriodaphnia dubia* than secondary MPs by increasing the temperature (Jaikumar et al., 2018).

A recent study to compare the direct and indirect toxic effects of MPs and nanoplastics toward zebrafish (*Danio rerio*) larvae locomotor activity demonstrated that 40μm MPs were themselves not toxic and had little effect. In contrast nanoplastics had a negative impact on larval locomotion which was associated with a reduction in growth. When larvae were co-exposed with α-ethynylestradiol (EE2) and either MPs or nanoplastics, all were associated with hyperactivity, reduced body length and oxidative stress (Chen et al., 2017).

The toxicity of MPs in zebrafish does seem to depend on the size of the particle. When zebrafish were exposed to different types of MPs sizes (0.1, 1.0, 5.0 and 70 μm), bigger particles size 70 μm had higher impact and caused intestinal damage including cracking of villi and splitting of enterocyte (Lei et al., 2018).

Table 1-3 Summary of selected freshwater organisms exposed to different (shapes, sizes and types) of MPs. To examine the uptake, depuration and the ecotoxicological effect for different exposure time.

Organism	Type of MPs	Size of MPs	Concentrations	Duration of exposure	End point examination	Observations	Reference
Cnidaria							
Hydra attenuata	PS flakes extracted from facewash	<400 µm	Concentrations 0, 0.01, 0.02, 0.04, 0.08 g ml^{-1}	30 and 60 min	Feeding rate, morphology and reproduction	No lethal effect, and effective ingestion of MPs	(Murphy and Quinn, 2018)
Arthropoda							
Daphnia pulex	PS nanoplastic	75 nm	2 mg L^{-1} (1.06X 10^9 particles ml^{-1})	48h - 21days	Survival, growth, and reproduction, oxidative stress	-(LC50)= 76.69mg/L -Decreased reproduction rate	(Liu et al., 2019)

						mediated and heat shock proteins	-Decrease growth rate -induce stress defence	
	Primary Fluorescent plastic microspheres Secondary irregular shapes Combined with three temperatures (18, 22, and 26°C)	1-5 µm and 1-10 µm	$10^3, 10^4, 10^5, 10^6, 10^7$ particles ml^{-1}	96h	Acute toxicity test by increase the temperature		-Increase mortality by increase the temperature	(Jaikumar et al., 2018)
Daphnia magna	Carboxylated PS MPs	20 and 100 nm	2 mg L^{-1}	30 min - 24 h	Uptake, accumulation and depuration		Accumulation in gut epithelial layer with faster depuration for larger beads	(Rosenkranz et al., 2009)
	Primary Fluorescent plastic microspheres Secondary irregular shapes Combined with three temperatures (18, 22,	1-5 µm and 1-10 µm	$10^3, 10^4, 10^5, 10^6, 10^7$ particles ml^{-1}	96h	Acute toxicity test		-Increase mortality by increase the temperature for both primary and secondary MPs	(Jaikumar et al., 2018)

and 26°C)					
PET microfibers With and without algal (*Desmodesmus subspicatus*) pre-feeding	300 μm	12.5-100 mg L^{-1} Algae (density 5 X 10^4 cells)	48 h	Uptake, feeding performance and immobilization	-No lethality in daphnids fed with algae. - unable to recover from microfibers induced stress after additional 24 h incubation period	(Jemec et al., 2016)
Micro and nano-plastics Combined with phenanthrene (chemical stressor)	50 nm and 10 μm	50 mg L^{-1} and 500 mg L^{-1}	14 day	Uptake Vector property of microplastics	-significant phenanthrene bioaccumulation, -dissipation and transformation is noted in daphnids coupled with 50 nm particles than 10 μm size demonstrating the higher adsorption rate of hydrophobic	(Ma et al., 2016)

						contaminants on smaller particle size	
	Primary and secondary MPs	1–5 µm	10^2 to 3×10^4 MPs mL^{-1}	12 min-21 days	Uptake and depuration	-Increased gut passage time with aggregates after secondary microplastic exposure -lower feeding and reproduction at high microplastic levels -secondary microplastics are more harmful than primary uptake	(Ogonowski et al., 2016)
	PE MPs	1 and 100 µm	12.5 to 400 mg L^{-1}	96 h	Uptake and immobilization	-1 µm MP ingested and cause immobilization -96 h EC50 for 1 µm	(Rehse et al., 2016)

						microplastics is 57.43 mg/L	
	Fluorescent PS beads with and without algae *Raphidocelis subcapitata*	2 μm and 100 nm Algae	$1.4X10^5$ and $3.1X10^8$ particles ml^{-1} $6.7X10^5$ cells ml^{-1}	21 days	feeding rate assessment	Five times higher ingestion rate for 2 μm; 21% decreased feeding rate with -no significant effects on reproduction	(Rist et al., 2017)
Daphnia galeata	PS nanoparticles	52 nm	5 mg L^{-1}	5 days	Survival, reproduction and growth rate	-Decrease in survival, -decrease in reproduction rate -abnormal development	(Cui et al., 2017)
Ceriodaphnia dubia	Primary Fluorescent plastic microspheres Secondary irregular shapes Combined with three	1-5 μm and 1-10 μm	$10^3,10^4,10^5,$ $10^6,10^7$ particles ml^{-1}	96h	Acute toxicity test	-Increase mortality by increase the temperature -Primary MPs was more toxic than secondary MPs	(Jaikumar et al., 2018)

		temperatures (18, 22, and 26°C)					
Hyalella azteca	fluorescent PE PP fibres	10-27μm 10-27μm	4.64X10^4 MPs mL^{-1} 71.43 MPs ml^{-1}	10 days And 42 days	Mortality, reproduction, growth and egestion	-polyethylene: decreased growth and reproduction -Polypropylene: slower egestion time, decrease growth -Fibres were more toxic than particles	(Au et al., 2015)
Hyalella azteca	PS MPs	20−500 μm	ranging from 0 to 40% sediment dry weight.	28 days	Survival, reproduction and growth rate	-No effect on survival, reproduction and growth rate.	(Redondo-Hasselerharm et al., 2018)
Asellus aquaticus	PS MPs	20−500 μm	ranging from 0 to 40% sediment dry weight.	28 days	Survival, reproduction and growth rate	-No effect on survival, reproduction and growth rate.	(Redondo-Hasselerharm et al., 2018)
Gammarus pulex	Polymethyl methacrylate	29.5 ± 26 μm		48h - 1week. 96 h-	Uptake	Active ingestion	(Hannes K Imhof et al., 2013)

				1wk			
	PS MPs	20−500 μm	ranging from 0 to 40% sediment dry weight.	28 days	Survival, reproduction and growth rate	- negative effect on growth	(Redondo-Hasselharm et al., 2018)
	Fluorescent PET fragments	10-150 μm	0.4, 40 and 4,000 particles ml^{-1}	24h-48 days	Body burden on (Juveniles and adults) Survival and feeding activity	-Juveniles uptake more MPs from adults -No affect on survival, development (molting), metabolism (glycogen, lipid storage) and feeding activity	(Weber et al., 2018)
Nematode							
Caenarhabditi s elegans	Polyamides (PA), PE, PP, PVC and PS	~70 μm 0.1 μm, 1.0 μm and 5.0 μm).	0.001, 0.01, 0.1, 1.0 and 10.0 mg L^{-1}	2 days	Survival, body length and reproduction	-Reduction in survival, -decreased body length -reproduction impairment	(Lei et al., 2018)

Annelida							
Tubifex spp.	PS MPs	20−500 μm	ranging from 0 to 40% sediment dry weight.	28 days	Survival, reproduction and growth rate	-No effect on survival, reproduction and growth rate	(Redondo-Hasselharm et al., 2018)
Mollusca							
Sphaerium corneum	PS MPs	20−500 μm	ranging from 0 to 40% sediment dry weight.	28 days	Survival, reproduction and growth rate	-No effect on survival, reproduction and growth rate	(Redondo-Hasselharm et al., 2018)
Chordata							
Cyprinus carpio	PE from cosmetic products		1-2 mg L^{-1}	21 days	blood biochemical parameters in	-Decrease in total protein, globulin, cholesterol and triglyceride levels -Decrease in Gamma-glutamyl transferase	(Nematdoost Haghi and Banaee, 2017)

						activity -Increase albumin and creatinine levels	
Danio rerio (zebrafish)	polyamides (PA), PE, PP, PVC and PS	~70 μm 0.1 μm, 1.0 μm and 5.0 μm).	0.001, 0.01, 0.1, 1.0 and 10.0 mg L^{-1}	10 days	survival, body size and reproductive, Toxicity, oxidative damage and calcium levels in the intestine were	PA, PE, PP and/or PVC microplastics: intestinal damage including cracking of villi and splitting of enterocyte Effect depend on the MPs size	(Lei et al., 2018)

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	low-density PE (LDPE) fragments	17.6 µm	5-500 mg L^{-1}	10 or 20 days	Length, weight, condition factor (CF), transcriptional level of antioxidant, anti and pro-apoptotic, and neurotransmitter genes, and the histopathology of the gill, liver, brain, kidney, and intestine	No significant impact	(Karami et al., 2017)
	PS	45 µm and 50nm	1 mg L^{-1}	2-5 days	Direct and indirect toxic effect	Reduction in Oxidative stress and body length	(Chen et al., 2017)

1.5 Effect of MPs on natural freshwater ecosystems

Microplastic environmental studies vary enormously in the type of MP (e.g. size, shape, density, and composition) as well as confounding factors (e.g. storms and wind), so that few conclusions can be made concerning the fate of MPs in ecosystems (Hidalgo-ruz et al., 2012; Lattin et al., 2004).

Rainfall and storm disturbance can increase the presence of MPs in the water column, particularly in shallow lakes and estuaries (Lattin et al., 2004; Yonkos et al., 2014). The behaviour of MPs in natural freshwater ecosystems will to some extent depend on their partitioning between the water column and sediment. High-density MPs, such as PVC and polyesters settle into the sediment and do not persist in the water column due to their negative buoyancy (Kowalski et al., 2016; Lozoya et al., 2016). However low-density MPs, such as PE and PS are positively buoyant and more likely to persist in the water column, making them accessible to filter feeders for accidental ingestion (Avio et al., 2017; Cole et al., 2013; Eerkes-Medrano et al., 2015a). Therefore any environmental studies on the impact of MPs on freshwater organisms should include benthic-dwelling organisms.

The lack of information outside of the standard ecotoxicology models extends to studies of community responses and effects in a more natural field environment where predator-prey interactions and competition exist. Here MPs are more likely to be fibres or non-spherical shapes generated from primary MPs, making it difficult to extrapolate from studies using single species and virgin MPs (Rummel et al., 2016).

1.6 *Daphnia magna* and its use as an ecotoxicological model to examine the impact of microplastics

Daphnia magna (Crustacea; Branchiopoda) is a keystone species in certain freshwater habitats (Shaw et al., 2008). They feed on bacteria, protozoa and algae and are a primary food source for fish. *Daphnia magna* is distributed across the world in habitats varying from temperate to arid and can survive in temperatures between 2 and 28°C and their optimum pH is 6.5 to 9.9 (Benzie, 2005). *Daphnia* are the most widely used freshwater aquatic indicator species for ecotoxicological studies (Heckmann et al., 2007). Among their many desirable features are a short generation time (in culture at

20°C the generation time is roughly one week, making it possible to track ontogeny response), ease of culture in a simple media, small body size, and sensitivity to a broad range of toxicants. Parthenogenic reproduction means comparisons can be made between treatment and genetic background, reducing experimental variation and increasing test validity (Ebert, 2005; Figure 1.4).
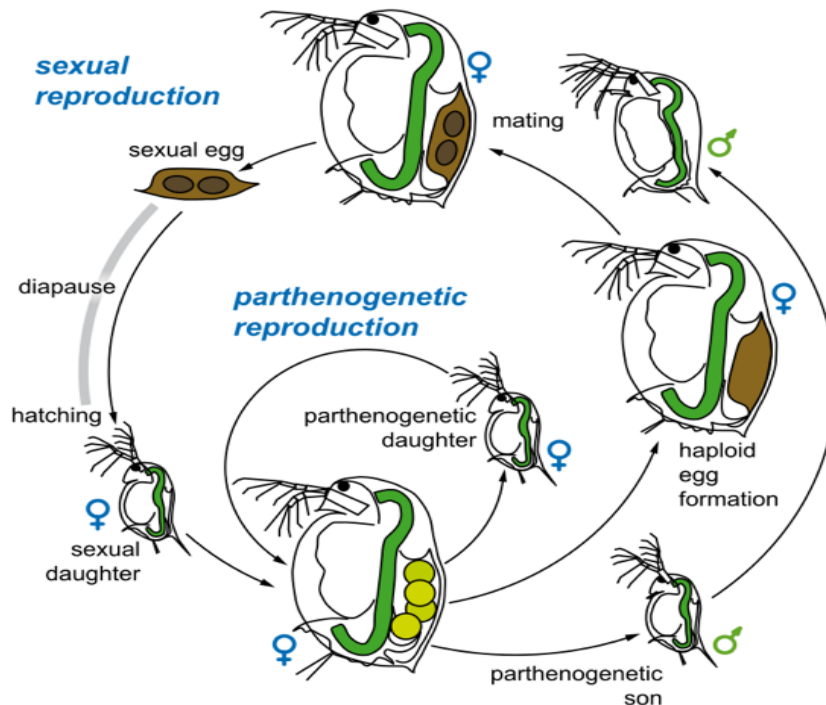
Figure 1-4 Cyclic parthenogenesis in *Daphnia magna*. Individuals can alternate between sexual and parthenogenetic reproduction. When reproducing parthenogenetically, diploid asexual embryos fully develop in the brood chamber of mature female daphnias, and are then directly released in the environment. Sexual reproduction requires the parthenogenetic (clonal) production of males that then mate with adult females to fertilize one to two haploid eggs that are encapsulated in a hard shell and can undergo a resting phase known as diapause (Ebert, 2005). Drawing by Dita B. Vizoso, Fribourg University.

Daphnia magna feed through the uptake of small suspended particles from the water and consume large quantities of algae by creating a current of water that runs through their carapace by beating the thoracic legs rhythmically (Dumont Henri, 2002; Lovern et al., 2007) (Figure 1.5). The current generated produces a feeding column which enables the *D. magna* to pick which items to ingest, selecting particles on the basis of size, shape, and texture (Lovern et al., 2007). The dynamics of *D. magna* food uptake

follow a type 1 functional response (FR; relationship between resource supply and resource use). This means that below a certain food concentration (the incipient limiting level), the food uptake from the water (feeding rate) is proportional to the food concentration, and the filtering rate (amount of water filtered per unit time) is maximal. Above this level, the feeding rate is constant because the filtering rate decreases with increasing food concentration in the water (Ebert, 2005).

Daphnia magna decrease feeding rates when exposed to low levels of food (Lovern et al., 2007). And this reduction in feeding may cause a reduction in growth, survival and reproduction dynamics (Lovern et al., 2007; Thompson et al., 1982). Any undesirable food for *D. magna* which might be toxic or indigestible could inhabits feeding or have deficiency in some nutrient results of decrease in growth and death (McMahon and Rigler, 1965). Although, that the filtration rate could play an important role in the selection of food particles (Dumont Henri, 2002), research found that in the presence of toxic large blue-green algae, *D. magna* have the ability to reduce the filtration rate through narrowing their valves to excluding the large particles and reduce movement of thoracic appendages (McMahon and Rigler, 1965; Wiedner and Vareschi, 1995).

The *D. magna's* gut consists of three parts; the esophagus, the midgut (lined with an epithelium and bearing microvilli) and the hindgut (Figure 1.5). In addition there are two small digestive ceca in the head section of the midgut (Ebert, 2005). Peristaltic contractions of the gut wall pass food through the gut inside a peritrophic membrane which prevents it from entering the ceca (Ebert, 2005). The food particles uptake occurs through mechanical sieves which dominant factor in filter-feeding (Gophen and Geller, 1984).

Food excretion process occurs through peristaltic movement in addition to pressure (Dumont Henri, 2002). Laboratory experiments shows the ability of *D. magna* to consume particles between 1-70 μm in size, and that they lack the ability to distinguish between particles size and quality (Dumont Henri, 2002).

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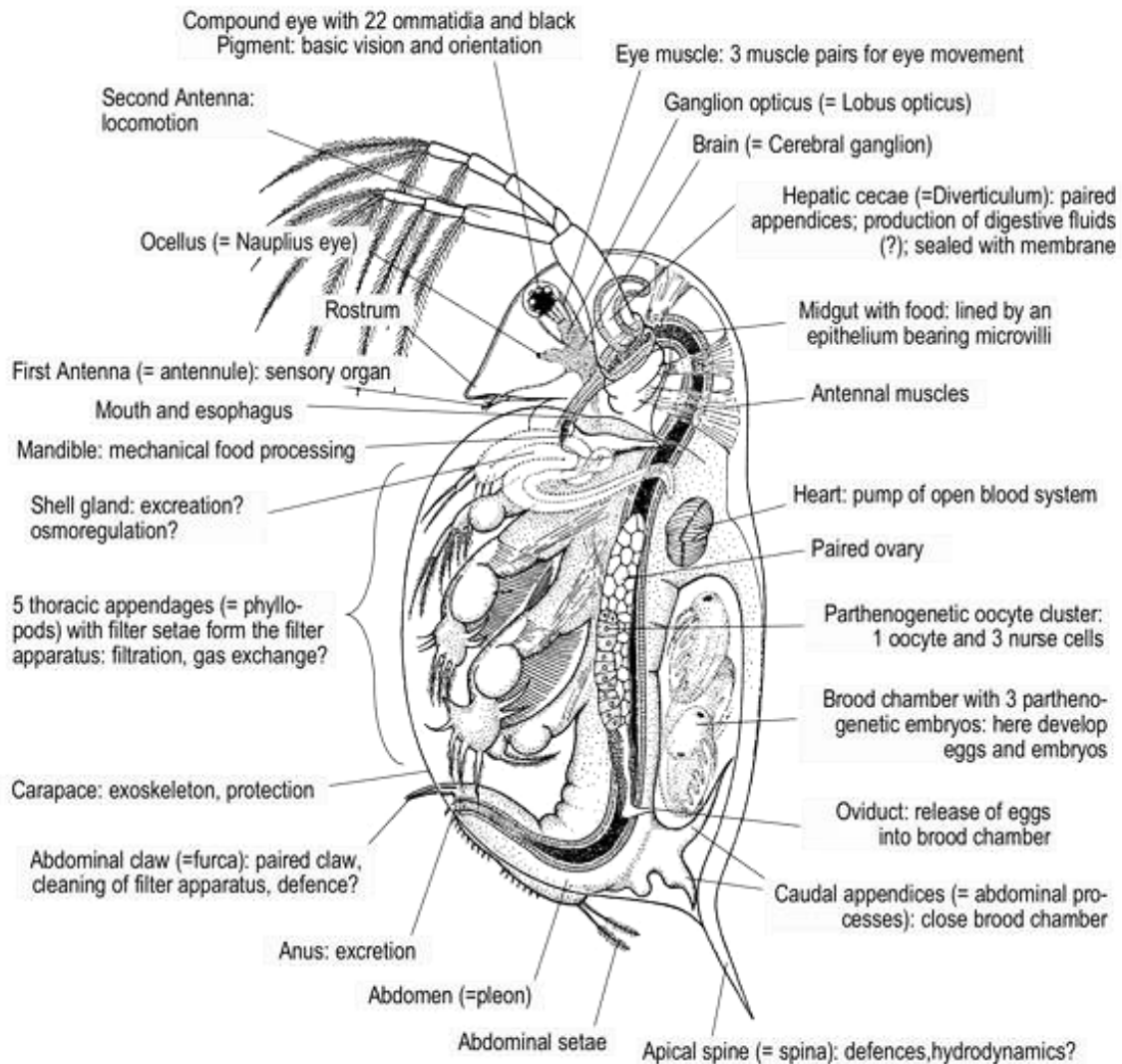
Figure 1-5 The anatomy of adult female *Daphnia magna* show the digestive system which consist of three parts (Esophagus, midgut, and hindgut) and the digestive ceca (diverticula) in the head section of the midgut (Ebert, 2005).

To date *D. magna* is the most examined freshwater invertebrate in laboratory studies and has been used as ecotoxicological model to examine acute and chronic toxicity effect of PS MPs of different sizes and concentrations (de Sá et al., 2018; Rist et al., 2017; Rosenkranz et al., 2009). *Daphnia magna* have been exposed to PE MPs (Ogonowski et al., 2016; Rehse et al., 2016) and to acrylic resin *(Booth et al.*, 2015) and to PET textile microfiber (Jemec et al., 2016). The effect of MPs varied from a

reduction in survival or reproduction rate to no effect at all, depending on MPs concentration and exposure rate (Anbumani and Kakkar, 2018). Although another study found that the exposure to high concentration of MPs increased mortality and growth rate, and *D. magna* needed several generations to recover (Martins and Guilhermino, 2018) Table 1.3.

1.7 *Culex pipiens* and its use as an ecotoxicological model to examine the impact of microplastics

Although MPs have been found in all aquatic ecosystems that have been sampled, very few studies have examined the uptake of MPs in freshwater dipteran larvae, although a recent study looked at the ability of *Chironomus riparius* to ingest carboxylated MPs sized 2.1, 6.2, 10.8, and 19.4 μm (Scherer et al., 2017).

Mosquitoes belong to the order Diptera (two winged flies) and are members of a single family, the Culicidae (Snow, 1990). The Culicidae family is a large and abundant group including some of the most important blood-sucking arthropods known (Lounibos, 2002). The family is classified into two sub-families: Anophelinae with 3 genera and Culicinae with 109 genera in 11 different tribes (Ngo et al., 2014).

Culex pipiens were used in this study as they are the most common mosquitoes in urban areas in Great Britain (Townroe and Callaghan, 2014). *Culex* species live in freshwater such as ponds, urban gardens containers (Townroe and Callaghan, 2014) and shallow waters (Dadd, 1971). Mosquitoes have a short life cycle; female mosquitoes lay their eggs on the water surface either individually or attached group called 'raft'(Clements, 1992). The eggs then hatch within few days depending on the temperature, to the larval stage, which consists of four substages known as 'instars' (Clements, 1992) (Figure. 1.6).
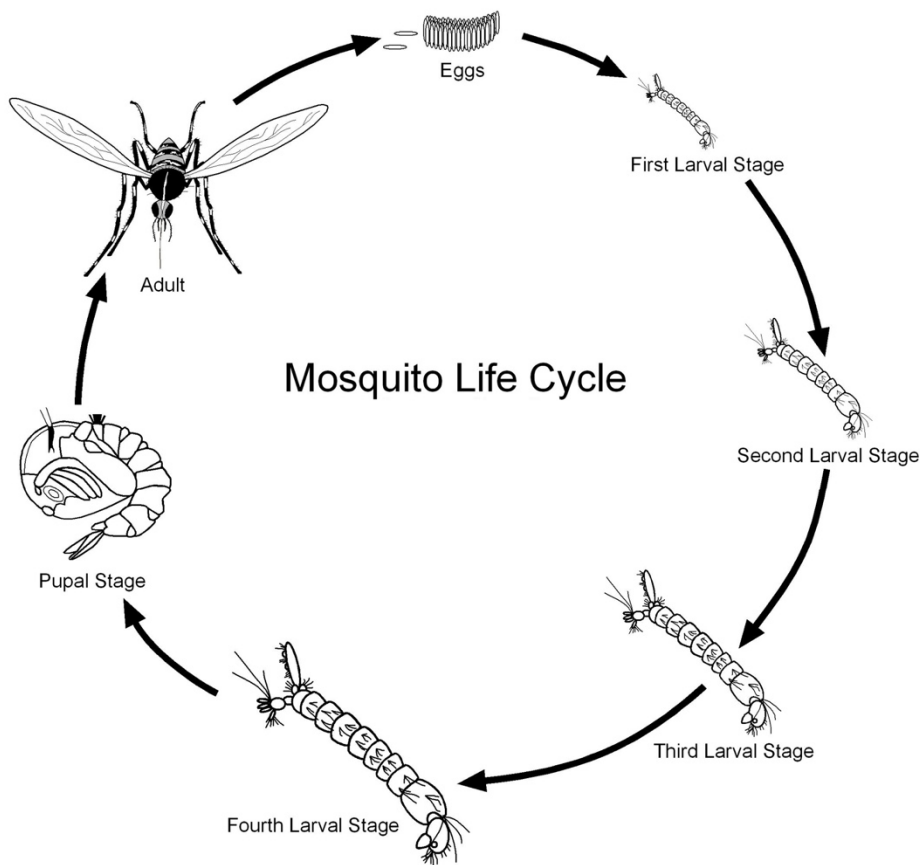
Figure 1-6 *Culex pipiens* **life cycle, adults lay their eggs on the water surface, hatch to larvae stage (1ˢᵗ, 2ⁿᵈ, 3ʳᵈ and 4ᵗʰ instars) to develop to pupal stage then flying adults.**

Larval *Culex* are filter feeders, hanging their body upside down with their respiratory siphons near the water surface. The mouth parts consist of lateral palatal brushes (LPB), mandibular brushes, combs, maxillary brushes and filaments (Clements, 1992). The beating of the LPB regulate the water flow and particle movement. During flexion, the LPBs form filament rows which move through the water as the flipped pages of a book help to carry the suspended particles towards the head the beating of the LPB regulate the water flow and the particles movement (Clements, 1992). Larvae are able to rotate their heads 180° which facilitates the uptake of food particles. The food particles then enter the feeding groove and are packed in to the pharynx where particles are sieved with the dorsal and ventral bounds (Rashed and Mulla, 1989). Food particles move to the esophagus which consist of three reservoirs, that connected directly to the midgut where the food nutrients are absorbed (Clements, 1992). The pupal life stage looks like a coma shape and is a non-feeding stage (Clements, 1992). After 1-4 days pupae

develop to flying adults either male or female, males adults feed on nectar and females feed on blood meal (Clements, 1992).

Mosquito larvae have been used as freshwater ecotoxicological models to examine the toxic effect of chemicals in freshwater ecosystem (Lal *et al*., 1983). Their additional value in this study is that previous research on their feeding behaviour means that there exists literature on their exposure to latex MPs (Dadd, 1971). Results shows that all larval instars are able to uptake MPs, however ingestion is relative to size with *Culex* able to ingest latex bead between 0.45μm-1mm (Dadd, 1971).

1.8 Aim and objectives of the study

When this study began in 2014, few studies had been conducted to investigate the effect of MPs on freshwater aquatic environments and the organisms therein (de Sá et al., 2018; Messinetti et al., 2018; Wagner and Lambert, 2018). There was almost no information on the ecotoxicological impact of PS MPs on freshwater invertebrates and the potential impact on the individual, population or community. We began in chapter two by asking questions regarding ingestion and effects of single virgin MPs on single standard toxicity model species. The aim was to add to the growing body of evidence of the impact of MPs through a thorough investigation of the impact of 2 μm micro-polystyrene on *D. magna* on reproduction, growth and mortality. This size was chosen since it is a similar size to the algae *Chlorella vulgaris* which is used to feed *D. magna*. This work was undertaken at a point where few had studied the impact of MPs on *D. magna* and freshwater ecosystems. We hypothesized that *D. magna* would be unable to differentiate between MPs and algae and that the uptake of both algae and MPs would be equivalent. Given an inability to distinguish between food and non-food, we predicted that the reduction in food ingested would have a significant impact on the fitness of the *D. magna*. Longer term impacts would also be dependent on excretion of MPs as well as general toxicity. I measured the impact on growth and reproduction using standard ecotoxicology 21 days' life history tests following OECD guideline 211 (OECD, 2012).

Chapter three built on the findings in chapter 2 by comparing the effects of the 2 μm MPs to those of a larger 15 μm PS MP using the same methodology. In chapter 2 I presented evidence that *D. magna* could distinguish between 2 μm MPs and algae

despite their similar size (Al-Jaibachi and Callaghan, 2018). Here, the much larger MPs ought to be more readily distinguishable and therefore if *D. magna* really are selectively eating algae, it would be possible that they would select against their ingestion.

The aim of chapters four and five were to expand my research by looking into a non-model species (*Culex pipiens*) with a different life history to the water flea *D. magna*. Both *D. magna* and C. *pipiens* mosquitoes have relatively short life-cycles and both filter feed. However their differences in life history mean that the impact of MP exposure may have different implications. The same 2 and 15 μm fluorescent PS beads were used to allow a direct comparison. The aim of chapter four was to determine whether MPs could transfer between insect life stages and, particularly, could move into the flying adult stage. Mosquitoes develop through four feeding larval instars and a non-feeding pupal stage, and finally emerge into a flying adult. In chapter five I concentrated on the relationship between MP concentration, consumption and ontogenic transference and also to see if the MPs have any detrimental effect on the mosquito.

The previous chapters looked specifically at ingestion and impact on a single species of freshwater invertebrate. In **chapter six** I moved the study out to a series of mesocosms, to look at the impact of MP exposure on *Daphnia* and *Culex* in a natural system where competition and predation are factors. Here I used the larger size of MP for ease of detection and measured population size over time and general pond community composition with an aim of comparing laboratory with field results.

The final aim of the study was to investigate trophic interactions and particularly the impact that MPs might have on feeding responses of freshwater invertebrate predators. **C**hapter **seven**, was devised to follow on from the previous chapter to determine whether MP exposure modulates interaction strengths between predators and prey, and whether trophic MP transfer can be related to predation rates. Here I used larvae of the predatory non-biting midge *Chaoborus flavicans* as a voracious predator of *C. pipiens*, which often co-occur in natural and artificial aquatic habitats.

1.9 Note on the structure of the thesis

All the experimental chapters have been written in research article style since four have already been published in peer-reviewed journals (*Science of the Total Environment, Biology Letters and PeerJ*) and the intention is to publish all chapters. Thus some

repetition occurs in the thesis reflecting introductions written for different audiences. The references cited have been displayed as throughout the entire thesis (i.e. Harvard style) and are listed at the end together with other citations. There is no separate materials and methods page, since the methods are either given in detail in each chapter or have been published elsewhere.

Chapter 2 IMPACT OF POLYSTYRENE MICROPLASTICS ON *DAPHNIA MAGNA* MORTALITY AND REPRODUCTION IN RELATION TO FOOD AVAILABILITY

This chapter has been published in its entirety:

2.1 INTRODUCTION

Plastics are used extensively worldwide since they are cheap, easy to manufacture and have properties that allow them to replace natural products, including wood, stone and glass (Cole et al., 2011). Around 90% of the world's plastics are low- or high-density polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS) or polyethylene terephthalate (PET) (Andrady and Neal, 2009). Few of these are released into the environment without some form of modification (additives) that can potentially leach into the water and many have properties that allow the adsorption of hydrophobic pollutants, so continuing their persistence and spread throughout the water (Andrady and Neal, 2009). Enormous amounts of plastic enter aquatic environments from the land, with an estimated 4.8–12.7 million tons of plastic entering oceans per year (Jambeck et al., 2015). Visible, larger plastic fragments pose well known risks to marine life and environments, but there is an increasing awareness of the impact of microplastics (MPs) which are defined as plastics less than 5 mm in size (Cole et al., 2011; Moore, 2008; Wright et al., 2013). MPs can be generated from the degradation of larger pieces but many are manufactured specifically, for example, for use in cosmetics products such as facial scrubs (Napper et al., 2015). Ingestion of MPs has been demonstrated for many marine organisms and there is considerable evidence to suggest that they are transferred up between different trophic levels (Eriksson and Burton, 2003). MPs in freshwater ecosystems (Besseling et al., 2017) are less studied and yet an increasingly important environmental issue (Dris et al., 2015; Wagner et al., 2014). MPs enter freshwater bodies through land-based sources or wastewater treatment plants in addition to the potential degradation of large plastic particles (Mason et al., 2016). Cosmetic products such as facial scrubs, toothpaste or body wash are a primary source of MPs with up to 100,000 MPs released into wastewater in a single use (Napper et al., 2015). Very recently, environmental lobbying in the UK has resulted in a ban of MPs in personal beauty products which will come into force in 2018 as well as a greater awareness of the issues. However, this does not extend to other sources of microplastic (MP) pollution. These include synthetic fibres in clothing (e.g., fleece) following machine washing (Fossi et al., 2014) as well as resin pellets used in plastics manufacture. A survey of the River Thames (UK) shoreline revealed 1–4 mm MPs at concentrations of 22–297 particles L^{-1} (Horton et al., 2016). The most dominant MPs were fibres, but in one site downstream of a storm drain, receiving urban runoff, many

of the plastics were derived from thermoplastic road-surface marking paints (Horton et al., 2016). In the US effluent from wastewater treatment plants was shown to contain an average of 0.05 ± 0.024 MP particles L^{-1} (Mason et al., 2016) and effluent feeding into lakes generated an average of 0.79 ± 0.88 mg/L to 1.56 ± 1.64 mg L^{-1} MPs (Lasee et al., 2017). Likewise, a survey of beach sediments from the subalpine Lake Garda in Italy revealed high concentrations of plastics including polystyrene (45.6%), polyethylene (43.1%) and polypropylene (9.8%) as well particles (9–500 μm) of polyamide and polyvinylchloride (Imhof et al., 2013a).

Recent investigations have argued that aquatic organisms have a limited ability to distinguish between food and MPs (Fossi et al., 2014; von Moos et al., 2012). For example, the copepod *Corvus typicus* could not differentiate between algae and 20.6 μm MPs (Cole et al., 2013). Similar results were found when *Acartia clausi* and *Calanus pacificus nauplii* were exposed to MPs in the presence of food (Cole et al., 2013). These factors have led to increasing concerns regarding MPs and have contributed to a recent increase in studies on the impact of MPs on marine and freshwater environments.

Studies on freshwater invertebrate species including *Lumbriculus variegatus*, *Daphnia magna*, *Notodromas monacha*, *Potamopyrgus antipodarum*, and *Gammarus pulex*, have confirmed that ingestion of MPs occurs (Imhof et al., 2013b). Ingestion and elimination of polyamide fibres and polystyrene MPs were demonstrated in the freshwater amphipod *Gammarus fossarum* and impacted on food assimilation (Blarer and Burkhardt-Holm, 2016).

