Microfibre effects on the freshwater amphipod *Gammarus pulex*

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The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

Microplastic pollution is an emerging global issue of growing concern in environmental research. A substantial area of microplastic pollution is the release of polymer microfibres associated with clothing and fabrics through wastewater. Generally, research has focussed on the sources and fate of microplastics and less research has been undertaken on the effects caused in various aquatic species.

This study set out to address some of the literature gaps highlighted and investigated the effects microfibres could have on a keystone freshwater species, *Gammarus pulex*. Ecotoxicity tests were carried out to assess the impact of microfibres on mortality, locomotion, ingestion, feeding and growth of individuals. These tests were carried out at high and low concentrations to observe short-term effects that microfibres had on individuals.

Significant mortality and reduction in locomotion was observed in the majority of Ecotoxicity tests even at low concentrations. All individuals were found to have readily ingested microfibres throughout the study which caused increased mortality and a reduction in locomotion. All microfibre types caused mortality and reduced locomotion, even natural cotton microfibres.

The study highlighted the negative impacts of microfibres on aquatic species especially when considering that microplastic pollution is growing and accumulating in aquatic environments. Further research in establishing current and future microplastic concentrations is required to further standardise ecotoxicity tests and allow more accurate testing at environmentally realistic conditions.

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1 Introduction

Microplastic pollution is an emerging threat to global ecosystems and research into plastic sources and the issues associated with plastic has been growing increasingly since 2004 as shown in Figure 1.1 (Geyer et al., 2017). Plastic is a synthetic polymer material that largely revolutionised the manufacturing industry and brought about substantial societal benefit (Thompson and Napper, 2018). Plastic is a highly durable and versatile material which since its discovery has led to an exponential increase in production and use, outpacing any other material (Kershaw, 2015; UNEP, 2018). 40% of plastic is produced as packaging and is designed to be used once and thrown away and replaced with new plastic leading to a damaging amount of waste (UNEP, 2018; Thompson and Napper, 2018). 14% of plastic created is used in the manufacture of clothing and fabrics (Napper and Thompson, 2016; UNEP, 2018).

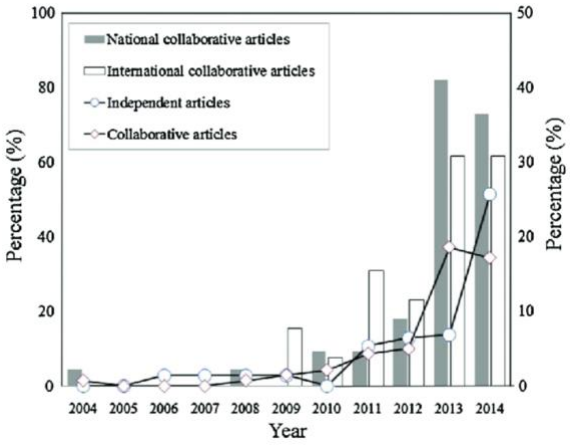

Figure 1.1 Independent and collaborative production of articles researching plastic pollution per year (2004-2014)(Barboza and Gimenez, 2015).

The rapid increase in plastic usage has in turn led to growing levels of plastic waste and the issue of where it all ends up. 86% of single-use plastics are thrown away with only 14% recycled and made into new products, 32% of single-use plastic waste is lost into the environment as leakage (UNEP, 2018). Furthermore, this increase in plastic waste is expected to rise, with peak plastic waste not predicted until the year 2100 (Jambeck et al., 2015). Discarded plastic waste has been unearthed all across the globe with plastic reaching as far as Himalayan mountains, Arctic environments and deep ocean trenches (Evangeliou et al., 2020; Jamieson et al., 2019; Peeken et al., 2018). A major issue with increasing plastic pollution is that the material persists in the environment and will never fully depolymerise, and instead will break down over long periods of time (Wagner et al., 2018). Plastic will breakdown into microscopic fragments of plastic which are penetrating ecosystems across the globe (Rochman, 2015).

Understanding of the issues surrounding microplastics is growing rapidly, however there are many knowledge gaps in the research area that are yet to be addressed (Thompson and Napper, 2018). One of these areas is the interaction microplastics have with aquatic ecosystems and how much of a threat this issue could have on biodiversity (Blair et al., 2017). Microplastics and larger plastic debris have been discovered in the guts of notable fauna globally and is estimated to affect over 600 aquatic species, with 15% of these being endangered (UNEP, 2018). Currently, the predicted rise in plastic use exceeds the increased efforts to mitigate plastic pollution (Borrelle et al., 2020). Research in this area suggests that plastic pollution will continue to increase and have a greater impact on aquatic and terrestrial species and contribute to the biodiversity crisis (UNEP, 2018).

Less is known about microplastics' effects on biodiversity, with the impact of smaller sized particles being less obvious (Kershaw, 2015). Aquatic species provide important ecosystem services, removal or a decline in aquatic species could negatively impact the wider ecosystem (Oliveira et al., 2019). Research has highlighted effects on aquatic invertebrates from microplastic pollution as an important area of research, where more specific work needs to be done (de Sá et al., 2018; Oliveira et al., 2019).

The increased demand for studies investigating how microplastics interact with aquatic species has influenced this topic of research. Experimental work has been designed to address some of the gaps in the literature. As previously mentioned, microplastics emerge from a number of sources; one that is particularly concerning is the amount of plastic associated with clothing and fabrics which are released directly into wastewater to riverine systems (Napper and Thompson, 2016). As a result, this study's experimental design will investigate the effects associated with microplastic fibres, used in clothing, on a keystone freshwater species.

1.1 Aims and objectives

The overall aim of this study is to gain a greater understanding of how microplastics affect aquatic species in the freshwater environment.

1.1.1 Project aims and objectives

- To evaluate published literature sources, concerning the fate and effects of microplastics in the aquatic environment, focusing specifically on freshwater systems.
- To establish key research knowledge gaps surrounding the fate and effects of microplastics in freshwater systems.
- To investigate the effects of plastic debris and microplastics on aquatic ecosystems throughout published literature.
- To conduct laboratory experiments to explore the effects of microplastic debris on the freshwater amphipod *Gammarus Pulex*.
- To determine if these effects are observed at current environmentally realistic microplastic concentrations.

1.1.2 Hypotheses

- Microplastic pollution is increasing in freshwater environments and effects upon freshwater ecology are being found more commonly in the wider literature due to an increase in pollution and research into the topic.
- Mortality rate will be higher when individuals are exposed to microplastics of high concentrations due to the increased ingestion of microfibres.
- Locomotive movement will be reduced in individuals when exposed to microplastics as a response to microfibre exposure.
- Current environmentally relevant microplastic concentrations will have reduced effects in laboratory experiments as effects are likely to be dose-dependent.

2 Literature Review: Sources, fate and effects of microplastics in freshwaters

2.1 Plastic as an emerging contaminant

In recent years there has been an emerging interest into plastic and the pollution associated with the material. Plastic is a material formed by synthetic polymers which can be made into a diverse range of products (Thompson and Napper, 2018; Wagner et al., 2014). Large amounts of plastic are lost into the environment and transported to rivers and lakes with the majority ending up in the oceans (Thompson and Napper, 2018). In particular, microplastic pollution is an emerging threat to aquatic systems and can impact wildlife and even humans through contamination of food chains (Dilkes-Hoffman et al., 2019). Plastic is appearing in environments all across the globe and has been often linked as a potential indicator of the Anthropocene epoch (Geyer et al., 2017).

A large surge in recent literature has focussed on the occurrence of microplastics which have been found in many wide ranging locations across the world. This review of the literature will cover the topic of microplastics from the production, use and waste of the material to the different sources of pollution. There will be a particular focus on the environmental fate and occurrence of plastic as well as the effects plastic has on aquatic ecology. There will also be a discussion about where the gaps are in the literature and where future research needs to be prioritised.

2.2 Plastic production, waste and degradation

2.2.1 Plastic production and usage

Since 1950, when plastic production began, global output per year has increased exponentially up to 350 million tonnes by 2015, shown in Figure 2.1 (Geyer et al., 2017). Plastic has greatly benefitted society as it is an unique material, being durable, strong and cheap (Andrady et al., 2009; Thompson and Napper, 2018). Plastic was originally used in a sustainable way for the military where plastic was regularly reused (Geyer et al., 2017). Since the First World War there has been a global transition in which plastic transitioned from a circular to a linear use (Geyer et al., 2017; UNEP, 2018). This is when materials are used once and discarded in an unsustainable manner and instead of being reused, new materials are produced (Geyer et al., 2017; UNEP, 2018). Plastic is now used in a wide range of different products and packaging across the world, 50% of which are intended to be disposable products (Derraik, 2002; Geyer et al., 2017; Jambeck et al., 2015).

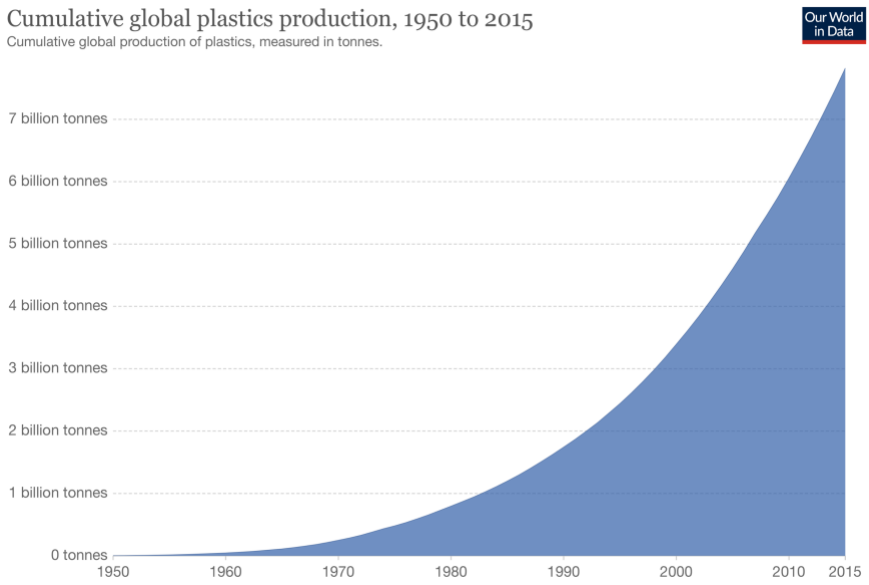

Figure 2.1 Global plastic production from 1950-2015 (Geyer et al., 2017).

2.2.2 Plastic waste and pollution

Global plastic waste has also increased dramatically as human use has grown, up to 275 million MT of plastic was sent to landfill globally in 2010 (Jambeck et al., 2015). Despite more recent attention to the amount of plastic use in everyday life, we are not predicted to reach peak plastic waste until closer to 2100 (Browne et al., 2011; Jambeck et al., 2015). The main issue in the rapid increase of single-use plastic packaging is what happens to the material once it has been used. Only a reported 9% of plastic packaging is recycled with a further 12% being incinerated, leaving 79% of this plastic to either be lost to landfill or enter the natural environment (Geyer et al., 2017). Plastic use in clothing, similarly to its use in packaging, has led to a disposable attitude developing over recent years. Plastic being so cheap and readily available has led to a reduction in clothing and material costs and a subsequent increase in clothing being bought and thrown away (Andrady and Neal, 2009).

The plastic pollution issue has been closely intertwined with the climate crisis since the start of the century and has been building in exposure in the literature in recent years. Recent public attitudes have called for the reduction of single-use plastics after recent exposure; it was rated by the Australian public as the most serious environmental issue amongst nine others including water pollution, climate change and water shortages (Dilkes-Hoffman et al., 2019). Plastic pollution and production are linked to climate change, with plastic production relying on fossil fuels, and making up 6% of oil and gas use in Europe (Alessi et al., 2018; Rabnawaz et al., 2017). Plastic pollution also contributes to climate change due to the breakdown of plastics releasing potent greenhouse gasses such as methane and ethylene (Royer et al., 2018).

2.2.3 Single-use plastic in response to COVID-19

There has been a substantial increase in single-use plastic as a response to the 2020 COVID-19 pandemic (Hale and Song, 2020; Prata et al., 2020). The COVID-19 pandemic has seen tremendous changes to society and affected the world population in many facets. The increased use of personal protective equipment (PPE) has been essential for many frontline workers in stopping the spread of the virus, and demand for PPE has been exceptional (Prata et al., 2020). There has been a rapid expansion in the demand for and use of single-use masks, gloves and plastic packaging following the outbreak despite plastic having the properties that make it a vector for viral infections and studies suggesting paper bags or washable fabrics would be a safer alternative (Hale and Song, 2020). With this increased use, there has been an greater misuse of PPE, with over a million discarded face masks and gloves reported (Sarkodie and Owusu, 2020). This increased discarded plastic waste has seen an influx of masks and gloves entering the aquatic environment and being found in rivers, lakes and coastal waters (Sarkodie and Owusu, 2020). Studies have found these accumulating in the aquatic environment in multiple places showing signs of degradation and releasing microplastic particles and fibres into the environment (Fadare and Okoffo, 2020).

2.2.4 Plastic Properties and Degradation

The physical properties of plastic give it the ability to persist in the environment across geological timescales (Botterell et al., 2019; Thompson and Napper, 2018). In combination with the exponential increase in its production, there is now a significant surplus of plastic waste in the marine and freshwater environments (Browne et al., 2011; Capolupo et al., 2020; Thompson et al., 2004). When plastic reaches freshwater and marine environments it is subjected to a number of processes that contribute to physical weathering of plastic debris (Wagner et al., 2014). Exposure to UV radiation, wave and water action as well as other debris can lead to the disintegration and breakdown of plastic into microplastics (Wagner et al., 2014; Welden, 2015). Furthermore, the processes of photo-oxidative degradation, catalytic and biodegradation, induced by ozone, promote the breakdown of microplastics in the aquatic and terrestrial environments (Evangeliou et al., 2020; Singh and Sharma, 2008).

Photo-oxidative degradation is the process in which polymers are degraded by UV radiation and visible light which breaks the chemical bonds of the plastic as well as increasing the embrittlement of the polymer meaning it breaks up more readily (Singh and Sharma, 2008). Catalytic breakdown is the process in which polyolefins (polyethylene, polypropylene, polyester) are chemically degraded, releasing gases and oils; studies have found plastic releasing greenhouse gases as a result of this (Royer et al., 2018; Singh and Sharma, 2008). Biodegradation is the process in which polymers are broken down by oxidation, hydrolysis and microbial mineralisation (Singh and Sharma, 2008). Importantly, when plastic is released into the environment there are a number of complex processes which will break the polymer down into increasingly smaller pieces over time, creating microplastics and nanoplastics but never fully decomposing (Klemchuk, 1990; Kershaw, 2015).

2.3 Plastics in the environment– materials, size and characteristics

Plastics occur in the aquatic and terrestrial environments in multiple sizes and have different characteristics, making them a complex issue.

2.3.1 Microplastics

The term microplastic, coined by Thompson et al. (2004), refers to plastic pieces smaller than 5mm, which have been found in wide ranging locations such as Antarctica, the Arctic and deep ocean trenches (Bessa et al., 2019; Jamieson et al., 2019). Microplastics originate in two different forms; primary microplastics and secondary microplastics. Primary microplastics are produced microscopic in size, such as microbeads from cosmetics or plastic fragments used in manufacturing (Jambeck et al., 2015; Geyer et al., 2017). Secondary microplastics originate from the breakdown of larger plastic debris and due to physical weathering become microscopic fragments and fibres (Thompson et al., 2004; Wagner et al., 2018). Many types of microplastic polymers exist in both the freshwater and marine environments as highlighted in Tables 2.1 and 2.2. The type of microplastics found in the freshwater environment is less extensively researched but data gathered from wastewater treatment works discusses the types and amounts of plastic entering and existing in the freshwater environment (Kay et al., 2018; Murphy et al., 2016).

Table 2.1 A table detailing the polymer type of microplastics commonly found in freshwater environments released from Wastewater treatment works and their abundance released at each stage of the treatment. SC=Sludge cake (Murphy et al., 2016).

	liquid fraction (303 MP)				solid fraction (79 MP)			24 h SC duplicate (48 MP)			
								day 1		day 2	
polymer	S1	S2	S3	S4	grit	grease	SC	09:30:00	14:30:00	09:30:00	14:30:00
acrylic	8.3	12.6	5.9	12.0	0.0	6.8	16.7	12.5	18.2	33.3	15.0
alkyd	28.7	17.2	20.6	8.0	54.6	13.6	16.7	0.0	27.3	33.3	5.0
PET	3.8	12.6	2.9	4.0	0.0	13.6	0.0	0.0	0.0	0.0	0.0
polyamide	4.5	2.3	14.7	20.0	9.1	0.0	0.0	0.0	0.0	0.0	10.0
polyaryl ether	0.0	1.2	0.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0
polyester	10.8	13.8	29.4	28.0	27.3	23.7	16.7	25.0	36.4	11.1	30.0
polyethylene	4.5	1.2	14.7	4.0	0.0	32.2	33.3	0.0	9.1	0.0	5.0
polypropylene	2.6	1.2	5.9	12.0	0.0	5.1	0.0	12.5	0.0	22.2	20.0
polystyrene	2.6	17.2	5.9	4.0	9.1	1.7	16.7	37.5	0.0	0.0	10.0
polyurethane	8.9	8.1	0.0	0.0	0.0	1.7	0.0	0.0	0.0	0.0	0.0
polyvinylfluride	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PS acrylic	19.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PV acrylate	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PVA	3.2	10.3	0.0	4.0	0.0	1.7	0.0	12.5	9.1	0.0	0.0
PVC	1.3	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PVE	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]S1 = influent, S2 = grit and grease effluent, S3 = primary effluent, and S4 = final effluent. [b]PET = polyethylenterephthalat, PS acrylic = polystyrene acrylic, PV acrylate = polyvinyl acrylate, PVA = polyvinyl acetate, PVC = polyvinyl chloride, and PVE = polyvinyl ethelene.

Table 2.2 Detailed polymer type and density of microplastics also how commonly each type is found in the aquatic environment from 42 different studies (Hidalgo-Ruz et al., 2012).

polymer type	polymer density (g cm^{-3})	no. of studies
polyethylene	0.917–0.965	33
polypropylene	0.9–0.91	27
polystyrene	1.04–1.1	17
polyamide (nylon)	1.02–1.05	7
polyester	1.24–2.3	4
acrylic	1.09–1.20	4
polyoximethylene	1.41–1.61	4
polyvinyl alcohol	1.19–1.31	3
polyvinylchloride	1.16–1.58	2
poly methylacrylate	1.17–1.20	2
polyethylene terephthalate	1.37–1.45	1
alkyd	1.24–2.10	1
polyurethane	1.2	1

[a]Data from a total of $N = 42$ studies.

2.3.2 Nanoplastics

Nanoplastics are plastic particles defined as less than 100nm in size (da Costa et al., 2016). Nanoplastics are a size category derived from microplastics and have slightly different properties; they have a smaller volume and a relatively large surface area making them more capable for penetrating tissue of species and being transferred through food chains (Chae and An, 2017).

Less is known about nanoplastics in terms of their fate and occurrence in freshwater environments, their toxicity, and how far they can penetrate species in comparison to other sized plastic debris (Koelmans et al., 2015). Nanoplastics are generated by products such as waterborne paints, adhesives and biomedical products as well as the breakdown from larger plastic debris and microplastics (Koelmans et al., 2015). Nanoplastics are less common in freshwater environments, however they have been deemed a contaminant of concern (Chae and An, 2017). They may not appear in many freshwater studies as they generally occur as a result of breakdown from larger pieces of plastic, which occurs more in the marine environment (Blair et al., 2017). The grading of sampling nets may also affect the nanoplastics content of surveys as common sampling nets are larger than nanoplastics at 330μm (Hidalgo-Ruz et al., 2012).

2.3.3 Natural Fibres and Debris

Natural textiles and fibres are underrepresented in the literature and generally are not considered when discussing plastic fibres and debris in aquatic systems (Stanton et al., 2019). This may be due to the common perception that natural fibres will not cause harm to organisms within aquatic environments (Ladewig et al., 2015).

Natural fibres have been discovered in animals such as birds, commercial fish and species of invertebrates (Compa et al., 2018; Remy et al., 2015; Zhao Shiye et al., 2016). Natural materials also breakdown and natural fibres will accumulate in the aquatic environment through sewage effluents and behave as a contaminant in the same way plastic fibres do (Dris et al., 2014; Dris et al., 2018). Natural fibres such as cotton are commonly associated with clothing and are released into the environment through washing machines and are abundant in the aquatic environment (Compa et al., 2018). These natural fibres can be readily ingested alongside microplastics; one particular study found that 43% of fibres ingested by fish species were natural fibres such as cotton, linen and silk (Halstead et al., 2018).

Much like plastic fibres, natural fibres are found to absorb pollutants and are ingested by aquatic species, causing trauma and harm to these organisms. Natural fibres are even found to be more absorbent to pollutants than polymer fibres due to their electrokinetic properties (Ladewig et al., 2015). More research is required into the comparitive toxicity of natural fibres; this will allow specific knowledge of whether the composition of a plastic fibre is toxic or if it is the fibre shape which harms aquatic species.

2.4 Sources and Pathways

Identification of sources of plastic has been at the forefront of microplastic literature, with many being identified on land as well as plastic sources in the ocean (Browne et al., 2011; Cole et al., 2011; Kershaw, 2015). As highlighted in Figure 2.2, there are multiple different sources of microplastic pollution from different sectors which will be discussed below.

Pathways are opportunities for pollutants to be transported from the source to the sink, in this case being plastic, flowing through streams, sewage or through the air, to rivers or other freshwater environments (Rodríguez-Navas et al., 2013).

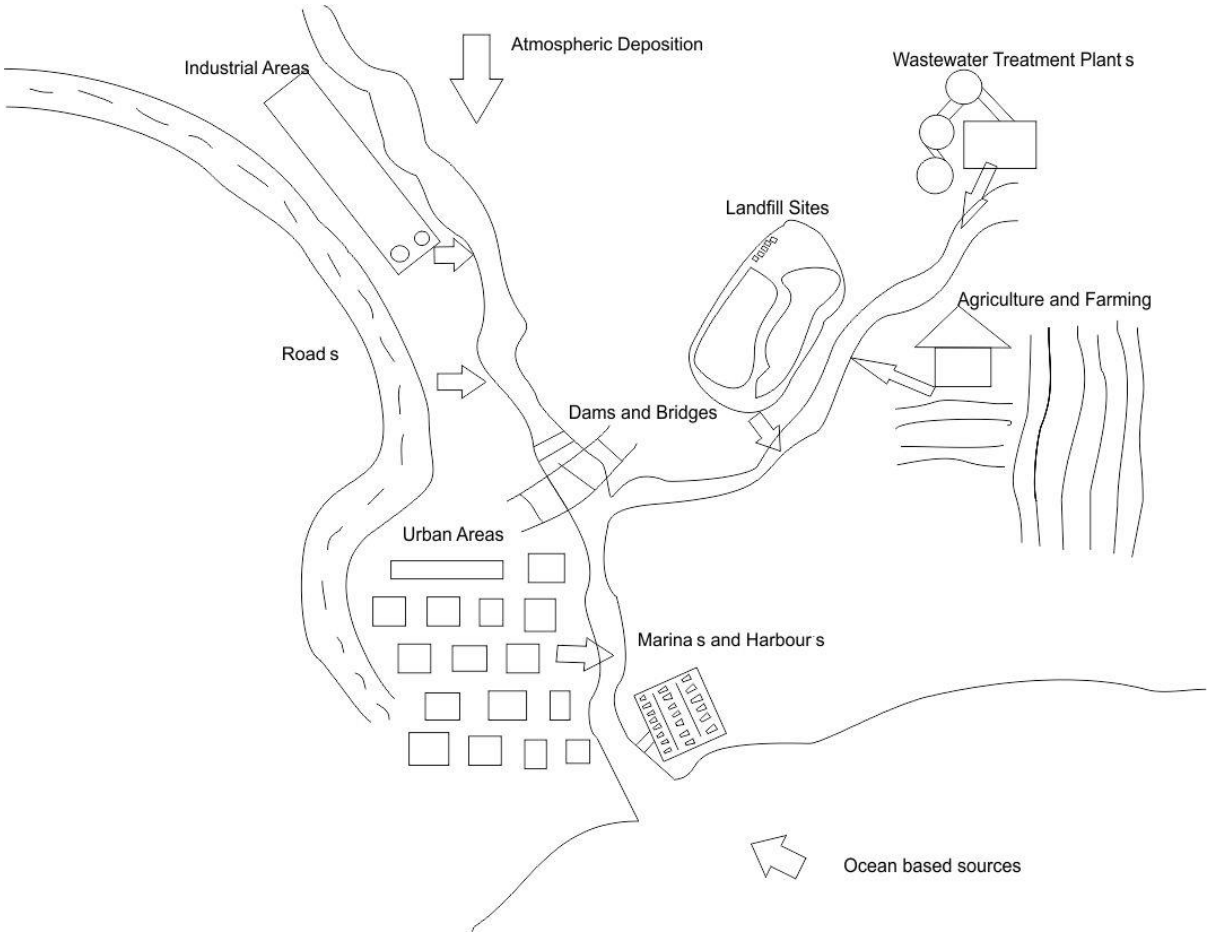

Figure 2.2 A model showing a basic view of the sources of microplastics on a river catchment discussed within the review of literature in this study.

