Assessing the influence of fibre type and biofilms on the ingestion and retention of microfibres by freshwater invertebrates.

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Content

1. Table of Figures

Table of Tables

Abstract:

Microfibre pollution is an escalating environmental issue, with natural fibres found in greater abundance than their synthetic counterparts in the environment. This study examines the ingestion and retention of microfibres by two freshwater macroinvertebrates, *Gammarus pulex* and *Corbicula fluminea*, under varying conditions of fibre type, biofilm presence, water turbidity, and exposure time. Ecotoxicity tests revealed significant ingestion of both polyester and cotton microfibres, with species-specific retention and ingestion patterns that differed with fibre type. For *G. pulex*, polyester fibres exhibited longer retention, while cotton fibres were ingested in larger quantities. The presence of biofilms enhanced fibre ingestion, indicating a key role in retention. Notably, *G. pulex* continued ingesting microfibres over one- and two-week exposure periods, suggesting no learning avoidance of microfibre consumption. C. fluminea ingested consistently low number of microfibres throughout all experiments, suggesting that selective feeding mechanism of suspension feeding bivalves may limit the risk of microfibre ingestion. These findings underscore the environmental risks posed by both natural and synthetic microfibres and highlight the need for further research, particularly on natural fibres, to assess their ecological impacts at environmentally relevant concentrations.

1.0 Introduction

Plastic fragments smaller than 5 mm, known as microplastics (MPs), can cause a variety of ecological and environmental problems, including for example, acting as a vector for pathogens (Wagner et al., 2014) and other pollutants (Tumwesigye et al., 2023), altering soil pH (Qi et al., 2020; Li et al., 2021), and having myriad impacts on the behaviour and physiology of organisms (Gola et al., 2021). MPs in the environment typically represent a range of polymer types, particle sizes and particle shapes. Of the different MP shapes, microplastic fibres (MPFs) dominate environmental MP samples (Dris et al., 2017). MPFs are commonly released from synthetic textiles; for example, as many as 90% of MPs in oceans are believed to be derived from washing and drying synthetic textiles (Gaylarde et al., 2021). However, their abundance does not represent the entire textile fibre pollution problem.

In addition to MPFs, the environment is also a sink for many natural microfibres (NMFs), which are originally derived from plant (e.g. cotton, hemp, jute) and animal (e.g. wool, cashmere, silk) products and processed for anthropogenic use. NMFs are also an area of environmental concern because they are typically more abundant than their synthetic counterparts in the environment (Dris et al., 2017; Stanton et al., 2019), prolific throughout marine (Barrows et al., 2018) and freshwater (Miller et al., 2017) ecosystems. However, research into the impacts of NMFs is much more limited than for MPFs, and environmental risks are poorly understood.

Of the environmental threats posed by microfibres of both natural and synthetic origin (MFs), their ingestion by organisms has generated significant scientific, public, and political concern. While the ingestion of MFs, both of natural and synthetic origin is under-researched, it has been found that MFs consistently cause greater toxicity when ingested than other MP morphologies (Gray and Weinstein, 2017; Ziajahromi et al., 2017; Hodgson, 2019; Qiao et al., 2019). MFs are also ingested in greater numbers than other shapes of MP in the field (Windsor et al., 2019; Bertoli et al., 2022); however, this is likely attributable to their abundance in the environment rather than MFs being preferentially consumed. Regardless of the cause, the greater ingestion of MFs and current toxicity findings suggest that they pose a greater threat to wildlife than any other shape of MPs. There is therefore a pressing need for further research to better understand the risks that all MFs pose.

Ingestion rates may also be influenced by environmental factors, such as water turbidity and the presence of biofilms on fibres. Turbidity can reduce the visibility and detectability of particles, potentially altering ingestion patterns in aquatic organisms. High levels of suspended solids have been found to affect ingestion rates in aquatic invertebrates (e.g. Arruda et al., 1983; Aldridge et al., 1987). Similarly, biofilms, that readily form on MF in aquatic environments, can modify the physicochemical properties of microparticles, which can alter their ingestion by organisms (Fabra et al., 2021). Given that ingestion is a critical factor in assessing the ecological impact of MFs, understanding how environmental conditions influence ingestion dynamics is essential for evaluating their broader environmental risks.

While the ingestion of MFs is an important determinant of their potential ecological effects, their egestion is potentially more important. This is because it determines the length of time such particles will remain in an organism and cause adverse effects (Klein et al., 2021), as well as the potential for trophic transfer through food webs (Windsor et al., 2019). Despite this, the egestion of anthropogenic particles, particularly MFs, is not well understood or reported.

Studies focused on the egestion of MPs have demonstrated that MPs are egested by a range of organisms from fish (Ory et al., 2018; Xiong, 2019) to macroinvertebrates (Redondo-Hasselerharm, 2018). However, the time taken from ingestion to egestion is significantly longer for MPs than food pellets (Ory et al., 2018), suggesting that these particles are processed differently, perhaps getting stuck in the gastrointestinal tract.

It has been suggested that the shape of the ingested particulate impacts their egestion, with irregularly shaped MPs likely to take longer to egest (Redondo-Hasselerharm, 2018). As MFs are irregularly shaped, it follows that they would take longer to egest than other particle morphologies, as supported by Xiong et al. (2019), who found that MP filaments took longer to be egested than other MP shapes. The long time for egestion may also be caused by MFs becoming tangled with one another, or other material, in the gastrointestinal (GI) tract. Ignoring the diversity in the varied characteristics of MFs could lead to oversimplified conclusions regarding their ecotoxicology, and potentially skew current sustainability narratives within the fashion industry. Therefore, assessments of all fibre types, both natural and

synthetic, is essential to avoid misrepresentation and minimise the risk of greenwashing, ensuring more accurate representations of the environmental risks of MFs.

As the shape of particles is known to impact their egestion, it can be hypothesised that there will be a difference in the egestion of natural and synthetic fibres. Cotton fibres have a twisted ribbon-like structure, different to the uniform cross-section and smooth surface of plastic fibres, such as polyester fibres, which are extruded. Other natural fibres such as wool have similarly irregular shapes, yet the difference in the egestion of NMFs and MPFs has not been compared, with little research looking at NMFs. This leaves a significant knowledge gap regarding the egestion of NMFs by primary consumers, of critical importance for understanding the potential for their biomagnification and adverse biological effects throughout the food chain. This is particularly true given that the abundance of NMFs has been shown to be much higher than MPFs across multiple studies (eg. Dris et al., 2017; Stanton et al., 2019). This MRes attempts to fill these gaps by investigating and comparing MPF and NMF egestion by freshwater macroinvertebrates at environmentally relevant MF concentrations, and considering fibre condition, water turbidity and environmental exposure time.

Aim and Objectives:

The aim of this MRes is to explore the influence of fibre type, biofilm formation and water turbidity on MF ingestion and retention by two freshwater invertebrates, representing differing feeding strategies. This will be achieved by addressing the following objectives:

1. To assess the time for MFs to be egested by *Gammarus pulex* and *Corbicula fluminea*.

2. To determine how MPF and NMF retention is related to ingestion.

3. To compare the ingestion and retention of NMFs and MPFs.

4. To assess the impact of biofilms on MF ingestion and retention.

5. To assess the impact of turbid water on MF ingestion and retention by a filter feeding bivalve.

2.0 Literature Review

2.1 Plastics:

Plastics are synthetic materials made from polymers, which are chemical compounds of repeated units known as monomers bonded together to form long chains (Gad, 2014). The properties of plastics can be greatly influenced by the number and type of monomers, the polymer's structure, and the additives used (Fergusson and Staudinger, 1973; Baker and Mead, 2000), resulting in a diverse array of polymers suitable for various applications. Most modern plastics consist of carbon-based monomers derived from petrochemicals, making them highly resistant to degradation through chemical and physical weathering processes.

The 20th century saw the advent of plastics after the invention of Bakelite by Dr Leo Baekeland in 1907 (Mossman, 1997), with innovations throughout the 20th and 21st century, resulting in thousands of different plastic polymers. The expansion of plastic production was rapid, and by the 1960’s plastics were commonplace in modern life (Mossman, 1997). In 2011, the demand for plastics was almost 200 times that in 1950 (PlasticsEurope, 2012). The increase in plastic production has not been matched by innovations in their end-of-life processing, meaning that vast quantities of plastics are now entering the environment due to inefficient and unsuitable waste management practices. Despite this, the cost-effectiveness, versatility, and durability of plastics has led to their widespread use as an alternative to other materials (Fergusson and Staudinger, 1973; Hammer et al., 2012). This includes natural fibres for textile applications, with 14% of plastic production attributed to fibres alone (Bartl, 2020).

2.1.1 Plastic Pollution

Initially celebrated as revolutionary materials, plastics are now associated with environmental pollution (Klemeš et al., 2021). Almost half of all non-fibre plastics are single use (Gibb, 2019), with most plastics having a life span of less than a year (Zaman and Newman, 2021). At the end of its useful life, only 9% of plastic waste is recycled (Ritchie et al., 2018), with the majority either incinerated or disposed of into unsuitable waste management systems that release up to 12 million metric tons of plastic to the ocean every year (Jambeck et al., 2015). By 2015, it was estimated that 79% of all plastic waste produced since 1950 was either in landfill or in the

natural environment (Geyer et al., 2017), and by 2050 it is projected that there will be more plastic in the ocean than fish (Ellen MacArthur Foundation, 2016).

Once in the environment, plastic debris can harm wildlife through an array of mechanisms; the two most notable being entanglement and ingestion. Entanglement is typically a more visible impact of plastic pollution, and is reported to suffocate and drown wildlife (Allen et al., 2012; Hiemstra et al., 2021), reduce their mobility (Dar et al., 2022), and impair their ability to catch prey (Derraik, 2002) and avoid predators (Gregory, 2009; Pawar et al., 2016). Although more difficult to detect and research, plastic ingestion has been documented since the 1960s (Kenyon and Kridler, 1969), harming organisms through mechanisms that include perforating and blocking digestive tracts (Mascarenhas et al., 2004; Wilcox et al., 2018; Panti et al., 2019; Foskolos et al., 2023), creating a false feeling of satiety leading to starvation (Pierce et al., 2004; Danner et al., 2009), and bioaccumulating toxic chemicals in organisms' tissues (Derraik, 2002; Besseling et al., 2013; Rochman et al., 2013; Peng et al., 2021). Plastics also adsorb chemicals and persistent organic pollutants (POPs) from the surrounding medium (Rios et al., 2010; Hirai et al., 2011; Rochman et al., 2013), concentrating them to levels potentially harmful to organisms (Andersson, 2014).In addition, hundreds of plastic-associated chemical compounds are toxic (Groh et al., 2018), although plastic additives have unknown or disputed environmental impacts (Andrady and Neal, 2009; Hahladakis et al., 2018). The ingestion of plastic debris is now reported in wildlife worldwide, with over 90% of seabirds having plastic in their stomachs (Van Franeker et al., 2011; Wilcox et al., 2015), and more than 300 fish species documented to have ingested plastics (Markic et al., 2020).

The abundance of plastics in the environment is not due to them being produced and disposed of in greater amounts than other anthropogenic products, but instead due to their persistence (Andrady, 2015). Most plastics do not biodegrade; instead, they break down through exposure to UV rays or fragment into smaller pieces through other mechanisms of chemical, physical, and biological weathering, reducing the size of polymer chains until they become metabolizable. This allows them to persist in the environment for decades. Consequently, even if inputs are stopped entirely, plastic pollution would continue to be widespread in global environments for centuries (Barnes et al., 2009).

2.1.2 MPs

The scale of plastic pollution has changed over time. While plastic accumulation in the environment and interactions with organisms have been increasing in the past few decades (reviewed by Barnes et al., 2009), the size of plastics in the environment is reducing (Barnes et al., 2009). The fragmentation of plastics through biological, chemical, and mechanical processes presents a further threat to ecosystems, creating tiny plastic particles (Rillig, 2012; Zettler et al., 2013; Andrady, 2015) that can be ingested by the smallest of organisms, and persist in every environmental niche on earth.

Microplastics (MPs) are plastic particles smaller than 5 mm in their longest dimension, originating from both primary and secondary sources (Frias and Nash, 2019). Primary MPs are those manufactured as such, for use in household and industrial products (Cole et al., 2011; Boucher and Friot, 2017), while secondary MPs result from the breakdown of larger plastic debris through biological, chemical, and mechanical processes (Rillig, 2012; Zettler et al., 2013; Andrady, 2015). The proliferation of MPs throughout the environment has been documented on every continent (Agamuthu, 2018; Cera et al., 2020), even in remote and uninhabited islands (Martins et al., 2020; Nichols et al., 2021). The impacts of MP pollution are similar to those of larger plastic debris but their small size presents a new threat. MPs are more biologically available, with organisms ingesting and inhaling them, facilitating the trophic transfer of MPs (Farrell and Nelson, 2013; Caruso, 2019), and damaging olfactory and respiratory systems (Verla et al., 2019; Shi et al., 2021).

Secondary microplastics typically constitute the majority of microplastics found in the environment (Burns & Boxall, 2018) and include plastic textile fibres. Despite their dominance in environmental samples, a greater focus has been placed on primary microplastics in the academic literature, the media, and government policies (Gouin et al., 2015). This focus is likely caused by the tendency to simplify MPs into a single, cohesive pollutant, leading the public and policymakers to believe that rinse-off cosmetic products and other now banned MP sources were the largest, if not only, contributors of MPs to the environment (Rochman et al., 2019). As a result, other sources of MPs, particularly from secondary sources, are often overlooked. The lack of clarity about different types of MPs may stem from the inconsistencies between studies, with the number of shape categories ranging from 4 to as many as

10 (Hidalgo-Ruz et al., 2012; Frias and Nash, 2019; Rochman et al., 2019; Edo et al., 2021) and lacking in standard definitions.

2.2 Fibres

There are three main categories of fibre - natural fibres (plant and animal-based fibres), synthetic fibres (mainly formed from extruding petrochemical products), and regenerated fibres (extruded fibres with a natural polymer, e.g., cellulose, starting material). The unique structures of each fibre type give them varied properties and uses, making them as ubiquitous in daily life as plastics.

While natural fibres have been utilized for over 10,000 years (Mwaikambo, 2006), synthetic fibres were only introduced in the past two centuries (Loasby, 1951). Today, synthetic and regenerated fibres account for 69% of global fibre production, compared to just 29% for natural cellulosic textile fibres (TextileExchange, 2020). Unfortunately, as with plastics, unsustainable processes of textile production and disposal mean large volumes of fibres pollute the environment every day.

2.2.1 Fibre pollution

Fast fashion has created a culture of frequent purchase and disposal of clothes (Birtwistle and Moore, 2007), with some items thrown out after just a few or even no uses (Laitala and Klepp, 2015). Previous estimates have suggested that, in the UK, the average consumer disposes of 30 kg of textiles annually (Allwood et al., 2006).

While some clothing and textiles are recycled and repurposed, with significant volumes in the UK donated to charity shops where they are either sold, recycled, or sent abroad to be reused (Birtwistle and Moore, 2007; Farrant et al., 2010; Allwood et al., 2006), textile recycling remains difficult. Only around 1% of textiles are recycled annually (Cotton et al., 2020). Complications in recycling textiles stem from issues such as sorting fibre blends (Hawley, 2009) and the degradation of fibres during the recycling process (Bukhari et al., 2018). This means most textiles at the end of their useful life ultimately end up in landfill. Even before disposal, fibre production causes significant environmental pollution with textile dyes released into aquatic environments known to be toxic throughout the food chain (Zhang et al., 2013; Kaur and Dua 2015), including for humans for whom dyes can be carcinogens (Mehra et al., 2021).

The textile industry, like the plastic industry has recently seen a move towards sustainable fibre alternatives, particularly for clothing, with a push for increased use of natural fibres. However, natural fibre alternatives are not always more sustainable. While the production of a cotton shirt has a carbon footprint of less than half that of a polyester shirt (Fairtrade Foundation, 2020), natural fibres such as cotton are highly water dependant and reliant on pesticides, needing almost 3000 L of water to produce just one t-shirt and accounting for 6% of global pesticide use despite using only 2.4% of global arable land (Fairtrade Foundation, 2015). There has been a move towards more sustainable growing practices of cotton, yet as of 2019 only 25% of cotton grown complied with at least one voluntary sustainability standard (IISD, 2023), and in 2021 only 1.4% was organic (TextileExchange, 2022). Therefore, a push towards greater use of cotton due to its status as a natural fibre may be misleading customers regarding sustainability.