Various studies have begun to look at the impact of MPs on growth and reproduction in the model ecotoxicology organism *Daphnia*. *Daphnia magna* (Cladocera, Crustacea) is a freshwater filter feeder with the ability to uptake and ingest small suspended particles from the water (Ebert, 2005). *Daphnia* usually feed on algae, although they are capable of feeding on bacteria and can consume particles between 1–70 μm in size (Ebert, 2005). *Daphnia* are said to be unable to distinguish between particles size and quality (DeMott, 1986), which implies a lack of selection and likely ingestion of MPs. Tiny carboxylate polystyrene MPs (20 and 1,000 nm) can cross the *Daphnia* gut epithelium and accumulate in lipid storage droplets (Rosenkranz et al., 2009). Bioaccumulation of these nano-polystyrenes is associated with a negative impact on the growth, mortality and reproduction of *D. magna* (Besseling et al., 2014b). MP fibres (ground polyethylene

terephthalate) from textiles were toxic to unfed *D. magna* but no mortality occured when animals had been fed (Jemec et al., 2016). Degraded macro plastics were also toxic to *D. magna*, increasing inter-brood period and decreasing reproduction at high concentrations whereas responses to cosmetic MPs found no such effect and effects were restricted to the level of nutrition (Ogonowski et al., 2016). This contrasts with the effect of polyethylene MPs where ingestion of high concentrations of 1 μm MPs led to the immobilisation of *D. magna* (Rehse et al., 2016).

The aim of the present study was to add to the growing body of evidence of the impact of MPs through a thorough investigation of the impact of 2 μm micro-polystyrene on *D. magna* on reproduction, growth and mortality. This size was chosen since it is a similar size to the alga *Chlorella vulgaris* which is used to feed *Daphnia* This work was undertaken at a point where few had studied the impact of MPs on *Daphnia* and freshwater ecosystems. We hypothesized that *Daphnia* would be unable to differentiate between MPs and algae and that the uptake of both algae and MPs would be equivalent. Given an inability to distinguish between food and non-food, we predicted that the reduction in food ingested would have a significant impact on the fitness of the *D. magna*. Longer term impacts would also be dependent on excretion of MPs as well as general toxicity. We measured the impact on growth and reproduction using standard ecotoxicology 21 days' life history tests following OECD guideline 211 (OECD, 2012).

2.2 Materials and methods

2.2.1 *Daphnia magna* and *Chlorella vulgaris* culture

Daphnia magna were obtained from the Water Research Centre (WRC, Medmenham, UK) and cultured at the University of Reading for more than ten years prior to this experiment. Full details of culturing methods are given in (Hooper et al., 2006). *Daphnia* were maintained in Organization for Economic Co-operation and Development (OECD) reconstituted water (media) and fed yeast and *C. vulgaris* var Viridis following the methods of (Hayashi et al., 2008). New cultures of *Daphnia* were prepared with 15 neonates in 1,200 ml beakers filled with OECD media (the progeny of these neonates are the first brood).

Juveniles were removed regularly from the culture and the media was changed once a week. The third brood produced by the original 15 neonates were used for experiments.

2.2.2 Preparation of MPs

MPs used for uptake and depuration experiments were supplied by Sigma-Aldrich, Dorset, UK, (Lot no. MKBQ9691: batch no. 1001856699) as 2 μm carboxylate-modified polystyrene, fluorescent yellow-green (excitation 470 nm; emission 505 nm), density $1.050g/cm^3$. MPs were stored as a stock suspension (2.5 mg mL^{-1}) in distilled water and mixed using a vortex prior to dilutions. MPs used for toxicity tests were also supplied by Sigma-Aldrich, Dorset, UK, (Lot no. BCBN6954V: batch no.78452) as 2 μm non-fluorescent polystyrene microplastics. These were stored as an aqueous suspension of 10% solids.

2.2.3 Microplastic uptake

Adult (18 days) *D. magna* were placed individually in glass beakers filled with 50 ml media and starved for 24 h. They were then exposed to 1.46×10^2 mg L^{-1} 2 μm MPs with and without algae (calculated based on carbon 1.00×10^{-1} mg L^{-1}) of *C. vulgaris*. Four replicates of each treatment were exposed for 15, 30, 60, 120 and 240 min in daylight conditions. at 20 ±2 °C. Following exposure *Daphnia* were washed with deionized water to remove MPs that had adhered to the carapace, dried on tissue and placed in an Eppendorf tube and stored at −20 °C.

2.2.4 Microplastic depuration

In order to evaluate depuration of MPs, 20 *Daphnia* from both treatments (see previous section) were exposed for one hour then transferred into clean media for 15, 30, 60, 120 and 240 min (replicated four times). *Daphnia* were washed twice with deionized water for one minute before freezing at −20 °C.

Frozen *Daphnia* were placed individually into a 1.5 ml Eppendorf tube filled with 500 μl distilled water and homogenised by crushing the *Daphnia* using a glass Kontes Pellet Pestle (Fisher Sciences, Loughborough, UK) for one minute. A further 500 μl distilled water was added to wash the pestle. A 0.2 ml aliquot of the homogenate was filtered onto a black background nucleopore track-etched membrane (Whatman, Kent, UK) <0.2 μm, by using a glass vacuum filter holder connected to a manual air pump. The membrane was examined under the epi-fluorescent microscope (Zeiss Axioskop) under (20×) to count the fluorescent MPs in each treatment.

2.2.5 Microplastic visual assessment

Four adult *Daphnia* from each treatment for each experiment were observed under an epi- fluorescent microscope (Carl Zeiss Axioskop, Wetzlar, Germany), at (10×) magnification with the main focus on the gut system. Images were taken through a blue filter (excitation 450–490 nm) to differentiate the MPs from algae which fluoresced under a green filter (excitation 510–560 nm).

2.2.6 Differential microplastic uptake under varying food regime

Adult *D. magna* were exposed individually for 60 min to a different combination of MPs and algae concentrations (Table 2.1) Each treatment was replicated six times and assigned to a randomized block design (where replicates were randomly situated on the laboratory bench to eliminate the contribution of factors other than treatment as an experimental error). Following exposure, *Daphnia* were washed twice with deionized water for one minute before freezing at −20 °C. MPs were quantified as described earlier.

Table 2-1 Concentrations (mg L^{-1}) of MPs and algae added to each treatment to study the uptake of microplastics by *Daphnia magna*.

Treatments	Volume μl	Algae concentration (mg L^{-1})	MPs concentrations (mg L^{-1})
Control MP only	50	0	6.93 x10^{-4}
	100	0	1.39x10^{-3}
	200	0	2.77x10^{-3}
	400	0	5.54x10^{-3}
	600	0	8.31x10^{-3}
	800	0	1.11x10^{-2}
Algae=MP	50	5.00x10^{-2}	6.93 x10^{-4}
	100	1.00x10^{-1}	1.39x10^{-3}
	200	2.00x10^{-1}	2.77x10^{-3}

	400	4.00×10^{-1}	5.54×10^{-3}
	600	6.00×10^{-1}	8.31×10^{-3}
	800	8.00×10^{-1}	1.11×10^{-2}
Algae>MP	50	5.00×10^{-2}	1.39×10^{-3}
	100	1.00×10^{-1}	1.39×10^{-3}
	200	2.00×10^{-1}	1.39×10^{-3}
	400	4.00×10^{-1}	1.39×10^{-3}
	600	6.00×10^{-1}	1.39×10^{-3}
	800	8.00×10^{-1}	1.39×10^{-3}
MP>Algae	50	1.00×10^{-1}	6.93×10^{-4}
	100	1.00×10^{-1}	1.39×10^{-3}
	200	1.00×10^{-1}	2.77×10^{-3}
	400	1.00×10^{-1}	5.54×10^{-3}
	600	1.00×10^{-1}	8.31×10^{-3}
	800	1.00×10^{-1}	1.11×10^{-2}

2.2.7 Chronic toxicity tests—adults

Daphnia (18 days old from the third brood) were placed individually into glass beakers filled with 50 ml media and exposed to one of eight treatments (replicated five times) (Table 2.2). Media and concentrations were renewed three times per week. In all treatments, life history traits (survival and reproduction) were monitored for 21 days. Neonates were counted daily and removed. Animals unable to swim after gentle stirring for 15 s were considered dead. The experiment was run under laboratory conditions at 20 ± 2 °C, and 16 h light :8 h dark.

Table 2-2 Concentrations (mg L^{-1}) of MPs and algae added to each treatment to study chronic toxicity in *Daphnia magna*.

Treatments	Algae concentration (mg L^{-1})	MPs (mg L^{-1})
Control low algae	1.00×10^{-1}	0
Control high algae	8.00×10^{-1}	0
Algae=MP (low)	1.00×10^{-1}	1.39×10^{-3}
Algae=MP (high)	8.00×10^{-1}	1.11×10^{-2}
Algae>MP	8.00×10^{-1}	1.39×10^{-3}
MP>Algae	1.00×10^{-1}	1.11×10^{-2}

2.2.8 Chronic toxicity test—neonates

A standard chronic toxicity test was conducted in compliance with OECD guideline 211 (OECD, 2012). Five third brood neonates (<24 h) old were placed in glass beakers, and exposed to different treatments in 50 ml media (Table 2.2). Media and concentrations were renewed three times a week and life history traits (survival, growth and reproduction) were monitored for 21 days. Body length (the area from the top of the head to the base of the tail spine) was measured under a stereomicroscope every other day. The experiment was run at 20 ± 2 ◦C, with 16 h light: 8 h dark

2.2.9 Statistical methods

Data were checked for normality using the Shapiro–Wilk test, SPSS 21.0 (SPSS Incorp., Chicago, Il, USA). The uptake and depuration experiments were normally distributed data and analysed by ANOVA whereas T-tests were conducted to compare between 15 and 240 min.

For the reproduction experiment the Kolmogorov–Smirnov test showed non-normally distributed data. Data were analysed using the Wald chi-square test, which is based on the linearly independent pairwise comparisons among the estimated marginal means of

the offspring. T-tests were used to compare the mean reproduction between treatments with and without MPs.

Growth rate data was normally distributed and analysed by UNIANOVA. A post-hoc pairwise comparison was undertaken with t-tests to measure the effect of MPs on the growth rate.

The mortality tests were normally distributed. Analysis was carried out using Minitab V. 17, general linear model and a probit analysis conducted, and the response curve for concentrations was made using a scatterplot.

2.3 RESULTS

2.3.1 Uptake of MPs

Daphnia that were fed with algae plus MPs ate significantly fewer MPs compared to those with just MPs ($F_{1,30}$ = 50.702, P < 0.001) (Figure 2.1) (Tables S1–S2). Daphnia treated with MPs and algae not only ate fewer MPs than *Daphnia* without algae, but there was a significant reduction in bead uptake over time ($F_{4,19}$ = 5.771, P = 0.005). There were significantly fewer MPs in *Daphnia* fed algae after 240 min compared to the number after 15 min' t (d.f. 6) = 2.5, P = 0.042. Although *Daphnia* without algae ingested significantly more MPs over time than those fed algae, ingestion over time did not increase significantly ($F_{4,19}$ = 1.244, P = 0335). The number of MPs ingested after 15 min did not significantly vary from the number of MPs ingested after 240 min t (d.f. 6) = −1.2, P = 0.27.
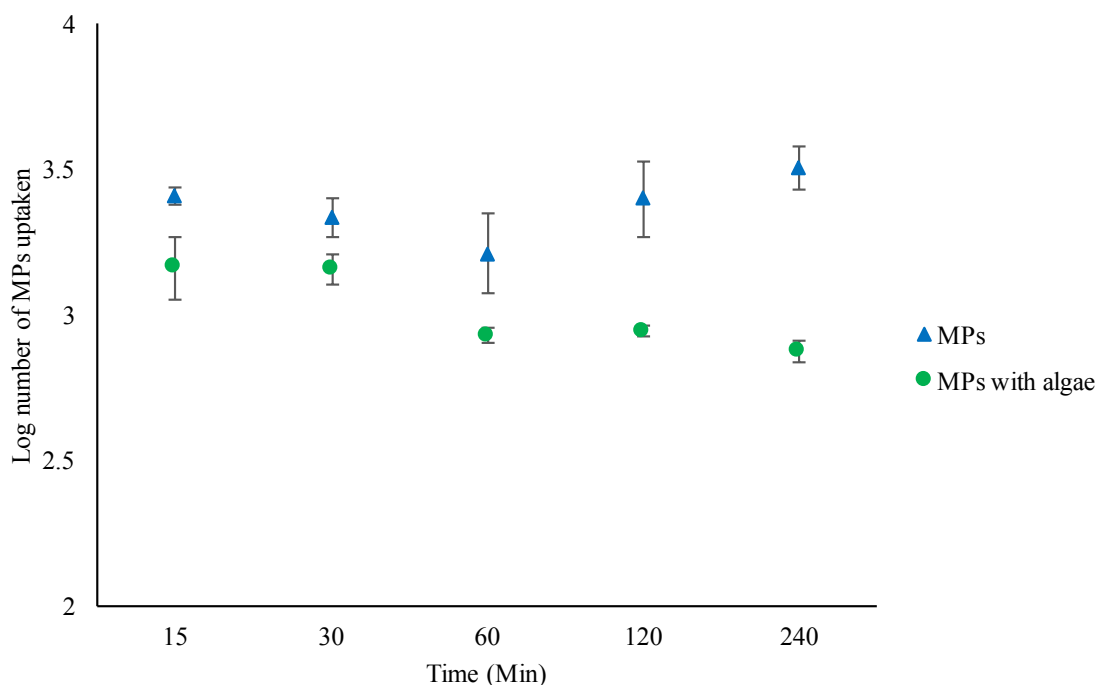
Figure 2-1 Uptake of 2 μm polystyrene MPs by *Daphnia magna* exposed to MPs only (1.46 ×10² mg L⁻¹) or MPs with algae (1.46 ×10² mg L⁻¹ and 1.00 ×10⁻¹ mg L⁻¹) over 240 min. Each point represents the mean ± the standard error.

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2.3.2 Depuration of MPs

As before, *Daphnia* fed algae contained significantly fewer MPs compared to those without algae ($F_{1,30}$ = 976.162, P < 0.001) (Figure 2.2) (Tables S3–S4). Bead excretion in *Daphnia* fed algae did not vary over time ($F_{4,19}$ = 1.006, P = 0.435). There were no significant differences in bead counts after 240 min compared to the number after 15 min t (d.f.6) = 0.978, P = 0.366. However, the unfed *Daphnia* excreted a significant number of MPs over time compared to fed *Daphnia* ($F_{4,19}$ = 5.452, P = 0.006). There were significantly fewer MPs in *Daphnia* after 240 min compared to the number after 15 min t (d.f.6) = 3.5, P = 0.013.
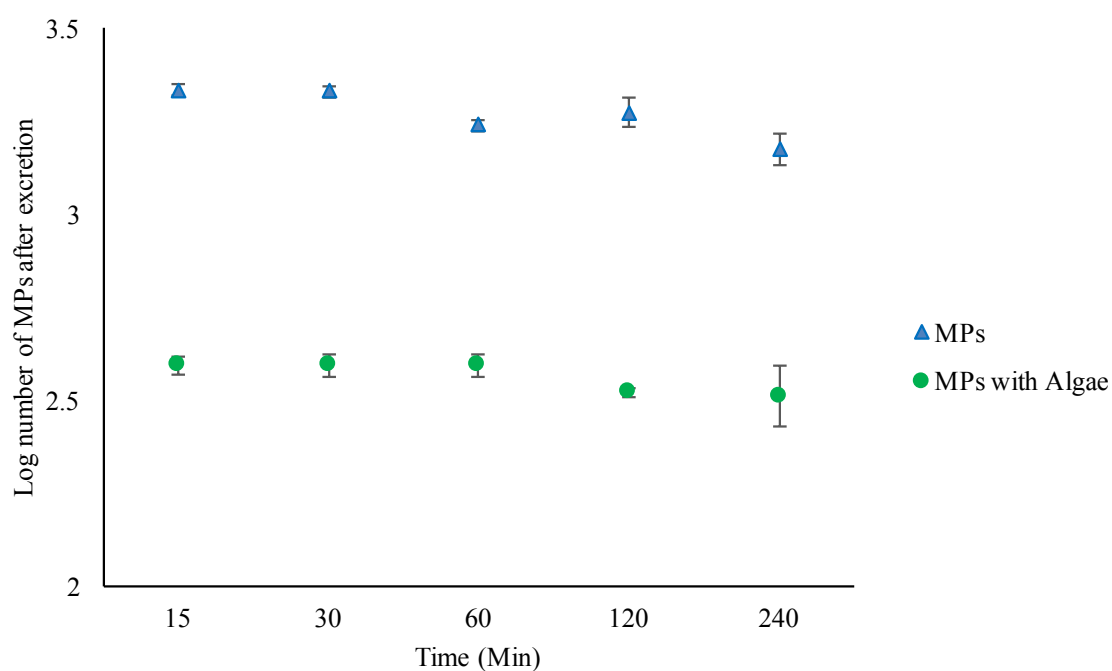
Figure 2-2 Excretion of 2 μm polystyrene MPs from the gut of *Daphnia magna* exposed to MPs only (1.46 ×10^2 mg L^{-1}) or MPs with algae (1.46 ×10^2 mg L^{-1} and 1.00 ×10^{-1} mg L^{-1}) over 240 min. Each point represents the mean ± the standard error.

2.3.3 Differential microplastic uptake under varying food regime

Four treatment regimes, which varied in the amount of either MPs, algae or both MPs and algae resulted in significant differences in the amount of MPs ingested ($F_{3,120}$ = 114.899, $P < 0.001$) (Figure 2.3). In unfed *Daphnia* treated with MPs (MPX), the internal bead count increased significantly as the concentration of MPs increased ($F_{5,120}$ = 12.849, $P < 0.001$). Where a low amount of food was introduced (MP>Algae) ingested MPs dropped significantly ($F_{3,120}$ = 20.788, $p < 0.001$) but did not change with increasing concentrations of MPs ($F_{5,120}$ = 1.207, $P < 0.310$). An equal dose of MPs and algae (MP = Algae) was likewise significantly lower than MPX and did not increase as both concentrations increased ($F_{5,120}$ = 1.131, $P < 0.348$). Where *Daphnia* were treated with a fixed low concentration of MPs and algal concentrations increased (Algae > MP), the number of MPs decreased significantly ($F_{5,120}$ = 4.242, $P < 0.001$).
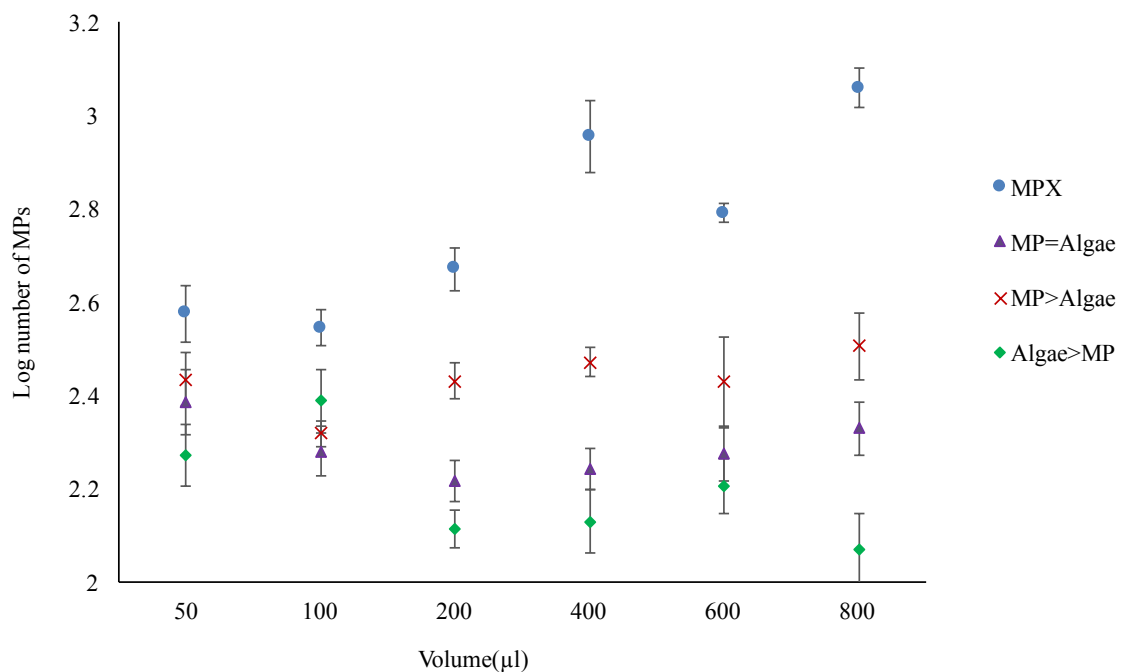
Figure 2-3 Uptake of 2 μm polystyrene MPs by *Daphnia magna* with and without algae in various volumes (μl) (see Table 2.1 for actual concentrations). Each point represents the mean ± the standard error.

2.3.4 Mortality test—adults

Mortality rates varied significantly between treatments ($F_{5,618}$ = 43.38, P < 0.001) (Figure 2.4). All treatments had significant mortality compared to the control (algae 8.00 $\times 10^{-1}$ mg L^{-1}) ($F_{5,618}$ = 48.97, P < 0.001). A pairwise comparison of mortality in *Daphnia* with restricted food (1.00 $\times 10^{-1}$ mg L^{-1})algae) plus or minus 1.39 $\times 10^{-3}$ mg L^{-1})MPs was significant t (d.f. 1) = −3.99, P < 0.001 and with ample food (8.00 $\times 10^{-1}$ mg L^{-1}algae) plus or minus 1.11 $\times 10^{-2}$ mg L^{-1} MPs was highly significant t (d.f. 1) = 4.59, P < 0.001 since there was no mortality in the treatment without MPs (Table 2.3).
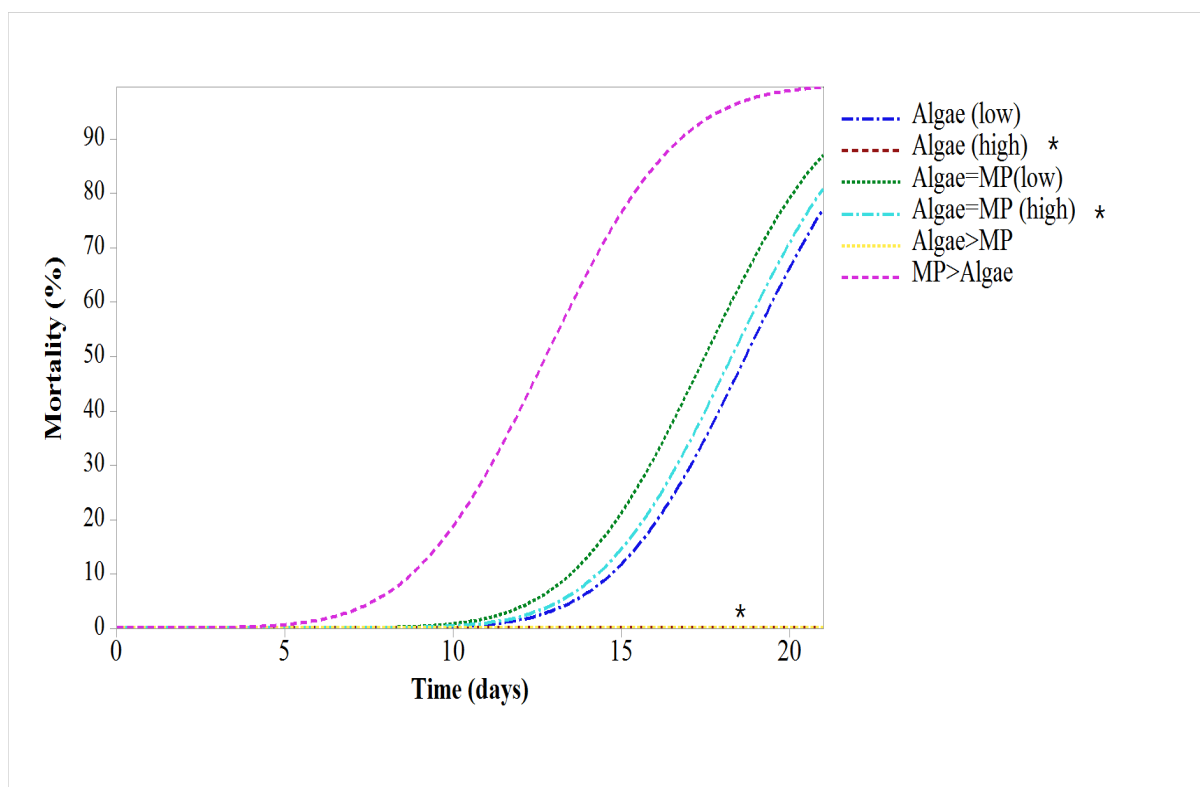
Figure 2-4 Mortality of *Daphnia magna* expressed as a function of time after chronic exposure to MPs under high and low food conditions for 21 days. Asterisks denote overlap between two treatments.

Table 2-3 Mean ± standard error (S.E.) of lethal time (LT10, LT50 and LT90) of adult *Daphnia magna* exposed to different concentrations (mg L^{-1}) of MPs and algae.

Treatments	Concentrations		Lethal Time % (Days)		
	Algae mg L^{-1}	MPs mg L^{-1}	LT$_{10}$ ±SE	LT$_{50}$ ±SE	LT$_{90}$ ±SE
Algae (low)	1.00×10^{-1}	---	7.33±0.825	12.47±0.69	17.6±0.85
Algae (high)	8.00×10^{-1}	---	39.4±0	44.5±0	49.9±50
Algae=MP (low)	1.00×10^{-1}	1.39×10^{-3}	11.22±0.79	16.36±0.69	21.5±0.87
Algae=MP (high)	8.00×10^{-1}	1.11×10^{-2}	12.35±0.76	17.48±0.70	22.6±0.92
Algae>MP	8.00×10^{-1}	1.39×10^{-3}	39.40±0	44.54±0	49.68±0
MP>Algae	1.00×10^{-1}	1.11×10^{-2}	4.95±0.85	10.09±0.70	15.23±0.83

2.3.5 Reproduction test—adults

A 21 day reproduction test on adult *Daphnia* resulted in no significant differences in the mean number of offspring between treatments (Figure 2.5) (X^2(5,N =30)=4.62, P =0.463). There was no significant effect on the mean reproduction between treatments with low algal concentration (1.00×10^{-1} mg L^{-1}) and those with low algal concentrations with the addition of 1.39×10^{-3} mg L^{-1} of MPs t (d.f. 8) = 0.971, P = 0.36. Similarly, there were no significant differences in mean reproduction between *Daphnia* treated with high algal concentrations (8.00×10^{-1} mg L^{-1}) and those with high algal concentrations with the addition of 1.11×10^{-2} mg L^{-1} of MPs t (d.f. 8) = 0.067, P = 0.948.

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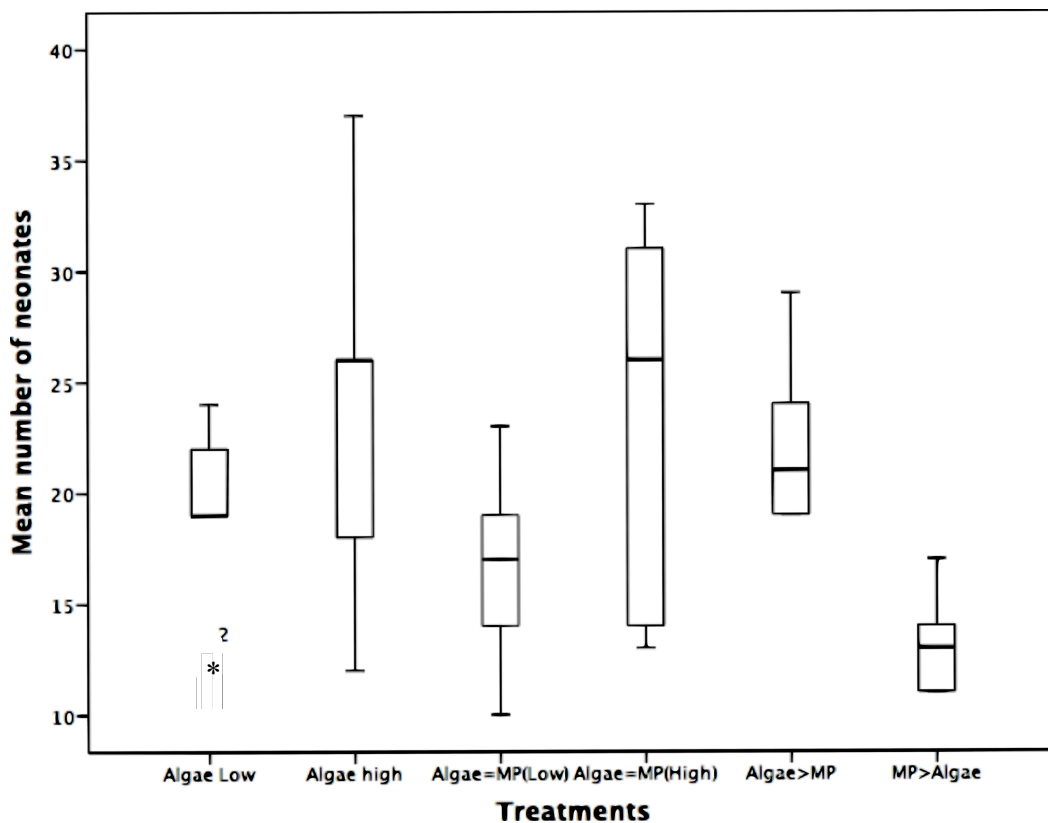
Figure 2-5 Effects of combinations of high and low MPs and algae concentrations on the mean number of offspring on *Daphnia magna*. Error bars indicate ± 95% confidence intervals and asterisks denote significant differences compared to the control *p* < 0.001.

2.3.6 Mortality rate—neonate

In neonates, there was a significant difference in the percentage mortality between treatments ($F_{5,618}$ = 26.86, $P < 0.001$) (Figure 2.6). The percentage mortality of *Daphnia* fed low algal concentrations and MPs was significantly higher than that of those treated with the same algal concentrations but no MPs t (d.f. 5) = 4.24, P = <0.001. In contrast, MPs had no impact on mortality when fed to *Daphnia* on a high algal concentration t (d.f. 5) = 4.51, P = 0.776. Calculated lethal times (LT_{10}, LT_{50} and LT_{90}) are presented in Table 2.4.
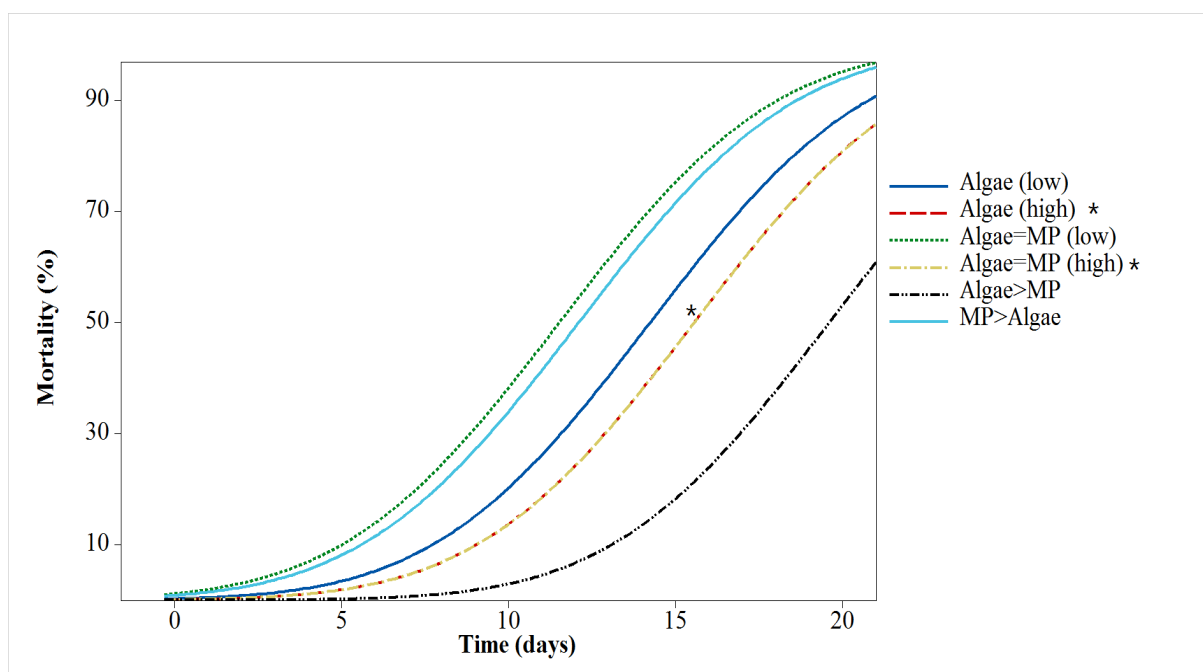
Figure 2-6 Mortality of neonate *Daphnia magna* after exposure to different treatments of MPs and algae over 21 days. Asterisks denote overlap between two treatments.

Table 2-4 Mean ± standard error of lethal time (LT10, LT50 and LT90) of neonate *Daphnia magna* exposed to different concentrations (mg L^{-1}) of MPs and algae.

