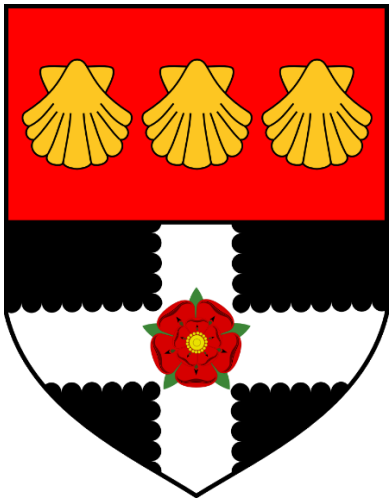

Impact of Microfibres on Feeding in the Freshwater Amphipod *Gammarus pulex*

A thesis submitted for the degree of

Doctor of Philosophy

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September 2021

Abstract

Microplastics (MP) are now a ubiquitous pollutant in aquatic environments, they can be manufactured (primary) or result from degradation of larger plastics (secondary). The most numerous MP found in aquatic environments are microfibres (MF), these MF occur from degradation of rope and textiles. A novel method for producing MF and exposing the amphipod *Gammarus pulex* to MF using algal wafers was devised, along with a protocol to compare feeding choices between two food options. Feeding choices between uncontaminated wafers and a variety of MF were investigated. It was found that all MF <50µm were ingested (acrylic, cat hair and cotton) were ingested, but none >50µm were (*Oris ovis* 70µm, Human hair 50-100µm). *Gammarus pulex* showed avoidance to feeding on acrylic MF when given a choice of uncontaminated wafers, this avoidance was not observed with either cat or cotton fibres. This implies that acrylic MF were either not recognised as food or were in some way repellent. When given the option between combinations of wafers contaminated with different fibres, *G. pulex* showed avoidance to acrylic when the other choice was either cat or cotton. When parasitised by *Polymorphous minutus, G.pulex* have been shown to alter their feeding behaviour. However, the same avoidance of acrylic MP was observed, although this avoidance was not statistically significant, no other impact from *P minutus* was observed. It was found that *G. pulex* would not ingest glitter in sizes 800 - 100µm. The ingestion of MF seems limited by size and composition impacts feeding preferences, with organic MF or no contamination being preferred to synthetic MF contamination. While feeding preferences were impacted, no MF had any impact on either growth or mortality during a 28-day exposure. The implications for these observed impacts upon *G. pulex* and the environment are further discussed.

Acknowledgements

I would like to thank many people for their assistance and support throughout my time conducting this research, without whom this thesis would not be possible.

Firstly, my wife Eilidh, who's unerring love and support has made it possible for me to spend the time and effort in completing this research, and tolerated all my detours to inspect water bodies for 'something interesting'. She was always happy to help wherever possible, sacrificing hair or acting as a sounding board, even during times of personal and professional difficulties.

My family as a whole, especially my parents for all of their help and advice throughout my life, who have always encouraged my interest in wildlife, and supported me going back into education. They have provided me not only the opportunities that have led to this, but also more hair to experiment with and time reading through work, making sense of my explanations.

My supervisor Amanda, for her seemingly unlimited patience and advise, teaching me not only new scientific and analytical skills, but also a greater appreciation of research, academia and life in general. Without the time you spent with me I am sure I would not have the publications and knowledge I now have, never misidentifying a lagomorph again!

To Glyn for his help as I attempted micro and molecular biology, and Eileen Harris from the Natural History Museum for her help in acanthocephalan identification.

Finally, to everyone from Suite 8, and the 11 o'clock coffee group, for your help, advise and companionship for the past four years, despite being an outsider geographically, you always made me feel welcome.

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List of Abbreviations

Acry	Acrylic
ANOVA	Analysis of Variance
ASTM	American Society for Testing and Materials
BFR	Brominated Flame Retardants
BPA	Bisphenol A
CAT	Catalase
Cott	Cotton
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
FTIR	Fourier-Transform InfraRed
GC	Gas Chromatography
HDPE	High Density Poly Ethylene
LC	Lethal Concentration
LD	Lethal Dose
LOEC	Lowest Observable Effect Concentration
MF	Microfibre
MP	Microplastic
MPF	Micro Plastic Fibres
MS	Mass Spectroscopy
NOEC	No Observable Effect Concentration
NP	Nano Plastic

OEC	Observable Effect Concentration
OECD	Organisation for Economic Co-operation and Development
OMF	Organic Micro Fibres
PA	Polyacrylamide
PCBs	Polychlorinated Biphenyls
PCR	Polymerase Chain Reaction
PE	Polyethylene
PET	Polyethylene Terephthalate
PHB	Polyhydroxy Butyrate
PLA	Polylactic Acid
PMMA	Polymethylmethacrylate
PP	Polypropylene
PPC	Polypropylene Carbonate
PPM	Parts Per Million
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinylchloride
RO	Reverse Osmosis
RPM	Revolutions Per Minute
SEM	Scanning Electron Microscope
SOD	Superoxide Dismutase
TBT	Tributyltin

UK	United Kingdom
USA	United States of America
UV	Ultraviolet
WG-GES	Working Group on Good Environmental Status
WWTP	Wastewater Treatment Plant

CHAPTER 1.

Literature Review

1.1 Plastics

Plastic is a term used to describe a broad range of substances, of which a hydrocarbon polymer is the main constituent. While plastics can be synthetic or semi-synthetic, the majority use fossil fuels as the basis for their polymer chains, although increasingly organic elements are being incorporated (Rujnić-Sokele and Pilipović, 2017). Other additives are incorporated into plastics, while not being chemically bound to the polymer. Additives include antioxidants such as nonylphenol, and bisphenol A (BPA), softening agents (plasticisers) nonylphenol and phthalates and flame retardants, including brominated flame retardants (BFR) (Hermabessiere et al., 2017). While the origin of plastics can be traced to the late 19th century, those products were easily degraded and lacked malleability (Knot and Mulder, 2001), the invention of Bakelite in 1907 is often considered the start of the plastic age (Porta, 2021).

The use of plastics has accelerated continuously throughout the past century, because of their advantages when compared to traditional materials, such as wood and metal. They are lightweight, cheap and quick and easy to form into almost any shape and as they are traditionally non-degradable or reactive, they seem to be non-toxic and suitable for food storage (Shashoua, 2012). Inevitably, due to their inherent usefulness to society and as the amount manufactured increased, so too did the amount of plastic-waste generated, currently around 10-15% of all waste generated is plastic (Idumah and Nwuzor, 2019). While 78% of plastic waste are thermoplastics, which can be recycled by being reheated and

reformed, the reality is that the vast majority of this is not recycled. This can be because of additives within the polymers, items being unclean or simply the infrastructure being insufficient to reclaim suitable items (Gu et al., 2017). Non recycled plastics are either incinerated or disposed of, most often in landfill (Gu et al., 2017). While the use of landfill is reducing across Europe and landfill is now being mined, for plastics suitable for recycling, this is not the case globally (Canopoli et al., 2018). One reason for the reluctance to continue dumping plastics in European landfills, is due to their persistence in the environment and the leaching of additives and plastics into the soil and water courses (Nurhasanah et al., 2021). While plastics are resistant to degradation, landfills are often hot, exposed to pH ranging from 4-9 and physical stress (He et al., 2019). As a result, they provide the physical and chemical conditions which greatly increase degradation and cause the formation of microplastics, this can be further exacerbated by the presence of microbes such as *Aspergillus*, which can use polymers as a carbon source (Zahra et al., 2010).

Most plastic in freshwater systems are from incorrectly disposed of litter, with packaging forming around 75% of riverine plastic debris (Schwarz et al., 2019). Rivers often have very high plastic pollution rates, due to their proximity to large human populations and the industry and waste that goes along with them (Best, 2019). The physical properties of the litter, along with the water flow-rate, will often determine its fate. Litter with a greater density, or in slow water, will be more likely to settle and embed in the sediment. Conversely less dense litter, or litter with a larger surface area to volume ratio, in faster water, will be more likely to be transported downstream (Castro-Jiménez et al., 2019; van Emmerik et al., 2018). From rivers, through to estuaries, plastic pollution is often discharged into the marine environment. While much of this plastic gets sequestered into coastal

sediment, a portion is also released into the open ocean (Duis and Coors, 2016; Lebreton et al., 2017; Xu et al., 2020). Estimates, for the mass released globally, range from 4.8 to 12.2 million tons (Law, 2017).

Plastics in the marine environment have been studied for longer than in freshwater, yet the topic is still under-researched, especially in the deeper and more remote regions (Law, 2017). One of the key difficulties of estimating plastic pollution, in the marine environment, is that there is no standardisation of sampling methods, different methods being optimised for collecting different MP, and the sheer volume of the marine environment makes any significant sampling unfeasible (Chiba et al., 2018; Liu et al., 2019; Pravettoni and Rekacewicz, 2019). While the proportion of plastic pollution from different origins varies, the most common source is from mismanaged coastal waste (8mT per year), followed by mismanaged inland waste (2mT per year), primary microplastics used in manufacturing (1.5mT per year) and discarded fishing equipment (0.6mT per year) (Billard and Boucher, 2019; Law, 2017).

Plastic litter has been broadly classified by its size, although different groups have used different definitions they generally agree on the terminology, for example, mega (>1m), macro (<1m), meso (<2.5cm) and micro (<5mm) (J. Wang et al., 2018). Alternatively mega (>100mm), macro (20-100mm), meso (20-5mm) and micro (<5mm) (Barnes et al., 2009). There have been two main risks to aquatic organisms from plastic litter, ingestion and entanglement (Nicolau et al., 2016). Ingestion of plastic litter can cause damage to the gastrointestinal tract, fill the stomach with indigestible material (Nicolau et al., 2016) or clog the respiratory system, causing suffocation (Thushari and Senevirathna, 2020). The other mechanism for harm is entanglement, most obviously from discarded fishing equipment,

this can hamper or prevent animals from being able to swim, thereby impairing their hunting or ability to escape predators (Ryan, 2018). However, this entanglement can also lead to suffocation or respiratory failure in organisms with gills or lungs, by preventing them reaching the surface or passing adequate water across their gills (Derraik, 2002; Galgani et al., 2018; Ryan, 2018).

1.2 Biodegradable and Compostable Plastics

An ever-increasing area of research and development in is biodegradable and compostable plastics, this is because they are advertised as having many of the advantages of traditional plastics, without the detrimental impact upon the environment (Iwata, 2015a). Biodegradable plastics are those which 'are capable of undergoing decomposition into carbon dioxide, methane, water, inorganic compounds, or biomass in which the predominant mechanism is the enzymatic action of microorganisms, that can be measured by standardized tests, in a specified period of time, reflecting available disposal condition' (American Society Testing and Materials standard D6813). Beyond this, a compostable plastic is one which is 'capable of undergoing biological decomposition in a compost site as part of an available program, such that the plastic is not visually distinguishable and breaks down to carbon dioxide, water, inorganic compounds and biomass, at a rate consistent with known compostable materials (e.g. cellulose)' (ASTM standard D996) (Song et al., 2009). While these seem to be a near perfect solution, several studies have shown that the observed degradation of these plastics varies greatly from what is advertised and that some biodegradable plastics, particularly those which require high concentrations of oxygen, will not biodegrade in aquatic environments (Müller et al., 2012; Nauendorf et al., 2016; Brine and Thompson, 2010). There have also been questions raised about whether the inorganic

chemicals which leach from biodegradable plastics, present a novel form of toxicity (Degli-Innocenti et al., 2001). If this is the case then it is possible that biodegradable plastics cause toxic additives to become more bioavailable than their non-degradable counterparts.

1.3 Microplastics

The typical criticism of plastics has been with macro plastics and the images of animals choking on and becoming caught up in them. The light has now been drawn to microplastics (MPs) which are small plastics, generally accepted as those less than 5mm (Cole et al., 2011), although other definitions have been suggested (covered in Chapter 3). These MPs are, unsurprisingly, far more numerous than larger plastic litter (Eerkes-Medrano et al., 2015) and have been found in all marine and freshwater environments (Costa and Barletta, 2015; Khan et al., 2020; Yin et al., 2019). Microplastics are a highly diverse group of pollutants, their structure and chemistry are dependent upon their origins. One distinction is between primary and secondary MP, primary being manufactured as microplastics for use in industry for plastic products (Auta et al., 2017) or as exfoliants in cosmetics (Crawford and Quinn, 2017), these are most often encountered as regular and spherical. Secondary MP are the result of degradation of other plastic products, and can be far more varied (Ogonowski et al., 2016).

When plastic bags, wrappers or thin containers degrade they produce thin, irregular MP with a relatively high surface area. The most common polymer in films is polyethylene (PE) both low and high density (Kalogerakis et al., 2017), the surface area they provide can act as a substrate for microbes (Arias-Villamizar and Vázquez-Morillas, 2018; Huang et al., 2019; Zahra et al., 2010). While most studies into microbes and microplastic films have been

conducted in terrestrial soil, marine benthic pathogens have also been shown to attach to MP and use them as a substrate, increasing their bioavailability (Bowley et al., 2021). In the same way that films are transported in water currents they are also easily transported in air currents, they are the second most frequent shape, after fibres, deposited by air currents (Enyoh et al., 2019).

Plastic micro fibres (MF) are long, thin MPs which are more than twice as long as they are thick. They most commonly result from the degradation of textiles, which are now predominantly synthetic, rather than cotton (Suaria et al., 2020). Fibres are the most numerous MP found in the marine environment, particularly in subsurface water (Desforges et al., 2014; Kanhai et al., 2018; Reineccius et al., 2020; Suaria et al., 2020) comprising up to 95% of MP and most commonly PE (Kanhai et al., 2018). Despite their ubiquitous nature and the fact they dominate the textile market, they have been found to be only 8% of oceanic fibres, with 80% being cellulose based and 12% being animal fibres (Suaria et al., 2020). The other main origin of MFs is through discarded fishing equipment, both nets and line, although it is estimated that these marine originating MF constitute less than 20% of total oceanic MF. The 80% of terrestrial based MF mainly originate from the washing of textiles, both industrially including manufacturing, and domestically.

Fragments, as the name suggests are hard, angular and irregular MP which are usually formed by the degradation of larger hard plastics, most commonly PE, polypropylene (PP) and polyethylene terephthalate (PET) (Capone et al., 2020; Tanaka and Takada, 2016), after fibres they are often the second most frequently ingested MP found in the guts of fish (Khan et al., 2020; Sanchez et al., 2014; Tanaka and Takada, 2016). They can also be primary MP,

where they are deliberately shredded to be used as exfoliating agents, because they had been deliberately manufactured to be like that (Kalčíková et al., 2017).

Foams are similar to fragments, the difference being they are low density and softer, they are most often polystyrene (PS), specifically expanded PS which due to it being soft and buoyant is particularly susceptible to degradation (Song et al., 2020). Being buoyant they are more exposed to ultraviolet (UV) radiation than other MP, and they also are more susceptible to mechanical wear in tidal regions, where they can release additives, but also due to their surface area are highly prone to adsorbing toxins (Zhang et al., 2018).

1.4 Sources of microplastics in the aquatic environment

Freshwater provides, either directly or indirectly, all four ecosystem services; provisioning, supporting, regulating and cultural (Aylward et al., 2005). Due to our reliance on it, economic valuing of freshwater is near impossible (Liu et al., 2010) yet the human population has contaminated it throughout history.

As a result of the popularity of plastics there are many sources of MP in the environment, for example wastewater treatment plants (WWTPs). These plants can be either domestic, industrial or a combination of both, where hazardous industrial waste is isolated and the rest is sent to a municipal treatment plant (Munter, 2003). Industrial sources of MP include microbeads, used directly in the production of plastic products and many processes use MP as a disposable resource (European commission, 2011). Other sources include microbeads used in cosmetic and scientific products (Ziajahromi et al., 2017) (Lasee et al., 2017) as well as MF from the washing of fabrics, be that industrial or domestic (Ziajahromi et al., 2017). Very recently the impact of glitter has been considered by several

organisations, who have banned the use of glitter in ‘arts and crafts’. Another source of glitter is cosmetics, where the particle size is generally much smaller <150µm as opposed to the >1000µm which can occur in craft glitter (Aardahl et al., 2005).

While it does seem evident that WWTPs are a source of MP discharged into water bodies (Murphy et al., 2016) given that the concentrations of MF and MP are significantly higher further downstream of WWTPs (Estahbanati and Fahrenfeld, 2016), it should also be noted that they are highly effective at removing MP. The primary and secondary settling processes within water treatment have been shown to remove up to 99% of MP (Ziajahromi et al., 2017)(Murphy et al., 2016), therefore, while they are a source into the ecosystem, they also play an enormous role in removing MP from effluent. Even this removal is not necessarily truly protecting the environment, as many WWTPs sell the slurry produced, from primary and secondary settlement, to agricultural businesses, where it is used as fertiliser (Stumpe et al., 2012). The high concentrations of plastics in slurry then becomes bioavailable in the terrestrial environment instead. It is well documented that agricultural run-off is an important factor in eutrophication of water ways (Poudel et al., 2010), therefore, it is reasonable that the same run-off could carry MP back into the aquatic environment. Effluent from WWTPs is easy to analyse, as it can be collected from pipes at regular intervals and flow rates are closely monitored, allowing accurate calculations of concentration (Leslie et al., 2017), however, run-off from slurry means that contamination could occur, at any point along a river close to agriculture.

MP run-off can also be caused by driving, as tyres are gradually worn down with use, they leave MP on roads. In the United Kingdom alone an estimated 63,000 tonnes of plastic per year are lost from tyres. Depending on where the road is, this loss will either be washed

to municipal WWTPs, or simply run-off into the environment (Jan Kole et al., 2017). A less obvious source of MP is atmospheric fallout (Dris et al., 2016), whereby plastic MF are transferred from their source by air movement. This atmospheric fallout can be as high as 313 particles / m^2 / day (Cai et al., 2017), for a major river such as the Thames with a surface area greater than 215,000,000 m^2 (Borges et al., 2004) this equates to a huge number of particles per day. Atmospheric fallout also means that ponds and lakes are susceptible to MP contamination, especially in urban environments (Magnusson et al., 2016).

1.5 Toxicology

Toxicology can be defined as 'the testing of substances for adverse effects to humans and the environment' (Hartung, 2009). While a simple definition, it encompasses a broad range of adverse effects. The most obvious and drastic, adverse effect is death. However, there are several ways to investigate how much of a substance it takes to kill; the most frequently used measure is the LD_{50}. The LD_{50} of a substance refers to the Lethal Dose, or the amount of a substance that kills 50%, or the median, of tested organisms (Bellas, 2007; Chaumot et al., 2015; Duke and Powles, 2008; Farré et al., 2008). The 50% is used because natural variation between individuals may result in some organisms being particularly susceptible, or resistant to the substance, as such the minimum dose needed to kill all organisms, and the minimum dose to kill one may be vastly different. That being said, the lethal dose for 1% of test organisms (LD_1) has been used if exposure is particularly likely (Karanth et al., 2004). The LD_{50} of any substance will likely vary between different species, as such they are often investigated for a range of species from mice to, at times, humans (Bailey et al., 2014). Tributyltin (TBT) used as an antifouling agent, is more bioavailable to invertebrates and fish, as it can be absorbed through their gills, therefore, becoming toxic at

lower concentrations than to other vertebrates, which are exposed through ingestion (Guardiola et al., 2012; Kotrikla, 2009; Lilley et al., 2012). Unfortunately TBT was also found to bioaccumulate and had an impact on the embryonic development of vertebrates (Antizar-Ladislao, 2008).

There are also variations in LD_{50} within individual animals, for example the lethal dose of morphine is different if it is ingested, given intra muscularly or intra venously (Powers et al., 2018), other exposure including skin and gill contact are also used. Even within a species, with a consistent exposure method, there are often differences in toxicity, most importantly is life stage; juveniles can often be more vulnerable to substances than adults (Adam et al., 2010a; Karbalaei et al., 2021; Lilley et al., 2012). Overarching all of the above is time, the LD_{50} often varies between acute and chronic exposures. There is much disagreement around the definition of chronic and acute, some define acute as 4h some as 24h or even 48h (Zaitsu et al., 2016) and so the definitions are largely arbitrary, however, as the actual exposure time is always given, this is an objective measure to use.

Most frequently the dose is recorded as mass of substance per unit mass of organism, for example mg kg^{-1}, however, if the organism is exposed continuously, for example through the air or water then this concentration is often used, without indication of per unit mass, for example parts per million (ppm) or mg ml^{-1}. When this concentration is used, it is referred to as lethal concentration or LC rather than LD.

While death is the most obvious adverse effect there can be many more, all of which are impacted by the variables described above, be it species, exposure method, life stage or exposure time. However, all else being equal, the impact on different organs or processes

may still be vastly different at different concentrations (Cikcikoglu Yildirim and Yaman, 2019).

Growth is commonly studied as a sublethal effect, because it is easily measured and is an indication of overall health (Bellas, 2007), if growth is reduced or increased, compared to a control, then it is evidence of some effect from a substance. Growth is not just an indicator of underlying effects, it is itself essential, many animals cannot reproduce until they attain a certain size (Clarke et al., 2013). Even if an animal is capable of reproduction, size is often a determining factor in intraspecific competition for mates and food sources (Bailly et al., 2018; Graça et al., 2001), as well as providing a degree of defence against predators (Frederiksen et al., 2006). Growth can be measured simply, by tracking the change in mass or length (Chambers et al., 2006; Critchell and Hoogenboom, 2018; Thomas et al., 2020), although a variation, assimilation efficiency, involves comparing the amount of food ingested and how much growth it leads to (Straub et al., 2017).

Behavioural changes have also been measured, and quantified as a sublethal effect. While any behaviour can be assessed, commonly investigated behaviours are feeding, mating and movement (Agatz and Brown, 2014; Anbumani and Kakkar, 2018; Kunz et al., 2010; Marriott et al., 1989) it is possible to investigate multiple behaviours, although the majority of studies only focus on one (Lee, 2000). Even a simple behaviour, distance, swam can become complicated, the water flea *Daphnia magna*, when exposed to nicotine, shows a significant reduction in movement for 20 min but then an increase in movement after 70 min (Zein et al., 2014). A reduction in movement can indicate a reduction in energy or general condition, in the environment this can make animals more prone to predation or less efficient predators (Sanches et al., 2018). Increases in movement are not necessarily

beneficial, when the carp *Labeo rohita* is exposed to the insecticide malathion they were found to become increasingly active, however, this movement was erratic and suggested a stress response (Patil and David, 2008). These impacts can be intentional, for example the feeding behaviours of the mosquito *Aedes aegypti* are reduced by 90% when exposed to d-allethrin (Lee, 2000).

There are various stress responses that can be used to assess sub lethal effects. As described above, behavioural modifications can be an indicator, but they can be difficult to quantify. Biochemical stress responses, while they may be more technically difficult to measure, can give clear, quantifiable markers (Ighodaro and Akinloye, 2018). Oxidative stress is found in both plants and animals, as is the antioxidant catalayse (CAT) and superoxide dismutase (SOD), the levels of these enzymes within tissue have been used to measure stress (Cikcikoglu Yildirim and Yaman, 2019; Yu et al., 2018). Fluctuations in these markers can be detected at lower concentrations and earlier than physical or behavioural changes can be identified.

Similar techniques can be used to investigate hormonal changes and gene expression. One of the most drastic impacts that have been found, during toxicology studies, have been on hormonal changes. Male fish have been shown to become feminised when exposed to oestrogens, meaning they develop female sexual characteristics (Huggett et al., 2002). It is not only hormones which can disrupt endocrine receptors, feminisation has also been observed in *Xenopus laevis* when exposed to polychlorinated biphenyls (PCBs), although in this case they also became demasculinised, where male characteristics are reduced (Qin et al., 2007). While these impacts have been found in adult organisms, juveniles are even more

sensitive to changes in their endocrine system and total sterility, or even change in sex can occur (Harvey and Everett, 2006).

In the case of these sublethal effects, LD_{50} is not the standard measurement, instead observable effect concentrations (OECs) are used. The most commonly used OECs are no observable effect concentration (NOEC) the highest concentration at which no effect could be observed, and lowest observable effect concentration (LOEC) being the lowest concentration at which an effect could be observed (Kooijman et al., 2009). As with LD and LCs, not all organisms will be effected in the same way, therefore $NOEC_{50}$ $LOEC_{50}$ can be used for when an effect is or isn't observed in 50% of the test organisms (Marinković et al., 2011). In theory, the NOEC and LOEC are almost identical and immediately either side of a value, however, until that point is discovered, they provide current limits to concentrations known to be safe, or unsafe.

Ecotoxicology is a branch of toxicology that focusses particularly upon the toxicology of substances in the environment and combines toxicology with ecology (Anbumani and Kakkar, 2018). One of the key factors in ecotoxicology is the trophic transfer of substances, from lower trophic levels to the higher, explaining the need to understand the food web, in order to identify which organisms are most at risk (Wang et al., 2019). Substances do not only transfer from prey to predator, but they can accumulate as they rise up the trophic levels, until they reach an OEC or LC. There have been some high-profile examples of this, diclofenac is a non-steroidal anti-inflammatory drug used for both humans and livestock, in mammals its toxicity is well understood. Even a low therapeutic concentration for a mammal (0.25 mg kg^{-1}) was found to cause fatal renal failure in oriental white-backed vultures (*Gyps bengalensis*), as the main food source for vultures was dead livestock they

rapidly ingested lethal doses of diclofenac, resulting in a population drop of 95% (Green et al., 2004; Oaks et al., 2004; Shultz et al., 2004). As a result of this discovery, diclofenac has been investigated in Africa, where a similar effect has been noted (Naidoo et al., 2009), in the Indian subcontinent, legislation restricting the use of diclofenac has resulted in local recovery of vulture populations (Prakash et al., 2012).

A similar case was found in the use of the insecticide dichlorodiphenyltrichloroethane (DDT), whereby since the 1970s it was observed that the populations of predatory birds were reducing, it was found that DDT had bioaccumulated through lizards which fed on dead insects, to toxic levels within birds of prey (Chu et al., 2006; Turusov et al., 2002). It was also found that a sublethal effect of DDT on birds was a thinning of their egg shells, resulting in reproductive failure (Hellou et al., 2013).

1.6 Use of macroinvertebrates in ecotoxicology

Macroinvertebrates are an essential element of the aquatic environment, as they are often low in the trophic levels, they can be some of the first animals to be exposed to pollutants. They are also the main dietary constituent of many fish species in both marine and freshwater environments (Kooijman et al., 2009; Levengood and Beasley, 2007). They are easy to observe and manipulate and have relatively short life cycles, allowing simple studies into mortality and intergenerational effects (Wallace and Webster, 1996). Invertebrates have a far wider range of functional feeding groups, when compared to vertebrates. Different functional feeding groups are more prone to different contaminants (Chaumot et al., 2015).

Filter feeders, such as mussels, are found in both marine and freshwater and are commonly used for toxicology studies (Angarano et al., 2007; Farrell and Nelson, 2013a; Leslie et al., 2017), mussels are also frequently used because they are directly used as a human food source. Due to their feeding mechanism, they are particularly susceptible to small suspended solids, which do not settle, or other pollutants which can adsorb onto these solids (Khandeparker and Anil, 2007). Antifouling products are used to prevent the growth of microbes and invertebrates on the hull of ships, many of them are self-polishing. In this the antifouling agent, a toxin, is contained within a matrix that slowly degrades, revealing more antifouling agent underneath, this lengthens the time before repainting is needed. As a result there are constant small solids, contaminated with a known toxin, falling on the benthos of harbours (Dafforn et al., 2011; Fitridge et al., 2012). These pollutants are of particular risk to filter feeders and grazers (Marcheselli et al., 2010).

Grazers, or scrapers, feed upon the biofilms that grow on substrate, be that sediment such as sand or mud, solid surfaces like rock and concrete or directly upon plants and algae (Wallace and Webster, 1996). The majority of gastropods are scrapers and found in the marine, freshwater and terrestrial environments (Akindele et al., 2019; Bal et al., 2017; Mohammad et al., 2021; Rothmeier et al., 2020). Scrapers are susceptible to pollutants that settle on the surfaces they graze, or that are incorporated into the biofilms they feed upon, bacterial tolerances can be far higher than invertebrates and bioaccumulation can occur within biofilms and plants (Geng et al., 2019; Harrison et al., 2004). As these biofilms are able to sequester contaminates, ecosystems which experience a high degree of pollution, such as harbours and waterways in industrial regions, have higher concentrations of contaminates within biofilms (Arnold et al., 2017; Walden and Zhang, 2018). These

contaminants need not only be chemically sequestered, antifouling products and MP can also be physically sequestered within the film (Wu et al., 2019; Yang et al., 2020).

Collectors search through sediment or substrate for edible material. These organisms can live on the benthos, such as the mayfly larvae *Baetis rhodandi* (Kelly et al., 2002a) which collects food from gravel and the surface of sediment. Collectors can also burrow and live within the substrate itself, for example oligochaetes (Huerta Lwanga et al., 2016). Burrowing collectors are particularly susceptible to contaminants which have become sequestered deep within sediment and no longer bioavailable to other feeding groups (Scherer et al., 2017a; Wagner et al., 2014).

Shredders use mouthparts or other appendages to break up detritus or living plant matter, allowing them to be ingested, as with other feeding groups, there are both marine and freshwater examples of shredders. Much of the detritus freshwater shredders feed upon is terrestrial in origin and so represents a route for terrestrial pollutants to enter the aquatic environment (Berenzen et al., 2005; Kunz et al., 2010). Due to their ability to process detritus, they often have varied diets and constitute a large portion of freshwater invertebrates (Kelly et al., 2002b; Pereira et al., 2017; Wallace and Webster, 1996). Many malacostraca are shredders, including amphipods, decapods and isopods, they are particularly suited to this role, due to the anatomy of their mouthparts and many have gnathopods which can assist in the processing of detritus (Štrus et al., 2019). Due to their varied diets and abundant nature they have often been used in ecotoxicological studies, especially those focussing on freshwater system (Auber et al., 2011; De Lange et al., 2006; Joyce et al., 2007; Kampfraath et al., 2012; Kelly et al., 2002b).

Finally, there are predators, which eat other animals, both invertebrate and vertebrate. Many predators are also scavengers and will feed on the carrion of large vertebrates, that may have bioaccumulated contaminants. This scavenging has the potential to re-expose lower trophic levels and magnify the concentration of contaminants (Elliott et al., 2014). Some large invertebrates, such as cephalopods, are truly predatory and so are directly at risk of bioaccumulated toxins. Cadmium, mercury and aluminium have been found in very high concentrations in cephalopods, at times above the safe limit for human consumption (Sangiuliano et al., 2017; Storelli and Marcotrigiano, 2004). The distribution of contaminates is far from homogenous and many seem to accumulate in the digestive glands, in the case of cadmium concentrations can reach 3500µg g^{-1}, which would be fatal to many organisms (Penicaud et al., 2017), this seems to be an adaptation in these predators.

1.7 *Gammarus pulex*

The *Gammaridae* are a family of crustaceans commonly used for the study of ecotoxicology. They are fairly speciose and individual species are numerous within different environments, making them highly environmentally relevant taxa (Armitage et al., 1995; Chaumot et al., 2015; Kunz et al., 2010).

In the UK *Gammarus pulex* is the most common of the freshwater Gammarids; it is numerous in most streams and rivers and is particularly prevalent in chalk streams (McGrath et al., 2007). As well as occupying a range of habitats, *G. pulex* also occupy at least two functional feeding groups, while predominantly shredders, which are drawn to the fungi and bacteria on decomposing leaves (De Lange et al., 2005a), they can also be voracious predators, targeting the mayfly *Baetis rhodani* (Kelly et al., 2002a), as well as exhibiting

cannibalistic tendencies (McGrath et al., 2007). Due to their diets, *G. pulex* are often exposed to natural fibres, particularly cellulose fibres, but also animal-based fibres from detritus.

The average life cycle of *G. pulex* is around 12 months and they reach sexual maturity at around 130 days. This is longer than other model organisms such as *Daphnia magna* and *Artemia marina* and unlike these species, there is no way to control when eggs will hatch, as they do not produce an ephippium (Daam and Rico, 2018; Jaikumar et al., 2019). However, there are methodologies using gravid females to produce groups of known age, essential if tests are undertaken upon juvenile individuals (Bloor, 2010).