Fibres are rarely bonded together to create a product and are instead woven or twisted. This allows fibres to shed from fabrics and break down over time into MFs. As with MPs, the small size of MFs increases their bioavailability, and their presence in the environment is now becoming an area of increasing concern.

2.2.2 Microfibres

Although lacking in a standardised definition, Liu et al., (2019b) proposed that MFs are fibres less than 50 μm in diameter and 5 mm in length. Despite being derived from natural sources, conventional natural textile fibres are not a natural product. The natural fibres used in modern applications undergo processing that alters their properties, such as the addition of dyes and additives, which removes them from their natural state (Wagaw et al., 2024), making them an anthropogenic product.

MFs dominate samples of MPs, often accounting for upwards of 70% of the particles recorded in microplastic studies (e.g. Desforges et al., 2014, Barrows et al., 2019; Suaria et al. 2020b; Kanhai et al., 2016; Brahney et al., 2020; Huang et al., 2021; Liu et al., 2019a; Wang et al., 2020; Welsh et al., 2022; Wright et al., 2020; Soltani et al., 2021, 2022). Although the environmental impacts of MF pollution are comparable to those of other MP shapes, MFs consistently exhibit higher toxicity when ingested (Gray and Weinstein, 2017; Hodgson, 2019; Qiao et al., 2019). MFs are thought to pose a larger threat to wildlife than other forms of MPs because of their greater

abundance (Windsor et al., 2019; Bertoli et al., 2022), therefore demanding research to better understand the risk they pose.

There are a wide variety of sources of MFs, from personal care products such as wet wipes (Briain et al., 2020) to carpets (Soltani et al., 2021). MF pollution increased sharply during the COVID-19 pandemic, partly due to the widespread use and improper disposal of face masks (Saliu et al., 2021; Shruti et al., 2020; Fadare & Okoffo, 2020). Cigarette butts are one of the most littered items globally (Kurmus and Mohajerani, 2020) and typically contain a cellulose acetate (CA) filter designed to limit the carcinogenic load on smokers, but are also a significant source of MFs in the environment (Shen, 2021). Cigarette filters each contain more than 10,000 filaments of poorly biodegradable CA (Hengstberger and Stark, 2009; Novotny et al., 2009), which fragments in the environment (Novotny et al., 2009), releasing around 100 CA MFs per butt per day (Belzagui et al., 2021). Once smoked, cigarette butts contain heavy metals and chemicals which leach into the environment when littered and are known to be toxic, altering the behaviour, growth rate, reproduction, and mortality of organisms who interact with them (Moroz et al., 2021). The total volume of MFs released from cigarette butts per year is potentially as significant as that from household laundry (Belzaugi et al, 2020), which is often considered as one of the greatest sources of MFs.

MFs are shed from clothing throughout their production (Zhou et al., 2020; Chan et al., 2021; Dhir et al., 2021), use (De Falco et al., 2020; Palacios-Mateo et al., 2021), washing (Napper and Thompson, 2016; Athey et al., 2020), drying (Kapp and Miller, 2020, Tao et al., 2022), and even after their disposal (Sun et al., 2021). The washing of textiles is a well-researched source of MFs, with upwards of 70% of people in developed countries owning a washing machine (Statista, 2023). Washing machines cause textiles to shed MFs, which are then fed into wastewater systems. Although wastewater treatment plants (WWTPs) have MP and MF removal rates reported to be upwards of 70% (Murphy et al., 2016; Talvitie et al., 2017; Magnusson and Noren, 2014; Carr et al., 2016; Sol et al., 2021), the sludge in which MPs and MFs are captured in during the secondary and tertiary treatment processes is often used as fertilisers on farmland. Agricultural soils are now suggested as a significant sink for urban MPs (Nizzetto et al., 2016), with MFs found to persist at sites where sludge has been applied 15 years later (Zubris and Richards, 2005). The relatively small

percentage of MPs and MFs that are not removed during treatment still represent a substantial volume of particles released into the environment, with MFs accounting for up to 90% of the total plastic mass in WWTPs (Gaylarde et al., 2021). In many countries sewage is untreated, with 80% of industrial and municipal wastewater globally remaining untreated before being released into the environment (Duis and Coors, 2016; WWAP, 2018). In developing regions where washing machines are less common, textiles are washed by hand (Götz and Tholen 2016), often releasing these fibres directly into the environment (KeChi-Okafor et al., 2023).

Even before reaching the WWTP, MFs from domestic washing of textiles pollute the environment, accounting for up to 35% of atmospheric MF pollution (Mishra et al., 2020). Once washed, clothes may be dried using a tumble-dryer which is itself a source of MFs which, depending on the type of dryer, can be released into wastewater treatment infrastructure or the atmosphere (O'Brein et al., 2020; Kärkkäinen and Sillanpää, 2021). The release of MFs from the domestic laundering of clothing is so significant, that as many as 90% of MPs in oceans are believed to be derived from washing and drying of synthetic textiles (Gaylarde et al., 2021).

Despite increased research into MF sources, there remain gaps in our knowledge due to the difficulties in quantifying and investigating non-point sources. Research by Carr (2017) suggests that the active use of textiles and MF shedding accounts for a larger component of MF pollution than WWTPs, supporting the theory that non-point sources are important contributors to MF pollution. Similarly, although the most researched source of MF is textiles, there has been research suggesting other products such as carpeting and personal care products may be greater sources than textiles (Belzagui et al., 2021); however, more research is needed to confirm this.

MFs and MPs are transported throughout and across environments through a variety of mechanisms. The small size of MPs allows them to be transported vast distances in the atmosphere (Gasperi et al., 2018), often deposited in rain (Dris et al., 2016), allowing them to contaminate even the most remote areas, and those protected from humans (Brahney et al., 2020). MFs are also easily transported once present in the aquatic environment due to their small size and weight, making them readily transportable by flowing water.

Despite MPFs accounting for over half of all textile fibres produced (TextileExchange, 2020), NMFs are being found in greater number than their synthetic counterpart in the environment (Dris et al., 2017; Stanton et al., 2019). However, research into the interactions between organisms and NMFs in the environment and their impacts is even more limited than for MPFs.

2.2.3 Natural Microfibres

Natural microfibres (NMFs) are fibres of natural origin that have been anthropogenically modified, often through the addition of dyes, additives, and other treatments. These fibres encompass a range of types, including protein-based animal fibres (e.g., wool, silk), cellulose-based vegetable fibres (e.g., cotton, hemp; often referred to as cellulosic fibres), as well as mineral fibres such as asbestos and glass. Cellulosic MFs in marine waters have been reported since the mid-20th century (Atkins et al., 1954; McAllister et al., 1960), while those of synthetic origin were first reported in water samples in the late 20th century (Bucanan, 1971). Because of the perception that NMFs degrade and are potentially even bioavailable, most research investigating MFs does not report the proportion of cellulose-based fibres found in their samples (Athey and Erdle, 2022; Finnegan et al., 2022). Although the reason for the abundance of NMFs is not understood, it may be in part due to cellulosic fabrics shedding in greater amounts, therefore producing more MFs than synthetics (Zambrano et al., 2021, Sillanpää et al., 2017). For example, cotton – a cellulose based fibre – is a staple fibre which are relatively short, while textile applications of polyester are regularly as long filament fibres. The tensile strengths of cotton and polyester fibres also differ (Napper and Thompson, 2016). The shorter length and lower tensile strength of some natural fibres therefore mean they can shed more easily from textiles. NMFs are also not as biodegradable as commonly stated, with natural and regenerated fibres able to persist for months to decades in the environment (Belzagui et al., 2021; Puls et al., 2011; Turner et al., 2019; Zambrano et al., 2020), as dyes and treatments alter biodegradability and increase fibre persistence in the environment (Park et al., 2004; Li et al., 2010; Puls et al., 2011; Sait et al., 2021; Sørensen et al., 2020; Zambrano et al., 2021). The abundance of NMFs in the environment is not mirrored by an abundance of research, so that significant gaps in our knowledge remain regarding the

ecotoxicology of these fibre types, and their interactions with organisms in the environment.

2.3 Geographies of MP and MF

Research into plastic pollution has been concentrated on developed nations (Blettler et al., 2018), but many of these countries export their plastic waste to nations with more lenient waste management policies (Liu et al., 2018). This has created disparate geographies of plastic pollution whereby the countries contributing the greatest volumes of plastic waste see the least impacts (Wang et al., 2020), and the countries seeing the largest environmental impacts, most often developing nations, are comparatively under-researched. This trend can also be seen with textile pollution with 70% of all clothes donated to charity exported to Africa, much of which ends up in landfill (Kubania, 2015). As a result, these regions may become hotspots for MP and MF pollution, as improper waste management and the disposal of textiles contribute to the accumulation of these pollutants in the environment.

Urban areas have been identified as hotspots for high MP and MF concentrations due to high population density and industrial activities (Barnes et al., 2009). Research on the Laurentian Great Lakes recorded concentrations ranging from 0 to over 450,000 particles km-2, depending on proximity to urban areas (Eriksen et al., 2013). Atmospheric deposition of MFs is also significantly higher in urban environments compared to suburban ones (Dris et al., 2018). That said, despite lower levels of human activity and reduced sewage input, rural and less populated areas also experience notable levels of MP and MF pollution, highlighting the pervasive and far-reaching nature of these pollutants (Lourenco et al., 2017).

Research into MP and MF pollution has predominantly focused on marine environments, with both recorded in every ocean and marine habitat on earth (do Sul et al., 2014; Lee, 2015; Gago et al., 2018). Only 3.7% of studies on MPs in 2017 focused on freshwater environments (Wagner & Lambert, 2018) despite rivers being known to act as a conduit transporting these pollutants from land to marine environments (Bowmer & Kershaw, 2010; Lebreton et al., 2017). Even fewer studies address terrestrial environments (Rillig, 2012) despite evidence that agricultural soils are significant sinks for urban MPs (Nizzetto et al., 2016) and that terrestrial sources account for the majority of plastic pollution in the oceans (Barnes et al., 2009; UNEP,

2009; Jambeck et al., 2015). Despite a recent growth in research on terrestrial and freshwater environments, large gaps remain in our understanding of plastic and textile pollution, and their micro counterparts, in these environmental compartments.

2.3.1 Typical Environmental Concentrations

MP concentrations have been studied in both freshwater and marine environments; however, the reported concentrations vary drastically between studies and regions. Research on MFs is comparatively limited. A lack of standardised methods for sampling MPs and MFs (Hidalgo-Ruz et al., 2012) and reporting findings also complicates comparisons between studies (Ryan et al., 2020). In some cases, researchers have deliberately excluded MFs from analysis due to the challenges in preventing contamination such as from atmospheric deposition and cross-contamination from other sources (e.g. Foekema et al., 2013; Kuhn et al., 2020). Estimating MP concentrations in an environment by extrapolating data that ignores temporal variations can misrepresent the actual abundance by orders of magnitude (Stanton et al., 2020). Changes in weather conditions have also been found to impact the reported MP and MF concentrations in marine samples, which again hinders our understanding of the true concentrations of these particles in the environment (Ryan et al., 2020).

2.3.1.1 Marine Concentrations

In marine environments, MP and MF concentrations show wide variability, even across the same ocean. For example, Desforges et al. (2014) reported MP concentrations ranging from 8 to 9,180 particles/m² in the northeast Pacific Ocean, with lower concentrations observed at offshore locations. MFs exhibit similar variability. Barrows et al. (2016) found average MF concentrations of 10 fibres/L across global oceans, with the Arctic Ocean exhibiting the highest (31.3 MFs/L) and the Indian Ocean the lowest levels (4.2 MFs/L). Notably, open ocean samples consistently showed higher concentrations than coastal samples (Barrows et al., 2018).

The concentrations of MP and MFs also vary vertically within the water column. MFs have been found to be more abundant in sea surface waters than when sampled in waters at depth (Reisser et al., 2015), with La Daana et al (2017) reporting less than 1 fibre/L at a depth of 11 m in the Atlantic Ocean. However, MFs have also been

found in higher concentrations in deep-sea sediments than at the ocean surface (Woodall et al., 2014), demonstrating the complexity of their distribution in marine environments. Despite differences in density, MPs of varying specific gravities can be found throughout the water column, caused by the cyclical movement of these particles through a number of processes, including the vertical and horizontal dispersion due to the stratification of the water (Isobe et al., 2014; Law et al., 2014), wind mixing events (Kukulka et al., 2012; Reisser et al., 2015), and biofouling (Moret-Ferguson, et al., 2010; Kooi et al., 2017).

2.3.1.2 Freshwater Concentrations

Different freshwater ecosystems have variable MP and MF concentrations. A review by Frank et al. (2022) on Russian inland waters highlights this range, reporting concentrations as low as 0.007 particles/m³ in the mouth of the Northern Dvina River, and as high as 11,000 particles/m³ (11 particles/L) in the Altai lakes. This wide variation is often attributed to differences in sampling methodologies. For instance, the Northern Dvina River study employed a 200 µm neuston net at a depth of 20 cm (Yakushev et al., 2021), inherently excluding particles smaller than 200 µm, whereas the Altai lakes were sampled at a depth of 30 cm using 5 L glass jars (Malygina et al., 2021), capturing particles of all sizes. This discrepancy in sampling techniques reflects the broader challenge of obtaining accurate and comparable data across freshwater systems globally. A review of research on MP concentrations in worldwide lakes found a median value of around 1.4 MPs/L (Dusaucy et al., 2021).

This variability highlights the limitations posed by inconsistent sampling protocols. The type of sampling equipment, mesh size, and depth all influence the recorded concentrations, making cross-study comparisons difficult. This inconsistency highlights the need for standardised methods in MP and MF research to provide a clearer understanding of their prevalence in freshwater ecosystems.

2.3.1.3 Sampling methods

The variability in estimated MP and MF concentrations can be partly attributed to the limitations of current sampling methods. Cutroneo et al. (2020) found that nets were the most common sampling tool for MPs in the marine environment, used in 61% of studies prior to 2019, with a mesh size of 333 µm being the most common. However, the use of these nets means that many studies are underestimating the total MP

abundance, as particles smaller than 333 μm are excluded. Studies using nets with smaller mesh sizes (<100 μm) have reported significantly higher MP abundances compared to those using larger meshes (Gorokhova, 2015; Noren, 2007; Enders et al., 2015; Nel & Froneman, 2015; Kang et al., 2015). Trawling using neuston and manta nets can also underestimate the concentration of MPs due to their bias against MFs which, being less than 50 um in diameter, can be thin enough to pass through even the smallest mesh sizes.

While MFs are widely agreed to dominate environmental samples of MPs, the proportion of natural fibres reported varies, with between 30-90% of microparticles in samples reported to be natural fibres (Suaria et al., 2020b; Barrows et al., 2018; Soltani et al., 2021). The variance in NMF abundance may be due to an inability of current techniques to identify either synthetic or natural fibres, particularly when a biofilm is present. The growth of bacterial and fungal communities on the surface of fibres can interfere with detection methods, distorting signals and altering surface morphology, which complicates accurate identification. Barrows et al. (2018) found that several fibres, initially classified as synthetic, were later reclassified after further analysis. This suggests that similar misidentifications may occur in other studies, leading to an underestimation of NMF prevalence.

NMFs are also often actively excluded from MP analysis due to difficulties in separating these particles. Chemical digestants used to isolate MPs can damage and degrade natural and semisynthetic fibres (Dehaut et al., 2016; Treilles et al., 2020). Because of the difficulties in working with NMFs, current environmental concentration estimates for NMFs are limited.

Due to the abundance of MPFs in the environment, as well as the difficulties involved in identifying and manipulating them, as well as in differentiating them from NMFs, it has been suggested that they should be considered as a distinct category of anthropogenic pollutant separate to other MP shapes (Ryan et al., 2020).