Treatments	Concentrations		Lethal Time % (Days)		
	Algae mg L^{-1}	MPs mg L^{-1}	LT10 ±SE	LT50 ±SE	LT90 ±SE
Algae (low)	1.00×10^{-1}	---	7.71 ±0.39	14.23 ±0.35	20.75 ±0.42
Algae (high)	8.00×10^{-1}	---	9.02 ±0.4	15.54 ±0.36	22.06 ±0.44
Algae=MP (low)	1.00×10^{-1}	1.39×10^{-3}	4.98 ±0.41	11.51 ±0.35	18.03 ±0.41
Algae=MP (high)	8.00×10^{-1}	1.11×10^{-2}	9.03 ±0.4	15.55 ±0.36	22.07 ±0.44
Algae>MP	8.00×10^{-1}	1.39×10^{-3}	13.07 ±0.42	19.59 ±0.42	26.11 ±0.52
MP>Algae	1.00×10^{-1}	1.11×10^{-2}	5.57 ±0.4	12.09 ±0.34	18.61 ±0.4

2.3.7 Reproduction test—neonate

The reproduction rate was significantly different between adults that had been treated since they were neonates with high and low algae concentrations (Figure 2.7) (X^2(5,N =28)=618, $P > 0.001$), this was because all treatments that included (8.00 $\times 10^{-1}$ mg L^{-1}) algae had significantly higher reproduction compared to those on (1.00 $\times 10^{-1}$ mg L^{-1}). There were no significant differences in reproduction between identical food regimes with and without MPs (low algae concentration (1.00 $\times 10^{-1}$ mg L^{-1}) in the presence of MPs (1.39 $\times 10^{-3}$ mg L^{-1}) t (d.f. 7) = 1.63, $P = 0.146$, high algal concentration (8.00 $\times 10^{-1}$ mg L^{-1}) in addition to MPs (1.11 $\times 10^{-2}$ mg L^{-1}) t (d.f. 8) = 0.46, $P = 0.652$).
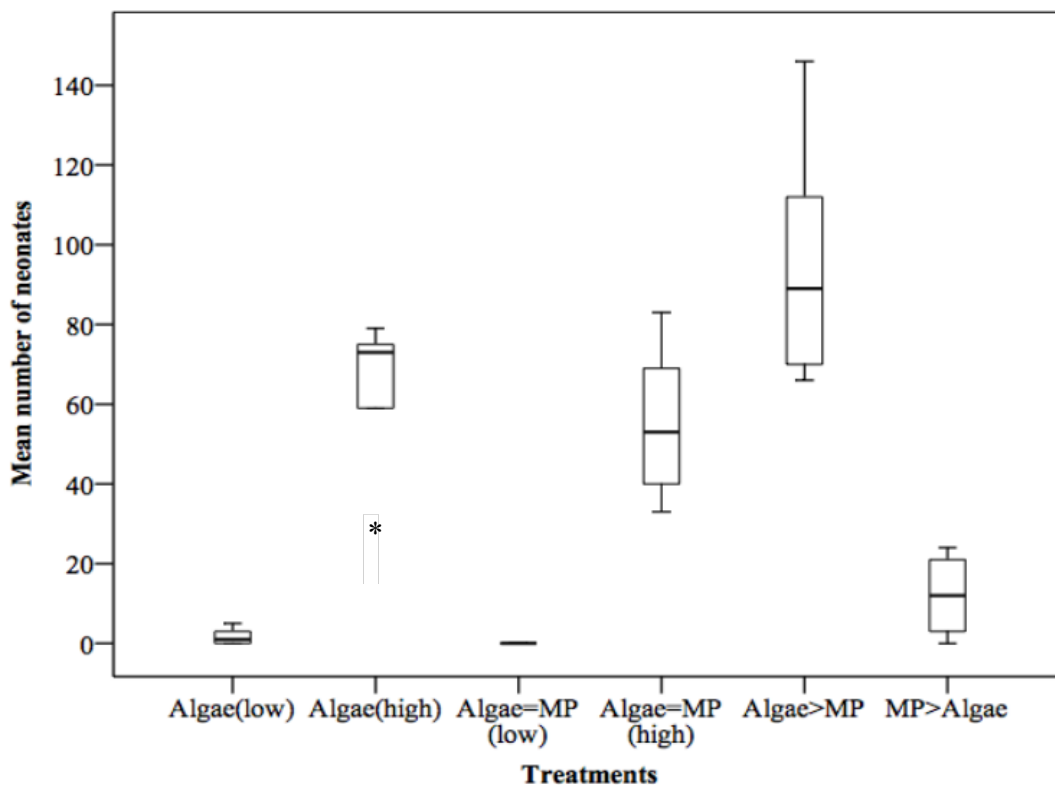
Figure 2-7 *Daphnia magna* reproduction (neonate production) after 21 days' exposures to a range of MP and algae treatments (algae (low), algae (high), Algae = MP(low), Algae = MP(high), Algae > MP, MP > Algae). Error bars indicate ± 95% confidence intervals and asterisks denote significant differences compared to the control *P* < 0.001.

2.3.8 Growth rate

Daphnia body length increased significantly in all treatments over 21 days compared to the initial size ($F_{5,274} = 166.8$, $P < 0.001$) (Figure 2.8). *Daphnia* growth was lower in animals exposed to low algae concentrations (1.00×10^{-1} mg L^{-1}) compared to those given high algae concentrations (8.00×10^{-1} mg L^{-1}) t (d.f. 278) $= -14.5$, $P < 0.001$. MPs significantly reduced growth in both algal food regimes (1.00×10^{-1} mg L^{-1}, $F_{2,126} = 3.009$, $P = 0.05$); (8.00×10^{-1} mg L^{-1}, $F_{2,149} = 0.63$, $P = 0.05$).
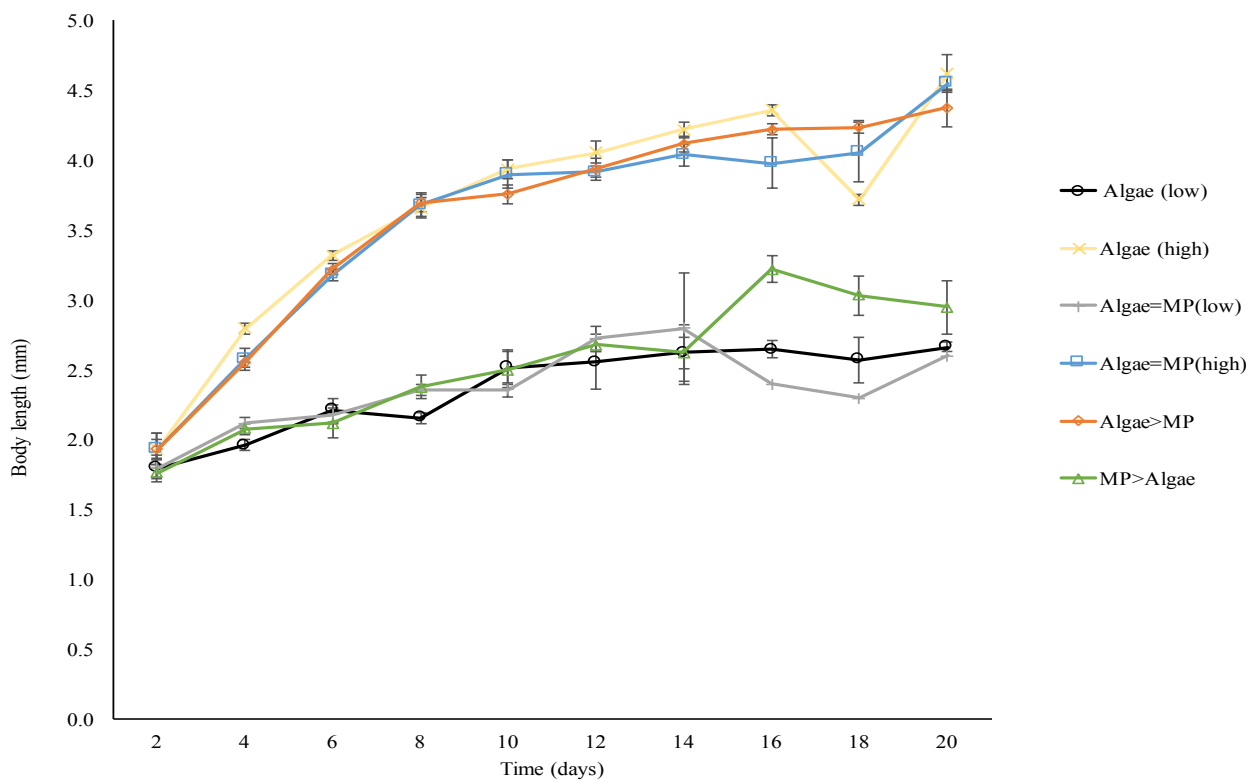
Figure 2-8 Effect of 21 days' exposure to different combinations of MPs and algae (Algae (low), algae (high), Algae = MP(low), Algae = MP(high), Algae > MP, MP > Algae) on body length of *Daphnia magna*. Each point represents the mean of five replicates ± standard error.

2.4 DISCUSSION

Previous studies have suggested that *D. magna* find it hard to distinguish between MP and food particles in the media (Wiedner and Vareschi, 1995). This study was therefore designed to look at the interaction between MP ingestion and food intake, with MP size chosen to approximately match the cell size of the algae. Algae concentrations were chosen based on the minimum and maximum normal daily feeding of *Daphnia* (Hooper et al., 2006), while MPs concentrations were chosen to be close to the algae concentrations volume/volume. When exposed to a single concentration of MPs *Daphnia* almost immediately ate them in large quantities. Previous studies have demonstrated that *Daphnia* will feed on polystyrene, beating their appendages at a constant rate regardless of the food concentration (Pavlaki et al., 2014). The same study also found that plastics of different sizes (1.1 μm and 5.7 μm) were ingested in proportion to their concentration (Pavlaki et al., 2014). This could explain the fact that the amount eaten was reduced when food (algae) was present, which suggests a simple competition for uptake. This hypothesis was further tested by mixing concentrations of algae and MPs. Here there was a direct correlation between MP uptake and concentration so that *Daphnia* ate more MPs as more were available. However, the presence of algae, even at low concentrations, had a significant negative impact on MP uptake. Although MP concentrations increased, intake did not if algae were present, even at higher concentrations of MPs. A high concentration of algae significantly reduced the uptake of a low concentration of MPs so that they were not ingested in direct proportion. Therefore, unlike the study looking at the uptake of different sized polystyrene, there is no evidence that MPs are eaten in proportion to their concentration when algae are present. This implies selectivity (Pavlaki et al., 2014). Previous studies have demonstrated that *Daphnia* are not blindly filtering particles from the water, since particles smaller than the mesh size of their appendages are still eaten (Pavlaki et al., 2014). The uptake of particles is strongly influenced by surface chemistry (negative charges increase uptake) and wettability (which can be reduced by the presence of surfactants to decrease uptake) (Pavlaki et al., 2014). There is also some evidence that *Daphnia* can discriminate artificial from natural particles by taste for instance selecting to feed on phytoplankton rather than clay particles (DeMott, 1986). A previous study noted that MPs tended to form aggregates with algae, thereby effectively increasing the food particle size, with a reduction in MP ingestion when algae were present (Long et

al., 2015). There was no evidence of aggregation in any of the experiments presented here pre-feeding.

Where no food was offered, the number of MPs in the *Daphnia* gut remained relatively stable with no significant difference between the number in the gut at the beginning and the end of the experiment. However, there was a significant reduction in MPs over time when food was present.

The excretion of particles was further investigated by measuring the depuration of MPs following a 1 h exposure to MPs followed by clean OECD water for 15, 30, 60, 120 and 240 min. The results showed that the number of MPs in the *Daphnia* decreased over time, suggesting excretion. This has been shown before, with small MPs clearing more rapidly than larger ones (Rosenkranz et al., 2009). However, in the individuals exposed to MPs and food particles, the number of MPs in the gut hardly decreased over time. It is not clear why this is the case and it could be that MPs and food aggregate in the gut, making it hard to excrete MPs. Additionally, it could be that *Daphnia* is re-ingesting the plastic particles after excretion into the media.

Daphnia exposed to MPs for 21 days showed mortality after seven days of exposure in all treatments compared to the controls. However, treatments with high MP concentrations and low food levels had the highest mortality compared to the control with low food concentrations. Treatments with high MPs and ample food showed lower mortality compared to the control with low food concentrations. This suggests that where ample food is present, MPs have little effect on adults.

There was also no impact on their reproduction. Again, food levels were more important than MPs concentrations. Similar results had been found recently by (Ogonowski et al., 2016) when exposed *Daphnia* to primary MPs or kaolin with low and high food concentrations, results showed life history traits more related to food concentration rather than MPs.

The neonate toxicity test confirmed previous results that mortality was linked to availability of food rather than MP concentrations. *Daphnia* exposed to high food concentrations show higher survival despite MP treatment and reproduction was dramatically decreased in treatments with low food concentrations. Only in the treatment with low food concentrations and low doses of MPs could we see a potential impact of MP with no reproduction. This issue had been investigated previously by

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exposing the *Daphnia* to metal and different food concentrations which shows that chronic toxicity is linked more to food availability rather than metals; however, that metal get more toxic when there is low food level (Pavlaki et al., 2014).

Growth rate was more effected by food concentrations rather that MPs, *Daphnia* treated with high food concentrations grow are twice as large as those with low food availability. Similar results were obtained elsewhere with both *Daphnia* and marine isopods Idotea emarginata (Hamer et al., 2014; Ogonowski et al., 2016). This is in contrast to work published on the effect of ~70 nm nano-polystyrenes (Nano-PS) on both *Daphnia* and algae (Scenedesmus obliquus) (Besseling et al., 2014). Nano-PS negatively impacted population growth and reduced chlorophyll concentrations in the algae. *Daphnia* exposed to Nano-PS had reduced body sizes and the numbers and body size of neonates were lower. Nano-PS also caused high numbers of neonate malformations. However the difference here, apart from the size, is that the authors didn't use pristine polystyrene but aged the nano-PS with the algae for 5 days (Besseling et al., 2014). Their comparison between aged and pristine nano-PS demonstrated that pristine MPs may not represent the full impact of the exposure.

2.5 CONCLUSIONS

Our research was designed to determine the effect of 2 μm MPs on *Daphnia magna* in the presence of algae *Chlorella vulgaris*. This was an experimental approach and was not intended to reflect environmental concentrations of MPs. There is no accurate measurement of 2 μm MPs in freshwater environment and this particular size of plastic can be generated from either a primary source such as cosmetics, or from the degradation of large plastic particles (Connors et al., 2017).

The uptake of MPs decreased in the presence of algae and excretion of MPs reduced. The concentration of MPs ingested did not increase with concentration when algae were available which indicates that the *Daphnia* is selectively eating the algae rather than MPs. Chronic toxicity tests (mortality and reproduction rate) found no toxic effect after a 96 h of exposure although seven days of exposure to high concentration of MPs increased mortality. Life history traits of neonates (mortality, reproduction and growth rate) was mainly linked to food concentrations rather than MPs that could confirm *Daphnia* select the food particles rather than MPs.

The study presented here was undertaken to look at the impact of the MPs themselves and as such our results have been obtained with clean MPs that have not been exposed to any contaminants. Environmental MPs are likely to mix with other contaminants, some of which could bind to them and alter their toxicity. Therefore, a future direction of research should include investigating the toxicity of MPs collected from the aquatic environment or in mixtures with known freshwater pollutants such as pesticides.

Chapter 3 IMPACT OF 15 μM POLYSTYRENE MICROPLASTICS ON *DAPHNIA MAGNA* MORTALITY AND REPRODUCTION IN RELATION TO FOOD AVAILABILITY

3.1 Introduction

Plastic pollution in aquatic habitats is a serious problem which has generated growing awareness worldwide of the scale of the issue. Much of the research focus has been on marine ecosystems and organisms: fewer than 4% of research papers have considered freshwater (Wagner and Lambert 2018). In recent years, however, interest in environmental occurrence and the effects of microplastics (MPs) has shifted towards freshwater ecosystems (Wagner and Lambert 2018; Wagner et al. 2014; Eerkes-Medrano et al. 2015).

Microplastics are defined as diverse plastics, including polyethylene and polystyrene, whose fragments are smaller than 5 mm in size which are created by the degradation of larger particles or are manufactured as microbeads for use in, for example, cosmetics and toiletries (Imhof et al. 2013; Eriksen et al. 2014; Andrady 2011). Their size results in them being easily ingested by many aquatic organisms at various trophic levels and stages of development, including freshwater invertebrates (Al-Jaibachi, et al. 2018a; Al-Jaibachi, et al. 2018b; Aljaibachi and Callaghan 2018; Cole et al. 2013; Besseling, Wang, Lürling, et al. 2014; Scherer et al. 2017). Measuring the impact of MPs in ecosystems is challenging and the identification of plastic polymers sampled from the field is difficult and requires sophisticated equipment (Klein et al., 2015). Therefore, many studies, including this one, rely on data obtained from controlled laboratory environments with known concentrations and MP types.

Daphnia magna is a standard ecotoxicity model and shows a high sensitivity to toxicants (Pablos et al., 2015). They are also used as models of filter feeders in the freshwater environment and have been utilised to examine the uptake and depuration of MP sizes from 1 nm to 2 μm (Besseling, Wang, Lu, et al. 2014; Aljaibachi and Callaghan 2018). Work has also been directed to life-history effects and both the acute and chronic toxicity of MPs on *D. magna.* This work has demonstrated that food availability is more important than the presence of MPs (Aljaibachi and Callaghan 2018; Rist et al. 2017; Canniff and Hoang 2018; Lithner et al. 2009). A very recent study has looked at fluorescent green polyethylene MPs of 63–75 μm in size and their impact on the ingestion and life history of *D. magna* (Canniff and Hoang 2018). Ingestion was found to increase in line with MP concentration and time, but no impact was seen on survival or reproduction. A surprising result was the positive impact that

MPs had on *Raphidocelis subcapitata* algae, which were found to grow more in the exposure media with MPs present than without them. These authors concluded that the algae use MPs as a substrate on which to grow and that MP ingestion, therefore, was another route for food assimilation (Canniff and Hoang 2018).

The aim of the present study was to add to the growing body of evidence regarding the impact (or lack of this) of MPs by carrying out a thorough investigation of the effect of a larger 15 μm polystyrene MP on *D. magna* in terms of reproduction, growth and mortality, including standard ecotoxicology 21 days' life history tests in accordance with the Organization for Economic Cooperation and Development (OECD) guideline 211(OECD, 2012). In our previous study, there was evidence that *D. magna* could distinguish between 2 μm MPs and algae, despite their similar size (Al-Jaibachi and Callaghan 2018). Here, the much larger MPs ought to be more readily distinguishable and, therefore, if *D. magna* really were selectively eating algae, it would be possible for them to selectively avoid their ingestion. Previous work in both *D. magna* and other freshwater organisms, such as the amphipod *Gammarus fossarum*, has given a clear indication that when MPs are ingested, but not absorbed into the body cavity, no negative impacts on survival, growth or reproduction are found (Blarer and Burkhardt-Holm 2016), with food availability having the greatest impact. Therefore, regardless of whether *D. magna* selectively avoids eating MPs or not, no negative effects of MP ingestion would be expected.

3.2 Materials and Methods

3.2.1 *Daphnia magna* Culture

Daphnia magna were obtained from the Water Research Centre (WRC, Medmenham, UK) and were cultured at the University of Reading for more than 10 years prior to this experiment. New cultures of *D. magna* were prepared with fifteen neonates from a single female in 1200 ml beakers filled with artificial freshwater and prepared as described in the guideline of Organization for Economic Co-operation and Development reconstituted water media (OECD) water (OECD, 2004). All cultures were fed, once daily, 2 ml of unicellular green algae, *Chlorella vulgaris* and 0.5 ml of yeast 10 mg/100 ml, cultured at 20 ± 2 ºC with a photoperiod light : dark of 16:8 h. Juveniles were removed regularly from the culture and OECD water was changed once a week.

3.2.2 Algae Culture

Chlorella vulgaris var Viridis was supplied as a culture collection of algae and protozoa, SAMS, UK, cultured and prepared according to the method of Hooper (Hooper et al., 2008). *D. magna* and algae were cultured under laboratory conditions at a temperature 20 ±2 ºC and a photoperiod light : dark 16:8 h. After 14 days, algae were centrifuged at 3000 rpm for 30 min at room temperature in 250 ml Sorval tubes. After discarding the supernatant, the cells were re-suspended in 100 ml of deionized water. Optical density was measured with a spectrophotometer (CECIL instruments, Cambridge, England), wavelength 440 nm. Algal suspension were frozen directly under -80 ºC to maintain algal growth.

3.2.3 Preparation of Microplastics

Microplastics used for uptake experiments were supplied by the Life Technologies Corporation, UK, (Lot no. 1555427) as actual particle size 14.8 ± 0.13 µm polystyrene microspheres, fluorescent red-orange (excitation 565 nm; emission 580 nm). MPs were stored as a stock suspension 1×10^6 MPs ml^{-1} in a medium of 0.15 M NaCl, 0.05% Tween 20 and 0.02% thimerosal. Microplastics used for the toxicity test were supplied by Bangs laboratories, Inc, USA (Lot no. 12980) as actual size 15.45 ± 1.1 µm polystyrene microspheres, density 1.06 g.cm^3 fluorescent dragon green (excitation 480 nm; emission 520 nm). Stock solution were suspended using vortex before one millilitre was decanted to ensure dispersion of plastic particles into a 1.5 ml Eppendorf tube and centrifuged at 9000 rpm for 10 min. The supernatant was discarded and 1 ml of distilled water was added. The solution was then re-suspended by using the vortex and centrifuged again at the same speed and for the same duration. This process was repeated two more times.

3.2.4 Microplastics Uptake

Individual adult (18-day-old) *D. magna* were placed in 50 ml beakers filled with media and starved for 24 h prior to exposure. They were then exposed to (200 MPs ml^{-1}) 15 µm MPs with and without algae 100 µl of *Chlorella vulgaris* (7×10^4 cells/100µl). Five replicates for each treatment were exposed for 30, 60, 120 and 240 min. The experiment was run at 20 ± 2 ºC, with a light : dark control of 16:8 h. Following exposure, the *D.*

magna were washed twice with deionized water for 1 min, to wash off any MPs stuck to the carapace then stored at -20 ºC.

3.2.5 Microplastic Excretion

In order to evaluate excretion of MPs, 20 *D. magna* were exposed for 1 h to MPs (200 MPs/ml), with or without 100 µl of algae, then transferred to 50 ml clean OECD reconstituted water for 30, 60, 120 and 240 min (replicated five times). The *D. magna* were washed twice with deionized water for 1 min, to remove any MPs stuck to the carapace, before freezing at -20 ºC.

3.2.6 Microplastics Uptake as a Function of Concentration

Eighteen-day-old *D. magna* were exposed to 40, 80, 160 or 320 µl of microplastics and algae in different combinations (Table 3.1) for 60 min. Concentrations were selected based on the individual daily feeding limit for *D. magna.* Treatments were replicated three times and assigned according to a randomized block design. Following exposure, *D. magna* were washed twice with distilled water for 1 min before freezing at -20 ºC.

Table 3-1 Concentrations of microplastics and algae added to each treatment to evaluate the uptake of MPs by *D. magna* as a function of concentration

Treatments	Microplastics concentrations (µl)	Number of MPs ml^{-1}	Algae concentrations (µl)	Number of Algae cells ml^{-1}
MPs only	40	2×10^5	0	0
	80	4×10^5	0	0
	160	8×10^5	0	0
	320	1.6×10^6	0	0
MP=Algae	40	2×10^5	40	28×10^4
	80	4×10^5	80	56×10^4
	160	8×10^5	160	1.12×10^6
	320	1.6×10^6	320	2.24×10^6
MP>Algae	40	2×10^5	80	56×10^4
	80	4×10^5	80	56×10^4
	160	8×10^5	80	56×10^4
	320	1.6×10^6	80	56×10^4
Algae>MP	80	4×10^5	40	28×10^4
	80	4×10^5	80	56×10^4
	80	4×10^5	160	1.12×10^6
	80	4×10^5	320	2.24×10^6

3.2.7 Quantification of Uptake and Excretion

Frozen *D. magna* were placed individually into a 1.5 ml Eppendorf tube filled with 500 µl distilled water and homogenised using a glass Kontes Pellet Pestle (Fisher Sciences Loughborough, UK). A further 500 µl distilled water was added to wash the pestle. Half of the homogenate, taken for counting the MPs under the microscope, was filtered onto a nucleopore track-etched membrane (Whatman, UK) 10 µm with a white background, by using a glass vacuum filter holder connected to a manual air pump. The membrane was examined under an epi-fluorescent microscope (Zeiss Axioskop) at a magnification of 10x, in order to count the fluorescent MPs in each treatment.

3.2.8 Microplastics Visual Assessment

Five adult *D. magna* from each treatment for each experiment were observed under the confocal microscopy (Nikon), at 4X magnification to investigate the gut system. Images were taken through a blue filter (550-600 nm), in order to differentiate MPs from algae, which fluoresce under red-orange illumination (565-580 nm).

3.2.9 Chronic Toxicity Tests – Adults

Third brood *D. magna* adults (18 days old) were placed individually into glass beakers filled with 50 ml media and exposed to one of eight treatments (replicated five times) (Table 3.2). Media and concentrations were renewed three times per week. In all treatments, life history characteristics (survival and reproduction) were monitored for 21 days. Neonates were counted daily and removed. Animals unable to swim after gentle stirring for 15 s were considered to be dead. The experiment was run under laboratory conditions at 20 ± 2 ºC, light : dark 16:8 h.

Table 3-2 Concentrations of MPs (MPs/ml) and algae (µl) added to each treatment to study chronic toxicity in *D. magna*

Treatments	Algae concentrations (µl)	Microplastics concentrations (MPs ml^{-1})
Algae (Low)	100	0
Algae (High)	800	0
Algae=MPs (Low)	100	100
Algae=MPs (High)	800	800
Algae>MPs	800	100
MPs>Algae	100	800

3.2.10 Chronic Toxicity Test - Neonates

A standard chronic toxicity test was conducted in accordance with OECD guideline 211 (OECD, 2012). Five individuals from third-brood neonates (< 24 h) were placed in 50 ml glass beakers and exposed to MPs and/or algae (Table 3.2). Media and concentrations were renewed three times a week and life history characteristics (survival, reproduction and growth) were monitored for 21 days. Body length (from the top of the head to the base of the tail spine) was measured every other day under a stereomicroscope. The experiment was run at 20 ± 2 ºC, light : dark 16:8 h.

3.2.11 Statistical analysis

Statistical analyses of uptake, depuration and effects of concentration were carried out using SPSS 21 (SPSS, 2012). Data were analysed following log10 transformation to meet normality and homoscedasticity assumptions (Shapiro-Wilk test, $p > 0.05$; Bartlett's test, $p > 0.05$) where applicable. Microplastic uptake as a function of time was analysed using Univariate Analysis of Variance (UNIANOVA) following pairwise, *post hoc* Tukey's comparisons (treatments × time).

Reproduction test data were analysed using a generalized linear model (GLM) following *post hoc* Tukey's comparisons. Growth rate data were analysed using

UNIANOVA (mixed model), followed by post-hoc pairwise comparisons (growth rate × treatments × time).

Probit analysis was conducted for the chronic toxicity test (mortality rate for adults and neonates) and response curves for different concentrations were produced as a scatter plot (Minitab V. 17).

3.3 Results

3.3.1 Uptake of Microplastics

Daphnia magna fed with both algae and MPs ingested significantly fewer MPs compared to those receiving no algae ($F_{1,32}$=39.53, *p*< 0.001) (Figure 3.1). *D. magna* fed MPs without algae ingested more MPs over time ($F_{3,32}$=18.47, P = 0.001), with significantly more MPs ingested after 240 min of exposure compared to after only 30 min ($F_{3, 32}$ = 5.137, *p*=0.005, post hoc p>0.001)(Figure 3.1, Figure 3.2). When algae were provided as food, MPs ingestion did not significantly increase over time ($F_{3,32}$=0.068, *p*=0.976) (Figure 3.1, Figure 3.3).
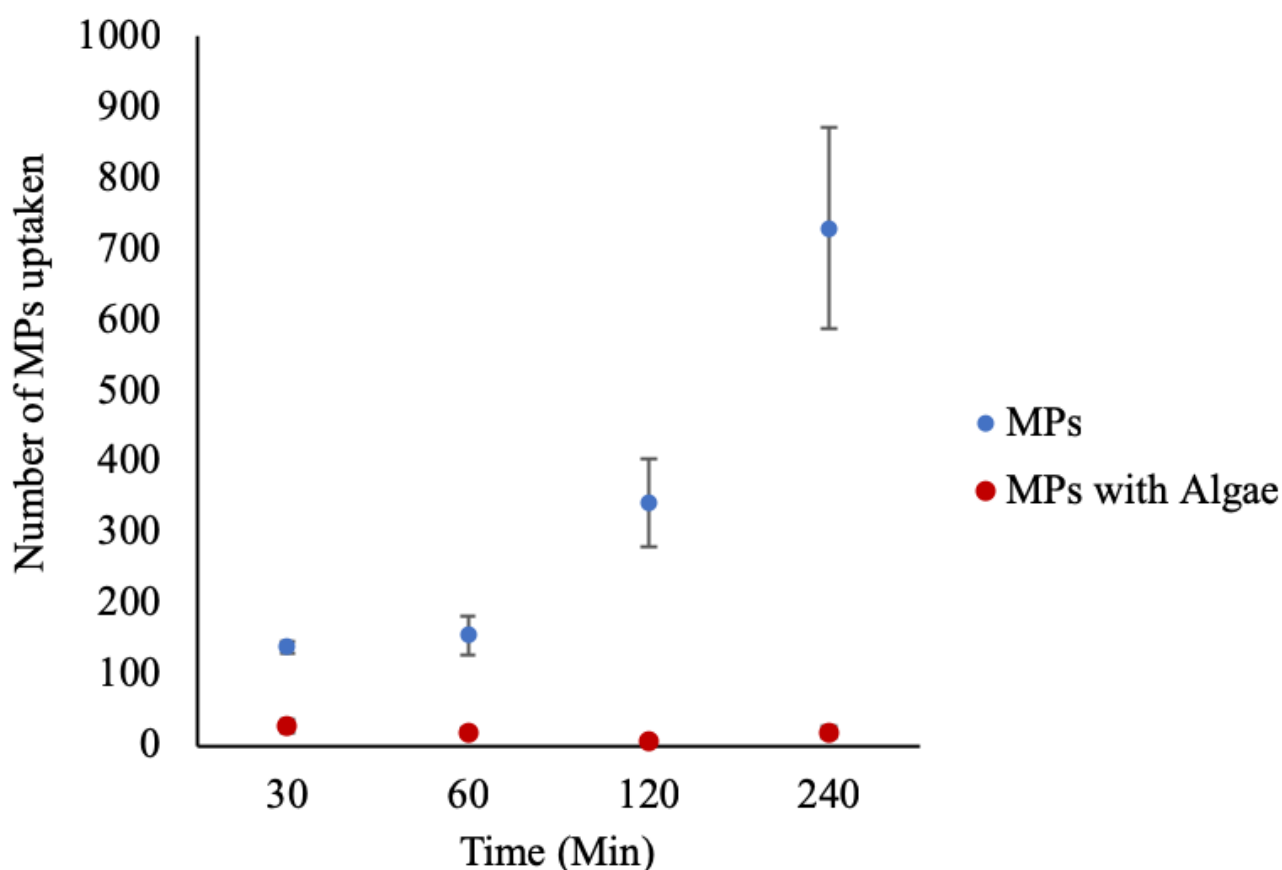
Figure 3-1 Uptake of 15 μm polystyrene MPs by *Daphnia magna* exposed to MPs only (200 MPs ml^{-1}) or MPs plus algae (200 MPs ml^{-1} with 7 x 10^4 cells 100μl^{-1}) over 240 min. Each point represents the mean ± the standard error (SE).

Figure 3-2 Uptake of polystyrene MPs size 15 µm by unfed *Daphnia magna* as a function of time; (A) uptake after 30 min, (B) after 60 min, (C) after 120 min and (D) after 240 min (E) control *D. magna* under a transmission light microscope.
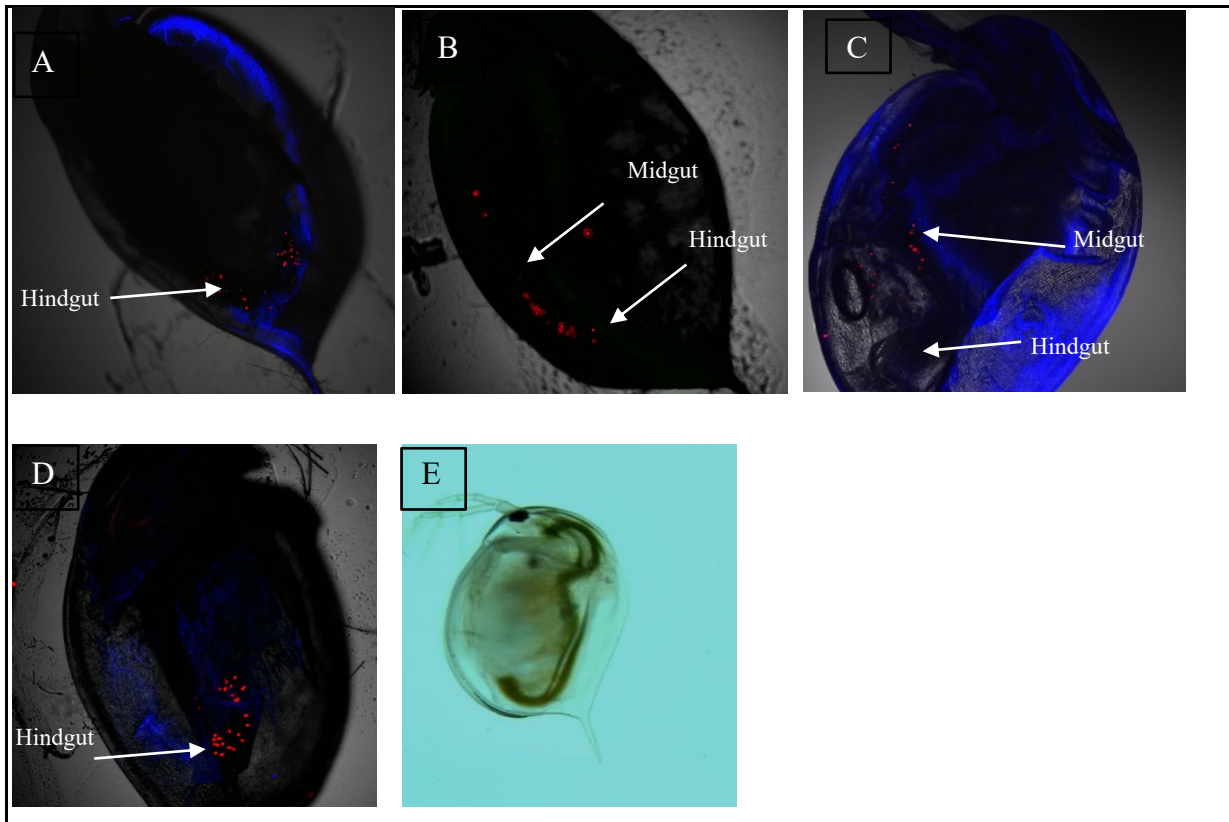
Figure 3-3 Uptake of polystyrene MPs size 15 µm by *Daphnia magna* in the presence of algae as a function of time; (A) uptake after 30 min, (B) after 60 min, (C) after 120 min and (D) after 240 min (E) control *D. magna* under transmission light microscope.