1.8 Ecotoxicology of Microplastics

MP ecotoxicological research has focussed mainly on the marine environment (Eerkes-Medrano et al., 2015; Wagner et al., 2014) and particularly around estuaries. Much of the research has been based around whether plastic particles are present within the tissue of various taxa (Peng et al., 2017). Several of these studies show that a wide range of taxa, including molluscs, arthropods and vertebrates, from around the globe, have been exposed to plastics and they have become incorporated into the tissue and therefore, the food web (Farrell and Nelson, 2013b; Kolandhasamy et al., 2018; Sanchez et al., 2014). However, studies into how the presence of these particles impact the individual organisms have differing results.

Some find that MP increase mortality or negatively impact development or endocrinology of both vertebrates and invertebrates (Huerta Lwanga et al., 2016; Oliveira et al., 2013). The 10d LC_{50} of isopod *Hyalella azteca* to PE particles <27µm was found to be

4.6×10^4 microplastics/mL, while for PP fibres it was only 71 fibres per ml (Au et al., 2015). A study into the ingestion of 10µm PS spheres by the caddisfly larvae *Sericostoma pyrenaicum* found that mortality was significantly increased when the concentration of MP was greater than 1800 parts per ml (López-Rojo et al., 2020).

Other studies show that plastics are largely inert within the body and apart from the space they take up they have limited impact upon the animals (Rainieri et al., 2018a; Weber et al., 2018). This should not be particularly surprising as many plastics are used medically within the body, for the very reason that they are non-reactive and have an extremely high persistence. However, a more consistent finding is that a compound combination effect is seen between plastics and other toxins such as heavy metals or antibiotics (Fonte et al., 2016; Kim et al., 2017; Oliveira et al., 2013; Rainieri et al., 2018a). It has been shown that, in some cases at least, MP act as a vector for toxic substances to enter the body through adsorption (Brennecke et al., 2016) it is, therefore, essential that their relationship with common contaminates is well understood, as this could impact what are considered safe concentrations of toxins to the environment.

Some species seem able to actively avoid MP, the waterflea *Daphnia magna* are functional filter feeders, when exposed to 2µm PS beads they were readily ingested. However, if their natural food of algae was present there was a reduction in ingestion of MP, even when the concentration of MP was increased (Aljaibachi and Callaghan, 2018a). This is significant because if organisms choose to avoid MP then the probability of harm is reduced.

A particularly worrying aspect of MP pollution is that there is extraordinarily little known about their fate in the environment (Anbumani and Kakkar, 2018; Paul-Pont et al.,

2018) due to their high persistence, it is possible that high concentrations will bio accumulate with fatal consequences to the organism, which then transfers the MP back into the food web via scavengers and so again bio accumulate in apex predators. As many large marine predators are endangered or threatened by human hunting, (Hellberg et al., 2019) it is possible that this added pressure from MP could push many species towards extinction.

1.9 MP ecotoxicology in gammarids

Lethal effects of MP to *G. pulex* or other gammarids have not been observed, despite being investigated numerous times (Lebrun et al., 2012; Mateos-Cárdenas et al., 2021, 2019; Redondo-Hasselerharm et al., 2018a; Scherer et al., 2017a; Weber et al., 2018). Sublethal effects have, however, been observed by numerous studies.

Gammarus fossarum, another species of freshwater gammarid were fed both biodegradable polyhydroxybutyrate (PHB) and the petroleum based polymethylmethacrylate (PMMA) in the size range 32-63µm, at concentrations 10 – 100000 particles per individual. It was found that MP were ingested proportionate to their concentration, and that there was no difference in the rate of ingestion or egestion between the two types of MP. What was identified was a difference in assimilation efficiency, whereby ingestion of PMMA lead to a decrease in assimilation efficiency compared to both the control and PHB. It was hypothesised that this was due to the PHB providing a food source, where the PMMA did not, and so lowering the quality of the food rather than an interaction with the PMMA (Straub et al., 2017).

Gammarids will readily ingest both natural and synthetic fibres, with no impact on survival, *Gammarus duebeni* was exposed to 60µm lengths of cellulose and PE fibres at 600

fibres per ml, and it was found that there was no difference in the ingestion between the natural or synthetic fibres and no difference in survival after 96h of exposure (Mateos-Cárdenas et al., 2021).This was an acute exposure, and so growth was not measured, but if there was a chronic exposure then an impact on growth may have been identified.

While *Gammarus pulex* has been shown to readily ingest MP, size does seem to play a role in ingestion preference, one study has suggested that they actively prefer larger MP, finding more 90µm PS beads were ingested than 10µm, and ingestion was proportional to concentration (Scherer et al., 2017b). It should be noted that this is unusual, and no other study has found MP ingestion of this size in *G. pulex*.

As with MP, nanoplastics (NP) are ingested, proportionate to concentrations in sediment, and similarly they have no impact on growth or mortality y (Redondo-Hasselerharm et al., 2021). One study on the closely related *G. fossarum*, which occupies a similar ecological niche in continental Europe, did show that ingestion of MF negatively impact the assimilation efficiency, however, this could be due to the use of fibre concentrations at 2680 fibre per cm^2 (Blarer and Burkhardt-Holm, 2016a). Given that the maximum environmental concentration found in freshwater is 40-100 fibres per cm^2 (Hanvey et al., 2017a) the use of such high concentrations seems somewhat excessive.

Perhaps most crucially, these species make up a substantial portion of the diet for both invertebrates and vertebrates, this means that they provide an ideal vector for MP to enter the food chain and any potential trophic effects within freshwater and potentially into the marine environment (Aditya and Saha, 2006; Ahlgren et al., 2011; Hölker and Stief, 2005).

1.10 Aims and objectives

The overarching aim of this research was to identify how the presence of MF, both plastic and natural in food sources, impacts the feeding activity of *G.pulex.* The following objectives were conducted in order to build understanding and ultimately achieve the thesis aim.

1) It has been shown that some invertebrates are able to avoid ingesting MP when given the option of food sources, however, nothing has been done to investigate ingestion of MP when invertebrates are given a choice. In order to investigate this, the difference in ingestion of microfibres by *Gammarus pulex*, with and without a choice of uncontaminated food was studied.
2) When exposed to pollutants, some animals have been shown to modify their movement and feeding behaviours. As there seems to be little evidence of mortality from MP the sublethal behavioural effects were studied. This will be acheived by observing the difference in feeding behaviour of *Gammarus pulex* when feeding on food sources with and without microfibres contamination,
3) It has been shown that there is no acute difference in ingestion and survival between natural and synthetic fibres, but previous studies have shown a reduction in growth when Gammarids were exposed to synthetic MP and MF. Therefore, the difference in ingestion and behaviour of *Gammarus pulex* when feeding on organic or plastic microfibres will be investigated.
4) A commonly used type of MP is glitter, used both in cosmetics and in decorations. They have been banned by several organisations due to concerns over their impact

on the aquatic environment. As they are relatively large MP it will be investigated as to whether they will be ingested by *G. pulex*.

5) During the course of my research, I noticed that a number of *Gammarus* were infected with a parasite. Given that parasites are known to sometimes change the behaviour of their hosts, and that invertebrates feeding behaviour was found to be impacted by MFs, this was investigated in relation to the ingestion of MFs.

1.11 Layout of the Thesis

The following six chapters comprise a more in-depth review of microplastics, an explanation of the common methodologies used in experimentation, three experimental chapters and a general discussion and conclusion. The in-depth review is Chapter 2 and is a review on microplastics in tropical freshwater, which has been accepted as a chapter in a textbook. The methodologies detailed in Chapter 3 will be common to the three experimental chapters, however, the process by which these methods were developed will also be discussed. The first experimental chapter is Chapter 4 and is a published article, in this the impacts of plastic microfibres on the feeding behaviour of *G. pulex* are discussed as well as publishing the main methods developed and used throughout the thesis. Chapter 5 is an article accepted for publication and explains the difference in ingestion of a range of microfibres, both synthetic and organic, by *G. pulex*. The differences in feeding behaviour when exposed to these fibres are also identified and finally the impact of chronic ingestion of these fibres on the growth and mortality of *G. pulex* are investigated. In Chapter 6 the combination of *Acanthocephalan* parasitism and plastic microfibres upon the feeding behaviour of *G. pulex* are explored. The final experimental chapter, Chapter 7, explores the ingestion of glitter of different sizes and with various lengths of starvation of *G. pulex*. Finally, Chapter 8 is a general discussion and conclusion. Because the experimental chapters of this thesis are presented as papers, there is some repetition of content, especially materials and methods.

CHAPTER 2.

Microplastics in freshwater ecosystems with special reference to tropical systems; detection, impact, and management.

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Yardy, L., Al-Jaibachi, R., Callaghan, A. Microplastics in freshwater ecosystems with special reference to tropical systems; detection, impact, and management. In: Dalu, T. & Tavengwa, N.T. Emerging Freshwater Pollutants: Analysis, Fate and Regulations. 2021. Elsevier

2.1 Abstract

Microplastics (MPs) are defined as diverse plastic fragments smaller than 5 mm in size. They are ingested by aquatic organisms at various trophic levels and stages of development, including freshwater invertebrates and fish. Microplastics are highly abundant in freshwater environments worldwide and yet we know little about their impact on biodiversity, movement through the food chain, ability to change community composition and alter predator-prey interactions. Many of these questions remain unanswered, with studies tending to focus on toxic effects in laboratory settings. Although there are studies to investigate the occurrence and abundance of MPs in freshwater environments including rivers and lakes, relatively few have looked at the impact in tropical ecosystems or with organisms found therein. This chapter will review research on MPs in freshwater systems, highlighting tropical waters where research has been undertaken. The chapter will include a review and comparison of detection and quantification methodology with some practical suggestions and recommendations for future work.

Keywords:

Microplastics, Pollutant, Freshwater, Fish, Invertebrates, Detection, Life history, Ecotoxicology, Lakes, Rivers, and streams

2.2 Introduction

As a result of poor waste management and improper 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, by number, most plastic waste is microscopic, including the plastic found in the Great Pacific Garbage Patch (Howell et al., 2012).Microplastics (MP) are most commonly defined as plastics < 5mm (Auta et al., 2017; Li et al., 2017) and is therefore definition used in this chapter, however, this definition is not universal. 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 'nanoplastics' $\leq$ 0.1 mm; 'microplastics' items $\leq$1 mm to > 0.1 mm; 'mesoplastic' for particles between $\leq$ 5 to >1 mm, and 'macroplastics' as >5 mm (Fendall and Sewell, 2009; Gigault et al., 2018; Moore, 2008).

Under environmental influences such as ultraviolet light and physical abrasion the degradation of larger plastic particles leads to the production of MPs (Wagner et al., 2014). Consequently, much of the plastic pollution found on the ocean surface is dominated by particles smaller than 4.8mm 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 rivers (Lechner et al., 2014; Morritt et al., 2014), lakes (Horton et al., 2017) , mangrove swamps (Kukulka et al., 2012) and even groundwater (Panno et al., 2019). Waste emanating from domestic sewage is often released into freshwaters where a multitude of organisms are exposed to the pollution. Despite widespread presence of MP worldwide little research had focussed specifically on tropical freshwater, especially in low economic value regions. This chapter will discuss the sources and types of MP, their occurrence within freshwater systems and organisms, the impact of waste water and will give some practical advice towards conducting studies.

2.2.1 Source and types of microplastics in freshwaters

Microplastics can be fibres, films, foams, fragments or pellets (Rochman et al., 2019) (Figure 2.1). Fibres are defined differently by individual studies, but as a rule, they are more than twice as long as they are thick, threadlike, and very thin (Cole et al., 2011), they can be found individually or as a bundle where individual fibres cannot be separated (Rochman et al., 2019).Foams are usually thick, soft and compressible, they can be smooth or angular. Pellets are regular and smooth such as nurdles (small, coloured plastic pellets used in the manufacturing of plastic products), when perfectly round they are referred to as spheres. Fragments are hard, irregular and angular, often resulting from the breakdown of larger pieces. Films are fragments, which are very thin and flat, and commonly result from the degradation of plastic bags (Kalogerakis et al., 2017; Quecholac-Piña et al., 2017).

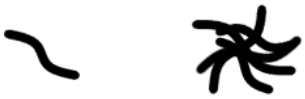
Shape	Example	Description
Fibre		Long, thin and threadlike Found individually or as a bundle
Fragment		Solid, hard and angular
Film		Thin and flat, often irregular
Foam		Thick, soft and compressible
Pellet		Solid, hard and regular, can be spherical

Figure 2.1 The most common types of microplastic found in aquatic environments

Microplastics are further defined as primary or secondary depending on their origin. Primary MPs are found in the environment in the same size and shape that they were manufactured. These MPs are mostly made of polyethylene but may also be made of polypropylene, polyamide, Teflon, or many other plastic polymers (Rochman, 2016). They are often added to consumer care products such as toothpaste or facial scrubs which can pass through filtration systems of the wastewater treatment plants (WWTP) and enter aquatic environments (Browne et al., 2007; Horton et al., 2017; Napper et al., 2015). A UK investigation into MP beads in cosmetic products found that a single use of a facewash containing MP beads could release up to 94,500 MPs into the wastewater system (Napper et al., 2015). Following on from campaigns from non-governmental organisations, many countries have now banned, or are proposing to ban, the sale of products that contain primary

microbeads, including many European countries, Taiwan, Australia, India, and South Korea (Xanthos and Walker, 2017).

Secondary MPs are formed by the breakdown of larger pieces of plastics; this can be due to mechanical damage or environmental weathering, for example a polyethylene terephthalate (PET) bottle that has broken down into many pieces (Duis and Coors, 2016; El Hadri et al., 2020; Jaikumar et al., 2019).

Our planet is home to over a billion cars on the road with tyres that shed plastic particles every time they are driven: these particles are in the range of 1-1000 µm (Baensch-Baltruschat et al., 2020) and in Denmark and Norway tyre wear accounts for >50% of microplastics in the water (Lassen et al., 2015; Peter Sundt, Per-Erik Schultze, 2014). Throughout Europe it is estimated that 1.3 million tonnes of MPs per year are released into the environment this way (Wagner et al., 2018) . As 90% of new roads are being built in tropical and subtropical regions (Dulac, 2013) and these areas being susceptible to flooding (Alamgir et al., 2017), tropical freshwater environments will be increasingly vulnerable. Once these particles have been produced, they enter the aquatic and terrestrial environment through surface run off and aerial deposition. Surface runoff is the process of material gathered on surfaces (particularly in an urban environment) being washed towards other environments, such as fields on the side of roads, or more often into drains which then discharge into waterways or WWTPs (Lambert and Wagner, 2018; Pariatamby et al., 2020).

Degradation of polymers by environmental conditions takes place through several routes including thermo-oxidative, photo and biological degradation. Thermo-oxidative degradation is a slow oxidative degradation under moderate temperatures (Andrady, 2011);

photo-degradation results from the exposure to sunlight over time, in which large polymer molecular weight is decreased and oxygen-rich functional groups are released (Andrady, 2011; Browne et al., 2007) and biodegradation refers to degradation by living organisms, such as microorganisms, which have the capacity to convert the polymers to carbon dioxide, although for most polymers only partial breakdown occurs (Andrady, 2011). All these processes can affect the plastic materials and cause continuous break-down to polymers over the time until they become tiny in size (Ryan et al., 2009). Due to these biotic and abiotic factors, plastics in different environments will degrade at different rates, this is evident in the marine environment, where degradation occurs much faster in epipelagic regions (Wang et al., 2021). The key factors associated with plastic degradation are UV exposure, temperature, moisture, pH and presence of microorganisms, with the exception of pH, all other factors are more conducive to plastic breakdown in freshwater (Wang et al., 2021). This is especially relevant to tropical regions which experience much higher UV exposure, temperatures and humidity, and so even the terrestrial environment has ideal conditions for degradation (Arias-Villamizar and Vázquez-Morillas, 2018).

Unfortunately, many plastics are non-degradable meaning they may fragment into secondary plastics but do not chemically degrade, and as such are extremely persistent within the environment. Biodegradable “plastics” that are broken down by microbes are often cellulose rather than petroleum based. Whilst switching to biodegradable plastics is seemingly an ideal solution, degradable and biodegradable plastics have limitations, as they are not suitable for exterior use where they encounter degrading conditions. These conditions are often limited within the environment, for example only surface water has sufficient ultraviolet concentrations for photodegradation, and the microbes required for

biodegradation can have specific requirements. Ultimately degradable and biodegradable plastics do not negate the need for waste treatment as they do not reliably degrade, and when they do the result is still to produce secondary MPs (Iwata, 2015b; Nauendorf et al., 2016; Rujnić-Sokele and Pilipović, 2017; Song et al., 2009).

Aerial deposition is where MPs are lifted into the atmosphere, then transported by air currents to fall out of the atmosphere elsewhere; this is facilitated by their small size and relatively low density (Allen et al., 2019). There is little data on the distance MPs travel in the air, or how high up they are in the air column. It is likely to be difficult to predict since the shape and type of MP is so variable. No significant differences were found in the concentration of MPs collected at 1.7 and 80 m above ground level (G. Chen et al., 2020). While the distance MPs are transported in the atmosphere is unknown, studies from the French Pyrenees (Allen et al., 2019) and Swiss Alps (Bergmann et al., 2019) suggest at least 95 km. While not a true source of MP, aerial deposition is an important transport mechanism within tropical freshwater because it explains the presence of MP in remote areas away from obvious sources of MP pollution.

Most MPs that are transported aerially are fibres; in Paris over 90% of MPs collected from atmospheric fallout on the roof of a university were fibres (Dris et al., 2015), a result replicated in London with 92% being fibres (Wright et al., 2020). In Dongguan, China, aerial samples of fibres were more often from non-synthetic textiles (84.6%), and yet the abundance of plastic fibres was still an order of magnitude higher than any other type of MP (Cai et al., 2017). Samples from Hamburg, Germany, however showed that most MPs were fragments (95%), likely to be secondary MPs (Klein and Fischer, 2019).

2.3 Occurrence of microplastics in freshwaters worldwide

The past ten years have seen a rapid increase in research looking at the presence of MPs in the water and sediment of freshwater environments including rivers ,lakes, ponds, and reservoirs (Lambert and Wagner, 2018). Surveys have demonstrated alarming quantities of MPs in these ecosystems, including within tropical regions (Table 2.1). Even a remote mountain lake in Mongolia has been contaminated with MPs (Free et al., 2014). Similarly, MP particles have been detected in the surface water of the Laurentian Great Lakes in USA (Eriksen et al., 2013), and Canada (Zbyszewski and Corcoran, 2011). Almost 99.9% of the microbeads found in the North American studies were particles less than 2 mm in diameter spherical and had a PE composition, resembling those used in facial products, suggesting this as the original source. In Africa, Lake Victoria, bordered by Kenya, Uganda, and Tanzania, is threatened by the input of raw sewage and the dumping of domestic and industrial waste. A survey of MPs in the surface waters of Northern Lake Victoria found that most appeared to be secondary MPs generated from larger plastic debris that may have resulted from poor waste management (Egessa et al., 2020b).

The concentrations of MPs reported in the various freshwater studies are difficult to compare since they used different sampling methods and units for quantifications (Horton et al., 2017). To compound the issue of comparability, studies vary enormously in the type of MP (e.g., size, shape, density, and composition) recorded as well as the abiotic factors (e.g., weather, season and equipment used) that may influence MP distribution (Hidalgo-ruz et al., 2012; Lattin et al., 2004).

Rainfall and storm disturbances 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 fate 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 polyvinyl chloride (PVC) and polyesters (PES) settle into the sediment and may be less prevalent in the water column due to their negative buoyancy (Kowalski et al., 2016; Lozoya et al., 2016). However low-density MPs, such as polyethylene (PE) and polystyrene (PS) are positively buoyant and more likely to persist in the water column, making them accessible to filter feeders for accidental ingestion, and increasing their potential distribution range (Avio et al., 2017; Cole et al., 2013; Eerkes-Medrano et al., 2015).

Rivers have historically, and continue to be, essential to human communities globally; they are relied upon for food, transport, industry, and tourism. It is therefore unsurprising that rivers have been highly impacted by plastic pollution. While previously studies on MP contamination had been largely limited to temperate, developed areas, in recent years studies have expanded to include tropical and lower- and middle-income countries. This new research is significant because the majority of continental (rather than oceanic) MPs that enter the marine environment come from Asian rivers (Lebreton et al., 2017; Ta and Babel, 2020) and of the top 20 plastic polluting countries only Turkey (14^{th}) and North Korea (19^{th}) are neither tropical or sub-tropical; China (1^{st}) and USA (20^{th}) are both partially subtropical (Jambeck et al., 2015).

A study of the Netravathi River in Southwest India investigated both macro and MP pollution in sediment and water samples and found that plastic abundance in the water column ranged from 56 pieces m^{-3} to 2328 pieces m^{-3} (Amrutha and Warrier, 2020). The amount of plastic increased downstream of towns with high populations but it was also higher upstream compared to uninhabited regions downstream, suggesting small to

moderate populations are a detectable source. Likewise, the sediment results showed a range between 253 pieces kg^{-1} and 9.4 pieces kg^{-1}, with the lowest values sourced from regions with low anthropomorphic pressure. Surprisingly, the region with the highest water pollution had a low sediment pollution 17.6 pieces kg^{-1}. This anomaly can be explained by the resuspension of MPs from sediment into the water column by turbulent currents caused by wind, rain, and monsoon (Amrutha and Warrier, 2020).

Plastic pollution in canals connected to the Saigon River in Vietnam was predominantly generated from plastic bags (37%), followed by food containers (14%), of which 79% were polyethylene (Lahens et al., 2018). The yearly mass of land-based plastic that entered the river per inhabitant was calculated to be 350-7270 g $inhabitant^{-1}$ yr^{-1}. Microplastics were also identified as either fibres or fragments; the smallest size group (50-250 µm and 0.5-50 10^3 $µm^2$) accounted for half of the MPs collected. The vast majority of these MP were fibres, up to 519,000 fibres m^{-3}, compared with a maximum of 23 fragments m^{-3}; while both fibres and fragments were lowest upstream and highest downstream, the difference was less than expected, given the difference in population between the sites (Lahens et al., 2018).

In many countries, lakes are essential to the local communities, and supply not only food and water, but also transport and recreation. Lakes typically have a much lower flow rate than rivers and therefore have the potential to act as a sink for many pollutants, including MPs. Lake Victoria is the largest tropical freshwater lake, bordered by Tanzania, Uganda, and Kenya, and it is estimated that 4 million people are dependent upon the fishing industry alone (Mkumbo and Marshall, 2015). When the surface waters were studied, all samples were found to have MPs ranging from 0.02 pieces m^{-3} to 2.19 pieces m^{-3} with an

average of 0.73 pieces m^{-3} (Egessa et al., 2020b). This was within the same range as the Great Lakes of America, but lower than large lakes in China (Lake Taihu, Dongting and Hong)(Su et al., 2016; Wang et al., 2018). Another study on Lake Victoria focused upon MPs in the sediment and found mean abundance of 0.9 to 239.8 pieces kg^{-1} in shoreline sediment and 0 to 14.5 pieces kg^{-1} in lakebed sediment. (Egessa et al., 2020a) It found that films were the dominant plastic polluting the shoreline whereas fibres were dominant in the lake sediment.

Lake Vembanad in Kerala, Southwest India is a freshwater estuarine system, with higher salinity north of the lake (4.5) and very low salinity (0.42) in the south. The north is fed by Kochi, the commercial capital of the state, whereas the south is fed by smaller populated centres. A study of various sites across the lake found an average MP contamination of 253 pieces m^{-2} throughout with higher concentrations (496 pieces m^{-2})in the north; this was attributed in part to pollution from Kochi but also to the marine influence the north experienced (Sruthy and Ramasamy, 2017). Higher salinity influencing MP pollution has been recorded in the Gulf of Mexico (Wessel et al., 2016). Here the locations that were more exposed to tides and currents were shown to have a greater abundance of MPs. There are several explanations for this: tidal action increases plastic degradation into secondary fragments by mechanical wear producing more MPs (Wessel et al., 2016); it also increases the residence time of plastics within the region as the water column moves in and out (Wessel et al., 2016). The higher salinity produces a higher density which enables larger and denser MP to stay in suspension rather than become sequestered into sediment, further explaining the increase in MP typically found in these regions. Salinity was a proposed factor for MP levels in a Spanish River Delta where the estuarine sediments

are significant sink areas for MPs, particularly fibres (Simon-Sánchez et al., 2019).

Table 2.4 Microplastics pollution in tropical freshwater ecosystems

Location	Average abundance	Reference
USA		
Californian rivers, USA	30-109 particles m^{-3}	(Moore et al., 1989)
Los Angeles river, USA	12000 particles m^{-3}	(Moore et al., 1989)
South America		
La Salada Lake, Argentina	Summer: 180 MPs m^{-3} Spring: 140 MPs m^{-3}	(Alfonso et al., 2020)
Amazon, Brazil	Sediment: 417-8178 pieces kg^{-1}	(Gerolin et al., 2020)
Pantanal wetlands, Brazil	Urban: 19.9 ± 5.8 x100 L^{-1} Rural: 4.5 ± 2.5 x100 L^{-1}	(de Faria et al., 2021)
Asia		
Netravathi River, India	Water: 288 pieces m^{-3} Sediment: 96 pieces kg^{-1} Soil: 84.5 pieces kg^{-1}	(Amrutha and Warrier, 2020)
Vembanad Lake, Kerala, India	Sediment: 96-496 particles m^{-2}	(Sruthy and Ramasamy, 2017)
Veeranam Lake, India	Water: 28 items km^{-2} Sediment: 309 items kg^{-1}	(Bharath K et al., 2021)
Red Hills Lake, Chennai, India	Water: 5.9 particles L^{-1} Sediment: 27 particles kg^{-1}	(Gopinath et al., 2020)
Sambarmati River, India	Sediment: 135 particles kg^{-1}	(Patel et al., 2020)
Poyang Lake, China	Water: 1064 ± 90 MP m^{-3}	(Jian et al., 2020)
Wei River, China	Water: 3.67 to 10.7 items L^{-1}	(Ding et al., 2019)
Pearl River, Guangzhou City, China	Water: 19860 items m^{-3}	(Yan et al., 2019)
Urban lakes in Changsha, China	Water: 2425 ± 248 to 7050 ± 1061 items m^{-3}	(Yin et al., 2019)
Xiangxi River of Three Gorges Reservoir, China	Surface water: 80-864 particles m^{-2} Sediment: 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: 11-35 particles kg^{-1}	(Su et al., 2016)
Africa		
Lake Victoria, Uganda	Surface water: 0.02-2.17 pieces m^{-3} Sediment: 0.8-240 pieces kg^{-1}	(Egessa et al., 2020b)
Lake Naivasha, Kenya	Surface water: 0.407 ± 0.135 pieces m^{-3}	(Migwi et al., 2020)
Ox-bow lake, Yenagoa, Nigeria	Surface water: 1000-8330 pieces m^{-3} Sediment: 347-4030 pieces kg^{-1}	(Oni et al., 2020)
Braamfontein Spruit, South Africa	Surface water: 705 pieces m^{-3} Sediment: 167 pieces kg^{-1}	(Dahms et al., 2020)

2.4 Ingestion and impact of microplastics in freshwater fish

There is a plethora of evidence in the literature showing that different animal taxa ingest plastics; this can be divided into intentional and unintentional, with unintentional being subdivided into incidental and trophic. Intentional is when an organism deliberately ingests plastic believing it to be a food source, while incidental is when an organism accidentally ingests plastic alongside their normal food source. Trophic is when an organism ingests another organism which itself has ingested plastic, thereby allowing the MP to transfer between trophic levels and can result in bioaccumulation in predators (Cedervall et al., 2012; Cole et al., 2011; Peters and Bratton, 2016).

Fish ingestion of MPs is a worldwide environmental issue that has been widely reported (Wang et al., 2019). Freshwater fish ingest plastics intentionally, mistaking them for food particles, or accidentally, where they are mixed with food (Roch et al., 2020). The feeding habits of fish are thought to be linked to plastic intake (Jabeen et al., 2017; Silva et al., 2018), with fish foraging on the sediment bed exposed to higher concentrations than predatory and omnivorous fish feeding in the water column (McNeish et al., 2018; Mizraji et al., 2017; Roch et al., 2020). In the tropical Goiana Estuary in South America high levels of fibres have been found in different life stages of fish linked to their feeding preference. Juveniles feed in the water column targeting zooplankton ,switching to feeding on benthic invertebratesas subadults, ingesting more fibres (Ferreira et al., 2018; Silva et al., 2018). The study found that fish have an increased intake of microfilaments if these contaminants are abundant in the same habitats and season of their preferred prey (Silva et al., 2018).

In Northeast Brazil 83% of *Hoplosternum littorale*, a common and regularly consumed riverine fish, contained plastics, most of which were MPs (Silva-Cavalcanti et al., 2017). The

most common type of MP was fibre (46%) and the authors found plastic load in the guts was correlated with a lower diversity of food type. Similar percentages have been found in South East Asia; a study looking into eight species from the Chi River in Thailand found between 50% and 86.7% of fish had ingested MPs with an average of 1.7 pieces per fish, and once again it was fibres that represented 86.9% of MPs (Kasamesiri and Thaimuangpho, 2020). A study from Malaysia in the Skudai River had greater variation, with MP ingestion between 19% and 100% for six species with an overall average of 40%.

In Lake Victoria, only 20% of Nile perch (*Lates niloticus*) and Nile tilapia (*Oreochromis niloticus*) were found to have ingested MPs, which is lower than found elsewhere, probably because they were limited to detecting MPs larger than 0.5 mm (Biginagwa et al., 2016). A more recent study investigated *O. niloticus* and a catfish *Bagrus bayad*, with the same limit of >0.5 mm and found that 79.5% of the Tilapia and 78.6% of the catfish had ingested MPs, consuming an average of 7.5 ± 4.9 and 4.7 ± 1.7 pieces respectively (Khan et al., 2020). Again, fibres were the most frequent MP, followed by films**.**

Whilst early studies were focussed on proving that fish were eating the plastics (Lambert and Wagner, 2018; Sanchez et al., 2014), research soon began to search for negative effects from MP ingestion. Physical effects reported include blockages in the alimentary system, inflammation and damage to the gastrointestinal tissues and associated impacts on nutrient absorption (Jabeen et al., 2018; Lei et al., 2018). There have now been many studies, mostly in the laboratory, looking at ecotoxicological effects of MPs on fish and there is some evidence that MPs may have various toxicological effects. For example, PS MPs had a negative impact on the activity of neurotransmitter enzymes in the brains of red tilapia (*Oreochromis niloticus*) (Ding et al., 2018) and in zebrafish (*Danio rerio*), they were

associated with oxidative liver damage and reductions in lipid and energy metabolism (Lu et al., 2016). These impacts, if also observed in the wild, would likely impact predator-prey interactions and organism growth and reproduction. There are concerns that some MPs, which have a large surface area and due to their hydrophobicity are known to bind to toxins, will concentrate toxic chemicals such as hydrophobic organic compounds, thereby magnifying their effect (Wang et al., 2020). The degree to which MP have a negative impact on aquatic animals have been questioned, with several studies finding negligible to no impact (Cole et al., 2011; Li et al., 2020; Xu et al., 2020), the risk of overfishing and climate change are suggested to be far greater than any risk posed by MP (Cunningham et al., 2020). It is also worth considering the role publication bias has to play in the proportion of publications finding significant impacts.

2.5 Ingestion and impact of microplastic on freshwater invertebrates

As with early fish studies, research on freshwater invertebrates initially focussed on detection and loading, an example being a study of a riverine valley in South Wales, UK, downstream from a WWTP, where half of the macroinvertebrates tested (Baetidae, Heptageniidae and Hydropsychidae) contained multiple fragments of plastic (Windsor et al., 2019). A large-scale survey of Asian clam, *Corbicula fluminea,* in the Yangtze river basin which included lakes, rivers and estuaries, demonstrated that levels of MP pollution (mostly fibres) in the sediment were closely correlated with MP load in the clam (Su et al., 2018). The authors have suggested that it could be used as an indicator species for MPs in freshwaters.

Most published research on freshwater invertebrates has been in the laboratory using model species, including the mollusc *Sphaerium corneum*, *Daphnia*, *Lumbriculus*, *Gammarus*, and *Tubifex* species, answering questions related to ingestion, uptake, depuration and ecotoxicological effect (Anbumani and Kakkar, 2018). Generally, research has revealed that MP uptake is concentration- and time-dependent (Bruck and Ford, 2018; Canniff and Hoang, 2018; Lambert and Wagner, 2018; Rehse et al., 2016).