2.4 Ingestion of MPs and MFs

MPs, MPFs, and NMFs are ingested by organisms through two primary mechanisms: accidental ingestion due to unselective feeding or mistaking them for food (Wright et al., 2013), and indirect digestion through consumption of

contaminated prey (Ryan, 2019). Once ingested, the smallest MPs and MFs have the potential to translocate, moving from the gut into the tissue and organs of organisms (Kershaw, 2015), with particles smaller than 150µm being most likely to translocate (Duis and Coors, 2016). The movement of MPs and MFs beyond the gut into other tissues can increase the risk of chronic and potentially more significant harm to organisms.

The dominance of MFs in the environment is mirrored by the particles ingested by organisms, with animals across trophic levels ingesting a greater number of MPFs than other MP shapes (Li et al., 2016; Khedre et al., 2023; Villagran et al., 2020 Compa et al., 2018 Hara et al., 2020; Rebelein et al., 2021; Lourenço et al., 2017). MPFs have also been found to cause higher mortality rates once ingested than other forms of MPs (Gray and Weinstein, 2017). While MP ingestion is often restricted by size, because organisms have limits to the size of particle they ingest, MFs can be ingested even when their length exceeds the size limit that the organism would typically consume because of their small diameter. For example, Jemec et al. (2016) reported a fibre of 1400 µm in length in the gut of Daphnia magna despite daphnids being thought to have an upper limit for MP ingestion of 50 µm. This suggests that MPFs pose a greater risk than other shapes of microplastics, as their length does not preclude their ingestion.

Predators are often regarded as being at high risk of MP and MF bioaccumulation (Kühn et al., 2015; Lipej et al., 2022; Roman et al., 2022) and MPs have been shown to transfer across trophic levels (Watts et al., 2014; Miller et al., 2020). However, the degree to which they bioaccumulate is debated. Garcia et al., (2021) suggest that these particles primarily are ingested through direct consumption, rather than bioaccumulating. There is also little evidence of MP and MF biomagnification, suggesting that higher trophic levels may not be at greater risk from MPs than lower trophic levels (Bour et al., 2018; Gouin, 2020).

A trend repeatedly found through the literature shows plastics of certain colours, shapes, and sizes being preferentially and intentionally ingested by organisms (e.g. Carpenter et al., 1972; Gramentz, 1988; Moser and Lee, 1992; Shaw and Day, 1994; Phaksopa et al., 2021; Rios et al., 2022). As examples, *Pimephales promelas* (fathead minnows) ingested up to 10 times more MPs between 63-75 µm than those

between 125-150 µm (Hoang and Felix-Kim, 2020) and marine bivalves *Mytilus edulis* and *Crassostrea virginica* selectively ingested more MF than similarly sized microspheres (Ward et al., 2019). Some organisms have even been shown to actively avoid ingesting MPs and MPFs, particularly when offered an alternative food source (Cole et al., 2013; Aljaibachi and Callaghan, 2018; Yardy and Callaghan, 2020). For some invertebrates this may be due to chemo-mechanical sensilla which allow them to determine whether an object is edible. *G. pulex* used in a study by Yardy and Callaghan (2020) are known to have these sensilla (Lange et al., 2005), and actively avoided feeding on acrylic MF contaminated algal wafers. The marine copepod *Centropages typicus*, similarly fed less on algae when microplastics were present, with higher MP concentrations correlating with lower algae ingestion rates (Cole et al., 2013). Despite this reported avoidance behaviour, field studies have found significant MP ingestion by crustaceans, mostly consisting of MFs. Murray and Cowie (2011) found that 83% of *Nephrops norvegicus* (Norway Lobster) contained MPs, predominantly MFs, while Devriese et al. (2015) found that 63% of sampled *Crangon crangon* (Brown shrimp) had ingested MPs, 96.5% of which were MPFs. This suggests that despite the ability to detect non-food particles, organisms may still ingest them, potentially due to their high abundance in some locations, or the potential of biofilms to mask their chemical cues.

The documented effects of MP and MF ingestion vary widely. Some studies show minimal impacts, with no changes in growth, pathology, or stress in organisms with long term exposure to virgin MPs, manufactured MPs that have not been weathered or recycled, (Jovanović et al., 2018). Others report sub-lethal impacts, such as impaired reproduction in oysters (Sussarellu et al., 2016), reduced resistance to parasitic infections (Masud et al., 2022) and reduced feeding activity (Besseling et al., 2013; Watts et al., 2014; Cole et al., 2013). Studies on chronic exposure to MPs and MFs suggest that environmentally relevant concentrations may cause sub-lethal and lethal effects (Tosetto et al., 2016; Hamm and Lenz, 2021; Walkinshaw et al., 2023). Research on the ingestion of MFs, particularly NMFs, remains limited (Ryan et al., 2020), however recent studies indicate that NMFs exhibit similar levels of toxicity as MPFs (Kim et al., 2021; Mateos-Cardenas et al., 2021).

While MPFs are now commonly reported to be ingested by organisms, this is not the case for NMFs which only recently garnered attention in research. As mentioned

previously, NMFs fibres are often actively excluded from MP studies due to the methods used to isolate them. Consequently, research into NMF ingestion has been limited until recently. However, recent studies indicate that NMFs are prevalent in aquatic organisms, found ingested in comparable numbers to MPs (Compa et al., 2018) and even exceeding the numbers of ingested MPs in some cases (Hou et al., 2023). Research also suggests that NMFs and MPFs exhibit similar levels of toxicity for freshwater and marine invertebrates (Kim et al., 2021; Mateos-Cardenas et al., 2021). Indeed, NMFs may pose a greater threat to organisms than MPs and MPFs due to their faster degradation, which could release absorbed pollutants into the environment more quickly than MPs as these chemicals are released after degradation (Ladewig et al., 2015). Cotton MFs, for example, have been shown to reduce the growth rate of mussels (Walkinshaw et al., 2023) and impact the growth and behaviour of *Menidia beryllina* (Inland Silverside fish) and *Americamysis bahia* (Mysid shrimp; Siddiqui et al., 2023).

Although the Ingestion of MPs and MFs is widely reported in marine, freshwater and terrestrial organisms, with some studies reporting ingestion in over 80% of the organisms investigated (e.g. Murray and Cowie, 2011) many studies report very low and even no particles ingested. Rummel et al., (2016) found plastics were present in only 5.5% of fish samples, while Foekema et al., (2013) found similarly low numbers in Atlantic herring (only 2% had ingested plastics) and Atlantic mackerel (no MPs were found). Even when studies find MPs in the GI tract of organisms, they often contain only one particle (e.g. Foekema et al., 2013; Beer et al., 2018; Budimir et al., 2018). This suggests that while these particles can be ingested in abundance by organisms, they may not be retained in their gut, instead being egested along with normal food. The retention of anthropogenic particles by organisms may be a more important determinant than their ingestion on the impacts that these particles will have, yet there remains limited research looking at the egestion of either MPs or MFs (Mateos-Cárdenas et al., 2021).

2.5 Egestion

Organisms have a range of evacuation methods for inedible and indigestible materials, some of which are passive (e.g. diffusion along concentration gradients) while others are active (e.g. regurgitation and the production of pseudo faeces).

Mammals and fish are known to have well-structured orders of egestion, with the least digestible food items being evacuated last; however, repeated ingestion slows down this process, leading to the accumulation of ingestible solids in the gut (Dos Santos and Jobling, 1991). Dos Santos and Jobling (1991) hypothesised that this would also be the case for plastics, with plastics being retained in the gut after ingestion and accumulating if repeated feeding occurs. Their study found this to be the case, with reduced egestion of plastics with repeated feeding by cod. However, more recent ingestion studies have found limited MP ingestion and accumulation in fish (Hermsen et al., 2017; Liboiron et al., 2018, 2016; Jovanović et al., 2018; Bosshart et al., 2020). This may be due to efficient MP egestion mechanisms, with freshwater fish able to egest MPs within hours of ingestion (Grigorakis et al., 2017; Hoang and Felix-Kim, 2020; Roch et al., 2021), along with normal food sources (Grigorakis et al., 2017; Roch et al., 2021). While less researched, there have been similar findings for MFs. As with MPs, freshwater fish have been found to egest MPFs at rates comparable to their normal food (Hou et al., 2023), with no significant retention of MPFs, or differences in egestion between MPFs and other MP shapes (Grigorakis et al., 2017). Therefore, MPs and MFs may not be retained longer than normal food and may have limited impacts on the organism that ingests them. However, even if MPs can be egested there are other mechanisms through which they can agglomerate in organisms. For example, hydrophobic and static attractions have been reported to cause MPs to be lodged between external appendages of live copepods (Cole et al., 2013), and in the gills of crabs (Watts et al., 2014; Villagran et al., 2020) and shrimp (Gray and Weinstein, 2017). MPs lodged in gills may impact their ability to breathe and impede their normal metabolic processes (Watts et al., 2016). Limited research has been performed investigating the egestion of NMFs.

As with ingestion, the egestion of MPs has been found to be size dependent, with larger particles being egested more quickly than normal food, while small particles are egested alongside normal food by *Oncorhynchus mykiss* (rainbow trout; Roch et al., 2021). Less is known about the effect of MP shape on egestion (Gray and Weinstein, 2017).

Invertebrates do not have a well-structured, ordered form of evacuation, with inedible material being passed in the same way as other ingested material. For example, MPs have been found to be egested within hours of ingestion by marine copepods

(Cole et al., 2013; Powell and Berry, 1990). Invertebrates may therefore be less prone to accumulating MPs, as they will be egested more readily and quicker than in mammals and fish.

Many laboratory studies have explored MP and MF egestion by invertebrates; however, these studies may not be representative of the conditions experienced by invertebrates in the field with implications for study findings. For example, when bivalves were exposed to higher MPF concentrations they had higher retention rates than when exposed to lower concentrations, suggesting that these bivalves were more able to reject and egest particles in lower MPF concentration environments (Woods et al., 2018). This is important, as laboratory experiments often use MF concentrations higher than those experienced in the environment, which could lead to results that are not comparable to behaviours in the environment. Therefore, ecotoxicology studies looking at the retention of MFs should use more environmentally relevant concentrations to show environmentally relevant results. Both Windsor et al., (2019), and Gusmão et al., (2016) found that macroinvertebrates sampled from the field were able to egest MPs and MFs with no observed physical damage caused. This suggests that at environmentally relevant conditions and concentrations, MPs and MFs may not cause significant harm.

Evacuation methods of MFs and MPs vary depending on the shape and size of the particles. For example, short MPFs were egested by *Palaemon varians* (comon ditch shrimp) whereas longer fibres were regurgitated (Saborowski et al., 2019). Beads were also egested, but not regurgitated (Saborowski et al., 2019). MPFs have also been found to be retained longer than food materials and MP spheres by the freshwater amphipod *Hyalella azteca* (Au et al., 2015). This may be because of the shape of MPFs. When ingested, MPFs tend to aggregate to form fibre balls, as shown in crabs and lobsters (Murray and Cowie, 2011; Devriese et al., 2015; Watts et al., 2015). The formation of these fibre balls poses a risk to organisms, as they may be more likely to form blockages and damage the GI tract. The egestion of MPs was not, however, found to be different between fragments, fibres and spheres for grass shrimp, with MP size being a greater determinant of the residence time (Gray and Weinstein, 2017). It is also interesting to note that Watts et al. (2015) found a reduction in the size of MPFs after egestion, supporting findings that invertebrates may be contributing to the formation of micro- and nano- plastics in the environment

through fragmentation caused by feeding mechanisms (Mateos-Cárdenas et al., 2020; Valentine et al., 2022). The known propensity of MFs to agglomerate once ingested, and the heterogeneous structure of MFs suggests that there is a great potential for the ingestion of MFs in large quantities to lead to GI blockages, and significant harm to the organisms that ingest them. These findings underscore the complexity of ingestion and egestion, which depends on multiple factors such as the material composition, particle shape, size, and condition, as well as the characteristics of the organism and its environment. Understanding this complexity is essential for accurate assessments of the risks of MF and MP pollution.

2.6 Ecology

2.6.1 Macroinvertebrates

There is a need to focus on macroinvertebrates (invertebrates larger than 500 um; Hauer and Resh, 2017) when looking at environmental pollutants, as many are keystone species (Kellert, 1993). In particular, the macroinvertebrate community is a critical food source for fish, and other predators (Baun et al., 2008), and macroinvertebrates are important environmental regulators and providers of ecosystem services, without whom ecosystems would collapse (Prather et al., 2013).

Macroinvertebrates are commonly used in ecotoxicology studies due to their abundance, sensitivity to pollutants, and short lifespan, which enables the study of pollution impacts across multiple generations. However, only recently has research explored the ingestion and egestion of MFs, both synthetic and natural, in macroinvertebrates. The small size of MFs makes them readily available for ingestion by macroinvertebrates; however, there is little understanding of how different characteristics of macroinvertebrates, such as their feeding strategy, can impact MF ingestion. As macroinvertebrates are thought to be an important source of MPs and MFs in higher trophic levels (Foley et al., 2018), there is a need to understand why and how they are ingested to understand the transfer and movement of these pollutants through the aquatic system.

The extent to which MPs and MFs are ingested and retained, respectively, is determined partly by the particle characteristics, but also the ecology of the organism that ingests them. The ingestion and egestion of MPs and MPFs has been found to vary between taxa (Bertoli et al., 2022; Garcia et al., 2021; McNeish et al., 2018;

Khedre et al., 2023) Potential reasons for this variation include inter- and intraspecific differences in structure and size of the GI tract (Jabeen et al., 2017; Hoang and Felix-Kim, 2020; MCNeish et al., 2018; Roch Continue et al., 2021), feeding mechanisms (Chaumot et al., 2015; Bour et al., 2018), and the capability to detect and avoid ingesting inedible participates (Khedre et al., 2023).For example, the ingestion of polystyrene beads by marine zooplankton varied between species, their life-stage, and MP size (Cole et al., 2013), and the ingestion of MPs by marine zooplankton was found to vary with MP shape (Botterell et al., 2020). While research has been performed on a variety of taxa from a range of functional feeding groups (FFGs), few directly compare FFGs and differences in ingestion.

2.6.2 Water turbidity

Water turbidity, primarily caused by suspended particulate matter, can influence the feeding behaviour of a range of aquatic invertebrates, but has the most significant effect on filter feeders who filter food from the water column. Many filter feeders rely on particle concentration and quality to regulate their feeding activity, with increased turbidity often leading to reduced clearance rates and altered food selection strategies (Aldridge et al. 1987; Tuttle-Raycraft and Ackerman 2019). In some cases, prolonged exposure to elevated turbidity levels may drive morphological adaptations in feeding structures, as seen in certain populations exhibiting interpopulation differences in feeding appendages (Payne et al. 1995).

Despite these findings, the role of suspended solids in MF ingestion remains unexplored. It is unclear whether high turbidity conditions enhance or inhibit MF ingestion by aquatic invertebrates, particularly filter feeders, which may either indiscriminately ingest particles or selectively reject non-nutritive materials. Given the increasing prevalence of MP and MF in aquatic environments, further research is needed to determine how suspended solids influence ingestion rates, potential retention times, and physiological impacts on invertebrate populations. Understanding these interactions is crucial for assessing the ecological risks associated with microfibre pollution in natural systems.

2.6.3 The presence of biofilms

As most MP and MF ecotoxicology studies use virgin particles (Lahtiniemi et al., 2018), the applicability of research findings to the natural environment is limited. This

is because MPs in the environment are quickly colonised by biofilms, i.e., a structured community of surface-associated microorganisms (Watnick and Kolter, 2000) including algae, fungi, and bacteria, embedded in a matrix of self-secreted extracellular polymeric substances that help the community of microorganisms to thrive (Wang, 2021). Biofilms are highly nutritious and the primary food source for many macroinvertebrates, which suggests that biofilms on the surface of MPs and MFs may disguise their inedibility and lead to their intentional ingestion. Biofilms have been found to increase the ingestion of MPs by marine organisms (Powell and Berry, 1990; Vroom et al., 2017; Hodgson et al., 2018; Weideman et al., 2020; Fabra et al., 2021). For example, *Eurytemora affinis* fed on MP beads spiked with bacterium more readily than sterile beads, and regurgitated sterile beads, whereas bacteria-coated beads were egested in faecal pellets, suggesting that they could not determine the inedibility beads when bacteria-coated (Powell and Berry, 1990).