2.4.1 Fishing

There are fewer ocean-based sources yet they are still significant; fishing and lost equipment generates a substantial amount of plastic, released into the environment (Alessi et al., 2018; Derraik, 2002). There are suggestions that ocean-based sources contribute to more plastic pollution in the oceans than land-based sources; however quantification of these sources is difficult and uncertain (Jang et al., 2014). Fishing debris consists of fishing nets, polypropylene rope for buoys and general debris lost from vessels, creating a significant amount plastic debris lost into the oceans (Jang et al., 2014; Gallagher et al., 2016). Fishing debris comprises a substantial amount of marine litter. For example, 14% of UK coastal plastic is generated from lost fishing equipment and is the second most common source of plastic in EU waters (UNEP, 2018). A study by Unger and Harrison (2016) investigating fisheries as a source of microplastics concluded that fisheries are responsible for considerable amounts of microplastic pollution in UK waters providing 63.5% of discarded plastic waste on beaches.

2.4.2 Shipping and marine activities

The shipping and marine industry also act as a source of plastic pollution, with debris lost from ships as well as paint and anti-fouling containing plastics (Dibke et al., 2021; Welden, 2015). Microplastics have been found in estuarine locations where shipping is common, however, polyester fibres and pellets found in a study point to clothing and manufacturing to be more likely sources (Gallagher et al., 2016). One study points to ships' paint and anti-fouling to be a considerable source of microplastics especially when abrasion took place in port (Dibke et al., 2021). These increases in plastic are expected to be attributed to increased marine and boating activity in estuarine locations and

that there is very little regulation for plastic waste from boats (Galloway, 2015). Furthermore, the rise in boating activity has increased the impact anti-fouling and boat paints have on releasing microplastics into the marine environment from the wear on jetties and abrasion over time of the water (Dibke et al., 2021; Magnusson et al., 2016; Verschoor et al., 2016). Commonly found plastic such as acrylic and polyester make up marine paints, as found in close proximity to these shipping and marine areas in relatively high concentrations (Gallagher et al., 2016).

2.4.3 Sources in urban areas

Land-based sources are generally regarded as the main sources of plastic waste, debris and microplastics entering the freshwater and marine environments globally (Jambeck et al., 2015; Thompson and Napper, 2018). There are a large range of sources of microplastics that are land-based which are shown in Figure 2.2; urban areas being a source of concern.

Large quantities of plastic reach the freshwater and marine domains from discarded waste and litter from urban areas, as well as waste management sites (Duis and Coors, 2016). Plastic waste generated by people and businesses is often lost into water courses and rivers, transported by rainfall events, and enters the natural environment at an increasingly rapid rate (Geyer et al., 2017; Jambeck et al., 2015). Jambeck et al. (2015) estimated that mismanaged waste in urban coastal areas is a significant source of plastic waste entering oceans with between 4.8 to 12.7 million megatonnes entering the oceans in 2010. Urban areas that are close to a water course are considered to contain a significant amount of sources of plastic debris, which will break down into microplastics in the environment, as highlighted by a study of the city of Paris and the microplastic content in its watercourses (Dris et al., 2015). Paris was found to have high microplastic contamination (Table 2.3) but also very variable results. The implication from this study is that urban areas have the potential to input substantial amounts of plastic debris to watercourses and that these levels can vary depending on a number of variables such as weather, direct dumping and river traffic (Dris et al., 2015). Tourism has a substantial impact on plastic entering the environment; it is suggested to create a 40% surge in marine litter in European urban areas (Alessi et al., 2018).

2.4.4 Wastewater treatment plants and domestic products

A large volume of plastic enters wastewater treatment plants, where larger pieces are typically removed from the sewage effluents. However, microplastics are often released in vast quantitites such as 65 million microplastics a day, despite removing 90-99%, from a single wastewater treatment plant (Murphy et al., 2016; Kay et al., 2018). Many of these microplastics are released through the abrasion of textiles and materials such as synthetic clothing and car tyres (Capolupo et al., 2020; Napper and Thompson, 2016). Another source which received widespread media attention were microbeads found in cosmetic products (Duis and Coors, 2016). There are a number of types of plastic from different sources that are released from wastewater treatment plants, as highlighted in Table 1. Polyester is one of the most abundant and is a very common plastic found in freshwater environments (Murphy et al., 2016; Wagner et al., 2018). Polyester is used in clothing and textiles primarily, and fibres from these are lost to the environment at a high rate; a typical wash cycle releases around 700,000 fibres comprised substantially of polyester (Napper and Thompson, 2016). Plastic has been used in creating textiles and clothing due to it being a cheap and durable material that can be formed into fibres which make up the textile (Björkner, 1995). However, a recently discovered issue with this is that these plastic fibres can be easily released into the environment, particularly when they are washed (Napper and Thompson, 2016). It is not just fibres released from wastewater with fragments; flakes and microbeads make up a significant amount of the microplastics released from treatment plants (Kay et al., 2018; Murphy et al., 2016). Fragments and flakes are often associated with larger plastic debris broken down, and higher amounts of these in systems may be a result of increased plastic debris and litter released into wastewater systems (Blair et al., 2017). Additionally, storm water runoff allows exceptional amounts of plastics to reach aquatic environments. Considering treatment plants remove

96% of microplastics sewage, around 620 million microplastics are estimated to enter rivers from storm drains in the UK each day they release sewage overflows (Murphy et al., 2016). It is noteworthy that treatment plants remove between 90-99% of microplastics under normal circumstances, so increased storm activity associated with climate change will increase the influx of microplastics entering marine environments (Myles et al., 2014). The main pathways for plastic debris to enter the environment are through sewage works and storm water passages flowing into rivers (Browne, 2015). Generally, larger debris will not pass through wastewater treatment works, meaning that for large debris significant rainfall is required to allow debris to bypass treatment works (Murphy et al., 2016). Approximately 98% of microplastics are also removed by treatment plants, increasing the noteworthiness of storm water as a pathway for microplastics (Magnusson et al., 2016). Despite the removal of these plastics, 65 million microplastics are released each day from a single treatment works (Murphy et al., 2016). Wastewater treatment plants allow microplastics from a number of sources reach the aquatic environment and can be considered as a source as well as a pathway.

2.4.5 Roads and bridges

Plastic waste and microplastics associated with roads and bridges have an important impact on the amount of plastic entering freshwater environments. A substantial reason for this is tyre wear, which is estimated to account for 5-10% of plastic reaching aquatic environments (Kole et al., 2017). When car tyres are used, synthetic rubber particles are released due to the friction created; this is then transported to aquatic zones from rainfall runoff (Verschoor et al., 2016). For example, it was estimated that 500 tonnes of tread wear microplastics from tyres was directly transported into river systems in 2012 in the Netherlands (Verschoor et al., 2016). The size and composition of these microplastic particles released depends on several factors including car speed, climate, road surface and driving speed (Kole et al., 2017). For example, driving on the motorway at 100 km/h was found to generate twice as many microplastic as on slower roads (Kole et al., 2017). The closer the road surface is to an aquatic area the more likely microplastic pollution will affect that zone, for example bridges and coastal roads are more likely to increase direct pollution from car tyres (Kole et al., 2017). It has also been discovered that synthetic polymers found in car tires can release potentially toxic chemicals and additives that leach out from the plastic such as Benzothiazole (CTR) and Phthalide (PVC) (Capolupo et al., 2020).

2.4.6 Landfill

Plastic waste sent to landfill also breaks down over time into microplastics which are then transported via leachtate to the aquatic environment (He et al., 2019a; Welden, 2015). A study by He et al. (2019a) investigated municipal solid waste landfill sites, and leachate samples were taken from these sites, discovered to contain substantial amounts of microplastics (621 microplastics in 12 samples). The study found that around 70% of this plastic was polypropelene and polyethylene fragments from broken down landfill (He et al., 2019a). Despite this, landfill sites have a more complex pathway of transporting plastics to the aquatic zones and will have a lower impact. However, microplastics can have a noteworthy impact upon soils and can impact agriculture and plant health such as alterations in soil biophysical properties, which have been observed (de Souza Machado et al., 2018b).

2.4.7 Industry

Microplastics and larger plastic debris are commonly found in industrial areas for uses in cleaning and air-blasting of paint, packaging and manufacturing processes (Mani et al., 2015; Thompson and Napper, 2018). Industry located close to watercourses such as shipyards and marinas, as well as general industry on a riverside, can have a direct input of these plastics into aquatic environments either as plastic debris or microplastics. Another notable source of microplastics is manafacturers that use and produce small plastic resin pellets and granules used for sand blasting and also as a raw material; these are often lost in use and transportation and found in the aquatic environment (Cole et al., 2011). A Swedish study found water samples with 102,000 microplastics per cubic metre close to

a plastic production facility whilst normal concentrations in Swedish waters tend to be between 105-2,400 particles per cubic metre (Lozano and Mouat, 2009).

2.4.8 Agriculture

It has been found that 2% of total plastic production globally is used in agriculture and horticulture for applications in greenhouse technology, as well as protection of crops and other uses (Rodríguez-Seijo, A. and Pereira, R.J.B.o.A.S., 2019). Plastic is commonly used in agriculture and is omnipresent on farms and purposes include for: tunnels, greenhouse covers, irrigation, reservoirs, mulching films, packaging, harvesting nets and films (Rodríguez-Seijo and Pereira 2019; DEFRA, 2010). A report by DEFRA (2010) found that 45,000 tonnes of non-packaging agricultural plastics are sold in the UK, which creates 85,000 tonnes of waste plastic that cannot be recycled due to soil contamination. This report gained a majority vote to not intervene meaning these numbers will only increase. Plastic use in agriculture has been increasing exponentially, making it a significant plastic source (Mormile et al., 2017). Many pollutants are released from farmland into riverine systems from rainfall run-off, meaning microplastics from the increased plastic used in farms will be transported to the aquatic environment (Novotny, 1999; Rodríguez-Seijo and Pereira 2019). Microplastics can also be transported to agricultural systems through sewage sludge added to crops as fertilizer (Habib et al., 1998). Considering roughly 98% of microplastics are removed in the sewage sludge, there is the potential for vast numbers of microplastics to be exposed to agricultural land (Magnusson et al., 2016). It is estimated that between 125 and 800 million tonnes of microplastics are added to agricultural land annually in Europe through the application of sewage sludge or processed bio-solids (Nizzetto et al., 2016). This could affect species in this environment and run-off from farmland may transport these microplastics to rivers and water bodies, however there is no such data of this currently (Magnusson et al., 2016).

2.4.9 Atmospheric deposition

Plastic particles have the ability to travel further distances via atmospheric transport and deposition than through aquatic systems (Allen et al., 2019). A study by Dris et al. (2016) investigated atmospheric deposition in urban and non-urban areas, concluding that atmospheric depostition should not be ignored as a source of microplastics. Atmospheric processes can transport microplastic particles over long distances, with microplastic and nanoplastic particles being found in Arctic samples penetrating one of the last pristine environments on Earth (Bergmann et al., 2019). This atmospheric route is a new pathway allowing microplastics to find their way into the oceans, soils and river catchments (Bergmann et al., 2019).

Knowledge of sources of microplastics is important in attempting to manage the influx of plastic entering our environment, however as seen in Figure 2.2, and as outlined above, the variety of sources is substantial and increasing rapidly as more research is undertaken. It is important to manage the inputs of plastic, and research into this has generally been the focus. Research into the effects of this pollution is necessary also to further assess the damage it is having (Chae and An, 2017).

2.4.10 Storm water runoff as a pathway

Large amounts and sustained periods of rainfall, coupled with impervious surfaces in urban areas, where lots of plastic resides, creates a large influx of plastic into rivers and this plastic debris will bypass sewage and water treatment (Magnusson et al., 2016). Storm water systems and combined sewage overflows exist in most urban areas and allow for large amounts of water to bypass treatment to reduce flooding, however this means pollutants including plastic can enter river systems (He et al., 2019a).

2.4.11 Atmospheric transfer as a pathway

Another pathway for plastic debris entering our oceans is wind-blown transport from coastal or urban locations either directly into the sea or rivers (Thompson and Napper, 2018). This can involve the transfer of plastic litter directly to rivers, often seen in urban areas, as well as the atmospheric transport of microplastics through the air and atmosphere (Allen et al., 2019).

2.4.12 Other pathways

The breakdown of the macroplastics into microplastics could be considered as a pathway as it is how microplastics enter the environment from larger debris (Browne, 2015). Pathways can also occur beneath the ground especially as plastics break down in landfill sites and are transported to rivers or the coast (Welden, 2015; He et al., 2019a). Transport through groundwater is important to consider alongside surface water transport.

The multiple sources and pathways highlight the complexity of the issue and the difficulty in preventing plastic entering the aquatic environment due to there being numerous avenues, as discussed above and highlighted in Figure 2.2 (Browne, 2015).

2.4.13 Key Knowledge Gaps for sources and pathways

The standout knowledge gap is the issue of how difficult it is to quantify these sources and make direct comparisons between them (Thompson and Napper, 2018). The number of sources is continuously growing with the addition of new research. Due to the increasing number of sources, estimations of the total quantities of microplastics released is more difficult. Quantifying microplastics released by sources will require more consistency in the measurement of microplastics in the aquatic environment and the quantity of microplastics released by sources (Geyer et al., 2017; Thompson and Napper, 2018).

2.5 Fate and Occurrence

The fate of plastics is determined by river, tidal and weather patterns as well as the type and size of plastics which are transported throughout the freshwater and marine environment (Eerkes-Medrano and Thompson, 2018; Eerkes-Medrano et al., 2015). The majority of plastic debris accumulates in the oceans, found from the Arctic and Antarctic regions as well as across all oceans and seas that have been surveyed (Bessa et al., 2019; Browne et al., 2011; Thompson and Napper, 2018). Plastic debris, including microplastics, are found at all levels of the water column from the sea surface to the pelagic zones and ocean and freshwater sediments (Jamieson et al., 2019; Wagner et al., 2014). Plastic debris is transported across oceans by sub-tropical ocean gyres and accumulates in large plastic patches across oceans, which breaks down and releases further microplastics (Kershaw, 2015). Microplastics also accumulate in freshwater sediments and are transported through rivers and freshwater bodies (Nel et al., 2018). Microplastics have become a consistent and substantial pollutant found at high concentrations and in a number of aquatic species (de Souza Machado et al., 2018a; Sanchez et al., 2014). Studies into freshwater species such as freshwater Gudgeon (*Gobio Gobio)* and the Asian Clam *(Corbicula fluminea)* have indicated that microplastics have been found in freshwater environments and have also be found in the species that inhabit these environments (Sanchez et al., 2014; Su et al., 2018).

2.5.1 Fate of microplastics

Microplastics in freshwater environments tend to be there temporarily as they travel from multiple sources to the marine environment until reaching oceanic cycles, deep sea sediments or are ingested by marine or freshwater organisms (Lambert and Wagner, 2018). However, some plastic is retained in rivers due to entanglement as a result of flow variation and entrapment by vegetation and sediment (van Emmerik and Schwarz, 2020). This means plastic remains in freshwater environments in the water column as well as in sediments (Eerkes-Medrano and Thompson, 2018). Therefore,

concentrations of microplastics in freshwaters can vary by large values, as highlighted in Table 2.3. Environmentally realistic concentrations are concentrations that are currently found in literature (and can then be applied in ecotoxicity tests), can also vary (Table 2.3 and 2.4). These variations depend on the distribution between the water column and sediment, importantly being higher in sediments (Castañeda et al., 2014).

Much of the fate of microplastics depends on the composition of the plastic, how much it has degraded and how the plastic persists in the aquatic environment (Kershaw, 2015). Generally, dense plastics will travel less distance and therefore are more likely found in sediment closer to sources (densities presented in Table 2.2). However, the density of a plastic also depends on its degradation, as plastics can have a larger relative surface area due to shape, which will affect where they occur within the water column (Chae and An, 2017). Plastic with a larger surface area tends to be transported further by physical forces such as wind and water movement; for example turbulent forces can re-suspend microplastics and transport them further (Eerkes-Medrano and Thompson, 2018). As microplastics will persist in the aquatic environment and only continue to breakdown, there is a great potential for changes in water movement to transport microplastics substantial distances with microplastics reaching ocean trenches, mountain summits and Arctic sea ice (Jamieson et al., 2019; Peeken et al., 2018; Zhang et al., 2021).

2.5.1.1 Water column distribution

Microplastics have been found in each level of the water column(see Table 2.3), from surface waters down to sediments (Kershaw, 2015). Microplastics are most commonly found between 200 and 600 meters in offshore waters and more frequently found in sediments in freshwater environments (Choy et al., 2019; Eerkes-Medrano and Thompson, 2018). This generally depends on the type of plastic and its density (common plastic densities are provided in Table 2.2), which will alter the fate of the plastic. Water currents and flow rates will also affect the fate of microplastics and their position in the water column, causing differences in types of plastic found in each zone (Geyer et al., 2017). Despite freshwater concentrations of microplastics being generally low, as seen in Table 3, a study of the Danube River found that in some parts of the water column plastic outnumbered natural particles (Lechner et al., 2014).

2.5.1.2 Sediment distribution

Microplastics and larger plastic debris are increasingly common in freshwater sediments with sediment sampling being frequently used to measure microplastic concentration in different locations (Ballent et al., 2016). Microplastics' density can change when colonised by sediment-dwelling organisms, meaning they are transported through the water column into sediments (Duis and Coors, 2016). Many studies suggest concentrations in sediments are much higher in freshwater environments (Castañeda et al., 2014; Eerkes-Medrano and Thompson, 2018).

Table 2.3. Concentrations of microplastics in freshwater environments from a number of sources. This table was adapted from (Horton et al., 2017)).

Study	Sample type	Water body	Particles found	Concentration (Particles/L^{-1})	Location
(Eriksen et al., 2013)	Water	Lake	43,000 km^2 particles	0.00027	Great Lakes, USA
(Free et al., 2014)	Water	Lake	20,264 km^2 particles	0.00012	Lake Hovsgol, Mongolia

(Corcoran et al., 2015)	Lake Sediment	Lake	26 particles in 42.2g 9 particles in 103.2g	616.1/kg 87/kg	Lake Ontario, Canada
(Imhof, Hannes et al., 2018; Imhof, Hannes et al., 2013)	Beach sediment	Lake	1108 and 108 m^2 Average particle abundance 75 m^2	17/kg 1.7/kg 1.2/kg	Lake Garda, Italy
(Faure et al., 2015)	Beach sediment	Lake	Average particle abundance 1300 m^2	20/kg	Lake Geneva, Switzerland
(Fischer et al., 2016)	Water and beach sediment	Lake	Average particle abundance 234 kg 3.02m^{-3} surface water 112kg 2.51m^{-3}	0.03 0.025	Lake Chiusi and Lake Bolsena, Italy
(Su et al., 2016)	Water and Lake sediment	Lake	3.4-25.8/L 11-234.6kg sediment	3.4-25.8/L	Taihu Lake, China
(Ballent et al., 2016)	Benthic and shore sediments	Lake	980kg Benthic 140kg Beach	-	Lake Ontario, Canada
(Baldwin et al., 2016)	Water	River	0.05-32m^{-3}	0.00005-0.032	Great Lake tributaries, USA
(Dris et al., 2015)	Water	River	30m^{-3} (Plankton trawl) 0.35m^{-3} (Manta trawl)	0.03 0.00035	River Seine, France

			35000 - 293000 particles m^{-3}	35-293	
(Faure et al., 2015)b	Water	River	7 m^{-3}	0.007	Various rivers, Switzerland
(Lechner et al., 2014)	Water	River	316.8 m^{-3}	0.32	River Danube, Austria
(Mani et al., 2015)	Water	River	892,777km^{-2}	0.005	River Rhine
(McCormick et al., 2014)	Water	River	2.4 m^{-3} 5.7 m^{-3}	0.002 0.006	9 Rivers in Chicago, USA
(Yonkos et al., 2014)	Water	River	155,374 km^{-2} 40,852 km^{-2} 67,469 km^{-2} 112,590 km^{-2}	0.001 0.00027 0.00045 0.00075	River Papatsco Corsica Rhode Magothy
(Klein et al., 2015)	Sediment	River	228-3763/kg	-	Rivers Rhine and Main, Germany
(Ballent et al., 2016)	Sediment	River	610/kg	-	Lake Ontario Tributaries, Canada
(Castañeda et al., 2014)	Sediment	River	13,759	70.6-105.8/kg	St Lawrence river, Canada
(Horton, A. et al., 2017)	Sediment	River	185 – 660/kg	-	River Thames, UK
(Wang et al., 2017)	Sediment	River	178 – 554/kg	-	Beijiang River, China
(Bordós et al., 2019)	Sediment and Water	Rivers, Ponds and reservoirs	Water 3.52-32.05/m^{-3} Sediment 0.46-1.62 p/kg	0.00352-0.0325	Various lakes, ponds and rivers, Hungary
(Estahbanati and Fahrenfeld, 2016)	Water	River	71.7/ m^{-3}	0.071	Raritan river, USA

(Forrest et al., 2019)	Water	River		0.12 (mean) 0.41 (max)	Ottawa River, Canada

2.6 Effects of microplastics on ecology

Plastic debris has a range of effects on ecology, sediment properties and people (Duis and Coors, 2016). It is a growing opinion of the public that plastic is used too frequently and improperly with recent media campaigns showing an increase in sustainability issues and aiming to reduce plastic usage across society (Wamsler and Brink, 2018). This section will review literature that has explored the effects of plastic on ecological end-points, as well as other impacts. It has been an emerging area of research into whether microplastics pose an imminent issue to ecologically significant species.

2.6.1 Effects of plastic on Aquatic Ecology

Table 2.4. A summary of the literature on studies regarding effects of micro- or nanoplastics on various freshwater species, adapted from Chae and An (2017).