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., 2018b). 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 some studies have not confirmed this observation (Lambert and Wagner, 2018), work on *Daphnia galeata* exposed to PS nanoparticles (52 nm) recorded the

transfer of particles from the external body to the internal organs as well as storage in lipid droplets (Cui et al., 2017).

Likewise, small PS MPs (2 µm) were found to transfer ontogenically from larvae to adult *Culex pipiens* mosquitoes, whereas larger MPs (15 µm) were not. The smaller PS MPs accumulated in the mosquito renal excretion system, where they stayed throughout the metamorphosis to pupae and then to adults, thereby transferring plastics from the freshwater to the terrestrial foodwebs (Al-Jaibachi et al., 2018a). Despite the retention of the PS MPs, there was no impact on the mosquito development or eclosion (Al-Jaibachi et al., 2019).

Although PS MPs (20-500 µm) decreased growth rates in *Gammarus pulex* (Redondo-Hasselerharm et al., 2018b), 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 (Aljaibachi and Callaghan, 2018b; Ogonowski et al., 2016). 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 fibres obstructing the gut system (Jemec et al., 2016). Immobilisation of *D. magna* is a parameter measured in ecotoxicology and smaller sized MPs (1 µm) were shown to have a concentration- and time-dependent immobilisation effect (Rehse et al., 2016) while deceased growth rate and induced stress defences were reported in *Daphnia pulex* (Z. Liu et al., 2019). The trophic transfer of MPs from two standard ecotoxicological invertebrate models (*Daphnia magna* and *Chironomus riparius* larvae) with and without a toxic pollutant chemical to zebrafish (*Danio rerio)* found no impact on liver enzymes as a measure of stress (Hanslik et al., 2020).

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).

2.6 Management of water waste systems and microplastics

Microplastics are known to enter the WWTPs through domestic products; clothes washing (Browne et al., 2011; Napper and Thompson, 2016), personal care products (Duis and Coors, 2016; Fendall and Sewell, 2009; Kalčíková et al., 2017), toothpaste (Leslie, 2014), and have recently been found in human stools (Schwabl et al., 2018). Microplastics, including microbeads, washed down domestic drains are transported in the raw effluent to the WWTPs. Studies suggest that this is the main source of MP pollution in freshwater ecosystems (Horton et al., 2017; McCormick et al., 2014; Windsor et al., 2019). The wastewater treatment processes currently used in the European Union are not specifically designed to capture MPs but where some form of tertiary treatment is operating efficiently, the removal of MPs from the water can be effective. However, if sludge is retained and spread onto agricultural land as a fertiliser, any MPs that it contains will be available for pollution of the natural habitat (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).

In studies from subtropical regions with high value economies (USA, South Korea, and Australia), the majority of MPs in both the incoming waste and effluent are fibres (Conley et al., 2019; Hidayaturrahman and Lee, 2019; Lasee et al., 2017; Ziajahromi et al., 2017, 2016). Whenever textiles are washed, they release fibres, hence the presence within incoming waste, and when compared to particles fibres do not settle as readily, due to greater surface area to volume ratio, and their ability to pass through pores more easily (a 100 µm long fibre will pass through a 25 µm pore). Samples from a textile manufacturing area in Eastern China showed that most fibres in both surface water and sediment were in the range of 0.1-

1 mm with the average size within surface water being smaller than in the sediment (Deng et al., 2020).

A recent survey on regulation of WWTPs and MPs found that worldwide there are no regulations that specify the maximum levels of MPs permitted in discharged wastewater (Freeman et al., 2020).

In tropical regions, including countries in South America, wastewater treatment is often limited and awareness of the issue with MPs has lagged behind other areas of the world. In Ecuador, the lack of effective enforcement of wastewater management policies along with poor management of solid waste and general water resources has resulted in exceptionally high levels of MP pollution (Donoso and Rios-Touma, 2020). Less than 10% of wastewater from the capital city Quito is treated through WWTPs and many major Andean cities are found on the headwaters of basins that drain into the Pacific or the Amazon-Atlantic basin (Donoso and Rios-Touma, 2020). In their 2020 study, Donoso and Rios-Touma (Donoso and Rios-Touma, 2020) found exceptionally high concentrations of MPs in wastewater samples, mostly fibres from clothing, that far exceeded those in countries such as Germany with effective wastewater treatment. The WWTPs are an essential tool in limiting MPs entering the environment, because they can be up to 100% effective in removing MPs from wastewater (Ziajahromi et al., 2017). While well designed and maintained plants can achieve these levels of efficiency, not all plants are created equal (Figure 2).

The most basic plants are a mesh screen which removes large solid matter from wastewater, the smaller the mesh the more efficient it will be in removing solids; however, it requires a more advanced and expensive clearing mechanism. Clarification tanks improve

MP removal efficiency by allowing suspended matter to settle out from the water. Dissolved solids will not be removed by clarifiers, they require either biological or chemical treatment. The effluent from a biological reactor must then go through another clarifier to remove the microbes and suspended solids into a sludge, some of this sludge is disposed of as waste activated sludge with the remainder being pumped back into the biological reactor. The effluent from this secondary clarifier can be discharged into the environment or go through a tertiary treatment. Tertiary treatment can include further filtration through sand filters, chemical treatment or reverse osmosis. Plants with these clarifiers, biological reactors and tertiary treatments have been shown to achieve >90% efficiency, whereas plants with simple mesh screening having a far lower efficiency (Ziajahromi et al., 2017, 2016) (Table 2.2).

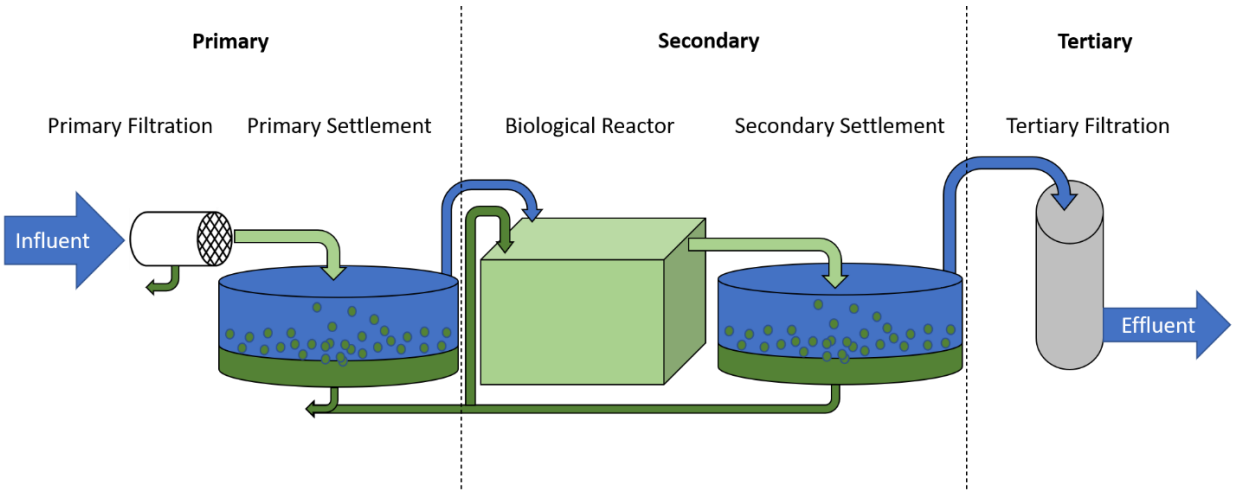


Figure 2.2 Waste water treatment plant showing Primary, Secondary and Tertiary Stages.

Table 5.2 Microplastic removal rate from WWTPs in the tropics

Country	Treatment Stage	Removal efficiency	Microplastics in effluent	Reference
Thailand	Secondary	84%	2 pieces L^{-1}	(Hongprasith et al., 2020)
South Korea	Tertiary	98.9-99.2%	33-297 pieces L^{-1}	(Hidayaturrahman and Lee, 2019)
South Korea	Secondary	75-91.9%	433-7863 pieces L^{-1}	(Hidayaturrahman and Lee, 2019)
South Korea	Primary	56.8-64.4%	1568-12580 pieces L^{-1}	(Hidayaturrahman and Lee, 2019)
Australia	Secondary	76.6%	2.76 ± 0.11 pieces L^{-1}	(Raju et al., 2020a)
China	Tertiary	64.4%	28.4 pieces L^{-1}	(X. Liu et al., 2019)
China	Tertiary	89.17-97.15%	0-447 pieces L^{-1}	(Sun et al., 2019)
Turkey	Secondary	73-79%	4.1-6.9 pieces L^{-1}	(Gündoğdu et al., 2018)

2.7 Practical Approaches to Microplastic Studies

2.7.1 Microplastic Collection and separation

Water

There are two main methods for sampling water, *in-situ,* and *ex-situ*. The optimum method depends on several factors including sample size required, equipment available, time available, nature of water body, type, and size of MP targeted (Prata et al., 2019). *Ex-situ* sampling involves collecting a defined volume of water from the test site and processing it in a laboratory by passing it through sieves of the required size or through a filter with vacuum filtration. The advantage of this approach is that MPs of all sizes can be collected and little specialised equipment is needed. However, the processing can take time and the sample volume is limited by the amount that can be transported from the sampling site to the laboratory (Dubaish and Liebezeit, 2013). When conducting *in-situ* sampling, MPs are removed from the water at the sample site; this is most often achieved using nets, although sieves can also be used. Specialised nets such as manta nets allow a large volume of water to be sampled rapidly, however they require a boat, and the mesh size imposes limits on which MP can be sampled. Nets with a finer mesh such as plankton nets allow smaller MP to be collected, but they are prone to clogging and so typically cannot be used to sample large volumes, as such a greater number of replicates are required to sample the same total volume (Prata et al., 2019). Microplastic fibres are selectively recovered depending on mesh size; an 80 µm mesh captured 250 times more fibres than a 333 µm mesh (Dris et al., 2018). A pump system allows water from a known depth to be sampled, and a set of metal sieves allow a greater volume to be sampled, by separating particulates across a range of mesh sizes. (Lenz and Labrenz, 2018).

The method used to collect MPs can have a significant impact on the estimate of MP abundance. A study based at Guangxi in China collected MPs from the Lijiang river using two collection methods: pumping 90L over a 25µm sieve and plankton nets (mesh sizes 75 and 300 µm) fitted with flow meters(Zhang et al., 2021). The abundance of MPs was 100-fold higher using the pumping method, due to the smaller mesh size. However, the total volume sampled using the pump was three orders of magnitude lower than the nets, possibly resulting in an overestimation of MP abundance (Zhang et al., 2021).

In order to separate MP, water samples are passed through a 5 mm sieve to remove large matter and then a finer sieve to remove suspended solids from the water, it is common to use a 0.3 mm sieve in this final stage, however, many nanoplastics and fibres will pass through (Dris et al., 2018), if these are of importance then a sieve of 20 µm should be used. The contents of the sieves are then transferred to a beaker and dried in an oven to remove the water; this allows the mass of suspended solids to be calculated.

Once dried a substance is added to the solids to remove the organic matter, this can be an oxidising agent, an acid, an alkali, or an enzyme. While acids and alkali will digest organic matter, they can degrade or discolour MP (Catarino et al., 2017; Karami et al., 2017). The risk of degradation is much lower using oxidising agents, most commonly hydrogen peroxide (Karami et al., 2017; Matthew and Richard, 2017). Enzymes have an even lower risk of damaging MP, and do not require the same safety precautions as other methods (Maes et al., 2017), however they are expensive, which limits their use.

A compound is added to the solution to achieve a density of 1.15 g mL^{-1} or greater, the solution is vigorously stirred to release MPs from the sediment and then transferred to a density separator (Masura et al., 2015). Sodium Chloride (NaCl) is the most common

compound because it is cheap and environmentally safe, however it is less effective at separating denser MPs such as High Density Poly Ethylene (HDPE),PVC or PET, for that Zinc Bromide (1.7 g cm^{-3}) has been shown to be the most effective (Quinn et al., 2017). Once separation is complete the denser sediment can be discarded, allowing the solution with the floating MPs to be run through a sieve or filter from which individual MPs can be identified and classified.

Sediment

Sediment from the lake shore can simply be collected with a shovel or trowel and kept in a glass container, but if submerged samples are needed, they must be collected using a sediment grab or corer (Shruti et al., 2019). Most studies investigating MP in sediment transport samples back to a laboratory, with only a few conducting a full field sieve, however, some do pre-screen samples using a 2-3 mm sieve that can remove large material(Hanvey et al., 2017b). Many studies have taken the depth of samples into account, rather than a simple 2-dimensional quadrat sampling regime, to allow for the differential settlement of various MPs (Hanvey et al., 2017b).

A similar separation process is used for sediment samples; however, an additional density separation is conducted to remove the majority of the sediment before oven drying, this is again most often performed with NaCl (Hanvey et al., 2017b).

If using gut contents or tissue samples, density separation may not be necessary as there is unlikely to be much sediment and depending on the amount of material in the gut simply sieving and manually sorting may be sufficient (Silva-Cavalcanti et al., 2017) Studies using tissue samples or gut contents with a high organic load have used digestion processes,

followed by sieving (Thiele et al., 2019). The increased time and temperature required to digest large volumes of organic matter can make MPs more prone to damage (Thiele et al., 2019). The use of enzymes alongside other agents, or using lower temperatures has helped reduce this damage (Catarino et al., 2017; Thiele et al., 2019).

Microplastic experiments involving organisms

To reliably expose organisms to MPs, their route into the organism must be established; this could be through ingestion or respiration. If through ingestion, then the functional feeding group of the organism must be identified and MPs suitably incorporated into their food source (Kampfraath et al., 2012; Yardy and Callaghan, 2020). Where the organism is a filter feeder such as *Daphnia spp.* and bivalves, MPs can be simply suspended in water at known concentrations and organisms left to ingest them, (Aljaibachi and Callaghan, 2018b; Thomas et al., 2020).

Where the organisms are scrapers and grazers such as snails, the preferred method is to allow the MPs to settle onto food so that they are ingested as the animal feeds (Song et al., 2020). If the food is plant material, it is possible that the MP adsorbs onto the plant surface, allowing trophic transfer to be investigated (Mateos-Cárdenas et al., 2019).

MPs have been directly incorporated into the food in known concentrations, for example using agar or algal waters mixed with the MP or tissues of an organisms that has been previously exposed to the MPs (Farrell and Nelson, 2013a; Kampfraath et al., 2012; Yardy and Callaghan, 2020)'. The advantage of this approach is that it ensures the MP is taken up as a result of feeding rather than MPs adhering to the body. Food does not have to be artificial to achieve this; live organisms exposed to MPs previously can be used as a source of MP exposure to follow trophic transference. Chae et al., (2018) exposed an alga,

Chlamydomonas reinhardtii to nanoplastic (NP) beads and demonstrated that the NPs were transferred into the invertebrate filter-feeder *D magna.* The authors continued with the trophic study by feeding the NP exposed individuals to a secondary-consumer Chinese rice fish, *Oryzias sinensis* which was itself then fed on to an end-consumer dark chub fish *Zacco temminckii* (Chae et al., 2018). The NPs were transferred all the way from the alga to the end consumer in the food chain. However, the study was more of a proof-of-concept paper, using concentrations that are much higher than those found in the environment and without the animals having a choice of food.

When MPs are being deliberately fed to organisms it is prudent to make them easily identifiable to the experimenter. This has been done simply and cheaply by choosing an easily recognisable shape or colour, which is distinct from the rest of the food source. This was achieved in a study of *Gammarus* ingestion by using black fibres which were easily distinguished from the green algal food (Yardy and Callaghan, 2020). Another method is to use fluorescent dye to stain MPs, allowing clear visualisation under a fluorescent microscope, however a low-cost alternative can be an ultraviolet torch which also allows real time observation of fluorescent MPs through tissue (Ehlers et al., 2020). Although using fluorescent dyes is a more expensive option it allows smaller MPs to be easily identified and differentiated from non-MP particles.

Once exposed, MPs in organisms can be studied in either the tissue of the organism or in the gut only. If small organisms are being investigated then the whole individual can be used, for larger organisms a tissue sample is likely to be the best approach (Silva-Cavalcanti et al., 2017). If the gut is being investigated then there are two options, the gut can be dissected out and investigated alongside its contents (Steer et al., 2017), alternatively the

gut contents can be removed and investigated separately(Grigorakis et al., 2017; Ory et al., 2018). Gut contents can be removed physically, or the animal can be left to defecate; either way this has the advantage that the organisms does not necessarily need to be killed (Coppock et al., 2019; Reynolds and Ryan, 2018). If animals have been deliberately exposed to fluorescent MP they can be identified easily using fluorescent microscopy and to date studies using this approach have been conducted with MPs of various materials and sizes with several freshwater invertebrates (Table 3).

Microplastics (from the environment, or deliberately exposed to the organism) can be separated using a similar method as for sediment samples. If using gut contents or tissue samples, density separation may not be necessary as there is unlikely to be much sediment and depending on the amount of material in the gut simply sieving and manually sorting may be sufficient (Silva-Cavalcanti et al., 2017) Studies using tissue samples or gut contents with a high organic load have used digestion processes, followed by sieving (Thiele et al., 2019). The increased time and temperature required to digest large volumes of organic matter can make MPs more prone to damage (Thiele et al., 2019). The use of enzymes alongside other agents, or using lower temperatures has helped reduce this damage (Catarino et al., 2017; Thiele et al., 2019).

Table 2.6 Fluorescent MPs exposed to freshwater invertebrates

Species	MP	Peak Fluorescence	MP Concentration	MP size/µm	Ingested	Which organ	Reference
Gammarus duebeni	PE	605 nm	60,000 mL^{-1}	10-45	Yes	Gut	(Mateos-Cárdenas et al., 2020)
Gammarus pulex	PET	465-495 nm	4,000 mL^{-1}	10-150	Yes	Gut	(Weber et al., 2018)
Gammarus pulex	PS beads	441 nm	3,000 mL^{-1}	1	Yes	Gut	(Scherer et al., 2017a)
Gammarus pulex	PS beads	441 nm	3,000 mL^{-1}	10	Yes	Gut	(Scherer et al., 2017a)
Gammarus pulex	PS beads	441 nm	300 mL^{-1}	90	Yes	Gut	(Scherer et al., 2017a)
Daphnia magna	PS beads	441 nm	3,000 mL^{-1}	1	Yes	Gut	(Scherer et al., 2017a)
Daphnia magna	PS beads	441 nm	3,000 mL^{-1}	10	Yes	Gut	(Scherer et al., 2017a)
Daphnia magna	PS beads	441 nm	300 mL^{-1}	90	No	Gut	(Scherer et al., 2017a)
Chironomus riparius	PS beads	441 nm	3,000 mL^{-1}	1	Yes	Gut	(Scherer et al., 2017a)
Chironomus riparius	PS beads	441 nm	3,000 mL^{-1}	10	Yes	Gut	(Scherer et al., 2017a)
Chironomus riparius	PS beads	441 nm	300 mL^{-1}	90	Yes	Gut	(Scherer et al., 2017a)
Physella acuta	PS beads	441 nm	3,000 mL^{-1}	1	Yes	Gut	(Scherer et al., 2017a)
Physella acuta	PS beads	441 nm	3,000 mL^{-1}	10	Yes	Gut	(Scherer et al., 2017a)
Physella acuta	PS beads	441 nm	300 mL^{-1}	90	Yes	Gut	(Scherer et al., 2017a)
Lumbriculus variegatus	PS beads	441 nm	300 mL^{-1}	1	Yes	Gut	(Scherer et al., 2017a)
Lumbriculus variegatus	PS beads	441 nm	300 mL^{-1}	10	Yes	Gut	(Scherer et al., 2017a)
Lumbriculus variegatus	PS beads	441 nm	300 mL^{-1}	90	No	Gut	(Scherer et al., 2017a)
Eriocheir sinensis	PS beads	488 nm	40,000 µg L^{-1}	5	Yes	Gill Liver Gut	(Yu et al., 2018)

Lumbriculus variegatus	Acrylic	Red range	1:10 Ratio MP:food	29.5 ± 26	Yes	Gut	(Imhof et al., 2013)
Daphnia magna	Acrylic	Red range	1:10 Ratio MP:food	29.5 ± 26	Yes	Gut	(Imhof et al., 2013)
Notodromas monacha	Acrylic	Red range	1:1 Ratio MP:food	29.5 ± 26	Yes	Gut	(Imhof et al., 2013)
Potamopyrgus antipodarum	Acrylic	Red range	1:10 Ratio MP:food	29.5 ± 26	Yes	Gut	(Imhof et al., 2013)
Gammarus pulex	Acrylic	Red range	1:10 Ratio MP:food	29.5 ± 26	Yes	Gut	(Imhof et al., 2013)
Culex pipiens	PS Beads	470 nm	$8x10^5$ mL^{-1}	2	Yes	Malpighian tubules	(Al-Jaibachi et al., 2018a)
Culex pipiens	PS Beads	480 nm	$8x10^2$ mL^{-1}	15	Yes	Malpighian tubules	(Al-Jaibachi et al., 2018a)

2.7.2 Detection methods

Once MPs have been isolated for study, a sample, or all of the material recovered, must be analysed to allow sample identification and quantification. Manual methods include visually inspecting each piece under 40X magnification to look for e.g., homogenous texture, straight lines, and unnatural shapes or colours (Elkhatib and Oyanedel-Craver, 2020; Prata et al., 2019; Shim et al., 2017; Wagner, 2018). Alternatively, a 'hot needle test' can identify plastic since holding an extremely hot needle close to the suspected item will melt or deform the MP (Karlsson et al., 2017). Similarly, the 'break test' is also used, where the suspected MP is poked and bent with forceps and needle to see if it will bend and not break (M. C. Chen et al., 2020). While these methods allow reliable identification, they are highly time consuming and result in the destruction of the particles sampled. The preferred method to identify and quantify MPs uses one of several spectroscopic methods.

Fourier-Transform InfraRed (FTIR) spectroscopy measures the infrared adsorption and emission of a sample which is compared to a database of known materials. This can not only differentiate between plastics and non-plastics but it can also identify the specific type of plastic. While this method is the 'gold standard' for identification, it requires examples of different plastics to be in its database, and it can also take several hours to analyse a 5x5 cm sample. Purchasing an FTIR microscope is expensive, and is not suitable for identifying particles <20 µm (Wesch et al., 2016). As such many studies manually sort through samples and then analyse a sub-sample of their suspected MPs with FTIR for quality assurance (Hanvey et al., 2017b; Prata et al., 2019).

Scanning Electron Microscopy (SEM) can identify particles using their physical structure, and can be used to confirm findings from standard microscopy (Eriksen et al., 2013), however, is time consuming and does not give information on polymer type.

Raman spectroscopy uses the interaction of the sample with monochromatic light; it will provide reliable data on the polymer type, yet has been used less than FTIR (Sun et al., 2019). This may be because while it provides resolution of MPs down to 1 µm (Ribeiro-Claro et al., 2017) and does not suffer disturbance from polar environmental compounds (Schymanski et al., 2018), it is disturbed by fluorescence (both organic and inorganic) (Elert et al., 2017) and the filters needed are hugely expensive (Prata et al., 2019).

Few studies have used gas chromatography / mass spectrometry (GS/MS) (Sun et al., 2019), because while it instantly gives data on polymer types and relative abundance (Zhang et al., 2020), it gives no data on the number, size, or shape of individual MP (Prata et al., 2019; Sun et al., 2019) and is destructive. A more advanced method includes the addition of a thermo-extraction and desorption process, enabling products from plastic thermal

degradation to be analysed separately, which allows a sample to be analysed without MP having to be pre-sorted (Dümichen et al., 2017; Elert et al., 2017).

2.8 Summary

Despite the ever-increasing number of studies of the effects of MPs on aquatic biota, individual studies have been biased towards to a particular type of polymer MP, with a widespread use of laboratory over field studies, and an overwhelming use of fish and crustaceans as model organisms (de Sá et al., 2018). It can be argued that in countries where research of the abundance and impact of MP is in its infancy, there is limited value in examining whether a novel species will ingest a novel polymer; the wealth of available data suggests that depending on the MP size, it will eat it accidentally or through confusion with food particles. Identification of polymer type and origin along with understanding its fate and transport will help to predict loadings in sediment and water.

A 2020 systematic analysis of global data identified seven main categories of MP in the environment: polyethylene terephthalate, polyethylene, polyvinyl chloride, polypropylene, polystyrene, polyurethane and miscellaneous plastic (Jones et al., 2020). The type of MP found in waters around the globe varied considerably between countries and research mostly concentrated on measuring total abundance with little evidence of any links of sources to particles in the environment. However, the prevalence of fibres from clothing points to a failure of WWTPs to remove the MPs and prevent them from entering the ocean.

In tropical regions, wastewater treatment may be unable to cope with the removal of MPs and a heightened awareness and better waste water management may help reduce

the release of MPs, particularly fibres, into the environment. The WWTPs are an essential tool in limiting MPs entering the environment, because they can be up to 100% effective in removing MPs from wastewater.

Before worldwide research can be pulled together in a meta-analysis, there is a need for standardized MP characterization methods and collection methods, using the types of guidelines already developed for chemical pollutants. We urgently need more studies looking at the behaviour of MPs in a variety of habitats, including partitioning and availability to organisms and the food chain. It is essential that studies can be effectively and informatively compared, and at the moment very few studies have a capacity to identify the smallest MP, which we know to be the most abundant, and there is limited value in comparing them with studies with a less sensitive methodology.

A consistent and comprehensive method for identifying the MP present in an ecosystem should form the basis for further investigations into the impact of these MP at both the individual, population and community levels. Key to making these investigations environmentally relevant would be to not only use relevant MP shapes and polymers, but to use them in environmentally relevant concentrations. By using consistent methods to base the experimental parameters, the impacts to different ecosystems would be more reasonably compared.

CHAPTER 3

General Methods and Method Development

3.1 Gammarus Collection

Gammarus were collected from Emm Brook within the Dinton Pastures Nature Reserve in Wokingham, a tributary of the River Loddon between the points (Decimal Degrees 51.440494, −0.874373 to 51.442274, −0.874359) (Figure 3.1). The region of the brook used had a gravel bed interspersed with larger >15cm stones covered in algae. The water depth varied across the brook and throughout the year, at the shallowest it rises gradually to the bank, at its deepest it varied from 72cm in February to 54cm in August. The brook is shaded by trees and riparian vegetation, however, there was no vegetation within the water body. In addition to *G. pulex*, there are many benthic macroinvertebrates including caddis, mayfly, stonefly and blackfly larvae, leeches, isopods snails and chironomids. During *Gammarus* collection, several species of fish were also caught, most commonly *Cottus gobio*, which is a well-known predator of *G. pulex* (Kaldonski et al., 2007).

Hessian nets were used with a kick sampling technique, focussed on likely areas, particularly around deep banks or downstream of woody debris. *Gammarus* were removed from the net by hand and placed in 1L bottles filled with stream water. Only *Gammarus* over 12mm were collected, any obviously gravid females were also not collected and no more than 40 *Gammarus* were placed in any single bottle.

Figure 3.1 Location of Dinton Pastures Nature Reserve, to the west of Reading (top), and the location of Emm Brook within the nature reserve, the sampling area highlighted in red (bottom).

3.2 *Gammarus* Identification

Bottles containing *Gammarus* were transported back to the laboratory within 30 mins, where they were emptied onto a 1mm sieve and rinsed with Reverse Osmosis (RO) water, to remove sediment and contaminants. They were then transferred into a deep tray, so they could be removed individually, using a plastic pastette and a plastic spoon with netting.

All *Gammarus* appeared to be the same species, *G. pulex*. Thirty individual *Gammarus* were then identified using two keys (Dobson, 2012; Eggers et al., 2016), in particular the eye figure 3.2 and uropod morphology Figure 3.3. As expected, considering the location and environment, all *Gammarus* were confirmed to be *G. pulex*. Due to the fact that *G. pulex* had to be killed in order to be identified, this was not repeated prior to each experiment, instead all *Gammarus* were assumed to be *G. pulex* and features were confirmed in test *Gammarus* during dissection. At no point was any species other than *G. pulex* identified.

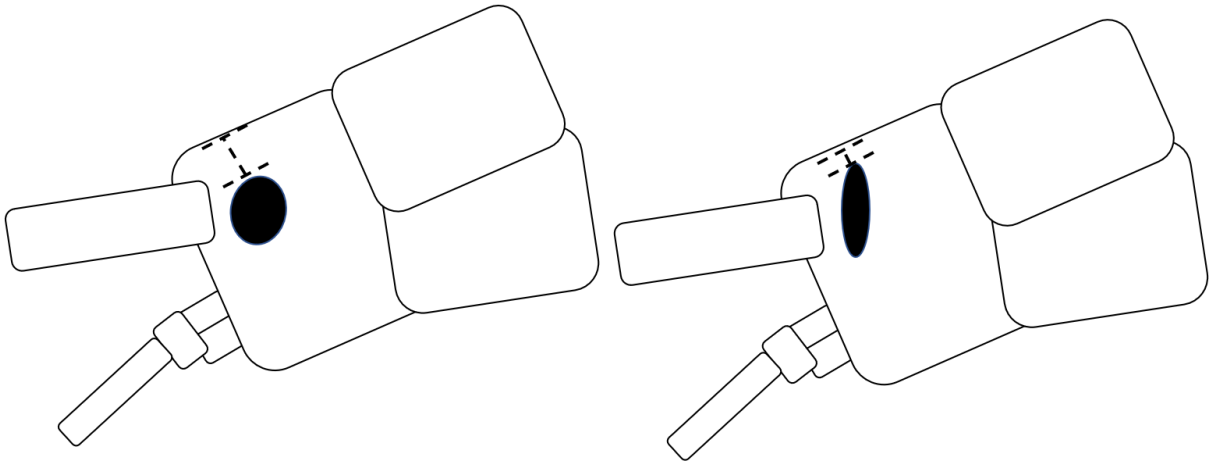


Figure 3.2 The heads of *G. pulex* (left) and *G. dubeni* (rght), *G. pulex* having a less elongated eye and the distance between the eye and edge of head is approximately the same as the diameter of the eye.

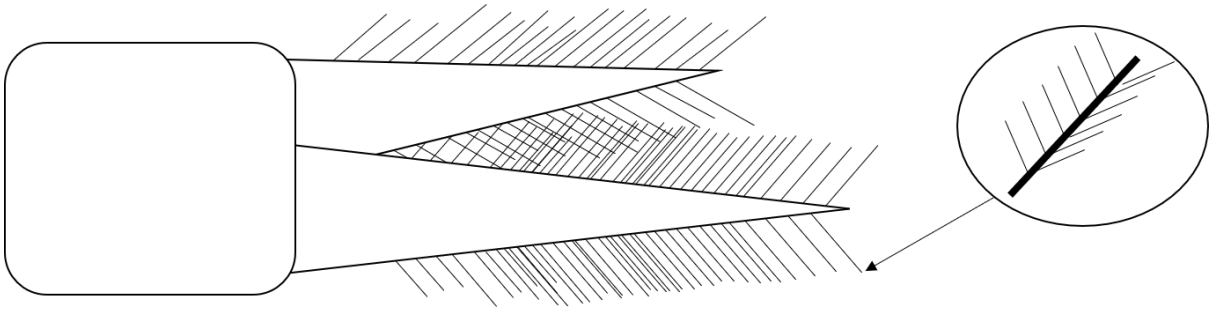


Figure 3.3 The 3rd (most posterior) uropod of *G. pulex*, the inner appendage (top) is more than half the length of the outer appendage (bottom), the hairs are regular and dense (not clumped) and the hairs on the outer edge of the outer appendage have secondary hairs.

3.3 *Gammarus* storage

Gammarus were stored in adapted 45L glass aquariums, with the inlets to the filter systems covered in fine mesh, to prevent *Gammarus* swimming into the filter and an aeration stone placed near the filter outlet, to ensure oxygenation. The base was covered with 2cm of aquarium gravel and then filled with 40L of Organisation for Economic Co-operation and Development (OECD) reconstituted water (Hooper et al., 2006). This reconstituted water was made up in 90L batches, by adding 50L of RO water to a container along with, 17628 mg of $CaCl_2 \cdot 2H_2O$, 7398 mg of $MgSO_4 \cdot 7H_2O$, 5832 mg of $NaHCO_3$, 522 mg of KCl and 0.18 mg of Na_2SeO_3. This was stirred vigorously until the salts had dissolved.