Biofilms not only alter the potential for MP and MF ingestion by organisms, but also alter the movement of these particles through the environment. Biofilms formed on the surface of MPs and MFs alter their density, which can cause positively buoyant particles to sink (Ye and Andrady, 1991; Lobelle and Cunliffe, 2011; Karami, 2017). It has been suggested that the accumulation of biofilms on MPs creates a cycle whereby a plastic accumulates a biofilm, sinks to the benthos, loses the biofilm through grazing and lack of sunlight (Ye and Andrady, 1991; Wright et al., 2013), and begins to refloat where the cycle begins again, exposing organisms in all areas of the water column to the pollutant.

Studies comparing weathered and virgin MP have found more severe ecological effects from weathered MPs (Hartmann et al. 2017; Seauront, 2018) with biofilms enhancing their toxicity (Karami, 2017). The greater toxicity may be due to the biofilm increasing the sorption ability of MPs, meaning that they can take up greater amounts of pollutants from the surrounding medium (Guo et al., 2019; Guan et al, 2020; Wang et al., 2020; Wang et al., 2021). Microbial communities on MPs are also often found to be different to those of the surrounding medium (Rosato et al., 2020; Kirstein et al., 2016), with some species found on MPs potentially being pathogenic (Kirstein et al. 2016). Biofilm communities also differ between polymers (Rosato et al., 2020) and between synthetic and cellulosic MFs (Zambrano et al., 2020), which may alter the toxicity of different MPs and MFs. As well as enhancing the toxicity of

MPs and MFs, biofilms also present their own environmental problems. Biofilms have been found to act as a vector for the transport of alien species (Derraik, 2002) and pathogens (Zettler et al., 2013) and potentially increase the formation of antibiotic-resistant genes in the environment (Wang et al., 2022). The ability for biofilms to enhance the toxicity of MPs and MFs highlights a need for more research investigating environmentally relevant particles.

Despite the toxicity of biofilms, it has been proposed that they may be beneficial to the plastic pollution issue, speeding up the degradation of plastic litter when hydrocarbon-degrading microorganisms are present (Wagner et al., 2014). However, such microorganisms are not always present in biofilms (Lobelle and Cunliffe, 2011). In contrast, biofilms may protect plastics from environmental factors that increase degradation rates, e.g., obscuring debris from ultraviolet light (Barnes et al., 2009; O'Brine and Thompson, 2010).

3 Method

3.1 Overview

The aim and objectives were achieved through a series of laboratory experiments, using two widespread macroinvertebrate animals as test organisms, to assess whether and how they ingest and egest MFs. Aquaria housing individual organisms were kept under controlled conditions to isolate the potential impact of MFs from other drivers of animal health and behaviour. MFs were then dosed in aquaria and the ingestion and egestion of animals compared between treatments. The duration of exposure to MFs varied from 4 hours to 2 weeks, and MFs were exposed in clean and turbid water, and with and without attached biofilm. The water was spiked either with MPF or NMF so a comparison could be made between the ingestion and retention of plastic versus natural fibres.

3.2. Invertebrate Selection and Collection

3.2.1. Gammarus pulex

G. pulex were selected as a test organism due to their extensive use in ecotoxicology studies (Kunz et al., 2010), including research looking at the impact of both MP and MF ingestion (Weber et al., 2018; Kratina et al., 2019; Yardy and Callaghan, 2020; 2021). The Gammaridae family of crustaceans are commonly used in ecotoxicology studies due to their importance in aquatic food chains, sensitivity to pollutants, and abundance in the environment (Felten et al., 2008; Kunz et al., 2010; Chaumot et al., 2015). *G. pulex* are both predators and shredders (Kelly et al., 2002), who mainly feed on living and detrital plant material in the benthic zone of rivers and streams across Europe and Northern Asia (Karaman and Pinkster, 1977). As *G. pulex* is an important food source for other invertebrates, birds, and fish (Gledhill et al., 1993), they can represent a key source of pollutants across trophic webs, also contributing to their importance as a study organism.

G. pulex were collected from Tottle Brook, Nottingham (52.5610°N, -1.1134°W) for a series of pilot experiments that were used to finalise the methodological design, including aquaria size and experimental duration (results not presented further). For reported experiments, *G. pulex* were collected from Holywell Brook, Loughborough (52.7584°N, -1.246694°W). The location changed from pilot work because a larger

and more accessible population was present at Holywell Brook. *G. pulex* were collected by kick sampling due to its efficiency and widespread use in invertebrate sampling (Mackey et al., 1984), using a standard square net with a 250 µm mesh. A minimum of 40 *G. pulex* were collected for each experiment by targeting areas of good *G. pulex* habitat, particularly slow flowing areas with leaf-litter accumulation. Individuals greater than 5mm were chosen for both G. pulex and C. fluminea. Specific sizes were not selected for due to unconfirmed relationships between body size and MP ingestion by invertebrates (e.g. Hoellein et al., 2021; Weber et al., 2021; Ritchie et al., 2025). Sex was not selected for. Once collected, *G. pulex* were taken to the laboratory and placed in a 10l bucket of dechlorinated water with a pond pump, where they were left for 24 hours to acclimatise without food (as per Yardy and Callaghan, 2020) to allow for any fibres ingested in the environment to depurate prior to the exposures.

3.2.2. Corbicula fluminea

C. fluminea are bivalve filter and deposit feeders, invasive to the UK, widely used in the investigation of MP and MF ingestion (Su et al., 2018; Li et al., 2019; McCoy et al., 2020; Li et al., 2022). Bivalves have been extensively studied in relation to microplastics, as they typically filter-feed by pumping large volumes of water across their feeding appendages. As such, there is potential for them to ingest large quantities of MPs during their filter feeding activity. However, it has been argued that bivalves may be poor biomonitors for MP pollution because of their ability to efficiently reject non-food particles (including MP particles) as part of selective feeding strategies (Ward et al., 2019; Weis, 2020; Woods, Stack, Fields, Shaw, & Matrai, 2018). Additionally, field studies have shown that filter feeders may be less exposed to microplastics than other FFGs (Bour et al., 2018). Despite this, bivalves and other filter feeders, remain a model ecotoxicological group for MPs and MFs, and an interesting comparator to the deposit feeding *G. pulex.*

C. fluminea were collected from the Great Western Canal in Loughborough (52.763808°N, -1.183517°W). A square net was run along the submerged canal bank, away from concrete-lined sections several times before rinsing the contents of the net to remove sediment. *C. fluminea* were then identified and picked out by hand. This was repeated along the canal until a minimum of 40 *C. fluminea* were collected. Once collected, the *C. fluminea* were taken to the laboratory and placed in a 10l

bucket of dechlorinated water with a pond pump, where they were left for 24 hours to acclimatise without food.

3.3 Fibre Preparation

Fabric swatches measuring 1 cm² were collected from a red 100% cotton t-shirt and a green 100% polyester t-shirt. These colours were selected because they are easily distinguishable from each other and would not be worn in the lab. Polyester and cotton were chosen as they are the most commonly used synthetic and natural fibres, respectively (Carmichael, 2015; Khan et al., 2020; TextileExchange, 2020). This makes them significant for research, as their prevalence in the industry reflects their environmental abundance. The t-shirts used were second-hand, as worn textiles are more representative of those found in the environment.

The squares were then prepared to create reservoirs of contaminated water to be used in the experiments. In order to maintain a similar concentration between reservoirs, the swatches were weighed, and only swatches weighing 0.015g (+-5%) were used. 2 squares of each fabric were carefully cut with scissors into a 500 µm sieve over a 10L bucket, one for polyester and one for cotton. A sieve was used to prevent large clumps of fibres entering the reservoirs. 10L of dechlorinated water was then poured through each sieve to release the fibres. The scissors were washed after cutting each disc to prevent contamination between reservoirs. The reservoirs were stored in a temperature chamber at 15°C prior to each experiment.

Although fibre preparation using a cryogenic microtome, as per Cole (2016), is now commonly used in MF investigations, this method was not utilised here because this approach could have damaged the biofilm matrix that was coating fibres in some experimental treatments. Cryogenic microtome has also been found to produce fibres that are not representative of those in the environment, resulting in fibres of heterogeneous lengths that are shorter on average than environmentally relevant particles (Detree et al., 2023).

In treatments with biofilmed fibres, 4 biodegradable pyramid teabags were emptied, cleaned and individually filled with either 4 cotton, or 4 polyester swatches. The tea bags were then sealed with waterproof tape. On 04/07/2024 the teabags were left in a garden pond to condition for 5 weeks. The teabags were transported to the

laboratory on the day of the experiment in pond water, followed by the immediate preparation of the conditioned swatches following the same protocol as the unconditioned fibres.

For each experiment, before the contaminated water was used, the water in each reservoir was stirred for 20 seconds to disturb any fibres that had settled.

3.3.1 Concentration of fibres

The fibre concentration in treatments was tested prior to the start of experiments by repeating the fibre preparation process and taking 10x 100ml aliquots from the reservoir using a glass pipette and filtering them through Whatman GF/F filters (0.7 µm, 47 mm) using vacuum filtration apparatus. Fibres were visually identified and counted under a dissecting microscope (10 and 40x magnification).

MF concentrations were recorded to be approximately 2000/L (Table 1). Although MF concentrations in the environment are suggested to be around 10/L (Ryan et al., 2020; Suaria et al., 2020), higher concentrations were chosen due to the significant variability in concentrations in different environmental compartments, for example near WWTPs where MP concentrations in effluent have been reported ranging between 0.2 and 6999 MP/L depending on the country and treatment processes used (Acarer, 2023). Whilst higher than many concentrations found in rivers, the concentration used is also lower than concentrations commonly used in similar laboratory dosing experiments.

Table 1: Average cotton and polyester microfibre concentrations measured in the reservoirs of contaminated water used in the mesocosm experiments.

	Concentration/L	
Fibre Type	Cotton	Polyester
Non-Biofilmed	2393	2087
Biofilmed	3150	2120

3.3.2 MF length

MF ingestion studies often use MFs that are homogenous in size and shape, which is not representative of the heterogeneous particles found in the environment (Phuong et al., 2016). Therefore, in this study, fibre length was not controlled for. Instead, the length of 100 randomly chosen fibres from each reservoir was measured

prior to the experiments and, where possible, 50 were measured after they were ingested, to determine if study organisms preferentially ingested fibres of certain lengths.

3.4 Mesocosm set-up and treatment conditions

Laboratory experiments were chosen to allow for the invertebrate's environment to be controlled, limiting the external impacts on MF ingestion to those being investigated. Laboratory studies are useful for initial, exploratory research as they can be repeated by others, which allows for research to be expanded in the future to include other treatments or, in this case, macroinvertebrate taxa.

Experiments took place in 100 ml glass beakers, each housing a single animal. Prior to experiments, the 100ml glass beakers were thoroughly cleaned with water and dish soap. Care was taken to remove soap residue prior to each experiment, as soap remaining in the water could harm the invertebrates by changing the properties of the water and reducing available dissolved oxygen (Alavaisha et al., 2019). These beakers were each filled with 80 ml of water from one of the four reservoirs, which contained unconditioned and biofilmed polyester and cotton fibres. These beakers were kept in a temperature chamber set a constant 14 °C for the duration of all experiments, which is within the preferred range for *G. pulex* (Gledhill et al., 1993). Beakers were exposed to a natural day/night cycle via an adjacent window for the duration of the experiment, which took place in July 2024. Reservoirs of water were also kept in the temperature chamber so when water changes took place, water was of the same temperature.

3.4.1 4-hour exposure experiments

A series of experiments investigated the ingestion and egestion of MFs by comparing the MF concentration in animals exposed to MFs for 4-hours to the concentration in animals exposed to MFs for 4-hours (ingestion period) followed by a 24-hour egestion period. The time duration of the ingestion (4-hours) and egestion (24-hour) periods are similar to time frames used in other microplastic (MF) studies. In experiments, half of the individuals from each treatment group (n = 10) were immediately sacrificed after the 4-hour ingestion period (Gray and Weinstein et al., 2017; Saborowski et al., 2019). The remaining invertebrates (n = 10) were rinsed to remove any loose fibres and transferred to individual beakers containing 80 mL of

uncontaminated, dechlorinated water, where they were left for the 24-hour depuration (i.e. egestion) period.

Depuration times for *G. pulex* vary between 0.5 and 48 hours depending on the food source (Monk, 1977; Sutcliffe et al., 1981; Willoughby and Earnshaw, 1982; Willoughby, 1983). In contrast, no specific depuration time for *C. fluminea* has been identified. To accommodate this variability, a 24-hour depuration period was chosen, as this exceeds typical food depuration times (around 8 hours, based on Monk, 1977; Sutcliffe et al., 1981; Willoughby and Earnshaw, 1982; Willoughby, 1983) and aligns with previous studies on invertebrate egestion of microplastics (e.g., Jemec et al., 2016; Saborowski et al., 2019).

Following the 24-hour depuration, the remaining invertebrates were sacrificed. *G. pulex* were preserved in 40% methanol sucrose, while *C. fluminea* were frozen at -40°C. All beakers were thoroughly washed after each experiment to avoid cross-contamination. The animals sacrificed immediately after 4-hour exposure to MFs and those sacrificed after a further 24-hour depuration time were dissected to assess MF contamination, described in section 3.5.

3.4.2 Experiments with biofilmed fibres and turbid water

Further experiments were performed to assess both the potential impact of water turbidity, and the presence of biofilms on fibres on the ingestion and retention nof MPFs and NMFs. For the biofilmed fibres experiment, the same procedure as the non-biofilmed fibres experiment (section 3.3.1) was followed using both *G. pulex* and *C. fluminea* but using fibres that had been exposed to environmental conditions for 5 weeks to allow biofilm accumulation (section 3.3). Turbid water and biofilmed fibre experiments were only conducted over a 4-hour exposure period, with long term exposures not investigated.

To evaluate microplastic ingestion in turbid water, the experimental protocol described in section 3.3.1. was once again followed, but with water taken from the Great Western Canal and held in reservoirs within temperature chambers, which provided turbid conditions. The water will also have other differences in water quality when compared to the experimental, spiked water used in other experiments but, given the short duration of exposure (4-hours) the impacts of other, parameters was expected to be negligible. Turbid water experiments were only performed with *C.*

fluminea, as they filter particles from the water column, which is known to be complicated by turbidity as filter feeders adjust their feeding strategy based on the turbidity of the water (Argente et al., 2014).

3.4.3 Accumulation Experiment

A further experiment was performed to assess whether MFs were accumulated within organisms when exposed to MFs for longer periods. In these experiments, animals were kept in 12x 1 L beakers for 2 weeks. The larger beakers were selected based on a pilot study, which indicated that smaller beakers (100 mL) displaced water when pond pumps were used to oxygenate water and caused overly forceful aeration which, in some cases, caused stress and mortality to animals. In larger beakers, aeration was more appropriate and less aggressive, and the airstream generated from pumps more spatially discrete and taking up a smaller proportion of the beaker volume.

Each beaker was fitted with a pond air pump connected to an air stone to ensure the pump remained submerged, prevent the pumping of large bubbles, and also dissuaded *G. pulex* from attempting to swim into the air tube. Additionally, two mineral gravel grains were placed in each beaker to provide shelter, disrupt water flow, and reduce stress caused by aeration (Karami, 2017). The 12 beakers were filled with 800 mL of contaminated water, half containing non-biofilmed cotton and the other half non-biofilmed polyester. Ten *G. pulex* or *C. fluminea* were added to each beaker.

The water in each beaker was changed every 3 days. After 7 days, 20 *G. pulex* and 20 *C. fluminea* (half from each treatment) were sacrificed, while another 20 from each group were transferred to uncontaminated water for a 24-hour depuration period before being sacrificed, following the protocol detailed in section 3.5.2.

3.5 Sample Processing

Although digestion techniques are commonly employed in MP research, they were avoided in this study due to concerns that chemicals like hydrogen peroxide could alter or damage the microparticles, particularly NMFs (Nuelle et al., 2014). Instead, invertebrates were dissected and MFs removed for analysis.