Author	Freshwater Species	Phylum	Type of experiment	Effects
(Malinich et al., 2018)	*Artemia naupli,*	Arthropoda	Microplastic exposure to larval fish species examining consumption and growth of individuals.	This study found no supporting evidence that microplastics had effects on this larval species.
(Batel et al., 2016)	*Artemia nauplii,* *Danio rerio*	Arthropoda Chordata	Microplastics were exposed to *Artemia sp.* and fed to zebrafish to assess the transfer up the trophic levels.	The study shows a clear transfer between trophic levels of microplastics being transferred up food chains. After 3 hours' exposure 95% of *Artemia* ingested microplastics with Zebrafish readily ingesting microplastics through the food chain, Zebrafish did not exhibit any adverse effects.
(Ziajahromi et al., 2018)	*Chironomus tepperi*	Arthropoda	Microplastic toxicity tests using microplastic spiked sediment measuring growth and emergence.	These tests suggested using environmentally realistic microplastic concentrations the test species growth and emergence were negatively affected. Also determined smaller sized microplastic particles had a greater impact upon growth, emergence and mortality. This study found microplastics sized between

				10-27µm exhibited the most significant effects.
(Nasser and Lynch, 2016)	*Daphnia magna*	Arthropoda	Toxicity assessment of *Daphnia magna* using nanoplastics.	The exposure resulted in the significant release of proteins. The NP's inside the individual's guts affected its ability to feed on algae after 6 hours of exposure but would need a more direct focus to further confirm.
(Rehse et al., 2016)	*Daphnia magna*	Arthropoda	Analysed the acute effects of microplastic PE - particles on *Daphnia magna*	At all concentrations microplastics were present in the guts of all individuals exposed. Ingestion of mp particles caused immobilisation of daphnids at high concentrations. After 72 hours 35% of individuals were immobilised.
(Booth et al., 2016)	*Daphnia magna, Corophium volutator,* *Vibrio fischeri*	Arthropoda Arthropoda Proteobacteria	Exposure to plastic nano partices at 3 environmentally realistic concentrations as well as at higher concentrations.	Negative effects on all species were observed. However, the ecotoxicity cannot be calculated reliably by using a single type of plastic so it is recommended to look and a multiple plastic type approach.
(Casado et al., 2013)	*Daphnia magna, Thamnocephalus platyurus, Pseudokirchneriella subcapitata,* *Vibrio fischeri*	Arthropoda Arthropoda Chlorophyta Proteobacteria	Ecotoxicity tests on species across the whole trophic level to assess impacts of silica and polyethyleneimine polystyrene nanoparticles.	The study found significant toxicity at a range of concentrations across trophic levels with larger size particles having a higher effect. The study found that size had a significant ($P<0.05$) impact on Eco toxicity effects.
(Weber et al., 2018)	*Gammarus pulex*	Arthropoda	Exposure of PET microplastics at different concentrations over 24hr and 48hr.	PET microplastics were found to have no significant effect on the individual's survival, development, metabolism or feeding activity. A 48-day exposure did not significantly affect

				feeding activity, energy reserves, moult or mortality.
(Au et al., 2015)	*Hyalella azteca*	Arthropoda	Polyethylene and polypropylene microplastics were exposed to freshwater amphipod.	Chronic exposures significantly decreased growth and reproducibility. Mortality, gut clearance and growth was negatively affected in a dose-dependent manner and produced significant results.
(Bhattacharya et al., 2010)	*Chlorella* and *Scenedesmus*	Chlorophyta	Exposure of nanosized plastic spheres to assess the effects of adsorption upon two algal species	The adsorption of plastic spheres was found to negatively affect algal photosynthetic activities and promote that ROS production.
(Sjollema et al., 2016)	*Dunaliella tertiolecta, Thalassiosira pseudonana, Chlorella vulgaris*	Chlorophyta Heterokonta Chlorophyta	Exposure of microplastic particles upon microalgae to determine effects upon photosynthesis and growth.	There were negative effects observed upon microalgae but only by the smallest particle size at high concentrations. The effects were clearly observed on growth of algae, with the cell density when exposed to polystyrene beads reducing by 45%.
(Besseling et al., 2014)	*Scenedesmus obliquus, Daphnia magna*	Chlorophyta Arthropoda	Toxicity test with polystyrene nanoplastics on the effects of growth and photosynthesis of algae as well as the growth, mortality, neonate production, and malformations of zooplankter	Nanoplastics reduced growth and the chlorophyll concentrations of the algae. Body size for zooplankter was lowered due to nano-plastics as well as causing severe altercations in reproduction.
(Cedervall et al., 2012)	*Scenedesmus sp., Daphnia magna, Carassius carassius* Chordata	Chlorophyta Arthropoda Chordata	Toxicity test on 3 levels to analyse the transfer of nanoplastics through a food chain and the effects.	Polystyrene particles transferred through the food chain will affect lipid metabolism and behaviour. Exposed fish moved slower and to a lesser extent than control variants. They allowed daphnia swim in and out of the mouths of the fish without being consumed.

				Significant weight loss was noted.
(Mattsson et al., 2015)	*Scenedesmus sp., Daphnia magna, Carassius carassius*	Chlorophyta Arthropoda Chordata	Toxicity test on 3 levels to analyse the transfer of nanoplastics through a food chain and the effects.	Polystyrene nanoplastics were found to have severe effects on behaviour and metabolism in fish. Significant differences in the speed of movement of fish exposed to nanoplastics particles.
(Karami et al., 2016)	*Clarias gariepinus*	Chordata	Exposure study to assess the impacts of virgin or phenanthrene low density-polyethylene on juvenile African catfish.	This study highlighted that exposure of microplastics to *C.gariepinus* can cause major altercations in biomarker responses and affected the species physiology.
(Khan et al., 2015)	*Danio rerio*	Chordata	This study assesses whether Ag uptake and localisation is affected by polyethylene plastic beads.	Adds to the growing body of literature that demonstrates plastics ability to affect an organism's relationship with the freshwater environment. Ag uptake was significantly increased when microplastics were incubated with Ag suggesting that microplastics can be vectors for chemical transfer in fish species.
(Lu et al., 2016)	*Danio rerio*	Chordata	This study investigated the uptake into tissues of polystyrene microplastics and toxic effects on the liver.	Microplastics accumulated in the gills, liver and gut. Microplastics were found to cause inflammation and lipid production in the liver.
(Lei et al., 2018)	*Danio rerio,* *Caenorhabditis elegans*	Chordata Nematoda	Toxicity was tested using 5 different types of microplastic at different sizes.	These conditions suggest that microplastics cause intestinal damage. The size is what determines this damage rather than composition.

(Wardrop et al., 2016)	*Melanotaenia fluviatilis*	Chordata	Exposing rainbow fish to microbeads, clean and spiked with PDBE.	Microbeads from cosmetic products with sorbed organic pollutants were exposed to fish species with microbeads bio-accumulating in the individuals they were exposed to. This study provided evidence that microbeads can transfer pollutants to the tissue of fish species.
(Manabe et al., 2011)	*Oryzias latipes*	Chordata	Individuals were exposed to latex nanoparticles and the uptake, excretion, and the effect of nanoparticle accumulation on survival rate were assessed.	Fluorescent nanoparticles were readily ingested in fish species and excreted much more slowly than larger particles. The results suggest these particles were not toxic however they did, through a combination of other effects, create a synergistic toxic effect especially on fish larvae species.
(Kashiwada, 2006)	*Oryzias latipes*	Chordata	Exposure of nano-particles to individuals.	The results show that nano-particles can penetrate the blood-brain barrier. Further research in this area was needed.
(Greven et al., 2016)	*Pimephales promelas*	Chordata	Exposure of polystyrene and polycarbonate nano particles on fathead minnow.	PCNPs and PSNPs can act as stressors to the innate immune response of fish by altering organismal defence mechanisms. Exposure caused significant degranulation increases and extracellular trap release also increased due to exposure to PCNPs and PSNPs.
(Murphy and Quinn, 2018)	*Hydra attenuata*	Cnidaria	Exposure analysis, measuring feeding rate, microplastic ingestion and reproduction.	This study shows that individuals are capable of ingesting plastics with some individuals completely stuffing their gastric cavities. The test found that exposure to microplastic had significantly impacted the feeding rates of the species exposed. Exposure also

				caused significant changes to morphology however these were non-lethal. There was found to be no effect on the reproduction of the individuals.

There are numerous processes in which plastic can affect different species, including large pieces of plastic being ingested by species such as sea birds and marine mammals, which can damage the intestinal tract and cause internal injuries (Duis and Coors, 2016; Ryan, 1987). Many species of fauna have also been documented to exhibit physical effects as a result of ingesting large plastic debris plastic in the literature (Gall and Thompson, 2015). Physical effects include entanglement, being trapped in plastic, which can cause loss of life and movement, to potentially important and at-risk species (Gall and Thompson, 2015; Li et al., 2018). Other physical impacts occur when plastic is ingested, which causes problems associated with breathing or feeding (Li et al., 2018). This has been recorded less commonly in freshwater environments, however there has been documented work showing plastic debris being consumed by freshwater birds in multiple species (Holland et al., 2016). Plastics of all sizes are reported to interact with aquatic organisms in all environments at all trophic levels, with effects varying from species to species (Capolupo et al., 2020). The type of plastic may also have differing effects on species, an example is that polyethylene terephthalate (PET) plastic was found to have no impact upon the freshwater Shrimp *Gammarus pulex* species (Weber et al., 2018). However, when PET fibres were exposed to freshwater crustaceans (*Daphnia magna)* they were ingested and this resulted in increased mortality of the *D. magna* (Jemec et al., 2016), (see Table 2.3 for further detail).

Microplastics' impact on ecology is less obviously observed than the impacts of macroplastics, due to their size and less research conducted in this area. Microplastics are commonly ingested by many species and passed up the food chain to other larger species, many consumed by humans (Kershaw, 2015; Miranda and de Carvalho-Souza, 2016). There are a number of ways that species can be affected by microplastics including through ingestion, translocation to other tissues, leaching of toxic additives such as CTR and PVC or by plastics acting as a vector for bacteria and pathogens (Duis and Coors, 2016; Kershaw, 2015; Capolupo et al., 2020). This can have a number of effects such as mortality, change in movement, change in food consumption and growth, changes in behaviour and can affect the species' breathing (Felten et al., 2008a). Numerous studies have found microplastics to negatively affect certain aquatic species. A study by Lei et al. (2018) found that microplastics of varying types will cause intestinal damage due to ingestion. Au et al. (2015) discovered that polyethylene and polypropylene negatively affected freshwater amphipods reproducibility and growth. Despite this, many studies have found no evidence to suggest negative effects of microplastics, however they discuss the need for further research (Malinich et al., 2018; Weber et al., 2018). A summary of the effects on freshwater species found in the literature is presented in Table 2.4.

2.6.2 Ingestion

Ingestion is the process in which microplastics are consumed by organisms and transported into the body of the organism (Cole and Galloway, 2015). Microplastic and natural fibres are easily ingested by organisms because of the fibres' lift-to-drag ratio (Dris et al., 2018; Kershaw, 2015; Remy et al., 2015). They are also often mistaken for food by smaller organisms and there have been studies suggesting that some organisms cannot differentiate between microplastics and algae (Lambert and Wagner, 2018). Ingestion has been observed to cause many different effects upon different species (Table 4.). (Au et al., 2015) discovered a very common effect of microplastic ingestion on *Hyalella*

azteca, where increased blockage of the gut and the disruption of digestion, led to a reduction in growth or possible mortality, as also observed in other organisms (Jemec et al., 2016). This can also affect the breathing by blocking airways of species which can cause mortality for individuals (Kershaw, 2015). The blockage by microplastics has physical impacts on organisms, most commonly the disturbance of the intestinal tract and the digestive system, causing a reduction in feeding and growth (Farrell and Nelson, 2013). Ingestion can also mean these microplastics are transferred through food chains and webs into other species and has been examined in the literature (Cedervall et al., 2012). This can allow larger species to indirectly ingest microplastics through normal feeding behaviour. In some of the literature where toxicity tests have investigated effects of microplastics, it has not been clarified whether or not microplastics were actually ingested (Table 2.3). There are numerous studies that investigate ingestion in aquatic species. Lu et al. (2016) discovered individual of the *Danio rerio* species ingested plastic into the gills, liver and gut which caused inflammation and lipid production in the liver.

2.6.3 Translocation

Translocation of microplastics is a process that happens after particles have been ingested and are transported from the gut via the intestinal lymphatic cells to other areas and tissues, where it can potentially cause harm (Kershaw, 2015; de Souza Machado et al., 2018a). Translocation generally occurs with smaller particles, including nanoplastics, and is the most common entry into other parts of the body, affecting aquatic species and mammals (de Souza Machado et al., 2018a). The size of particles that can translocate is generally specific to the species studied, however, it tends to be around 150µm and below (Duis and Coors, 2016). Translocation of plastic particles is important as it may cause internal damage to cells, inflammation and bioaccumulation (Schür et al., 2019). However, literature on definitive effects from translocation is lacking, importantly at environmentally realistic concentrations. Translocation of microplastics does support the hypothesis that plastic can be transferred up through food chains (Cedervall et al., 2012). Also, due to plastic moving from the gut to elsewhere this could mean humans are more likely to consume plastic through consuming tissues of fish that have plastic inside them (Cox et al., 2019).

2.6.4 Facilitated transport of chemical pollutants

Leaching of toxic additives occurs when plastics released to the aquatic environment begin to break down and release chemicals, either on the surface of the plastic or within the physical structure of the plastic (Koelmans et al., 2014). These additives can be leached into water courses or after being ingested by species, the latter having a greater impact (Koelmans et al., 2014). Leachates have been found leaching from plastic that can cause chemical toxicity to aquatic species which have included additives, monomers and residues from the production process (Lambert and Wagner, 2018). Car tyre rubber has been found to leach harmful chemicals which include benzothiazoles, which are the most common, as well as phthalates and phenols (Capolupo et al., 2020). High numbers of different chemicals can be released by plastic debris, however this appears to be at low concentrations which are often too low to be picked up by detection methods (Capolupo et al., 2020).

2.6.5 Vectors for bacteria

Bacteria and viruses can attach to plastic particles in sewage and treatment plants and when released can potentially affect species and bring in new pathogens to freshwater ecosystems (de Souza Machado et al., 2018a). Kirstein et al. (2016) discovered that microplastics act as a vector for diseases emerging in aquatic environments, affecting multiple species. A study by Zettler et al. (2013) found that microbial communities can form on plastics in the marine environment, which therefore theoretically should be also possible in freshwater environments. Microbial communities include numerous bacteria as well as opportunistic pathogens from the *Vibrio* genus (Zettler et al., 2013). Multiple bacterial assemblages have been identified on microplastics in urban rivers and have the potential to transfer through food chains with plastics (R et al., 2016). The effect of these microbial

and viral communities on species are relatively unknown, however it can be assumed if these bacterial communities could enter food chains and affect humans this could be damaging to health.

2.6.6 Nanoplastics

Nanoplastics are considerably smaller than microplastics and therefore their effect on species is much more complex as they can reach new areas and cause new effects (Chae and An, 2017). The effects from nanoplastics are even less known than microplastics as toxicity tests have been less frequent and are more difficult to carry out (Eerkes-Medrano and Thompson, 2018). There have been studies conducted that have drawn conclusions, however it has been reported that nanoplastics will stay in the body of organisms and marine life for significantly longer periods than microplastics (Chae and An, 2017; Ward and Kach, 2009). Other studies have found that nanoplastics have impacts on a number of species affecting; filtering activities, embryo toxicity, feeding, motility and multiple moting (Bergami et al., 2017; Della Torre et al., 2014; Wegner et al., 2012). Chae and An (2017) concluded that nanoplastics do not only affect single species, but have an impact on populations and ecosystems.

2.6.7 Knowledge gaps on effects literature

The literature on effects of microplastics is less established than the areas on sources, fate and occurrence of microplastics and there are more areas to address. Key areas to address are microfibres' effects on important ecological species for toxicity and sub-lethal effects. The majority of studies (Table 2.4) have focussed on marine species, and greater research needs to be undertaken on freshwater species (Haegerbaeumer et al., 2019). Literature in regards to ecotoxicity of microplastics has foccussed on microplastic particles and spheres (Table 2.4), whereas microfibres associated with clothing have been neglected in ecotoxicity studies (Henry et al., 2019). Of the few studies that have investigated microfibres, natural fibres have been neglected from these studies despite natural fibres comprising a large proportion of microfibre pollution (Stanton et al., 2019). There is also a key gap in the literature in that the majority of ecotoxicity tests with microplastics have used high concentrations which is important, but have not assessed the level of toxicity caused by environmentally realistic concentrations (Haegerbaeumer et al., 2019).

2.7 Species for ecological testing

Invertebrates have been used for many of the studies that assess effects of microplastics on non-target organisms, as they are the most likely to interact with plastic in the freshwater environment (Table 3). Invertebrates make up a very large and diverse group of species in the aquatic environment (Baun et al., 2008). Crustaceans are the most commonly used group for toxicity testing largely due to being ubiquitous, small and easy to keep in laboratory conditions, also can grow very quickly and readily reproduce in laboratory conditions (e.g. *Ascellus aquaticus, Gammarus pulex* and *Daphnia magna*) (Baun et al., 2008; Bloor, 2011). Crustaceans work as leaf shredders and aid the breakdown process of organic matter. As well as this, they are valuable food sources for fish and other larger predators (Baun et al., 2008). As the majority of these species feed on sediment and the aquatic floor, they are often found to have ingested plastic that has settled on the sediment floor (Baun et al., 2008). Non-target organisms are therefore at risk of ingesting plastic at a rate which increases in line with plastic pollution, and thus allow this plastic to enter the food chain and move up trophic levels to higher predators, as illustrated in Figure 2.3 (Baun et al., 2008; Kershaw, 2015).

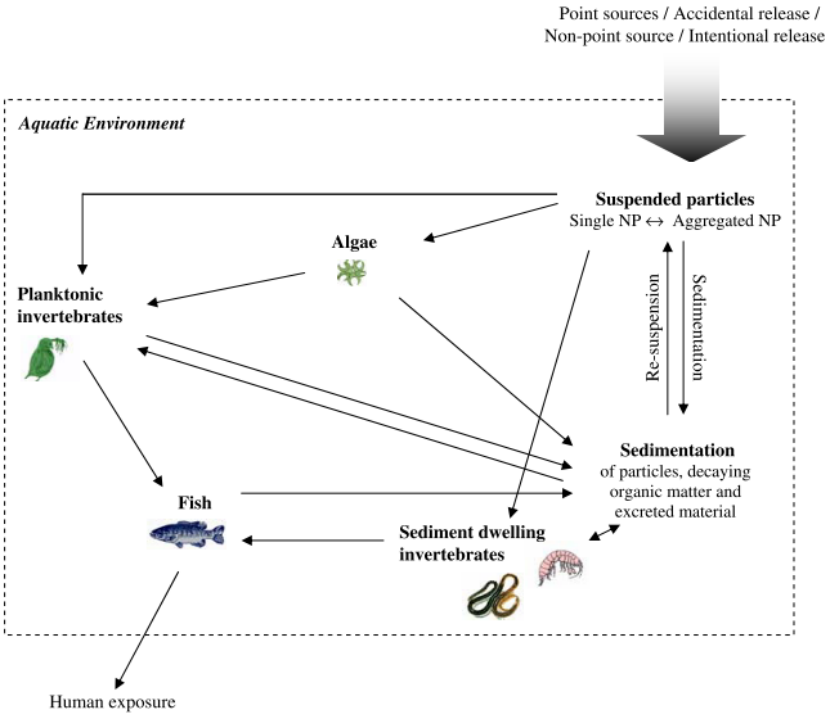

Figure 2.3. A model highlighting the significance of invertebrate organisms to the aquatic food web as well as how they can be exposed directly to plastic particles in this case nanoparticles (Baun et al., 2008).

2.7.1 Gammarus pulex

The freshwater amphipod *Gammarus pulex* are a very common leaf-shredding detritivore found in the majority of freshwater environments (Chaumot et al., 2015). *Gammarus pulex* are a keystone species which means they have a large effect on ecosystems relative to their size and occurrence (Bond, 1994). This means that *Gammarus pulex* play a critical part in sustaining the ecological community, by regulating the numbers and types of different species of the freshwater community (Bond, 1994; Chaumot et al., 2015). If *Gammarus pulex* are being affected by microplastics this could therefore have a causal effect on other species and the freshwater ecological community as a whole. The removal of the *Gammarus pulex* could have further effects to predators above them in the food chain and have a potential effect on the ecosystem they are involved in (Feroz Khan et al., 2012). *Gammarus pulex* are often a sediment dwelling species as well as being found in the water column, which means individuals will also be exposed to concentrations in sediments which tend to be higher than in the water column (Castañeda et al., 2014; De Lange et al., 2006).

2.7.2 Effect measures and endpoints in *Gammarus pulex*

Gammarus pulex are a commonly used species in ecotoxicity tests for the reasons stated above and are an ideal test species to be used for these reasons (Bloor, 2011; De Castro-Català et al., 2017; De Lange et al., 2006; Felten et al., 2008a; Felten et al., 2008b; Maltby, L. and Crane, 1994; Weber et al., 2018). There are a number of measures and end points examined in the literature that will form this study and the experimental section.

Mortality, or whether the individual dies, is investigated in almost all toxicity tests and is the endpoint that will indicate how toxic the pollutant is (Maltby, L. and Crane, 1994). Mortality is an easy-to-use endpoint as it is simple to quantify and clearly indicates that the contaminant is having a marked effect on the test species, if all other measures are controlled (Felten et al., 2008a). However, using only mortality is not enough in this case, as more environmentally realistic concentrations of

microplastics have not been found to commonly cause mortality in *Gammarus pulex* individuals (Weber et al., 2018).

Locomotion of *Gammarus pulex* has been examined in previous ecotoxicity tests, seeing decreases in the movement of individuals under toxic conditions (De Lange et al., 2006). Locomotion is the act of the individual moving by swimming and stretching the body which will generate a signal of 1-2V and frequencies between (0.8 to 1.2Hz) (Felten et al., 2008b). This can also be observed and measured by counting the number of times an individual will swim past a certain point, as a more functional and easy to quantify measurement (Felten et al., 2008b). When *Gammarus pulex* individuals' locomotive activity decreases this is seen as a negative effect and a behavioural reaction to stress, which is exhibited when exposed to harmful pharmaceuticals (De Lange et al., 2006).

Growth and feeding rate are also valid measures used in ecotoxicity tests on *Gammarus pulex,* with contaminants often having an effect on whether the individual is consuming food and gaining weight as a result of growth (Felten et al., 2008a). It has been discovered that feeding rate and growth are similarly linked with locomotive activity and decrease in *Gammarus pulex* individuals' when exposed to a toxic substance (De Lange et al., 2006). This is important because if growth, feeding rate and locomotive activity all decrease then this will be strong evidence that individuals are being affected negatively due to the measured toxic contaminant.

Other aquatic species can also be justifiably tested on in toxicity tests, however there is a lack of studies focussing on *Gammarus pulex* which are a keystone and a good focus for a study in a shorter timeframe. Many other species are commonly used such as *Daphnia magna, Hydra attenuata, Oryzias latipes* as well as many more which are mentioned in Table 2.4. (Chae and An, 2017).

2.8 Summary of literature and knowledge gaps

Literature on the topic of sources and fate of microplastics is extensive and there is a relatively clear picture of where microplastics come from and where they end up, as highlighted in Figure 2.2. (Thompson and Napper, 2018). The state of microplastic pollution is complex and there are many sources and pathways that release plastic and additives from primarily urban areas to the marine environment (Kershaw, 2015). There is a plethora of research to tell us plastic production, use and waste is rising and the issue with what to do with the waste is becoming increasingly concerning (Evangeliou et al., 2020). It is also widely recognised that plastic of varying types and composition is being discovered widely across the globe in remote and varied locations (Eriksen et al., 2013). Microplastics have been discovered in high enough concentrations to present potential impact on ecological species, which presents a growing source of literature on the subject (Chae and An, 2017).