3.3.2 MPs Excretion

As before, *D. magna* fed algae contained significantly fewer MPs compared to those without algae ($F_{1,32}$=120.204, p< 0.001) (Figure 3.4). MP excretion in *D. magna* fed algae did not vary over time ($F_{3,32}$=0.185, p=0.906) (Figure. 3.5 A-D). However, the unfed *D. magna* excreted a significant number of MPs over time ($F_{3,32}$=7.069, p= 0.001)(Figure 3.6 A-D).
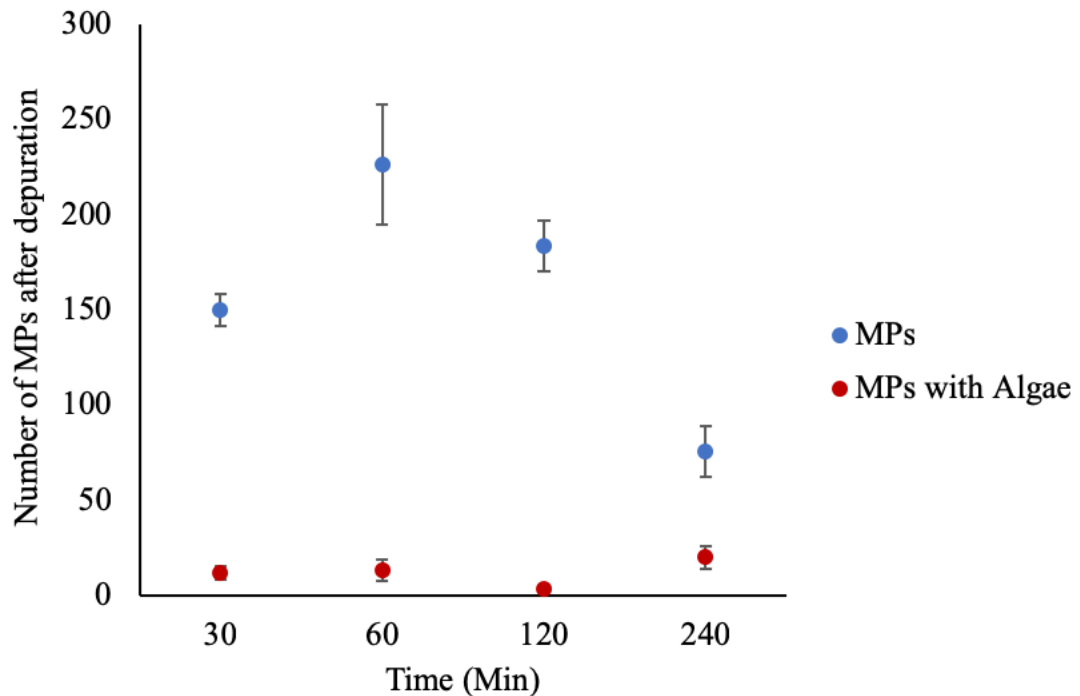
Figure 3-4 Mean number (± SE) 15 μm polystyrene MPs detected in *Daphnia magna* over time following 60 min exposure to MPs (200 MPs ml⁻¹) or MPs with algae ((200 MPs ml⁻¹ and 7 x 10⁴ cells 100μl⁻¹) with subsequent transfer on to clean media
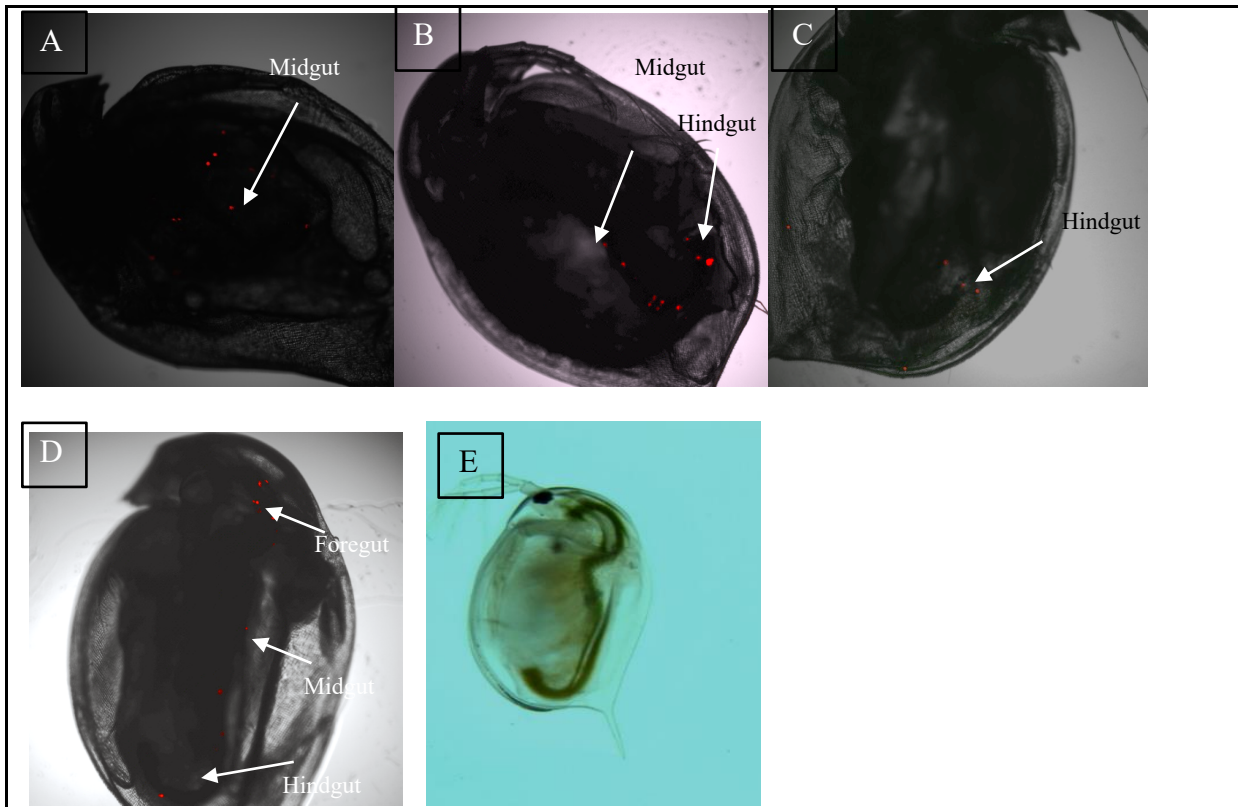
Figure 3-5 Depuration of polystyrene microplastics 15 µm in size by _D. magna_ in the presence of algae as a function of time; (A) after 30 min, (B) after 60 min, (C) after 120 min and (D) after 240 min (E) control _D. magna_ under transmission light
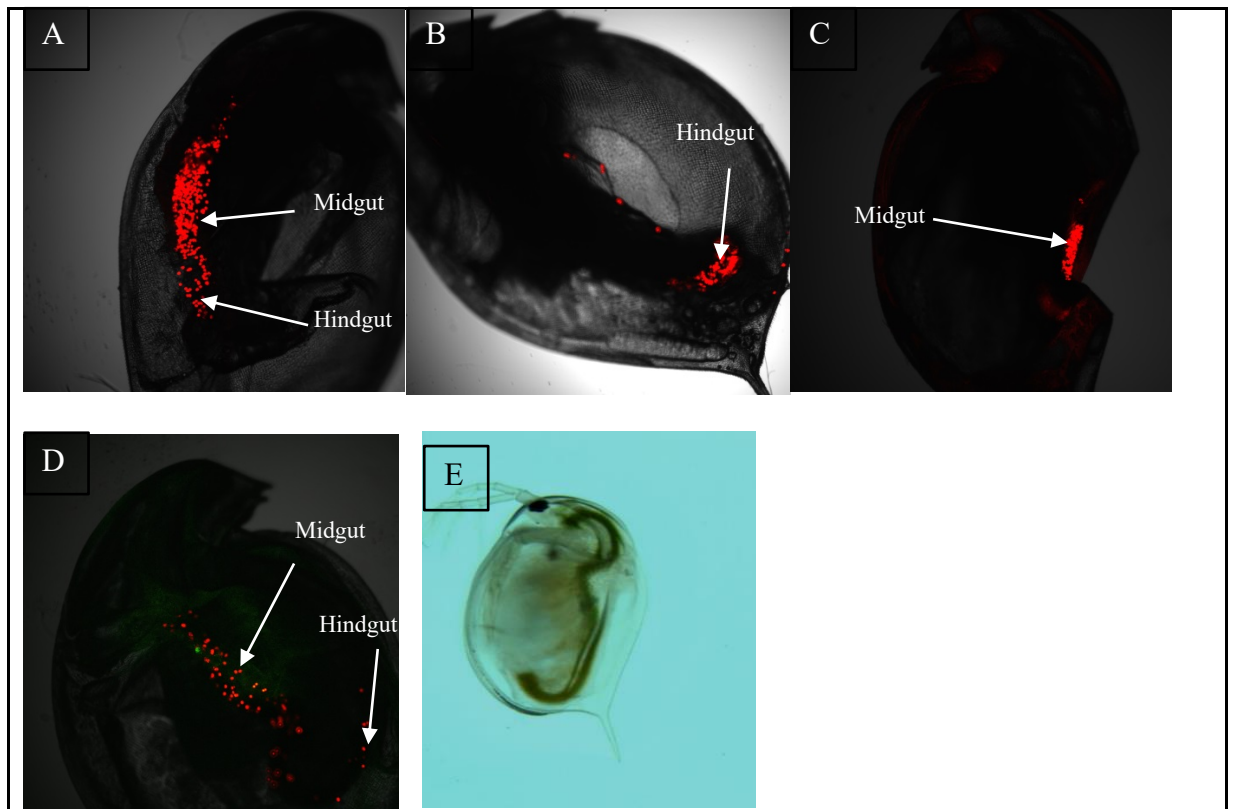
Figure 3-6 Depuration of polystyrene microplastics 15 µm in size by non-fed *D. magna* as a function of time; (A) after 30 min, (B) after 60 min, (C) after 120 min and (D) after 240 min (E) control *D. magna* under transmission light

3.3.3 Uptake as a Function of Concentration

Microplastics ingested by *D. magna* without algae increased significantly as MP concentration increased (F $_{3,32}$=14.12, *p*< 0.001, Figure 3.7). The same was true for treatments to MPs> algae (F $_{3,32}$=29.20, *p*< 0.001). When *D. magna* were exposed to equal amounts of MP and algae (MP=algae), there was no increase in ingestion with increasing concentration (F $_{3,32}$=0.415, *p*=0.743). The mean number of MPs ingested by *D. magna* exposed to algae>MPs significantly decreased as algal concentration increased (F $_{3,32}$=148.63, *p* < 0.001).
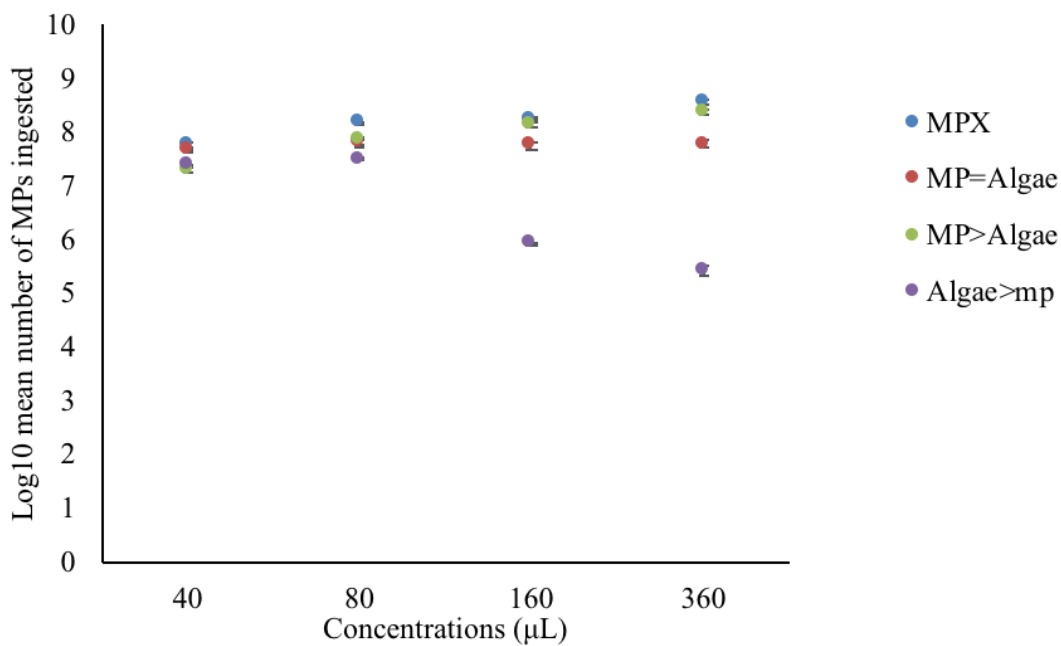
Figure 3-7 The mean (+SE) number of 15μm polystyrene MPs in *Daphnia magna* adults exposed to various concentrations of MP and algae (see Table 3.2 for details).

3.3.4 Adult Mortality Test

The presence of algae had a highly significant effect on adult mortality ($X^2(5, n=30)$ =17.4, $p=0.004$) (Figure 3.8). However, the mortality of adults exposed to low algae concentrations did not significantly differ from those exposed to the same algal concentrations and MPs ($U= 10$, $p=0.317$) or between groups exposed to high concentrations of algae and those with the same concentrations of algae and MPs ($U=10$, $p=0.513$).
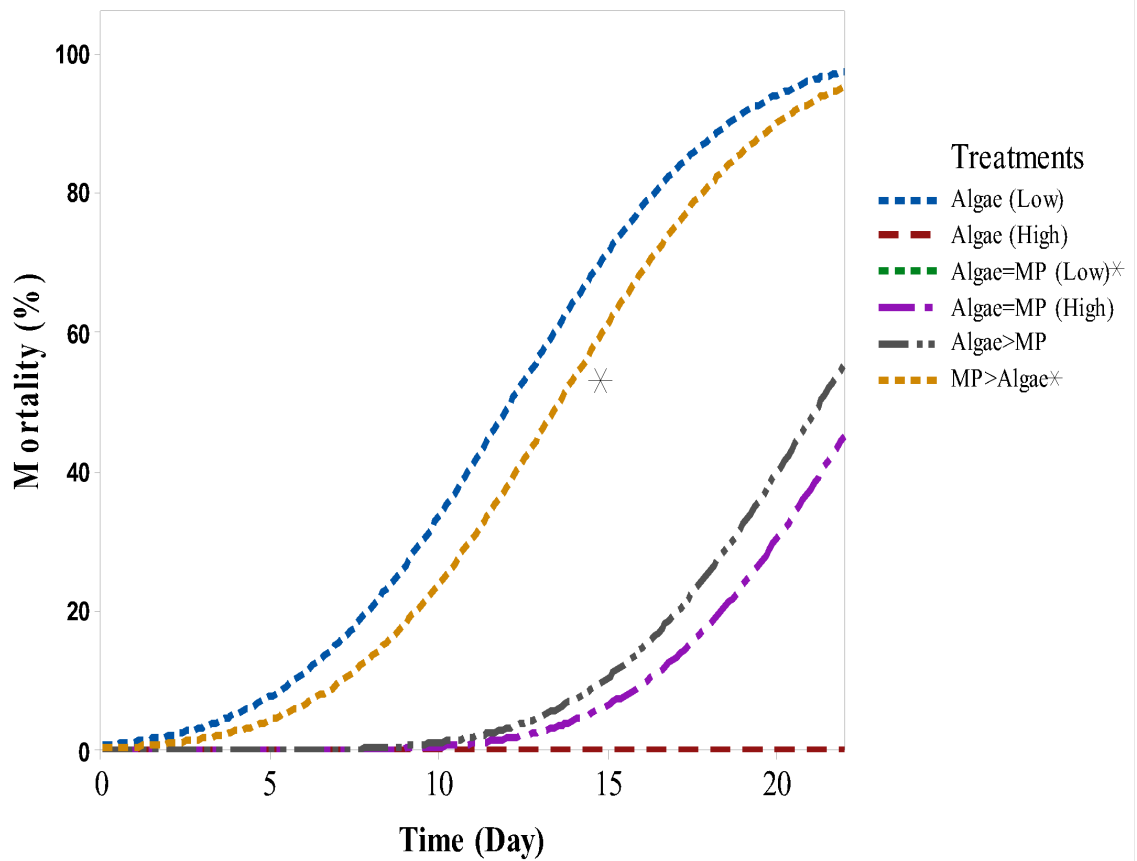
Figure 3-8 Mortality of *Daphnia magna* for 21 days expressed as a function of time after chronic exposure to MPs under high and low food conditions.

3.3.5 Reproduction Test- Adults

A 21-day reproduction test of adult *D. magna* revealed significant differences in the mean number of offspring between treatments ($X^2(5, n=30) = 216.1$, $p= 0.001$) (Figure 3.9). These differences were not related to MPs. Table 3 shows a pairwise comparison between low algal concentration (control) and other treatments.

Table 3-3 Pairwise comparison between algae (Low) and other treatments, using mean differences ± standard error (SE), degrees of freedom and the *p*-value for each treatment

Treatment	Mean differences	Standard Error (SE)	DF	*p*-value
Algae (High)	-35.20	3.655	1	< 0.001
Algae=MP (Low)	-5.20	2.713	1	0.221
Algae=MP (High)	-30.00	3.510	1	< 0.001
Algae>MP	-10.40	2.898	1	0.002
MP>Algae	3.00	2.392	1	0.419
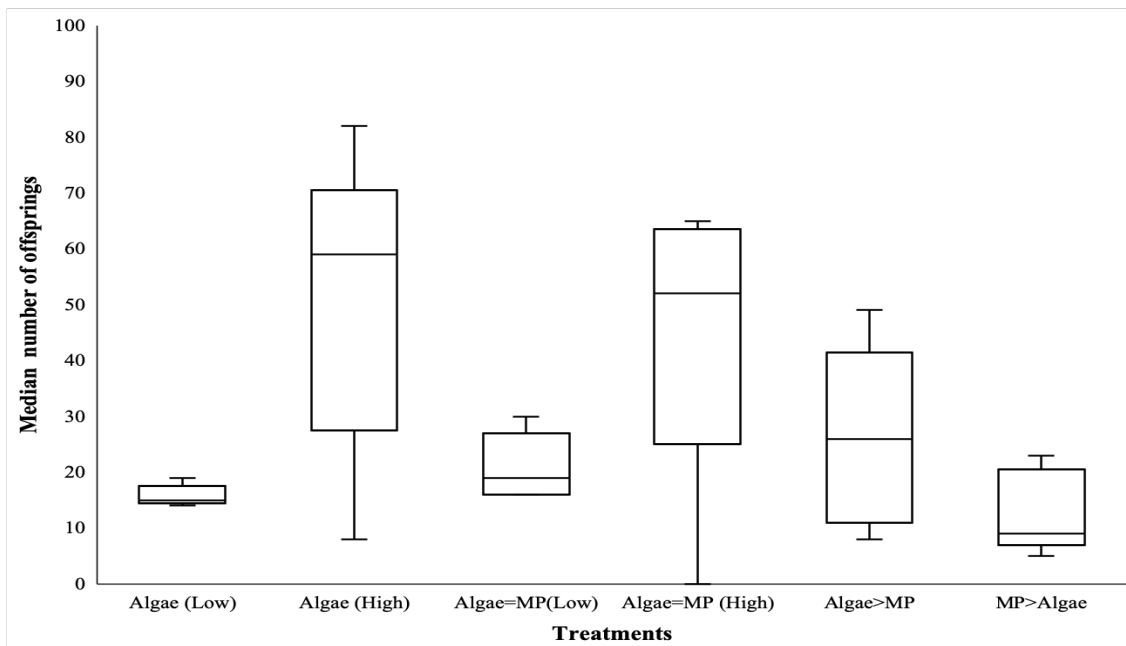
Figure 3-9 Effects of combinations of high and low MPs and algae concentrations on the mean number of offspring of *Daphnia magna* compare it to the control Algae(Low). Error bars indicate ± 95% confidence intervals.

3.3.6 Mortality Test- Neonates

Mortality tests were significantly different between treatments exposed to low and high algae concentrations, irrespective of the presence of MPs (X^2(5, n=30) =17.79, p = 0.003) (Figure 3.10).
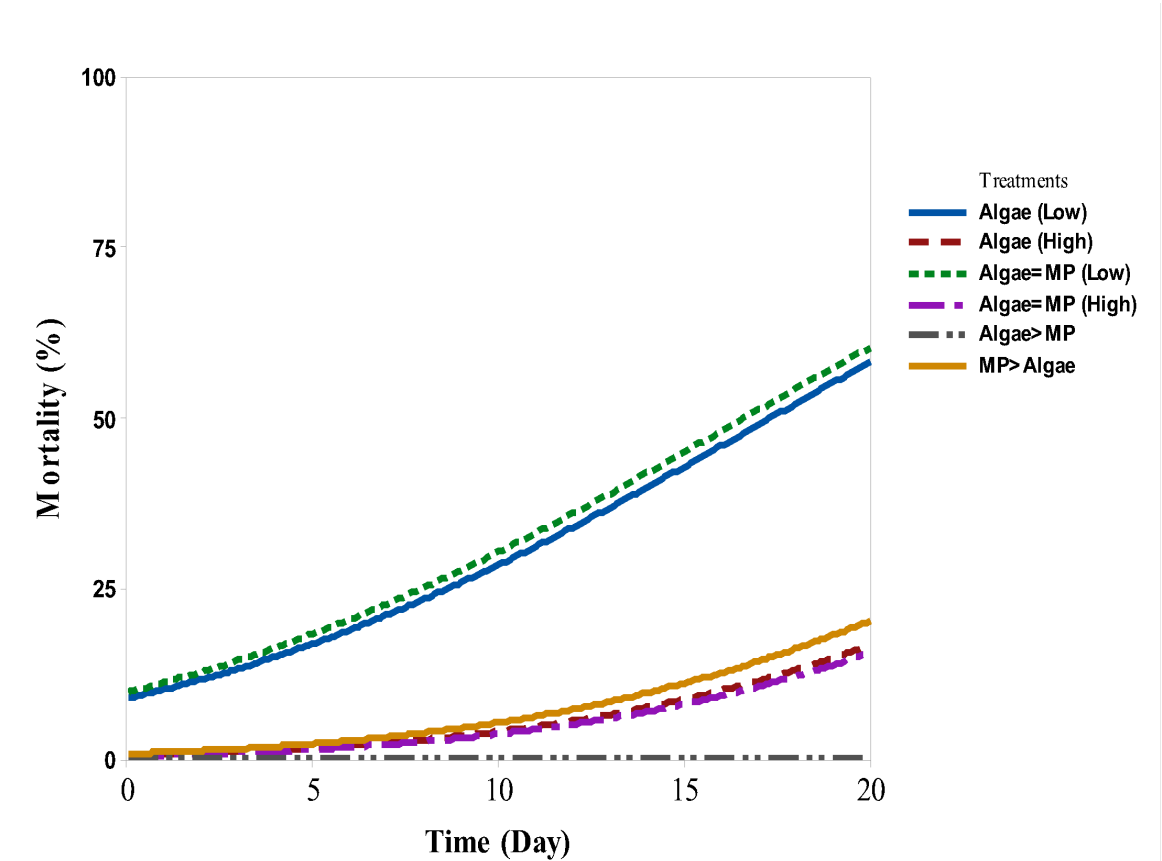
Figure 3-10 Mortality rate over 21 days for neonate *Daphnia magna* after exposure to different treatments of MPs and algae

3.3.7 Reproduction Test- Neonates

There were significant differences in reproduction between treatments ($X^2(5, n=30) = 1032$, $p < 0.001$, Figure 3.11). These differences were due to the amount of algae present: MPs had no impact. Table 3.4 shows pairwise comparisons between algae in low concentrations (control) and other treatments.

Table 3-4 Pairwise comparisons between control algae (low) and other treatments, mean differences ± standard error (SE) and statistical significance as a *p*-value.

Treatment	Mean differences	Standard Error (SE)	DF	*p*-value
Algae 800	-55.76	3.17	1	< 0.001
Algae = MP (Low)	0.99	1.77	1	1
Algae = MP (High)	-65.2	3.4	1	< 0.001
Algae > MP	-55.9	3.10	1	< 0.001
MP > Algae	2.499	1.57	1	0.452
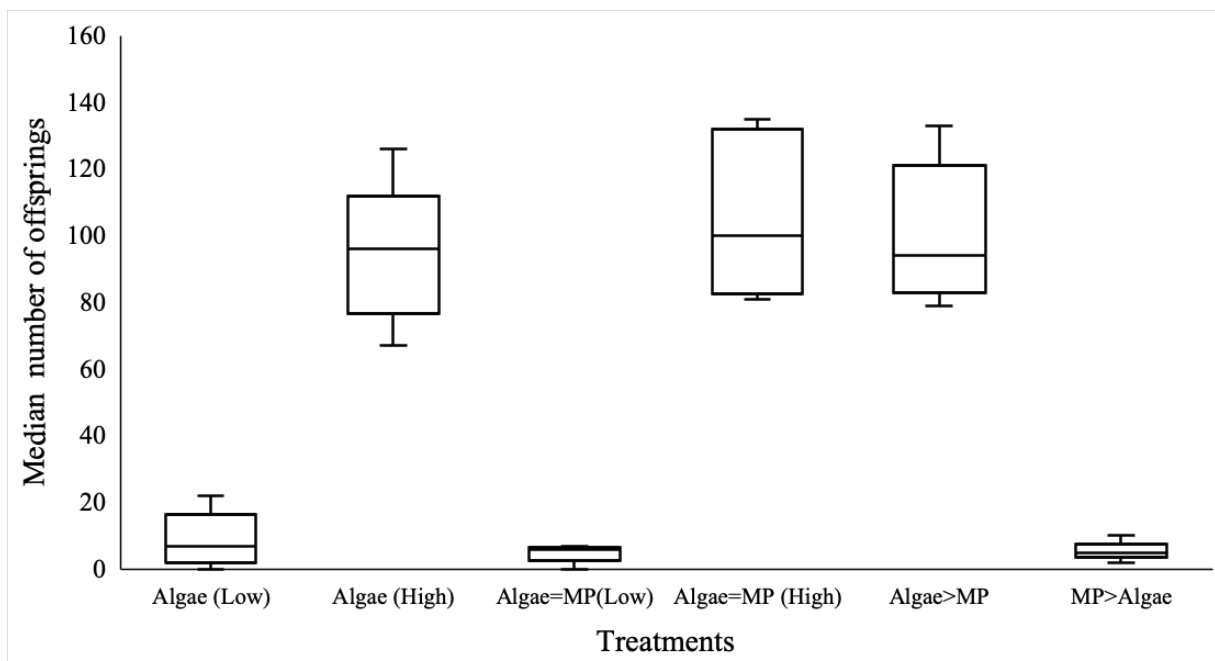
Figure 3-11 Effects of combinations of high and low MPs and algae concentrations on the mean number of offspring of *Daphnia magna* compare it to the control Algae(Low). Error bars indicate ± 95% confidence intervals.

3.3.8 Growth Rate

The results show highly significant differences in growth rate between the treatments (F $_{45,283}$=3.455, p< 0.001) (Figure 3.12). Pairwise comparisons between a control with algae (Low) and other treatments (Table 3.5)

Table 3-5 Mean differences $\pm$ standard error and p-value comparisons between control algae (Low) and other treatments

Treatment	Mean differences	Standard Error (SE)	DF	p-value
Algae 800	- 0.48	0.38	1	<0.001
Algae=MP (Low)	0.03	0.39	1	0.957
Algae=MP (High)	- 0.44	0.38	1	<0.001
Algae>MP	- 0.48	0.38	1	<0.001
MP>Algae	- 0.09	0.38	1	0.153
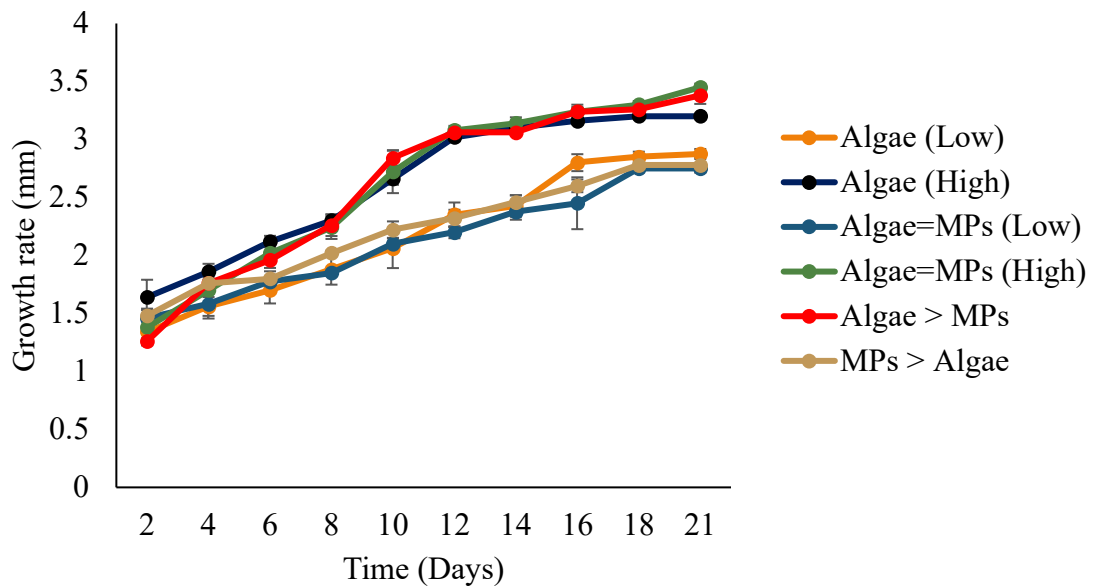
Figure 3-12 Effect of 21 days exposure to different combinations of MPs and algae (Algae (Low), Algae (High), Algae = MP(Low), Algae = MP(High), Algae >MP, MP> Algae) on the body length of *Daphnia magna*. Each point represents the mean of five replicates ± standard error (SE).

3.4 Discussion

The present study followed on from our recent work on the effect of microplastics of 2 μm in size on *D. magna* (Al-jaibachi and Callaghan 2018). In the earlier study we found that MPs ingestion was reduced in the presence of food in the media and that there was a reduction in MPs excretion from the gut when food was ingested with MPs. In addition, chronic toxicity tests (mortality and reproduction) on adult or neonate *D. magna* carried out after 21 days of exposure showed that mortality and reproduction were mainly linked to the availability of food, not the presence of MPs, although mortality increased when *D. magna* was exposed to high concentrations of MPs and low levels of food. Also, neonate growth rates were also linked to the availability of food rather than the ingestion of MPs, for which no effect of MPs was found.

The aim of the current study was to evaluate the effect of a larger polystyrene size (15 μm) on uptake, depuration and toxicity in adult and neonate *D. magna,* particularly on whether concentrations of food particles had an impact on MP ingestion. I also wanted to determine whether there is any aggregation of food particles with MPs in the gut which might reduce excretion over time. The uptake and ecotoxicity of MPs has been studied in both marine and freshwater environments using a variety of aquatic organisms (Chae and An 2017). However, studying various MP sizes, in order to examine their effect on invertebrate biology, is vitally important, since most invertebrates are exposed to different sizes, concentrations, materials and shapes of plastics in the aquatic environment.

Daphnia magna are filter feeders with specialized filtering structures to strain suspended particles. The minimum ingested particle size is mainly determined by the mesh size of the filtering apparatus which, in *D. magna*, ranges from 0.2 to 75 μm (Scherer et al. 2018). The maximum size of MP which could be ingested is limited by the morphology of the mouthparts and the opening width of the carapace and is around 100 μm in *D. magna* (Scherer et al. 2017). Since MPs have a similar size range to their natural food, including primary producers such as unicellular algae or bacteria as well as particulate organic matter (POM), there is no anatomical reason why they should not ingest MPs. Therefore the expectation was that *D. magna* would ingest the 15 μm MPs.

Epi-fluorescent microscopy clearly demonstrated that 15 μm MPs are ingested and pass through the digestive system of *D. magna*. As exposure time increased, so did the

number of MPs in the gut, a result which is in agreement with previous work on *D. magna* with larger (63–75 μm) MPs (Canniff and Hoang 2018; Rehse et al. 2016). The uptake of smaller MP sizes (1, 2 and 5 μm) has been more extensively studied and the results revealed concentration, time-dependent patterns (Rehse et al. 2016; Rosenkranz et al. 2009; Aljaibachi and Callaghan 2018). However, the uptake of small MP sizes is greater than larger sizes, as has been shown by Rosenkrans et al. 2009, in a study on *D. magna* exposed to two MPs sizes (20 nm -1000 nm). The study revealed accumulation in the gastrointestinal tract after 1 h of exposure and a greater uptake of MPs 20 nm in size than those of 1000 nm in size (Rosenkranz et al., 2009).

Our results indicate that, in the presence of food, the amount of MPs in the gut was significantly lower than in *D. magna* given only MPs. This effect increased over time, with the number of MPs actually dropping in the food treatment condition. This suggests the ability of *D. magna* to distinguish between MPs and food particles, so that *D. magna* prefer to take up food versus MPs over time, which is commensurate with previous outcomes for *D. magna* with smaller MPs of 2 μm in size that food was chosen in preference to plastics (Aljaibachi and Callaghan 2018).

The excretion of MPs was investigated by measuring the evacuation of MPs from the gut following a 60 min pulsed exposure to MPs with and without food particles, followed by the introduction of clean OECD water for 30, 60, 120 and 240 min. The drop in MP numbers in the gut, particularly in the MP-only treatment condition, can be explained simply by excretion. This has been shown before, with small MPs clearing more quickly than larger ones (Ogonowski et al. 2016; Rosenkranz et al. 2009) and was also investigated when *D. magna* were exposed to MPs 2 μm in size, with and without the presence of food particles (Aljaibachi and Callaghan 2018). The Gut evacuation time had been evaluated earlier and showed its are more related to the length of the daphnid and depends on food density (Kooijman and Evers, 1988). However, in both ingestion and excretion experiments, the presence of food resulted in an increased excretion of MPs up to 120 min, with a subsequent rise at 240 min. The epi-fluorescent pictures show that the MPs are sitting in the foregut, midgut and hindgut at 240 min, whereas they were mainly to be found in the hindgut at 120 min. This sudden rise in MP numbers at 240 min is responsible for what would have been a significant effect on MP

numbers with food. We conclude that *D. magna* had re-ingested the MPs excreted from the gut.