3.5.1 Gammarus pulex

In the laboratory, *G. pulex* were rinsed thoroughly to remove any loose MFs and those caught between their legs and other appendages before being placed into a clean petri dish for further examination. Each individual was dissected under a stereo microscope at magnifications between 10 and 40x to expose the contents of the digestive tract. It was found that fibres were often intertwined with each other and other ingested materials (figure 1). In order to count fibres in intestinal tracts, these clumps of material requiring careful manipulation to separate out the individual fibres and separate them out across filter papers to reduce the risk of fibre underestimation (Haap et al., 2019). The samples were then filtered using Whatman GF/F filters (0.7 µm, 47 mm) in a vacuum filtration system, and the fibres were subsequently counted under the microscope.

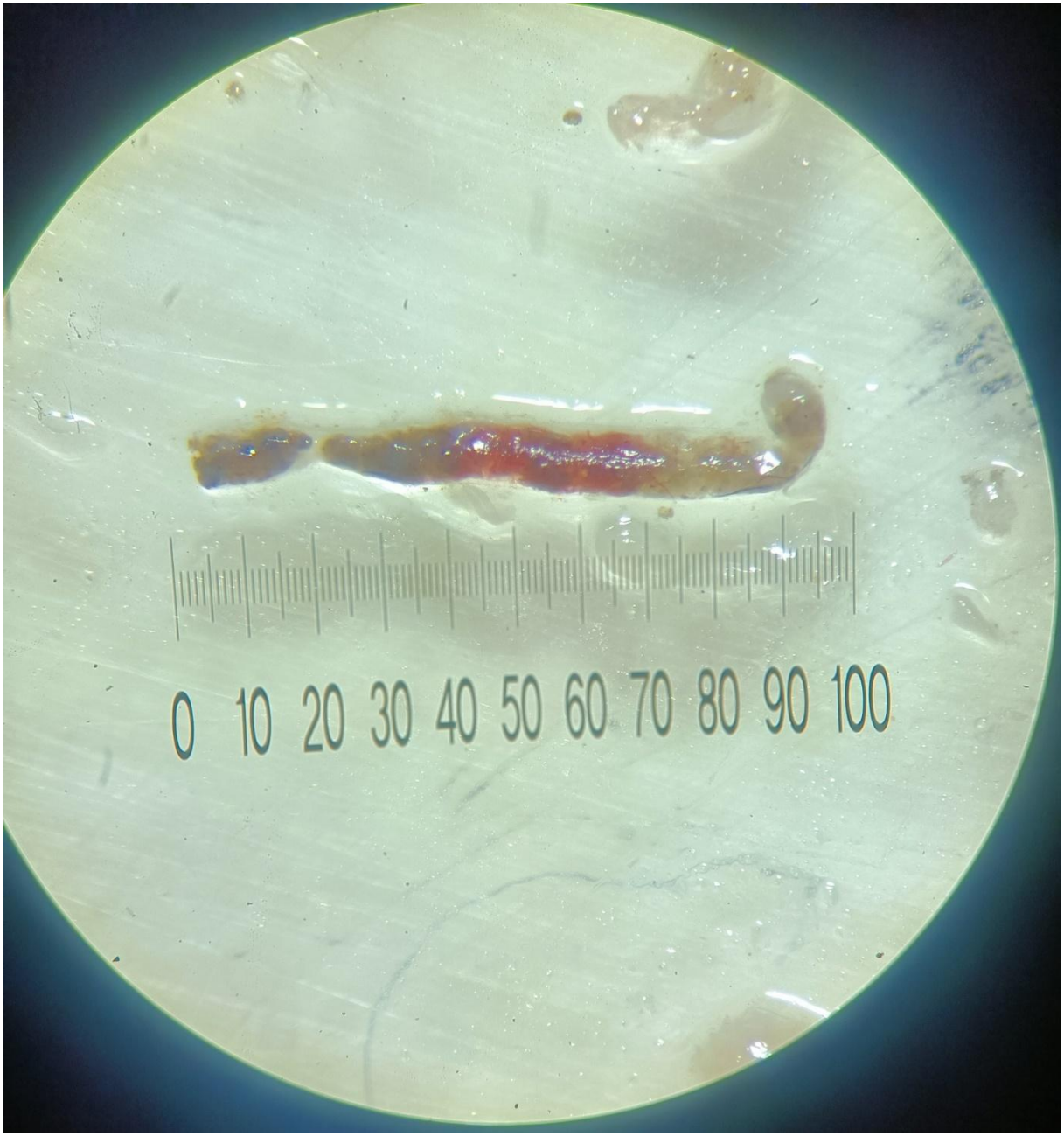

Figure 1: Red cotton fibres in the GI tract of G. pulex

3.5.2 Corbicula fluminea

The same preparation procedure was applied to *C. fluminea*, although the larger size of these organisms meant that the filtration process had to be adjusted. *C. fluminea* were dissected, and the contents of their GI tract were released before the dissected clam was poured into a 200ml beaker, which was topped off to 200 ml with deionised water. Aliquots of 20 ml were extracted using a graduated pipette and processed through the vacuum filtration system, with fibres counted for each aliquot. To assess whether two aliquots were sufficient to estimate the total number of fibres ingested,

the fibre counts from the first 40 ml were used to predict the fibre content of the entire 200 ml sample.

3.6 Contamination Control

Contamination from aerial deposition can impact MP, and particularly MF, research. During experiments, all beakers and water reservoirs were in sealed temperature chambers, limiting the potential for aerial deposition of MPs meaningfully altering the concentrations in beakers.

To limit sample contamination during the processing of invertebrate samples, all samples remained in sealed sample tubes until processing, and were only exposed during dissection, which typically took 5 minutes for each animal. For each individual, a new petri dish was used, and the filtration apparatus was washed thoroughly between each use. A white cotton lab coat was worn whenever handling or processing samples, distinct from the red and green samples used in spiked experiments.

3.7 Statistical Analysis

Data analysis was performed using IBM SPSS Statistics Version 29.0.1.0. Tests for normality and homogeneity of variance were conducted which showed the data was non-normal (table 2 & 3). Therefore, Kruskal-Wallis and Mann-Whitney U tests were conducted.

Table 2: Shapiro-Wilk test for normality. A non-significant p-value (p > 0.05) suggests that the data follow a normal distribution.

Normality (Shapiro-Wilk)								
Experiment			G. pulex			C. fluminea		
			W-value	df	sig	W-value	df	sig
4 hour exposure	Ingestion	Cotton	0.873	10	0.110	0.662	10	< .001
		Polyester	0.785	10	0.010	0.731	10	< .001
	Egestion	Cotton	0.905	10	0.246	0.713	10	0.001
		Polyester	0.811	10	0.020	0.366	10	< .001
1 week exposure	Ingestion	Cotton	0.986	5	0.964	0.771	5	0.046
		Polyester	0.927	5	0.574	0.684	5	0.006
	Egestion	Cotton	0.860	5	0.230	0.552	5	< .001
		Polyester	0.998	5	0.999	0.552	5	< .001
2 week exposure	Ingestion	Cotton	0.991	5	0.985	0.881	5	0.314
		Polyester	0.935	5	0.628	0.771	5	0.046
Biofilmed Fibres	Ingestion	Cotton	0.931	10	0.459	0.859	10	0.074
		Polyester	0.925	10	0.402	0.509	10	< .001
	Egestion	Cotton	0.844	10	0.049	0.623	10	< .001
		Polyester	0.863	10	0.084	0.650	10	< .001
Turbid Water	Ingestion	Cotton				0.694	10	< .001
		Polyester				0.781	10	< .001
	Egestion	Cotton				0.802	10	0.015
		Polyester				0.650	10	< .001

Table 3: Levene's test for homogeneity of variance across treatment groups. A non-significant p-value (p > 0.05) indicates that the assumption of equal variances is met.

Homogenity of Variance (levene's Test)									
Experiment		*G. pulex*				*C. fluminea*			
		Statistic	df1	df2	sig	Statistic	df1	df2	sig
4 hours	Ingestion	0.082	1	18	0.778	0.643	1	18	0.433
	Egestion	0.885	1	18	0.359	17.455	1	18	<.001
1 week	Ingestion	0	1	8	1	0.182	1	8	0.681
	Egestion	3.928	1	8	0.083	0	1	8	1
2 weeks	Ingestion	3.349	1	8	0.105	0	1	8	1
Biofilmed	Ingestion	18.517	1	18	<.001	6.231	1	18	0.022
	Egestion	6.321	1	18	0.022	0.395	1	18	0.538
Turbid Water	Ingestion					0.218	1	18	0.646
	Egestion					0.13	1	18	0.722

4 Results

4.1 Mortality and Microfibre Ingestion

No mortality was recorded for *G. pulex* or *C. fluminea* during the experiments.

Both cotton and polyester MFs were ingested and retained by *G. pulex* and *C. fluminea* for all experiments. 56.25% of *C. fluminea* ingested no MFs and 67.14% retained no fibres. 11.67% of *G. pulex* ingested no fibres and 16% retained no fibres.

4.2 Ingestion and Retention of MFs

4.2.1 Four-hour exposure

Mann-Whitney U tests showed that the retention of non-biofilmed MF by *G. pulex* and *C. fluminea* after a 24-hour depuration period did not significantly differ from the number ingested following a 4 hour exposure. For *G. pulex,* polyester (U = 34.0, p = .247), and cotton fibres (U = 64.5, p = .280, see figure 2) showed no significant reduction. Similarly, Mann-Whitney U tests showed no significant differences in the number of ingested and retained fibres in *C. fluminea* for polyester (U = 55.5, p = .684), or cotton fibres (U = 34.5, p = .247, see figure 3).

4.2.2 One week exposure

The retention of non-biofilmed MF by *G. pulex* and *C. fluminea* after a 24-hour depuration period did not significantly differ from the number ingested following a one-week exposure. For *G. pulex*, polyester (Mann-Whitney U = 21, p = 0.095) and cotton fibres (Mann-Whitney U = 4, p = 0.095) showed no significant reduction. Similarly, for *C. fluminea*, no significant difference was observed for polyester (Mann-Whitney U = 9.5, p = 0.548) or cotton fibres (Mann-Whitney U = 10, p = 0.690). (See Figure 3.)

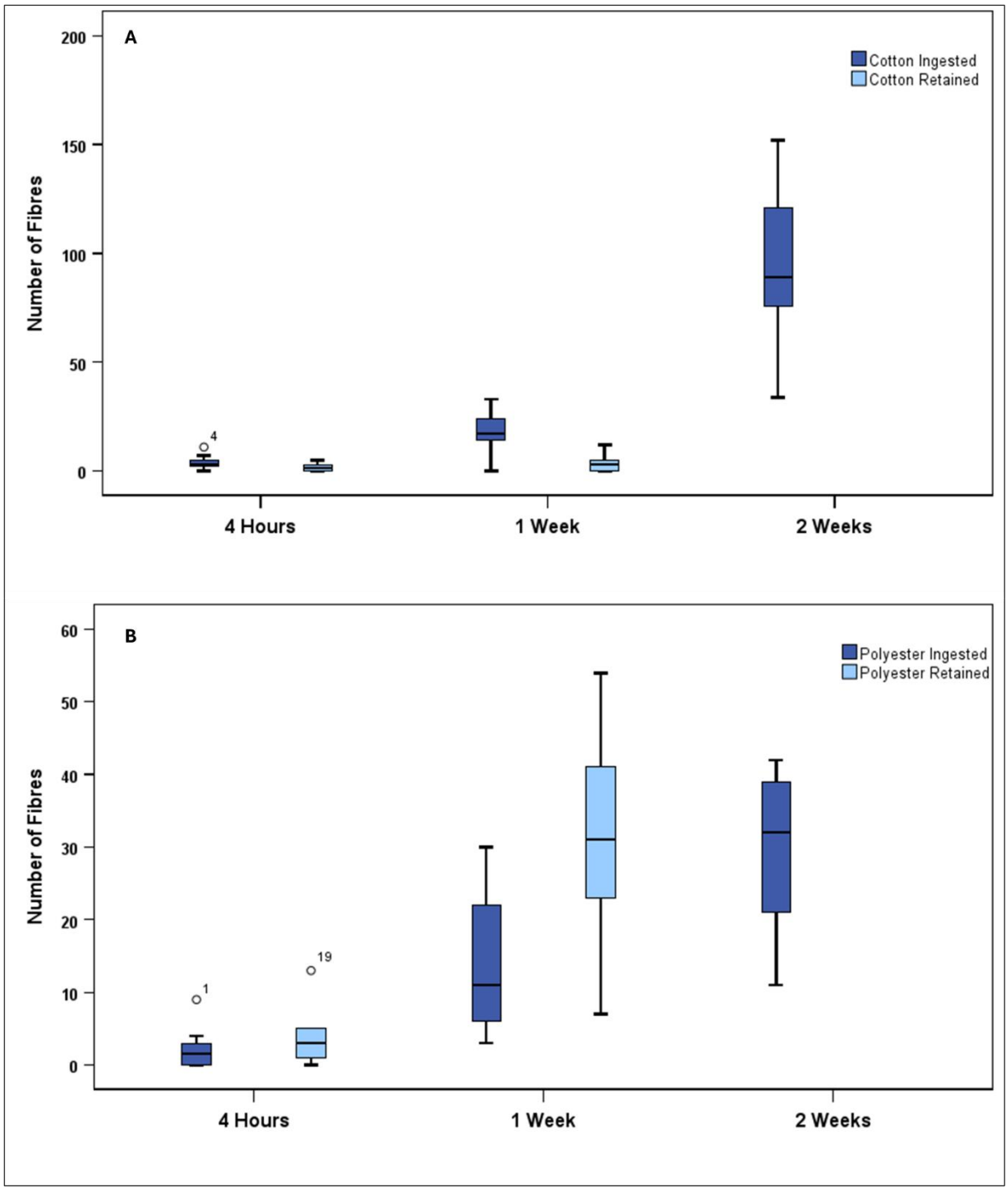

Figure 2: Number of ingested and retained cotton (A) and polyester (B) fibres by Gammarus pulex after exposure periods of 4 hours, 1 week, and 2 weeks and a depuration time of 24 hours. Dark blue represents ingested fibres, while light blue represents retained fibres. Outliers are indicated by open circles. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR.

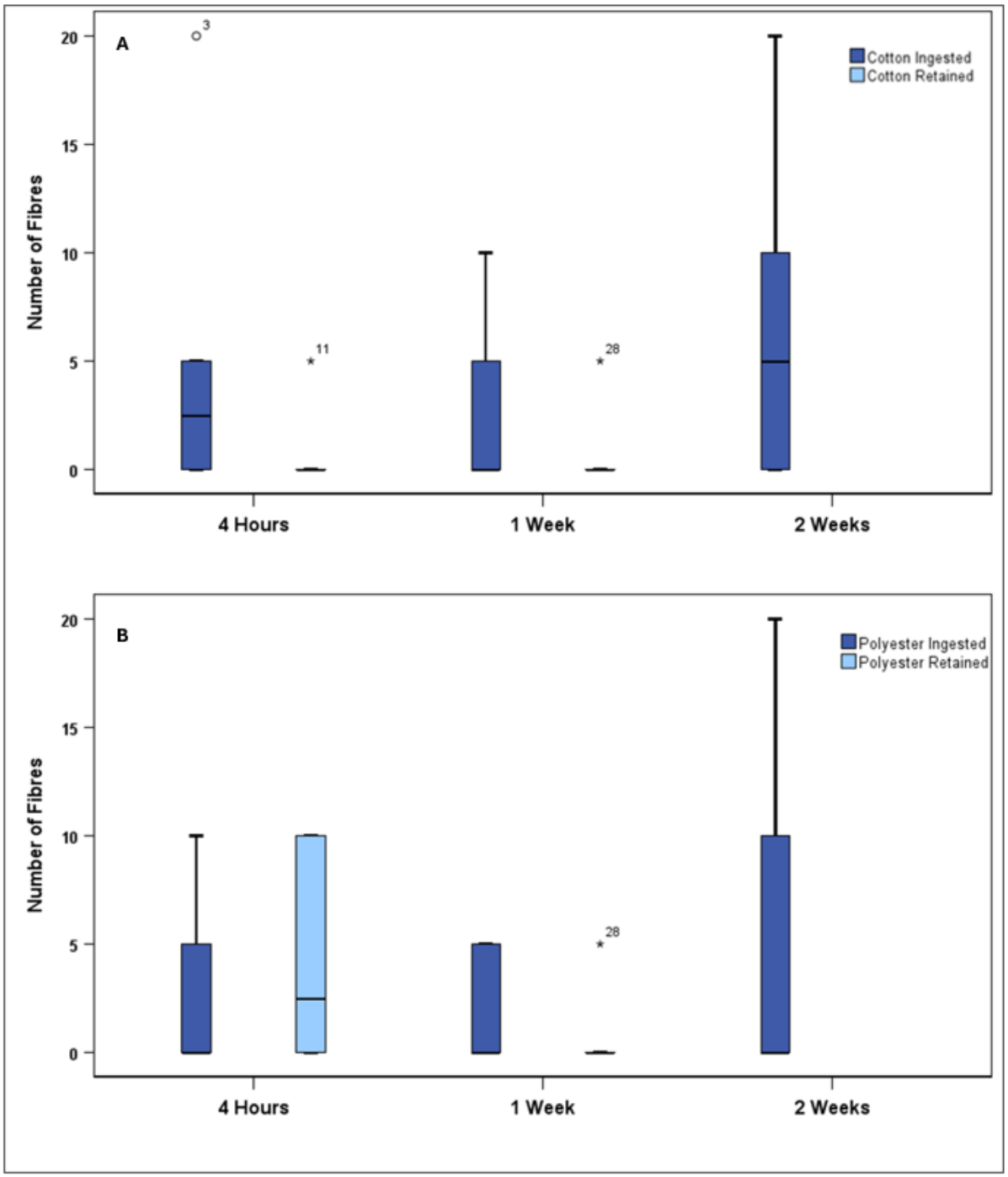

Figure 3: Number of ingested and retained cotton (A) and polyester (B) fibres by Corbicula fluminea after exposure periods of 4 hours, 1 week, and 2 weeks and a depuration time of 24 hours. Dark blue represents ingested fibres, while light blue represents retained fibres. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR. Outliers are indicated by open circles and extreme outliers are marked with a star (*).