From thoroughly examining the literature surrounding the topic of microplastics it is clear that a significant knowledge gap concerns the effects of microplastics on freshwater species and what this means at environmentally realistic concentrations. In the literature previously compiled, *Gammarus pulex* has undergone less testing with fewer of the most common plastic types exposed to the species (Table 4.). Conversely, with other contaminants such as pharmaceuticals, *Gammarus pulex* are commonly used to determine the ecological status of a watercourse (Borja et al., 2007). Importantly, a missing link in the effects literature is Polyester and Acrylic, commonly found and dense plastics found in sediments and released in wastewater are not found much in Table 4. (Hidalgo-Ruz et al., 2012). Therefore, ecotoxicity testing using these common microfibres is recommended to be replicated across different important species.

Little is known about how future microplastic levels will affect the ecosystem and the keystone species that are relied upon. Therefore, this study aims to address some of these gaps by running ecotoxicity tests upon *Gammarus pulex* at high concentrations as well as environmentally relevant concentrations to determine the effects and if they align with observations previously reported in the literature.

3 Methodology

3.1 Methodology introduction

This methodology outlines a quantitative laboratory approach using microcosms and *Gammarus pulex* individuals that were cultured in a laboratory environment. This was to ensure no prior contamination, allowing any potential observed effects to be a result of microplastic exposure. The individuals used in this study were acclimatised to laboratory conditions, therefore when exposed to microplastics the responses observed will be measureable and reliable.

3.2 Test species

This study used *Gammarus pulex* as a test species due to them being an important environmental indicator and a keystone species, which has been used in many different ecotoxicity studies previously (Feroz Khan et al., 2012). Being a keystone species, means if an effect is observed in an exposure test there is the potential for subsequent knock-on effects on other species in the ecosystem, ultimately manifesting in a greater ecosystem impact (Feroz Khan et al., 2012). The species were purchased from Blades Biological Ltd, which is a specialist laboratory that sources and raises live specimens in laboratory conditions, meaning all *Gammarus pulex* individuals are handled carefully at all times to keep contamination to an absolute minimum.

3.3 Laboratory Culture

The individuals were cultured in the laboratory for at least one month to give them the opportunity to acclimatise to the conditions. The conditions were a constant temperature of 17ºC with a light/dark cycle of 12:12 hours and humidity at 30%, which is considered to be the standardised conditions for *Gammarus pulex* (Agatz and Brown, 2014). Italian Alder *(Alnus cortada)* leaves taken from a site close to the laboratory, which were cleaned with deionised water to reduce any potential airborne contamination, were used to feed the individuals. These leaves were cultured in organically rich water for at least one month to enable bacterial and algal growth upon the leaf surface to breakdown the leaf and allow *Gammarus pulex* to have food available. The leaves were cut into discs using scissors and weighed when dry before and after each experiment and around 100mg of leaf discs were made available per individual. The individuals were exposed in Artificial Pond Water (APW) shown in Table 3.1 which allows for a close comparison to the water and mineral content of an unpolluted river in the environment where the species naturally resides (Naylor et al., 1989).

Table 3.1 Artificial Pond Water (APW). 5ml of each of these stock solutions were made up to 1 litre of APW using distilled water (Naylor et al., 1989).

Stock Solution	g L^{-t}
$CaCI_2$ H_2O	58.80
$MgSO_4$ $7H_2O$	24.65
$NaHCO_3$	12.95
KCI	1.15

3.4 Experimental design

Ecotoxicity experiments were undertaken to assess the potentially damaging effects of a range of microplastics and natural fibres upon the test species *Gammarus pulex*.

3.4.1 Experimental set up

The experiment was set up in a Weiss Gallenkamp Fitotron SGC097 Plant Growth Chamber kept at constant conditions the same as to those outlined in section 3.3 for the laboratory culture. 12 *Gammarus pulex* individuals were weighed alive before the exposure; the method involved drying the individual with blotting paper and then using a Sartoris Cubis Premium Balance, the individuals averaged 30mg ± 5mg and were chosen as close to 30mg as possible. They were then added to an individual 300ml beaker containing 200ml of APW, added to the chamber and connected to an aerator to keep the water oxygenated as shown in Figure 3.1. 6. Individuals were exposed to microfibres; the other 6 individuals acted as controls, without a contaminant, to ensure the experiment conditions were not affecting the endpoints being measured. The aerators and food source were removed from the beakers ten minutes prior to the time of recording any end points. The Ecotoxicity experiments took place sequentially due to factors such as space available in the growth chamber meaning all experiments could not occur at once. Additionally, due to outside influences there was a substantial delay to experiments so the dates they took place varied. The sequence of the experiments is shown in Table 3.2.

Figure 3.1 A picture taken of the experimental set up in the growth chamber of the beakers and aerators.

Table 3.2 A Gantt chart highlighting the sequence of the experiments including the start date of each experiment.

	Week 1 (04/02/20)	Week 2 (11/02/20)	Week 3 (18/02/20)	Week 4 (25/02/20)	Week 5 (12/10/20)	Week 6 (19/10/20)	Week 7 (26/10/20)
Polyester high							
Polyester low							
Cotton high							
Cotton low							
Acrylic high							
Acrylic low							
Nylon high							
Nylon low							
Mix high							
Mix low							
Mask							

3.4.2 Microfibre preparation and concetrations

Experiments were undertaken using a range of fibre types of different colours to be able to differentiate between polymer types. These were blue polyester, white cotton, red acrylic, purple nylon, a combination of these plastics and microfibres generated from a single-use PPE mask. These materials are commonly found in clothing, fabrics and lost plastic debris, commonly found in wastewater effluents entering environments (Murphy et al., 2016). The microfibres were made by breaking down large pieces of polymer thread into microscopic fibres. Fibres were cut and broken up in a pestle and mortar until separated into individual fibre strands 1mm or less. To ensure these particles were uniform in size, they were passed through a 1mm sieve shaker to guarantee all microplastic particles were less than 1mm in size. This means there is the potential for them to be easily ingested (Cole and Galloway, 2015). The particles were weighed dry to determine the concentration for each experiment. The particles were then added to APW in a Schott bottle creating a stock solution and shaken for 1 minute to avoid the aggregation of fibres or fragments. These solutions were then added, at a range of concentrations, to beakers containing the test species and food source.

An additional experiment was run alongside the ecotoxicity to generate an understanding of the threat single-use PPE is having in terms of a microplastic pollutant. A single-use mask made from cotton, polyester and polyethylene was exposed to a beaker of aerated APW and left for a 7-day period. Once complete, the mask was removed, the water filtered and the microplastics released counted to quantify the fibres released over a 7-day exposure to water. These fibres, along with more generated from a

physical break-up of the mask, were subsequently used to form an ecotoxicity test with *Gammarus pulex* individuals exposed to the contaminant.

Individuals were exposed to concentrations of 440mg/L (High) and 57.5mg/L (low). These are derived from the division of stock solutions prepared with the microplastics and comparable with other similar studies (Murphy and Quinn, 2018). A mix of the four materials of microfibre types at equal parts were also prepared to be used at the low concentration which is environmentally realistic in sediment samples (Chae and An, 2017). The experiment using mask fibres used $1/6^{th}$ of a brand new PPE mask made of polyester and cotton with a concentration of 1200mg/L. This concentration was purposefully high to account for greater clumping of fibres and difficulty breaking down the mask into individual fibres.

3.5 Experimental endpoints

3.5.1 Mortality

The individuals were checked at least every 24 hours throughout the 72-hour test, which is in line with the standardised methodology of ecotoxicity tests of this nature (Chae and An, 2017). More frequent mortality tests were taken throughout the daytime when the laboratory could be accessed to provide a more accurate time of mortality. The individuals were observed over at least three occasions over the space of an hour to check if they were still undertaking ventilator and locomotive activity and clearly alive. If they were no longer alive they would be removed and examined. LC50 and LC99 values were obtained using the ecotox package for R adapted for *Gammarus pulex* Eco toxicity tests (Hlina Benjamin L et al., 2019).

3.5.2 Feeding rate and Growth

The individuals were weighed dry before and after each 72-hour test cycle on a high resolution balance as well as the food source which was also weighed dry before and after the experiment. This produced a growth value to show how much additional weight has been gained in size by the individual. These values were then also used to determine the feeding rate, as shown in the following equation adapted from (Agatz and Brown, 2014). The 72-hour test is short for a feeding rate study, however 3-35 day tests have been published in the literature and due to access issues, extensive chronic exposures could not be carried out (Consolandi et al., 2019).

$FR = (\Delta F/G) \times d$

ΔF = Difference in weight of food/ weight of leaf consumed (mg)

G= Average weight of individual (mg)

d= Time (days)

However, certain other factors could not be controlled, for example; the change in weight of an individual due to moulting could affect the feeding rate by substantially altering the average weight of one individual compared to one that does not moult (Kunz et al., 2010). To counter this, if an individual moulted and the weight change was substantial enough to affect the feeding rate it was counted as an anomaly and not counted in the calculation (shown in Figure 13).

3.5.3 Locomotive activity

Locomotive activity, or the motion in which animals move from one place to another, has been previously used as a measure of toxic stress (Felten et al., 2008a; Felten et al., 2008b; Nørum et al., 2010). This was measured in a similar way to these studies in observing how often individuals crossed a line across the base of the beaker of water; a modified method as seen in previous studies (Felten et

al., 2008a). This was measured over the space of 3 minutes for each individual, to gain an average reading, at the same time in the morning and afternoon of each day of the study. This was measured with the microplastics kept in the water at every stage so that the viscosity of the water would not be changed in different measurements as this could affect the individual's ability to move freely. Locomotion was measured across 72-hours providing average readings across 4 consistent times each day the individuals were exposed. Each treatment was tested and measured in the same way and the only change factor was the material and its concentration.

3.5.4 Ingestion

Ingestion of particles is an important measure to analyse whether plastic is being consumed and if there are any disparities between different plastic types being consumed by the individuals. After the 72-hour experiment is complete the individuals exposed, including those that perished earlier in the experiment, were cleaned with ethanol and then dissected using needle tweezers with the gut being removed and inspected under a microscope. The plastic particles or natural fibres were then counted under a microscope three times and then an average attained to increase reliability and can then be compared across all the other treatments.

3.5.5 Data Analysis

The results of the experiments were recorded in Microsoft Excel and then analysed using Rstudio for the figures and values calculated. LC50s were calculated and tabulated. The data was tested for normality using Shapiro-Wilk tests using R. All statistical comparisons made on normal data were executed using ANOVA tests on R; data that was non-normally distributed would be analysed using Kruskal-Wallis significance tests to allow P-values to be established. P–values in this data with a probability number less than 0.05 were deemed to be significant. The variance tools gave further analysis of the relationships and variance of the data to ascertain the significance of the data. ANOVA tests were used to compare the variance of locomotion in individuals' exposed and the controls at each time point, to allow for complete analysis of the data and its significance.

4 Results

Exposure to microfibers resulted in significant impacts on the behaviour of individual *Gammarus pulex*. The majority of these differences are statistically significant when tested using ANOVA variance testing. Measured endpoints including locomotive activity, feeding rate and growth were significantly reduced when individuals were exposed to microfibers at concentrations of 0.44g/L and 0.0575g/L. In addition, microfibres were ingested by every individual exposed across a range of fibre types, colours and concentrations. Finally, mortality of individuals was discovered in all treatments, which allowed for the determination of estimated LC50 values, which is the concentration that would cause mortality to 50% of individuals (Table 4.1).

4.1 Mortality and LC50 Estimates

The following section will discuss mortality as an endpoint with Table 4.1 highlighting the survival rate for each test and table 4.2 with estimated LC50 values.

Table 4.1 Survival rate of the exposed individuals in each treatment of the ecotoxiciy tests.

Treatment	Survival Rate (%)
0.44gl Polyester	50
0.0575gl Polyester	83.3
0.44gl Cotton	66.6
0.0575gl Cotton	83.3
0.44gl Acrylic	66.6
0.0575gl Acrylic	83.3
0.44gl Nylon	66.6
0.115gl Nylon	83.3
0.44gl Mix	50
0.0575gl Mix	66.6
Mask	83.3

The two lowest survival rates and highest mortality comes in the high concentration 0.44g/L polyester test and the high concentration 0.44g/L mix test, which is a combination of all fibre types used in the study. The general trend shows mortality began to behave in a dose-dependent manner as it was observed in all tests that greater mortality occurred in the high concentration 0.44g/L treatments. However, a wider range of concentrations would be needed to confirm does dependency of the results.

Table 4.2 Estimated LC50 and LC99 with a 95% confidence interval values for each contaminant.

Type of fibre	LC50 (g/L)	LC99 (g/L)
Polyester	0.62 ± 0.35	4.87 ± 2.76
Acrylic	1.19 ± 0.67	19.81 ± 11.21
Nylon	1.20 ± 0.68	88.82 ± 50.25
Mix	0.57 ± 0.32	44.71 ± 25.30
Cotton	1.41 ± 0.81	500.42 ± 283.13

Table 4.2. specifies the estimated LC50 and LC99 values, which is a concentration that would cause mortality to 50% and 99% of replicates, respectively. This was carried out for each polymer type used in the ecotoxicity tests, however as only two concentrations' were used per fibre type. The results

gathered in the table are strictly estimates and would require replicating in further studies which specifically uses a larger concentration range and incorporates concentrations that result in complete mortality of all individuals. Due to this, the values show large disparities between the polymer types. Despite the variation of result, the LC50 values fall between the values of 0.5 and 1.5 g/L which suggests a concentration of microfibres between these levels would likely cause mortality to 50% of replicates.

4.2 Locomotion

The locomotion data highlights significant changes to the individual's behaviour over the 72-hour time period, with differences observed from the onset of the exposure and throughout the duration of the experiment. The overall trend displays individuals exposed to a contaminant had lower locomotive activity in comparison to the control individuals who experienced the same conditions except the contaminant (Figures 4.1-4.11). An individual that does not survive is shown when the line stops before the 72-hour point on the Figure. Asterisks included in the figures denote significant difference in comparison to the controls.

Table 4.3 ANOVA variance between the exposed individuals and controls at the beginning of the Eco toxicity tests and at the end of the 72-hour exposure. Significant variance is determined by p values < 0.05.

Fibre type	Initial ANOVA P-Value	End ANOVA P-Value
Polyester High	0.7	0.0015
Polyester Low	0.92	0.000081
Acrylic High	0.39	0.0000000106
Acrylic Low	0.4	0.00035
Nylon high	0.63	0.0000386
Nylon Low	0.73	0.00000535
Cotton High	0.29	0.000039
Cotton Low	0.15	0.00024
Mix High	0.11	0.00000918
Mix Low	0.62	0.00013
Mask	0.39	0.0014

ANOVA tests were carried out to validate any trends in the data and show significant changes as a result of exposure to microfibres. The ANOVA test p-values included in the locomotion results is a comparison of treatment to control at each time point and allowed for comparison over time to see if 0-hour locomotion was significantly different to 72-hour. This was performed after Shapiro-Wilk tests confirmed the normality of the data. As shown in Table 3, subsequent ANOVA tests were conducted on the data for a single time point at the start and end of the tests to ensure that the variance at the start of the test was not significantly different between all the individuals. This strengthens the data, as the variance at the beginning of each test was not significant. However, at the end of each test the variance of locomotion between individuals exposed to microfibres and controls was significant in every test.

4.2.1 Acrylic

The following figures examine changes in locomotion, measured by number of line crossings made by an individual *Gammarus pulex* exposed to acrylic fibres below 1mm in size. The results show the response of individuals at two concentrations a high exposure of 0.44g/L and a low concentration of 0.0575g/L

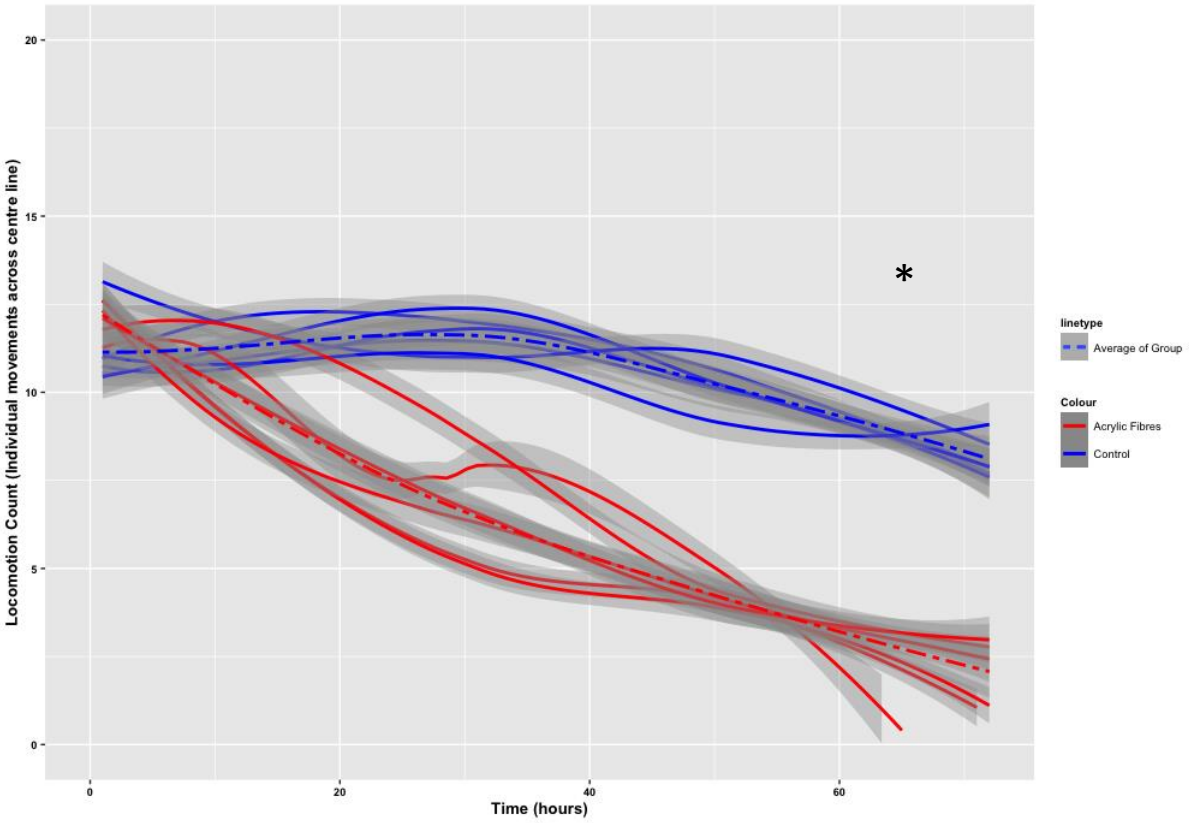

Figure 4.1 Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included. ANOVA variance analysis test was conducted to determine the significance of the variance, ($p < 0.05$). The treatment for this test was a concentration of 0.0575g/L of acrylic fibres. The grey areas surrounding the lines demonstrate the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

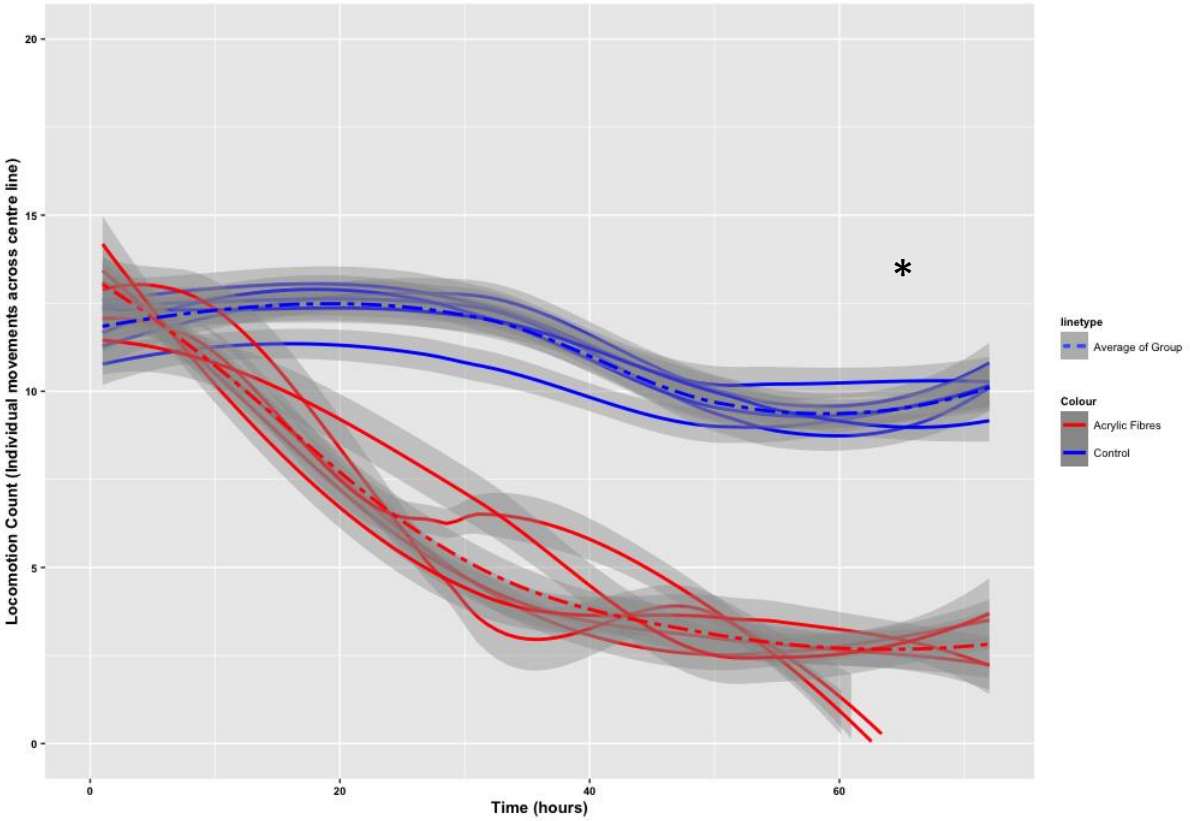

Figure 4.2 Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was done to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.44g/L of acrylic fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

Gammarus pulex individuals in both treatments had significant reduced locomotion in comparison to the controls ($P = 1.2e^{-5}$) when the concentration is at 0.44g/L and ($P = 0.00016$) at 0.00575g/L. In both treatments the reduction in locomotion increased as the experiment progressed. In the 0.0575g/L treatment, the locomotion for the average exposed to acrylic reduced by 75% with the reduction of the controls being 18.18%. The change factor increased over the time period; at 20 hours the change factor between control and treatment was 4, and at 72 hours this increased to 7. For the 0.44g/L treatment, after 20 hours the difference factor was 5 and by 72 hours the average difference factor was 8 individual movements across the centre line. The high concentration test showed an 80% reduction in locomotive activity for the average individual exposed to acrylic, whereas the controls' locomotive activity only reduced by 17%. Despite the high concentration having an increased impact on the locomotive activity by 5%, this does not display evidence of a dose response to the acrylic fibres. Figures 4.1 and 4.2 show that the variability between replicants was low with the separation of lines with the 95% confidence interval surrounding being small.

4.2.2 Cotton

The following figures examine the changes in the locomotion of *Gammarus pulex* individuals exposed to cotton fibres below 1mm in size. The results show the response of individuals at two concentrations a high of 0.44g/L and a low concentration of 0.0575g/L.