The container was then topped up to 90L, this was necessary as the stirring necessary could not be done when the container was filled to 90L. The water was aerated with an aeration stone attached to an air pump and covered with a black cloth to prevent algal growth.

Every fortnight 15L was removed from aquariums and fresh reconstituted water added, in addition, every three months the *Gammarus* were removed from aquariums and placed in a 5L jug. The aquariums were then totally emptied of water and stones and cleaned with warm tap water. The stones were also rinsed under hot tap water to remove the faeces that built up on the base and filter medium rinsed or replaced, before the stones and then fresh reconstituted water were added prior to reintroduction of the *Gammarus*.

3.4 Gammarus Feeding

Finding suitable food proved to be a challenge, the literature suggested using conditioned leaves as food sources (Bloor, 2011, 2010; Redondo-Hasselerharm et al., 2018a), meaning shed leaves left in stream water for up to six weeks and then frozen for use throughout the year. Oak, elm, sycamore and nettle leaves both from the stream and the banks were collected and used as food. With the exception of well-conditioned sycamore leaves, none were fed upon by *Gammarus* (although they were used as shelter from light) despite animals being starved for several days. These experiments were first conducted in late February, as such there was insufficient conditioned sycamore to last through to autumn, when more could be collected. Algae collected from the stream was eaten, however, it was fragile and contaminated with sediment that could not be washed off, without disintegrating the algae. For this reason, algal fish food wafers were trialled and found to be readily ingested.They were easy to grind down into a powder, manipulate by adding fibres and then reform into wafers of exact sizes.

3.4 Fibre production

Fibres of a known length needed to be reproducibly generated, other studies achieved this using cryogenic microtomes (Cole, 2016), however, this equipment was not readily available and fibres were not required to be as short as 100µm. To produce uniform fibres, a jig was formed by placing two screws 10cm apart in a length of wood, the screws were then trimmed so that 5cm extended above the surface of the wood. Two 5cm lengths of brass tube with an internal diameter of 5mm were cut and placed over the protruding screws (Figure 3.4), this prevented fibres from catching on the threads of the screws. Another jig was produced by attaching a plate of stainless steel with a perfect 90˚ bend, perpendicular to the surface of a length of wood. A second, flat, steel plate was then fixed to the length of wood opposite the 90^{o} angled piece. A 1cm length of the brass tubing was then soldered onto the second steel plate leaving a 500µm gap between the tube and the bend, this gap was made with a 500µm thick piece of steel (Figure 3.5).

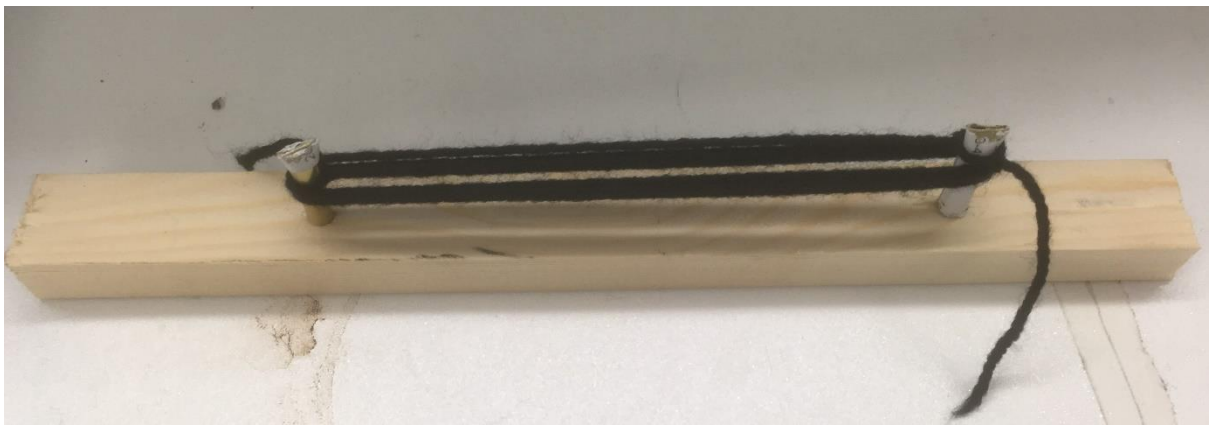


Figure 3.4 First jig with acrylic wool wrapped around prior to freezing.

Figure 3.5 Second jig with frozen piece of acrylic wool being cut into 500µm lengths.

The fibres were wrapped around the brass tubes on the screws (Figure 3.4), 5 times for acrylic wool, sheep wool, cat and human hair, 30 times for the cotton. The fibres were tied off around one end of the jig and soaked with RO water. The jig was then placed into a -80˚C freezer for one hour, after that the fibres were cut off 1cm from the brass tubes using a scalpel leaving perfectly straight frozen lengths of fibres. These lengths were divided in two to form 4 x 5cm lengths, this was necessary as the fibres quickly thawed and so the other lengths could be stored on ice while the first was being processed. The fibres were processed into microfibres by inserting one end of the frozen fibre through the soldered tube until it touched the perpendicular piece of steel and then running a type 11 scalpel through the 500µm gap, down the edge of the brass tubing to remove 500µm lengths of fibre (Figure 3.5). These fibres were then brushed with the back of the scalpel into a petri dish (Figure 3.6), placed on a hotplate at 40˚C with small hole in the petri dish lid to allow the fibres to dry. Once dried they were placed in lidded glass beakers, to prevent contamination. Different fibres can be seen in figure 3.7. Placing both jigs in the freezer and using thick leather gloves allowed up to 2cm of each 5cm length of fibre to be used before it

thawed too much to cut cleanly. Fibres were measured against a micro-ruler in order to measure them to the closest 100 µm, out of 500 93% were found to fall between 200 and 500 µm, with those falling outside this range all being longer than 800µm and likely occurring when the fibres started to thaw and cuts were less clean.

Figure 3.6 Acrylic microfibres removed and placed in a petri dish prior to drying.

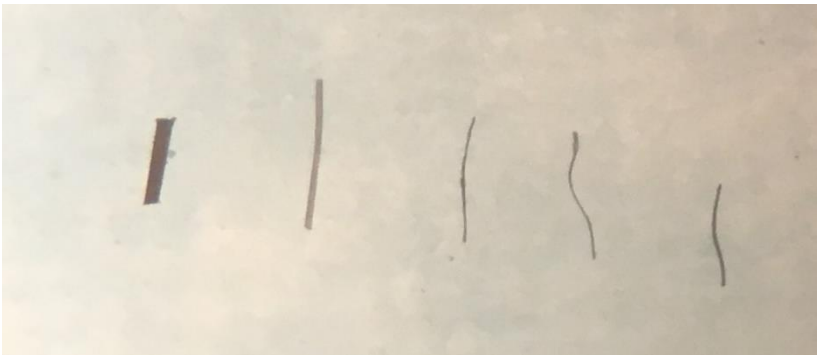


Figure 3.7 Microfibres generated for use in experiments, from left to right human scalp hair (100µm), human scalp hair (50µm), *Felis catus* hair (30µm), *Gossypium* (20µm) and acrylic (30µm).

3.5 Wafer production

Test wafers were produced by grinding algae wafers with a mortar and pestle until a homogenous powder was produced, this powder was stored in a lidded glass container to prevent contamination. Wafers were produced in 1g batches, initially 5% by mass wafers were produced by adding 0.05g fibres to 0.95g algae power, homogenised in a mortar and pestle and then 0.5mL RO water added to produce a wafer that was then dried on a hotplate at 70˚C for 2 hours. However, when inspected it was noticed that the fibres had clumped together, different methods of homogenising prior to adding the water were trialled, but clumping always occurred. Therefore, the percentage of fibre was dropped until the clumping no longer occurred; this seemed to occur at 3%. In order to confirm this and to record how many microfibres were in each wafer, a wafer was made at 0.5%, 1%, 1.5%, 2%, 2.5% and 3%. These wafers were divided into 0.05g test wafers by cutting cubes approximately 2mm x 2mm x 2mm with a scalpel and then shaving the cubes until they were the correct mass. For each concentration, ten, 0.05g test wafers were made and each of these was then divided into quarters: each of these quarters was then crushed and individual fibres counted under 10X magnification. The data for each concentration fit the assumptions for normality and was analysed using a two-way analysis of variance between wafers and between quarters, Table 3.1 shows that there was no significant difference within concentrations, and Figure 4.1 in Chapter 4 shows that there was a proportionate difference in fibre numbers between concentrations.

Table 3.1 The F and P Values of Two-Way ANOVAs, on the mean number of 200-500µm Acrylic fibres between wafers and wafer quarters at several nominal concentrations (% by mass MF)

	F and P Values	
Nominal Concentration	Quarters	Wafer
0.5	F(3,15)=1.156, P=0.359	F(1,15)= 0.004, P=0.951
1	F(3,15)=0.455, P=0.718	F(1,15)= 0.261, P=0.617
1.5	F(3,15)=0.597, P=0.627	F(1,15)= 2.570, P=0.130
2	F(3,15)=0.409, P=0.749	F(1,15)= 4.322, P=0.055
2.5	F(3,15)=0.646, P=0.597	F(1,15)= 0.163, P=0.692
3	F(3,15)=0.156, P=0.924	F(1,15)= 1.559, P=0.231

3.6 Ingestion experiments

In order to count the number of fibres ingested by *Gammarus* reliably, they were starved for 24 hours, this ensured that they all wanted to feed and that their guts were empty, preventing cross-contamination (Scherer et al., 2017b). In order to identify the gut retention time of *Gammarus*, 10 were placed in individual 5L aquariums with 2L of reconstituted water and a 0.05g wafer 3% microfibre contamination by mass, they were checked every 15 minutes until faecal pellets were observed. These pellets were first observed at four hours 30 minutes, as such four hours was chosen as the timeframe for experiments to ensure no fibres were excreted before dissection.

The method above was repeated for ingestion experiments, with wafers of different test concentrations of fibres, described in detail in each chapter's methods section. After four hours *Gammarus* were euthanised in 50˚C water in a 5mL beaker. Euthanising allowed them to be dissected effectively, without disturbing the gut contents (Figure 3.8). The

dissection was conducted by removing the head, immediately behind the eye with a type 10 scalpel, and then removing the telson, this allowed the gut to be pulled from the body cavity and then pulled apart in order to find and count all fibres (Figure 3.9).

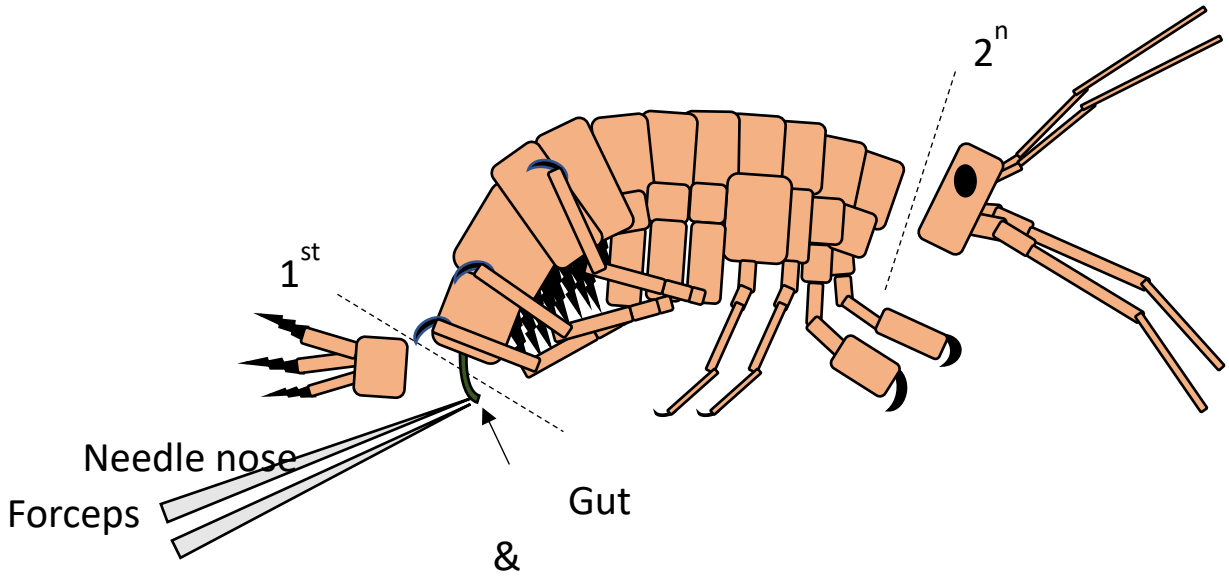


Figure 3.8 The location of incisions in order to remove the gut of euthanised *Gammarus pulex.*

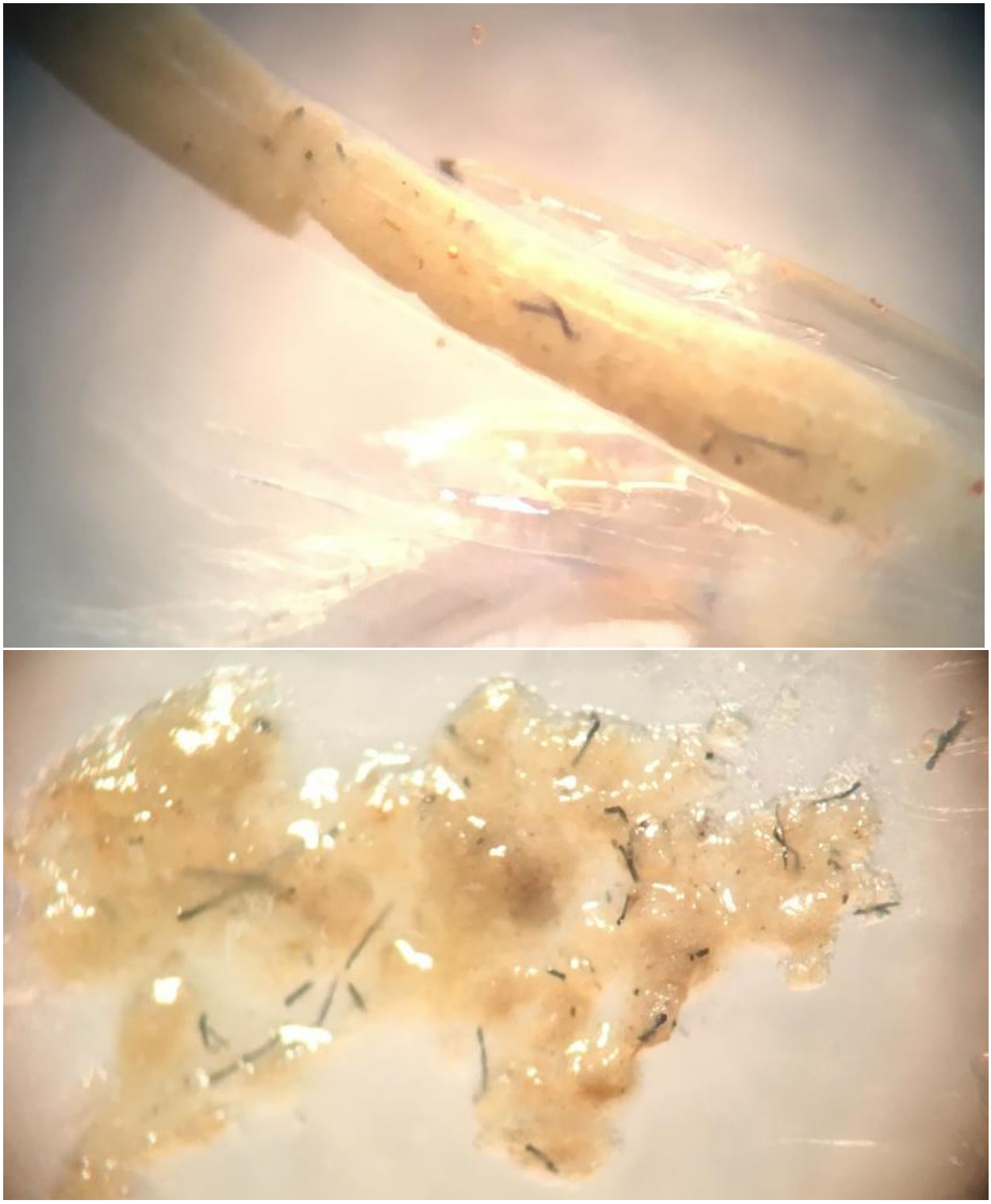


Figure 3.9 The gut of a *Gammarus pulex* fed 3% by mass acrylic wool, being removed from the body (top) and having been pulled apart to count the fibres (bottom)(10X magnification)

3.7 Behaviour experiments

In order to investigate whether *Gammarus* prefer feeding from one food source, over another, the same protocol was used as in section 3.6, however, alongside a 0.05g test wafer, another 0.05g test or control wafer was added to the 5L aquarium. The *Gammarus* was then observed constantly for four hours, the number of times a feeding event occurred and the amount of time spent feeding on each wafer was recorded using a stopwatch to give a cumulative feeding time. A feeding event was counted when a *Gammarus* was actively feeding on a wafer, or had removed a piece of wafer and was feeding from it. Regularly, *Gammarus* would simply sit alongside a wafer but not feed from it, this was not counted as a feeding event.

Up to four *Gammarus* in four aquariums could be observed and recorded at one time, however, due to the size of tanks and workbench it was found to be much easier to have three experiments running at once. Trials were conducted using videography to film the experiments, and then rewatch them to record the feeding events. No more than four experiments could be filmed at once and it was harder and more time consuming to confirm whether a feeding event was occurring, as the film had to be paused and zoomed in. For this reason, experiments were conducted in person.

CHAPTER 4

What the fluff is this? *Gammarus pulex* prefer food sources without plastic microfibers

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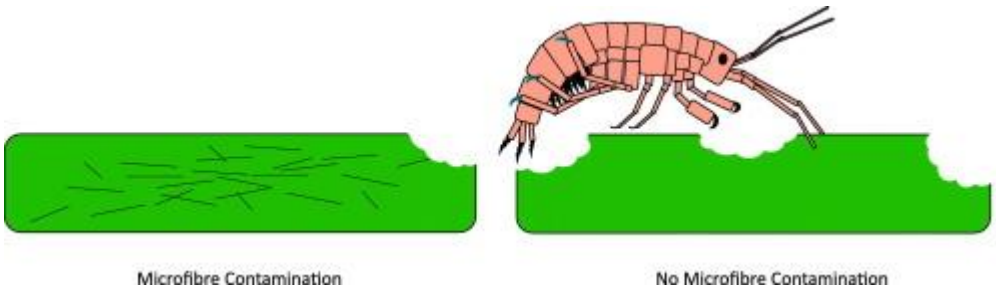
Yardy, L., Callaghan, A. 2020. What the fluff is this? *Gammarus pulex* prefer food sources without plastic microfibres. *Science of the Total Environment*, 715: 136815

4.1 Abstract

Investigations into the impact of micro plastics (MP) and microfibers (MFs) upon the freshwater aquatic environment are still in their infancy despite our growing awareness of their importance. *Gammarus pulex* have long been used as a study organism for ecotoxicology and several studies have already used them to investigate the impact of MFs. One area of research which has not been exploited is the extent to which *G*. *pulex* can detect MFs and whether or not they avoid eating them. To answer this question we developed a reliable and accurate method of exposing *Gammarus* to known amounts of MF embedded in algal wafers. Here we show that when given the choice between control wafers and those contaminated with 2% or 3% MF *Gammarus* ingest fewer MF than would be expected if a random choice was made (2% W = 7, P = 0.017; 3% W = 13, P = 0.034). Their feeding behaviour also changes, with a significant reduction in time feeding ($F_{1,18}$ = 21.3 P < 0.001) as well as significantly fewer visits to contaminated wafers ($F_{1,18}$ = 5.312 P = 0.033). This suggests that *G*. *pulex* are able to detect MF in the 200-500 µm range and are partially repelled by them.



Keywords: Microplastics, Microfibres, Pollution, Amphipoda

4.2 Introduction

Approximately 70% to 80% of microplastics (MPs) in marine environments are thought to originate from inland sources and be transported out from rivers to the oceans (Andrady, 2011). Microplastics are defined as diverse plastics, including polyethylene and polystyrene, whose fragments are smaller than 5 mm in size, they can be particles or fibres, fibres being more than twice as long as they are thick and generally thinner than human hair (Cole et al., 2011). They can be produced by the degradation of larger particles, for example through clothes washing (Browne et al., 2011; Napper and Thompson, 2016), or are manufactured as microbeads for use in personal care products including toothpaste, sunscreen and facial scrubs (Duis and Coors, 2016; Fendall and Sewell, 2009; Kalčíková et al., 2017; Leslie, 2014).

The highest volumes of MP pollution have been found in the Northern Hemisphere at water fronts and in enclosed waters near to urban areas (Cózar et al., 2014; Barnes et al., 2009). As well as accumulation in the environment (Cózar et al., 2014), MPs can accumulate in individuals (Browne et al., 2008) and they have even been found in human stools (Schwabl et al., 2018). Their size results in them being easily ingested by many aquatic organisms at various trophic levels and stages of development, including freshwater invertebrates (Cole et al., 2013; Scherer et al., 2017; Al-Jaibachi et al., 2018a, Al-Jaibachi et al., 2018b; Aljaibachi and Callaghan, 2018). By entering the food chain MPs can be readily transferred between trophic levels (Chua et al., 2014; Betts, 2008; Farrell and Nelson, 2013; Setälä et al., 2014; Davarpanah and Guilhermino, 2015).

Studies to determine the impact of ingested MPs in smaller invertebrates such as copepods, isopods and zooplankton have concluded that MPs have no detrimental effect following ingestion, possibly because the MPs were too large to cross the midgut wall and

were eliminated in faeces (Cole et al., 2013, Cole et al., 2015). This was found in the isopod *Idotea emarginata* (Hämer et al., 2014), cladoceran *Daphnia magna* (Aljaibachi and Callaghan, 2018) and dipteran mosquito *Culex pipiens* (Al-Jaibachi et al., 2018a, Al-Jaibachi et al., 2018b; Aljaibachi and Callaghan, 2018). In studies using the larger *Gammarus fossarum*, the impact of MP ingestion varied depending on the type of plastic (Straub et al., 2017). Petroleum-based MPs significantly reduced the assimilation efficiency of MP contaminated food in the long-term, whereas biodegradable plastic did not, although ingestion of both types of plastic led to significantly reduced growth compared to the control (Straub et al., 2017). In other studies, Irregular MP fragments of polyethylene terephthalate (PET) had no negative effects on feeding in *Gammarus pulex* (Weber et al., 2018).

A meta-analysis on the impact of MP on the aquatic environment revealed that most studies had focussed on particles rather than fibres (Foley et al., 2018). Microfibres (MFs) have been investigated in several marine crustaceans, including Sand Hoppers (*Orchestia gammarellus*), Shore Crabs (*Carcinus maenas*, *Carcinus aestuarii*) and Langoustine (*Nephrops norvegicus*) concluding that MF between 1 and 5 mm were ingested (Piarulli et al., 2019; Watts et al., 2015; Welden and Cowie, 2016). Welden and Cowie (2016) found that the number and length of MF retained in the digestive tract of *N*. *norvegicus* was related to the gastric mill, an organ used to grind food in the upper gut, larger specimens had larger gaps and so more and larger fibres could pass through the gut and be excreted. They found that the only way for these trapped fibres to be lost was through moulting, where their gut lining and gastric mill was shed.

Most studies into MF have focussed on the marine environment and have found that the majority of fibres from the deep sea benthos were of cellulose origin (80%) with the remainder being polyester or acrylic. Degradation in the ocean is linked to UV action, so that plastic MFs in the deep sea tend to persist for hundreds if not thousands of years (Browne et al., 2011; Sanchez-Vidal et al., 2018). As the UV absorbance of freshwater is greater than saltwater, and as there is likely to be turbidity, there is likely to be a similar problem in deeper rivers and lakes (Markager and Vincent, 2000).

The freshwater shrimp *G*. *pulex* has been used as a model organism for investigating a range of topics within ecotoxicology, for example hormonal responses (Gismondi, 2018), metabolic responses (Lebrun et al., 2012), the effect of pesticides (Auber et al., 2011), and heavy metals (Duddridge and Wainwright, 1980). *Gammarus pulex* are especially useful for investigating the impact of MP because of their variable diet (Bloor, 2010, Bloor, 2011; Kunz et al., 2010). While predominantly shredders feeding on leafy detritus, they will predate several invertebrate taxa as well as feed upon carrion. In addition they are an essential food source for many small fish (Kunz et al., 2010; MacNeil et al., 1999) and represent a vector for plastics to enter the vertebrate food chain. *Gammaridae* are a diverse family of amphipod crustaceans with representatives in freshwater, brackish and marine environments. Therefore conclusions drawn from studying them are applicable all over the globe (Costa et al., 2005; Kunz et al., 2010).

No recent studies have investigated how MF may affect feeding behaviour and may cause selective feeding in *G*. *pulex*, *n*or have *G*. *pulex* been exposed to MF. Previous studies have shown that several macroinvertebrates, including *G*. *pulex*, will ingest MP in a variety of presentations, from as a suspension to settled on food (Weber et al., 2018).

One difficulty in many studies into MFs has been that they are often studied without being incorporated into food sources and in concentrations well above environmentally relevant levels (Hanvey et al., 2017; Wagner et al., 2014). While some studies have produced a method for exposing invertebrates to a reliable dose of MP alongside plant matter (Straub et al., 2017), it is unknown how well they work with MF or larger MPs. It has been shown that algae and grasses provide a vector for MP into taxa not obviously at risk of MP ingestion (Goss et al., 2018; Gutow et al., 2016), therefore this relationship must be thoroughly investigated.

In this study we have adapted a method for dosing food with MFs that was originally developed to study plant litter decomposition and invertebrate consumption (Kampfraath et al., 2012). Our new method permits a reliable quantifiable method for exposing benthic macroinvertebrates to MFs. We used the method to identify whether *G*. *pulex* show any preference or repellence towards MF when they are part of a food source. This understanding is of utmost importance because it gives an idea as to the potential for environmental MF to enter the food chain. In order to gain a greater understanding behaviour must be investigated, previous studies have suggested that chronic exposure to MP impacts growth (Straub et al., 2017), thus making it less nutritious and could be a driver for food choice (Carrasco et al., 2019). However, if such avoidance is detected during the first exposure to MP then avoidance cannot be due to the lower nutritional value, as this has not yet been learned by individual organisms.

4.3 Materials and methods

4.3.1 *G. pulex* collection site

The *G. pulex* were collected from Emm Brook, a tributary of the River Lodden, within Dinton Country Park in Reading, between the points (Decimal Degrees 51.440494, −0.874373 to 51.442274, −0.874359). This site was chosen for its good population of *G. pulex*, ease of access and because of its relatively shallow depth of <90 cm. Animals over 12 mm in length were collected by kick sampling using a hessian net, placed in plastic bottles filled with stream water and transported to the laboratory. The animals were briefly rinsed with reverse osmosis (RO) water in the laboratory to remove silt and river water and then species confirmed using a key (Eggers et al., 2016).

In the laboratory *G. pulex* were placed in 45 L plastic tanks (150 per tank) containing 40 L aerated Organisation for Economic Co-operation and Development (OECD) reconstituted water (Hooper et al., 2006),maintained at 17 °C with 12:12 light to dark ratio and fed algae wafers (Wafer Algae Eater Fish Food, API).

4.3.2 Microfibre preparation

Black 100% acrylic wool (Hayfield Bonus DK product code 5723101001, Hobbycraft, Farnborough) was used to generate MFs. The wool was cut into pieces to generate lengths of <5 mm by wrapping a length 5 times around two nails placed into a piece of wood 10 cm apart to generate ten parallel lengths. The wool was sprayed with RO water until it was saturated and then frozen at -80 °C for 1 h. After an hour the wool was removed and the first and last cm removed using a metal scalpel (Swann-Morton No 11 blade) and then cut into 5 cm lengths which were stored on ice until ready to be used. The wool lengths were further sliced into <500 µm lengths and dried on a hot plate.

4.3.3 Wafer production

Algae wafers, were ground using a mortar and pestle for 1 min until they were powder and stored in an airtight lidded glass beaker to prevent contamination. To make the wafers, 1 g of the algae powder was added to 0.5 mL of RO water and mixed to form a paste. The paste was shaped into a flat cake 5 mm thick and placed on a hot plate at 70 °C for 2 h to dry. Test wafers were prepared by adding 0.5%, 1%, 1.5%, 2%, 2.5% and 3% MF fibres by weight to the powder and then homogenized by grinding for a further 1 min before adding the RO water.

Once dried each cake was cut up into 0.05 g wafers with a scalpel and placed in a separate lidded container to prevent contamination. To test the accuracy of this method for exposure of animals to known amounts of fibre, ten of each nominal concentration of test wafer were cut into quarters. Each quarter was crushed with a spatula and placed under a 10× binocular microscope (Optech Microtech) for counting.

4.3.4 Execution of tests

Eight individual *Gammarus* 12-20 mm in length were placed in a 5 L aquarium filled with aerated 2 L reconstituted water and starved for 24 h. The *Gammarus* were then individually placed into an aerated 5 L aquarium filled with 2 L reconstituted water along with one 0.05 g wafer (either control or treatment) and left for 4 h to feed. After 4 h each *Gammarus* was removed from its tank, placed in a 5 mL beaker and killed with 50 °C water. Eight tanks were used per day for 5 days, with concentrations distributed randomly across the period, resulting in 10 replicates per treatment. Each day the aquariums were rotated in order to ensure that there was no impact from position.

Guts were removed from dead *Gammarus* under a binocular dissection microscope at 10× magnification. To remove the gut, the telson was removed with a second cut immediately behind the eyes. The gut was then pulled whole from the body using fine point forceps and picked through, counting the number of fibres.

Choice experiments were conducted using the same protocol, except each test aquarium had one 0.05 g control wafer as well as a 0.05 g test concentration wafer. The amount of time each *G*. *pulex* spent feeding on each wafer and the number of visits to each were recorded over 4 h, this was referred to as behavioural data.

4.3.5 Data analysis

All data analysis was conducted using R and R Studio. Shapiro-Wilkes tests were used to test for normality. The wafer data met the assumptions for normality and Two Way Analyses of Variance were conducted to see if there was any significant difference between wafers or wafer quadrants within each concentration. ANOVA was conducted between the concentrations in order to confirm significant difference in the number of MF between the concentrations.

The ingestion data met assumptions for normality therefore ANOVA was conducted to identify the relationship between the number of MF ingested and the concentration of MF in wafers.

The choice data did not meet the assumptions for normality, therefore Kruskall-Wallis tests were used in place of ANOVAs to investigate MF ingestion between concentrations. It was expected that the number of MF ingested would be half that of the non-choice experiment, however it was found that approximately half *G*. *pulex* ingested no MF, these

were ignored and Wilcoxon Rank tests were used to investigate the difference between the treatments of choice and no choice of those *G*. *pulex* which did ingest MF.

Behaviour data fit the assumptions for normality and so ANOVAs were used to identify the functional response.

4.4 Results

4.4.1 Wafers

All wafers dried and set as expected and were easily dissected. There was no significant difference in acrylic fibre counts between wafers or wafer quadrants within each concentration (Table 3.1). Fibres were measured against a microruler in order to measure them to the closest 100 µm and 93% were found to fall between 200 and 500 µm.

The number of fibres were directly proportional to the % of MF by mass added Figure 4.1), and significantly different between concentrations $F_{1,118} = 14{,}766$ $P < 0.0001$.