The number of MPs counted in *D. magna* increased with increasing concentration. When algae were present, there was no associated increase in MP content with increasing MP concentration. This result is similar to our previous results in Chapter 2 with 2 μm sizes, which showed that MPs were not ingested in line with increasing concentration when algae were present (Al-Jaibachi and Callaghan, 2018). It contradicts previous findings that *D. magna* did not selectively feed on algae when polystyrene microspheres were present with a suggestion that they had no taste preferences (de Mott, 1986). However the same author found that the cyclopoid *Tropocyclops* selectively fed on the alga *Chlamydomonas*, ignoring MPs of the same size (deMott 1986). Studies using the marine isopod *Idotea emarginata* showed that they were unable to distinguish between MPs and food particles (Hamer et al., 2014; Gutow and Saborowski 2014). Exposure to MPs at different concentrations in the presence of food showed a significant uptake of MPs and a rapid accumulation in the gut.

Chronic toxicity tests for adults and neonates exposed to two MP concentrations (100 and 800 MPs/ml) for 21 days have shown that the mortality rate was more associated with the availability of food than the toxicity of MPs, which is in agreement with previous studies, including mine in Chapter 2, that found MPs had no toxic effect on *D. magna* (Aljaibachi and Callaghan 2018; Canniff and Hoang 2018; Ogonowski et al. 2016).

Reproduction tests for adults showed a similar effect in that food availability had an impact, but MPs did not. Although in neonate toxicity test the presence of high MPs concentration and low food availability show low offspring level which is an indication of MPs effect. This study results agreed with those in Chapter 2 using 2 μm MPs, for which high MPs concentration had a negative effect on the number of offspring (Aljaibachi and Callaghan 2018; Ogonowaski, 2016). *Daphnia magna* growth rate also indicated that the effect was mainly on the availability of food, rather than the presence of MPs, which is in contrast to our previous study using a MP size of 2 μm (Aljaibachi and Callaghan 2018). This suggests that *D. magna* can distinguish between food and MPs, and that the presence of MPs does not interact with those nutrients essential for growth. There is evidence that MPs fed to some laboratory organisms have practically

no impact in the confines of the systems used. This could be because conditions are ideal and animals have very little stress linked to competition for food or predation. Certainly, MPs of 15 μm in size appear not to be toxic or have any impact on nutrition. Given that the MPs used were pristine and had not been in contact with any toxins which might adhere to their surface, we can say with confidence that, in themselves, these particles have no important effect on laboratory *Daphnia*. This is not to imply that MPs in the natural environment would act in such a neutral way, nor is it the case for all MPs. Deposit-feeding marine lugworms, *Arenicola marinara*, fed on plasticised polyvinylchloride under laboratory conditions at concentrations found in the environment suffered depleted energy reserves which were probably linked to a reduction in feeding and an inflammatory response (Wright et al., 2012). Likewise marine mussels *Mytilus edulis* fed factory clean high-density polyethylene up to 80 μm in size displayed toxic effects including a strong inflammatory response (von Moos et al 2012). Here there was evidence that the MPs were taken up into cells, something that we have not found in the much smaller *D. magna*. The contrast in findings between the marine studies above and those on *Daphnia* are perhaps related to the parameters used. In this study I used the standard ecotoxicology approach that is used for *D. magna* as a model organism. Here the growth, mortality and reproduction are the important factors to measure impact. However it could be that the plastic itself is less toxic than other MPs used in studies. Or the dispersion of MPs in the water body is altered as the spreading of particles are more related to the density and the sizes of MPs. In our study MPs might settled on the floor of the beaker rather than dispersed equally in the water body. Although 20 μm PS MPs seemed to impede feeding in the marine copepod *Calanus hegolandicus*, there was no effect on egg production or survival (Cole et al 2015). However other studies using PS MPs and marine organisms have demonstrated a negative impact. Adult oysters, *Crassostrea gigas*,exposed to PS MPs suffered from a reduction in absorption efficiency, fecundity and offspring growth (Sussarellu et al 2016). A recent review has highlighted some of the issues with studies on the impact of MPs on aquatic organisms. These include, a focus on marine over freshwater, the use of laboratory over field studies, the limited choice of MP type used and the overwhelming use of fish and crustaceans as model organism (de Sa et al 2018). The review concludes with a plea to vary the use of test organism, plastic type and to increase the amount of fieldwork over laboratory studies. Whilst I don't disagree with this account, I believe

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there is also a need for a more thorough and comparative body of evidence to compare the impact of one MP type on several organisms.

Despite the ever increasing number of studies of the effects of MPs on aquatic biota, there is a possibility that effect studies may be biased towards to a particular type of polymer without due consideration of reported occurrence in organisms and the environment, estimated release to the environment and bioavailability. The same possibility should also be considered for the model organisms used in laboratory assays.

Chapter 4 Up and away: ontogenic transference as a pathway for aerial dispersal of microplastics

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Statement of contribution:

The planning and experimental work was all undertaken by me with some assistance with writing and statistical analysis from my supervisor Professor Callaghan and Ross Cuthbert.

4.1 Abstract

Microplastics (MPs) are ubiquitous pollutants found in marine, freshwater and terrestrial ecosystems. With so many MPs in aquatic systems, it is inevitable that they will be ingested by aquatic organisms and be transferred up through the food chain. However, to date, no study has considered whether MPs can be transmitted by means of ontogenic transference, i.e. between life stages that use different habitats. Here, we determine whether fluorescent polystyrene beads could transfer between *Culex* mosquito life stages and, particularly, could move into the flying adult stage. We show for the first time that MPs can be transferred ontogenically from a feeding (larva) into a non-feeding (pupa) life stage and subsequently into the adult terrestrial life stage. However, transference is dependent on particle size, with smaller 2 μm MPs transferring readily into pupae and adult stages, while 15 μm MPs transferred at a significantly reduced rate. MPs appear to accumulate in the Malpighian tubule renal excretion system. The transfer of MPs to the adults represents a potential aerial pathway to contamination of new environments. Thus, any organism that feeds on terrestrial life phases of freshwater insects could be impacted by MPs found in aquatic ecosystems

4.2 Introduction

Microplastics (MPs) are ubiquitous pollutants found in marine, freshwater and terrestrial ecosystems (Mason et al., 2018; Sighicelli et al., 2018; Wagner and Lambert, 2018). There is little doubt that plastic and MP pollution is a major environmental concern globally. Despite this, there is relatively little research into the impact of MPs on freshwater ecosystems, with most research concentrating on marine systems and organisms (Wagner and Lambert, 2018). MPs have been defined as plastic particles smaller than 5 mm in size (Eriksen et al., 2014; Hannes K Imhof et al., 2013). However, this simple description covers a wide range of types, including, among others, polypropylene, polyethylene and polystyrene MPs entering the environment in different shapes and sizes, including fibres, pellets and cosmetic beads (Andrady and Neal, 2009; Rocha-Santos and Duarte, 2014). MPs are categorized based on their origin as primary or secondary types, depending on whether they were released into the environment as MPs (primary) or have degraded to that size in the environment (secondary) (Barnes et al., 2009; Moore, 2008). MPs pass through terrestrial environments in household

(Mason et al., 2016; Wagner and Lambert, 2018). Rivers can subsequently deliver MPs into the sea and lakes, where they can be found in high concentrations (Eriksen et al., 2013; Fischer et al., 2016; Su et al., 2016). MPs are ingested by aquatic organisms and can be transferred through the food chain in both freshwater and marine environments (Aljaibachi and Callaghan, 2018; Cole et al., 2013; Messinetti et al., 2018; Scherer et al., 2017). However, to date, no study has considered whether MPs can be transmitted by means of ontogenic transference, i.e. between life stages that use different habitats. Freshwater environments are inhabited by insects that spend their juvenile stages in water but their adult stages in the terrestrial environment. Such insects include mayflies, dragonflies, midges and mosquitoes, most of which are eaten by terrestrial vertebrates. This raises the potential for MPs to enter terrestrial ecosystems from freshwater habitats aerially via transference to adult invertebrate life stages. Here, we thus determine whether 2 and 15 μm fluorescent polystyrene beads could transfer between insect life stages and, particularly, could move into the flying adult stage. Fluorescent beads were selected to enable MPs to be easily detected in the non-feeding stages and also to allow an investigation of location within the body during metamorphosis. The *Culex pipiens* mosquito complex was selected as a model for this study given their worldwide distribution and broad habitat preference (Dow et al., 1994). Mosquitoes develop through four feeding larval instars and a non-feeding pupal stage, and finally emerge into a flying adult.

4.3 Material and methods

Two types of MPs were used: a 2 μm fluorescent yellow-green carboxylate-modified polystyrene (density 1.050 g cm^{-3}, excitation 470 nm; emission 505 nm, Sigma-Aldrich, UK) and a 15.45 +1.1 μm fluorescent dragon green polystyrene (density 1.06 g cm^{-3} (5 x 10^6 particles ml^{-1}), excitation 480 nm; emission 520 nm, Bangs Laboratories, Inc., USA). Four treatments were used: a control with no MPs, a treatment of 8 x 10^5 2 μm particles ml^{-1}, a treatment of 8 x 10^2 15 μm particles ml^{-1} and a 1 : 1 mixture of both treatments. Each replicate (five per treatment) contained 10 third instar *C. pipiens* larvae in a 50 ml glass beaker filled with 50 ml of tap water. The control and all treatments contained 100 mg of pelleted guinea pig food. Treatments were assigned randomly to a position on the laboratory bench to reduce experimental error.

One random individual was removed from each beaker when every mosquito had moulted into the fourth instar, and again when they pupated or emerged as adults. All samples were then placed in separate 1.5ml Eppendorf tubes and stored at -20 ºC prior to examination. MPs were extracted from mosquitoes by homogenization and filtration. The filter membrane was examined using an epi-fluorescent microscope (Zeiss Axioskop) under a 20 x lens to count the number of fluorescent MPs. Adults were further dissected under a binocular stereo microscope (0.7x – 4.5 x) to extract the gut and quantify the numbers of MPs under the epi-fluorescent microscope (Coleman et al., 2007).

All data were analysed using the statistical software R v. 3.4.2 (R Development Core Team, 2017). MP counts were analysed using generalized linear models assuming a quasi-Poisson distribution. Uptake of MPs was examined with respect to 'particle size', 'treatment' and 'life stage'. We performed model simplification via stepwise removal of non-significant effects. Tukey's tests were used post hoc for multiple comparisons.

4.4 Results

No MPs were found in control groups of any mosquito life stage. Densities of MPs were significantly different between life stages ($F_{2,56}$=160.42, $p < 0.001$), with MP numbers significantly falling as mosquitoes moved between successive ontogenic levels (all $p < 0.001$) (Figure 4.1; electronic supplementary material, table S1 and S2). MP transference to adults was confirmed by fluorescent microscopy where the beads were detected in the adult abdomen, specifically inside the Malpighian tubules (figure 4.2).

Significantly more 2 µm particles were found in mosquito life stages than 15 µm particles overall ($F_{1,58}$=303.98, $p < 0.001$). MPs uptake was also significantly greater overall in mixed exposure treatments ($F_{1,55}$ = 6.00, p = 0.02). Although 2 µm particles were transferred to adults in all instances, we found no transference of 15 µm particles following single treatment exposures. However, in the mixed MPs treatment, transference to adults of both 2 and 15 µm particles was evidenced (figure 4.1).
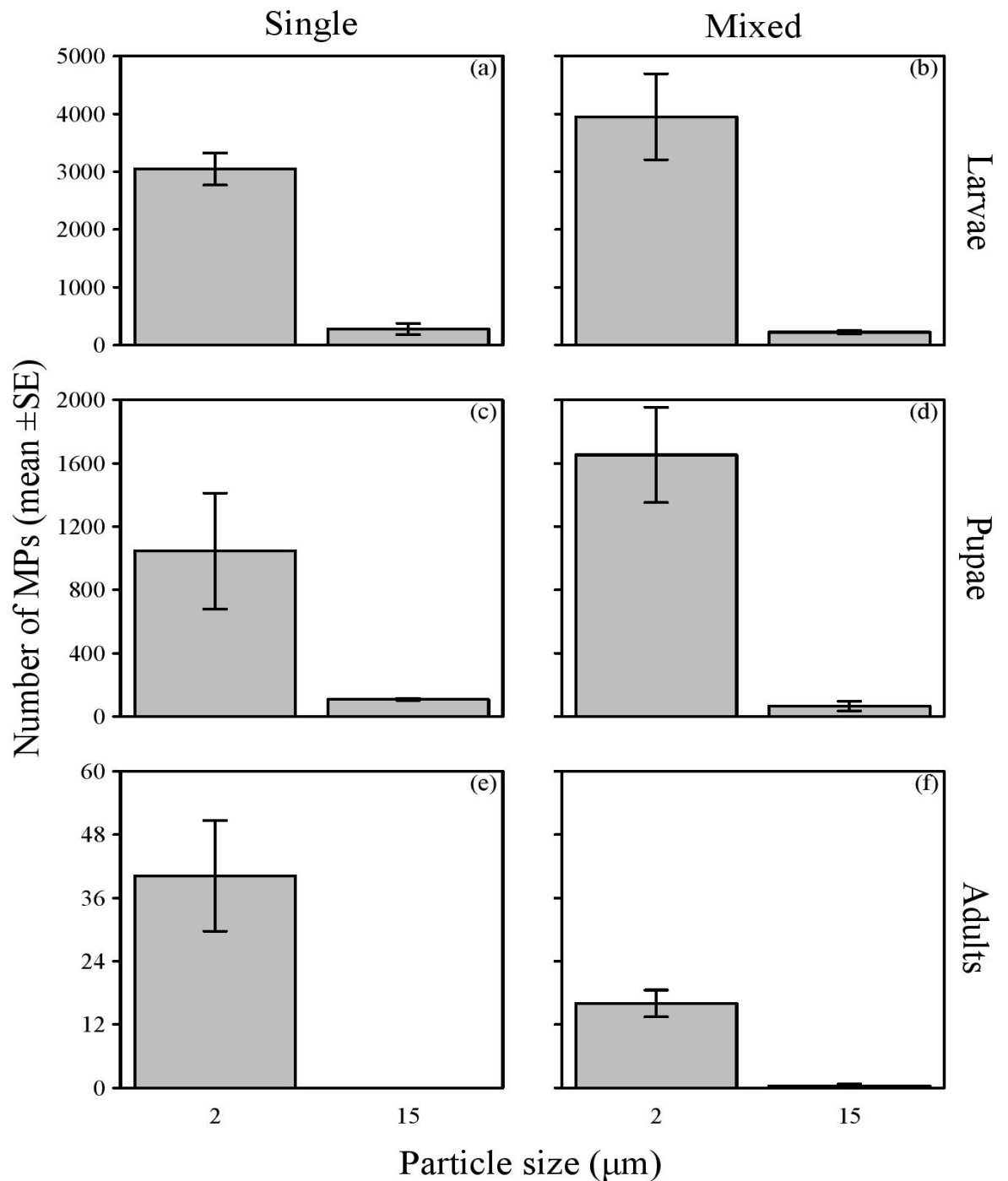
Figure 4-1 Uptake counts of MPs across larval (a,b), pupal (c,d) and adult (e,f) *Culex* **mosquito stages following single (a,c,e) and mixed (b,d,f) exposures to 2 and 15 μm beads. Means are ± s.e. (n = 5 per experimental group).**

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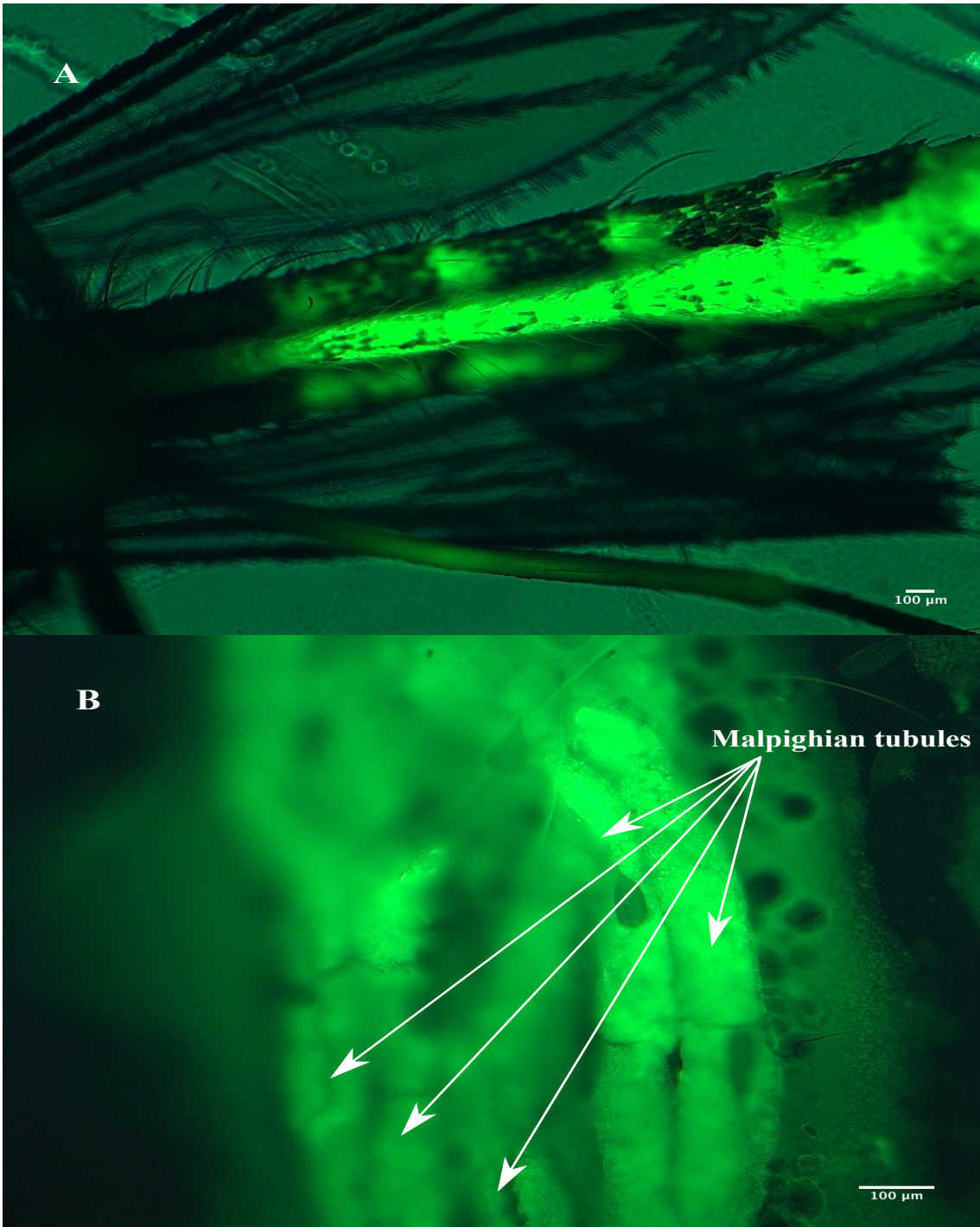
Figure 4-2 Epi-fluorescent microscope images showing fluorescent MP particles within (a) the abdomen of an adult mosquito before dissection and (b) the abdominal Malpighian tubules following dissection.

4.5 Discussion

Here, we show for the first time that MPs can be transferred ontogenically from a feeding (larval) into a non-feeding (pupal) life stage and subsequently into the flying (adult) life stage. Transference through to adults was found in both MP sizes, although the larger 15 μm MPs were not ingested as readily as the 2 μm MPs. Dissection of mosquito adults showed that 2 μm MPs accumulated in the renal excretion system of Malpighian tubules which, unlike the gut, pass from larvae to adult stages without visible reorganization (Clements, 1992). This has been demonstrated previously to provide a physical transport system between stages during metamorphosis for Pseudomonas bacteria and seems to be important for ontogenic transmission from larvae to adults (Chavshin et al., 2015).

Few 15 μm MPs were transferred into adults, suggesting that MP size is an important factor in ontogenic transfer which could be related to the transfer and accumulation of MPs in the Malpighian tubes. Although the translocation mechanism of MPs to the Malpighian tubules is unclear in mosquitoes, analysis of fish, fiddler crab and marine mussels has demonstrated that MPs can be translocated from gastro-intestinal tracts into other tissues in a wide range of phyla (Avio et al., 2015; Brennecke et al., 2015; von Moos et al., 2012). Malpighian tubules have an entry point to the gut between the mid- and hindgut of mosquitoes, but the flow of fluid is from the Malpighian tubules to the hindgut (Piermarini, 2016). Diptera are known to produce structures called concretions in the Malpighian tubules which have been shown to sequester heavy metals (Leonard et al., 2009). However, it is unlikely that this pathway would operate with a solid MP.

Our results have important implications because any aquatic life stage that is able to consume MPs and transfer them to their terrestrial life stage is a potential vector of MPs onto novel aerial and terrestrial habitats. Ingestion of MP-contaminated organisms by terrestrial organisms is not new (Piermarini, 2016). Indeed, the widespread distribution of MPs in marine environments has meant that animals such as fish and shellfish sold for human consumption are contaminated with a range of plastics with a consequent transference of MPs between trophic levels (von Moos et al., 2012). Unlike MP fibres, which are common in the air and atmosphere, there has been no evidence for MP beads being transported into the air (Dris et al., 2016). We have demonstrated here that species with aquatic and terrestrial life stages can harbour MPs through their life history. Adults

are predated on emergence by many animals including dipteran flies Empididae and Dolichopodididae, while resting predominantly by spiders and in flight they are the prey of dragonflies, damselflies, birds (such as swallows and swifts) and bats (Medlock and Snow, 2008). Where many insects are emerging from a highly contaminated site, the possibility of contamination of these predators could be high. While mosquitoes were used here as a model organism, any freshwater insect that can ingest MPs will likely equally transmit plastics into a terrestrial adult stage. This has implications for organisms that feed on adult stages with aerial and terrestrial animals accordingly open to MP exposure and transference appearing to occur at a higher rate for smaller MPs.

Chapter 5 EXAMINING EFFECTS OF ONTOGENIC MICROPLASTIC TRANSFERENCE ON *CULEX* MOSQUITO MORTALITY AND ADULT WEIGHT

This Chapter has been published in its entirety:

Statement of contribution:

The planning and experimental work was all undertaken by me with some assistance with writing and statistical analysis from my supervisor Professor Callaghan and Ross Cuthbert.

5.1 Abstract

Microplastics (MPs) continue to proliferate and pollute aquatic and terrestrial environments globally. The impacts of MP pollution on ecosystems and their functioning remain poorly quantified, with most research hitherto focusing on marine ecosystems. There is a paucity of information on the impacts of MPs in freshwater ecosystems, despite the broad range of pathways through which MPs can proliferate and the extensive range of species which actively ingest MPs in these systems. Of particular interest are organisms that bridge aquatic and terrestrial habitats. The present study thus examines the uptake, ontogenic transference and effect of different concentrations (0, 50, 100 and 200 MPs mL^{-1}) and sizes (2 and 15 μm) of polystyrene MPs between aquatic and terrestrial life stages of *Culex pipiens* complex mosquitoes. Both 2 and 15 μm MPs transferred from the aquatic larval to terrestrial adult stage of *Culex* mosquitoes, and uptake correlated tightly with initial exposure concentration. However, neither concentration nor size of MPs significantly influenced mortality rates between the aquatic larval and terrestrial adult stage. There was also no impact of MPs on the weight of emerging mosquito adults. We thus demonstrate that MPs can be transferred ontogenically through organisms with complex life histories, presenting a potential pathway for dispersal of MPs into terrestrial environments. We also show that MPs exposure does not affect mortality rates between life stages of freshwater *Culex* populations. This suggests that MPs do not impact nutritional uptakes, with unhampered development to adulthood facilitating subsequent dispersal of MPs aerially and between freshwater and terrestrial habitats.

5.2 Introduction

Microplastics (MPs) continue to proliferate in marine, freshwater and terrestrial ecosystems, with biotic impacts frequently unknown (Sighicelli et al., 2018). Microplastic pollution has been detected from the poles to the deep ocean, and more recently in bottled drinking water (Mason et al., 2018; Wagner and Lambert, 2018). Whilst there is little doubt over the enormity of plastic and MP pollution in scale, the vast majority of research has concentrated on marine environments whilst neglecting other ecosystems (Redondo-Hasselerharm et al., 2018; Wagner and Lambert, 2018) To date there is a paucity of information on the impacts of MPs in freshwater ecosystems,

despite the broad range of pathways through which MPs can proliferate (Mason et al., 2016), and the extensive range of species which actively ingest MPs in these systems (Canniff and Hoang, 2018; Imhof et al., 2016; Nel et al., 2018; Qu et al., 2018).

Microplastics have been defined as plastic particles of 5 mm or less in size (Eriksen et al., 2014; Hannes K Imhof et al., 2013). This is a broad definition as MPs manifest in a variety of forms, such as fibres, pellets and cosmetic beads, which all routinely enter the environment (Watts et al., 2014). Microplastics differ in their chemical composition, and can consist of various polymers such as polypropylene, polyethylene and polystyrene (Andrady and Neal, 2009; Rocha-Santos and Duarte, 2014). Further- more, MPs can either be primary or secondary in origin, with the former released directly into the environment as MPs, and the latter having de- graded over time to reach the MP size class (Barnes et al., 2009; Moore, 2008). Despite such inherent variation, there has been little work to compare differential impacts of the varied types of MPs on recipient organisms.

Movement of MPs through terrestrial and aquatic environments has been investigated, and several pathways have been suggested. For ex- ample, movement through the air due to (Dris et al., 2016) or directly through water courses from wastewater treatment plants into rivers and eventually the marine environment (Mason et al., 2016; Wagner and Lambert, 2018). Rivers can also deliver MPs into lakes, where they can be found in high concentrations and presumably fall into the sediment (Vaughan et al., 2017). In North America the highest MP concentrations were found in Lake Ontario-Erie, with an average range of 90,000–6,700,000 MPs km^{-2} (Fischer et al., 2016). In Europe, Lake Geneva contained the highest MP concentration with a mean of 220,000 ($\pm$SD: $\pm$160,000 MPs km^{-2}) (Eriksen et al., 2013), and in Asia, Lake Taihu contained a range of 10,000–6,800,000 MPs km^{-2} (Su et al., 2016).

Given the densities of MPs in freshwater ecosystems, it is likely that they will be ingested by aquatic organisms, and, in turn, probable that they will be transferred up through the food chain (Cole et al., 2013; Redondo-Hasselerharm et al., 2018; Scherer et al., 2017; Sussarellu et al., 2016). Laboratory experiments have demonstrated the uptake of MPs, and it is well established that they are ingested by many invertebrates in both freshwater and marine environments (Aljaibachi and Callaghan, 2018; Hannes K. Imhof et al., 2013). However, considerations of whether MPs can be transmitted by

means of ontogenic transference, i.e. between life stages within an individual, have remained scarce. In- sects comprise an important component of freshwater environments and are often highly abundant (Macadam and Stockan, 2015). Many in- sects have complex life histories, consisting of successive aquatic and terrestrial stages. Examples of such insects are stoneflies, damselflies, midges and mosquitoes, most of which are eaten by birds in their terrestrial stage. We have recently shown that MPs can be transferred into mosquito adults following ingestion as larvae (Al-Jaibachi et al., 2018b). Thus, ontogenic transference of MPs presents a further pathway for MPs to enter new ecosystems from aquatic environments, with the potential to enter organisms that do not feed on the aquatic stages of fresh- water or marine organisms.

The present study was undertaken to determine whether MPs which transfer between insect life stages of species with complex life histories could affect survival and adult size, which is linked to fecundity (Takken et al., 2013). Mosquitoes (Diptera: Culicidae) are ideal for this study since they go through four feeding larval instars, a non-feeding pupal stage and finally emerge into a flying adult that feeds on nectar and/or vertebrate blood depending on the sex and species. Here, we investigate the ingestion of 2 ± 0.2 and 15 ± 1.1 μm fluorescent polystyrene beads, and whether consumption is concentration-dependent. Fluorescent beads were selected to enable MPs to be easily detected in the non- feeding stages and also to allow an investigation of location within the body during metamorphosis. *Culex pipiens* complex mosquitoes were selected for this study because they exhibit a global distribution and colonise a broad range of aquatic habitats, such as stream pools, lake edges, marshes and shallow permanent ponds, alongside both natural (phytotelmata) and artificial containers (Townroe and Callaghan, 2015). The group is also known to be an important food source for birds and other terrestrial organisms in the adult stage (Dow et al., 1994). We hypothesise that: (1) MPs will move ontogenically from larval to pupal stages, and subsequently into adult mosquito stages, and that transference is both MP concentration- and size-dependent; (2) up-take of MPs will reduce the survivability of larval mosquitoes to the adult stage; (3) exposure to MPs will affect the nutrition and thus development of larval mosquitoes, resulting in smaller-sized adults upon emergence.

5.3 Materials and methods

5.3.1 Preparation of microplastics (MPs)

Two types of MPs were used: a 2 ± 0.2 μm fluorescent yellow-green carboxylate-modified polystyrene (density 1.050 g cm^{-3}, excitation 470 nm, emission 505 nm; Sigma-Aldrich, UK) and a 15.45 ± 1.1 μm fluorescent dragon green polystyrene (density 1.06 g cm^{-3}, excitation 480 nm, emission 520 nm; Bangs Laboratories Inc., USA).

The 2 μm MPs were stored as a stock suspension (2.5 mg mL^{-1}) in distilled water and mixed using a vortex (Whirlimixer Cyclone, UK) prior to dilutions. The 15 μm MPs were also stored as stock suspension (1% solid) polystyrene microspheres. Particles were washed prior to use by adding 1 mL from the stock solution into a 1.5 mL Eppendorf tube and then centrifuging at 9000 rpm for 10 min. The supernatant was discarded and 1 mL of distilled water was added. The solution was then resuspended by using the vortex and centrifuged again at the same speed and duration. This process was repeated two more times.

5.3.2 Mosquito colonies

Larvae of the *C. pipiens* mosquito complex were obtained from colonies reared at the University of Reading, UK following the methodology of (Cuthbert et al., 2018a). This colony originated from individuals collected in Cyprus in 2005 by A. Callaghan and have been reared in laboratory conditions since then. Adult *C. pipiens* were fed overnight twice a week with defibrinated horse blood (TCS Biosciences, UK) using artificial membrane feeder (Hemotek, UK). Cotton pads soaked in 10% sucrose solution were provided for additional sustenance.

5.3.3 Experimental protocols

We exposed *C. pipiens* larvae to one of two MP sizes (2 and 15 μm) under one of four concentrations (0, 50, 100 and 200 MP mL^{-1}) in a crossed design in the same laboratory where the colonies are maintained (25 ± 2 °C, RH $70 \pm 5\%$, 16:8 light:dark) for 12 days. In each of five replicates per treatment group, ten third instar *C. pipiens* larvae were placed in a glass beaker (60×80 mm) filled with 120 mL of tap water and

100 mg of ground pelleted guinea pig food for sustenance. Treatments were assigned randomly to a position on the laboratory bench to reduce experimental error. Microplastic concentrations were quantified at the start and the end of the experiment by taking 5×1 mL from different points of each beaker.

5.3.4 Uptake and ontogenic transference

One individual was randomly removed from each beaker once all mosquitoes in the beaker had moulted into the 4th instar, and again when they pupated or emerged as adults. All samples were then washed twice with distilled water to remove MPs from the surface of the mosquito and placed in separate 1.5 mL Eppendorf tubes, before being stored at $-20\ ^{\circ}$C prior to examination.

Microplastics were extracted from mosquitoes by homogenization and filtration. Mosquitoes were homogenized using a glass pestle in Eppendorf tubes containing 500 µL distilled water. Individuals treated with 2 µm MPs were filtered through a nucleopore track-etched mem- brane (Whatman, UK) of b1 µm and 25 mm dia.. Those exposed to 15 µm MPs were filtered through a nucleopore track-etched membrane (Whatman, UK) of b10 µm and 25 mm dia. using a glass vacuum filter holder connected to a manual air pump. The MPs captured by both fil- ters were quantified under a 20× epi-fluorescent microscope (Zeiss Axioskop, USA). Adults were further dissected under a binocular stereo microscope (0.7×–4.5×) to extract the gut and quantify the numbers of MPs under the epi-fluorescent microscope (Coleman et al., 2007).

5.3.5 Mortality rates

Mortality of successive stages was monitored and recorded daily over the course of the 12 day experimental period. We thus deduced overall proportional survival to the adult mosquito stage in *C. pipiens*.

5.3.6 Emerging adult weights

Emerging adult mosquitoes from each treatment were weighed using a microbalance (Thermo Cahn, USA), then examined under the epi-fluorescent microscope to ensure that no MPs were attached to the body.

5.3.7 Statistical methods

All data were analysed using the statistical software R v3.4.2 (R Development Core Team, 2017). Quantities of MPs in larval, pupal and adult *Culex* mosquito stages were analysed separately using generalised linear models (GLMs) assuming a quasi-Poisson error distribution as counts were found to be overdispersed compared to degrees of freedom. Microplastics were absent from all control groups, and so we excluded this treatment from statistical analyses here. The effects of MPs on proportioned survival of *Culex* from the larval to the adult stage were then analysed separately using GLMs assuming a quasi-binomial error distribution. Then, the effects of MP treatments on adult weights were analysed using ANOVA following log10 transformation to meet normality and homoscedasticity assumptions (Shapiro-Wilk test, p N 0.05; Bartlett's test, p N 0.05). In all models, we initially incorporated 'concentration' and 'MP size' of MPs as explanatory variables factorially. We then performed backward stepwise deletion of insignificant terms and interactions to facilitate the most parsimonious model fits (Crawley, 2007). We performed post hoc Tukey's comparisons where terms significantly affected a response variable at the 95% confidence level (Lenth, 2016).

5.4 Results

No MPs were found in control group replicates. Microplastics of both sizes were found in larval, pupal and adult life stages of mosquitoes, however abundances were strongly related to initial exposure concentration and MP size at each ontogenic stage. Abundance of MPs in larval mosquitoes was significantly influenced by initial exposure concentrations ($F_{(2, 27)} = 84.55$, $p < 0.001$), with quantities of MPs significantly higher across all increasing concentration increments (all $p < 0.001$; Figure 5.1a), and abundances up to a maximum of 255.8 (SD: ±8.7) MP larva^{-1}. Significantly greater quantities of 2 μm MPs were found in larval *C. pipiens* than 15 μm MPs ($F_{(1, 26)} = 28.53$, $p < 0.001$). Furthermore, there was a significant interaction effect between 'concentration' and 'MP size' ($F_{(2, 24)} = 6.44$, $p = 0.006$), reflecting emergent effects between the variables (Figure 5.1a). Whilst there were no significant differences in MP quantities in *Culex* larvae at 50 MPs mL^{-1} or 100 MPs mL^{-1} between the two MP size classes (both $p > 0.05$), uptake of the smaller 2 μm MPs was significantly ($p < 0.001$) higher at concentrations of 50 and 200 MPs at the same concentrations.