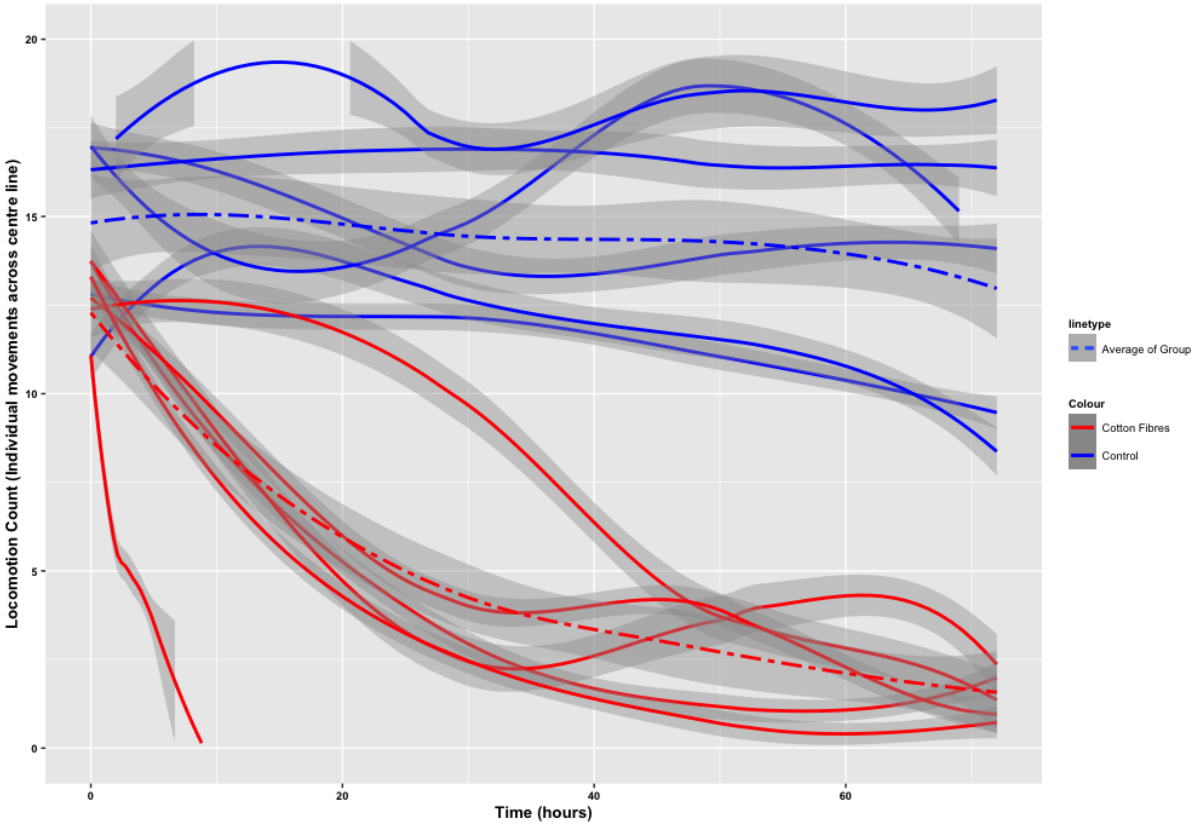

Figure 4.3. Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was conducted to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.0575g/L of cotton fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval.

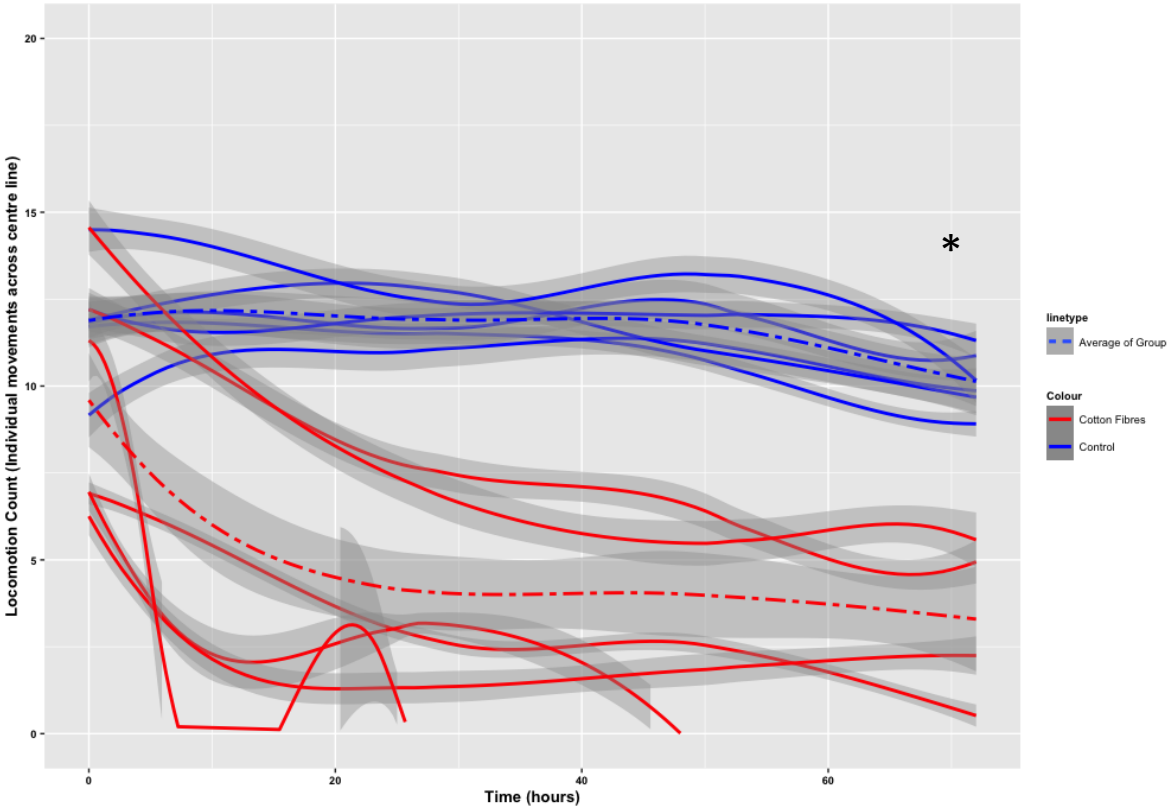

Figure 4.4. Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was carried out to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.44g/L of cotton fibres. The grey areas surrounding the lines demonstrates the 95% confidence. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

The low concentration exposure of cotton fibres was the only test to not show a significant difference in comparison to the control individuals over 72-hours (P = 0.29). Locomotion decreased significantly in the 0.44g/L study (P = 0.021) between the beginning and the end of the experiment. The average results for the group follow the same trend as Figures 4.1 and 4.2, with the 0.0575g/L treatment showing an average reduction in locomotion of 83% and the 0.44g/L treatment showing a reduction of 67%. The change factor for the averages at 72 hours is greater for the low concentration test; being 11 for the 0.00575g/L whereas it is 7 for the 0.44g/L treatment. Despite this, the 0.0575g/L was not significant (P = 0.29) in the variance between control and treatment. The variability in the controls is much greater for the 0.0575g/L treatment, whereas the 0.44g/L treatment showed low levels of variation between the controlled indiviuals which is similar to Figures 4.1 and 4.2. The response to cotton also does not show a dose-dependent relationship between cotton and a reduction in locomotion. The greater variation in Figure 4.3 would suggest a lower accuracy of result for the treatment which is reflected in the insignificant p-value (P = 0.29).

4.2.3 Mask

The 7-day degradation experiment using the single-use mask released 5,678 fibres in total, which were counted under a microscope; this equates to the release of roughly one fibre every other minute in water that is barely moving. The remainder of the mask was split into 6 equal parts and broken up into fibres consisting of cotton and polyester. These were exposed to *Gammarus pulex* individuals. The response from the individuals follows the same trend with a significant (P = 0.0018) reduction in locomotive activity for individuals exposed.

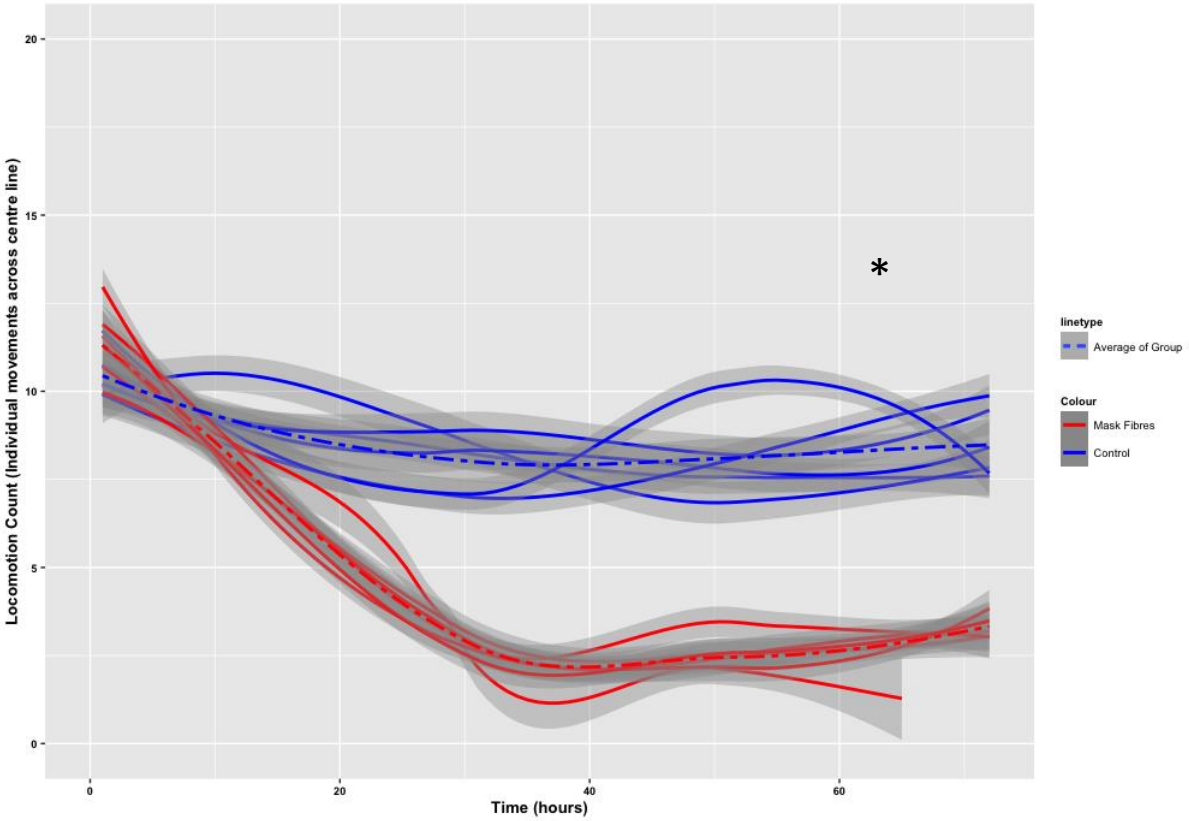

Figure 4.5. Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was conducted to determine the significance of the variance, significant p-values < 0.05. The treatment of this test was using a disposable face mask made with cotton and polyester fibres each individual was exposed to fibres from 1/6th of the mask. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

Figure 4.5 presents the locomotive data resulting from individuals being exposed to fibres released from disposable face masks. The trend is similar to previous treatments with *Gammarus pulex* individuals experiencing a significant reduction (P = 0.0018) in locomotive activity, as seen in individuals exposed to the fibres in Figure 4.5. The variability between replicates for both controls and exposed individuals is very low as displayed in Figure 4.5. The percentage change for the average exposed individual is a decrease of 64% whereas the controls only decreased by 23%. The reduction in locomotion increased as the experiment progressed, similar to the previous tests. The difference factor at 20 hours is 3 movements and then at 72 hours increases to 6 movements. However, it also appears that the locomotion reduces up to 40 hours afterwhich it reaches a plateau, which is different to Figures 4.1 and 4.2 where a consistent reduction in locomotion was observed.

4.2.4 Nylon

The following figures examine the changes in the locomotion, measured by number of line crossings made by an individual of *Gammarus pulex* individuals exposed to nylon fibres below 1mm in size. The results show the response of individuals at two concentrations a high exposure of 0.44g/L and a low concentration of 0.0575g/L.

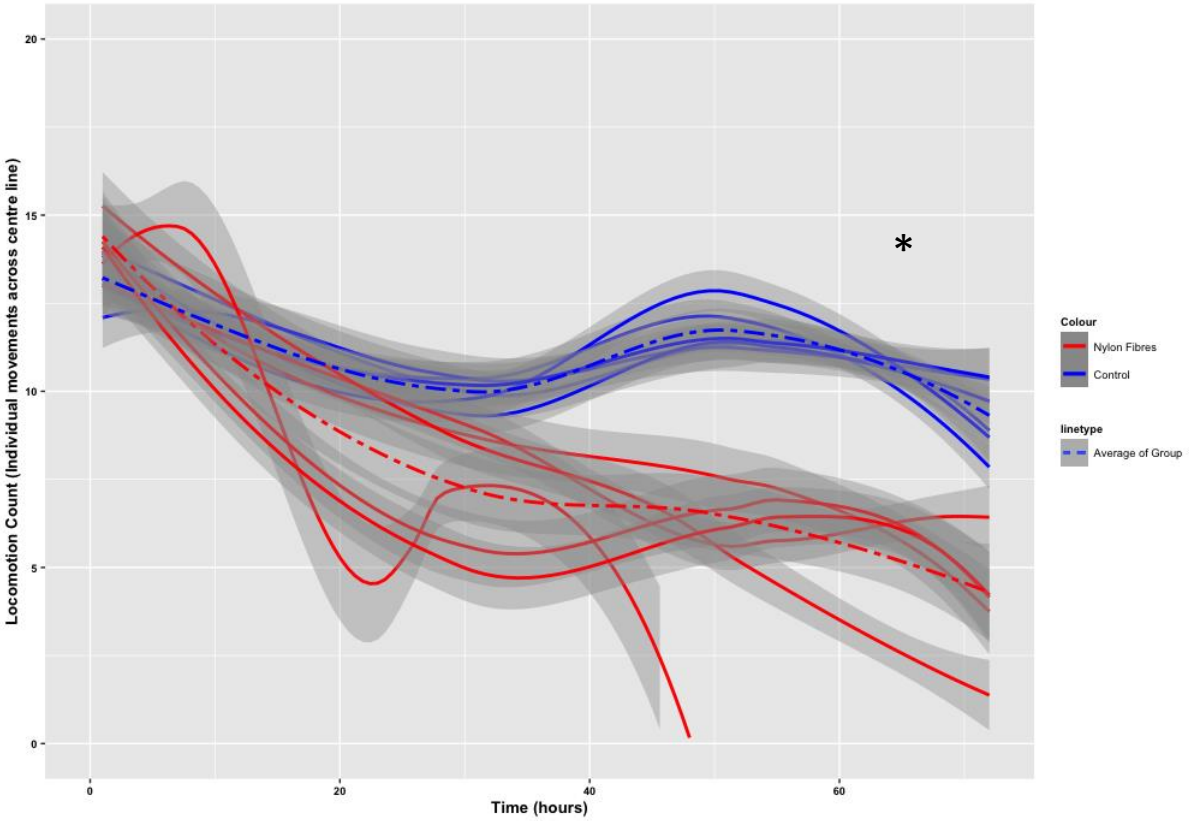

Figure 4.6 Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was done to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.0575g/L of nylon fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

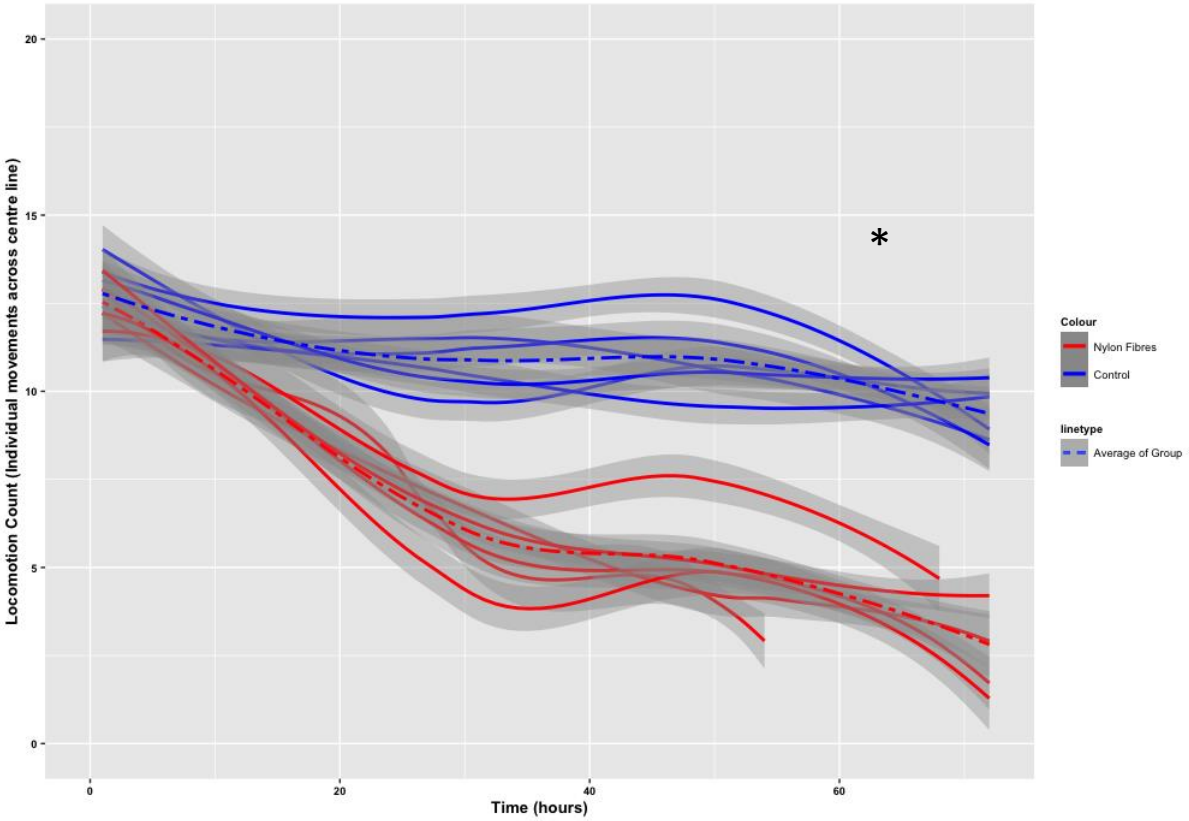

Figure 4.7 Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was done to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.44g/L of nylon fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

The nylon trend (Figures 4.6 and 4.7) is again similar, with both treatments displaying significant ($P=0.0045$ and $1.4x10^{-6}$ respectively of low and high concentrations) reductions in locomotive activity for individuals exposed to plastic fibres, in this case nylon. In the 0.0575g/L treatment there was a 71% decrease in the exposed individuals, compared to a 30% decrease in the control individuals, which is the greatest percentage decrease observed across all low concentration treatments. In the 0.44g/L treatment, locomotive activity decreased by 75% compared to the control, which decreased by 25% for the average. In a similar manner to the acrylic tests (Figures 4.1 and 4.2) the reduction in the locomotion increases as the experiment progressed. At 24 hours the difference between controls and exposed individuals is 3 and 5 movements for the low and high concentration treatments, respectively, which increased to 5 and 6 movements, respectively. One individual in the lower concentration test displayed particularly erratic changes before mortality at 48 hours. The low concentration test also displayed more variation between control and exposure than other fibre types.

4.2.5 Polyester

The following figures examine the changes in the locomotion, measured by number of line crossings made by an individual of *Gammarus pulex* individuals exposed to polyester fibres below 1mm in size. The results show the response of individuals at two concentrations a high exposure of 0.44g/L and a low concentration of 0.0575g/L.

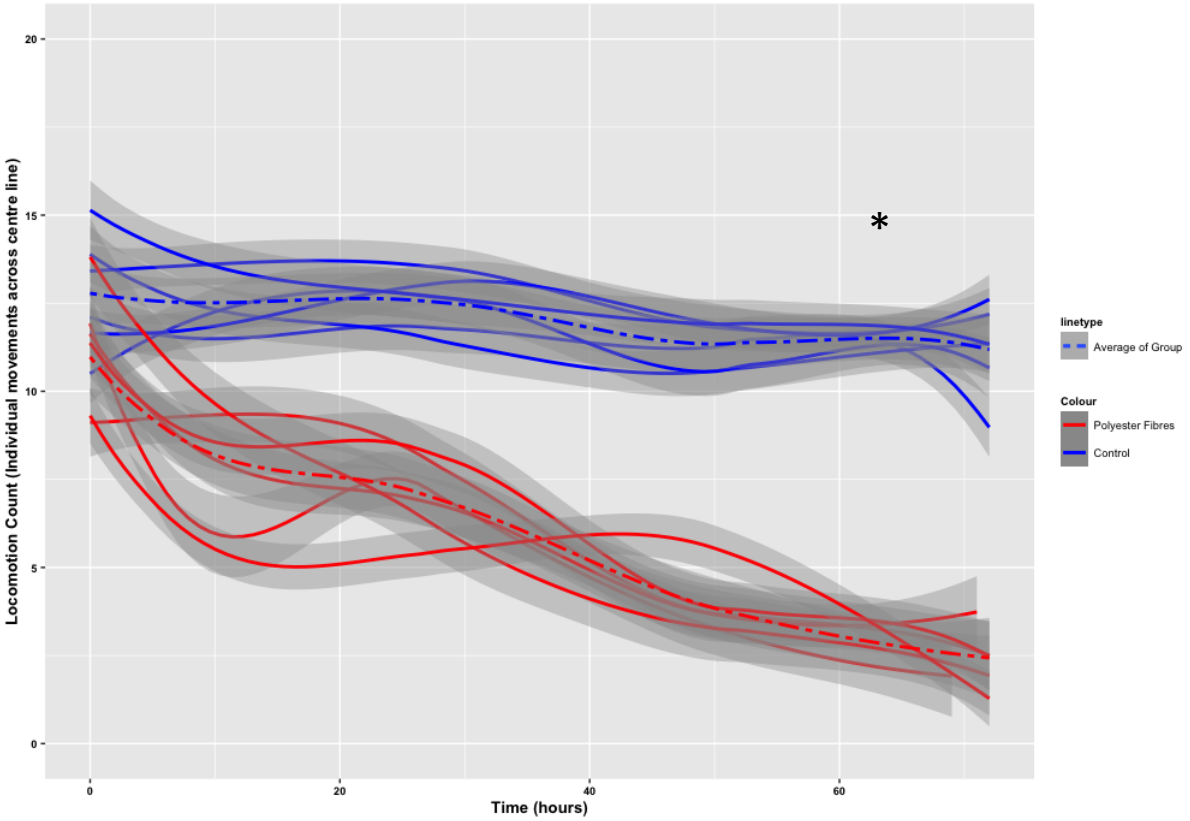

Figure 4.8 Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was conducted to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.0575g/L of polyester fibres. The grey areas surrounding the lines demonstrates the 95% confidence. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

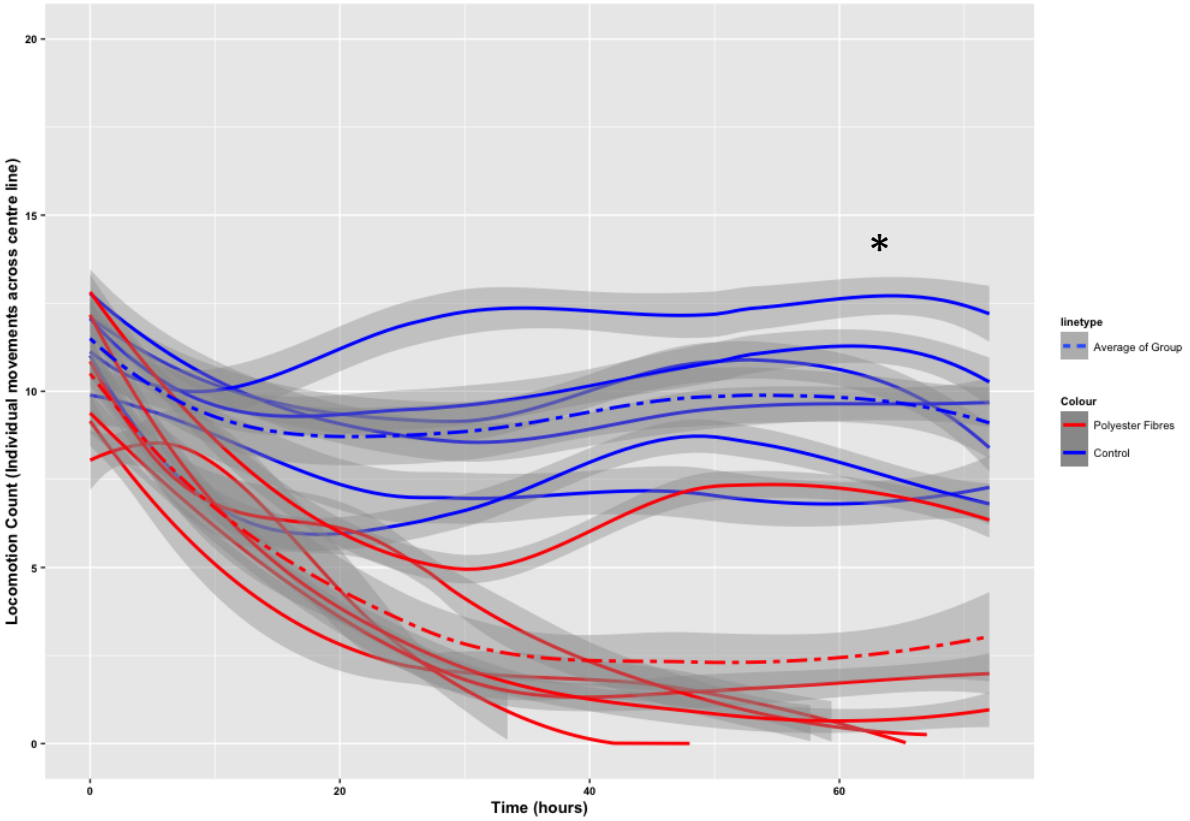

Figure 4.9 Locomotive response of each Gammarus pulex individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was conducted to determine the significance of the variance, significant p-values < 0.05. The treatment for this test was a concentration of 0.44g/L of polyester fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

The trend that the polyester follows is generally the same as described overall for other plastics too, in that the control individuals display greater locomotive activity. The key trend is that exposure to plastic causes a significant impact to movement in both experiments. During the polyester high test (Figure 4.9), the variation between control individuals is much greater than previously observed for control *gammarus*. Initially, the locomotive response for the individuals exposed to polyester declined rapidly over the first 24-hour period, with the high concentration test remaining constant for the remaining exposure (Figure 4.9). The lower concentration, however, increased in the 20 to 40-hour window and followed a similar trend of the control variables. Despite this, they clearly exhibited a lower overall locomotive response than the control individuals. Additionally, one individual out of the six exposed to polyester fibres follows the control individuals and seemed unaffected in terms of locomotive activity. The low concentration ANOVA variance is still significant (P = 0.02) when variance is measured between controlled and exposed individuals. The average lines give a considerably clearer view of the variance between individuals exposed to polyester and the controls, with Figure 4.2 displaying a greater difference. The variance between controls and treated individuals in this ecotoxicity test changed drastically throughout the study, with the initial p-values for both tests being insignificant and much higher than p-values for other materials (Table 4.1).