4.3 Comparative Ingestion: *Corbicula fluminea* vs. *Gammarus pulex*

4.3.1 Ingestion and retention of polyester fibres

Mann-Whitney U tests showed significant differences in the ingestion of non-biofilmed polyester MFs by *G. pulex* compared to *C. fluminea* after one week of

exposure (U = 2, p =0.032), with a greater average ingestion of MF by *G. pulex* (Median = 11, n = 5) compared to *C. fluminea* (Median = 0, n = 5, see figures 2b & 3b). Similarly, after two weeks of exposure, ingestion of non-biofilmed polyester fibres remained significantly different (U = 1, p =0.016), with a greater average ingestion of MF by *G. pulex* (Median = 32, n = 5) than *C. fluminea* (Median = 0, n = 5, see figures 2b & 3b). No significant difference was found after a 4-hour exposure (U =46, p = .796). There were also significant differences in the ingestion of biofilmed fibres after a 4-hour exposure between taxa (U = 13, p =0.004), with a greater average ingestion of polyester MF by *G. pulex* (Median = 5.5 n = 10) than *C. fluminea* (Median = 0, n =10, see figures 2b & 3b).

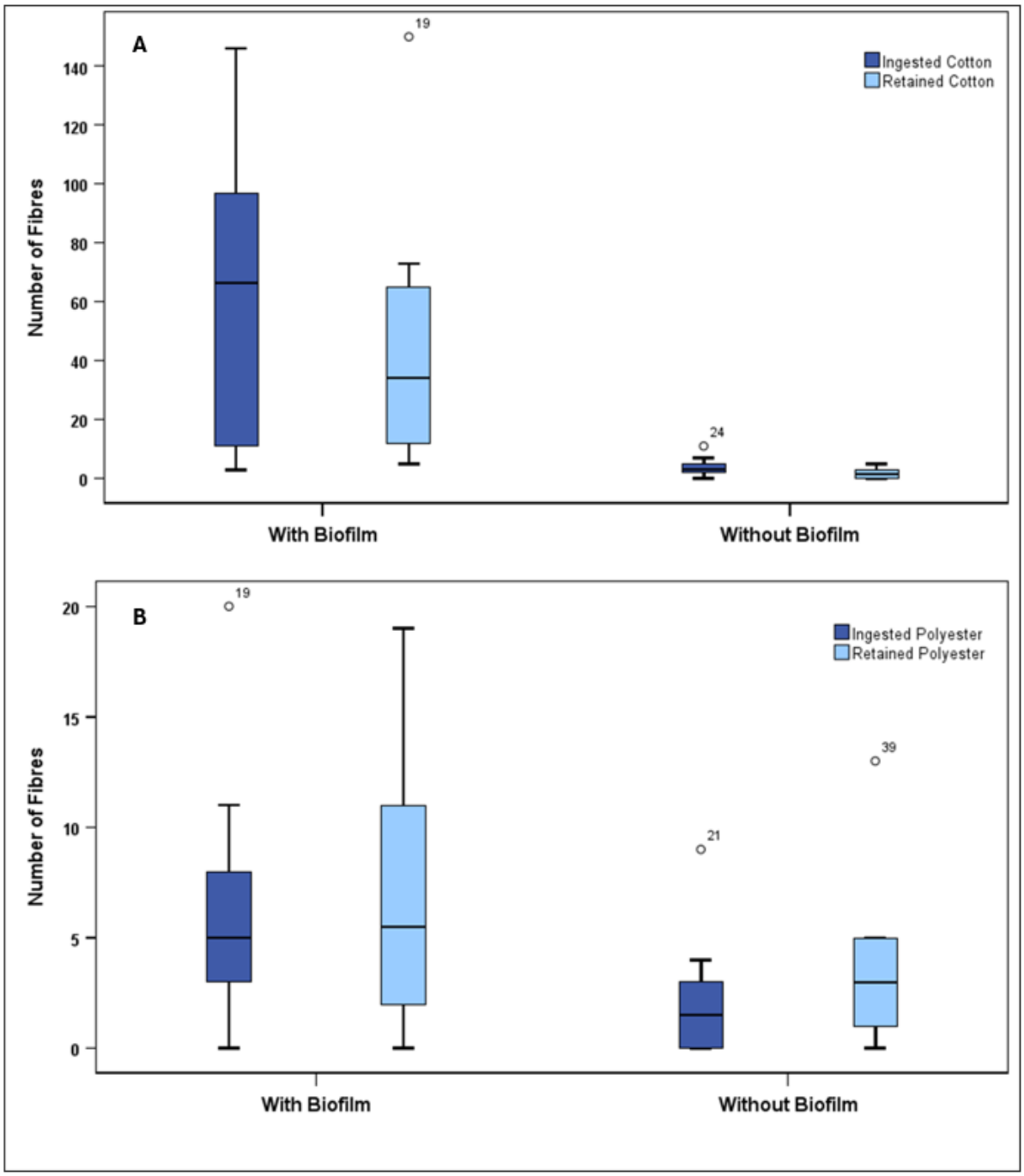

Figure 4: Number of ingested and retained cotton (A) and polyester (B) fibres by Gammarus pulex after a 4-hour exposure to biofilmed and non-biofilmed fibres and a depuration time of 24 hours. Dark blue represents ingested fibres, while light blue represents retained fibres. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR. Outliers are indicated by an open circle.

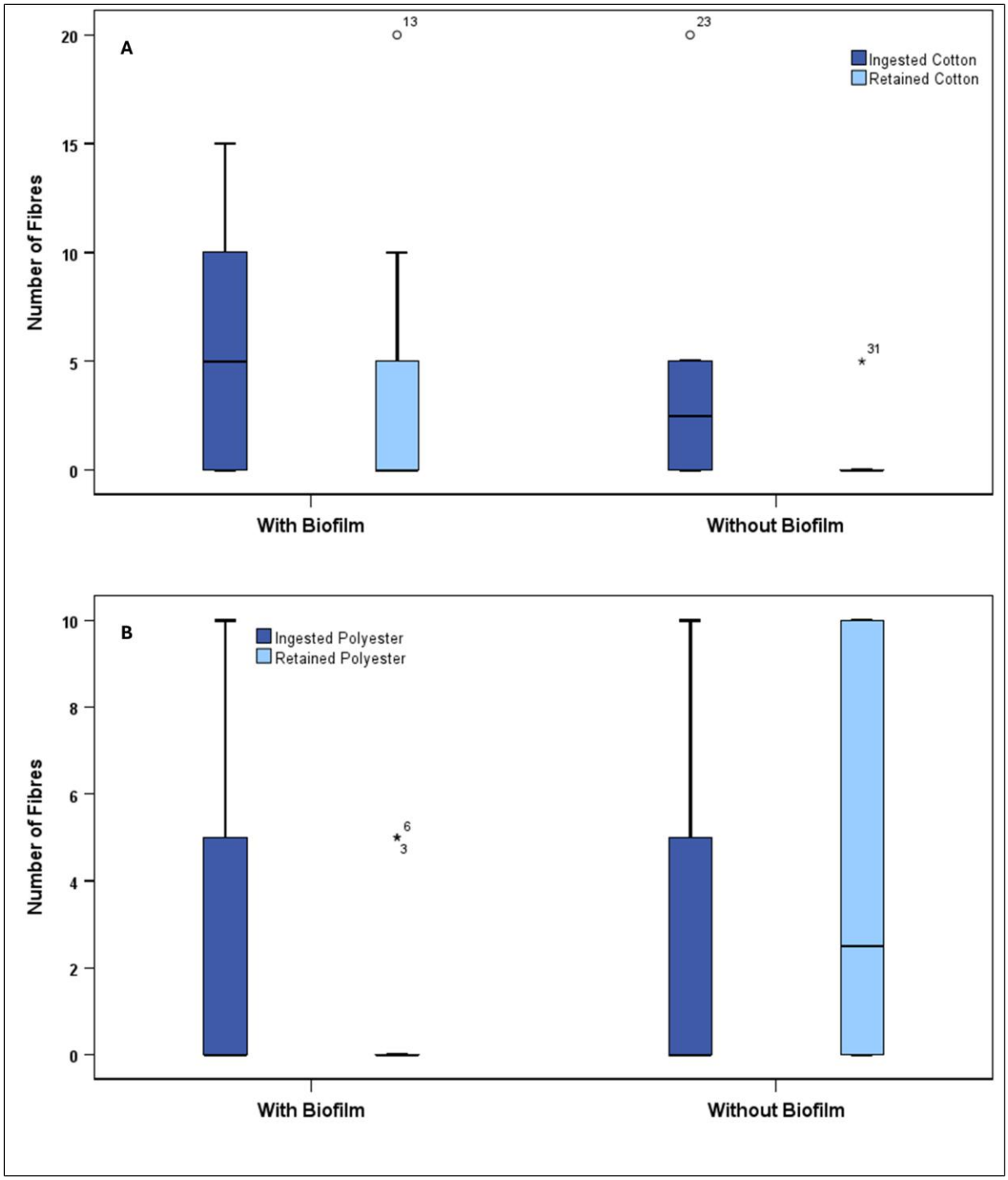

Figure 5: Number of ingested and retained cotton (A) and polyester (B) fibres by Corbicula fluminea after a 4-hour exposure to biofilmed and non-biofilmed fibres and a depuration time of 24 hours. Dark blue represents ingested fibres, while light blue represents retained fibres. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR. Outliers are indicated by open circles and extreme outliers are marked with a star (*)

There were also significant differences in the retention of unconditioned (non-biofilmed) polyester fibres between *G. pulex* and *C. fluminea* (Mann-Whitney U, U = 17, p =.011, figures 2b & 3b), with *G. pulex* retaining a greater average number of fibres (Median = 3, n = 10) than *C. fluminea* (Median = 0, n = 10). After one week, of exposure, the number of ingested unconditioned polyester MFs between animals

remained significantly different (Mann-Whitney U, U = .000, p =.008, figures 2b & 3b); *G. pulex* median = 31, n = 5; *C. fluminea* median = 0, n = 5). Similarly, there was a significant difference in the number of biofilmed polyester fibres ingested between taxa after a 4 hour exposure (Mann-Whitney U, U = 17, p =.011, figure 8), with *G. pulex* retaining a greater number of fibres (Median = 5 n = 10) than *C. fluminea* (Median = 0, n = 10).

4.3.2 Ingestion and retention of cotton fibres

Mann-Whitney U tests showed significant differences in the ingestion of cotton MFs between *G. pulex* and *C. fluminea* for unconditioned cotton fibres and after two weeks of exposure (U = .000, p =.008), with a greater average ingestion of MF by *G. pulex* (Median = 89, n = 5) than *C. fluminea* (Median = 5, n = 5, figures 2a and 3a). No significant difference was found after a 4-hour exposure for unconditioned cotton fibres (U = 45, p = .739) or after a 1 week exposure (u = 3.5, p = 0.56). For biofilmed cotton fibres there was also a significant difference between taxa (U = 11.5, p =0.002), with a greater average ingestion of MF by *G. pulex* (Median = 66.5, n = 10) than *C. fluminea* (Median = 5, n = 10, figures 2a and 3a).

No significant differences were found in the number of fibres retained between taxa for unconditioned cotton fibres after a 4 hour (Mann-Whitney U, U = 45, p = .631) or 1 week exposure (Mann-Whitney U, U = 7.5, p = .310). There was a significant difference in the retention of biofilmed cotton fibres between *G. pulex* and *C. fluminea* (Mann-Whitney U, U = 4.5, p < .001) with *G. pulex* retaining a greater average number of fibres (Median = 34, n = 10) than *C. fluminea* (Median = 0, n = 10, figures 2a and 3a).

4.4 Ingestion and Retention with Exposure Time

4.3.1 *G. pulex* ingestion and retention

The average MF ingestion by *G. pulex* was statistically different between exposure times for both cotton (Kruskal-Wallis, H(2, 20) = 13.046, p = .001) and polyester fibres (Kruskal-Wallis, H(2, 20) = 13.523, p = .001). Post hoc comparisons were conducted using Mann-Whitney U Tests with a Bonferroni adjustment. The difference in the ingestion of cotton fibres after a 4-hour and two week exposure was

statistically significant (mean rank 4 hours = 6.4, mr 2 weeks = 18.0, p = .001, figures 2a and 3a). The difference in ingestion of polyester MFs was also statistically significant between 4 hour and 2 week exposure periods (mean rank 4 hours = 5.85, mr 2 weeks = 17.1, p = .001, figures 2b and 3b) None of the other comparisons were significant after the Bonferroni adjustment (table 3 & 4).

Table 4: Bonferroni-adjusted pairwise comparisons of the number of ingested cotton fibres after a 4 hour, 1 week and 2 week exposure period.

Pairwise Comparisons					
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
4 hours-1 Week	-4.800	3.223	-1.489	.136	.409
4 hours-2 Weeks	-11.600	3.223	-3.599	<.001	.001
1 Week-2 Weeks	-6.800	3.722	-1.827	.068	.203

Table 5: Bonferroni-adjusted pairwise comparisons of the number of ingested polyester fibres after a 4 hour, 1 week and 2 week exposure period.

Pairwise Comparisons					
Sample 1-Sample 2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj. Sig.[a]
4 hours-1 Week	-7.350	3.231	-2.275	.023	.069
4 hours-2 Weeks	-11.250	3.231	-3.482	<.001	.001
1 Week-2 Weeks	-3.900	3.730	-1.045	.296	.887

The average retention of MF (concentration after the exposure period and 24 hour egestion period) by *G. pulex* was significantly different between the 4 hour and 1 week exposures for polyester (Mann-Whitney U, U = 49, p = .001), with a greater average retention of fibres after 1 week (Median = 31, n = 5) than 4 hours (Median = 3, n = 10, figures 2b and 3b), but was not significantly different for cotton (Mann-Whitney U, U = 29.5, p =.594, figures 2a and 3a).

4.4.2 *C. fluminea* ingestion and retention

The average MF ingestion by *C. fluminea* was not significantly different between exposure time for cotton (Kruskal-Wallis, H(2, 20) = .768, p = .681) or polyester (Kruskal-Wallis, H(2, 20) = .315, p = .854). The average MF retention by *C. fluminea* was also not significantly different between 4 hour and 1 week exposures for either cotton (Mann-Whitney U, U = 15.5, p =.254) or polyester (Mann-Whitney U, U = 27.5, p =.768).