For pupae, exposure concentration also had a significant effect on MP abundance in *C. pipiens* ($F_{(2, 27)}$ = 4.56, p = 0.02), with abundances up to a maximum of 54.8 (SD: ±15.2) MP pupa^{-1}. Whilst there were similarities in uptake between 50 MPs mL^{-1} vs 100 MPs mL^{-1} (p = 0.31), and 100 MPs mL^{-1} vs 200 MPs mL^{-1} (p = 0.28), MP abundances in pupae were significantly greater at 200 MPs mL^{-1} compared to 50 MPs mL^{-1} (p = 0.01). Moreover, significantly lower quantities of 15 μm MPs were found in pupae as compared to 2 μm MPs ($F_{(1, 26)}$ = 133.25, p < 0.001; Figure 5.1b), and this effect was consistent across exposure concentrations as there was no significant 'concentration × MPs size' interaction effect ($F_{(2, 24)}$ = 0.87, p = 0.43).

Microplastics were detected in the adult stage of *C. pipiens* mosquitoes, and MP abundance was significantly greater under increasing initial MP exposure concentrations overall ($F_{(2, 27)}$ = 14.07, p < 0.001) and for 2 μm MP compared to 15 μm MP ($F_{(1, 26)}$ = 4.71, p = 0.04). However, we found no incidence of MP transfer at 50 MPs mL^{-1} to adults across treatments, whilst MPs were transferred to adults at 2 μm MP concentrations exceeding 100 MPs mL^{-1}, and 15 μm MP concentrations of 200 MPs mL^{-1} (Figure 5.1c). There were similarities across the 'concentration' and 'MPs size' interaction here ($F_{(2, 24)}$ = 2.09, p = 0.15).
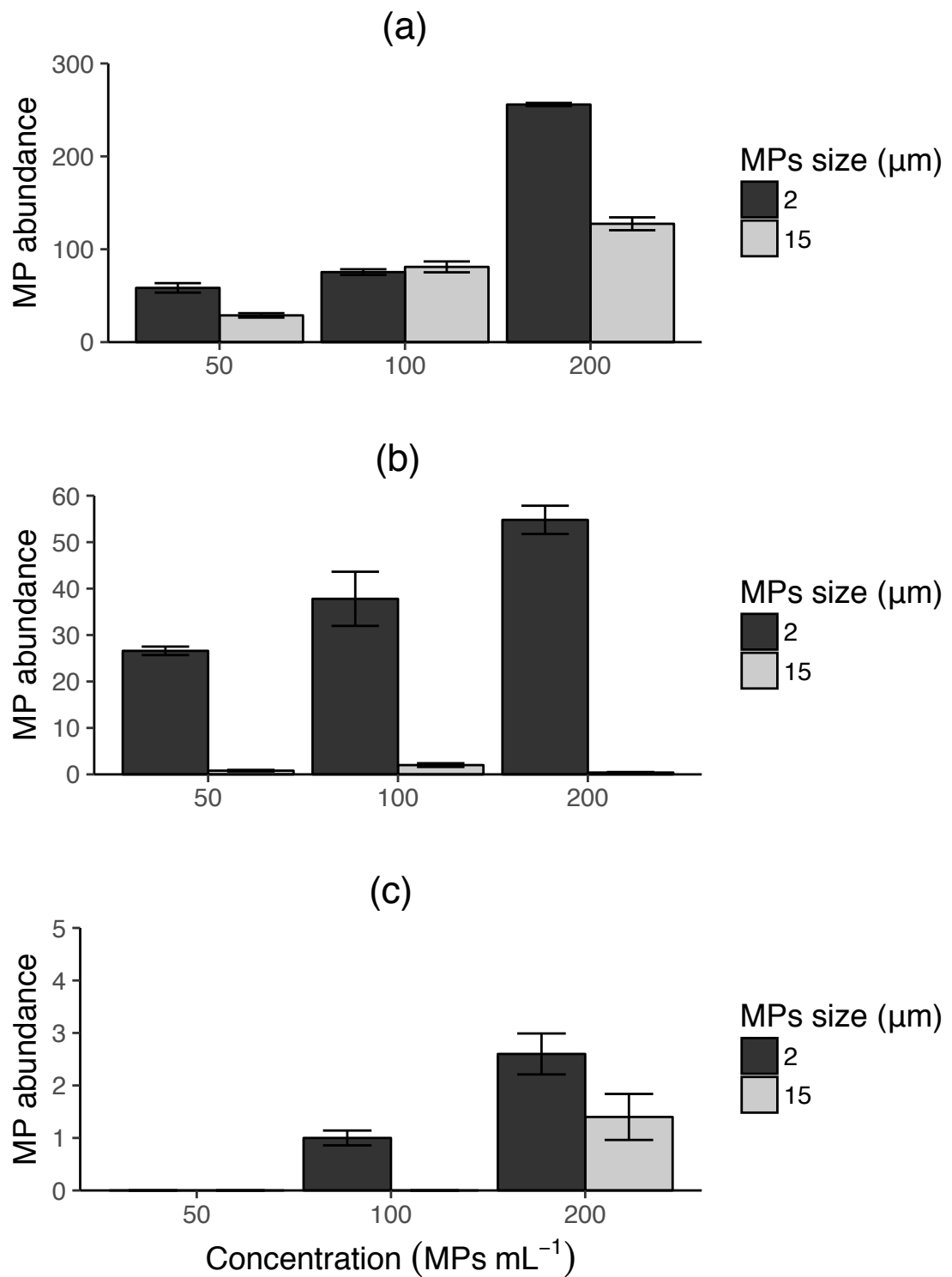
Figure 5-1 The number of microplastics (MP) across different exposure concentrations (50, 100 and 200 MPs mL^{-1}) and sizes (2 and 15 µm) in (a) larval stage, (b) pupal stage and (c) adult stage *Culex pipiens*. Means are ±SE.

Survival to the adult stage was not significantly affected by MP concentration ($F_{(3, 36)}$ = 0.78, p = 0.52; Figure 5.2) or by MP size ($F_{(1, 35)}$ = 0.31, p = 0.58). There was no significant 'concentration × MP size' interaction effect on survival to the adult stage ($F_{(3, 32)}$ = 2.60, p = 0.07).
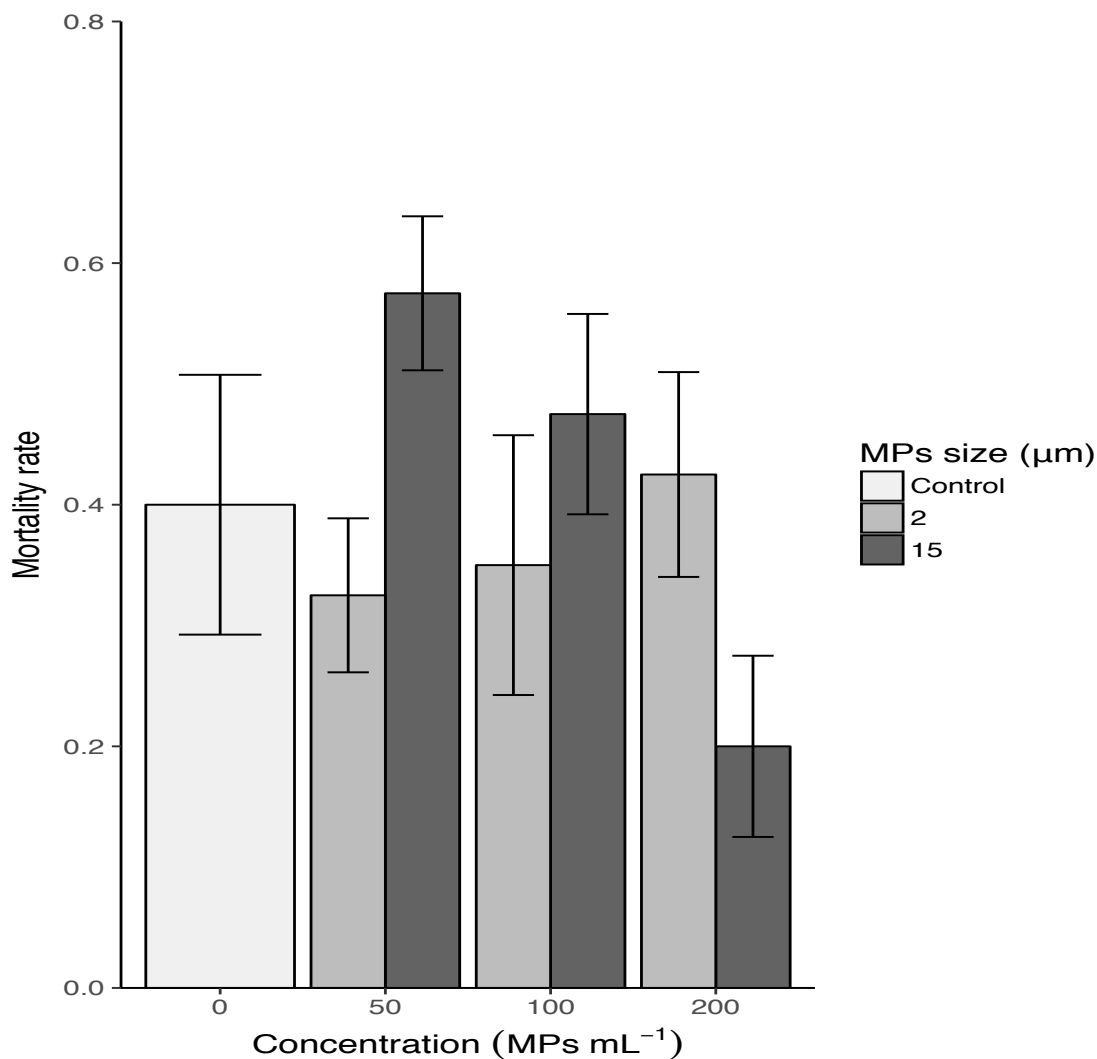
Figure 5-2 Mortality rates between larval and adult stage of *Culex pipiens* exposed to MPs under different concentrations (0, 50, 100 and 200 MPs mL^{-1}) and of different sizes (2 and 15 μm). Means are ±SE.

Exposure concentration of MPs did not have a significant effect on the weight of adult *C. pipiens* mosquitoes ($F_{(3, 36)}$ = 1.46, p = 0.24), and weight was not significantly influenced by MP size used during exposure ($F_{(1, 35)}$ = 0.76, p = 0.39; Figure 5.3).

Similarities were observed for the interaction between 'concentration' and 'MP size' on the weight of adult *C. pipiens* following exposure to MPs ($F_{(3, 32)} = 0.48$, $p = 0.71$).
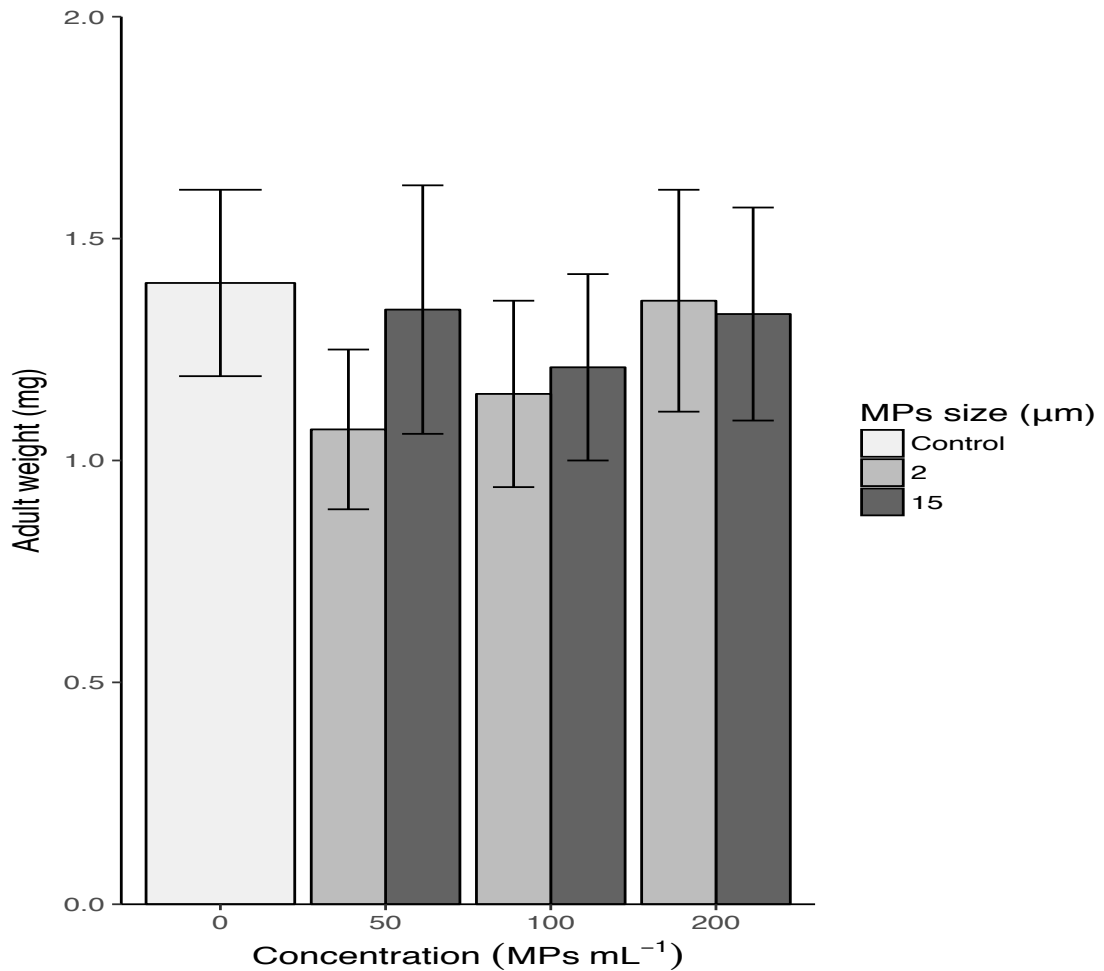
Figure 5-3 Weights of emerging Culex pipiens adults following exposure to MPs of different concentrations (0, 50, 100 and 200 MPs mL^{-1}) and sizes (2 and 15 µm). Means are ±SE.

5.5 Discussion

Microplastic pollution has increased concurrently in the aquatic environment with human population growth (Rocha-Santos and Duarte, 2014), alongside the high production and consumption of plastic materials (Andrady and Neal, 2009). Investigations have shown that the freshwater environment, including rivers, lakes and streams, contain substantial levels of MP pollution, with biotic impacts largely

unquantified and pathways for MP dispersal poorly understood (Horton et al., 2017; Wagner and Lambert, 2018). The present study demonstrates the potential for MPs to move ontogenically from feeding larval *C. pipiens* mosquito complex stages, into non-feeding pupal stages, and subsequently into flying adult stages. We also show concentration- and size-dependence of MP uptake, with greater numbers of particles taken up where MP concentrations were higher, and MP sizes were smaller. Here, MPs did not significantly influence the survival of larval mosquitoes to the adult stage, and the size of adults was not significantly influenced by prior MP exposure.

It was no surprise to discover that mosquitoes readily ingested MPs. Larvae of *Aedes aegypti* (Linnaeus), *Anopheles albimanus* Wiedemann, *Anopheles quadrimaculatus* Say and *Culex quinquefasciatus* Say have all been shown previously to ingest polystyrene latex beads (Aly, 1988; Dadd, 1971). We show that larvae did not ingest as many of the larger 15 μm MPs compared to 2 μm MPs which confirms previous work that suggests that filter feeding mosquitoes ingest particles based on their own size (Merritt et al., 1992). This does not infer selection, but probably reflects physical limitations; for example first instar larvae are unable to ingest latex beads as small as 45 μm in diameter (Dadd, 1971). Mosquito larvae feed using lateral palatal brushes to generate a current that causes water containing food or MPs to approach the mouth (Merritt et al., 1992) and it is possible that either the current or the mouth are not capable of easily dealing with larger fragments. However, MP ingestion at earlier life stages is not relevant for MPs to be passed ontogenically into the adult stage; it is only necessary for the fourth in- star to ingest the plastic.

Both 2 and 15 μm MPs were transferred from a feeding (larval) into a non-feeding (pupal) life stage and subsequently into the flying (adult) life stage during metamorphosis. Generally, where MPs were presented at higher concentrations, greater numbers of MPs were taken up by larval mosquitoes, and this differential abundance was sustained throughout their ontogenic development. Although MPs of both sizes were shown to transfer through to the adult stage, this transference only occurred under higher experimental concentrations (100 and 200 MPs mL^{-1}). It is difficult to compare these concentrations to those in the environment since detection is often of a mixed size group of MPs; reporting varies in units used and very small MPs are often not measured (Wagner and Lambert, 2018) (de Sá et al., 2018) (Hurley et al., 2018).

As with Al-Jaibachi et al. (2018), we found that the MPs accumulated in the Malpighian tubules of adults, however the number of MPs was substantially less than the number of MPs in the two previous life stages. Given the exposure conditions of the present study, it is likely that depuration or excretion reduces MP concentrations over time be- tween mosquito life stages, and particularly immediately post- emergence when adults evacuate their guts (Gillett, 1982). We suggest that MP size is a very important factor to ontogenic transfer. Small MP sizes can transfer and accumulate faster than large MPs, and in the present study smaller 2 μm MPs were able to transfer to the adult stage at a lower concentration than larger 15 μm MPs.

The extent to which MPs can enter new environments through metamorphosing insects is closely related to plastic toxicity; if MPs have lethal effects on immature stages, then transference and dispersal by adult life stages will not be possible. There was no evidence that MPs had any significant impact on the survival rate of aquatic larval *C. pipiens* through to the terrestrial adult stage. The absence of any negative impact on the weight of emerging adults suggests that the larvae may not have suffered from a lack of nutrition during development. Similar results have been found with studies of MP ingestion in *Daphnia* (Al- Jaibachi and Callaghan, 2018; Canniff and Hoang, 2018). Mosquito larval nutrition determines the extent of metabolic reserves as well as the size of adults upon emergence which, in turn, has a strong impact on fitness (Takken et al., 2013). Although the exposure duration was limited given that third instar larvae were used as the starting point of the experiment, the presence of MPs did not seem to influence the overall nutritional uptake of larvae to the extent where it affected adult growth. Thus, given the sustained survivability and development in the presence of MPs, it is highly likely that mosquitoes which uptake MPs will subsequently disperse MPs aerially into terrestrial food webs from the aquatic environment.

In conclusion, ontogenic transference presents a pathway for MP pollution to disperse from aquatic to terrestrial environments, vectored by mobile organisms with complex life histories. The efficacy of MP transfer is, however, dependent on both MP concentration and MP size, with considerable proportions of MPs lost between larval, pupal and adult *Culex* mosquito stages. Adult weights and mortality were not impacted by MPs, and so the presence of MPs does not seem to in-fluence the viability, development or nutritional uptake of mosquitoes. Future studies should examine the effects of MPs on ontogenic transference in other aquatic insect larvae, along with a

search for evidence of transference into predators. In addition, quantification of MP loss within exuviae would further elucidate the mechanism for MP reductions between ontogenic stages.

Chapter 6 THE FATE AND EFFECT OF 15μM POLYSTYRENE MICROPLASTICS ON A FRESHWATER POND COMMUNITY

6.1 Introduction

Although there are numerous studies to investigate the occurrence and abundance of MPs in freshwater environments including rivers and lakes, relatively few have looked at the impact on the organisms being exposed (Sighicelli et al., 2018; Wagner and Lambert, 2018). The majority of research on the uptake and effect of MPs in freshwater organisms has been conducted in the laboratory which does not reflect the many variables found in the environment (de Sá et al., 2018; Phuong et al., 2016). Laboratory studies are nearly all undertaken on individual organisms which ignores the interactions that occur in the natural environment. However these studies do give useful information on the uptake and ecotoxicity of MPs. Recent work on *D. magna* has shown that MPs can enter their gut system and show concentration-time dependent patterns (Aljaibachi and Callaghan, 2018; Canniff and Hoang, 2018; Jemec et al., 2016; Martins and Guilhermino, 2018; Nasser and Lynch, 2016; Ogonowski et al., 2016; Rosenkranz et al., 2009) Similar results have been found in *Gammarus fossarum* (Blarer and Burkhardt-Holm, 2016), annelids (*Lumbriculus variegatus*), crustaceans (*Gammarus pulex*), ostracods (*Notodromas monacha*), mosquitoes (*Culex pipiens*), and gastropods (*Potamopyrgus antipodarum*) (Imhof et al. 2013; Al-Jaibachi et al., 2018a; Al-Jaibachi, et al. 2018b). These studies are important since initial ingestion is more likely in lower trophic organisms which could enhance the transfer through the food chain (Cole et al. 2011; Anbumani & Kakkar 2018; Al-Jaibachi, et al. 2018a). The results of chapters 4 and 5 demonstrated that MPs can also be transferred ontogenically through the life stage which exposes a greater number of organisms to the MP contamination (Al-Jaibachi, et al. 2018a; Al-Jaibachi, et al. 2018b). The relationship between these laboratory results and the behaviour and interaction of MPs and invertebrates in the natural environment must be determined using more natural exposure methods.

Microplastics entering a natural environment are unlikely to remain stationary but will instead be transported between environmental compartments (Lambert and Wagner, 2018). The fate and movement of MPs will depend on hydrology and vegetation (Lambert and Wagner, 2018) and in lakes is likely to depend on sediment disturbance. There is no doubt that MP pollution is widespread, with a growing body of evidence to suggest that much higher MP concentrations are found in sediments compared to the water column. In Lake Taihu (China) the average number of MPs found in the water

body was 3.4 - 25.8 MPs L,$^{-1}$ while 11– 234.6 MPs kg^{-1} was found in the benthic sediment (Su et al., 2016). Similarly in Lake Chiusi (Italy) an average of 0.03 MPs L^{-1} were found in the surface water whereas 234 MPs kg^{-1} found in the sediment (Fischer et al., 2016). Higher levels of MPs have also been measured in river sediments including sediment of the River Thames, found to contain up to 660 MPs kg^{-1} (Horton et al., 2016). It is almost certain that the organisms living in these waters are ingesting MPs. However it is premature to generalise on whether the sediment or water column will have higher numbers of MPs since the data collected, as illustrated above, use very different methodologies.

The abundance of MPs in most freshwater environments investigated highlights questions about their impact on the biota biodiversity, food chain, community composition and predator-prey interactions and the possibility to accumulate in the food chain or transfer ontogenically to different environment (Al-Jaibachi, et al. 2018a; Al-Jaibachi, et al. 2018b; Rillig 2012; Wright et al. 2013).

Here studies of the impact of PS MPs are taken out into the field to study the abundance and impact of 15μm MPs on a community of freshwater invertebrates. Freshwater ponds are widely recognised as supporting greater regional invertebrate diversity than most other freshwater ecosystems in the UK and across Europe and can be rapidly colonised by variety of organism The small ponds chosen to implement the experiment have been studied previously and have demonstrated their value in rapidly measuring the impact of environmental stressors on freshwater communities in a controlled but natural environment (Ortiz et al, in review).

Fluorescent 15 μm PS MPs were chosen as they had already been examined in the laboratory for uptake and ecotoxicological effect in Chapter 3 (Al-Jaibachi, et al. 2018b). The larger size MP was used because of concerns regarding their detection in the ponds. The impact of MPs on the population size and community were examined by manipulating the ponds so that at the start of the study that had the same population size and composition. The animals used were all taken from the ponds where they had naturally colonised and included the two test species used in previous chapters, *C. pipiens* and *D. magna* as well as predators and animals that dwell in the sediment. The ponds were monitored for 12 weeks. We hypothesized that MPs would sink to the

sediment and be unavailable to animals in the water column with a consequent lack of effect on population size or community composition.

6.2 Materials and Methods

6.2.1 Preparation of microplastics

The MPs used in this experiment were supplied by Bangs laboratories, Inc, USA (Lot no. 12980), size 15.45 ± 1.1 μm PS microspheres, concentration (5×10^6 MPs ml^{-1}), density 1.06 g cm^{-3}, fluorescent dragon green (excitation 480nm; emission 520nm). MPs were prepared and washed prior to their exposure following the method in (Al-Jaibachi et al, 2018a). This was done by taking 1ml from the original solution, which was at a concentration of 5,000,000 MPs ml^{-1}, transferring it in to a 1.5 ml Eppendorf tube then centrifuged using an Eppendorf 5402 microcentrifuge for 10 minutes at 9,000 rpm. The resulting supernatant was then disposed of, and 1ml of distilled water was added to the Eppendorf tube. The tube was then thoroughly mixed using a Fisherbrand Whirlimixer® Cyclone Vortex Mixer ser. no. 29200 to re-suspend the MPs. 0.5 ml then added to each pond which contains 2,500,000 MPs per 25 litter in a final concentration of 100 MPs per ml as its consider more environmentally related concentration.

6.2.2 Study site and ponds

Thirty two ponds were artificially dug in experimental grounds at the University of Reading, Berkshire, England (51 26 15N and 00 56 29W). in 2012 (Figure 6.1). Fifteen of these ponds were randomly selected for use in this study. Each pond consisted of a sunken bucket lined with pond liner, (diameter 48 cm depth 30 cm).

Figure 6-1 Photograph of the artificial ponds taken during the initial setup of the experiment (Reading, UK).

The ponds had been colonised by macroinvertebrates over the five previous years. These were all removed by passing the pond water through a sieve (dimensions 6 x 12 cm; 250 μm pore size) and placing contents onto a white plastic sampling tray (25 x 35 x 5 cm) with some water. Previous analysis of abundances determined that five species could be reintroduced in the same proportion into each pond, in numbers that reflected the natural populations at the time (Table 6.1). The water level of each pond was filled with rain water to the same height, which represented 25L of water.

Table 6-1 Classification and number of the species added to each pond.

Species	Habitat and feeding	Classification	Number in each pond
Daphnia magna	Water column Filter feeder	Class: Branchiopoda Order: Cladocera Family: Daphniidae	1000
Culex pipiens	Water column and surface Filter feeder	Class: Insecta Order: Diptera Family: Culicidae	15
Chironomus plumosus	Sediment Filter feeder	Class: Insecta Order: Diptera Family: Chironomidae	30
Jenkins spire-shell Potamopyrgus antipodarum	Water surface and sides Herbivore	Class: Gastropoda Order: Littorinimorpha Family: tateidae	15
Water mite Hydrachnidia	Water column Predator	Class: Arachnida Order: Trombidiformes Family: Hydrachnidiae	15

6.2.3 Preparation and sampling of the community ponds

Five randomly selected ponds were left untreated as a control. Another five were treated with 500 µl of the original washed MP stock (5,000,000 MPs ml^{-1}). The ponds were then left for one week to allow for any disturbance and stress to organisms caused by setting up the experiment.

Weekly sampling then followed using a standardised technique; using a pond net of approx. 60mm x 120mm, with a small enough mesh size to collect both the zooplankton such as *D. magna* and other invertebrates such as mosquito larvae (*Culex* spp.). The net was swept through the water using a figure of 8 motion for a total of four times 10-15 cm below the surface of the water. Samples were then placed in a 1 litre plastic bottle and removed to identify in the laboratory using a stereo microscope and a number of

keys (Croft, 1986; Greenhalgh and Ovenden, 2007; Dobson et al, 2013). Identified organisms were counted and then returned to the pond from which they came. All members of each species were individually counted, except for *D. magna*, numbers of which were estimated by counting the number of individuals in a 1ml, and then multiplying this by the number of ml in the sample due to the very high numbers of individuals. This process was repeated for 12 weeks, with the initial set up on and addition of the MPs on the 12th June 2017, first data collection on the 19th June 2017, and the final samples taken on the 29th August 2017. Samples were taken between 10am and 12pm weekly.

6.2.4 Partitioning of microplastics in ponds

Five randomly selected ponds were emptied of all water and organisms and to each was added 2 kg of soil and 25 L of rain water. A random number of in *D. magna* and *Culex* larvae added to the ponds along with 500 µl of stock MP solution as detailed in section 6.2.3.

The ponds were left undisturbed for a week, samples were taken weekly started from the 22nd June 2017 until the 10th August 2017. The ponds were re-filled with rain water to 25 L weekly after samples were taken, because of environmental conditions causing high levels of evaporation.

For each sample per pond, 10 ml of water sample was taken from two depths (5cm under water surface and 5cm above sediment), using 1 ml pipette from 5 different points of the ponds and then water samples were mixed together before being filtered onto a nucleopore track-etched membrane (Whatman, Kent, UK) <10 µm, by using a glass vacuum filter holder connected to a manual air pump.

Five grams of sediment were collected using a spatula and placed on to 5 ml tube. A 2.5 g aliquot of the sediment spread directly on to slide and MPs counted directly. Furthermore, 5 *D. magna* and 5 *Culex* were collected from each pond (*D. magna* and *Culex* spp. were not present in every pond for the whole duration of the experiment so were discounted from analysis). Collected *D. magna* and *Culex* were placed into 1.5 ml Eppendorf tube then prepared to count the MPs following the method outlined in chapters 2 and 4 (Aljaibachi & Callaghan, 2018; Al-Jaibachi et al, 2018a).

6.2.5 Statistical analysis

The effects of MPs on pond macroinvertebrate communities were explored using Redundancy Analysis (RDA) in (Canoco v.5.10) (Smilauer and Lepš, 2014). A pooled record of all the macroinvertebrates recorded throughout the sampling period was used to test the effects of MP treatment whilst also taking into account the effect of season (Early (from June to July 2017) /late summer (from July to August 2017))

The abundance of invertebrate groups were analysed using R v3.4.2 (R Development Core Team, 2017). Generalized Linear Model (GLM) using lme4 package, quasi-Poisson error distribution since they were not normally distributed, as assessed by Shapiro-Wilk's test ($p < 0.05$), the mean number of each group in the treated ponds with MPs compared to the control week by week.

The abundance of MPs were analysed using (GLM) were chosen for analysis assuming a quasi-Poisson error distribution as counts were found to be over dispersed compared to degrees of freedom. Correlation analysis ggpubr package was used to evaluate the abundance and the relation of MPs in the *D. magna* and *Culex* spp. to compared it with the abundance in the water body, also used to evaluate the relation between temperature and rainfall to the abundance of MPs.

6.3 Results

6.3.1 Redundancy analysis on pond community

The non-metric multidimensional scaling (NMDS) (Figure 6.2) shows how each taxa was affected by the different variables of treatment type; control ponds and MPs treated ponds "Treatment", and time period; early summer "Early", being the first five weeks of sampling (from June to July 2017) and late summer "Late", being the last five weeks of sampling (from July to August 2017). The length and direction of the arrow indicates the strength and cause of population changes. The NMDS analysis showed significant differences in community between control and ponds treated with MPs (P= 0.003).

Daphnia magna and *Hydrachnidia* (water mites) were significantly negatively affected by the presence of the MPs, and they were associated with early summer (Figure 6.2). Whereas *Culex* mosquito larvae showed high temporal variation with much larger populations in early summer compared to late summer. *Potamopyrgus antipodarum* (Jenkins spire-shell) populations were weakly associated with control ponds and early summer, and *Chironomini* (non-biting midge larvae) populations were weakly associated with treated ponds and early summer.

After four weeks, two new species of invertebrate colonised the ponds. *Corixa punctata* (lesser water boatman) showed a weak association with control ponds and late summer, whereas *Leptophlebia* (mayfly larvae) populations seemed to prefer MPs ponds, but had little temporal variation.
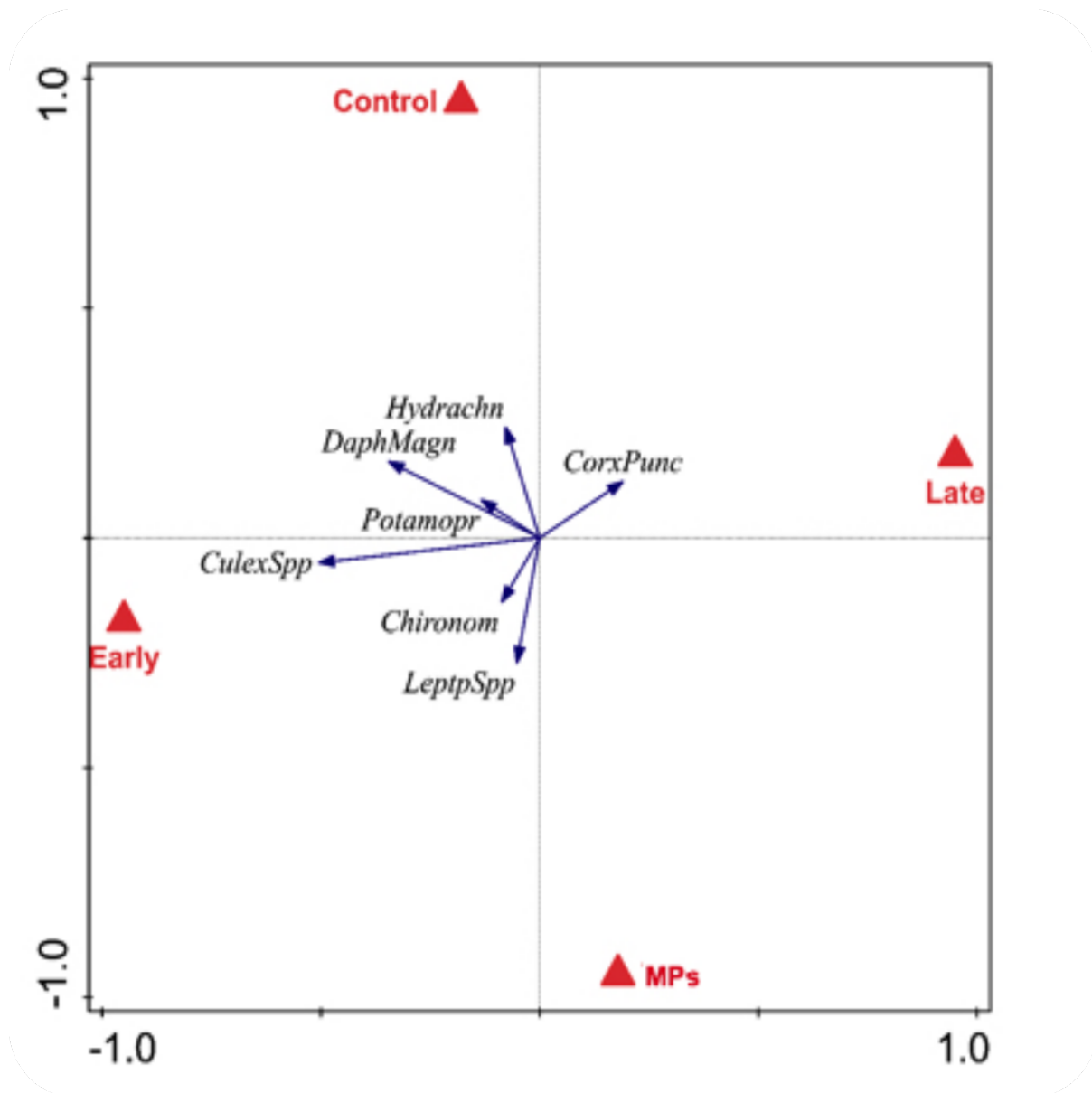
Figure 6-2 Non-metric multidimensional scale (NMDS) showing how each taxon was affected by time period ("Early" and "Late" summer) and condition ("Control" or untreated ponds and "MPs" or ponds treated with MPs). Species key: *D. magna* **(DaphMagn), Mosquito larvae/Culex spp. (CulexSpp), Non-biting midge larvae/***Chironomini (***Chironom), Jenkins spire-shell** /***Potamopyrgus antipodarum*** **(Potamopr), Water mite/***Hydrachnidia (Hydrachn***), Lesser water boatman/***Corixa punctata*** **(CorxPunc), Mayfly larvae/***Leptophlebia*** **spp. (LeptpSpp).**

6.3.2 Effects of microplastics on pond's community

6.3.2.1 *Daphnia magna* (Water flea)

Daphnia magna (water flea) numbers fluctuated between weeks but ultimately there were no significant differences between controls and ponds exposed to MPs at the end of 12 weeks (Figure 6.3) (Z=0.918, p=0.36). However on a week by week basis, there were some highly significant differences, with lower *D. magna* numbers in the MP treated ponds in the first half of the experiment (Figure 6.3, Table 6.2).