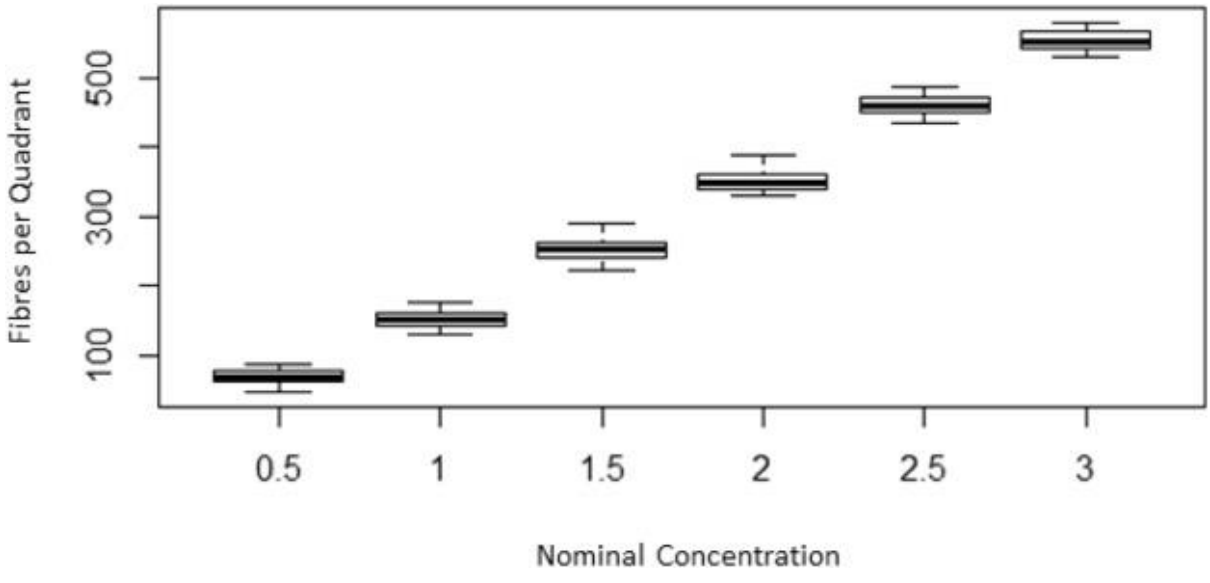


Figure 4.1 The number of fibres per quadrant of algae wafers made using different percentages (by mass) of 200-500 µm Acrylic fibres, N at each concentration = 40, median, quartiles and range shown.

Ingestion.

The *G*. *pulex* readily fed on the test wafers and ingested MFs. Thirty percent of the 1% treatment and 10% of the 2% treatment ingested no MF. There was a direct relationship between wafer concentration and the number of MF eaten (Figure 4.2), with a significant difference between test concentrations $F_{1,28} = 54.21$ $P < 0.0001$.

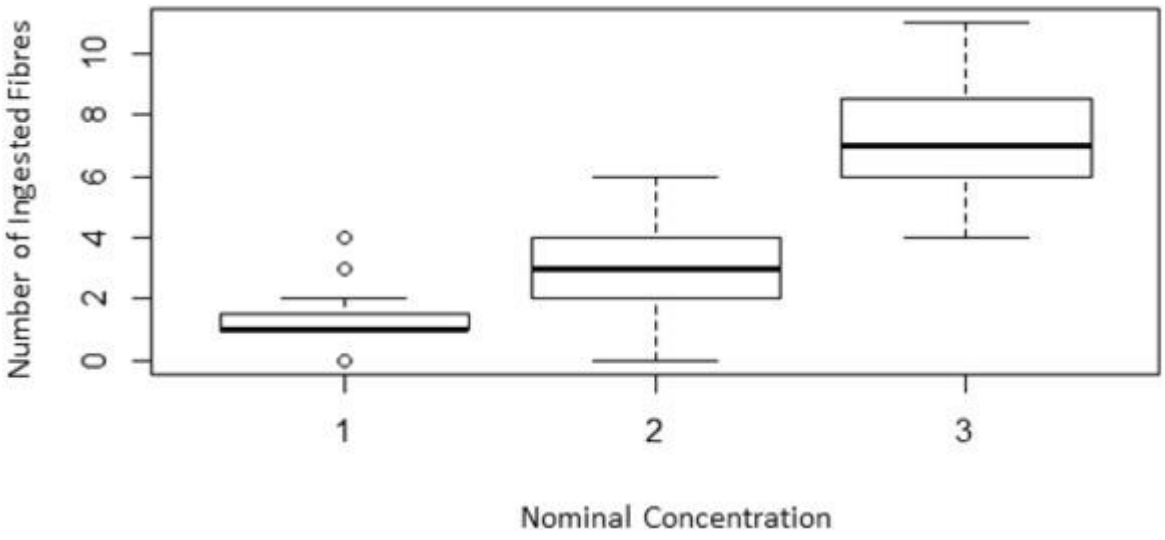


Figure 4.2 The number of 200-500 µm Acrylic fibres ingested by *G. pulex* in 4 h at 3 test concentrations. N for each concentration = 10, median, quartiles and range shown.

4.4.2 Choice experiments

Gammarus ingestion of MF approximately halved when animals were given a choice between contaminated and uncontaminated food (Figure 4.3). There was no significant difference in the number of MFs ingested between the concentrations when given a choice of uncontaminated food $H(2) = 3.028$ $P = 0.22$. Of the 12 *G*. *pulex* at each concentration, 4 of the 1%, 6 of the 2% and 5 of the 3% had ingested no MF, equating to approximately half of each concentration. When those that had ingested no MF were removed from the data and the remaining results were compared to the no-choice data, those *G*. *pulex* with a choice ingested significantly fewer MF than those without a choice Figure 4.4 (2% $W = 7$ $P = 0.017$, 3% $W = 13$ $P = 0.034$).

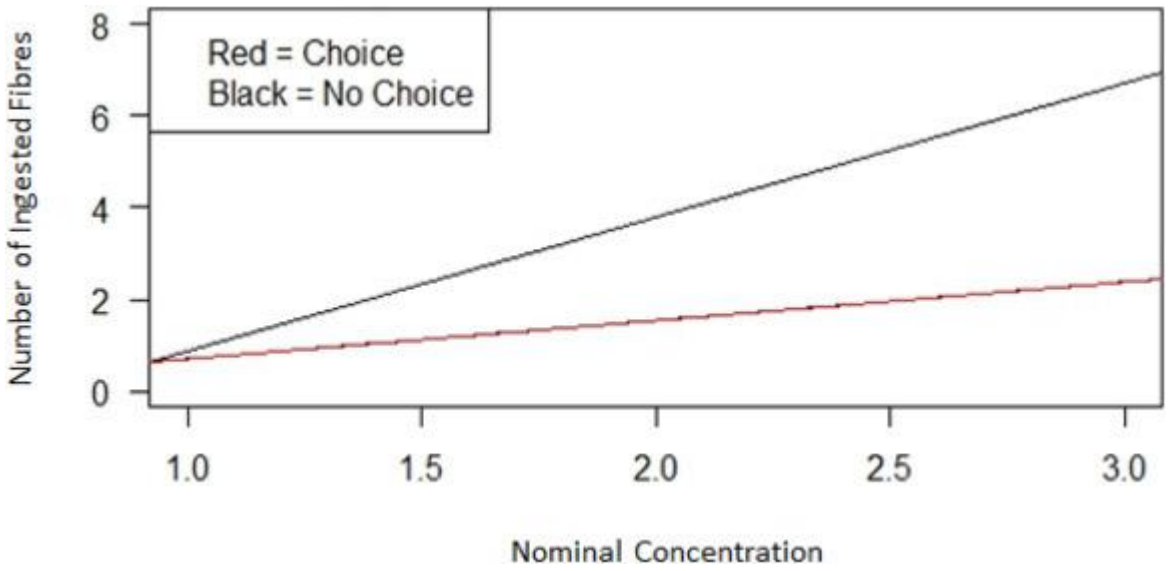


Figure 4.3 Linear Regressions for the ingestion of 200-500 µm Acrylic fibres by *G. pulex*, with and without the choice of non-contaminated food. N for each concentration = 12.

The observation tests revealed that *G. pulex* spent significantly less time feeding ($F_{1,18}$ = 21.3 P = 0.0002) on and significantly fewer visits ($F_{1,18}$ = 5.312 P = 0.0333) to contaminated wafers (Figure 4.5).

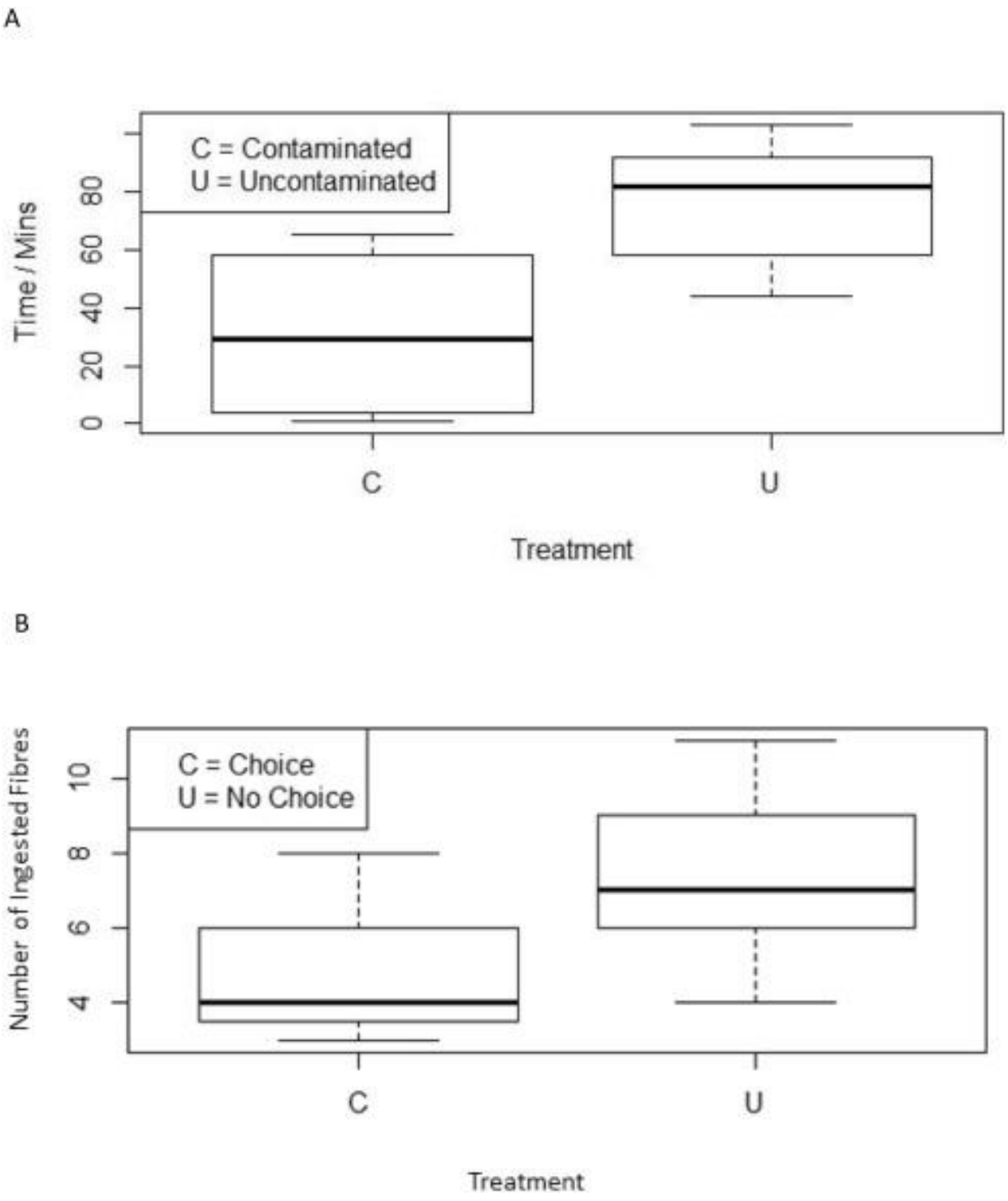
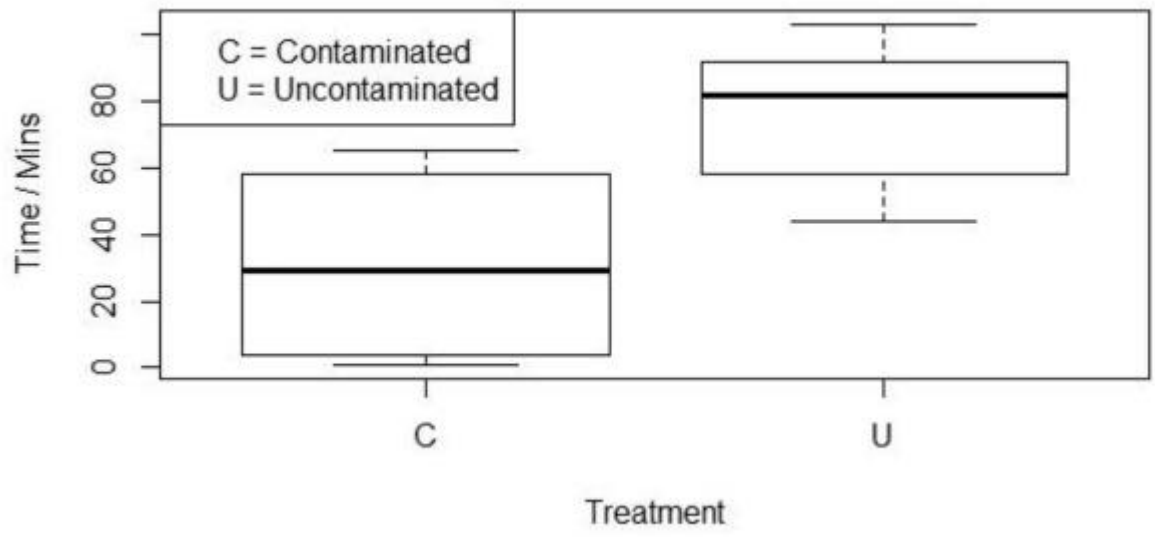


Figure 4.4 The ingestion of 200-500 µm Acrylic fibres by *G. pulex* with and without the choice of uncontaminated food at fibre concentrations (by mass) of 2% (A) and 3% (B), median, quartiles and range shown.

A



B

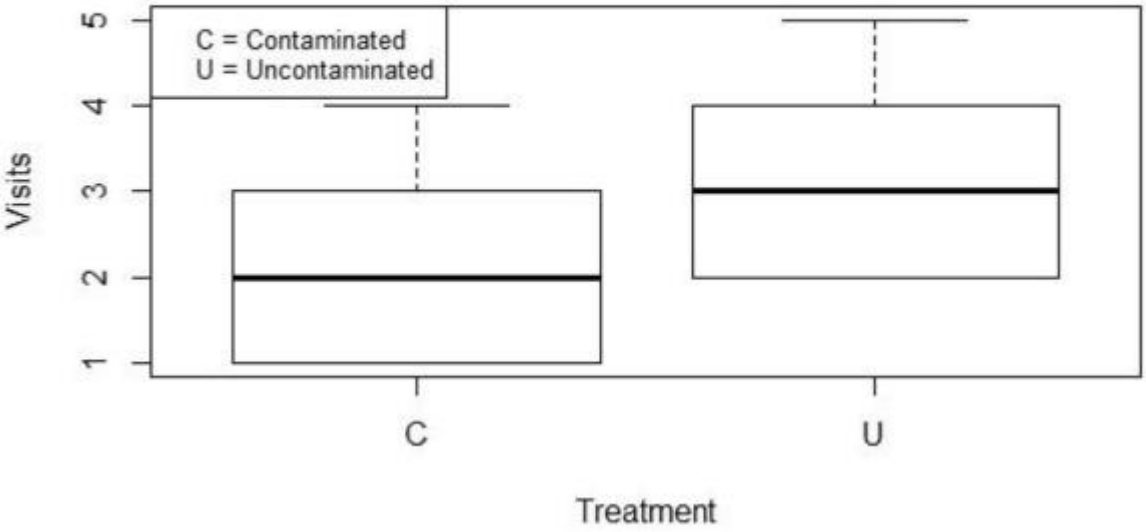


Figure 4.5 The amount of time in minuets *G. pulex* spend feeding from uncontaminated wafers and wafers contaminated with 200–500 µm Acrylic fibres (A) and the number of visits to each type of wafer (B), median, quartiles and range shown.

4.4 Discussion

We have developed an accurate, cheap and easy method to produce wafers to investigate the impact of MFs on aquatic invertebrates based on the method of (Kampfraath et al., 2012). The wafers produced were homogenous within a concentration and MF counts were directly proportional to the % of MF used to produce the wafer. Therefore, we can be confident that this method allows reliable dosing of MF which show a tendency to clump together without a solid matrix. *G*. *pulex* ingest plastic MFs in lengths up to at least 500 µm in proportion to the concentration present.

This method allows researchers to instigate worst case scenarios where invertebrates may be unable to avoid MF and can be used to study preference between different MFs. This method would work for smaller MF and MP, and should be suitable for other organisms which will feed upon algae wafers, enabling a standardised method for understanding the impact of various MPs upon a range of environments.

There are several reasons why invertebrates may detect and avoid plastics in food, there could be chemical cues (De Lange et al., 2006) or it could be they can physically feel their presence (Carrasco et al., 2019). If the main driving factor is the difference in texture between food and MP then the main food media texture should match the natural food texture as much as possible. An agar based gelatinous food source such as is used by Straub et al. (2017) produces a greater contrast between the food and the MP texture compared to this new method or natural food sources.

When given a choice between contaminated and uncontaminated food, *Gammarus* significantly avoided eating food with MFs, with fewer visits to the food and a reduction in time feeding. These observations were supported by quantitative data

demonstrating a significant difference in MFs ingested. *Gammarus* have previously avoided eating contaminated food including when chemical cues to bacteria and fungi are present (De Lange et al., 2005; De Lange et al., 2006). Furthermore there is evidence that animals can detect and avoid MPs. Carrasco et al. (2019) exposed *Orchestoidea tuburculata* to artificial food containing 8 µm particles of polystyrene MP spheres at 3 different concentrations (0%, 5% and 10%). The animals consumed significantly more food when no MPs were present compared to food contaminated with 10% MPs. As this study was a relatively short exposure (15 days) it is possible that the avoidance mechanism is physical rather than biochemical.

In the current study contaminated wafers were eaten with no evident repulsion when no uncontaminated food was available. This is in line with other studies which have recorded MF ingestion of fibres of up to 5 mm in length by taxa larger than *Gammarus*, including crustaceans, molluscs, annelids and fish, (Farrell and Nelson, 2013; Foley et al., 2018; Straub et al., 2017; Watts et al., 2015). Similar results have been found in the smaller *Daphnia magna* with many studies showing that there is a positive relationship between concentration of MP and the number ingested (Canniff and Hoang, 2018; Jemec et al., 2016; Rehse et al., 2016). However, Aljaibachi and Callaghan (2018) found that *Daphnia* seemed to be able to selectively ingest algal cells and avoid 2 µm MP particles.

These results are important in understanding the risk to the environment. It suggests that, at least *Gammarus* is able to avoid MF contaminated food, meaning that as long as their environment is not totally saturated with MF they could be ingested in rates lower than one might assume given environmental concentrations. As macroinvertebrates are the main vector for MP entering the higher trophic levels (Foley et al., 2018), including

vertebrates and ultimately humans, their ability to limit MP ingestion would in turn limit the amount entering higher trophic levels. There is already a highlighted knowledge gap in this area (Horton et al., 2017) and its understanding would help direct mitigation processes.

Gammarus produce copious amounts of faecal pellets which are eaten by other freshwater macroinvertebrates and are important sources of organic matter for bacteria (Joyce et al., 2007). Microfibres were clearly observed in faecal pellets with no evidence of being shortened which means that not only could *G*. *pulex* act as a vector for MP to enter higher trophic levels if they are eaten by fish or other invertebrates, but their faeces provide a source of MP to enter lower trophic levels through faecal ingestion (Kelly et al., 2002; Ladle and Griffiths, 1980) (Kelly et al., 2002).

Despite their apparent ability to avoid ingesting MF contaminated wafers, it remains to be seen whether *G*. *pulex* predation on differentially contaminated prey would vary.

CHAPTER 5.

Microplastic and Organic Fibres in Feeding, Growth and Mortality of *Gammarus pulex*

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Yardy, L., Callaghan, A. 2021. Microplastic and Organic Fibres in Feeding, Growth and Mortality of *Gammarus* pulex. *Environments*, 8, 74

5.1 Abstract

Microplastic fibres (MPFs) are a major source of microplastic pollution, most are released during domestic washing of synthetic clothing. Organic microfibres (OMF) are also released into the environment by the same means, with cotton and wool being the most common in the UK. There is little empirical evidence to demonstrate that plastic fibres are more harmful than organic fibres if ingested by freshwater animals such as *Gammarus pulex*. Using our method of feeding *Gammarus* MPFs embedded in algal wafers, we compared the ingestion, feeding behaviour and growth of *Gammarus* exposed to 70µm sheep wool, 20µm cotton, 30µm acrylic wool, and 50µm or 100µm human hair, and 30µm cat hair at a concentration of 3% fibre by mass. *Gammarus* would not ingest wafers containing human hair, or sheep wool fibres. Given the choice between control wafers and those contaminated with MPF, cat hair or cotton, *Gammarus* spent less time feeding on MPF but there was no difference in the time spent feeding on OMFs compared to the control. Given a choice between contaminated wafers, *Gammarus* preferred the OMF to the MPF. There were no significant differences in growth or mortality among any of the treatments. These results conclude that MPFs are less likely to be ingested by *Gammarus* if alternative food is available and are not more harmful than OMFs.

Keywords: Microplastic, Fibres, Animal hair, Wool, Cotton, *Gammarus pulex*, Feeding, Growth

5.2 Introduction

Microplastic pollution is no longer an obscure concern of environmental scientists. The level of public awareness and concern has resulted in changes to individual behaviours as well as governments updating legislation (Andrady, 2011; Huerta Lwanga et al., 2016; Leslie, 2014). Microplastics were first discussed in the marine environment, but there are now a substantial number of studies on the presence and impact of MP in freshwater (FW) environments (Eerkes-Medrano et al., 2015; Foley et al., 2018; Horton et al., 2017).

Microplastics (MPs) are defined as plastic particles of under 5mm in size. They are either manufactured as such (primary MPs) or are produced when plastic products break down into smaller fragments (secondary MPs) (Andrady, 2011). Secondary MPs are categorised into fragments, fibres, foams films and pellets (Rochman et al., 2019). Microplastic fibres (MPF) are defined as more than twice as long as they are thick (Cole et al., 2011).

MPs in the freshwater environment originate from many sources including effluent from factories (Lasee et al., 2017), surface water runoff (D.D. Poudel et al., 2010) , aerial dispersal (Dris et al., 2016; Prata, 2018) and slurry runoff. A significant contribution to MP pollution comes from microplastic fibres (MPF) which are copiously shed during machine washing of synthetic clothing (Browne et al., 2011; Napper and Thompson, 2016). The combination of vigorous machine washing, a massive shift from clothing materials made from natural fibres to plastics and disposable 'fast-fashion' has resulted in a serious pollution issue(Henry et al., 2019).

Studies on MPs tend to be divided into those looking for evidence of ingestion and those looking at the impact of the MP on some aspect of the organism's biology, with mixed and sometimes conflicting results (Al-Jaibachi et al., 2018b; Canniff and Hoang, 2018; Hämer et

al., 2014; Straub et al., 2017; Windsor et al., 2019). While the majority of studies have focused upon MP particles, those which focus upon MPFs have found similar results with good evidence for ingestion in marine crustaceans, *Orchestia gammarellus, Carcinus maenas, Carcinus aestuarii* and *Nephrops norvegicus* (Farrell and Nelson, 2013b; Hodgson et al., 2018; Piarulli et al., 2019; Welden and Cowie, 2016). Meta analysis has shown that virtually every taxa investigated has been shown to ingest MPFs, and they have been shown to be harmful, especially to juveniles of both vertebrates and invertebrates (Foley et al., 2018). An interesting question is why certain organisms would eat MPFs in the first place and there is an assumption that ingestion is accidental, indeed, this incidental feeding does seem to be the predominant cause of ingestion in fish (Peters et al., 2017; Peters and Bratton, 2016). This assumption was not fully upheld in our previous study, where we observed that *Gammarus pulex* selectively preferred food that was not contaminated with acrylic MPF (Yardy and Callaghan, 2020). That study concluded that the presence of the MPF was immediately detected, and it was the presence of these fibres that deterred feeding, however, some fibres were still ingested suggesting incidental feeding did occur.

Most MPFs are released during domestic washing of synthetic clothing, where mechanical and chemical stress can cause the detachment of fibres(De Falco et al., 2019). However, organic microfibres (OMF) are also released into the environment by the same means (Estahbanati and Fahrenfeld, 2016; Sanchez-Vidal et al., 2018), with cotton and sheep wool being the most common in the UK (WRAP, 2019). Both human and animal hair are commonly released into wastewater and hair, wool and cotton are all similar in thickness to many MPFs (Kshirsagar et al., 2009).

Whilst wool can refer to the hair products of several taxa, in the UK the majority is from sheep in the genus *Ovis* which have a thickness of 70-90µm (Kshirsagar et al., 2009). Cotton fibres range from 10-20µm (Huber and Müssig, 2008) and pet hair such as dog or cat can range between a 19 and 120 µm (Sato et al., 2006). Given that these fibres are in the same size range as MPFs, and that it is the physical presence of MPF in the guts of invertebrates displacing food that can cause negative impacts (Au et al., 2015; Blarer and Burkhardt-Holm, 2016a; Weber et al., 2018; Yardy and Callaghan, 2020), we decided to use our previous methodology to investigate OMF ingestion in the freshwater shrimp *Gammarus pulex*(Yardy and Callaghan, 2020) . *Gammarus pulex* is a standard ecotoxicological model organism and is important to many freshwater ecosystems across Europe and Asia (Blarer and Burkhardt-Holm, 2016a; Bloor, 2010; Neuparth et al., 2002), operating as a prey species, predator and shredder of organic material (Bloor, 2010; Kelly et al., 2002a, 2002b; Kennedy et al., 1978).

We previously demonstrated that *Gammarus* will eat acrylic MPFs embedded in an algal wafer when given no choice, but prefer not to eat the MPFs if uncontaminated wafers are available(Yardy and Callaghan, 2020). What is not known, and what this study aims to identify is whether the same behaviour of avoidance is observed when OMF are used rather than MPF. If it is simply the physical presence deterring feeding then it is expected that feeding will be indirectly proportional to the thickness of the fibres, OMF or MPF.

5.3 Materials and Methods

Gammarus pulex were gathered using kick sampling from a tributary of the River Lodden, Emm Brook (Decimal Degrees 51.440494, -0.874373 to 51.442274, -0.874359). This location provided a healthy population of *G. pulex* in a river with safe and easy access, reliable flow throughout the year and shallow depth. Hessian kick nets were used for collection and only

individuals greater than 12mm in length were taken and transported in plastic (PET) bottles from the collection site to the laboratory.

Once in the laboratory *G.pulex* were rinsed with reverse osmosis water to remove any contaminants from the brook and then placed into 45L tanks of Organisation for Economic Co-operation and Development (OECD) reconstituted water (Hooper et al., 2006), aerated with diffusion stones.

5.3.1 Fibre Preparation

A variety of different fibres that might be found in the aquatic environment following clothes washing were chosen (Table 5.1).

All fibres were soaked in RO water for 24 hours and then rinsed with RO water to remove surface contamination. The cat and human hairs were twisted into a thread similar to the cotton and acrylic (Figure 5.6) , allowing them to be prepared using the methodology of (Yardy and Callaghan, 2020), whereby the thread is saturated with Reverse Osmosis (RO) water frozen at -80˚C, and then inserted into a jig, allowing 500µm lengths to be cut off and collected.

The wafers were produced by homogenising 0.03g of the manufactured MPF and OMFs with 0.97g ground algal wafers (Wafer Algae Eater Fish Food, API) in a mortar and pestle. After 1 min 0.5ml of RO water was added to reconstitute the mixture into a paste. This paste was then pressed to a 5mm thick cake and dried on a hotplate. Once dried the cake was divided into 0.05g wafers. Ten of each of the OMF wafers were selected, divided into quarters and crushed: the number of fibres within each wafer were recorded to calculate an average number of fibres per wafer.

Table 5.1 Size, colour and source of fibres used

Fibre	Size	Colour	Origin
100% cotton thread *Gossypium arboreum*	≈20µm	Black	DMC black special embroidery thread product code 6404211000, Hobbycraft, Farnborough
100% acrylic wool	≈30µm	Black	Hayfield Bonus DK product code 5723101001, Hobbycraft, Farnborough
Cat hair *Felis catus*	≈30µm	Dark brown	Calico – author's cat.
Human hair *Homo sapiens*	≈50µm	Dark brown	Female – author's mother
100% Jacob Wool yarn *Ovis aries*	≈70µm	Black	West Yorkshire Spinners Brown Black Fleece Jacob Aran Yarn product code 6223481003, Hobbycraft, Farnborough
Human hair *Homo sapiens*	100µm	Dark brown	Female – author's wife

5.3.2 Acute exposures

Two methods were used to expose *G.pulex* to either one or two wafers. Fourteen *Gammarus* were placed into a 5L aquarium and starved for 24 hours before exposure. For the comparative ingestion study individual *Gammarus* were placed in a 5L aquarium with 2L reconstituted water and exposed to one of the six different fibre wafers (3%) or a control wafer (no fibres) for 4 hours. After this the *Gammarus* were killed with 50˚C water, dissected under 10X magnification, the number of ingested fibres were clearly visualized and recorded. Each day two *Gammarus* were exposed to each treatment, this was repeated for 5 days providing 10 replicates a total of 70 *Gammarus*.

5.3.3 Choice experiment

For the choice experiments the same experimental design was used, except *Gammarus* were exposed to each of the six different fibre wafers as well as a control for four hours, and

the time spent feeding on each wafer and the number of visits made to each wafer were recorded, as were the number of fibres ingested. Due to the need for constant observations, and the length of time the experiments required, two rounds of experiments with three individuals in each could be performed per day. Each treatment was performed once per day and repeated for 10 days, giving 10 replicates.

Gammarus were exposed to the following combinations of fibre, using the methodology given above; cat/cotton, cat/acrylic, cotton/acrylic. The *Gammarus* were observed continuously number of feeding visits and the time spent feeding on each wafer were recorded, a feeding event was decided if a *Gammarus* could be seen feeding on the wafer, or removing part of the wafer and holding it while feeding. Each day two replicates for each combination could be run, this was repeated for 5 days for a total of 10 replicates. The human and sheep wool fibres were not used in the choice experiments because no fibres were ingested in the initial exposures.

5.3.4 Chronic Exposures

As before, *Gammarus* were starved and conditioned prior to initial weighing. Individuals were removed from the aquarium, dried by gently pressing them between paper towels and weighed so that only individuals between 0.1g and 0.2g were used. Fifty individuals were allocated randomly a treatment using the Excel RAND and ROUNDUP functions (Microsoft Office). The treatments used were acrylic 1%, acrylic 3%, cat 1%, cat 3% and control, with 10 replicates for each treatment

Test aquariums were made using 250ml round PET containers with 1cm of aquarium gravel and 200ml of reconstituted water. One *Gammarus* was placed in each aquarium

alongside 1 0.05g wafer of whichever treatment was allocated. A rolling 7-day regime was followed for 28 days;

Day 1 – Weigh *Gammarus*, clean aquarium and add wafer

Day 4 – Remove old wafer and replace with new

Day 7 – Remove wafer

Gammarus were dried prior to weighing. The aquariums were cleaned while the *Gammarus* were weighed, this was done by pouring the contents of the aquarium into a 1 mm sieve and then rinsing with tap water to remove remnants of MFs, wafer and waste. The aquarium itself was then wiped with a paper towel and rinsed with tap water, the contents of the sieve were then tipped back into the aquarium and 200ml of reconstituted water was added along with a new suitable wafer, finally the *Gammarus* was replaced in the aquarium.

A block design was used: samples were divided into 5 groups A – E, with two of each treatment in each group. *Gammarus* within each group were allocated a number 1-10, thereby all 50 individual *Gammarus* could be identified with a number e.g. B5. The 7-day regime was staggered by one day, day 1 group A was Monday, day 1 group B was Tuesday etc. On day 1 it was also recorded if any *Gammarus* had died.

5.3.5 Statistical Analyses

All data was analysed using R Studio (Martin, 2021). Number of fibres ingested were corrected for the number available, and presented as % of available fibres ingested. As the block design was consistent day on day and each individual was in its own aquarium and

totally independent, all individuals were treated as true replicates. Shapiro-Wilk tests were used to test for normality within the data. The assumptions for normality were met in the comparative ingestion experiments and the time recordings for the choice experiments. The assumptions were also met in the acrylic / control and cotton / control fibre ingestion recordings. As such one-way ANOVA tests were used. The assumptions were not met for any of the choice experiment visit recordings or the cat/control fibre ingestion experiment so Kruskal-Wallace tests were used.

The chronic growth data was found to meet the assumptions for normal distribution, and one-way ANOVA tests were used. Due to the categorical nature of mortality results, the data was not normally distributed, as such McNamars test was used.