4.2.6 Combined Fibres exposure

The following figures present the changes in the locomotion, measured by number of line crossings made by a *Gammarus pulex* individual exposed to a combination of fibre types below 1mm in size. This consists of equal parts acrylic, cotton, nylon and polyester. The results show the response of individuals at two concentrations a high exposure of 0.44g/L and a low concentration of 0.0575g/L.

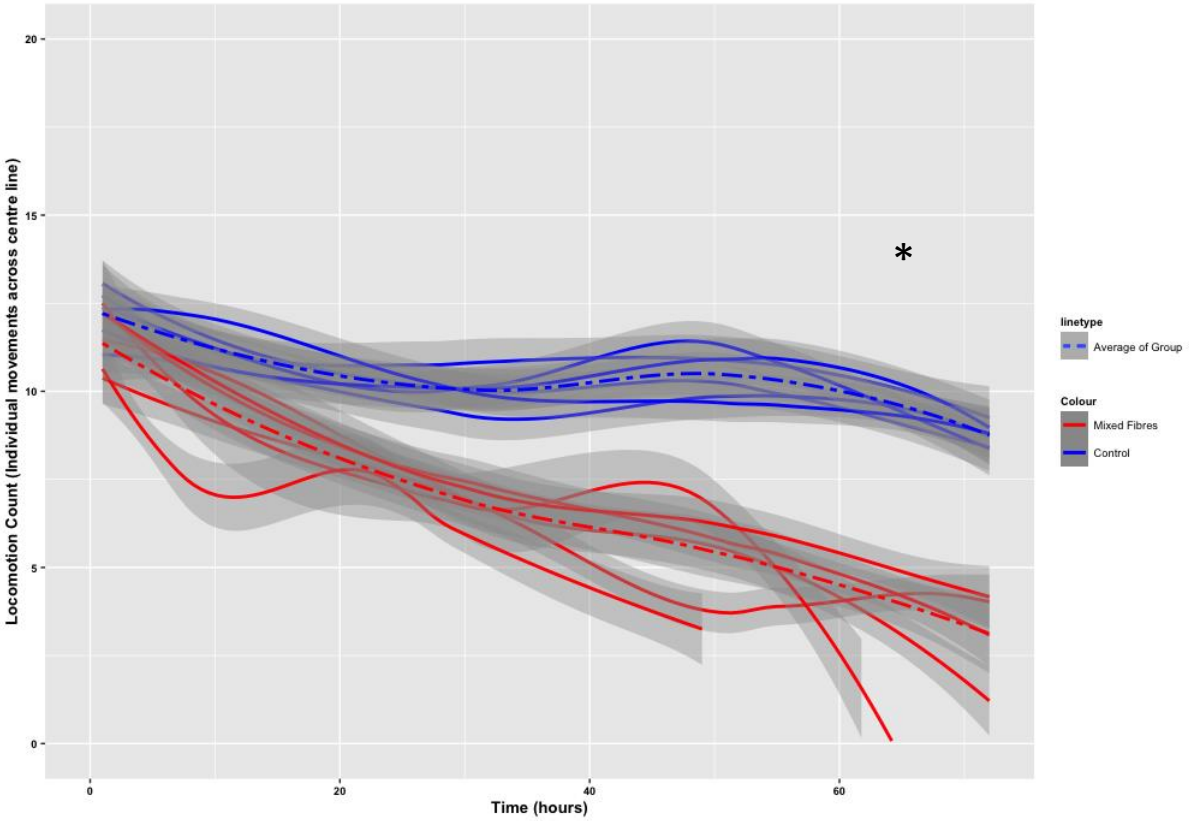

Figure 4.10 Locomotive response of each individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was carried out to determine the significance of the variance, significant p-values < 0.05. The treatment of this test was of a concentration of 0.0575g/L using a combination of polyester, acrylic, nylon and cotton fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

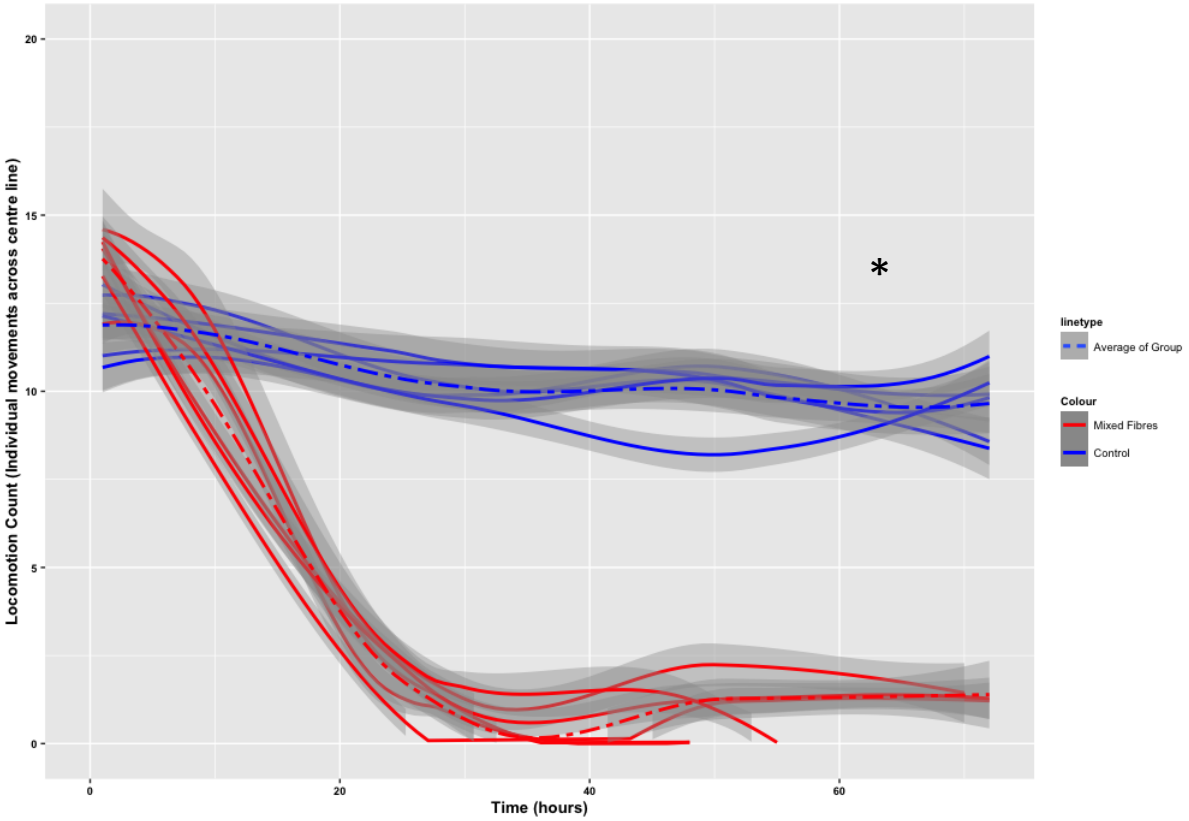

Figure 4.11 Locomotive response of each individual in a 72-hour ecotoxicity test. Average lines are also included and a ANOVA variance analysis test was carried out to determine the significance of the variance, significant p-values < 0.05. The treatment of this test was of a concentration of 0.44g/L using a combination of polyester, acrylic, nylon and cotton fibres. The grey areas surrounding the lines demonstrates the 95% confidence interval. The asterisk denotes significant variance between replicates and controls over the whole time period of the experiment.

As with the previous tests, locomotive activity of *Gammarus pulex* was significantly impacted in both the low concentration (P = 0.017) and high concentration (P = $1.1e^{-5}$) exposures. Locomotion behaviour was affected at a faster rate in the high concentration, with all replicates reducing locomotive counts by 0.5 every hour for the first 24 hours. In comparison, the lower exposure treatment decreased by a rate of 0.16 counts per hour over 24 hours. Greater *Gammarus pulex* mortality was observed in the high concentration treatment in comparison to the low concentration where 3 individuals died (Figure 4.11). The percentage of reduction in locomotive activity is the greatest of all tests in the 0.44g/L combined test, with an 86% decrease with the controls decreasing by only 17%. The 0.0575 g/L treatment decreased by 67%, compared to the controls which only decrease by 25%. The variability between replicates for the controlled and exposed individuals remains low throughout and shows good levels of consistency in both treatments.

4.3 Microfibres Ingested

The following section presents a boxplot showing the numbers of microfibres ingested by the *Gammarus pulex* individuals.

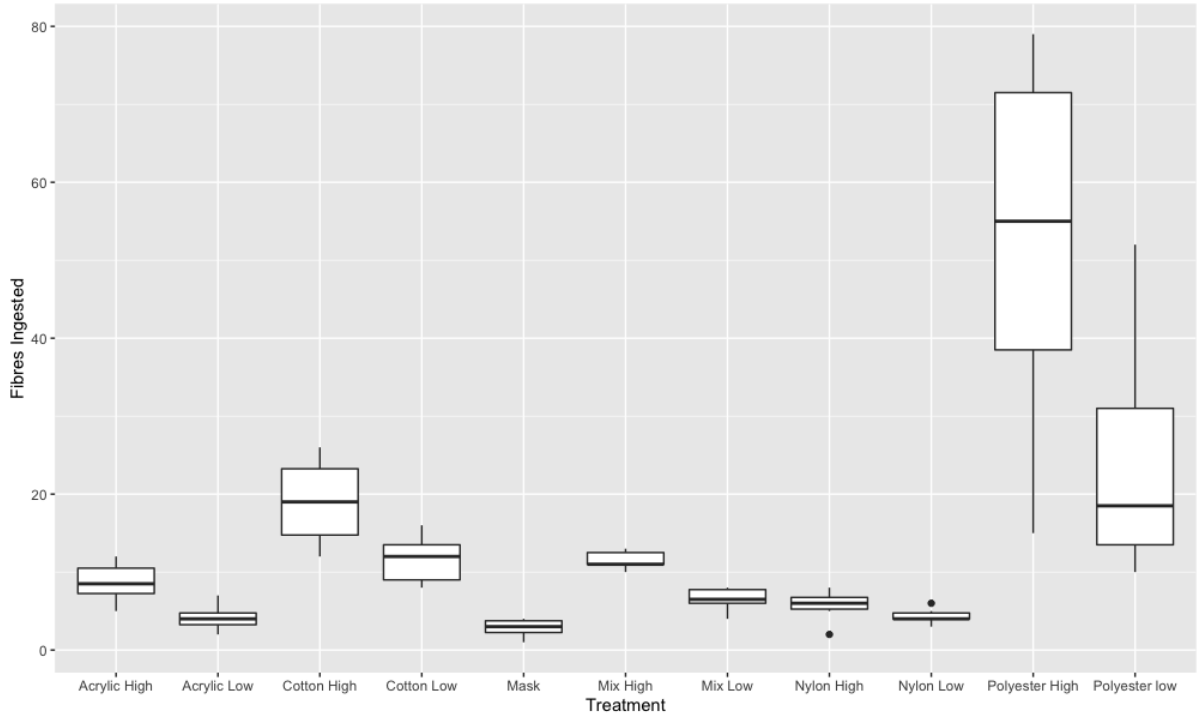

Figure 4.12 A boxplot displaying the microfibres ingested by Gammarus pulex individuals within a 72-hour Eco toxicity test. The tests displayed show different fibre types at low (0.0575g/L) and high (0.44g/L) concentrations the same as in Figures 1-11. The dots included display outlying results.

The boxplot highlights that there was greater ingestion of microfibres in individuals exposed at the higher concentrations, in comparison to the lower concentrations, including the fibre mix suggesting a dose-dependent relationship for ingestion. Specifically, for the polyester exposure, increased ingestion of polyester microfibres was evident in individuals exposed to the high concentration with a range of microfibres ingested from 16 to 78 microfibres per individual (Figure 4.12). However, the range of values, excluding outliers, as demonstrated by the size of the box plot, is greater than any other treatment (between 38 and 72). This demonstrates large variation in the numbers of microfibres ingested in the polyester tests; similarly, variability was also observed in the low concentration exposure but to a lesser extent. The polyester results seem exceptional in comparison to the other treatments.

The results with the polyester removed are more comparable with the range from all tests being 23 fibres. The cotton treatments saw a high amount of ingestion ranging from 9 to 25 fibres across both concentrations. When the concentrations reduced in the cotton treatment from 0.44g/L to 0.0575g/L, the average number of fibres ingested decreased by 37%. In comparison, in the acrylic treatments this same decrease was 38%, for nylon it was 33% and for the combined mixture of microfibres the percentage reduction was 36%, which is comparable for all of these tests. For reference, the percentage decrease in the polyester treatments was 65%, which is much greater than the other microfibre types.

All individuals exposed to plastic fibres across the concentration ranges explored were found to have ingested microfibres, generally in the range of 2-20 fibres per individual. Nylon low concentration, acrylic low concentration and the mask microfibres all generally were comparable; ranging between an average of 4 to 5 microfibres ingested per individual. The mask fibres were the only fibres created

in a different process to the other polymer types and had the least microfibres ingested with an average of 4 fibres per individual (which ranged from 2 to 5).

4.4 Feeding Rate

The following section presents the results for the feeding rate of individuals across the ecotoxicity tests.

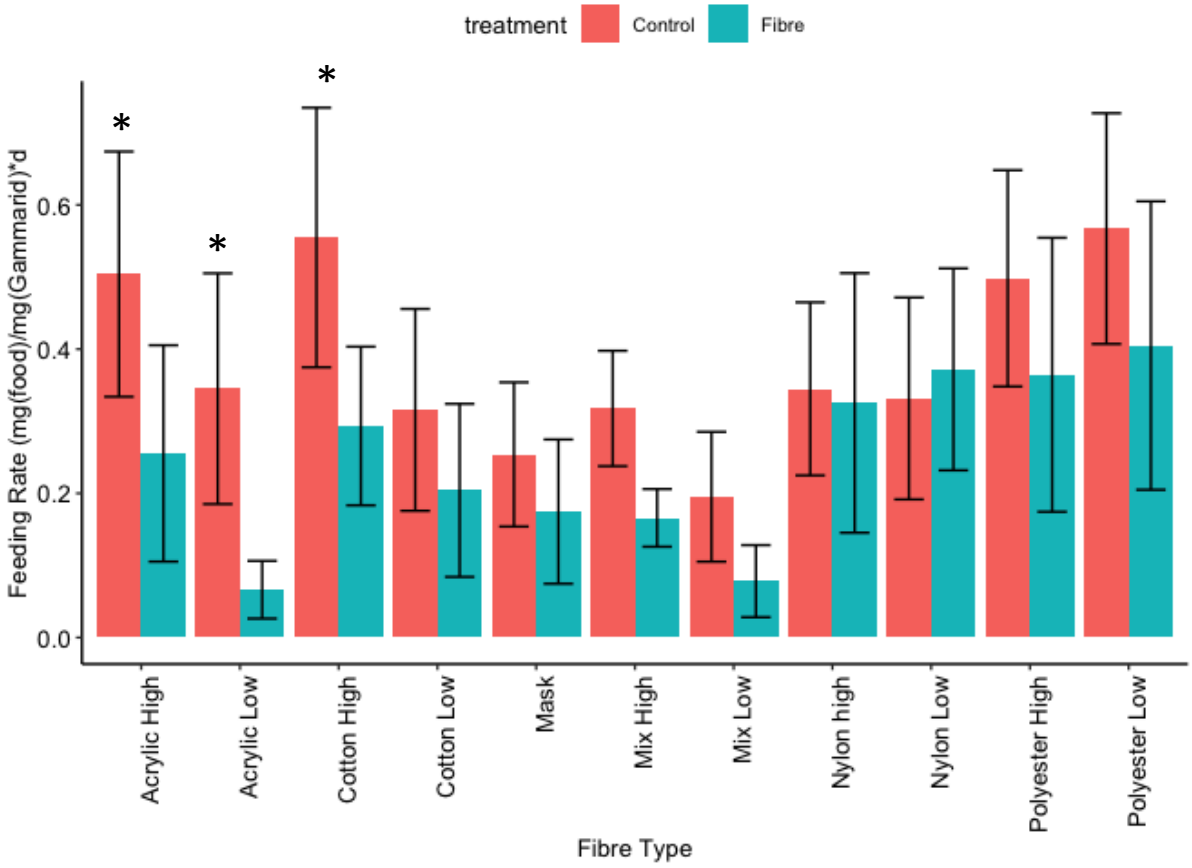

Figure 4.13 A bar plot that shows average feeding rate for both the control and fibres at each concentration. ANOVA analysis was conducted to test the variance between control values and those exposed to fibres. Error bars are displaying standard deviation. Asterisks denote the significant variance between fibre and control.

The Feeding rate demonstrates a substantial difference for the individuals exposed to plastic or natural fibres and the controls (Figure 4.13). The greatest reduction in feeding rate was as a result of the 0.0575g/L acrylic treatment with an 86% reduction in feeding rate which was significant (P=0.005). In the acrylic 0.44g/L treatment a decrease of 52% was detected which was significant (P=0.03) and the second largest decrease. The third largest decrease in feeding rate was observed in the cotton 0.44g/L treatment, with a decrease of 49% which was significant (P = 0.04). The other exposures showed decreases in feeding rate between treatment and control but significant variance was not measured by ANOVA tests.

The tests show a large standard deviation in data as denoted by the error bars; the larger error bars seen in the polyester, nylon, acrylic and cotton treatments suggest large deviation between replicates and suggest unreliable data. The smaller bars seen on treatments such as the mix low and acrylic low are smaller due to the reduced feeding rate, they still suggest large fluctuations in the data. The control individuals ranged from just under 0.2 up to above 0.5, and showed greater variance between treatments than the individuals exposed to microfibres. This suggests the feeding rate data is less reliable as the controls are expected to remain constant throughout treatments.

4.5 Growth

The following section presents the results from the growth of individuals from the Ecotoxicity tests.

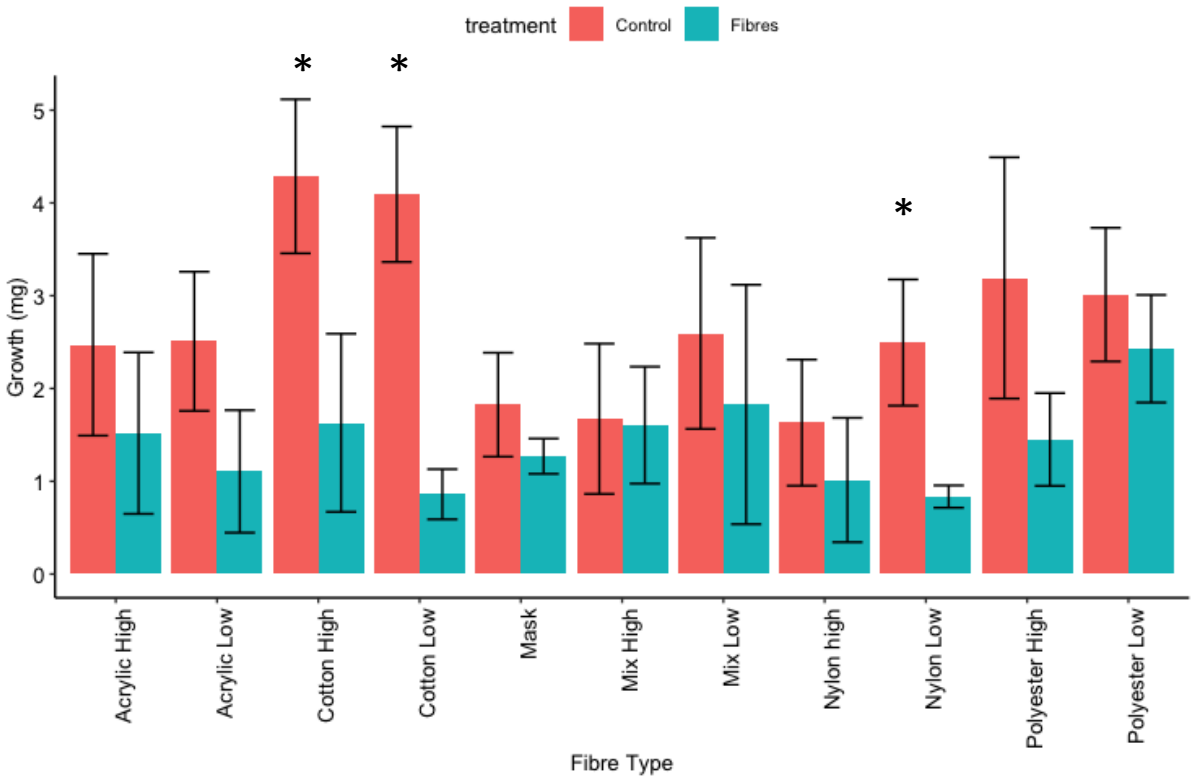

Figure 4.14 A bar plot that shows average growth in mg per individual rate for both the control and fibres at each concentration. ANOVA analysis was conducted to test the variance between control values and those exposed to fibres. The error bars are representing standard deviation. Asterisks denote the significant variance between fibre and control.