Figure 6-3 Mean abundance of *Daphnia magna* (water flea) over the experimental period in relation to treatment. The error bars indicate the standard error (±SE) of the mean.

Table 6-2 The analysis of *Daphnia magna* (water flea) abundance between control ponds and ponds treated with MPs $\pm$ standard error (SE) per week.

Weeks	Estimate$\pm$ SE	Z-value	P-value
1	-0.03$\pm$0.16	-0.2	0.93
2	-0.99$\pm$0.16	-5.99	<0.001*
3	-0.05$\pm$0.16	-0.35	0.83
4	0.77$\pm$0.16	4.66	<0.001*
5	0.68$\pm$1.67	4.075	<0.001*
6	1.93$\pm$0.167	11.52	<0.001*
7	0.65$\pm$0.166	3.90	<0.001*
8	-0.49$\pm$0.166	-3.002	0.2
9	-0.22$\pm$0.166	-1.327	0.2
10	-0.02$\pm$ 0.16	-0.121	0.90
11	-0.168$\pm$0.168	-1.005	0.4
12	-0.27$\pm$0.16	-1.62	0.18

6.3.2.2 Culex pipiens (Mosquitoes)

Culex pipiens (Mosquitoes) numbers fluctuated between weeks but ultimately there were no significant differences between controls and ponds exposed to MPs at the end of 12 weeks (Figure 6.4) (*Z*=-1.055, *p*=0.291). However on a week by week basis, there were some highly significant differences, with lower *Cluex* spp. numbers in the MP treated ponds in the 7th and 12th week of the experiment (Figure 6.4, Table 6.3).
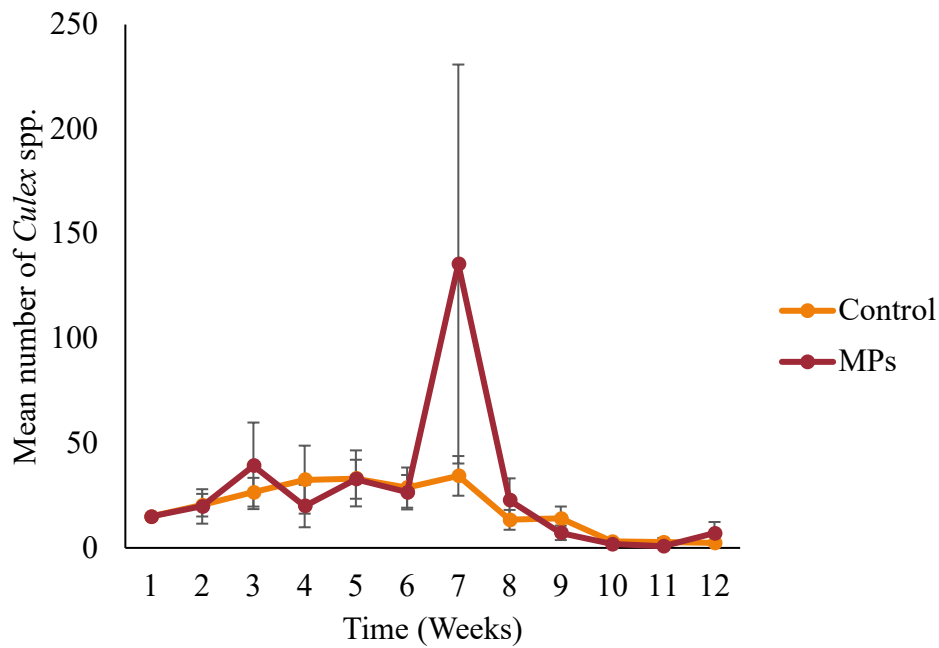
Figure 6-4 Mean abundance of *Culex* spp. over the experimental period in relation to treatment (MPs). The error bars indicate the ± standard error (SE) of the mean.

Table 6-3 the analysis of *Culex pipiens* (mosquitoes) abundance between control and MPs ± standard error (SE) per week.

Weeks	Estimate ± SE	Z-value	P-value
1	0.01 ±0.44	-0.02	0.98
2	0.019 ±0.44	-0.045	0.96
3	-0.398 ±0.43	0.933	0.35
4	0.478±0.430	1.110	0.27
5	0.008±0.43	0.004	0.99
6	0.069±0.426	0.161	0.87
7	-1.38±0.420	-3.288	<0.001*
8	-0.54±0.439	-1.233	0.21
9	0.65 ±0.459	1.42	0.15

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10	0.500 ±0.588	0.850	0.39
11	1.242 ±0.70	1.77	0.076
12	-1.08±0.53	-2.03	0.041*

6.3.2.3 *Chironomus plumosus* (Non-biting midge larvae)

Chironomus plumosus (non-biting midge larvae) numbers fluctuated between weeks but ultimately there were no significant differences between controls and ponds exposed to MPs at the end of 12 weeks (Figure 6.5) (Z= -1.168, P = 0.24). However, on a week by week basis, there were some highly significant differences in some of the weeks were more dominant in the ponds treated with MPs such as in week 3, 8 and 12 (Z=-3.052; P = 0.002), (Z=-3.623; $P < 0.001$), (Z=-2.364; P = 0.018). while in week 11 was they were more dominated in control ponds (Z=2.579 ;P = 0.009) (Figure 6.5, Table 6.4).
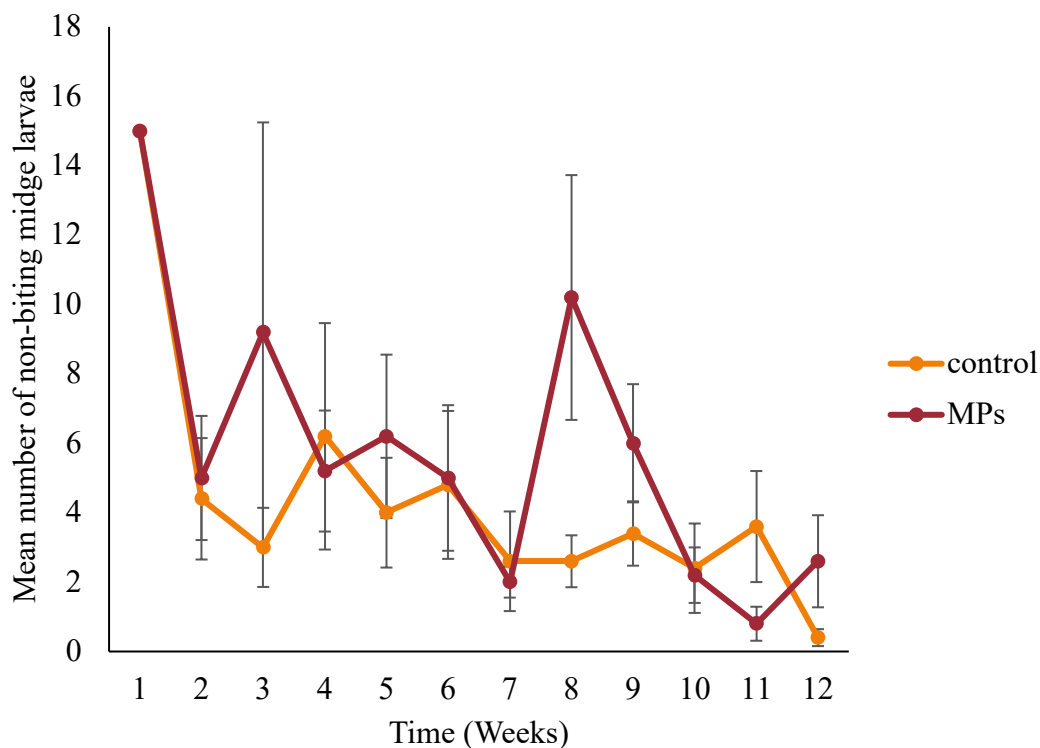
Figure 6-5 The mean abundance of *Chironomus plumosus* (non-biting midge larvae) over the experimental period in relation to treatment. The error bars indicate the standard error (±SE) of the mean.

Table 6-4 The analysis of *Chironomus plumosus* (non-biting midge larvae) abundance between control and ponds treated with MPs ± standard error (SE) per week.

Weeks	Estimate± SE	Z-value	P-value
1	0.015± 0.26	0.059	0.953
2	-0.11±0.35	-0.314	0.753
3	-1.10± 0.36	-3.052	0.002*
4	0.19± 0.33	0.568	0.570
5	-0.42±0.35	-1.196	0.231
6	-0.025±0.35	-0.072	0.942
7	0.27±0.46	0.594	0.552
8	-1.35± 0.37	-3.623	<0.001*
9	-0.55± 0.36	-1.505	0.132
10	0.10± 0.46	-0.220	0.825
11	1.52± 0.60	2.579	0.009*
12	-1.85± 0.78	-2.364	0.018*

6.3.2.4 *Potamopyrgus antipodarum* (Jenkins spire-shell)

Potamopyrgus antipodarum (Jenkins spire-shell) mean numbers fluctuated over the weeks but ultimately there were no significant differences between controls and ponds exposed to MPs at the end of 12 weeks (Figure 6.6, Table 6.5) (Z= 1.596; P = 0.110). However on a week by week basis, there were some highly significant differences between control and ponds treated with MPs. In week 7 and they were more dominant in the control ponds (Z=-3.52; P < 0.001), (Z=2.565; P= 0.01) respectively. while in week 12 was more dominated in ponds treated with MPs (Z=-2.256; P = 0.02).
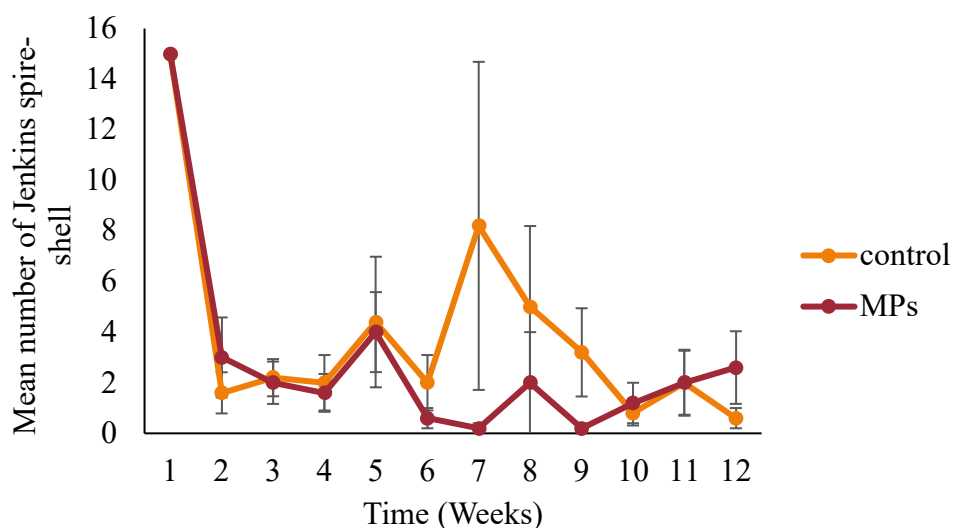
Figure 6-6 The mean abundance of *Potamopyrgus antipodarum* (Jenkins spire-shell) over the experimental period in relation to treatments. The error bars indicate the standard error (±SE) of the mean.

Table 6-5 The analysis of *Potamopyrgus antipodarum* (Jenkins spire-shell) abundance between control and ponds treated with MPs ± standard error (SE) per week.

Weeks	Estimate± SE	Z-value	P-value
1	-0.071± 0.29	-0.246	0.805
2	-0.69± 0.49	-1.407	0.159
3	0.02± 0.49	0.049	0.961
4	0.15± 0.52	0.287	0.773
5	0.024± 0.38	0.063	0.950
6	1.13± 0.69	1.624	0.104
7	3.64± 1.035	3.519	<0.001*
8	0.84±0.44	1.910	0.056
9	2.70±1.05	2.565	0.010*

Weeks	Estimate± SE	Z-value	P-value
10	-0.47± 0.68	-0.695	0.487
11	-0.070±0.50	-0.140	0.888
12	-1.53± 0.68	-2.256	0.02*

6.3.2.5 Hydrachnidia (Water mite)

Hydrachnidia (Water mite) numbers fluctuated between weeks but ultimately there were no significant differences between controls and ponds exposed to MPs at the end of 12 weeks (Figure 6.7, Table 6.6) (Z= 0.005; P = 0.996). However on a week by week basis, there were some highly significant differences, for instance in some of the weeks were more dominant in the control ponds such as in week 7 and 9 (Z=3.025; P = 0.002) and (Z=2.187; P = 0.028).
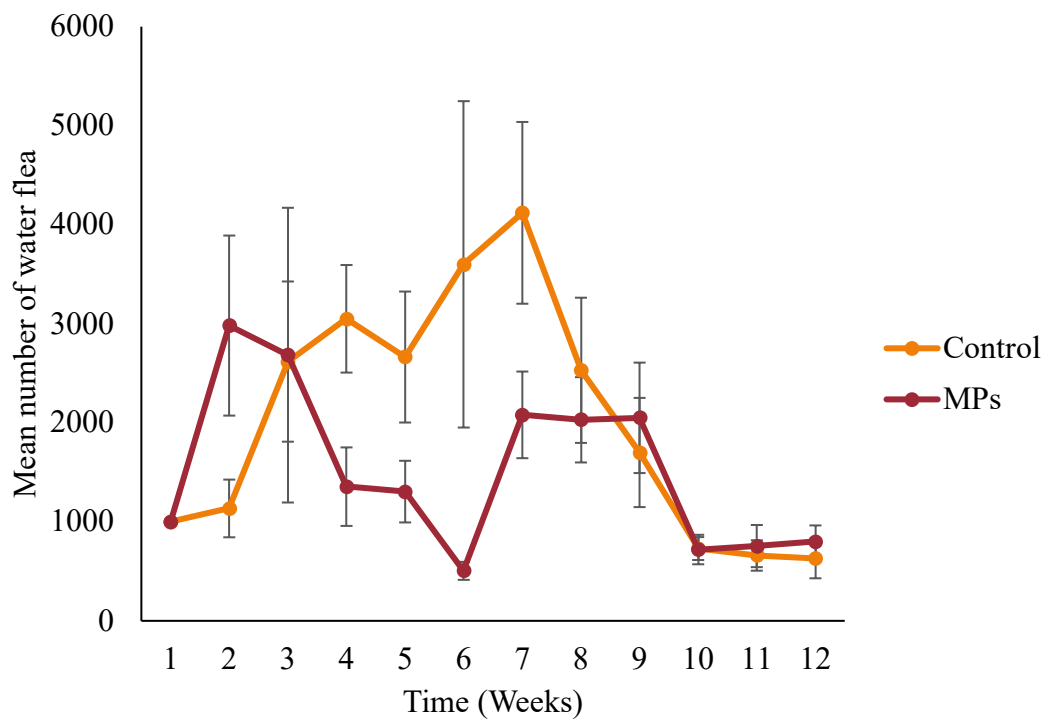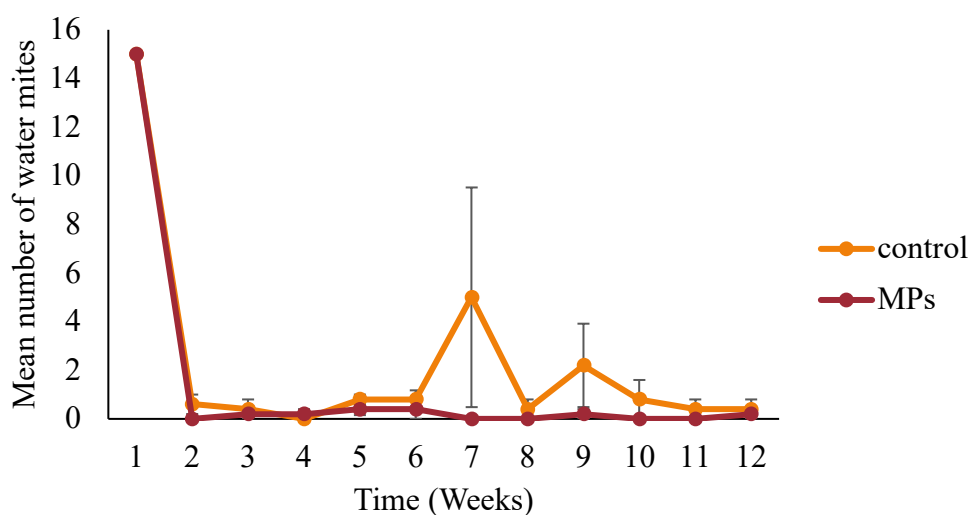

Figure 6-7 The mean abundance of *Hydrachnidia* (Water mite) over the experimental period in relation to treatment. The error bars indicate the standard error (±SE) of the mean.

Table 6-6 The analysis of *Hydrachnidia* (Water mite) abundance between control and ponds treated with MPs ± standard error (SE) per week.

Weeks	Estimate± SE	Z-value	P-value
1	-0.065±0.25	-0.255	0.798

2	18.55 ± 0	0.003	0.997
3	0.62± 1.24	0.504	0.613
4	-17.69± 0	-0.003	0.997
5	0.62±0.89	0.705	0.481
6	0.62± 0.89	0.705	0.481
7	3.15± 1.042	3.025	0.002*
8	18.11± 0	0.003	0.997
9	2.33± 1.06	2.187	0.02*
10	18.39± 0	0.004	0.997
11	17.67± 0	0.003	0.997
12	0.62± 1.24	0.504	0.613

6.3.2.6 *Corixa punctate* (Lesser water boatman) and *Leptophlebia* spp.(mayfly larvae)

New species had found in the ponds during sampling. *Corixa punctate* (Lesser water boatman) was more dominated in the control ponds, Lesser water boatman started to appear in the fourth week of sampling. However, there was non-significant differences between the mean number in control and ponds treated with MPs over 12 weeks $X^2(1)=$ 0.683, p =0.40 (Figure 6.8).

Leptophlebia spp.(Mayfly larvae) also started to appear from the fourth week of sampling and it was significantly dominated in ponds treated with MPs $X^2(1)=$ 5.62, p =0.018 (Figure 6.9)
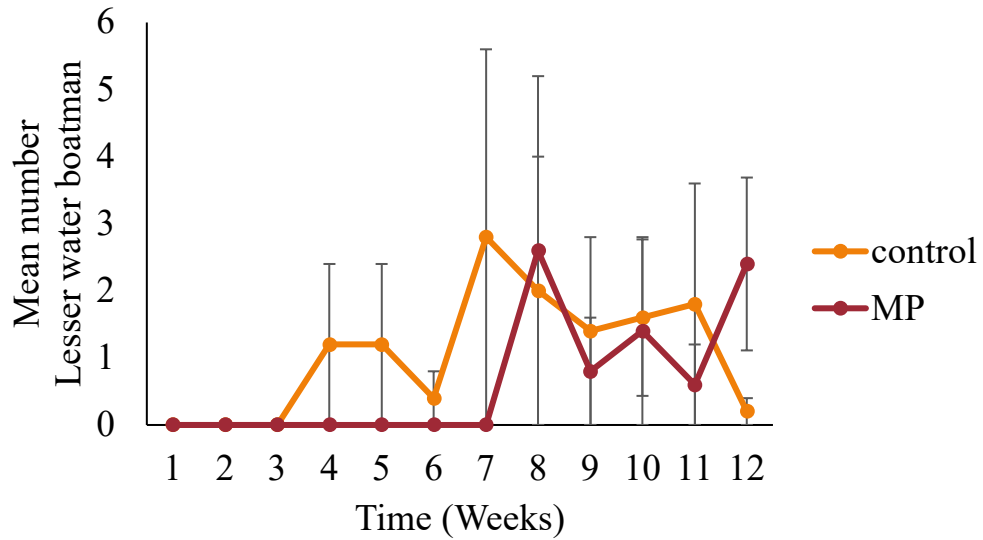
Figure 6-8 The mean abundance of *Corixa punctate* (Lesser water boatman) over the experimental period in relation to treatment. The error bars indicate the standard error (±SE) of the mean.
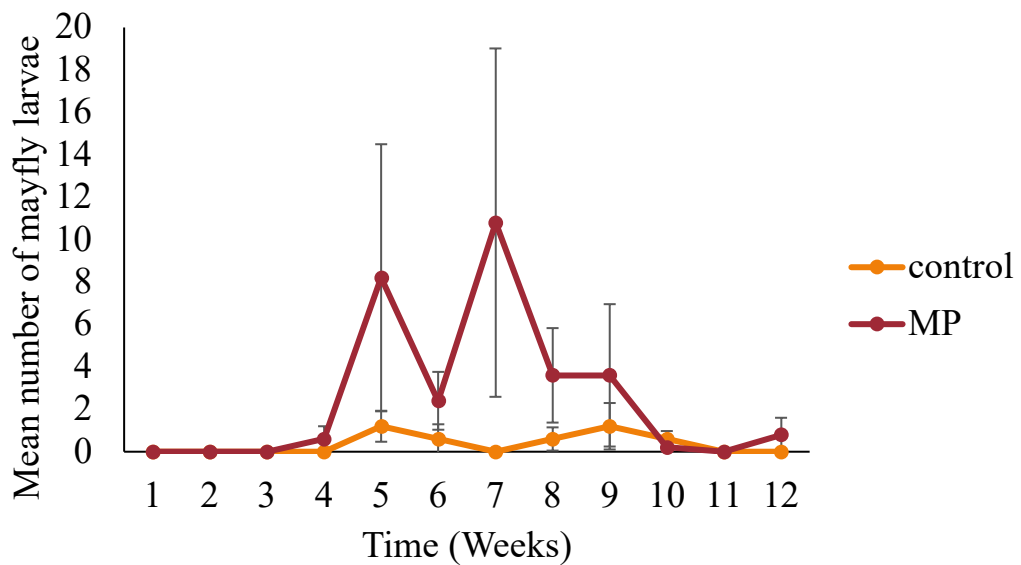
Figure 6-9 The mean abundance of *Leptophlebia* spp.(Mayfly larvae) over the experimental period in relation to treatment. The error bars indicate the standard error (±SE) of the mean.

6.3.3 Partitioning of microplastics between the water and sediment

Significantly more MPs were measured in sediment compared to water over time $F_{(2,68)}=59.4$, $p <0.001$ (Figure 6.10). The number of MPs in the water body remained constant over time, with no evidence of a change in number $F_{(1,33)}=0.33$, $p= 0.567$ (Figure 6.10).
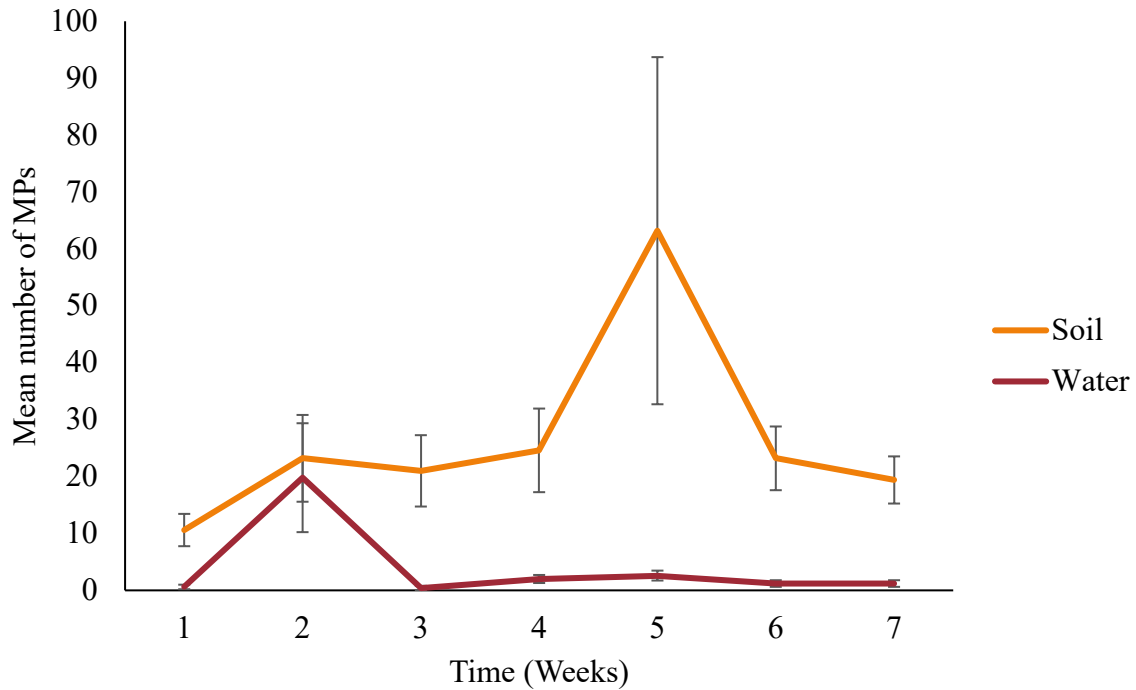
Figure **6-10** The mean number of MPs in the sediment and water body ± Standard Error of the mean (SE). Data collected weekly over 7 weeks.

6.3.4 External factors

The abundance of MPs in the water column showed a non-significant negative correlation with increase the temperature, correlation = - 0.06 ; $F_{(1,5)}$= 0.018, p= 0.898 (Figure 6.13). Similarly with rainfall over the sampling weeks with a non-significant negative correlation of = - 0.70; $F_{(1,5)}$= 0.1361, p= 0.727 (Figure 6.14).
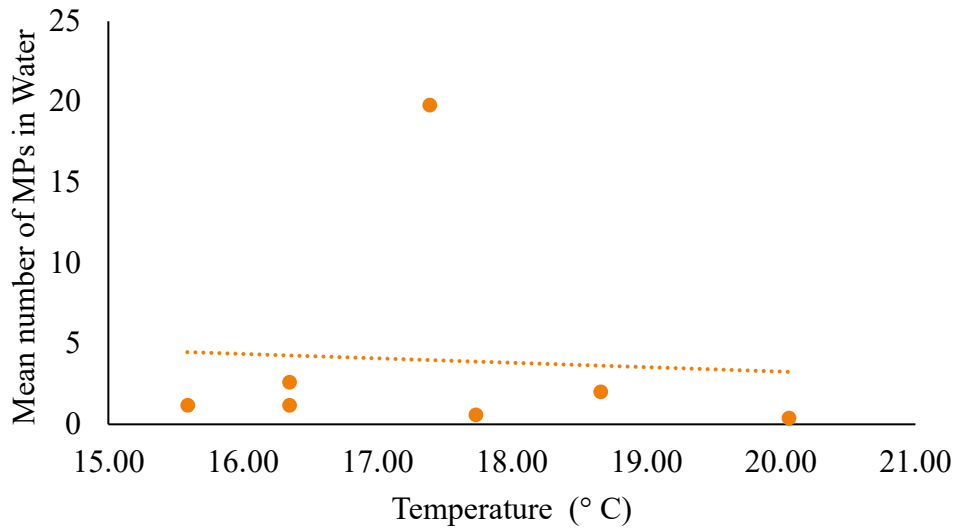
Figure 6-11 The mean number of MPs in the water column in relation to the temperature (°C) in Reading, UK over 7 weeks of sampling.
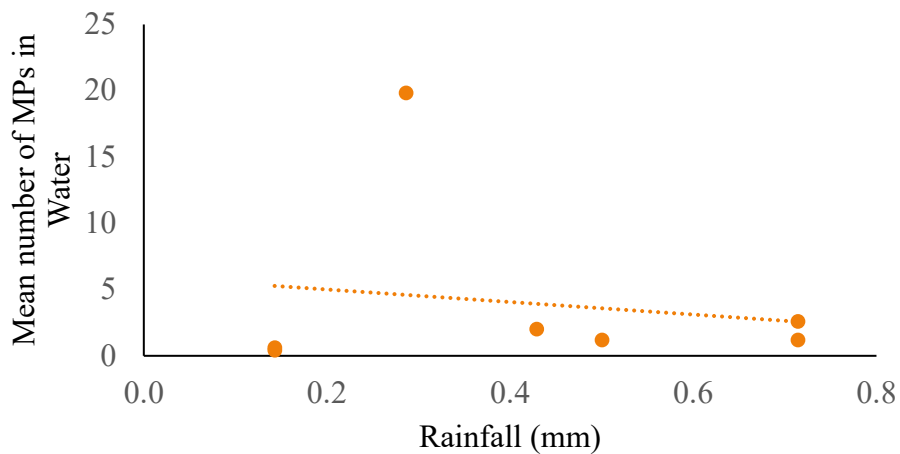
Figure 6-12 The mean number of MPs in the water column in relation to the rainfall (mm) in Reading, UK over 7 weeks of sampling.

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6.4 Discussion

MP pollution in freshwater environment is a global challenge to ecosystem and human health, and the long-term effect are still not very well understood (Horton et al., 2017). Many studies have focused on laboratory experiments to examine the uptake and toxicity of MPs on freshwater invertebrates while fields studies are still limited (Wagner and Lambert, 2018). Here, for the first time we examine the abundance and effect of 15 μm MPs on freshwater organisms in small naturally colonising ponds and measure the impact on biota abundance and community composition.

Our results demonstrated that from the first week of the experiment MPs fell to the bottom of the ponds and there were highly significant numbers of MPs in the pond's sediment rather than the water column. This agrees with previous work to examine the abundance of MPs in lakes (Su et al., 2016)

There are some external factors that can play a role to the disturbance and abundance of MPs, such as temperature. From our records (Figure 6.13) we found that no significant correlation between the temperature and the abundance of MPs in the water body. Although, the increase in temperature decreased the number of MPs in the water column. The ponds also experienced an extremely hot and dry summer and evaporation of water from the ponds was an issue. Observations suggested that when the temperature was high the organisms swam to the bottom of the ponds and when the temperature decreased the invertebrate swam closer to the surface (not formally recorded). We also recorded the rainfall not significantly correlated with the abundance of MPs (Figure 6.14).

Our study also examined the uptake of MPs by freshwater invertebrates to evaluate the relationship with MPs abundance in the surrounding environment, something which is still unclear and needs more investigations (Qu et al., 2018).

Also current results shows that the populations of the taxa fluctuated during the experimental period of 12 weeks. The only one taxa is positively significantly affected by the MPs pollution, the mayfly larvae (*Leptophlebia spp.*). Previous studies have suggested that MPs are taken up from sediments by freshwater species and the ability of freshwater invertebrates to uptake MPs depends on their feeding type (Redondo-Hasselerharm et al., 2018)

Previous laboratory studies showed that MPs had no effect on *D. magna*'s fitness while the mortality were more related to the availability of food rather than MPs (Aljaibachi and Callaghan, 2018; Besseling et al., 2014a; Canniff and Hoang, 2018; Rist et al., 2017). Also our current results found out that MPs size 15 μm had non-significant effect on *D. magna* population after 12 weeks of exposure. However, *D. magna* population numbers had been reduced in ponds exposed to MPs compared to the control ponds for the first six weeks of exposure. We could argue that MPs had an impact on the population of *D. magna* from the first week of exposure before precipitating into the sediment and that the effect of the initial exposure last for 6 weeks. The impact of MPs in the field show different results from laboratory studies, it could be to a number of other external factors involved. As in the field experiments cannot rely on only one stressor in freshwater ecosystems (Scherer et al., 2017). Also it may be that the effect of MPs pollution is only seen when such they are combined with other stressors and external factors such as water temperature. This approach recently had been investigated in the laboratory by exposing *D. magna, D. pulex* and *Ceriodaphnia dubia* to primary and secondary MPs combined with three temperatures 18°, 22°, and 26 °C: the results show the impact of MPs influenced by the temperature (Jaikumar et al., 2018). Also the field results are more in line with previous research where *D. magna* were exposed to (0.1 mg/l) pristine microspheres size 1–5 μm in diameter, and suffered effects on mortality, reproduction and the population growth rate up to third generations post exposure (Martins and Guilhermino, 2018).

In contrast, the population growth of *Culex pipiens* fluctuated over 12 weeks in both control and ponds exposed to MPs; its well known that mosquito populations are very seasonal (Ortiz-Perea et al., 2018; Townroe and Callaghan, 2015). The abundance of *Culex pipiens* was not significantly affected by the presence of MPs which agrees with the laboratory data on *Culex* mosquitoes showing that MPs had no effect on mortality or growth rate (Al-Jaibachi et al, 2018).