4.5 Polyester vs Cotton MFs

4.5.1 G. pulex ingestion and retention of polyester and cotton

Mann-Whitney U tests showed significant differences between the ingestion of unconditioned polyester and cotton MFs after a two week exposure period (U = 2, p =0.032), with a greater average ingestion of cotton MF (Median = 89, n = 5) than polyester (Median = 32, n = 5, figures 2 and 3) but not significant difference after a 4 hour (U = 33, p = .218), or 1 week exposure (U = 10, p = .69) to unconditioned fibres. There was also a significant difference for biofilmed fibres after a 4h exposure period (U = 15, p =0.007), with a greater average ingestion of cotton MF (Median = 66.5, n = 10) than polyester (Median = 5.5, n = 10, figures 2 and 3).

There was also a significant difference in the retention of unconditioned polyester and cotton fibres for *G. pulex* after one week of ingestion (Mann-Whitney U, U = 24, p = .016) with *G. pulex* retaining a less cotton fibres (Median = 3, n = 5) than polyester (Median = 31, n = 5, figures 2 and 3), but no significant difference in the retention of unconditioned polyester and cotton after a 4 hour (Mann-Whitney U, U = 64, p = .315) exposure period. There was also a significant difference for biofilmed fibres (Mann-Whitney U, U = 7.5, p < .001) with *G. pulex* retaining a greater average number of cotton fibres (Median = 34, n = 10) than polyester (Median = 5, n = 10, figures 4 and 5).

4.5.2 C. fluminea ingestion and retention of polyester and cotton

Mann-Whitney U tests showed no significant differences in the ingestion of unconditioned polyester and cotton MFs by *C. fluminea* after a 4 hour (U = 45, p = .739), 1 week (U = 11.5, p = .841), or two week exposure (U = 11, p = .841, figures 2 and 3). There were also no significant differences in the retention of unconditioned polyester and cotton fibres by *C. fluminea* after a 4 hour (U = 28, p = .105) or 1 week exposure (U = 12.5, p = 1, figures 2 and 3). Similarly, there was no significant difference in the ingestion (U = 27, p = .089), or retention (U = 48, p = .912), between biofilmed cotton and polyester fibres after a 4-hour exposure period (figures 4 and 5).

4.6 Turbid and non turbid

Mann-Whitney U tests showed no significant differences in the ingestion of cotton MFs by *C. fluminea* between turbid and clean water (Mann-Whitney U, U = 47.5, p = .853) or the retention of fibres under the same conditions (Mann-Whitney U, U = 54.5, p = .739, figure 6). Similarly, there were no significant differences for polyester

MFs between turbid and clean water (U = 45.5, p = .739 for ingestion; U = 60.5, p = .436 for retention, figure 6).

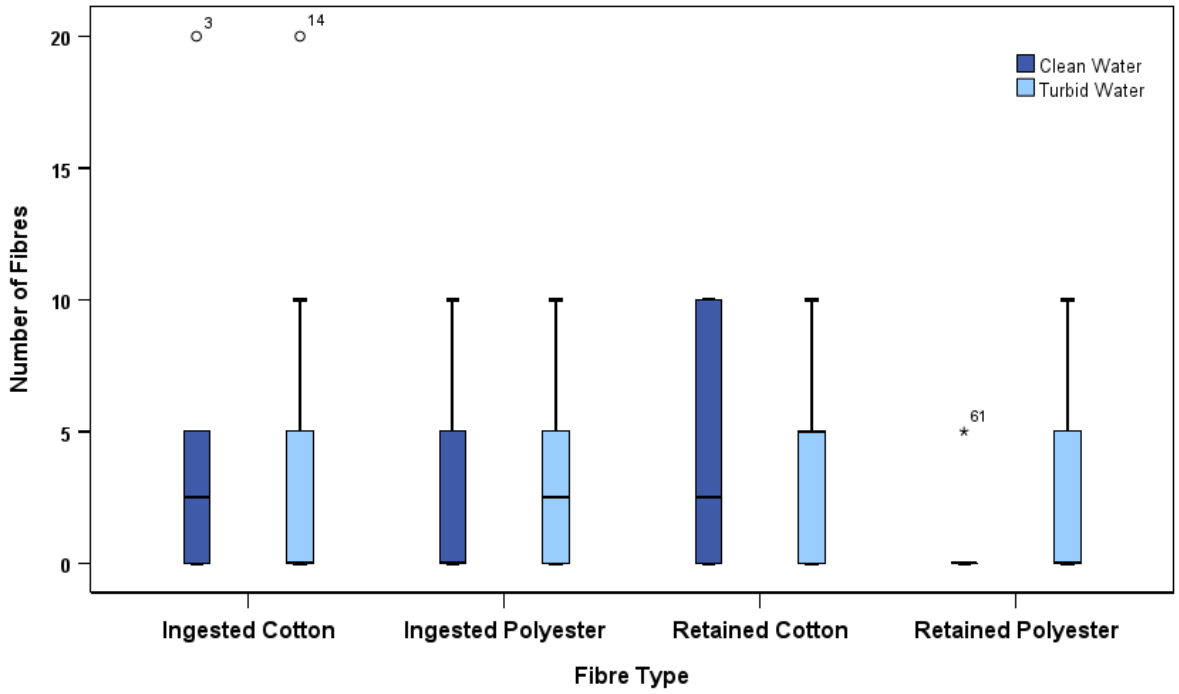

Figure 6: Ingested and retained fibres by C. fluminea after a 4 hour exposure and 24h depuration period respectively for clean and turbid water conditions. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR. Outliers are indicated by open circles and extreme outliers are marked with a star (*)

4.7 Biofilmed vs Non-biofilmed Fibre Ingestion and Retention

4.7.1 *G. pulex* ingestion

Mann-Whitney U tests showed significant differences in the ingestion of MFs by *G. pulex* between biofilmed and non-biofilmed polyester MFs (U = 77, p =0.043), with a greater average ingestion of biofilmed (Median = 5.5, n = 20) than non-biofilmed polyester fibres (Median = 1.5, n =20, figure 4b). Similar results were found for cotton fibres (U = 92, p < .0.001), with a greater average ingestion of biofilmed (Median = 66.5, n = 20) than non-biofilmed cotton fibres (Median = 3, n =20, figure 4a).

4.7.2 *G. pulex* retention

Mann-Whitney U tests showed significant differences in the retention of MFs by *G. pulex* for biofilmed and non-biofilmed cotton MFs (U = 99.5, p < .001), with a greater average ingestion of biofilmed (Median = 34, n = 20) than non-biofilmed cotton fibres (Median = 1.5, n = 20, figure 4a). However, there was no significant difference in the

retention of polyester MFs between biofilmed and non-biofilmed MFs (U = 67.5, p = .190, figure 4b),

4.7.3 *C. fluminea* ingestion

Mann-Whitney U tests showed no significant differences in the ingestion of biofilmed and non-biofilmed MFs by *C. fluminea* for polyester (U = 39, p = .436) or cotton fibres (U = 58, p = .579, figure 5).

4.7.4 *C. fluminea* retention

Mann-Whitney U tests showed no significant differences in the retention of biofilmed and non-biofilmed MFs by *C. fluminea* for polyester (U = 41, p = .529), or cotton fibres (U = 60.5, p = .436, figure 5).

4.8 Fibre Length

4.8 Fibre length between biofilmed and non-biofilmed experiments

The length of cotton fibres in the reservoirs in the biofilm experiment (Median = 324.3, n = 100) were significantly shorter than equivalents in the non-biofilmed experiments (Median = 513.5, n = 100, figure 25 Mann-Whitney U, U = 2379.5, p < .001, figure 7). There was no significance difference with fibre length for polyester experiments (Mann-Whitney U, U = 5529, p = .195, figure 8).

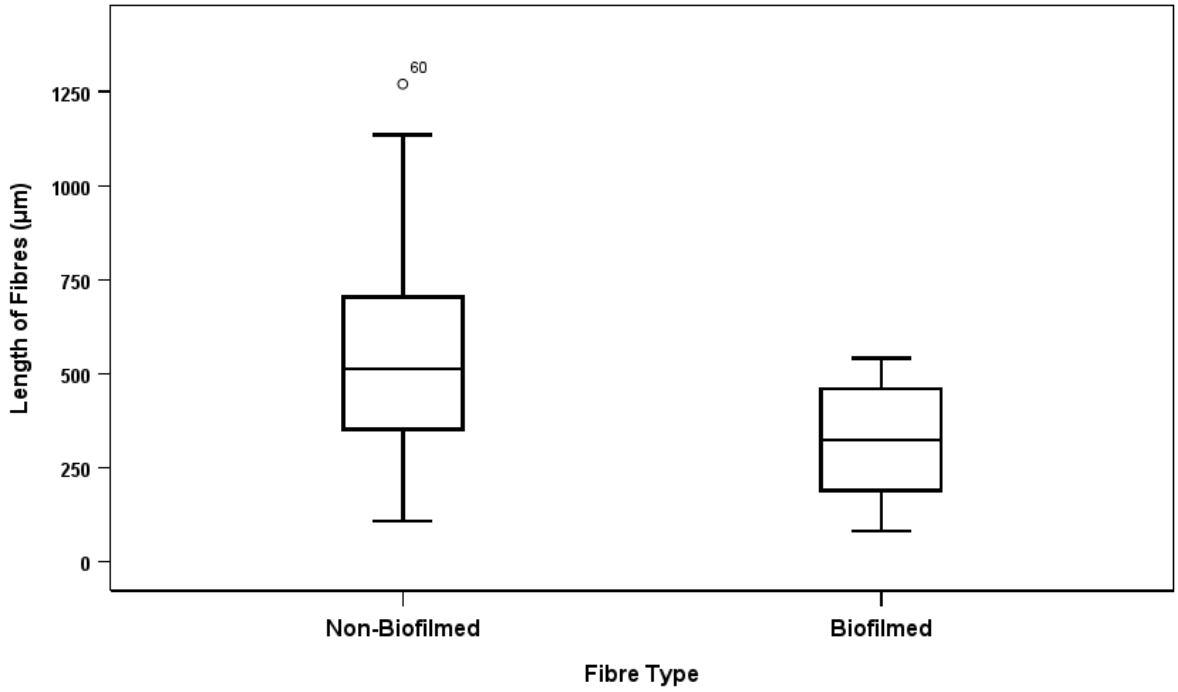

Figure 7: Length of non-biofilmed and biofilmed cotton fibres in the reservoirs of contaminated water used in the mesocosm experiments. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR. Outliers are indicated by open circles.

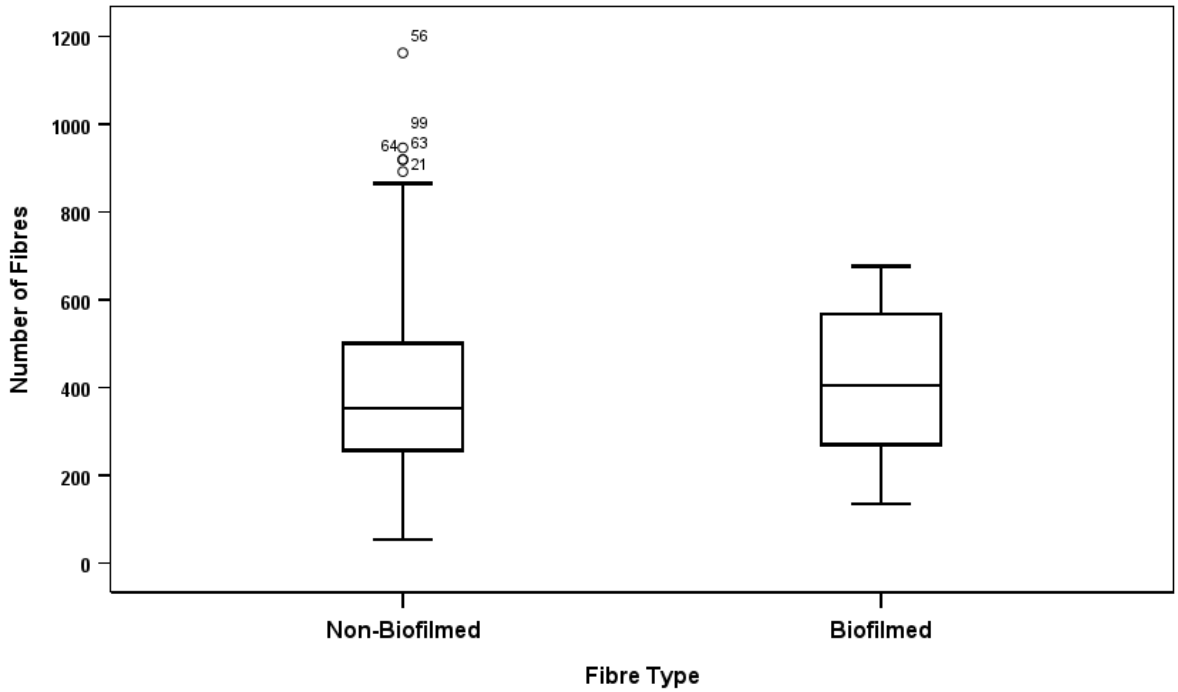

Figure 8: Length of non-biofilmed and biofilmed polyester fibres in the reservoirs of contaminated water used in the mesocosm experiments. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR. Outliers are indicated by open circles.

4.8.2 Fibre length of original, unconditioned particles compared to after ingestion

Mann-Whitney U tests showed no significant differences in the length of non-biofilmed fibres in reservoirs prior to spiking in experiments, and those ingested by *G. pulex*, extracted after dissection, for either cotton (U = 2012, p = .051) or polyester (U = 2488, p = .963).

Mann-Whitney U tests showed no significant difference in the length of non-biofilmed polyester fibres in reservoirs prior to spiking, and those ingested by *C. fluminea* (U = 852, p = .297). However, a significant difference was identified in the length of non-biofilmed cotton fibres in reservoirs prior to spiking in experiment, and those ingested by *C. fluminea* (U = 448, p <.001) with cotton fibres in the reservoir (Median = 513.5, n = 100) significantly longer than those ingested (Median = 324.3, n = 20, figure 9).

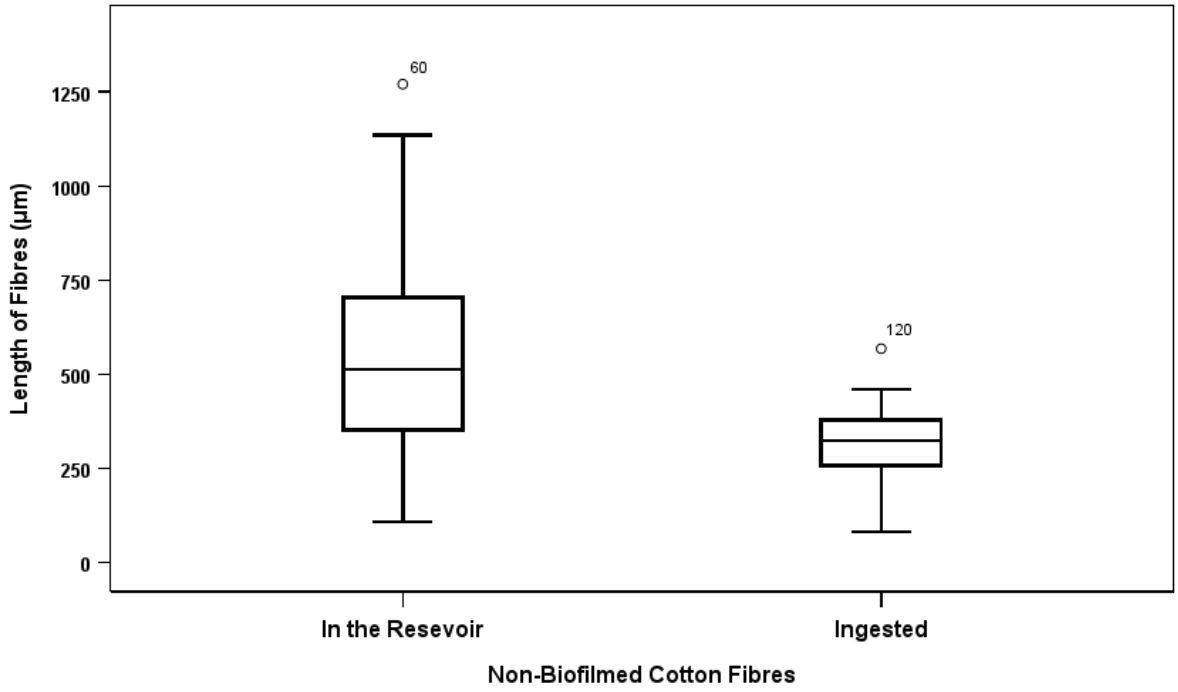

Figure 9: Lengths of cotton fibres in the non-conditioned reservoir of contaminated water used in the mesocosm experiments, and those extracted from C. fluminea. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR

4.8.3 Fibre length of original, biofilmed particles compared to after ingestion

Mann-Whitney U tests showed no significant differences in the length of fibres in the biofilmed reservoirs compared to those ingested by *G. pulex* for either cotton (U = 2276, p = .371) or polyester (U = 2488, p = .292).