5.4 Results

5.4.1 Acute Fibre Ingestion

Gammarus pulex ingested wafers containing acrylic, cat hair and cotton but would not ingest wafers containing human hair, or sheep wool fibres. Where fibres were ingested, they were observed within the gut and faecal pellets (Figure 3.9). *Gammarus pulex* ingested significantly fewer cotton fibres than either acrylic or cat (Figure 5.1) $F_{2,27}$=5.737 P=0.0084 (Acry/Cat = 0.7491 Acry/Cott = 0.0047 Cat/Cott = 0.0103)

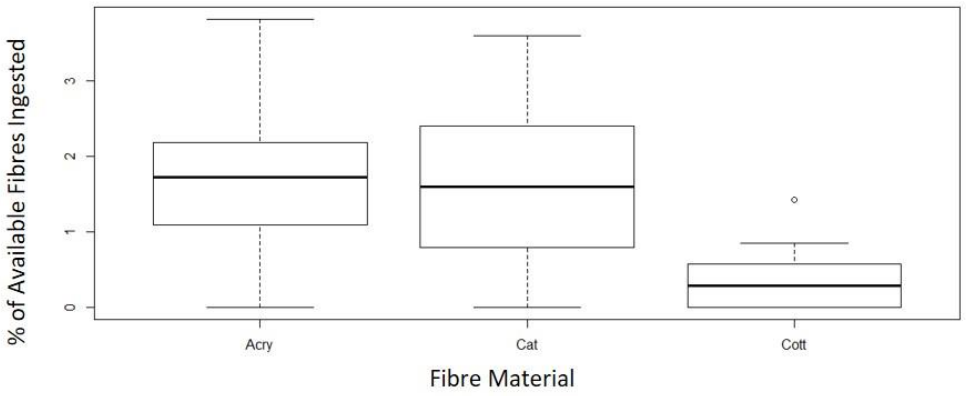

Figure 5.1 The percentage of available 200-500µm fibres ingested by *Gammarus pulex* in 4 hours. Acry = Acrylic, Cat = *Felis catus*, Cott = Cotton (n=10), median, quartiles and range shown.

5.4.2 Feeding Behaviour

When given a choice between a control or a contaminated wafer, there were no differences in the number of *Gammarus* visits made to any of the wafers (acrylic W=5.193 P=0.158 cat W=4.886 P=0.18, cotton W=1.507 P=0.680) or in the time spent feeding (cat $F_{1,18}$=0.487 P=0.494, cotton $F_{1,18}$=0.076 P=0.786) with the exception of the acrylic wafers. *Gammarus pulex* spent significantly less time feeding on the acrylic wafers (Figure 5.2 A,B,C,) ($F_{1,18}$=8.541 P=0.0084).

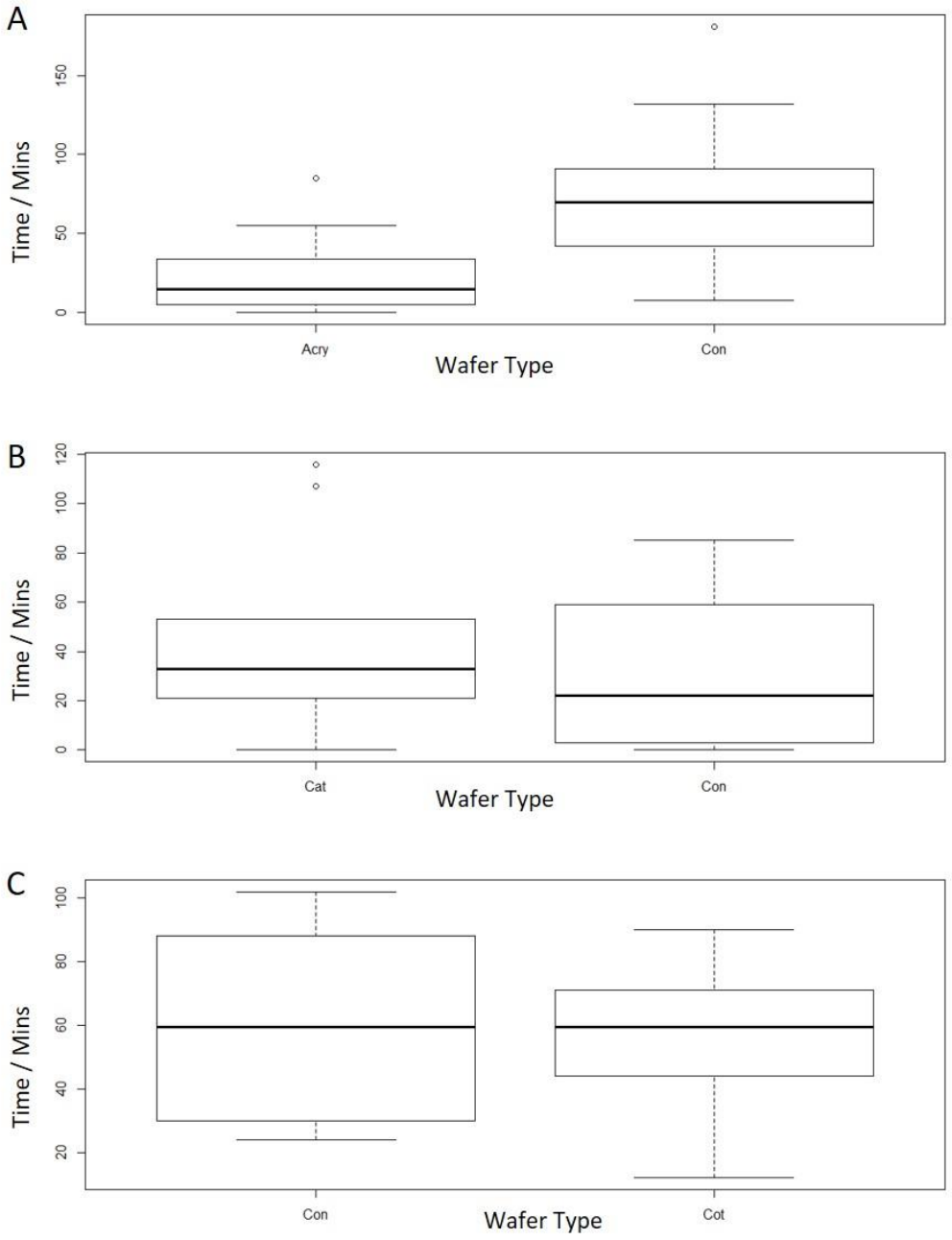

Figure 5.2 Time spent feeding on test or control wafers by *Gammarus pulex* in 4 hours. Test wafers contaminated with 200-500µm fibres Acry = Acrylic, Cat = *Felis catus*, Cott = Cotton (n=10), median, quartiles and range shown.

When given the choice between cat and acrylic or cotton and acrylic, *G.pulex* spent significantly less time feeding on acrylic wafers $F_{1,18}$=19.59 P>0.001 (Cat/Acrylic) $F_{1,18}$=20.71 P>0.001(Cott/Acrylic) (Figure 5.3A and B respectively) . Choice experiments between

contaminated wafers found no difference in time spent feeding between cat and cotton ($F_{1,18}$=0.077 P=0.785) (Figure 5.3 C).

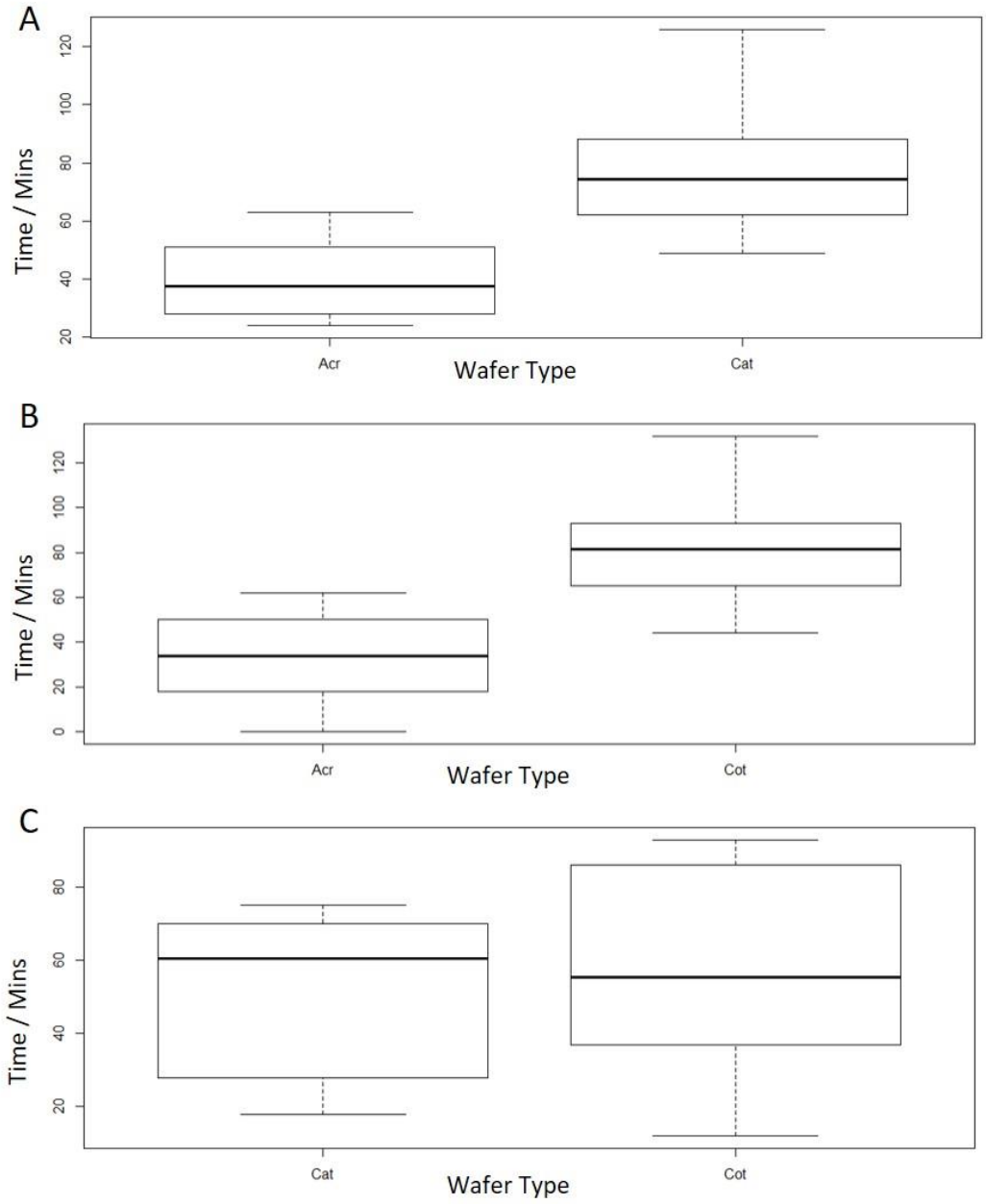

Figure 5.3 Time spent feeding on wafers by *Gammarus pulex* when given a choice between Acrylic & Cat (A), Acrylic & Cotton (B) and Cat & Cotton (C) (n=10). Contamination was 3% by mass 200-500µm fibres, median, quartiles and range shown.

It was found that given the choice between contaminated and control wafers, several organisms did not ingest any fibres 4/10 (Acrylic & Cat) and 6/10 (Cotton), these were removed from the analysis. Several organisms also did not ingest any fibres even without a choice of non-contaminated wafers 1/10 (Acrylic & Cat) and 4/10 (Cotton), these were also removed from the data set. When the results were analysed (Fig 5.4) it was found that significantly fewer acrylic fibres were ingested when given a choice $F_{1,13}$= 8.524 P=0.012, but there was no significantly difference in the number of OMF ingested with or without non contaminated wafers.

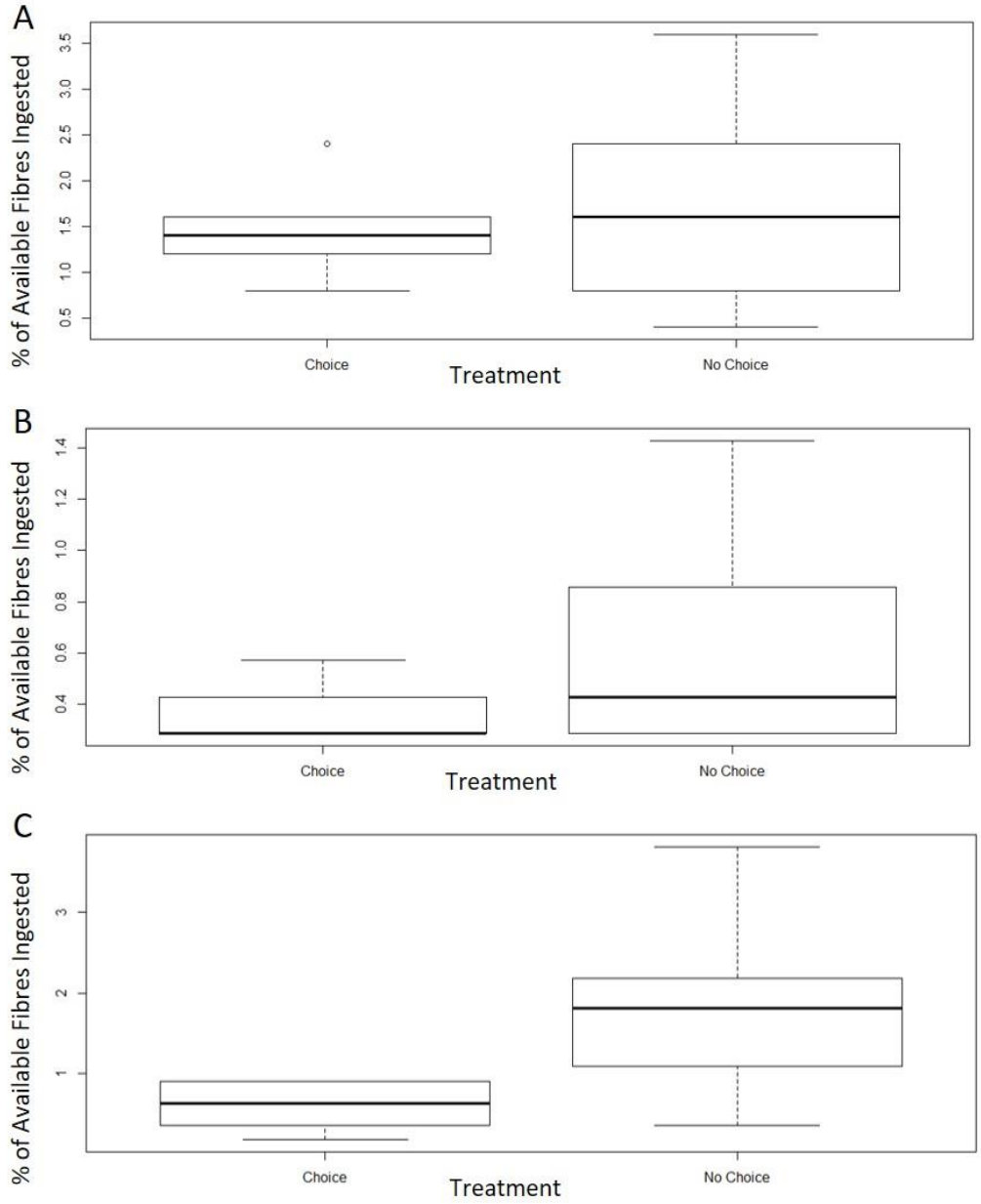

Figure 5.4 % of available fibres ingested with and without the choice of uncontaminated food sources by *Gammarus pulex* (n=10). Contamination was 3% by mass 200-500µm fibres from cat (A) cotton (B) and acrylic (C), median, quartiles and range shown.

5.4.3 Chronic ingestion

There was no significant difference in the starting mass of individual *Gammarus* between treatments ($F_{4,45}$=0.312 P=0.869). After the 28 days, there was still no significant difference in mass ($F_{4,45}$=0.812 P=0.524), or growth (change in mass) of *Gammarus* between treatments (Figure 5.5) ($F_{4,42}$=0.761 P=0.557). The greatest growth was found in the Control (7.7mg ±3.8 n=9) and Cat 3% (7.7mg ±4.0 n=10), followed by Acrylic 3% (7.2mg ±3.1 n=10), Acrylic 1% (5.3mg ±3.8 n=9), with smallest growth in Cat 1% (4.6mg ±6.0 n=9).

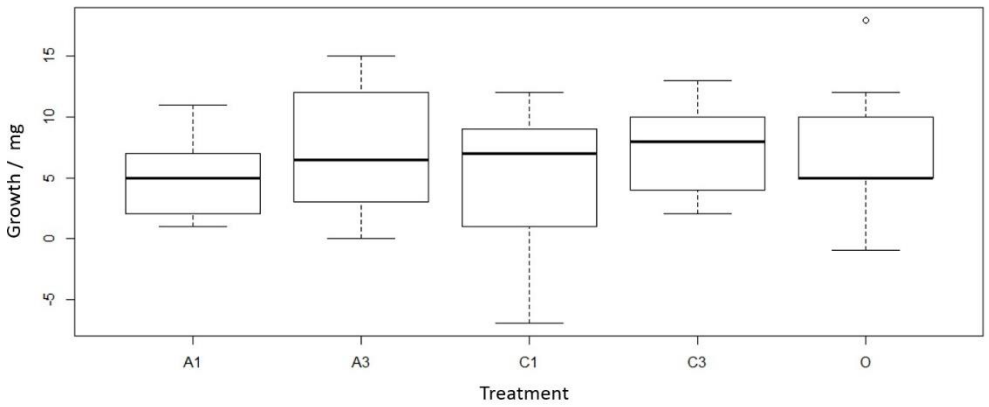

Figure 5.5 Growth as change in mass / mg of *Gammarus pulex* after 28 day exposure to different fibre treatments. A1 – Acrylic 1% by mass, A3 – Acrylic 3% by mass, C1 – Cat 1% by mass, C3 – Cat 3% by mass, 0 – Control, median, quartiles and range shown.

While there was greater mortality in the acrylic treatments compared to cat treatments (1% 2/10 vs 1/10, 3% 4/10 vs 1/10) these were found to be not significantly different (1% Chi^2_1=1 P=1, 3% Chi^2_1=1.33 P=0.248).

5.5 Discussion

When given a choice between food contaminated with acrylic fibres and those without, *G. pulex* avoided eating the acrylic-contaminated wafers. This is a repeat of the result published by Yardy and Callaghan(Yardy and Callaghan, 2020) using the same technique applied here. However, unlike our previous study, there was no evidence in a reduction in the number of visits to contaminated wafers, only the number of fibres ingested and the time spent feeding on contaminated wafers.

In contrast, the *Gammarus* would not eat wafers containing human hair or sheep wool, preferring to starve. These OMFs were larger in diameter than MPFs, cat hair or cotton, which were all ingested. This suggests either avoidance or a functional size limit to ingestion of fibres, with a potential maximum thickness of between 30µm and 50µm. The latter seems most likely and is similar to the findings of (Weber et al., 2018) who found PET fragments <53µm were most ingested. A study on *Gammarus fossarum* (Blarer and Burkhardt-Holm, 2016a) also found that MP 20µm were readily ingested.

Where fibres were ingested, they were observed within the gut and faecal pellets. When given no choice of food, *Gammarus pulex* ingested significantly fewer cotton fibres than either acrylic or cat hair. When not given a choice *Gammarus* did not ingest fewer acrylic fibres that cat hair, this suggests as with our previous study and others that *Gammarus* will readily ingest PMF (Blarer and Burkhardt-Holm, 2016a; Yardy and Callaghan, 2020). Cotton fibres, which were both the thinnest and as plant fibres, arguably the closest to the natural diet of *G. pulex* were ingested at the lowest rates. Size is unlikely to be a factor. Blarer and Burkhardt-Holm(Blarer and Burkhardt-Holm, 2016a) found that 20µm PA fibres were readily

ingested by *G. fossarum*. Other than the potential upper size limit, thickness does not explain fibre ingestion rates.

Chemical cues are important factors in *G. pulex* feeding (De Lange et al., 2005b). It is possible that the cotton contained unpalatable chemicals, possibly the black dye. While it has been shown that sublethal exposure to dyes does trigger a stress response in *G. pulex* (Cikcikoglu Yildirim and Yaman, 2019), this was at concentrations 1/16th of LD50, far higher than would have been experienced in this study. There was nothing within the behavioural data that suggested that *Gammarus* were repelled from feeding on cotton contaminated wafers, and so there must be another factor. As already stated, cotton fibres were identified in both gut and faeces, without any signs of degradation, therefore it cannot be that cotton was simply digested, hence its apparent absence. Another possibility is that the cotton fibres have a higher tendency than the other fibres to clump together, thereby making them easier to avoid, although none of these clumps were observed post feeding, in the gut or remaining wafer. It is possible that there were pollutants adsorbed onto the purchased fibres (acrylic, cotton and sheep wool), however, these were purchased new and stored in sealed containers, therefore any adsorbed substances would have been from manufacturing. The human and cat hairs were washed without soap prior to collection and then rewashed in the laboratory. In either case, any residual pollutants would be expected to remain had the fibres been in freshwater.

Studies with MP beads have demonstrated that size does matter. Whilst *G. pulex* ingested 90µm polystyrene (PS) beads, smaller 1µm beads that were ingested at lower rates (Scherer et al., 2017b). The relationship between size and ingestion frequency was explained by the

larger beads settling on the food rather than being suspended in the water column, so this cannot explain the differences of fibre ingestion in wafers.

Fibres of all types that were ingested in this study remained intact and were excreted whole in the faecal pellets of *G. pulex*. The literature suggests that the negative impacts from MP and MPF in *G. pulex* are related to their taking up space in the gut, thereby reducing food intake and influencing growth (Cole et al., 2011; Scherer et al., 2017a; Weber et al., 2018). Based on this we predicted that OMFs that are not digested, but egested from the gut, will have a similar impact on *Gammarus* growth as MPF.

The chronic ingestion data supported this and although there was lower growth in animals fed MPFs, there were no significant differences in growth between the fibre treatments and there was no evidence for increased mortality following MPF exposure. This study supports the findings of a previous study on *G. fossarum* (Blarer and Burkhardt-Holm, 2016a) where a significant difference was found in assimilation efficiency between those exposed to PA fibres and control. While this study found no significant difference in mortality, the discrepancy between acrylic and cat at 3% should not be ignored, and it is possible that at greater concentrations there would be a greater mortality. Importantly it is unknown whether this increased mortality would be found if uncontaminated food was available as well as acrylic contaminated, this would be more applicable to environmental conditions.

Gammarus pulex show an avoidance to MPF when given a choice, but no avoidance to OMF, and as these are far more numerous (Sanchez-Vidal et al., 2018; WRAP, 2019) it follows that they are more likely to ingest OMFs (deBruyn and Rasmussen, 2002). If OMFs are more likely to be ingested and they do not elicit avoidance behaviour, yet they have a similar impact, it could be argued that it is the lower diameter OMF not MPF are a greater risk to at

least some invertebrate species. We have been releasing processed OMF into the environment for centuries, and in very high rates through WWTPs for decades (Murphy et al., 2016; Ziajahromi et al., 2017, 2016). Many individuals and organisations have suggested that as a society we should swap to organic based fabrics rather than synthetic to limit the release of microfibres. Whilst this seems to be a worthy goal, this study has found no significant difference in mortality between MPF and OMF, it is easy to assume that plastics are always more of a problem in freshwater, however this may not be the case.

5.6 Conclusions

The current, quite relevant, concern over the release of MPFs into our waterways (Browne et al., 2011; Napper and Thompson, 2016), assumes that MPFs will have a more detrimental impact than organic fibres released through the same processes. However, there is little empirical evidence to demonstrate that plastic fibres are more harmful than organic fibres which have been released from washing machines for as long as they have existed.

Figure 5.6 Cat hair twisted into a yarn ready for processing using the method described in Chapter 3

CHAPTER 6.

Impact of *Polymorphus minutus* parasitism and plastic microfibres on *Gammarus pulex* feeding

6.1 Abstract

The relationship between parasites and hosts has long been investigated, parasites can impact not only the physical health of their hosts, but also their behaviour. The freshwater amphipod *Gammarus pulex* has several known acanthocephalan parasites, with different final hosts, one of these is *Polymorphous minutus*. It has been shown that *P. minutus* parasitism causes a change in behaviour of *G. pulex*.

Using our method of algal wafers with plastic microfibres incorporated, we investigated how both parasitism by *P. minutus*, and contamination of food sources, impacted the time spent feeding on, number of visits and number of MF ingested. It was found that parasitised *G. pulex* did ingest more MF, despite making fewer feeding visits, however, none of these differences were statistically significant. As with previous experiments, *G. pulex* spent less time feeding on contaminated food sources, although unlike previous experiments this difference was not statistically significant.

Despite there being no significant impact of parasitism upon microfiber ingestion, the difference in other behaviours, make parasitised *G. pulex* more likely to be ingested, and so still a vector for MF into higher trophic levels. Due to the final host of *P. minutus* being water fowl, this is also a vector for MF into the terrestrial environment

6.1 Introduction

Plastics, especially microplastics (<5mm) are now ubiquitous in the environment. Their presence and impact has been identified at every trophic level, from biofilms (Roager and Sonnenschein, 2019; Rogers et al., 2020) to invertebrates (de Sá et al., 2018; Ogonowski et al., 2016; Windsor et al., 2019) and vertebrates (Grigorakis et al., 2017; Lehtiniemi et al., 2018; Phillips and Bonner, 2015). These impacts have been found to be varied across all trophic levels, they have decreased growth rates in algae, isopods and amphipods (Au et al., 2015; Besseling et al., 2014; Blarer and Burkhardt-Holm, 2016b). Feeding behaviour in *G. pulex* leads to avoidance of MFs (Yardy and Callaghan, 2021, 2020), fish larvae of *Perca fluviatilis* change feeding preferences and no longer respond to olfactory cues of predators, increasing mortality (Lönnstedt and Eklöv, 2016).

Biochemical changes within organisms have also been observed, larval zebrafish suffer oxidative damage due to MP ingestion (Lei et al., 2018; Sökmen et al., 2020). Following MP ingestion, tilapia experience a 37% inhibition of acetylcholinesterase and a great increase in superoxide dismutase activity in the liver (Ding et al., 2018). These impacts are deeply contested, with many studies having shown no impact, particularly on growth (Critchell and Hoogenboom, 2018; Thomas et al., 2020; Weber et al., 2018; Yardy and Callaghan, 2021). Because the presence of MP seems to be ubiquitous, in aquatic environments and in being ingested by a huge range of organisms, the impacts observed above might be expected to lead to impacts on the environment (Shen et al., 2020).

In the process of collecting *Gammarus* for experiments, including those in Chapters 4 and 5, it was noticed that some *Gammarus* had a small elliptical pellet in their gut. After researching potential causes, A*canthocephalan* parasitism seemed the most likely. This was

due to the size and shape of the pellets, the species they were found in, and that Emm Brook has populations of fish and waterfowl that act as the final host for British Acanthocephalans.

Acanthocephalans are a phylum of parasitic worms (spiny head worm), named after their spiked proboscis, which utilise trophic transfer to their final host, which is most often a vertebrate (Nicholas, 1973). Several acanthocephalans are native to Britain, including *Pomphorhynchus* spp. (Kennedy et al., 1989). This genus often uses freshwater invertebrates such as *G. pulex* as intermediate hosts before it moves to the final host, which include the common chub *Leuciscus cephalus,* the three-spined stickleback *Gasterosteus aculeatus* and the brown trout *Salmo trutta* (Bakker et al., 1997; Kennedy et al., 1978; Sures and Siddall, 1999). The life cycle of the parasite is complicated and they typically have an intermediate and an end host. Eggs are laid by females in the gut of the final host, and are excreted in faeces. From here they are ingested by an arthropod, such as *Gammarus*. The larva, called an acanthor, hatches from the egg and enters the haemocoel through the gut wall, where it develops into an acanthella while internal organs develop. Once a fully developed larvae they are referred to as cystacanth, in this form they have a fully developed proboscis which is inverted within the body, which is enclosed in a cyst. At this stage, the Acanthocephalan is infective and when ingested by a final host the cyst opens and the proboscis hooks onto the gut and the adult stage begins (Figure 6.1).

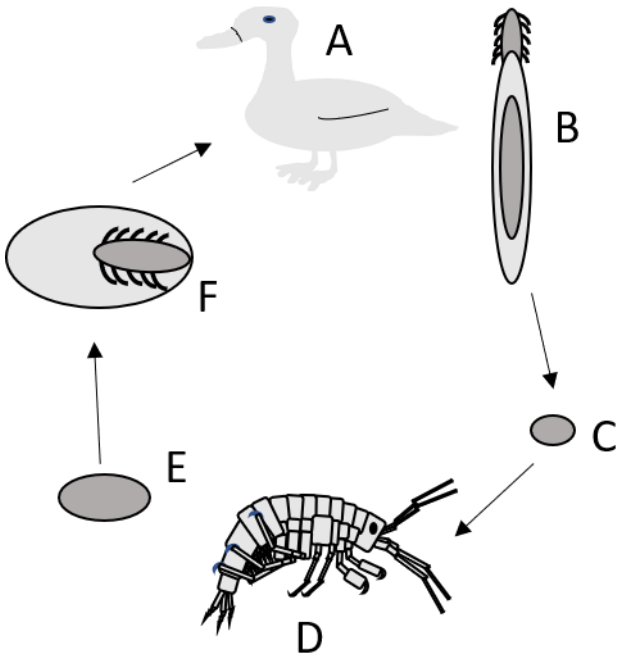

Figure 6.1 The lifecycle of *Polymorphous minutus*. In the final host (A) the adult worm (B) lays eggs (C) these are ingested by the intermediate host (D) once ingested the eggs hatch into an acanthor (E), then develops until internal organs are formed the proboscis is inverted and the larvae is an infective cystacanth (F).

The relationship between parasites and their hosts has long been an important topic within ecology, since parasites can impact the behaviour and condition of their host organisms. The freshwater amphipod *Gammarus pulex* is commonly parasitised as an intermediate host by three acanthocephalan worms in the UK, each with a different end host; *Polymorphous minutus* – waterfowl (Bauer et al., 2005)*, Pomphorhynchus laevis* - course fish (Kaldonski et al., 2007)*,* and *Echinorhynchus truttae* – Salmonid*s* (Fielding et al., 2003). Studies have shown that these parasites have an impact, not only upon the biochemistry of their intermediate hosts, such as increasing glycogen levels (Plaistow et al., 2001) and increasing respiration (Rumpus and Kennedy, 1974), but also upon their behaviour, such as altering the swimming of *G. pulex*, so that infected individuals are more

likely to be in the drift of a river and hence predated (McCahon, Maund and Poulton, 1991) . This behaviour may be due to infected *G. pulex* becoming photophilic (Bauer et al., 2000). It has also been shown that *G. pulex* infected with another acanthocephalan, *Echinorhynchus truttae* , show a significant increase in predatory feeding behaviour (Laverty et al., 2017). The presence of the parasite will potentially have an impact on both the likelihood of an individual Gammarid being eaten, along with the MPs it contains, but also, it may impact the feeding behaviour of the animal, so influencing the change in feeding observed when MFs were present in wafers.

Some of the main findings of Chapters 4 and 5 were that fibres impacted the feeding behaviour of *G. pulex* namely that the presents of synthetic fibres in food sources reduces the amount of time spent feeding on food sources when given a choice of uncontaminated food (Yardy and Callaghan, 2021, 2020). Given that parasitism also potentially impacts feeding behaviour this posed an interesting question as to what the implications of a combination of parasitism and presence of fibres would be. If there is an antagonistic relationship between parasites and the avoidance behaviour exhibited in Chapters 4 and 5 then we would expect parasitised *Gammarus* to ingest more MF. On the other hand, if the relationship is complimentary then we would expect even fewer MF to be ingested by parasitised *Gammarus*. Either way, this would not only impact the parasitised species but also directly impact its predators, through trophic transfer, and indirectly to both predators and prey, if there is a change in abundance of the parasitised species.