The growth rate results follow a similar trend as the feeding rate, with exposed individuals experiencing less growth than controls over the 72-hours. Cotton Low showed the greatest negative difference in growth by 79% which was a significant difference (P = 0.003). Cotton High also demonstrated a greater negative difference in the growth, with a 63% difference which was also significant (p = 0.03). The only other significant result (P = 0.001) was Nylon Low which saw a negative difference of 61%. This suggested that individuals exposed to microfibres grew significantly less in these tests. The variation of the controls is large again here, with growth ranging from 1.5mg to above 4mg in the cotton test. The variation in the individuals exposed to microfibres is less wide ranging between 0.8 and 2.4mg. The error bars are large in Figure 4.14, which denotes a large standard deviation between replicates which makes the data gathered here less reliable. Future experiments of this nature would be greatly improved by increasing the number of replicates.

5 Discussion

5.1 Introduction

The following section has reviewed the findings presented in the results section and will synthesise this with external literature to discuss trends and themes. The section will cover each lethal and sub-lethal response, discussing the validity and relevance of the data, any causes and reasons behind the data. This section will also present future recommendations for future research in this area.

5.2 Mortality and Lethal Concentration analysis

5.2.1 Lethal Concentration findings

Lethal concentration is an important measure to calculate when undertaking ecotoxicity testing as it demonstrates toxicity by calculating the concentration that will cause mortality to 50% of the individuals over the time period, in this case 72 hours (Gad, 2014).

The results displayed in Table 4.1 are noteworthy and conclusions can be drawn from them. Firstly, there is a positive correlation between *Gammarus pulex* individuals exposed to increased concentrations of microfibres and a subsequent increase in mortality. 81% of ecotoxicity tests in the literature have tested mortality on various species when exposed to microplastics (Haegerbaeumer et al., 2019). Multiple studies have investigated various *Gammarus* species and no significant mortality occurred as a result of different sized and shaped microplastics (Blarer and Burkhardt-Holm, 2016; Redondo-Hasselerharm et al., 2018). Notwithstanding, mortality did occur due to ingestion of microfibres and beads, however, it was not found to be a significant result of mortality (Blarer and Burkhardt-Holm, 2016). Despite this, other amphipod species (*Hyalella azteca*) have been found to react dose-dependently to microplastics particles in terms of mortality, which is a similar response as this study (Au et al., 2015). In Table 4.2 of this study, both polyester fibres and the mixture of fibres had comparatively lower LC50 values to the other polymer types, thus having higher mortality, both being below 1g/L, and the results suggest that a combination of microfibre types correlates with the greatest levels of mortality. This could be due to an increase of stressors on the individuals as a result of more fibre types and colours inducing stress reactions in the individuals (Felten et al., 2008a). Browne et al. (2013) recounted a similar increase in mortality of *A. marina* exposed to multiple plastic types. Polyester is generally absent from the majority of the ecotoxicity tests despite being commonly found in wastewater outflows and a common result of clothing fibres (Haegerbaeumer et al., 2019). LC50 values for other plastic types tested in comparison to polyester are higher (Table 4.2) which would support the suggestion that polyester is a more toxic microfibre. Additionally, other than the combined fibre treatment, the polyester treatment had the lower survival rate (Table 4.1), supporting suggestions that it is more toxic. The lowest LC50 and LC99 values were recorded for individuals exposed to polyester, aligning with Figure 4.12., which displays polyester having substantially more fibres ingested. Ingestion is a major factor that has been reported to effect further lethal and sub-lethal effects, such as reduced growth; increased oxidative stress and; increased mortality (Huerta Lwanga et al., 2016; Hurley et al., 2017). This suggests greater ingestion leads to greater mortality, which in the case of polyester in this study is true.

Cotton microfibres have a higher LC50 of 1.41g/L and a notably high LC99 of 500.4g/L, suggesting that when *Gammarus pulex* individuals were exposed to cotton fibres a higher concentration is required for mortality to positively correlate. This could be due to fibres having a less toxic composition, however this has not generally been researched. It has largely been speculated that plastic fibres would be more harmful, with suggestions to use more natural materials in the textile industry to combat microplastic pollution (Henry et al., 2019).

5.2.2 Comparison of microplastics and other pollutants

The LC50 values calculated in this study are still substantially higher than for other contaminants, for example cadmium which was found to have an LC50 of 0.494 mg/L (Felten et al., 2008b). Other common pollutants of *Gammarus pulex* which have LC50 values over a similar length of exposure are Dicholoroniline (17.4mg/L), Atrazine (14.9mg/L), Copper (0.047mg/L) and Lindane (0.079mg/L) (Taylor et al., 1991). Microfibres are unlikely to have the same effects as these chemicals because of the lethality, mainly caused by fibre size and shape blocking breathing and feeding processes (Au et al., 2015). Microfibres could exhibit similar effects to the aforementioned chemicals if they were acting as vectors, carrying these and other similar chemicals (Blarer and Burkhardt-Holm, 2016).

These commonly found pollutants cause toxicity at significantly lower concentrations, suggesting microplastics are less toxic and therefore much higher microplastic concentrations would be required to cause harm to *Gammarus pulex* individuals in the environment. The lowest concentration LC50 calculated (0.579g/L) is a much higher concentration than anything that has been currently found in the current environment (Burton, 2017). An average wastewater treatment plant can release around 65 to 120 million microplastics each day and in certain fluvial conditions high concentrations of microplastics near these sites could be possible (Blair et al., 2019; Murphy et al., 2016). However, there are no reports of concentrations as high as those presented in this study, with the highest concentrations in freshwater being around 600 particles per kg. More sampling close to wastewater treatment plants should be carried out to confirm any possibility of these concentrations and subsequent invertebrate mortality could occur in freshwater environments. In reality, current microplastic concentrations in the environment are much lower than the estimated LC50 values included as shown in Table 5.2.

The comparisons between microplastic ecotoxicity studies and subsequent results are difficult to make directly to some other studies as many used number of fibres instead of weight as this study did. The only way for this study to keep concentrations constant between studies was to keep the weight constant as counting fibres would be unrealistic. It is difficult to calculate how concentrations calculated in Table 3 equate to LC50s of 71,000 microplastic particles per litre, as the number of fibres that combines to a certain weight is unknown and depends on multiple factors such as density and size (Au et al., 2015).

An important consideration of the results in this study is that the individuals used were not taken from the environment, in order to rule out any prior contamination. A consideration of these results needs to be made for this, as it has been reported that laboratory cultured *Gammarus pulex* individuals may have a higher tolerance to contaminants by up to 10 times (Love, 2018; McCahon and Pascoe, 1988a; McCahon and Pascoe, 1988b). On the other hand, *Gammarus pulex* in freshwater environments could have a higher resistance due to building up their tolerance to pollution over time (Shahid et al., 2018). Additionally, *Gammarus pulex* are a moderately tolerant species and are commonly found in areas of poor water quality; a more sensitive species may react differently to microplastics. Species that are important for ecosystem health and are an environmentally important species that are scarce or protected may be impacted differently due to different sensitives to pollutants (He et al., 2019b). These could include stoneflies or mayflies often found in good quality water and are sensitive to pollution (Czerniawska-Kusza, 2005).

5.2.3 Alternative explanations for the results and future directions

The limitations of using LC50 analysis is that there are a number of factors that can influence the results, aside from the toxic effects of the tested contaminant (Hlina Benjamin L et al., 2019). Previous studies have stated that the differences in results between studies can vary due to the individuals' different life stages, such as: life cycle stage; moult cycle; or reproductive period (Felten et al., 2008b; McCahon and Pascoe, 1988a; McCahon and Pascoe, 1988b). This is why test organisms that were cultured in a laboratory were selected for these tests, because individuals are provided in the same life stage.

Despite any limitations that LC50's may pose, the standout discovery from the results in this study is that microplastic fibres can result in serious lethal effects, which could be environmentally significant if future microplastic pollution continues exponentially.

5.3 Impacts of exposure to microfibres on *Gammarus pulex* Locomotion

5.3.1 Use of locomotion as an ecotoxicological endpoint

Locomotive activity is a suitable indicator of physical wellbeing as it reacts sensitively to external changes and as a result has previously been used as a measure of *Gammarus pulex* for ecotoxicity tests (Arce Funck et al., 2013; Felten et al., 2008a; Felten et al., 2008b). In past studies, when *Gammarus* have been exposed to substances considered harmful such as lead, cadmium and pharmaceuticals, locomotive activity significantly decreased (De Lange et al., 2006; Felten et al., 2008b). A number of studies have measured locomotive response to a toxic substance and noted a significant reduction which demonstrates a negative response to a toxic stimulus; see Table 5.1 (Arce Funck et al., 2013; Iltis et al., 2017; Vellinger et al., 2012).

Table 5.1. Studies that show effects of locomotive activity in Gammarus pulex individuals in ecotoxicity testing.

Study and method of measurement	Substance exposed to individual	Effect on locomotion
(De Lange et al., 2006) **MFB method**	Fluoxetine, ibuprofen, carbamazepine, cetyltrimethylammonium bromide (CTAB)	When exposed to Fluoxetine locomotive activity reduced from 55% to <20%. Exposure to ibuprofen reduced activity from 60% to 30%. Locomotion was reduced slightly from 65% to 45% when exposed to carbamazepine. CTAB reduced locomotion from 65% to 10%.
(De Lange et al., 2009) **MFB method**	Fluoxetine, ibuprofen, carbamazepine, cetyltrimethylammonium bromide (CTAB)	Exposure of individuals to all substances at high concentrations caused high ventilation and low levels of locomotion.
(Felten et al., 2008a; Felten et al., 2008b) **Line crossing or individual movement measurement**	Cadmium, sulfuric acid	Exposure to cadmium led to a significant drop in locomotion of up to 39%. The line crossing reduced by 72%, 93%, 99% when pH levels were 6, 5.1 and 4.1 respectively.
(Vellinger et al., 2012; Vellinger et al., 2013) **Individual movement measurement**	Arsenate, cadmium	Locomotion was significantly reduced by all of the exposures by between 15.6% and 35.3% reductions in locomotive activity.
(Thurén and Woin, 1991)	Di-2-ethylhexylphthalate and dibutylphthalate	Exposed *gammarus* individuals did not respond to environmental changes that would normally influence locomotive activity, when activity was expected to

Infra-red lightbeam interruptions method		increase individuals did not react. Non-exposed individuals did respond to these changes and locomotion increased. The effects on locomotion persisted after the experiment.

A limitation of the data included in this report, in comparison to other studies, is the unavailability of a Multispecies Freshwater Biomonitor (MFB); a piece of equipment that measures frequency impedance changes due to an organism's movements (De Lange et al., 2009; Gerhardt and Schmidt, 2002). The benefits of a MFB is that no movements are missed and more data can be gathered without the need of someone watching the individuals. However, this apparatus was not available for this study and the line crossing method has been replicated a number of times and as recently as 2017 (Vellinger et al., 2012; Iltis et al., 2017). The line crossing method allows the same data to be gathered in terms of noting changes in the locomotion of individuals and has been replicated to gather reliable data, as long as the method remains consistent.

The objective of this endpoint was to determine if it was possible to see behavioural responses in locomotive activity in individuals exposed to microfibres similarly to past substances. Additionally, this report uses locomotion data to compare significant changes in locomotion, with the changes displayed by known contaminants such as pharmaceuticals and cadmium to demonstrate the toxicity of microplastics (De Castro-Català et al., 2017; Felten et al., 2008b). Locomotion is a key behavioural response of *Gammarus pulex.* Therefore, this is evidence of microplastics negatively affecting the behaviour of a keystone species. Locomotion of *Gammarus pulex* individuals having a negative correlation to pollution may have negative effects on populations; locomotion is important for upstream movement to stop the washing out downstream for individuals (Thurén and Woin, 1991). This can affect foraging and feeding behaviour and reduce availability for mating which could affect species populations (De Lange et al., 2006)

Changes in locomotion have been recorded before, but as a result of a change in water viscosity with the influence of material (Bolton and Havenhand, 1998). The influence of matter, such as leaves and fibres in the water can affect individual's movement through the water due to changes in the viscosity of the water. It could be suggested that the influence of microfibres could affect the water's viscosity, as the control individuals would not have the added fibres, therefore allowing easier movement through the water for the control individuals. However, the effect of this was not evident in this study and it has not affected the results as seen in Table 4.3, as the variance between control and exposed individuals for the initial locomotion is insignificant. The composition of the water remained constant throughout the study and therefore, viscosity of the water was unchanged throughout the study.

5.3.2 Effects on locomotion in *Gammarus pulex* and other species at different concentrations

The data produced in this study (Figures 1-11) overwhelmingly shows significant decreases in locomotive activity as a result of exposure to microfibres. The results display similar reductions in previous studies of toxicity on *Gammarus pulex,* in that exposed individuals suffer a significant decrease in locomotion while controls remain constant (De Lange et al., 2009; Felten et al., 2008b). Reductions of around 70% in locomotive activity were observed in both this study (Figures 4.1-4.11) and the studies presented in Table 5.1 (Felten et al., 2008b). Importantly, it contributes to a growing accumulation of data showing significant impacts of microplastics on locomotion such as nanoplastics exhibiting significant locomotion reductions in the roundworm species *C. elegans* (Zhao et al., 2017). Another study on *C. elegans* analysed four types of locomotion (head thrash, body bend, forward and backward movement) and discovered nanopolystyrene caused damage to locomotion behaviours and

the development of dopaminergic neurons (Qu and Wang, 2020). Additional research into zebrafish larvae noted microplastics and nanoplastics causing significant reductions in locomotor activity by between 18 and 27% (Chen et al., 2017). Freshwater tadpoles were exposed to polyethylene microplastics, which caused a reduction in locomotion, with swimming distance and speed reduced, and also increased anxiety levels, the differences between exposed groups and control groups were significant (Araújo and Malafaia, 2020).

5.3.2.1 High concentrations

The high concentration tests discuss treatments with a concentration of 0.44g/L, this is substantially higher than concentrations currently found in the freshwater environment as highlighted by Table 5.2. These tests display significant changes in the locomotive response in each exposure. All tests displayed insignificant variance at the beginning, and significant variance at the end of the 72-hours (Table 4.1). This suggests that with an increased amount of fibres available the effect on the individuals is greater (Figure 12). The decrease in locomotive activity is a compensatory behavioural reaction to a contaminant and has similarly been observed in multiple studies on *Gammarus pulex,* as shown in Table 1. Previous literature attributes these changes to be driven by compensatory mechanisms induced to maintain homeostasis of the individual; this causes individuals to reduce locomotion to preserve energy (Felten et al., 2008b).

5.3.2.2 Low Concentrations

From the data gathered (Figures 1-11) there is significant evidence that even low, environmentally relevant, concentrations of microfibres caused a decrease locomotive activity. This data provides evidence that the effect decreased in the low concentration tests but is still significant which is consistent with other literature; locomotion decreases that were significant were also apparent at the lowest concentration test on *C.elegans* (Zhao et al., 2017). This was at $10\mu gL^{-1}$, substantially less than the low concentration of 0.0575 gL^{-1} ($57500\mu gL^{-1}$) used in the current study and shows that low concentrations of micro and nanoplastics can cause effects. These concentrations vary from study to study depending on the test species, as *Gammarus pulex* individuals are more resilient and therefore are unlikely to show effects (Love, 2018). Further studies would be improved with repetition and using a wider range of concentrations to establish a dose-response relationship and calculate no observed effect concentrations (NOEC). The concentrations in which no effects are observed are important to discover as they provide knowledge of safe levels of a contaminant.

5.3.2.3 Environmentally realistic concentrations

The lower concentrations of 0.0575g/L have been found in some sediment samples in freshwater environments, making the results environmentally relevant (Castañeda et al., 2014). However, these concentrations are not consistently found and are not an accurate representation of the average freshwater environment. They are still very rarely found to be as high as 0.0575g/L (Table 5.2).

Importantly, the experiments in this study were water-only exposures and therefore the availability of microfibres was greater than the same concetration in the aquatic environment, as microfibres would potentially be bound up to the solid matrix of the sediment and therefore be less bioavailable. A further improvement of this study would be to try and replicate these concentrations in an ecotoxicity test that replicated the aquatic environment more and included sediment in the experimental set up.

It is estimated that current microplastic and nanoplastics concentrations in sediment could reach up to an equivalent concentration of 0.162g/L in some conditions (Besseling et al., 2014). With similar conditions to the low concentrations tests being found in environments in some areas of the world, these effects could be happening to native *Gammarus pulex* species. These concentrations are predicted to increase due to demand for plastic use in clothing and fabrics and an increase in natural environments, with concentrations of microplastics at similar levels to this study is likely (Blair et al.,

2017). It is unrealistic to predict what concentrations could be possible but more studies are increasingly finding higher concentrations (Dibke et al., 2021).

As previously mentioned, concentrations in other studies are substantially lower than used in this study, are more likely to be found in the freshwater environment and provide evidence that effects of microplastics can be seen in freshwater species (Zhao et al., 2017).

5.3.3 Effects of different composition and form of microplastics

5.3.3.1 Different polymer types

Generally, the types of fibres did not cause results to vary extensively and it is much more likely that the shape affects the individuals than polymer type. A study by Weber et al. (2018) demonstrated that *Gammarus pulex* exposed to PET microplastic particles were unaffected. The study measured moult cycle and mortality and not additional sub-lethal effects, such as locomotion, that may precede more toxic endpoints such as lethality. However, the study was over 48 days and no toxic effects were observed. The highest concentration test organisms were exposed to was 4,000 particles per litre, which is very low in contrast to this study, although direct comparisons between weight and numbers of particles is difficult is difficult as previously mentioned. Due to *Gammarus pulex* being a hardy species, it is expected that it is unlikely for toxic endpoints occur at low concentrations (Maltby et al., 2002).

PET has been documented to not affect *Gammarus pulex,* and different polymer types could have different effects and variations of toxicity (Weber et al., 2018). Numerous species across freshwater and marine environments have been exposed to micro- and nanoplastics of different types showing effects, however disproportionately more research has been carried out using polyethylene and polystyrene (de Sá et al., 2018). Studies have examined effects on locomotion of invertebrate species exposed to plastic types and seen significant reductions from both polystyrene and polyethylene (Araújo and Malafaia, 2020; Chen et al., 2017; Qu and Wang, 2020; Zhao et al., 2017). There is a gap in the literature showing significant reductions in locomotion of species as a result of other polymer types, but the data (Figures 1-11) show no substantial differences between polymer types. However, this may be due to polyethylene and polystyrene being the most commonly found plastics, with 31% of plastic discovered being polyethylene and polystyrene (de Sá et al., 2018). The Weber et al. (2018) study does not present a lack of effects of PET against other polymer types, so there is not sufficient evidence to suggest effects are not possible by PET as opposed to other polymer types, as identified in the data from this study (Figures 4.1-4.1).

5.3.3.2 Different polymer forms

From increasingly extensive sampling campaigns, there are a number of forms which plastics can take: fibres (23%), fragments (21%), spheres (11%), films (8%) and pellets (4%). Despite this, the majority of ecotoxicity tests used spheres over the more commonly found forms of plastic in the environment (de Sá et al., 2018). Therefore, fibres and fragments are underrepresented in the effects literature. A study found that pre-prepared plastic spheres are less readily ingested than plastic fragments (Lehtiniemi et al., 2018). The study did not expose individuals to fibres directly, however fibres were inadvertently found in the experiments due to contamination, and ingestion of fibres was as common as that of fragments (Lehtiniemi et al., 2018). This highlights the importance of microplastic fibres and fragments.

It could be the case that fibres have more of an effect on individuals than particles. This is supported by the data that shows natural cotton fibres at both concentrations significantly affected locomotive response (Figures 9 and 10). However, the data in this study (Figures 1-11) did not expose individuals to fragments or spheres to compare effects of plastic of different sizes. Further research into this area is required as there is a lack of literature pertaining to the effects on locomotion at different size and

shape of microplastic particles. As this study did not use fragments or particles, it cannot confirm different responses as a result of the composition of the plastic, but there is evidence to suggest both fibres and fragments affect locomotion (Rehse et al., 2016).

A study by Rehse et al. (2016) found that at high concentrations microplastic particles had an immobilising effect on *Daphnia Magna* individuals over a short-term exposure. As immobilising effects were observed in this study (Figures 1-11) this supports that microplastic particles can result in an immobilising effect across different aquatic species. *Daphnia Magna* have also been observed to become immobilised due to a number of fibres, both synthetic and natural, similarly to the results in this study (Dave and Aspegren, 2010). Similar effects in movement were observed in the fish species *Carassius carassius* when exposed to nanoparticles (Cedervall et al., 2012). Therefore, the data gathered and observations made in this study adds to a growing consensus that microplastic particles and fibres can negatively affect movement throughout food chains and important invertebrate species.

5.3.3.3 Effects of microplastics associated with the COVID-19 pandemic

The COVID-19 pandemic has created an excessive use of throwaway single-use PPE, including masks and gloves that release fibres and fragments respectively (Patrício Silva et al., 2021). These were used in this study to determine whether the same impacts as the other microfibre treatments can be displayed as a result of these fibres. Figure 4.6 demonstrates that high concentrations of fibres derived from these masks have the same negative effects on locomotion of individual *Gammarus pulex*. This is likely because the microfibres produced from the mask were of a similar composition, readily made from polyester and cotton, as well as other polymer types such as polypropylene, polyethylene and polystyrene (Fadare and Okoffo, 2020). The majority of the fibres were the same size, thus readily ingested by individuals and likely to affect individuals this way.

This provides some early evidence that the increase in plastic waste created by the pandemic can affect ecosystems and species. This ecotoxicity test (Figure 4.6) was generated the same as other tests however, as the fibres were derived from masks, they were less likely to be evenly distributed in the water and therefore the fibres were potentially not as available as other tests. Despite this, the concentration was consistent with other high concentration tests. This corresponds in the data with similar trends being displayed in the mask study (Figure 4.7) and the other significant data (Figures 4.1-4.11).

5.3.4 Future recommendations on the use of locomotion

It is clear that locomotion is affected by microplastics; the results display a significant change in locomotion from when the ecotoxicity test starts in the majority of the experiments undertaken (Figures 4.1-4.11).

These tests were conducted in confined and controlled conditions which may display similar conditions to current hotspots of microplastic pollution. However, in freshwater environments these concentrations are unlikely to be common place at current levels of pollution, so experiments that replicate common environmental concentrations are required still. This could be carried out in an experimental design that is closer aligned to the conditions found in a river, with sediment present as well as water flow with lower, more environmentally relevant concentrations. Further studies with other test species that are more sensitive to pollution would be useful, especially at environmentally realistic concentrations that have been found in multiple studies.

5.4 Fibres Ingested

5.4.1 Ingestion of microfibres in *Gammarus pulex*

Ingestion of microplastics is generally regarded as the initial cause of effects associated with microplastics on species and commonly causes blockage of guts, internal damage and affects breathing in multiple species (Cole and Galloway, 2015; Hurley et al., 2017).

Ingested microplastics have been discovered in the guts of amphipods from some of the deepest trenches in the world and attempting to quantify and analyse the effects is an important knowledge gap to fill (Jamieson et al., 2019; Lusher et al., 2017). 84% of deep sea amphipods found in a study had ingested microfibres, suggesting that more amphipods will ingest microfibres at current environmentally realistic concentrations (Jamieson et al., 2019). The amphipods found in deep sea trenches ingested microfibres, ranging from 1 to 8 fibres per individual; additionally, a study in Svalbard found amphipods had ingested on average 72.5 microplastics (Iannilli et al., 2019; Jamieson et al., 2019). In both of these studies, amphipods ingested microplastics in similar quantities to those observed in this study (Figure 4.12) which provides evidence that current concentrations of microplastics may pose a risk to health of amphipods.