Although not added at the start of the experiment, the mayfly *Leptophlebia* spp rapidly colonised the ponds. The mayfly seemed to prefer ponds with MPs but the data were not statistically significant However, one possible explanation for this is due to many *Leptophlebia* species being detritivores (Sweeney et al, 1986), the negative effect on *D. magna* in the first 6 weeks and *Hydrachnidia* populations may result in a greater number of dead organisms sinking to the sediment, providing a more nutrient-rich food

than the organic matter that the *Leptophlebia* larvae typically feed on. This may have resulted in more individuals surviving and therefore being collected during sampling. Predatory *Hydrachnidia* (water mites), were significantly affected by the presence of MPs in a couple of weeks. These results indicated that although the risks of microplastics may be low, they still may affect the biodiversity and the performing of aquatic communities which after all also depend on the sensitive species. Future research needs to involve more taxonomic groups to reveal gaps in existing knowledge on the impact of MPs through the investigation of longer term effect and the uptake of MPs through the food chain.

Chapter 7 THE INFLUENCE OF MICROPLASTICS ON TROPHIC INTERACTION STRENGTHS AND COLONISATION PREFERENCES OF DIPTERANS

This chapter has been published in its entirety:

Statement of contribution:

The planning and experimental work was all undertaken by myself and Ross Cuthbert with some assistance with writing and statistical analysis from the other authors.

7.1 Abstract

Microplastic (MP) pollution continues to proliferate in freshwater, marine and terrestrial environments, but with their biotic implications remaining poorly understood. Biotic interactions such as predation can profoundly influence ecosystem structuring, stability and functioning. However, we currently lack quantitative understandings of how trophic interaction strengths and associated behaviours are influenced by MP pollution, and how transference of MPs between trophic levels relates to consumptive traits. We also lack understanding of key life-history effects of MPs, for example, reproductive strategies such as oviposition. The present study examines s when the latter are exposed to MPs, using a functional response (FR) approach. Transfer of MPs occurred from larval mosquitoes to larval midges via predation. Microplastics transfer was significantly positively related to predation rates. Predation by *C. flavicans* followed a Type II FR, with average maximum feeding rates of 6.2 mosquito larvae per hour. These and other FR parameters (attack rates and handling times) were not significantly influenced by the presence of MPs. Further, *C. pipiens* adults did not avoid ovipositing in habitats with high concentrations of MPs. We thus demonstrate that MPs can move readily through freshwater food webs via biotic processes such as predation, and that uptake correlates strongly with consumption rates. Further, as MPs do not deter adult mosquitoes from ovipositing, our experiments reveal high potential for MP exposure and transference through ecosystems.

7.2 Introduction

Microplastic (MP; <5 mm in size) pollution is prolific in terrestrial and aquatic ecosystems globally (Mason et al., 2018; Sighicelli et al., 2018; Wagner and Lambert, 2018). Whilst the enormous scale of MP pollution is unequivocal, we currently have a poor understanding of how MP presence influences interaction strengths between trophic groups (Wagner and Lambert, 2018). Interaction strengths between predators and prey can profoundly impact the infrastructure of ecosystems through the determination of predator population growth and prey population stability (Gilbert et al., 2014; Paine, 1980). Thus, understanding factors that influence predation are crucial to predicting eco- system structure and functioning. Trophic transfer via predation has been identified as a potentially major pathway through which MPs can move through

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food webs (Batel et al., 2016; Chae and An, 2017; Nelms et al., 2018; Provencher et al., 2018), however quantifications of how exposure to MP pollution influences trophic interaction strengths are lacking, especially in highly vulnerable, understudied freshwater environments (Blettler et al., 2018). Functional responses (FRs) (Holling, 1959; Solomon, 2018) quantify resource use under different resource densities and are powerful predictors of interaction strengths between consumers and resources. Three FR types have been broadly characterised: Type I FRs are filter-feeder specific (Jeschke et al., 2004), wherein consumption increases linearly with resource densities; Type II FRs exhibit a decelerating intake rate, with high proportional consumption at low resource densities potentially leading to resource extirpation; Type III FRs are sigmoidal, characterised by low proportional consumption at low densities, thus potentially facilitating refugia for prey (Holling, 1959). For predators and prey, FRs can quantify how prey populations are regulated by predators across different context-dependencies (e.g. Cuthbert et al., 2018). Shifts in the FR form and/or magnitude of predators, for instance from destabilising Type II to stabilising Type III, are known to be driven by environmental contexts (Alexander et al., 2012). However, we currently have little quantitative bases to predict how pollutants such as MPs will influence predator-prey dynamics. Furthermore, distributions of prey populations are often reliant on selective processes relating to quantifications of risk and reward between habitat patches, especially for reproductive decisions such as oviposition sites (Nonacs and Dill, 1990). Indeed, the ability to avoid potentially harmful habitats can benefit the fitness of progeny and influence the success of populations. However, there has been little research to quantify the influence of MPs on selective behaviours, such as oviposition, which can drive species distributions and influence biotic interactions (Goldstein et al., 2012; Majer et al., 2012). In the present study, we thus examine whether MP exposure modulates interaction strengths between predators and prey, and whether trophic MP transfer can be related to predation rates. We then discern MP implications for ovipositional behaviour. Focal organisms were larvae of the predatory non-biting midge *Chaoborus flavicans*, and filter-feeding larvae of the mosquito complex *Culex pipiens*, which often co- occur in natural and artificial aquatic habitats.

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7.3 Materials and methods

7.3.1 Experimental design

Fluorescent 2 μm yellow-green carboxylate-modified polystyrene MPs (density 1.050 g cm^{-3}, 88 excitation 470 nm; emission 505 nm, Sigma-Aldrich, UK) were used in all experiments. Microplastics were stored as a stock suspension (2.5 mg mL-1)and mixed as per Al-Jaibachi et al. (2018a). *Chaoborus flavicans* (1.0–1.2 cm) larvae were purchased commercially (Northampton Reptile Centre, UK) and acclimated for 6 days in a laboratory at the University of Reading (19 ± 1 °C, 16:8 light:dark) on a diet of *C. pipiens* larvae in 5 L dechlorinated tap-water. Wild *C. pipiens* were collected from the Whiteknights campus of the University of Reading (51°26′12.2″N, 0°56′31.2″W). Egg rafts of *C. pipiens* were sampled from artificial container habitats and, upon hatching, fed ad libitum on crushed rabbit food pellets in the same laboratory in 10 L dechlorinated tap-water. Gravid adult *C. pipiens* were collected overnight using modified gravid box traps with a hay and yeast infusion used as bait (see Townroe and Callaghan, 2015). In experiment 1, in the laboratory (19 ± 1 °C, 16:8 light:dark), groups of 400 *C. pipiens* larvae (0.15–0.20 cm) were exposed to one of two MP treatments (0 particles mL^{-1}, 100 particles mL^{-1}) in 500 mL arenas for 22 h, whilst predators were simultaneously starved. Following treatments, *C. pipiens* larvae were rinsed in dechlorinated tap water and introduced at 5 densities (2, 5, 10, 20, 40; n=5 per treatment group) into 20 mL arenas containing 10 mL dechlorinated tap-water. After 2 h of prey acclimation, predatory *C. flavicans* were introduced and allowed to feed for 2 h. Predators were then removed and remaining live prey counted. Controls consisted of a replicate at each MP treatment and prey density in the absence of predators. Individual predators and prey were frozen at −20 °C before homogenisation and filtration using nucleopore track-etched membranes (Whatman, UK) of > 0.1 μm, with the MPs on filter membranes subsequently counted using an epi-fluorescent microscope (Zeiss Axioskop, Germany). In experiment 2, thirty wild gravid adult *C. pipiens* were transferred

into each of six 30 cm^3 cages in a laboratory (25±1 °C, 16:8 light:dark). Mosquitoes were allowed to oviposit in one of two paired 200 mL arenas containing different MP treatments (0 particles mL^{-1},100 particles mL^{-1}), placed randomly in opposite corners of the cages, over 3 days. Egg rafts were enumerated and removed daily.

7.3.2 Data analysis

 In experiment 1, the relationship between MP uptake via predation in *C. flavicans* and number of prey killed was examined using a generalised linear model (GLM) assuming Poisson error distribution. A GLM with quasi-Poisson error distribution was used to examine overall prey killed with respect to the 'MP treatment' and 'prey supply' factors, owing to residual over dispersion. Functional responses (FRs) were modelled using 'frair' in R (Pritchard et al., 2017) with Rogers' random predator equation (Rogers, 1972). Attack rate and handling time parameters were non-parametrically bootstrapped to generate 95% confidence intervals and compared according to MP treatment using the delta method (Juliano, 2001). Handling time estimates were used to generate maximum feeding rates over the total feeding period (1/h). In experiment 2, total egg raft counts were analysed using a generalised linear mixed model with negative binomial distribution between paired MP treatments, owing to residual overdispersion. Cage number was included as a random effect to account for the paired experimental design.

7.4 Results

In experiment 1, *C. pipiens* larvae exposed to 100 particles mL^{-1} contained 5.8 ± 2.7 (mean ± SD) MPs, whilst prey not exposed to MPs did not contain MPs. Whilst MPs were not detected in predators following consumption of unexposed prey, transference occurred in all *C. flavicans* that killed MP-exposed *C. pipiens*. Microplastic transference from *C. pipiens* to *C. flavicans* via predation was significantly positively related to the number of prey killed (z =1.972, p =0.049). Survival of prey was 100% in predator-free controls, and so all prey deaths were assumed to be due to predation. Predation by *C. flavicans* did not differ significantly according to prey MP exposure (t = 0.959, p =0.343). Prey killed increased significantly with greater prey supplies (t =4.938, p < 0.001) and under both MP treatments given no significant interaction (t = 0.721, p = 0.472). *Chaoborus flavicans* exhibited Type II FRs irrespective of prey MP exposure, given that first order terms were significantly negative in both treatment groups (Table 7.1). Attack rates (initial curve slopes) did not differ significantly between MP exposure treatments (z = 1.694, p = 0.090), but trended towards being higher where larval *C. pipiens* were exposed to MPs prior. Handling times did not differ significantly between MP treatments (z = 1.087, p = 0.277), although, reciprocally, maximum feeding rates

(curve asymptotes) tended to be higher towards prey not exposed to MPs (Table 7.1). Confidence intervals for attack rates and handling times overlapped overall (Table 7.1), and across all prey densities between MP treatments (Figure 7.1), further indicating a lack of significant difference in FRs. In experiment 2, a total of 43 egg rafts were oviposited in MP-treated water and 38 egg rafts in controls. There was no significant difference in oviposition between these treatment groups ($z = 0.380$, $p = 0.704$) (Figure 7.2). High statistical power, and thus low probability for Type II error, was found for both predation (power = 0.94) and oviposition (power = 0.93).

Table 7-1 First order terms from the proportion of prey killed as a function of prey density according to MP exposure treatments. Attack rate and handling time parameters from Rogers' random predator equation and bootstrapped ($n=2000$) 95% confidence intervals (CIs).

MP exposure (particles ml^{-1})	First order term, p	Attack rate (a), 95% CIs	Handling time (h), 95% CIs	Maximum feeding rate (1/h
0	-0.024, 0.005	0.549, 0.320–0.955	0.068, 0.014–0.144	14.706
100	-0.040, <0.001	1.070, 0.545–1.98	0.101, 0.056–0.169	9.901
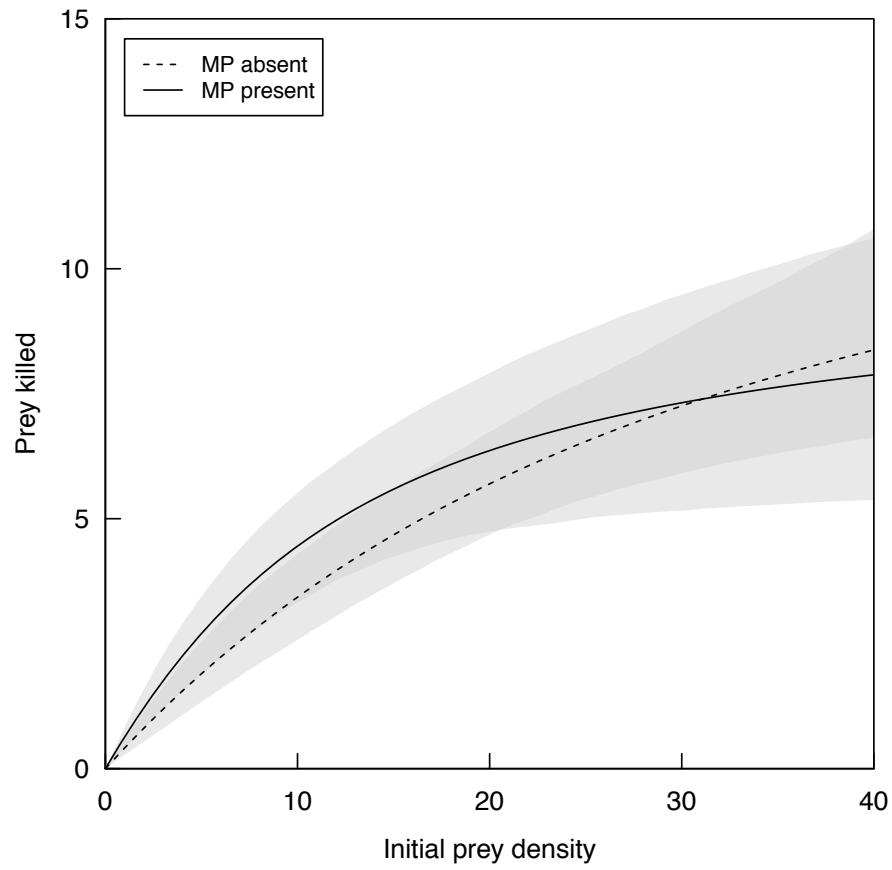
Figure 7-1 Functional responses of larval *Chaoborus flavicans* towards larval *Culex pipiens* following exposure to different microplastic (MP) treatments (absent, 0 particles mL^{-1}; present, 100 particles mL^{-1}). Shaded areas represent bootstrapped (n = 2000) confidence intervals.
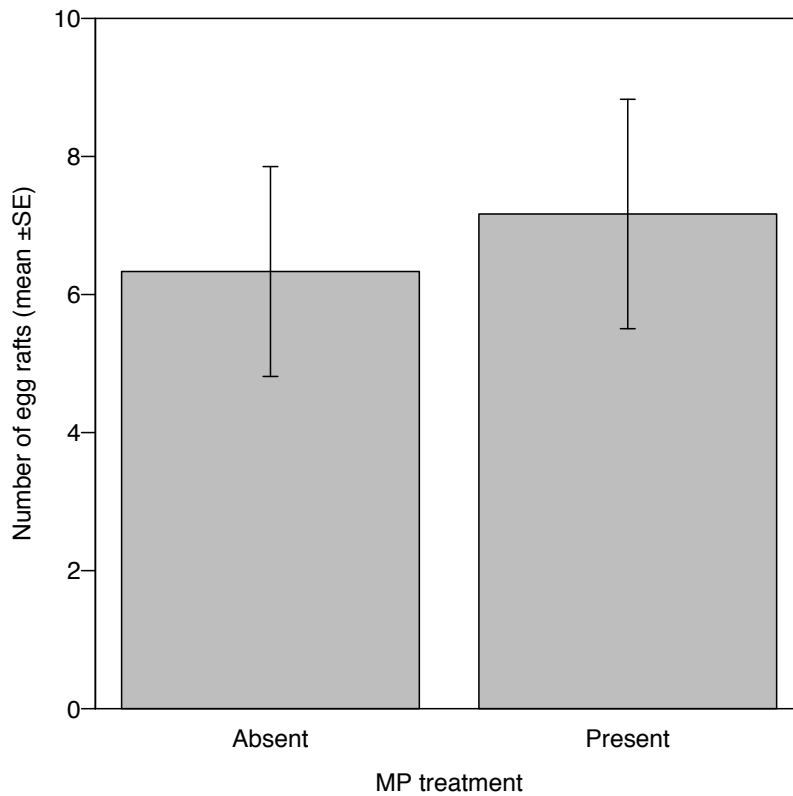
Figure 7-2 Number of egg rafts oviposited by _Culex pipiens_ between paired treatments with microplastics (MPs) absent (0 particles mL^{-1}) or present (100 particles mL^{-1}).

7.5 Discussion

Microplastics continue to proliferate across the biosphere, with ecological implications frequently unknown (de Sá et al., 2018; Mason et al., 2018; Sighicelli et al., 2018; Wagner and Lambert, 2018). In the present study, we further demonstrate active uptake of MPs through filtering by larval mosquitoes (Al-Jaibachi et al., 2018a, 2018b). Furthermore, we demonstrate that MPs can be transferred and retained trophically from filter feeding organisms to higher predators, and that trophic transference relates to consumption rates. Predation by larval _C. flavicans_ towards larval mosquito prey was significant irrespective of prior prey exposure to MPs. Neither search efficiency (attack rate) nor time taken to subdue, capture and digest prey (handling time) was significantly affected by prey MP exposure. Whilst both the area of attack rate and handling time parameters have been shown to be heavily context-dependent e.g. (Barrios-O'Neill et

al., 2016; Cuthbert et al., 2018b), here we show that the presence of MP pollution does not elicit changes to predation rates. Therefore, MPs are likely to be readily transferred to predators from prey in MP-polluted systems. Larval *Culex* mosquitoes actively filter and retain MP particles, and MPs have been shown to transfer ontogenically from larval to pupal stages, and then to the terrestrial adult mosquito stage (Al-Jaibachi et al., 2018a, 2018b). As with mosquitoes, *C. flavicans* exhibits both aquatic and terrestrial life stages, and thus the potential for ontogenic transference of MPs via this species from aquatic to terrestrial environments is high, and could accordingly impact terrestrial vertebrates. Strong potential for MPs to move further through food chains and impact organisms has been demonstrated in other freshwater systems (Chae et al., 2018). In the present study, as transference across trophic stages was positively related to predation rates, uptake of MPs via predation may be related to intraspecific or intraindividual variations in consumptive traits. Indeed, such variabilities are often naturally present within populations, and could influence MP pollution impact (Alexander et al., 2015). Oviposition by mosquitoes is selective across gradients of risk and reward (Pintar et al., 2018). The present study demonstrates that gravid adult *Culex* mosquitos are not deterred from ovipositing in aquatic habitats with MPs. Therefore, there is a high potential for larval stages to be exposed to MPs throughout their aquatic life stages, before subsequently transferring MPs trophically or ontogenically (Al-Jaibachi et al., 2018b). Although concentrations of MPs in the present study were high (but see (Fischer et al., 2016; Su et al., 2016), the lack of effect of MPs on predation rates and oviposition suggests that similar observations could occur in environments with lower MP concentrations. It is probable that colonists are naïve to potential risks of MPs to fitness. However, Al-Jaibachi et al. (2018a) found that MPs have little influence on the survival and fitness of *Culex* mosquitoes across their life history. Whilst this is the first study to quantitatively link MP transfer with predation rates in a FR framework, work is required to further explore potential trophic accumulation of MPs, alongside behavioural implications, and to discern the potential for aerial dispersal of MPs by freshwater insects aside from mosquitoes (Al-Jaibachi et al., 2018b). Previous work has demonstrated the direct exploitation of MPs particles as oviposition sites by insects aside from mosquitoes in aquatic systems (Goldstein et al., 2012; Majer et al., 2012), and MPs are ingested by a range of other aquatic invertebrates (Nel et al., 2018; Windsor et al., 2019). Here, as larval chaoborids ingest prey whole before regurgitating

undigested, solid material, examinations of physiological restrictions on MP retention are required for this group (Moore, 1988), particularly given their ecological importance in freshwater environments (Riessen et al., 1984). Elucidations of environmental context-dependencies which modulate MP uptake and transfer rates would additionally be of value, alongside the time-dependent effects of depuration.

Chapter 8 SUMMARY AND GENERAL DISCUSSION

8.1 General overview

Microplastics (MPs) are ubiquitous with potential direct or indirect impacts to organisms in the aquatic environment when ingested (Fossi et al., 2017). Most MP research is focused on the marine environment since it represents their largest sink (Rochman, 2018). Few studies have been conducted to investigate the effect of MPs on freshwater or terrestrial environments and the organisms therein (de Sá et al., 2018; Messinetti et al., 2018; Wagner and Lambert, 2018). This is of concern since an estimated 80% of MP marine pollution originates from the land where rivers are the most important carrier of plastic to the oceans (Rochman, 2018). Studies show that MPS are found in the surface water and the sediment of freshwater environments including rivers and lakes (Tibbetts et al., 2018). Although approximately 160 different marine organisms have been shown to ingest MPs, only 39 species have been investigated in the freshwater environment (de Sá et al., 2018). Additionally, knowledge on the impact of MPs on freshwater organisms is limited and conflicting (Chae and An, 2017).

The primary objective of this research was therefore to investigate the ingestion and excretion of polystyrene MPs (2 and 15 μm) on freshwater organisms (*D. magna, C. pipiens*) including interactions between species in a mesocosm. In addition I investigated the ecotoxicological impact of these MPs on reproduction and/or growth using *D. magna* as a model organism and applying standard ecotoxicology 21 days' life history tests following OECD guideline 211 (OECD, 2012).

The second objective was to investigate the fate of MPs in organisms that live part of their life in the water and part in the terrestrial environment. I chose to work with the ubiquitous mosquito *C. pipiens* as an example of a holometabolous insect. Additionally I measured the impact of MPs on survival rate and weight of adults.

In order to evaluate the impact of MPs in a more natural setting, I decided to move my research to the field by examine the effect of MPs on freshwater animals in mesocosms. Here I measured the impact of MP exposure on *Daphnia* and *Culex* in a natural system where competition and predation are factors. I used the larger size of MP for ease of detection and measured population size over time and general pond community composition with an aim of comparing laboratory with field results. Results from this

work led me to consider predator prey interactions and I undertook a final experiment to determine whether MP exposure regulated interaction strengths between predators and prey, and whether trophic MP transfer can be related to predation rates. Here I used larvae of the predatory non-biting midge *Chaoborus flavicans* as a voracious predator of *C. pipiens*, which often co-occur in natural and artificial aquatic habitats.

8.2 Uptake of microplastics by freshwater invertebrates

Results obtained in chapter 2 and 3 showed that two sizes of MPs (2 and 15 μm) are ingested and pass through the digestive system of *D. magna*, although the uptake of 2 μm MPs was considerably higher than 15 μm. As exposure time increased, so did the number of MPs in the gut. The results indicated that, in the presence of food, the amount of MPs in the gut was significantly lower than in *D. magna* given only MPs. This effect increased over time, with the number of MPs actually dropping in the food treatment condition. This suggests that *D. magna* have the ability to distinguish between MPs and food particles, so that *D. magna* prefer to take up food versus MPs over time. This is counter to other studies suggested that *D. magna* no able to distinguish between food and non-food particles (Wiedner and Vareschi, 1995). Additionally, the excretion of MPs was investigated by measuring the evacuation of MPs from the gut following a 60 min pulsed exposure to MPs with and without food particles, followed by the introduction of clean OECD water for 30, 60, 120 and 240 min. The drop in microplastic numbers in the gut, particularly in the MP-only treatment condition, can be explained simply by excretion. In both sizes the number of MPs excreted when there were no food particles was higher than in the presence of food. The ingestion and egestion of MPs had previously been investigated using *D. magna* exposed to MPs of different sizes and types, however the early studies in this field had neglected the presence of food and the measure of uptake and depuration was mainly examined on MP-only treatments which doesn't represent what happens in the natural environment (Rosenkranz et al., 2009). Increasingly more studies were conducted to examine the uptake of MPs in the presence of food particles and all those studies confirmed the uptake of MPs is time-dependent and concentration- dependent (Canniff and Hoang, 2018; Jemec et al., 2016; Ogonowski et al., 2016; Rist et al., 2017; Scherer et al., 2017) Although, some previous studies used one concentrations of MPs and algae (Canniff and Hoang, 2018; Rehse et al., 2016; Scherer et al., 2017) which did not examine the

selectivity of food particles but it was more focused on the bioaccumulation of MPs in the gut system.

From these results it can be concluded that it is possible for MPs to be ingested and accumulated readily in the gut in significant concentrations, and in cases where there is poor food availability *D. magna* will fill their gut with MPs. This accumulation of MPs in the gut could lead to transfer of considerable amounts to higher trophic levels in the food chain of the aquatic environment although to date this has not been investigated.

The results presented in chapter 4 and 5 show that the uptake of MPs not only occurs in *D. magna* but also in other invertebrates living in polluted freshwater environments. The uptake of 2 and 15 µm MPs in the dipteran insect *C. pipiens* mirrored results with the crustacean *D. magna*.

Both arthropod species are filter feeders, taking particles from the water column, and can graze (Kaufman et al., 1999; Sarnelle, 2005). *D. magna* grow incrementally by shedding their exoskeleton as both juvenile and adult, mosquitoes only shed their skin to grow as juveniles, escaping the water as adults which no longer grow. Of interest here was whether the MPs were retained in the mosquito gut during complete metamorphosis and whether they interfered with the metamorphosis process, causing increased mortality. In fact MPs were readily transferred from larvae into pupae, particularly the smaller sized MPs and had no impact on the success of metamorphosis. Fewer 15 µm MPs were transferred into adults, suggesting that MP size is an important factor in ontogenic transfer which could be related to the transfer and accumulation of MPs in the Malpighian tubes. This result alone is the first to show such an ontogenic transfer and as such has caused considerable concern and interest in the media.

A recent review highlighted that we don't know how MPs move from freshwater to terrestrial ecosystems (Eerkes-Medrano et al., 2015b). The results presented here have important implications because any aquatic life stage that is able to consume MPs and transfer them to their terrestrial life stage is a potential vector of MPs onto novel aerial and terrestrial habitats. Each mosquito adult had relatively few MPs and it is possible that they would be excreted early on in their adult life. However this study was a proof of principle and it is inevitable that other species with similar life histories and ability to ingest MPs will also be carrying them into the terrestrial environment, with the potential to transfer the pollutants to other animals. As mentioned previously, other invertebrates,

bats and birds all eat flying insects including mosquitoes (Medlock and Snow, 2008). Whilst each individual insect might not be harbouring a heavy load of MPs, a high availability to predators could lead to their bioaccumulation. This is particularly of concern where chemicals leach from plastics or adsorb onto the surface of the plastic, since this could expose other animals to high concentrations of toxins (Diepens and Koelmans, 2018). Where these animals are part of a human food chain, there are clear concerns about human health. That said, it is unlikely that the pathway discovered here is going to be important in human health, particularly considering that more obvious routes of exposure, such as fish and bottled water are in evidence (Andrade et al., 2019; Mason et al., 2018).

8.3 Ecotoxicological effect of microplastics on freshwater invertebrates

In an attempt to evaluate the effect of MPs uptake size 2 and 15 μm on *D. magna*, chronic toxicity tests (mortality and reproduction rate) were carried out for adults and (mortality, reproduction and growth rate) for neonates by exposing them to MPs in the presence of low and high food levels. This approach suggested ingestion selectivity of food particles by *D. magna.* The results indicated that the chronic exposure to high concentration of MPs size 2 μm in the presence of low food particles increased mortality and reduced number of offspring. while the 15 μm was more related to the availability of food. This results could be due to the higher concentrations used for MPs size 2 μm compare to the 15 μm. Detrimental impacts on life history traits of neonates (mortality and growth rate) were mainly linked to food concentrations rather than MP exposure and confirmed that *D. magna* selectively ingest food particles rather than MPs. These results agreed with other studies that have examined the chronic toxicity of polystyrene, polyethylene and pristine MPs in *D. magna* and show that toxicity mainly related to the availability of food (Ogonowski et al., 2016; Rehse et al., 2016; Rist et al., 2017) although some studies reported that the smaller particles size have higher chronic toxicity (Rist et al., 2017).

The work on MP toxicity in *C. pipiens* shows that adult weights and mortality were not impacted by MPs, and so the presence of MPs does not seem to influence the viability, development or nutritional uptake of mosquitoes. This was a surprise since there was an

assumption that MPs would be more likely to impact a holometabolous insect which undergoes a rearrangement of its tissues during metamorphosis compared to the *Daphnia* (Scriber and Slansky, 1981). The implication is that MPs are themselves not necessarily an issue for the organism but these results may not reflect the impact out in the natural environment where environmental conditions, competition, predation and resources are an issue. The studies presented here were undertaken to look at the impact of the MPs themselves and as such our results have been obtained with clean MPs that have not been exposed to any contaminants and under controlled laboratory conditions. Environmental MPs are likely to mix with other contaminants (de Sá et al., 2018) , some of which could bind to them and alter their toxicity, or external environmental factors may have different combined effect (Ma et al., 2016).

Another result to mention here is the oviposition data of mosquitoes given a choice between water with or without MPs (Chapter 7). There was no significant difference between treatments which suggests that the mosquitoes are not deterred from laying eggs in habitats with MPs. The implication here is that where MPs are present in the natural environment, they are unlikely to deter colonisation.

8.4 Microplastics effect on freshwater community under natural environment

Once in the ocean, many MPs sink to the sea bed (Rochman, 2018) and they are likely to behave in a similar way when in freshwater ecosystems (Ballent et al., 2016; Nel et al., 2018). Although it is known that MPs in the freshwater system are more abundant in the sediment than the water body, there remain few investigations of MPs under 300 mm because it's very difficult to analyse and quantify them with the current available methods (Hurley et al., 2018; Rehse et al., 2016).

The presence of small mesocosms at the University of Reading afforded the opportunity to research MPs in a more natural environment in order to evaluate the fate, abundance and biological effect of MPs on freshwater communities and to test whether methods applied in the laboratory to count the MPs would work with field collected samples. Microplastic detection was no more difficult than in the laboratory. The polystyrene MPs (15 µm) settled in the mesocosm sediment from the first week of exposure, and there were few MPs remain floating in the water column (Chapter 6). The weekly

examination of MPs in the gut system of *D. magna* and *Culex spp.* showed the presence of MPs in the gut systems and the ingestion of MPs by *D. magna* correlated with the abundance of MPs in the water body.

The MPs had little impact on the pond's community. *D. magna* was most effected by the presence of MPs in the mesocosms and show higher ecotoxicological stress than in the laboratory environment, especially in the first weeks of exposure. which could be due to the combined effect of MPs with other external factors such as increase in the temperature. Only one other study could be found to refer to determine the influence of temperature on MPs as an additional stressor, increasing the temperature had effect on the survival rate of *D. magna*, *Daphnia pulex* and *Ceriodaphnia dubia*, (Jaikumar et al., 2018). Research in the impact of MPs on the freshwater environment still limited. Most of the research investigated the impact of specific polymer type on model organism such as polystyrene (Jemec et al., 2016; Nasser and Lynch, 2016; Redondo-Hasselerharm et al., 2018; Rosenkranz et al., 2009; Scherer et al., 2017; Veneman et al., 2017), polyethylene (Rehse et al., 2016) or pristine particles (Martins and Guilhermino, 2018; Ogonowski et al., 2016) under laboratory controlled environment (de Sá et al., 2018). This doesn't represent what happen in the real natural environment.

8.5 Bioaccumulation of MPs on the food chain

Bioaccumulation of MPs is of real concern and has been shown elsewhere in marine environments (Botterell et al., 2018; Browne, 2015; Nelms et al., 2018; Setälä et al., 2014). However there is a paucity of information on bioaccumulation in the freshwater environment and how transference of MPs between trophic levels might influence e.g. predator consumptive traits (Wagner and Lambert, 2018). Predation can profoundly influence the structure of an ecosystem, impacting on its stability and function through determination of population growth and stability (Gilbert et al., 2014; Paine, 1980). Therefore it is important to understand how trophic interaction strengths and associated behaviours are influenced by MP pollution.

Transfer of MPs from mosquitoes to *C. flavicans* was via predation and counts were positively correlated with predation rates. Furthermore, attack rates, handling times and feeding rates of *C. flavicans*, which followed a Type II FR response, were unaffected by MPs. These results demonstrate that MPs can move through trophic levels whilst, in this

example, having no impact on either the predator or the prey. Although undertaken in the laboratory, these results are significant because we know that MPs are in the environment and are found in freshwater macroinvertebrates. A very recent study has demonstrated that 50% of macroinvertebrates, including mayflies and caddisflies, sampled from a waste water treatment plant in Wales contained up to 0.14 MP/ mg tissue (Windsor et al., 2019). The authors showed that presence of MP was independent of feeding guild or ecological niche and demonstrates that the problem is real and present (Windsor et al., 2019).

8.6 Conclusion and Future work

Plastic pollution is a critical environmental problem and yet the study of microplastics in freshwaters has only arisen in the last few years. There are many gaps in our knowledge, not least in basic information on their presence and distribution in the environment and the extent and relevance of their impacts on aquatic life. Surprisingly there is little data available on the chronic toxicity of different shapes and sizes of MPs collected from the aquatic environment or in mixtures with known freshwater pollutants such as pesticides. Interestingly there are few, if any, studies that use positive controls by exposing the *D. magna* to natural organic particles of similar sizes. With a move away from measuring the impact of MPs in the laboratory, more interest is likely to develop on the effect of environmental factors such as temperature on the impact of MPs. Arguably the most pressing issue is the use of standard methods and units to measure MPs concentrations, although this will be difficult to regulate given the large variation in technology used to detect and measure MPs (Li et al., 2018).

Given the interest in my work on ontogenic transfer, it is likely that it will open a new field of future studies to examine ontogenic transference of MPs in other aquatic insect larvae. An obvious follow up would be to look for insects with similar life history to mosquitoes and follow through to adults, collecting larvae or nymphs from known polluted waters where MPs have been found in the insect guts, an example being the recent study of macroinvertebrates in Wales (Windsor et al 2019). The transfer of MPs into terrestrial environments may follow routes other than predation, for example excretion of MPs from adult mosquitoes onto plants.

Whilst this is the first study to quantitatively link MP transfer with predation rates, work is required to further explore potential trophic accumulation of MPs. MPs are ingested by a range of other aquatic invertebrates and these interactions are likely to be studied in greater detail in the future (Nel et al., 2018; Windsor et al., 2019).

Finally, of increasing concern is if and how MPs may affect human health. This represents a major emerging issue since human populations have a high dependency on freshwaters for water and food. The near future is likely to see new policies and management tools being developed to attempt to combat the issue and it is important that these are informed by good quality research. The author hopes that the results, suggestions and ideas presented in this thesis will contribute to future research in the area of ecotoxicology of microplastics.

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