Changes in behaviour have been identified with parasitism in *G. pulex* by both *P. minutus* and *P. laevis* (Bollache et al., 2002; Cezilly et al., 2000; Kaldonski et al., 2007), in the case of *P. minutus,* geotaxism was reversed, and the normally benthic *Gammarus* swam

close to the water surface. When parasitised by *P. laevis*, their normal photophobia was inhibited, and they were attracted to chemical cues from bullhead fish (*Cottus gobio)*. All three parasites have been shown to decrease the feeding of *G. pulex* (Agatz and Brown, 2014; Fielding et al., 2003), this has been shown in increased time spent swimming rather than feeding, and a general decrease in amount of food ingested. In addition, both *P. minutus* and *P. laevis* had a negative impact on the mating of male *G. pulex* (Bollache et al., 2001).

The aim of this study was to compare the feeding behaviour and number of fibres ingested by *G. pulex* when parasitised by *P. minutus*, and when exposed to polyacrylamide (PA) microfibres. This will allow us to compare whether either or both factors will impact behaviour. In order to achieve this aim, the following objectives were required;

- Compare the number of microfibres ingested by parasitised and non-parasitised *G. pulex*;
- Compare the number of visits made to algal wafers with and without PA microfiber contamination, by parasitised and non-parasitised *G. pulex*;
- Compare the time spent feeding on algal wafers with and without PA microfiber contamination, by parasitised and non-parasitised *G. pulex*;

6.2 Method

6.2.1 Collection of Gammarus

Gammarus pulex were collected from Emm Brook, a tributary of the River Loddon (Decimal Degrees 51.440494, -0.874373 to 51.442274, -0.874359), this location is part of the Dinton Pastures Country Park, which has a good population of various waterfowl, a range of freshwater fish, including a large number of *C. gobio*, but no significant population of salmonids. *Gammarus* were collected by kick sampling with hessian nets and PET bottles as described in Chapter 3. Only individuals greater than 12mm and no obviously gravid females were taken.

Acanthocephalan parasitised individuals were identified in the field by eye. A red dot was clearly visible next to the gut (Figure 6.2). Infected *G. pulex* were collected and placed in a separate bottle to non-parasitised *G. pulex* and transported to the laboratory. The proportion of parasitised *G. pulex* was found to be influenced by season, with the lowest proportion found from November through to February, with parasitism rates <1%. The highest rates were found between mid-April through to late May at around 7%.

At the laboratory, all *Gammarus* were rinsed with Reverse Osmosis (RO) water and inspected under a dissecting microscope to confirm whether they were parasitised with an acanthocephalan or not. They were then placed into two aquariums of 45L tanks of Organisation for Economic Co-operation and Development (OECD) reconstituted water (Hooper et al., 2006) alongside aeration stones.

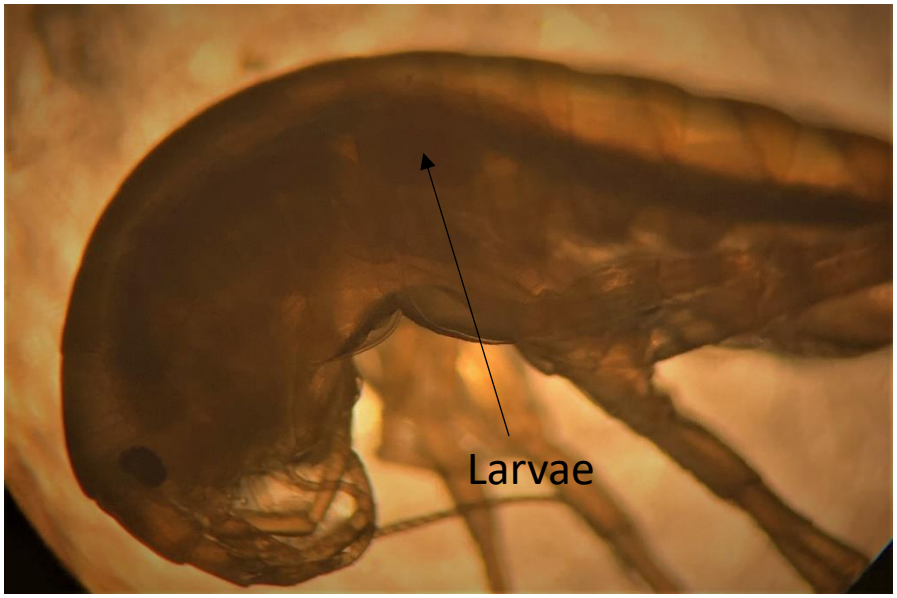

Figure 6.2 *Gammarus pulex* with *Acanthacephalan* larva ventral to gut

6.2.2 Parasite identification

Polymorphous minutus was identified by a red dot and *Pomphorhynchus laevis* by a yellow dot (Kaldonski et al., 2007; Thünken et al., 2010). These red dots identified encountered parasites as *Polymorphous minutus*. After dissection the acanthella were removed from *G. pulex* (Figure 6.3) where even under 100X magnification no structures could be identified. In order to confirm they were *Acanthacephalan* the internal structures needed to be visualised, in particular the spiked proboscis which is inverted within the body as an acanthella and cystacanth. In order to be able to identify this structure the cuticle needed to be cleared, this is because the cuticle is very tough and internal organs very soft, preventing normal dissection. The cuticle was cleared by placing one worm on a cavity microscope slide and adding 300µl (4 parts glycerin, 6 parts 70% ethanol). Once cleared the inverted proboscis was clearly identified (Figure 6.4), however, due to not being a fully developed cystacanth, the morphology of the proboscis could not be used to confirm species.

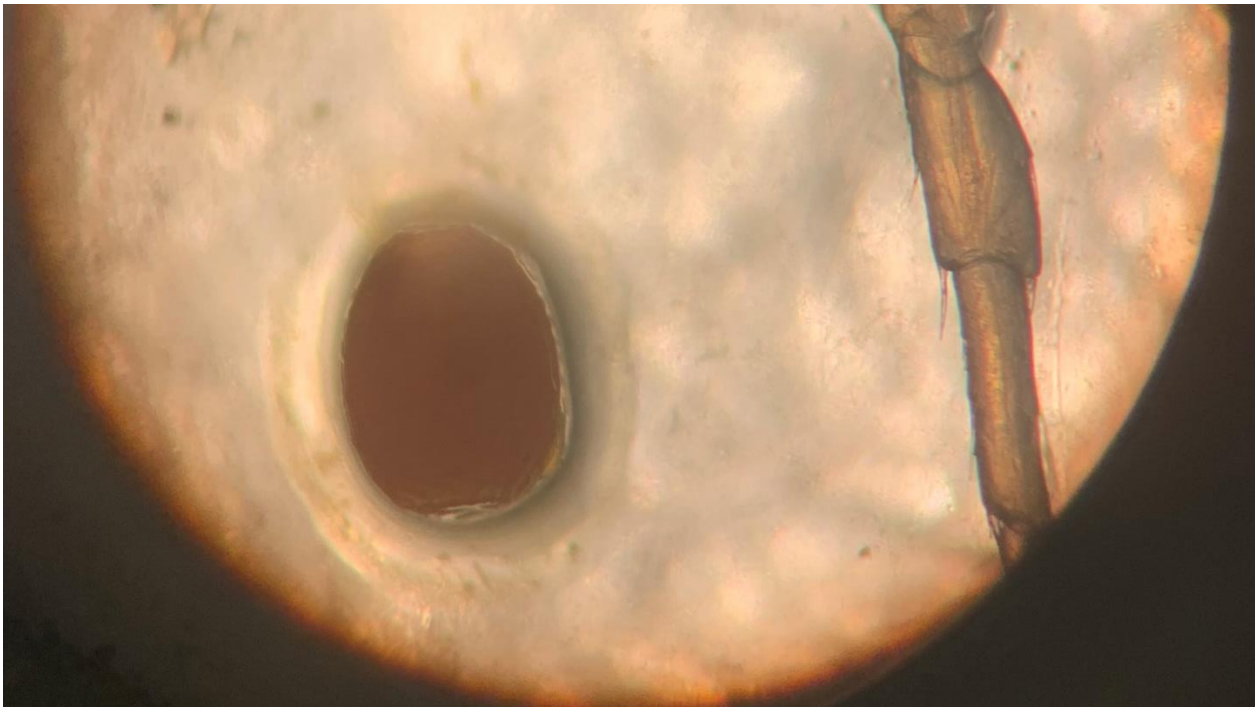

Figure 6.3 *Acanthacephalan* larvae after removal from *G. pulex*

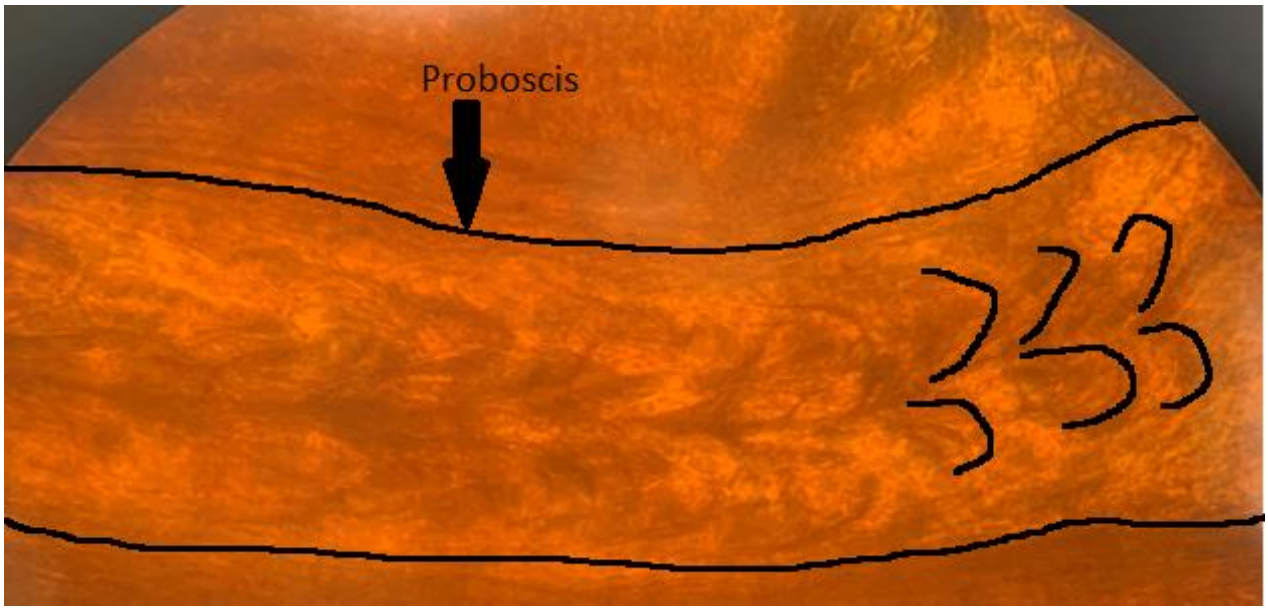

Figure 6.4 *Polymorphus minutus* cleared with 40% glycerine showing proboscis inverted within the body, several hooks and outline of proboscis have been marked for clarity.

6.2.3 Fibre preparation

Black 100% acrylic wool ≈30µm (Hayfield Bonus DK product code 5723101001, Hobbycraft, Farnborough) was processed into 500µm fibres using the same method as in Chapter 3, in which wool is soaked in Reverse Osmosis (RO) water and then frozen at -80˚C, inserted into a purpose-built jig, removing 500µm lengths.

The produced fibres were then added to crushed algal wafers, 0.03g fibres to 1g wafer (Wafer Algae Eater Fish Food, API), and mixed in a mortar and pestle for 1 min. Once homogenised 0.5ml of RO water was combined with the mixture to produce a paste. A cake 5mm thick was produced from this paste, which was dried and then divided into 0.05g test wafers.

6.2.4 Ingestion

Twenty parasitised *G. pulex* were placed into a 5L test aquarium filled with 2L of reconstituted water and twenty non-parasitised placed into an identical 5L test aquarium; they were then left for 24 hours to starve. After their starvation time, 15 each of the parasitised and non-parasitised *G. pulex*, were transferred, individually, into separate 5L test aquariums, filled with 2L reconstituted water, and then exposed to test wafers (3% acrylic fibres by mass). The remaining 5 parasitised and non-parasitised *G. pulex* were each placed in their own test aquarium, as above and given a control wafer, without fibres. They were left for 4 hours and then placed into 50˚C water to kill them before being dissected. The gut was removed by removing the head and telson. Ingested fibres were counted and if a parasite was found it was preserved in 80% ethanol.

6.2.5 Choice

Three parasitised *G.pulex* were placed into 5L test aquariums filled with 2L of reconstituted water and three non-parasitised placed into an identical test aquarium, they were left for 24 hours to starve. They were then transferred into individual test aquariums where they were exposed to a 0.05g test wafer and a 0.05g control wafer (Figure 6.5). The experiment was run for four hours, during which the three *Gammarus* were observed continuously, the number of feeding visits made to each type of wafer, as well as the total time spent feeding on each wafer was recorded. Feeding visits were defined as one in which the *G. pulex* was observed to be feeding from (rather than just holding) a wafer (as described in Chapter 3). If a piece of wafer was removed and was being fed on, this was also counted as time spent feeding.

After four hours, *Gammarus* were killed in 50˚C water and then dissected, with any fibres found within the gut easily visualised and counted, as described in Chapter 3. This process of observation, recording and dissection of the three *Gammarus* was repeated over five days to give a total of 15 replicates.

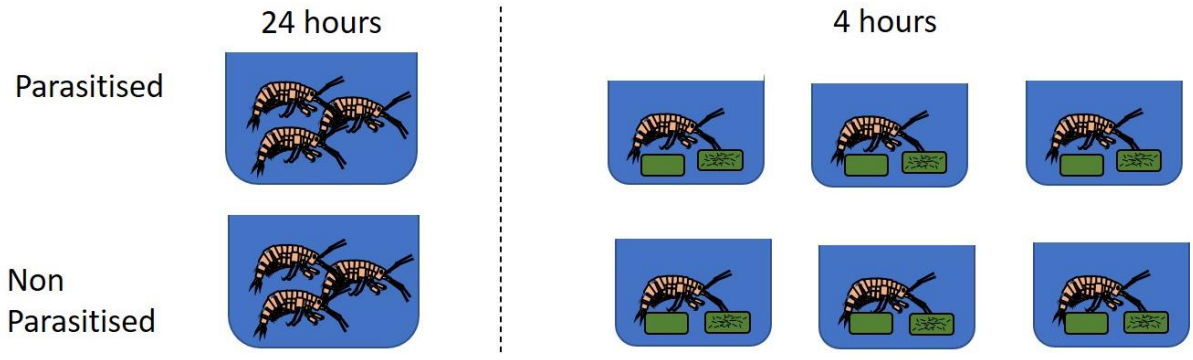

Figure 6.5 The experimental setup for investigating the feeding behaviours of parasitised and non-parasitised *G. pulex* when exposed to contaminated and non-contaminated food sources.

6.2.6 Analysis

All analysis was performed using R and R Studio (Savita and Verma, 2020), none of the results met the assumptions for normal data, except for fibres ingested without a choice, for which a one-way ANOVA was used. For all other analyses, Kruskal-Wallis tests were used.

6.3 Results

Parasitised *G. pulex* did ingest more, with an average of 6.9, compared to 6.7 for non-parasitised. However, there was no significant difference found in the ingestion of fibres, between parasitised and non-parasitised *G.pulex* ($F_{1,28}$=0.023, P=0.881).

Parasitised *G. pulex* spent 22 minutes feeding on contaminated wafers, less time than non-parasitised at 24 minutes. The reverse was true for uncontaminated wafers, with parasitised feeding for 53 minutes, and non-parasitised for 38 minutes. There was no significant difference in time spent feeding on either contaminated or non-contaminated wafers, between parasitised and non-parasitised *G. pulex* (W=16.7, P=0.669, W=25.1, P=0.399). Nor was there was a significant difference detected in the amount of time spent feeding on contaminated or uncontaminated wafers, by either parasitised or non-parasitised *G. pulex* (W=25.1 P=0.455, W=24.5 P=0.378).

Parasitised *G.pulex* made fewer visits to both contaminated and uncontaminated wafers at 2.1 and 2.8 respectively, than non-parasitised *G. pulex* with 2.5 and 3.2 visits respectively. There was no significant difference in number of visits to either contaminated or non-contaminated wafers, between parasitised and non-parasitised *G. pulex* (W=1.59 P=0.903, W=7.09, P=0.527). There was also no significant difference between the number of

visits to contaminated and uncontaminated wafers, by either parasitised or non-parasitised *G. pulex* (W=4.51 P=0.607, W=5.32 P=0.621).

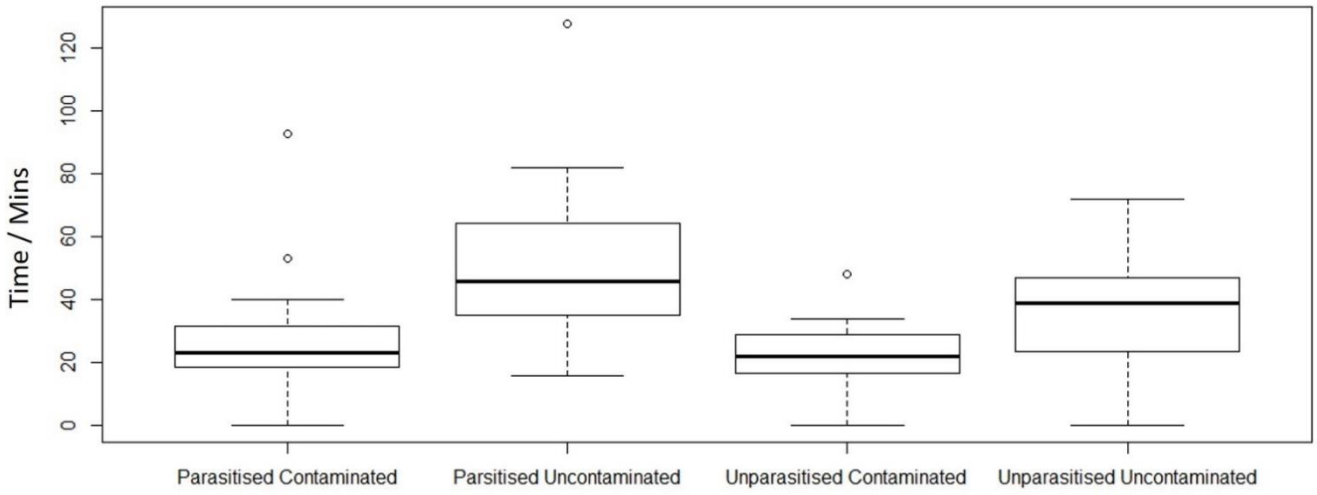

Figure 6.6 Time in minutes spent feeding on acrylic fibre contaminated and uncontaminated algal wafers by *Gammarus pulex* parasitised by *Polymorphous minutus* and non-parasitised *Gammarus pulex* median, quartiles and range shown.

6.4 Discussion

This study found no significant difference, in any feeding behaviour, between parasitised and non-parasitised *G. pulex*, as such we can state that *P. minutus* parasitism does not appear to impact the chance of microfibre ingestion by *G. pulex*. This study did find that both parasitised and non-parasitised *Gammarus* spent more time feeding on uncontaminated food sources, as shown in our previous study (Yardy and Callaghan, 2020) although in this study the difference was not statistically significant. Boxplots of the data (Figure 6.6), support there being a difference but, due to the nonparametric nature of Kruskal-Wallis a statistically significant difference could not be detected. Whilst we found no reduction in time spent feeding, one of the reasons this was found in previous studies was

that *Gammarus* spent more time swimming higher in the water column (Marriott et al., 1989). The experimental setup of this study meant that even when swimming on the surface they were no more than a few cm away from food. It is possible that in natural environments, the greater distance between the surface and food sources would result in decreased feeding times.

The fact that there was no reduction in feeding identified and that parasitised individuals ingested the same number of fibres as non-parasitised, means that the final host birds of *P. minutus* ingest no greater or lesser number of MP from each individual *G. pulex*. However, a high rate of parasitism in a waterway and the change in *G. pulex* behaviour, may increase the total number ingested and therefore, the percentage of diet that is made up of *G. pulex*. If these *G. pulex* have themselves a greater number of MP than other prey items then the final host will ingest a greater number of MP.

Seasonality has been noted in *Acanthacephalans* parasitism of Gammarids, with one study finding peak parasitism from July through to October, parasitism rates of 30% - 71% (Dudiňák and Špakulová, 2003). A much older study found that there was no overall seasonality of *P. minutus*, with different water bodies having maximal infection at totally different periods, one having highest rates from April through to September and another from September through to November (Hynes and Nicholas, 1963). This older study surmised that it is the abiotic factors, from particular water bodies, that dictates the growth and peak of infections by *P. minutus*.

What is unknown is the impact of MP upon these parasites; acanthocephalans have been shown to concentrate heavy metals in their bodies, in concentrations up to 2700 times that in the host's muscle (Siddall and Sures, 1998). While it is unlikely that MP would be

absorbed into the parasite, it is possible that despite their tolerance of chemical contamination, the physical presence of the plastic is enough to irritate them and prevent them from thriving.

Parasites have been shown to be an important factor in bioaccumulation (Sures et al., 2017) and have been shown to act as a sink for marine contaminates. As they can have several hosts, from a range of spatial and trophic levels, they can accumulate toxins from across this range, making them useful indicators of contamination (Nachev and Sures, 2016). *Acanthocephala* have been especially useful in contamination monitoring, due to their lack of gut, all contaminants must have crossed the parasite's tegument and are, therefore, highly bioavailable (Nachev et al., 2013; Sures et al., 2017). Unlike more conventional contaminates, such as heavy metals, the MP used in this study are not able to cross tissue barriers (although nanoplastics can and have been shown to cross both gut and placenta (Banerjee and Shelver, 2021; Braun et al., 2021; Yong et al., 2020)), therefore, the parasites themselves will not bioconcentrate MP within themselves.

It has been shown that juvenile vertebrates are particularly vulnerable to MP and that their presence can irritate the gut, skin and gills, increasing mortality and having a negative impact on condition (Critchell and Hoogenboom, 2018; Karbalaei et al., 2021; Naidoo and Glassom, 2019). The biology of the final host of *P. minutus* is such that, immediately from hatching, they feed themselves, meaning juvenile birds will also be exposed to MP and as such, are more likely to be negatively impacted. This is even more so in the case of piscivorous birds, which may not be a host but still a predator of *G. pulex* or the final host of *P. laevis*. these birds feed their chicks by regurgitation. Pellets regurgitated by Northern Fulmar (*Fulmarus glacialis*) chicks have been investigated with the findings that up to 28% of

chicks had MP in their regurgitate (Acampora et al., 2017). This shows that, even before entering the marine environment, chicks are exposed to marine litter. A study on the regurgitate of common kingfishers (*Acledo atthis*) shows this is also the case for freshwater piscivores, with 7.5% of pellets having MP (Winkler et al., 2020). Worryingly it has been shown that 77% of king penguin (*Aptenodytes patagonicus*) faecal samples contained microfibres (Le Guen et al., 2020).

Ultimately the increased risk of MP ingestion by predators is not restricted to *P. minutus* and *G. pulex*. Any intermediate host that ingests a disproportionately high or low number of MP and whose parasite make them more likely to be ingested, will result in a greater or lesser number of MPs ingested by the final host. This would be the case, even if the parasite has no impact upon MP ingestion rates, only if the parasite reduced ingestion rates of MP would the ingestion rate of MP by the final host be reduced. It also effects not only hosts, but all predators more likely to target the intermediate host, which can extend beyond the aquatic environment.

CHAPTER 7

Ingestion of Glitter by *Gammarus pulex*

7.1 Abstract

While the topic of microplastics in the aquatic environment is becoming increasingly understood, glitter (small fragments or films, often with metallic foil) has seldom been investigated, their discovery in wild organisms being incidental, alongside other MP. Despite the lack of ecotoxicity studies into glitter, there have been many forensic and water quality investigations, meaning their presence in aquatic environments is relatively well understood.

Using an adapted methodology to the other experiments, substituting microfibres for glitter, *Gammarus pulex* were exposed to various sizes and concentrations of glitter. 800µm, 400µm and 100µm were incorporated into wafers, in order to understand whether there was a relationship between the size of glitter and number of pieces ingested. Concentrations of 1%, 5% and 10% of 100µm glitter were then used to investigate the impact of concentration of glitter and number ingested.

It was found that even when starved for 96h, no glitter was ingested by any *G. pulex*, despite wafer matter being found in their guts. Attempts to process 100µm glitter down to smaller particles failed. It remains unknown whether size and concentration impacts ingestion of glitter by macroinvertebrates, what is known is that *G. pulex* will not ingest glitter of 100µm and larger.

7.1 Introduction

As the impacts of microplastics are becoming increasingly studied and reported on, new types and sources are being investigated (Thushari and Senevirathna, 2020; Yurtsever, 2019). One of the types of MP, that has been investigated by only a few studies, is what is generally referred to as glitter. Glitter is formed from rigid plastic sheets, most commonly PET, laminated with foil and then shredded or punched, to produce shiny disks or particles (Yurtsever, 2019). Larger glitter (>400µm diameter) is generally used as decoration, for a variety of surfaces, while glitter <400µm is often used in the cosmetics industry; because they are manufactured in these sizes, they are primary MP (Tagg and Ivar do Sul, 2019; Yurtsever, 2019). Despite the lack of information within the literature, the presence of glitter in the environment has distressed society, to the degree that it has been banned in several regions and has encouraged the development of biodegradable glitter (Green et al., 2021). The impact of both PET and biodegradable glitter has been investigated in one study, using 100µm PET glitter, a cellulose based 150µm glitter and natural and synthetic mica <200µm. Both the PET and the cellulose glitter had aluminium as the reflective layer. Mesocosms with two species, the duckweed *Lemna minor* and the New Zealand mud snail *Potamopyrgus antipodarum*, were produced. After 36 days of exposure to 435mg kg^{-1}, it was found that the root length of *L. minor* was significantly shorter, when exposed to all glitter, other than natural mica. There was also a reduction in chlorophyll in the water column for all glitter treatments, compared to the control. The abundance of *P. antipodarum* was twice as high in mesocosms with cellulose based glitter than PET glitter (Green et al., 2021).

There have been several incidental findings of glitter being ingested by aquatic organisms. Polyurethane (PU) glitter was identified in the gut of marine bluefish *Pomatomus saltatrix*, although this was only a single particle in 965 individual fish (Neto et al., 2020). Glitter has also been found in in two species of Decapod in the Thames estuary, the shore crab *Carcinus maenas* and the invasive Chinese mitten crab *Eriocheir sinensis*. While glitter was identified, it only constituted 0.6% of ingested plastic found in 145 individuals (McGoran et al., 2020).

While glitter has not been heavily investigated, with regards to ecotoxicology, there is a broad literature on the use of glitter in forensic science (Tagg and Ivar do Sul, 2019) because of the distinct morphology in different products (Zellner and Quarino, 2009). Partly due to the forensic interest in glitter, it is known that WWTPs are a major source of glitter in the environment (Li et al., 2018; Tagg and Ivar do Sul, 2019; Yurtsever, 2019). In activated sludge, glitter can constitute up to 25% of all MP, due to their extensive use in cosmetic products (Raju et al., 2020b).

Glitter is generally relatively large and many studies have shown that smaller MP are ingested far more than larger MP (Abbasi et al., 2019; de Sá et al., 2018; Mateos-Cárdenas et al., 2021; Ogonowski et al., 2016). Glitter is unusual, however, with it incorporating a metal film, most commonly aluminium, in its manufacture (Yurtsever, 2019). Aluminium is a well-known toxin and has been heavily studied, for several decades (Stockdale et al., 2014; Waters and Webster-Brown, 2013; Weatherley et al., 1988). Although there are only a matter of nanograms of metal in each particle of glitter, by being in a form that is ingestible, glitter works as a potential vector, for aluminium and other toxic metals, into the gut of aquatic organisms, both vertebrate and invertebrate (Tagg and Ivar do Sul, 2019; Yurtsever,

2019).As glitter has been ingested by other macroinvertebrates and as *G. pulex* has been shown, in at least one study, to ingest spherical particles up to 90µm in diameter, this chapter investigates whether glitter is ingested by *G. pulex,* in proportion to the size and concentration of glitter.

This was achieved by running two experiments:

The first compared the ingestion of glitter in three different sizes, 800µm, 400µm and 100µm, all at the same concentration, so see whether there was a preference for ingestion;

The second compared the ingestion of 100µm glitter at three concentrations, 1% 5% and 10% to investigate whether ingestion was dose dependant, as was found in MF in Chapter 4.

7.2 Materials and methods

7.2.1 Gammarus collection

Gammarus pulex were collected from Emm Brook, as described in Chapter 3. They were transferred to a 45L tank of reconstituted water and left to condition for 7 days, while being fed on algal wafers (API). Only adults over 12mm were retained for experiments and no obviously gravid females were used.

7.2.2 Glitter processing

Three sizes of black PET Glitter were purchased, (Own brand, Hobbycraft Farnborough UK). Sizes were measured using a microrule to the closest 100µm and found to be 800, 400 and 100µm in diameter. The glitter was coated in an oily residue, in order to remove this the glitter was washed, this was cleaned by pouring 0.5 ml of glitter into a 1.5ml Eppendorf

tube, followed by 1ml 80% ethanol. This mixture was vortexed for 30 seconds and then placed in a centrifuge for 1 minute at 6000 rpm, the supernatant was discarded and replaced with another 1ml of 80% ethanol, vortexed for 30 seconds and then centrifuged again for 1 minute at 6000 rpm. This process was repeated twice more using distilled water rather than ethanol. Four Eppendorf tubes of glitter were processed together and then once the final supernatant had been removed the glitter was placed onto a glass dish and placed on a hotplate at 40˚C for an hour.

Previous tests, using green glitter, revealed that the usual glint of glitter was not obvious under microscopy, light had to be shone from above and moved in order to reveal the shine and even then, it was not obvious. The glint of the black glitter was equally unreliable, however, it contrasted clearly against the rest of the wafer.

7.2.3 Test wafers

The same basic method, as described in Chapter 3, was used to produce the test wafers. The powder from ground algal wafers, was combined with glitter and 1ml RO water, in the mortar and pestle and mixed for 1 minute. Test wafer were produced at concentrations of 1% 5% and 10% glitter by mass and unlike fibres, even at 10%, the glitter wafers were visually homogenous and showed no evidence of clumping.

7.2.4 Comparison of Ingestion by Size

To compare the ingestion of different sizes of glitter, twenty *G. pulex*, five for each size and five for control, were placed in individual 5L aquariums with 2L of reconstituted water and left to starve for 24 hours. The water was then replaced with fresh reconstituted water and one 5% 0.05g test wafer, five replicates for each glitter size. The *G. pulex* were left to

feed for 4 hours and then killed with 50˚C water and then the gut dissected out, to inspect for the presence of glitter, under 40x magnification.

7.2.5 Comparison of Ingestion by Concentration

In order to investigate whether glitter was ingested at rates proportional to concentration, it was decided to use only the 100µm glitter. Twenty *G. Pulex*, five for each concentration and five for control, were transferred to individual 5L test aquariums and starved for 24 hours. The *G. pulex* was removed, the water was replaced with 2L of reconstituted water and one 0.05g test wafer was then placed in each aquarium, three control, three 1%, three 5% and three 10%, and the *G. pulex* returned. The *Gammarus* were left for 4 hours to feed on the wafers and then killed by immersing into 50˚C water, before being dissected and the gut removed. The gut contents were then inspected for presence of glitter, at 40x magnification and any glitter identified was recorded.

The whole experiment was then repeated, with the exception of starvation time, five 5L aquariums had 3L of reconstituted water and 10 *G. pulex* placed in each, alongside an aeration stone, attached to an air pump. The *Gammarus* were starved for 96 hours, twenty surviving *G.pulex* were selected, 5 replicates of each concentration and the experiment was repeated.

7.2.6 Further Processing of Glitter

Glitter could not be sourced smaller than 100µm, that was not a constituent of a cosmetic product and extracting glitter from products was deemed unfeasible. Therefore, attempts were made to further process glitter down into smaller particles. The 100µm

glitter was chosen to trial methods, as individual particles could not easily be seen by the naked eye, microscopy was needed to assess the success of methods.

Two methods were used; the first was to simply grind the glitter using a ceramic mortar and pestle. The glitter was ground for five minutes, being inspected every minute, however, there was no change. The glitter was then frozen at -80˚C and then re-ground for five minutes, but again, there was no change.