As *Gammarus pulex* is a keystone species and a commonly found amphipod, microplastic ingestion could have knock on effects on the aquatic ecosystem by being passed up the food chain to other species (Cedervall et al., 2012; Gerhardt et al., 2011). A study confirmed the possibility of this microplastic transfer through species by testing microplastic ingestion through algae, amphipods and fish species where nanoplastics were transported into fish species through food chains (Cedervall et al., 2012). These fish species (Atlantic Salmon (*Salmo salar))* then found negative effects in behaviour and fat metabolism (Cedervall et al., 2012).

Ingestion was measured by dissecting the individuals and counting the fibres found using a microscope, as shown in Figure 5.1. This may be less accurate than using FTIR spectroscopy, which analyses materials found and their composition to accurately confirm microplastics presence (Horton et al., 2017). However, this requires a lot of resources and handling of samples. An effective and well documented alternative is to count plastic fibres under a microscope identifying a plastic fibre where no cellular structure is evident (Hidalgo-Ruz et al., 2012).

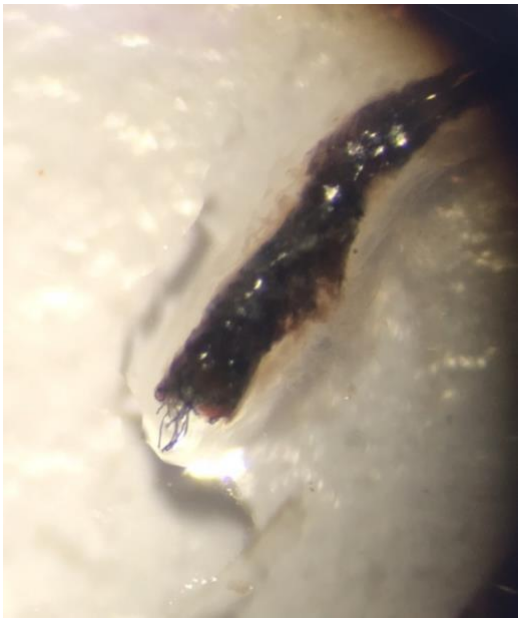

Figure 5.1 The removed gut of a Gammarus pulex individual dissected with polyester fibres in the gut.

5.4.2 Discussion of findings and comparisons

The boxplot shows fibres ingested (Figure 4.12) with polyester fibres being ingested to a greater extent than other fibre types, especially in the high concentration test. One factor that may influence the larger number of polyester fibres is they have a greater density (1.24-2.3g/cm^3), than acrylic (1.09-1.2gcm^{-3}) and nylon (1.02 to 1.052g/cm^3) (Hidalgo-Ruz et al., 2012). This means polyester fibres are more likely to settle on the bottom and therefore are more available where the individuals feed. Additionally, cotton fibres which had a density of 1.54 to 1.562g/cm^3 were ingested in a greater amount than acrylic and nylon. Polyester is found to have one of the highest specific gravity for common synthetic plastics which implies it would be the most readily available plastic for sediment dwelling species such as amphipods (Kershaw, 2015).

Another factor is that polyester fibres were dark navy blue in colour and therefore blended in more to the leaf discs compared to the brightly coloured red and purple of the acrylic and nylon, respectively. This potentially suggests that *Gammarus pulex* individuals avoid consuming brightly coloured fibre types as the second highest cotton also was a less vibrant colour. There is evidence that some crustacean species have colour vision; *Dapnhia magna* were one species, as well as common shrimp (*Crangon vulgaris*), showed strong indications that they used colour vision for particular behaviours such as locating items (Araújo and Malafaia, 2020). This does not prove that *Gammarus pulex* individuals can visually see coloured fibres and actively avoid them, however changes in light from fibres may induce stress in individuals (Araújo and Malafaia, 2020). Studies have revealed that crustaceans and other invertebrates can also detect objects and communicate through visual stimulus (Labhart, 2016). Invertebrates have been found to detect colours and light changes, however this is limited and in water this vision is decreased and therefore it is unlikely individuals would decipher between colours and choose to ingest a particular colour (Warrant and Nilsson, 2006). From this, it is possible that individuals could detect different colours and avoid the red of acrylic, however it is more likely that the greater number of polyester fibres were ingested due to their availability. Individuals may have favoured the polyester due to the colour; a study on deep sea amphipods were found to have ingested blue fibres of different types in greater numbers than other colours (Jamieson et al., 2019). This, however, is likely due to the greater abundance of blue plastic found in the ocean, commonly associated with fishing nets and other common microplastic sources (Desforges et al., 2014).

When removing the polyester results, the ingestion between fibre types is consistent and the data clearly shows that all fibre types and colours are readily ingested by *Gammarus pulex*. Multiple other studies have found amphipod species have ingested microplastic fibres and fragments (Blarer and Burkhardt-Holm, 2016; Halstead et al., 2018; Jamieson et al., 2019; Weber et al., 2018). Blarer and Burkhardt-Holm (2016) concluded that more plastic fibres were ingested as concentration increased, which is confirmed in the results with each higher concentration study having more fibres ingested (Figure 4.12). It was also shown that up to 67 plastic fibres were found in the gut of an individual, similar to the high values found in the Polyester High test in Figure 12 (Blarer and Burkhardt-Holm, 2016). The table below demonstrates that the amount of microplastics ingested in this study is comparative to other literature. The table indicates that the number of ingested microplastics in this study is similar too other ecotoxicity studies and also to microplastics ingested by species in the environment. A similar study on *Gammarus fossarum* species found individuals can either not discriminate between microfibres and alder leaves or can simply not avoid ingesting them when they occur close to feeding locations (Blarer and Burkhardt-Holm, 2016).

Table 5.2 Ingestion of microplastics in a range of species across experimental and occurrence studies.

Study	Species	Ingestion count
This Study (Figure 12) - Experimental	*Gammarus pulex*	Averaged 15.3 fibres ingested over the 72-hour exposure.
(Blarer and Burkhardt-Holm, 2016) - Experimental	*Gammarus fossarum*	10.28 ± 6.27 after 32hours exposure per individual.
(Halstead et al., 2018) - Occurence	Multiple Fish species; Yellowfin Bream, Silverbiddy, and Sea Mullet	249 particles ingested by 40 fish, average of 6 particles per individual over an unknown time period.
(Hurley et al., 2017) - Occurence	*Tubifex tubifex*	A total of 131 particles ingested by 300 worms sampled.
(Iannilli et al., 2019) - Occurence	*Gammarus setosus*	Average of 72.5 microplastics per specimen ranging from 65 and 90. 100% of individuals were found to have ingested microplastics.
(Lehtiniemi et al., 2018) – Experimental	*Gasterosteus aculeatus* and *Mysis relicta*	5.5 ± 6.6 and 3.25 ± 0.6 microplastics ingested on average for species respectively.
(Murphy and Quinn, 2018) - Experimental	*Hydra attenuata*	Averaging 3.44 per individual over an hour's exposure.
(Jamieson et al., 2019) - Occurence	*Hirondella gigas*, *Hirondella dubia*, and *Eurythenes gryllus*	Of the 90 individuals examined 65 contained at least one or more microplastic particles with a total of 122 particles being found in deep sea trench individuals.

(Cole et al., 2015) - Experimental	*Calanus helgolandicus*	Copepod exposed to microplastics ingested 3,278± 306 PS beads per day on average.
(Lusher et al., 2013) - Occurence	*Micromesistius potassou*, and *Aspitrigla cuculus*	50% of fish identified contained ingested plastic. There was on average 1.9 microplastics per individual.

5.4.3 Alternative explanations and future directions

The ingestion of polyester fibres in a much higher quantity than any other polymer or fibre type may be due to differing gut retention of these fibres, which occurs in amphipods (Weber et al., 2018). Gut retention may be different for individuals, meaning they will pass fibres more quickly or slowly, meaning some individuals had large numbers of fibres in their guts when dissected. Gut retention may depend on what the individual has ingested and its composition but also the toxicity of the substance (Taipale et al., 2011). Au et al. (2015) discovered that egestion of the gut contents took 2 to 4 times more than normal when exposed to microplastics in a dose-dependent manner. This means that microplastics will remain in the guts of amphipods longer than organic matter (Au et al., 2015).

Amphipods have been found to have ingested microplastics in a number of areas in aquatic environments, including in deep ocean trenches (Jamieson et al., 2019). This study provides further evidence that *Gammarus pulex* individuals readily ingest microplastic and natural fibres, however future research should focus on the effects of this ingestion to further analyse why ingestion is a problem.

5.5 Feeding Rate

5.5.1 Relevance of using feeding rate in ecotoxicity tests

The analysis of feeding rate in ecotoxicity tests is very important as it allows analysis of sub-lethal effects on *Gammarus pulex* individuals and analysis of more sensitive effects from toxic stimulus (Agatz and Brown, 2014; Felten et al., 2008b). Feeding rate in amphipods can be influenced through environmental and anthropogenic stressors (Nyman et al., 2013). Feeding studies conducted on a few individuals can be adjusted to determine potential trends and threats to whole populations, allowing greater understanding of pollutants to a whole ecosystem (Consolandi et al., 2019). It is important to examine any reductions in feeding for individuals as it effects energy levels and impacts growth, reproduction and population success, therefore is an important endpoint to measure (De Lange et al., 2006).

5.5.2 Discussion of findings and comparisons with the literature

The data highlighted a number of significant reductions in feeding rate, suggesting microfibres are having some sub-lethal effects on the individuals that are exposed. In the Cotton High concentration test and acrylic tests, fibres caused dose-dependent changes in feeding between short-term exposure, for which the difference between control and exposure was significant. This is replicated in literature, with a number of studies into different benthic invertebrate species and microplastics exhibiting similar dose-dependent changes (Haegerbaeumer et al., 2019). In the arthropod species *C. typicus* and *C. helgolandicus* when exposed to polystyrene spheres the feeding rate reduced in a dose-dependent manner (Cole et al., 2015; Cole et al., 2013).

Conversely, 9 out of 11 treatments displayed insignificant differences between control and treatment. This supports previous findings that suggest microplastics do not affect the feeding rate of *Gammarus*

pulex individuals (Weber et al., 2018). Microplastic fibres have been found to not affect feeding rates previously in the literature, such as a study on the marine isopod species Small Scallop (*Idaea emarginata)* (Hämer et al., 2014). The freshwater amphipod *Gammarus fossarum* was exposed to polyamide fibres over a 28 day chronic exposure and feeding rate was not affected (Blarer and Burkhardt-Holm, 2016). For the nylon and polyester tests (Figure 14), the low concentration studies had a greater impact on feeding than the higher concentrations, suggesting this relationship is not dose-dependent, which contradicts findings from a number of toxicity tests on *Gammarus* species (Agatz and Brown, 2014; Felten et al., 2008b). This is the opposite of previous findings in that a dose-dependent effect occurs (Haegerbaeumer et al., 2019).

The variability of feeding rates for the controls was large, from 0.2 to 0.55, similar to the treatments ranging from 0.1 to 0.4 which is a much greater range compared to similar studies (Agatz and Brown, 2014). This could be due to differences in the individuals such as weight and sex or inaccuracies in the measurements, however these were all controlled where possible. Again, this could be due to the shorter duration of the toxicity test which would potentially not allow for trends to fully show in the data, affecting the variation to a greater extent and not allowing a more consistent feeding rate to occur. Reductions of feeding were observed for all but one test but only four tests were significant. This is potentially due to a comparatively shorter exposure time of three days to more commonly seen six or seven days (Agatz and Brown, 2014). The reason for the shorter duration tests was to allow close and regular checks on experiments which would not have been possible over weekends and longer time periods due to laboratory restrictions. The insignificant changes may become significant over a longer exposure period; however additional longer tests would be needed to confirm this.

There are a number of environmental and anthropogenic factors that affect feeding behaviour of *Gammarus pulex* individuals such as temperature, light cycles and humidity, which were controlled as much as possible in the ecotoxicity tests. However, it is noteworthy to examine feeding over a short period as the shock of the contaminant may reduce feeding from the outset of the experiment and some results are significant (Agatz and Brown, 2014). Feeding and growth rate experiments have been carried out from 3-42 day exposures in previous literature (Consolandi et al., 2019). Examining feeding rate changes over a short period has not been carried out extensively for microplastics and the results are noteworthy as it can be concluded that these findings would suggest that microfibres do not have significant effects on feeding over short periods.

5.6 Growth

5.6.1 Relevance of growth and acknowledgements of limitations

Growth is another important sub-lethal effect that can be affected by *Gammarus pulex* individuals being exposed to toxic substances and has been used by other important studies (Maltby and Naylor, 1990; Willoughby and Sutcliffe, 1976). A reduction in growth is expected in ecotoxicity tests involving microplastics as a result of any blockage or obstruction in the gut creating a reduction of feeding (Weber et al., 2018). A study on exposure of imidacloprid to *Gammarus pulex* discovered that changes in feeding and subsequent growth was negatively impacted and as a result of stress responses in individuals which caused mortality (Nyman et al., 2013). These will have less relevance in relation to microfibres, however, the study shows how a toxic substance can reduce growth in *Gammarus pulex* individuals.

Growth is an important factor of a healthy ecosystem as it demonstrates the availability of food sources; reduction in growth of species such as algae has been deemed to have an impact on ecosystem health (Besseling et al., 2014). Changes in growth validates a sub-lethal impact of toxic substances commonly used as an endpoint in ecotoxicity tests (Capolupo et al., 2020). The effects of microplastics and nanoplastics which affect growth on different species will result in less food

availability for keystone species such as *Gammarus pulex*, compounding the issue further up the food chain and affecting the whole ecosystem (Feroz Khan et al., 2012).

5.6.2 Discussion of findings and comparisons

Changes in growth of the individuals are presented in Figure 14; only 4 tests showed a significant effect and the majority of these being low concentration tests. This is similar to the feeding rate, with the high concentration tests not showing significant changes. The tests using cotton both show significant differences between control and treatment, however when comparing these to the rest of the data the growth in the controls was greater than the controls in other tests. This demonstrates that some controls experienced more growth than others, rather than reduced growth in the exposed individuals. This is likely due to anomalies coupled with a short exposure time that may yield different results. The results for synthetic microfibres, other than cotton, show insignificant changes in the data between exposed individuals and the controls, which is likely due to the short exposure time of three days. It could also be that feeding and growth are not affected by the microfibres and additional longer exposures would help clarify the effects of microfibres on *Gammarus pulex*.

In other studies, amphipods have been found to have significant decreases in growth when exposed to microplastics (Au et al., 2015; Blarer and Burkhardt-Holm, 2016; Chae and An, 2017; Ogonowski et al., 2016). *Hyalella azteca* when exposed to acute polypropylene fibres concentrations demonstrated dose-dependent effects on growth as well as ingestion and mortality (Au et al., 2015). Growth in the amphipod *Daphnia Magna* was also found to incur a 3.1% reduction in body size and up to 10.8% reduction in body size in high concentration tests (Besseling et al., 2014). The reduction in body size and growth was attributed to differences in survival strategy (Besseling et al., 2014). In *Chironomous tepperi* negative growth effects were discovered, and found to effect individuals in a size-dependent manner, with smaller particles of polyethylene having greater effects (Ziajahromi et al., 2018). The growth effects on algae species *Dunaliella tertiolecta* were also size-dependent, with nanoplastics having more of a negative impact on growth (Sjollema et al., 2016).

No significant negative effects on amphipod growth were discovered in the amphipod species *Daphnia Magna* when exposed to PET microplastics, for both short term (24hr) or chronic (48d) exposures (Weber et al., 2018). Exposures to other species exhibited negative effects, for example *Xenopus laevis* tadpoles were exposed to high concentrations of polystyrene microplastics but no significant changes in growth occurred (De Felice et al., 2018). Microplastics were also found to have no negative growth effect of larval *Pimephales promelas* (Malinich et al., 2018). Despite these, and other studies, not showing negative effects it is still clear that more research in this area is necessary in response to increasing microplastic pollution (Weber et al., 2018).

5.6.3 Alternative explanations and future directions

Growth is affected greatly by a range of factors, but particularly over a short period by moulting periods that will influence the dried weight of the individual, which is how growth is measured (McCahon and Pascoe, 1988a). However, all individuals were treated in the same way to avoid any potential anomalies. Similarly, the relatively short exposure for this ecotoxicity test means that growth may not be the most appropriate measure, as growth may not be affected over short term (Willoughby and Sutcliffe, 1976).

6 Conclusions

6.1 Summary of research and experimentation

Microplastics are an emerging source of concern due to microscopic fragments and fibres of plastic being found to inhabit the most pristine parts of the world; acting as an anthropogenic marker of human impact on the planet (Ross et al., 2021). In recent years, literature has focussed on the

occurrence and fate of microplastics with the emergence of this research occurring at the beginning of the 21st century and building at fast pace more recently (Thompson and Napper, 2018). Research focusing on the effects of microplastics on ecological species is an area of research that requires further emphasis with gaps in key knowledge areas. This study highlighted effects on freshwater species to be lacking, especially on sub-lethal effects and effects at environmentally realistic concentrations.

The research focus of this study was to address some of these shortcomings by investigating the effects common microfibres, released in wastewater, are having on freshwater ecology, with a specific interest into a test species *Gammarus pulex*. To examine this, ecotoxicity tests were constructed for a range of common microfibres at high and low concentrations. To determine the effects of microfibres on mortality, locomotive activity, ingestion rate, feeding rate and growth rate were all measured over 72 hours.

6.2 Key Findings

From the data gathered there are some key findings. Microfibres at high and potentially environmentally significant concentrations can affect *Gammarus pulex* behaviour. Mortality occurred in all 11 ecotoxicity tests with a potentially dose-dependent trend continuing in the data. High concentrations (0.44g/L) for polyester and the combined fibres incurred the greatest mortality of individuals, with both treatments having a 50% rate of survival (Figure 4.1). LC50 values were made as estimates using the information from the ecotoxicity tests (presented in Table 4.2), giving an idea of the concentrations required to affect populations of *Gammarus pulex* individuals. However, these LC50 values were not examined over a large enough range of concentrations to be accurate and further work is necessary to confirm these results. Mortality was also significantly affected through exposure to cotton, which demonstrates that exposure to natural fibres also has the potential to result in negative effects and needs to be considered in future research.

Microfibres significantly reduced locomotion in all individuals across 10 out of 11 treatments, with low concentrations of cotton fibres being the only test which did not display significant results. The greatest effect on locomotion, an 85% reduction, occurred in the combined fibres treatment (Figure 4.11). This corresponds to the mortality results (Tables 4.1 and 4.2), with the combination of microfibres having the greatest negative affect. Despite significant effects found in all microfibre types, these reductions generally were not dose-dependent, with similar percentage reductions being observed for both concentrations. The locomotive effects were observed when *Gammarus pulex* individuals were exposed to cotton, a natural fibre, which is further evidence that natural microfibres can have similar effects to plastic microfibres and they should not be discounted in future studies.

Microfibres were ingested by all individuals in the exposed treatments, which contributes further evidence to other studies that amphipod species readily ingest microplastics. Polyester fibres were ingested in much greater numbers than any other fibre types.

Feeding rates were reduced in most treatments, however only 3 out of 11 of these reductions were significant. Growth rates followed a similar trend with only 3 out of 11 showing significant reductions.

As relatively little research in this area has been undertaken, the data gathered in this study presents some new contributions that hold significance in the current climate. Specifically, the effects on locomotion in *Gammarus pulex* individuals is significant and a clear indicator of sub-lethal effects of microplastics on a keystone species. Another interesting contribution from this study is that, although at a lesser extent, natural fibres such as cotton also exhibit similar trends as the polymer microfibres indicating that both natural and plastic microfibres are a potential cause for concern.

6.3 Further Recommendations for future work

Microplastic fibres are clearly negatively impacting a keystone species in this study, which at current concentrations could feasibly occur in the aquatic environment. Moreover, microplastic pollution is increasing rapidly with more plastic produced and polluted daily and not set to peak until 2100 (Jambeck et al., 2015). Weber et al. (2018) proposed that future research should attempt to identify common traits in groups of taxa that are affected by microplastics. Additionally, further work should be carried out to standardise methods for microplastic ecotoxicity testing; for example, forming standard units, replication of individuals and methods of calculating toxicological endpoints.

6.3.1 Environmnetally relevant research

A key knowledge gap this study intended to address was the issue of ecotoxicity testing at environmentally realistic concentrations not being carried out in many studies. This study attempted this by using low concetration tests for each microfibre type. However, further improvements can be made and are recommended as a result of this study. A key point brought out was that these studies are water-only tests and did not include sediment in the experimental design. More emphasis in future studies should include a more realistic simulation of current aquatic environmental conditions, taking into account the sediment profiles and water movement. Additionally, aquatic environments include multiple individuals and future studies should look at this aspect to observe competition and accumulation effects. These further tests are important as they would allow a real observation of the current and future potential impacts microplastics are having on aquatic species.

6.3.2 Full dose-dependent toxicity tests

In response to the results in this study, additional work could be undertaken to improve the conclusions drawn. LC50 values could be calculated using a minimum of 5 concentrations with a full dose response and mortality ranging for 0-100% across all treatments. This would be an interesting approach to understanding fully if microplastics have the potential to affect large communities of aquatic species. This could be replicated for all endpoints in this study as creating a full dose profile would allow these relationships to be fully confirmed. These dose-dependent LC50 values could then be used to establish water quality thresholds for water bodies and rivers allowing regulation of microplastic pollution.

6.3.3 Length and standardisation of study

Longer ecotoxicity studies should be carried out when studying feeding and growth responses to gain more complete and thorough results. Additionally, chronic tests over longer periods should be carried out to fully understand long-term effects on mortality and locomotion. Undertaking upwards of 48 day ecotoxicity tests will allow more time for comparable patterns to form in the data collected and will therefore improve the reliability. Conclusions drawn from the data collected in this and other studies could be improved by replicating these experiments and standardising the methodologies to improve comparisons across studies. Specifically, standardisation is required when comparing concentrations used in ecotoxicity tests to the concentrations found in the current environment. This would allow more direct analysis of effects on species that are currently realistic which at a current level is difficult in ecotoxicity studies. Additionally, feeding rate formulas and LC50 calculations can vary greatly, and standard methodologies should be used going forward to allow comparison between studies (Agatz and Brown, 2014).

6.3.4 Other recommendations of research

Further research into the characteristics of microplastics used would help reduce literature gaps. A comparison of fibres, spheres and fragments that are found in the natural environment would be useful to identify which microplastics are most of a concern in aquatic species. Also, ecotoxicity tests using

microplastics have tended to concentrate on the plastic itself causing the lethal and sub-lethal effects; future research should focus on microplastics as vectors for chemicals and bacteria.

In the future, greater numbers of species will likely be impacted by microplastics and gaining knowledge about the effects on more species is important, especially if they provide a significant ecosystem benefit. The research presented in this study, with some changes, could be replicated on other important species which act as biomarkers for water quality. Stonefly and Mayfly species that are commonly found in areas of good water quality with low levels of pollution could be exposed to microplastics to gain greater understanding of how microplastics affect more pollution sensitive species.

Finally, further human impact on the world is a certainty, despite growing calls to improve society's sustainability and reduce climate impacts. Current strategies in place are not drastic enough to reduce plastic pollution and its contribution to ecological breakdown and the anthropegenic impacts humans are causing. The COVID-19 pandemic has provided proof that humans' response to global issues hinges on the use of unsustainable and disposable products which further indicates society's inability to significantly reduce plastic pollution. Predicted growth in plastic use is widely expected to far outweigh mitigation of this issue and microplastic pollution will increase for the future decades (Borrelle et al., 2020; Geyer et al., 2017). Vital work should be carried out on predicting future microplastic concentrations and levels of pollution, as well as researching more dedicated waste solutions to reduce the amount of plastic released into the aquatic environment in the future.

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