The second method was to use steel ball bearings and a vortex. Half a ml of glitter was placed in an Eppendorf tube alongside five 4mm steel ball bearings, the tube was vortexed for two minutes but there was no change. The tube, with glitter and ball bearings was then frozen at -80˚C and vortexed again for three minutes, this resulted in the top of the tube being damaged, but when the glitter was observed under 40X magnification it was confirmed that the glitter was still around 100µm.

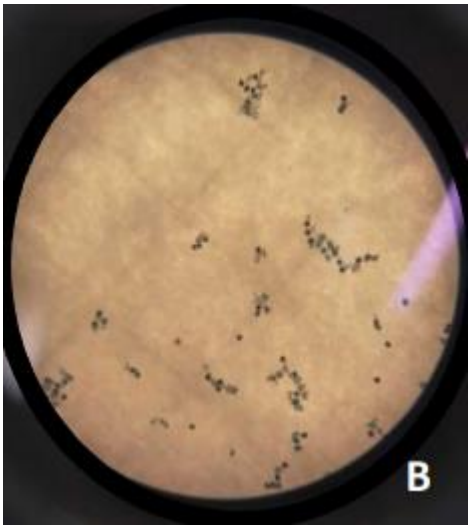

Figure 7.1 Glitter 40X at magnification, still present after exposure to 70% nitric acid

7.3 Results

7.3.1 Comparison of ingestion by size

After 4 hours of feeding, when exposed to the different sized glitter, it was found that all but one of the twenty *G. pulex* had ingested wafers, as there was algal matter found in their guts. The one that had not ingested anything was part of the control group.

7.3.2 Comparison of ingestion by concentration

After 24 hours, all but 3 of the *G. pulex* had fed on test wafers, of the three that did not, one was from the control, and two from the 5% group. It was evident that they had not fed because there was no matter in their guts, and no evidence of faecal pellets.

While all others had fed and there was matter in both their fore and hindgut, there was no evidence of glitter at any stage of the gut.

After 96 hours of starvation, 34 *G. pulex* had survived, of those that had died seven came from a single aquarium, therefore none of the live *Gammarus* from that aquarium were used. No other aquarium had more than three dead *Gammarus*, therefore 5

individuals from each of these aquariums were pooled and used in the ingestion experiments. Of these, one died in the 1% group, and after dissection it was found that it had not ingested any wafer. All other *G. pulex* had ingested wafer, however, as before, there was no evidence of glitter ingestion at any concentration.

7.4 Discussion

The presence of glitter, of any size, did not dissuade feeding by *G. pulex*, out of a total of 60 across all experiments, only 5 did not feed, and two of those were in control groups without glitter, this is in accordance with previous chapters and the literature in general (Scherer et al., 2017b; Yardy and Callaghan, 2021, 2020). However, the findings of this study diverge from the findings of (Scherer et al., 2017b), specifically that *G. pulex* show a preference for ingestion of larger MP, around 90µm, the smallest MP used in this study were only slightly larger, and yet none were ingested. This study does however, support our findings in Chapter 5, and the ingestion size limit of *G. pulex* does seem to be less than 100µm, at least for fragments. Assuming the size limit for MP particles is between 90 and 100µm, this is at least double the thickness limit for fibres by *G. pulex* (Yardy and Callaghan, 2021), which is explicable by the fact that while fibres are thinner, they are much longer, at least 500µm. Given the ideal opportunity for glitter ingestion in this study, proven by the success of previous chapters (Yardy and Callaghan, 2021, 2020), no glitter was ingested, as such it seems that glitter larger than 100µm is simply not at risk of being ingested by *G. pulex*. However, as glitter is just fragments of MP smaller glitter may well be ingested, along with other MP of similar size. At least two species of crustaceans have been found to ingest glitter (McGoran et al., 2020), although both of these were orders of magnitude larger than

G. pulex. Glitter, in the sizes investigated here, may well be ingested by larger invertebrates, possibly even other Gammarid species (Dangles and Guérold, 2000).

While other MP can have toxins adsorbed onto their surface, glitter is unique in that it has a film of aluminium or other toxic metals attached to them, providing a far higher dose than other adsorbed toxins (Bouwmeester et al., 2015; Brennecke et al., 2016; Gandara e Silva et al., 2016). Due to this relatively large amount of metal, on each particle of glitter, far fewer would need to be ingested in order to provide a harmful exposure. In addition, rather than MP adsorbing toxins from the environment, the metal from glitter could leach out into substrate or water, thereby exposing animals via contact, rather than just ingestion (Gandara e Silva et al., 2016).

Other functional feeding groups, especially filter feeders, may be even more vulnerable to glitter ingestion than shredders like *G. pulex*. Two species of bivalves, the oyster *Crassostrea virginica* and the mussel *Mytilus edulis* were exposed to MP from 1 to 1000µm, while rates of ingestion were inversely proportionate to size and no 1000µm MP were ingested, many 200-500µm MP were ingested (Phuong et al., 2018; Ward et al., 2019). As the majority of glitter are around 200µm (Tagg and Ivar do Sul, 2019) and filter feeders will readily ingest MP of this size, glitter may be of enhanced risk. As many of these bivalves are used as a food source by humans, the risks glitter poses may well extend to human health (Phuong et al., 2018), especially if metal contamination, associated with glitter, is also ingested.

CHAPTER 8.

General Discussion

This thesis contributes to the ever-growing knowledge of the impact of MP in *G. pulex*, and therefore, the corresponding impacts on the freshwater environment. While MP are being increasingly studied, and freshwater amphipods such as *G. pulex* have long been a model organism used in toxicology. The MP utilised in this thesis are lesser studied, both fibres and in particular, glitter. The use of a variety of MP is imperative, this is due to the highly varied nature of MP and the difference of ingestion and impact of these MP on different organisms (Scherer et al., 2017b). The use of organic MF to compare with plastic MF was especially important, to investigate the assumption that organic fibres are natural and thus safer than plastic MF (Mateos-Cárdenas et al., 2021).

8.1 Methodology

The method, described in Chapter 3, is an accurate and reliable method for exposing *G. pulex* to known concentrations of microfibres, both organic and synthetic. These exposures can be both chronic and acute, as demonstrated in Chapter 5. Whilst other studies have had success in mixing nanoplastic into substrate and observing their eventual fate (Redondo-Hasselerharm et al., 2021, 2018a), that method does not allow comparison between contaminated and non-contaminated food sources. As discussed in Chapter 2, virtually all animals will ingest MP if exposed to them, however, it was also shown that the ingestion depended on type of plastic and feeding method (Lehtiniemi et al., 2018; Scherer et al., 2017a). Because of this, comparisons between different plastics facilitate a greater

understanding into the relative risk of different MP, to different species. The capability to investigate feeding preferences is not limited to the scope investigated in this thesis; by exposing *G.pulex* that had been fed contaminated wafers, the behaviour of their predators could be investigated, to identify trophic transfer and the behaviour involved. Experiments into the trophic transfer of MP have, so far, focussed on only nano or very small <50µm spheres (Chae et al., 2018; Farrell and Nelson, 2013a; Gambardella et al., 2018; Hanslik et al., 2020). While useful to identify fate of MP at this size, feeding behaviour, especially of higher trophic levels, is unlikely to be impacted.

There are some limitations within this methodology. Wafers, whether they be contaminated or not, left in water, will have started to disintegrate into smaller clumps at 24 hours. Although the clumps are still easily visible to the naked eye and it can be observed whether they are being fed on, it is more difficult to remove wafer remnants and re-weigh them, in order to calculate how much had been ingested than agar based food (Weber et al., 2018). This would make studying assimilation efficiency, with our method, more time consuming and prone to greater errors, if small particulates were missed. This disintegration did not occur during the first four hours of wafers being immersed, any fragmentation that occurred in this time was due to *G. pulex* removing pieces to feed on.

Another limitation of this method is in the type of invertebrates that can be investigated. Most obviously, shredders are suitable, although freshwater snails (species unknown) were observed to feed on control wafers during conditioning of *G. pulex*. If they do ingest MP, when presented in this way, the true amount of MP they are exposed to would be known (Akindele et al., 2019; Gutow et al., 2016). When glitter and MF not incorporated into wafers were left to settle in water, it was observed that they did not settle

homogenously on the bottom of aquariums, meaning different regions of the aquariums had, in effect, different concentrations of MP.

8.2 Synthetic vs organic

All three experimental chapters, that investigated feeding behaviour, found a decrease in feeding when *G. pulex* were offered PMF contaminated food sources. Two found the difference statistically different and as explained in Chapter 6, the lack of statistical significance in the other chapter was due to the non-parametric test used. Another study on the ingestion of MP by invertebrates, found avoidance behaviour, in this case by the earthworm *Eisenia fetida,* at concentrations >4% PE polylactic acid (PLA) and polypropylene carbonate (PPC) (Ding et al., 2021). As discussed previously, the behaviours reported in this thesis are unlikely to have been learned, because *G. pulex* were collected from a brook, that was not downstream of a WWTP and therefore, a limited opportunity for exposure to a significant concentration of MF (Yardy and Callaghan, 2020).

The hypothesis put forward in Chapter 4, that the size of MF, rather than their chemical composition, that would impact feeding preferences and ingestion by *G. pulex* was, in part, disproved. While undeniably there is a maximum cut off, for the size of fibre, that *G. pulex* will ingest, around 50µm as supported by other studies (Blarer and Burkhardt-Holm, 2016b; Weber et al., 2018), it does also seem that the composition of the fibre matters too. As Discussed in Chapter 5, preference was shown by *G. pulex* for all organic fibres, rather than the synthetic fibre and no preference was shown to control over organic fibres. There was no difference in the number of synthetic or cat MF ingested and both were ingested at greater rates than cotton fibres were. This is in line with a study into the

mortality of *Gammarus duebeni* , when exposed to synthetic and organic micro-fibres, which found there was no significant difference in number of synthetic vs organic microfibres ingested, but more synthetic fibres were ingested (Mateos-Cárdenas et al., 2021). While that study and this thesis focussed upon different species, they are closely related and occupy the biological niche, freshwater amphipods, which are both predators and functional shredders. They also live in near-identical climates, *G. pulex* being indigenous to Great Britain and *G. duebeni* indigenous to Ireland, as such they can, reasonably, be compared. The importance of investigating organic fibres, or non-plastic particles, is their prevalence in the environment, being far more numerous than MP (Le Guen et al., 2020). Given many of these non-plastic particles and fibres constitute the diet and environment of not only *G. pulex* but macroinvertebrates in general, they are factors these organisms have evolved with. If, as several studies indicate, they have the same impact as MP, then while MP are a fairly novel contaminant, they are simply a novel source for longstanding impacts organisms must cope with.

8.3 Mortality & growth

The findings of this thesis are that neither organic nor synthetic fibre ingestion have a negative impact on *G. pulex* mortality. This is in line with other studies into organic and plastic contamination in *Gammarids*, which have broadly found no difference between the two contaminants. A study on *G. duebeni* investigated the acute toxicity of 60µm lengths of cellulose and polyester microfibres over 96h and found no impact on mortality (Mateos-Cárdenas et al., 2021), the same as was found in Chapter 5 over 28 days. The cellulose and polyester fibres used had diameters of 15µm and 17µm respectively, while only slightly thinner than those used in Chapter 5, the exposure method was very different, MF were

dispersed freely in water and so comparisons of concentrations are not viable. Another study using a similar method, with PE MP adhered to the duckweed *Lemna minor* was fed to *G. duebeni* ,to investigate the trophic transfer of MP, as well as the impact on mortality (Mateos-Cárdenas et al., 2019). After 48 hours of exposure, there was no impact on mortality on *Gammarus* and equally there was no impact on the growth of the duckweed. While these methods better replicate the environmental conditions and a realistic trophic transfer, it is difficult to ascertain the actual concentration of MP that *Gammarus* are exposed to.

Not only was no impact on mortality identified, but there was also no impact on the growth of *G. pulex*, as this was investigated over 28 days and growth occurred for all treatments, we can be confident in the method. Again, this is supported by the literature, in which no negative effect on growth has been identified in *G. pulex* (Weber et al., 2018)(Redondo-Hasselerharm et al., 2018b). In the closely related *G. fossarum*, negative impacts on growth have been identified (Blarer and Burkhardt-Holm, 2016a; Straub et al., 2017) using MP varying in size from 1.6µm to 250µm, which, while smaller than fibres used in this thesis, are larger than fibres used by another study on *G. pulex* (Mateos-Cárdenas et al., 2021). This negative impact on growth has been identified in other benthic macroinvertebrates, including *Hyalella azteca,* where mortality has also been identified (Au et al., 2015; Redondo-Hasselerharm et al., 2018b). Given that negative impacts have been identified in other freshwater macroinvertebrates, including other *Gammarids*, it seems prudent to continue investigations into sublethal effects in *G. pulex*. Organisms studied in the laboratory do not have the same risk factors as those in the wild, they have plenty of

food, no predators and a stable environment. As such the results of sublethal impacts may be less visible than they would in a more competitive, natural environment.

8.4 Trophic Transfer

While identifying the impacts of MP on invertebrates is essential for understanding the impact on the whole ecosystem, it is the presence and impacts on vertebrates which catches the attention of the public. This is because fish are an essential food source for much of the world and so humans are directly involved in the trophic transfer of MP in fish (G. Chen et al., 2020). Another group which are given attention are marine mega vertebrates (Jefferson et al., 2014), this is because they have attributes appealing to society, largely because they are well known and charismatic.

While plastic ingestion, including MP, by mega vertebrates has been identified and meso / mega plastics are known to have had lethal impacts (Müller et al., 2012; Nicolau et al., 2016), there have been no studies into the impacts of MP. There have been several investigations on the impact of MP in fish; juvenile fish, in particular, seem to be susceptible to negative impacts, particularly on the gut and gill, demonstrated in zebrafish (*Danio rerio*) (Rainieri et al., 2018b) and rainbow trout (*Oncorhynhcus mykiss*) (Karbalaei et al., 2021). Microplastics used in these investigations varied from 16 - 250µm and so were unable to penetrate tissues and so contact with the surface of the gut or gill caused damage. However, nanoparticles are able to penetrate the gut wall and enter the circulatory system, from here they can accumulate in other organs. A study using 20nm PS found accumulation in the brain of embryonic *D. rerio,* thisdamage was not only physical, but also oxidative damage to DNA (Sökmen et al., 2020), identifying the risk of NP to Vertebrates. With these

differences in mind there are two types of trophic transfer of MP, one in which MP don't pass through the gut or gill tissue and damage is limited to those organs, the other where NP does pass through tissues and can bioaccumulate in other organs. As both invertebrates and vertebrates are able to pass ingested MP, with the exception of certain invertebrates with morphological adaptations such as gastric mills (Welden and Cowie, 2016), the risk of true bioaccumulation is low in MP rather than NP.

8.5 Use of *G. pulex* and other freshwater invertebrates in MP ecotoxicology

This thesis used *G. pulex* as a model organism for several reasons, for this region of the UK, *G. pulex* are one of the most abundant macroinvertebrates (Bloor, 2010; Kelly et al., 2002b) and so findings that impact them will be relevant to the regional ecosystems. This is the case in much of Europe, with *G. pulex* being an ecologically important species (Adam et al., 2010b; Graça et al., 2001; Laverty et al., 2017), as a result there is a comparatively large literature for the impacts of MP on Gammarids, making comparisons between polymers and shapes possible (Redondo-Hasselerharm et al., 2018b; Scherer et al., 2017b).This is useful to give a general idea of the differences between types of MP and could be used as an indicator of greater environmental risk (Bloor, 2010). However, *G. pulex* is only a relevant species for European rivers and streams, while there are similar Gammarid species in other ecosystems, which will be relevant to their environments, sizes of MP ingested will differ, species to species (Scherer et al., 2017b; Straub et al., 2017). However, other key taxa, such as mayflies (Ephemeroptera) have been virtually unstudied, with only one study investigating the ingestion of MP by mayfly (Baetidae, Heptageniidae) and caddisfly larvae in the environment, finding that they had ingested MP. It was found that there was no

difference in ingestion between filter feeders, grazers and shredders (Windsor et al., 2019). Another study investigating the behaviour of both mayfly and caddisfly larvae, found that caddisfly *Odontocerum albicorne* readily utilised MP, alongside natural matter, to build cases (Gallitelli et al., 2021). The burrowing mayfly *Ephemera Danica* was exposed to substrate contaminated with MP and found to preferentially burrow in MP (1-5mm) fragments, rather than natural sediment or a combination of MP and natural sediment (Gallitelli et al., 2021).

8.6 Recommendations for further research

Future studies into MP should have greater standardisation with regard to MP sampling and exposure methods. In order to compare or simply monitor MP pollution in different aquatic systems, an agreed and consistent method should be adopted. Different methods have different capture efficiencies for different sizes and types of MP and those which are efficient in capturing small MP and fibres are often more time consuming, resulting in a smaller sample able to be processed.

By using standardised methods, the most abundant MP in any specific environment can be identified, allowing these particular MP to be investigated using relevant model organisms. In doing so, locally relevant conclusions can be drawn. If this were followed, there would be a greater number of studies using microfibres, rather than the preponderance of the more convenient and commercially available PS microbeads (Mateos-Cárdenas et al., 2021).

Using the methods described in this thesis, a greater range of synthetic microfibres should be investigated, so far only acrylic has been used and given the findings of Chapter 5,

chemical composition, rather than simply dimensions, do impact feeding behaviour and so different polymers may produce different responses.

In addition to a greater variety of MP types, a greater range of organisms should be investigated. Once the most prominent MP from an ecosystem have been identified, it would be prudent to expose them to animals found in those ecosystems in order to make environmentally relevant deductions.

Gammarus pulex is not only a functional shredder but also a predator. Its shredding, feeding behaviour has been investigated, however, the impact of MP contamination on its prey and how that impacts food choices, would give interesting insights. Not only into whether contaminated prey is avoided, similar to algal wafers, but also if MP contamination changes the proportions of diet comprising prey.

The gut microbiome of *G.pulex* and other freshwater invertebrates, is poorly understood. If the presence of MP has an impact on this microbiome it would provide another possible method for MP contamination to impact any animal with significant MP in their gut. This research was attempted alongside the other experimental chapters. The microbiota of the guts and faeces of *G. pulex* , pre and post conditioning, (as described in Chapter 3) were investigated, using microscopy and then DNA extraction and PCR replication. However, I was unable to perform a successful PCR or produce a product suitable for sequencing, due to financial and time constraints this research had to be abandoned (Appendix A).

8.7 Conclusions

- Microplastics are found in all water systems investigated, will be ingested and have been found in most taxa studied.
- There is a need for increased standardisation in environmental MP sampling, to be able to draw meaningful comparisons.
- The methods described in this thesis are effective and replicable when exposing aquatic macroinvertebrates to know concentration of microfibres.
- *Gammarus pulex* show a preference for food sources un-contaminated with acrylic microfibres, but no such aversion to contamination by cat hairs or cotton fibres.
- Contamination of food sources with 500µm acrylic or organic fibres at 3% by mass, has no impact on either growth or mortality of *G. pulex* after 28 days of exposure.
- *Polymorphous minutus* parasitism has no impact on the feeding preferences of *G. pulex* to acrylic microfibre contaminated food sources.
- *Gammarus pulex* will not ingest glitter fragments 100µm in size and are able to avoid them in contaminated food sources.

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APPENDICES

Appendix A

Is the gut microbiome impacted by microplastic ingestion?

Introduction

The microbes which live within the gut of animals have historically been referred to as 'gut flora' or 'gut fauna'. These terms are now redundant and inaccurate, the modern terminology is the gut microbiome (Koch, 2015). Gut microbiomes have been extensively investigated in mammals, both for medical and agricultural development. It is known that much of the digestion which occurs in the gut of mammals is due to the microbes, in particular bacteria, within their guts (Muegge et al., 2011). When these bacteria are removed or change it can cause potentially fatal illnesses, millions of humans a year die from gastroenteritis caused by a change in their gut microbiome (Mawatari and Kato, 2014). One well known cause of the loss of gut bacteria is the use of broad-spectrum antibiotics (Dethlefsen and Relman, 2011), when these bacteria are lost the host organism cannot replace them unless they are re-ingest them, in humans this is most often done by way of a faecal transplant (Fuentes et al., 2014), however, most non-domesticated organisms must rely on encountering the correct microbes within their environment and the beneficial bacteria re-establishing a colony.

The research into the gut microbiomes of invertebrates has been far less comprehensive. While there has been a moderate amount of research conducted upon terrestrial invertebrates, there has been far less upon aquatic invertebrates (Dillon and Dillon, 2004). It has been suggested, that due to the nature of aquatic environments, microbes do not rely upon a host organism and instead live freely within their environmental medium (freshwater or marine), greatly reducing the need and probability of symbiotic relationships forming between microbes and host guts (Harris, 1993). Regardless of whether symbiotic relationships have developed some aquatic invertebrates, including at least one Gammarus sp, does benefit from enzymatic activity of microbes within their guts (Harris, 1993).

As microplastics have been shown to act a vector for transporting adsorbed substances into the bodies of invertebrates (Brennecke et al., 2016), they also facilitate exposure of gut microbiome to these potential toxins. The plastic itself, while not toxic to invertebrates could be toxic to the gut microbiome, therefore the change in the gut microbiome must be investigated, alongside the impact on the host organism. It is possible that in the relatively stable and ideal conditions of laboratory studies the loss or change of gut microbiome may have no discernible impact on the host, however if that host were in the more competitive and unstable conditions of its natural environment such changes could have a significant impact on health (Baümler and Sperandio, 2016)

In order to investigate the impact of MP on the gut microbiome, and due to the lack of current literature, the normal gut microbiome first needs to be identified by sequencing the products of a PCR using 16s ribosomal primers. A sample of gut contents of *G. pulex* will be first sequenced when collected, to give the natural state. The *G.pulex* will be conditioned

and fed on algal wafers for several days, after which gut contents, and algal wafers will be sampled and sequenced, to give an experiment baseline state.

Gammarus pulex will then be exposed to MP for 24, 48 and 96 hours, after which the gut contents will be sequenced and compared against the experimental baseline state.

Methods

Initially agar plates were used to identify the types of microbes within the guts of *G.pulex* fed with uncontaminated alage wafers. Individual *G.pulex* were immersed in 50% ethanol for 5 seconds and then rinsed in two successive vials of sterile water, in order to remove external microbes (agar plates inoculated with this rise water produced no growth, confirming that the ethanol removed external contamination). The guts were then dissected under sterile conditions, homogenised using a homogenising pestle and 600µl nucleotide free water, this homogenate was diluted 1:10 and 1:100 and then used to inoculate agar plates (10% nutrient agar 90% water agar). Ten distinct types of colonies grew, 6 coming from 1:10 dilution, these individual colonies were triple spread and then identified with microscopy. As the only positive identification was for a yeast strain it was decided that RNA sequencing would be needed to identify microbes.

Using the pathogen protocol for Qiagen stool extraction kits, several extractions were run using whole *G.pulex,* guts, faeces and alagal wafers. Absorption spectroscopy was used to confirm nucleic acids were present in the extractions, with peaks around 230nm. As the volume of tissue that could be extracted from *G.pulex* guts was well below the capability of the kits, the guts of ten individuals were used, however, this was not enough to reliably extract DNA (only 1 in 5 samples showed peaks at 230nm) and it was decided that what

little DNA was extracted was likely to be *G.pulex* rather than from the microbiome. Success was gained from extractions from faeces, all 5 samples using 4cm of faeces produced peaks around 230nm. All 0.5 algal wafer extractions produced peaks at 230nm, with the highest peaks around 250nm (Figure A1).

It was decided to use faeces to identify the microbiome, and then the algal wafers as a control, therefore these were taken forward to PCR.

The initial PCR reagents used were 12.5µl Green Master Mix (Thermo-Fisher), 1µl each of forward and reverse 16s primers, 1µl DNA extraction sample *Escherichia coli* DNA for positive control and nucleotide free water for negative, 8µl nucleotide free water.

The cycles used were, 95^{o}C denaturing for 1 min, followed by 30 cycles of 55^{o}C for 2 min and 73^{o}C for 3 mins. The resulting products were run on gel electrophoresis at 99v for 1.5 hours, gel composition was 35ml buffer with 0.35g agarose and 2µl Gelred.

When observed all products had run to the end and so the gel was thickened by using 0.6g of agarose and only running for 1 hour. With this the positive control was clearly visible and approximately in the centre of the gel.

Results

Repeated attempts to rerun the PCR varying the denaturing time and temperature, along with increasing the volume of extracted DNA to 2µl. The algal PCR product and positive control showed bands in similar loci, however the faecal PCR product produced nothing (Figure A2).

PCR was then run using both 16s and 18s primers, this produced the same result, with the algae showing bands for both 16s and 18s, and the positive control showing bands only for the 16s, still nothing for the faecal products. Unfortunately, due to lack of available funds and inability to product reliable PCR products this research had to be abandoned.

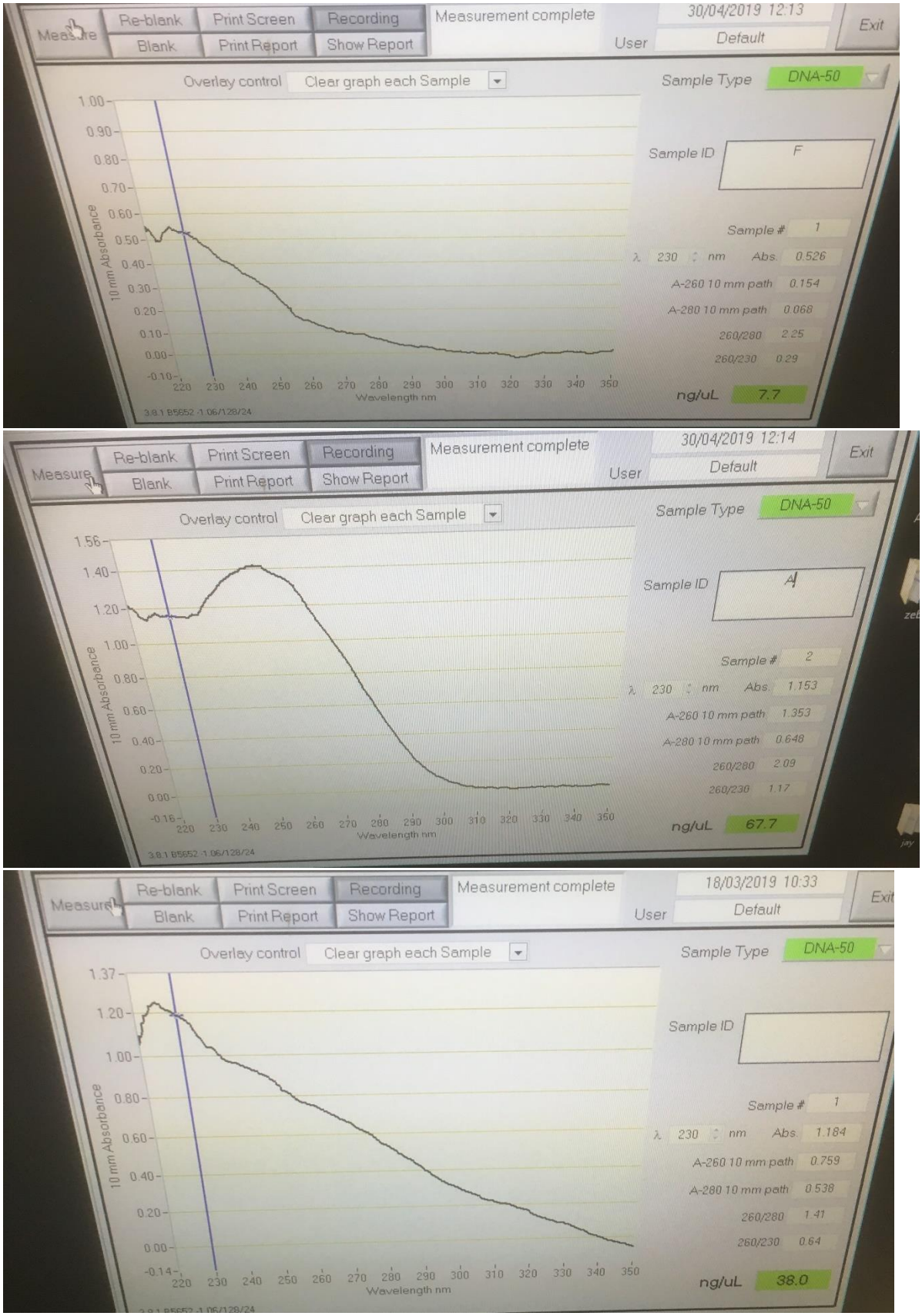

Figure A1 – Absorption Spectroscopy traces, showing peaks around 230nm, suggesting presence of DNA in samples

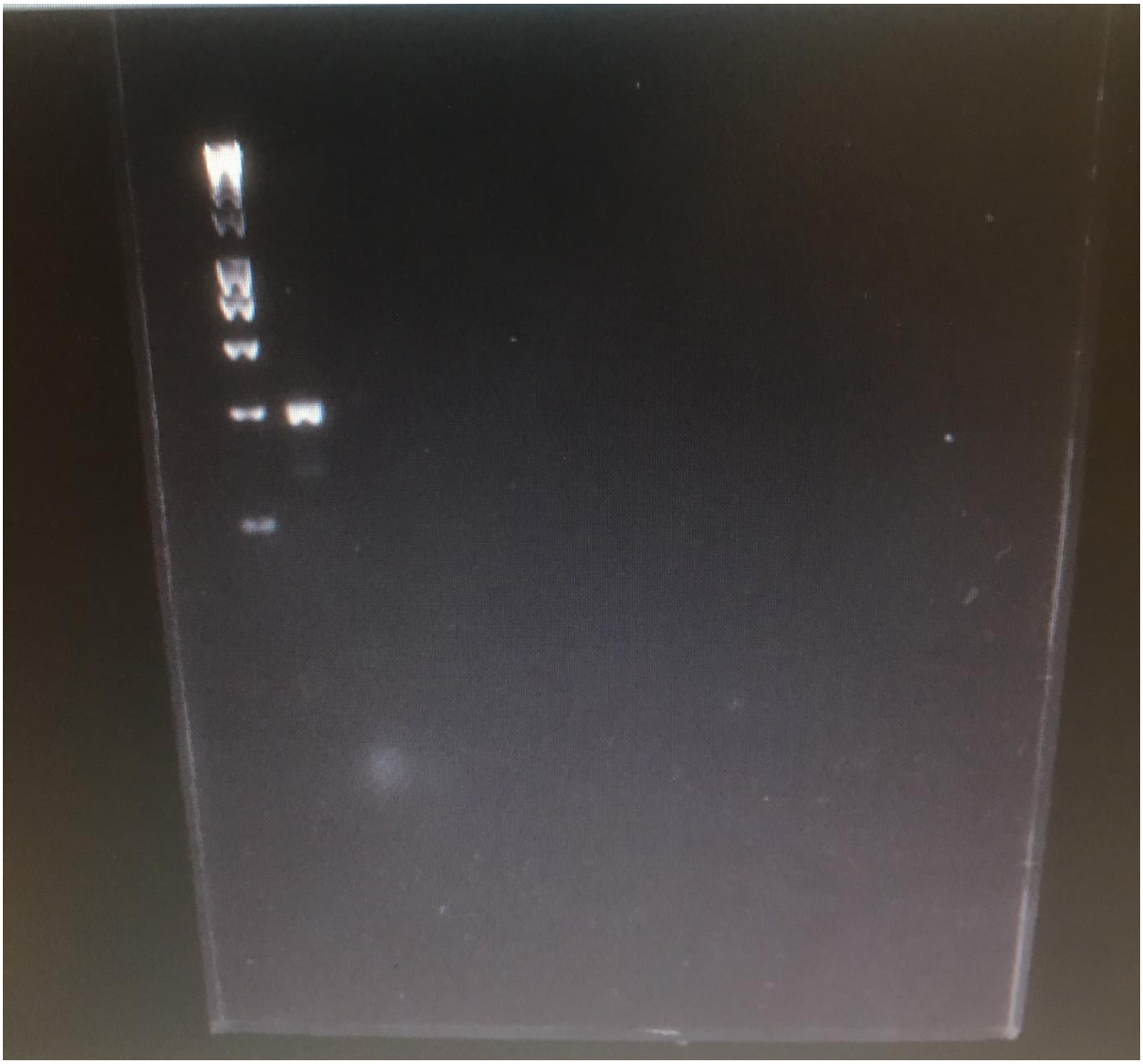

Figure A2 Gel electrophoresis of PCR products from faecal DNA extractions, wells from left to right are; ladder, positive control, negative control, sample.