Mann-Whitney U tests showed a significant difference in the length of fibres in the biofilmed reservoir, and those ingested by *C. fluminea* for polyester (U = 687.5, p = .028) with polyester fibres in the reservoir (Median = 431.4, n = 100) significantly longer than those ingested (Median = 310, n = 20, figure 10). No significant difference was found for biofilmed cotton in reservoirs and after ingestion (U = 986.5, p = .924).

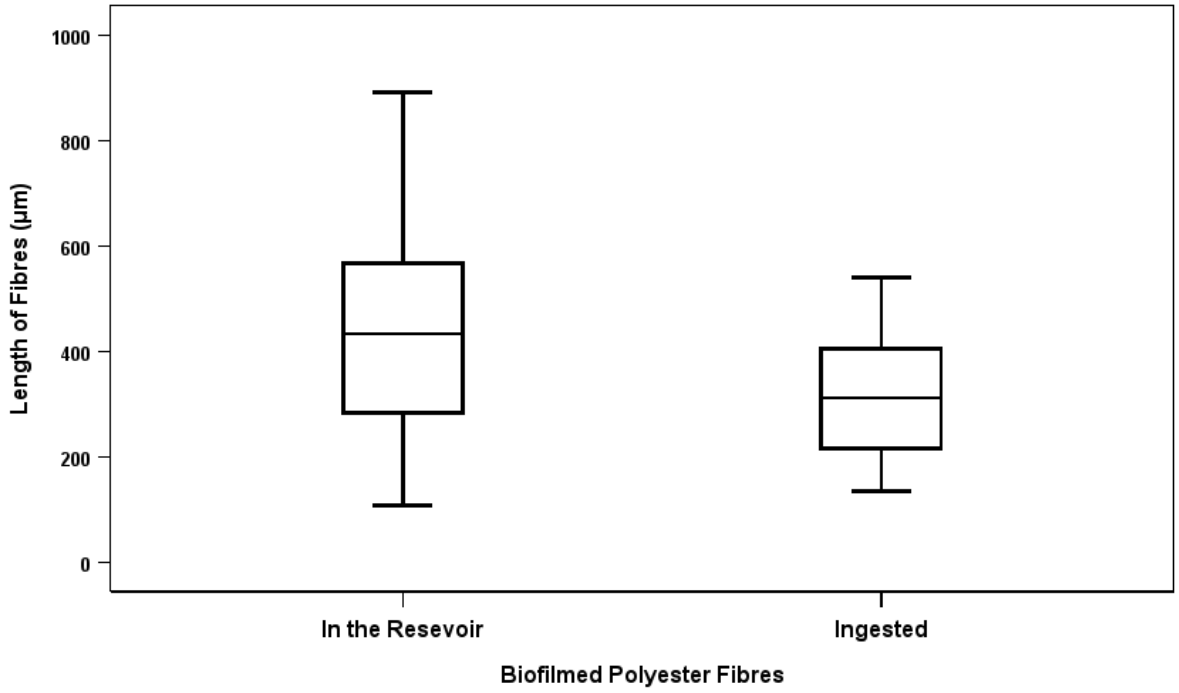

Figure 10: Length of polyester fibres in the biofilmed reservoir of contaminated water used in the mesocosm experiments, and those extracted from C. fluminea. The box represents the interquartile range (IQR), the horizontal line inside the box indicates the median, whiskers extend to 1.5 times the IQR.

5 Discussion

5.1 Mortality and Microfibre Ingestion

The lack of mortality recorded for *G. pulex* or *C. fluminea* is consistent with studies indicating that environmentally relevant concentrations of MPs do not typically cause mortality in *G. pulex* (Weber et al., 2018).

5.2. Ingestion and Retention of MFs

The lack of significant difference in the number of polyester or cotton fibres in the gut after both a 4 hour and 1 week ingestion period followed by a 24 hour egestion period for both *G. pulex* and *C. fluminea* (figures 2 & 3) suggests that MFs in this experiment were not easily egested by either species. For *G. pulex*, this is unexpected, as the species usually egests natural food items within 8 hours (Monk, 1977; Sutcliffe et al., 1981; Willoughby and Earnshaw, 1982). This prolonged retention may indicate that both MPFs and NMFs persist longer than natural food in the gut of *G. pulex*. Egestion times for C. fluminea are not know, however the findings that both cotton and polyester fibres are not efficiently egested after a 24 hour depuration period conflicts with a number of studies on bivalves. For example, marine bivalves *Crassotrea virginica* and *M. edulis* have been shown to egest the majority of ingested MPs and MPFs after 2, and 9 hours respectively (Craig et al., 2022; Weber et al., 2021), and the freshwater bivalve *Dreissena polymorpha* egested the majority of ingested particles after 12 hours (Weber et al., 2021).

The fact that, in contrast to other bivalves, *C. fluminea* did not efficiently egest fibres within the 24-hour period could suggest species-specific differences in the processing of MFs. *C. fluminea* may therefore be less able to egest MFs than other bivalves, or as limited research is available on the egestion of MFs it could be that MFs are egested more slowly than MPs. Such prolonged retention might increase the likelihood of physical harm, obstruction, or even translocation of MFs to other tissues, leading to more severe physiological impacts than seen in species that expel fibres more readily. These findings highlight the need for further research into the mechanisms of MF retention in different species.

The results found agree with studies that have shown MPs to be retained longer in invertebrates than vertebrate animals (e.g. Jemec et al., 2016), although this varies

between species. For example, while *Palaemon varians* (shrimp) expelled fibres within 24 hours (Saborowski et al., 2019), species like *Chironomus sp.* (non-biting midge larvae) and *Aeshna sp.* (dragonfly larvae) retained microfibres for longer periods (Khedre et al., 2023). These results highlight the need for understanding species-specific ecological traits to assess the impact of MPF and NMF ingestion.

The extended retention of MPFs in comparison to typical food sources in *G. pulex* may be influenced by the experimental set up, as during the depuration period *G. pulex* were without food, which has been shown to increase gut passage time (Monk, 1977). This aligns with findings by Moore (1975), where gut passage time for *G. pulex* ranged from 16 to 47 hours, depending on temperature when left to depurate without food. Given that other studies, including Gray and Weinstein (2017), also found MP residence times exceeding 24 hours, further investigation with longer depuration periods, including those where normal food is available, is needed. This is particularly significant given the importance of macroinvertebrate faecal pellets as a food source for other aquatic organisms (Joyce et al., 2007), as the egestion of MFs by macroinvertebrates could also lead to MF ingestion by other organisms.

Given the documented toxicity of these MFs (Jovanović, 2017; Ziajahromi et al., 2017; Gray and Weinstein, 2017; Hodgson, 2019; Qiao et al., 2019), and their propensity to be ingested and retained demonstrated in this study, it is essential to further investigate the mechanisms by which invertebrates ingest and retain them. Understanding these processes will be key to assessing the broader ecological impacts of MF pollution, including their movement and accumulation throughout the environment.

5.3 Comparative Ingestion: Corbicula fluminea vs. Gammarus pulex

Significant differences were observed in the ingestion of cotton and polyester fibres between *G. pulex* and *C. fluminea*. *G. pulex* ingested more polyester fibres than *C. fluminea* after biofilm accumulation, and after one and two weeks of exposure (figure 4 & 5). However, after just four hours of exposure, ingestion rates were not significantly different between the species. The same trends were seen for cotton fibres, with *G. pulex* ingesting more fibres than *C. fluminea* in all treatments except during the 4-hour and 1 week exposure (figure 6 & 7). The higher number of ingested fibres by *G. pulex* could be attributed to their greater mobility (Redondo-

Hasslerham et al., 2018), which enables them to encounter more particles than the more sessile *C. fluminea*. It could also be related to differences in feeding strategy, with *G. pulex* typically detrital feeding by sorting through deposited sediments for organic material, in contrast to *C. fluminea* that typically filter-feed.

G. pulex also retained more fibres across most treatments (figures 8, 9, 10, 11), likely due to higher ingestion rates. However, research indicates that bivalves may be efficient in selectively ingesting particles based on size, shape, and chemical properties (Tamburri & Zimmer-Faust, 1996; Woods et al., 2018; Ward et al., 2019). *C. fluminea* ingested very few fibres across all treatments. Therefore, the reduced retention of fibres compared to *G. pulex* may be due to their ability to avoid consuming MPFs and NMFs. This may also be due to the ability of *C. fluminea* to adjust its filtration rate in response to the concentration of suspended particles (Way et al., 1990). The absence of typical food (e.g. suspended algae particles) in the experiments likely prompted *C. fluminea* to conserve energy by limiting filtration. Bivalves like *C. fluminea* are known to selectively ingest and egest food, with previous research on MF ingestion by Woods et al., (2018) finding that 71% of MF in *M. edulis* were observed in their pseudofeces. This ability to reject inedible particles may have contributed to the comparatively low ingestion of both cotton and polyester by *C. fluminea*. These results highlight the species-specific differences in MF ingestion and retention between *G. pulex* and *C. fluminea*. The higher ingestion and retention of MFs by *G. pulex* suggest that this species may be at greater risk of fibre accumulation compared to *C. fluminea*. Understanding the ecological differences between species is crucial in assessing the broader environmental impact of MF pollution.

5.4 Ingestion and Retention Trends with Exposure Time

5.4.1 Gammarus pulex

Fibre ingestion by *G. pulex* increased significantly with longer exposure times ($p = .001$), from 4 hours to 2 weeks. Figure 12 shows a trend of increased fibre ingestion after 1 and 2 weeks of exposure, which may suggest a continuous buildup of fibres in the organism rather than a constant ingestion rate. This also suggests that the egestion of MFs does not occur at the same rate as egestion. Experiments with longer duration will be important to assess whether fibre ingestion continues to

increase with prolonged exposure. Additionally, the persistence of fibres suggests that *G. pulex* do not learn to avoid these particles over time, as they continue to ingest fibres throughout the exposure period.

The significant increase in the number of polyester fibres retained after 1 week exposure compared to 4 hours ($p = .001$) in combination with no equivalent significant difference in cotton fibre retention, suggests that unconditioned polyester fibres are more likely to bioaccumulate than comparable natural fibres (figure 13). These results also suggest that polyester fibres are more likely to be retained after long exposure in comparison to cotton fibres, which are more readily egested, potentially meaning that MPFs such as polyester represent a greater threat to *G. pulex*.

Although not investigated here, the presence of a biofilm will likely impact the ingestion of MFs over a long exposure period. This is particularly true given the significant differences in the ingestion and retention of biofilmed fibres by *G. pulex*. Therefore, further research should consider the combined effect of the presence of a biofilm and long exposure periods on the ingestion and retention of MPFs and NMFs, particularly given the environmental relevance of this scenario.

5.4.2 Corbicula fluminea

In contrast to *G. pulex*, *C. fluminea* did not show significant differences in fibre ingestion or retention after one or two weeks of exposure. Similar results have been found for another freshwater bivalve, *Mytilus edulis* (blue mussel), which show no MP bioaccumulation in their tissues after 14-days of exposure (Ward et al., 2019, cited in Ward et al., 2019b). The lack of ingestion and retention of MFs could be due to their feeding strategy, as *C. fluminea* filter feed and may be more selective in filtering out particles, as has been found for other suspension-feeding bivalves (Ward et al., 2019b).

5.5 Comparative ingestion and retention of polyester and cotton

5.5.1 Gammarus pulex ingestion

G. pulex consistently ingested greater numbers of cotton fibres compared to polyester fibres, with significant differences in their ingestion for biofilmed fibres ($p = .007$, Figure 15), and for non-biofilmed fibres after a two-week exposure ($p = .032$,

figure 14). This suggests that for *G. pulex* cotton fibres may be easier to ingest than polyester, or that they are intentionally consumed. The consistently lower ingestion of polyester may be due to *G. pulex*'s ability to detect chemical cues from the plastic MFs, allowing them to avoid ingesting polyester fibres. The higher tensile strength and longer length of polyester fibres (Skokan et al., 2020) also likely makes them harder to break down and ingest than cotton.

Interestingly, this finding conflicts with research by Yardy and Callaghan (2021), which showed that *G. pulex* ingested a greater number of synthetic MFs than cotton. However, previous research by Yardy and Callaghan (2020) reported that *G. pulex* avoided consuming plastics. These variations in findings may be due to differences in experimental conditions, such as the presence of food, shelter or pressures, or fibre characteristics, such as fibre length and conditioning.

Processed cotton fibres contain potentially toxic dyes and additives and are known to persist in the environment much longer than would be expected of a sustainable product. Combined with the greater ingestion of cotton fibres than polyester by *G. pulex, this* suggests that the use of natural alternatives to plastic products may not be a significantly better option.

5.5.2 Gammarus pulex fibre retention

Despite the higher ingestion of cotton fibres (median = 17) than polyester (median = 11), *G. pulex* retained more polyester fibres than cotton after one week of exposure ($p = .016$), suggesting that polyester may be harder to egest than cotton. Despite this, after a one-week ingestion period followed by a 24-hour depuration period, no significant difference in the retention of cotton fibres was found. A similar pattern was observed for polyester, where the number of retained fibres dropped (median ingestion = 17 fibres, median retention = 3 fibres), but this was not statistically significant. This suggests that there was no significant difference in the number of fibres ingested or retained for cotton or polyester. The greater retention of polyester fibres after a one week exposure contrasts with the finding that a significantly greater number of cotton fibres were retained when fibres were biofilmed than polyester ($p = .001$), likely caused by the significantly higher ingestion of biofilmed cotton fibres (median = 66.5, polyester ingestion median = 5.5).

For both *C. fluminea* and *G. pulex* the impact of other properties of the polyester and cotton fibres used here were not considered. For example, as the colour of MPs has been shown to have an impact on their ingestion (Rios et al., 2022), likely due to the different chemical compositions of dyes, there is the potential that the ingestion and retention trends found here are not entirely reflective of the fibre type and are impacted by physiochemical properties not investigated here.

These results therefore suggest that when ingested in comparable numbers, polyester fibres are retained for longer. However, cotton fibres are consistently ingested in greater numbers. An understanding of the relative toxicity of fibres is needed to assess the ecological risk of these fibres given the greater ingestion of cotton, and longer retention of polyester.

5.5.3 Corbicula fluminea

In contrast to *G. pulex*, *C. fluminea* showed no significant differences in the ingestion or retention of cotton and polyester fibres. Both fibre types were ingested and retained in similarly low quantities, indicating that *C. fluminea* do not exhibit a preference for either fibre type, and again suggests that *C. fluminea*'s selective feeding mechanism limits fibre ingestion and retention. This finding contrasts with research by Li et al., (2019) who found the ingestion of MPFs by *C. fluminea* varied depending on the polymer. As *C. fluminea* are therefore suggested to uptake MPFs depending on their chemical properties (Lie et al., 2019) a difference in the ingestion of cotton and polyester fibres would be expected. However, the long length of fibres used here may have impacted the ingestion of both fibre types by *C. fluminea* (discussed in section 5.7.2), causing their consistently low ingestion which may outweigh impact of the differences in chemical properties of the fibres.

5.6 Turbid and Clean Water Conditions

No significant differences in the ingestion or retention of MFs were observed by *C. fluminea* between turbid and clean water conditions. This indicates that the presence of suspended particles in the water column did not significantly impact MF ingestion by *C. fluminea*. This finding contrasts with previous studies, which found that the presence of normal food sources to increase or decrease the ingestion of microplastics in various invertebrate species (e.g., Scherer et al., 2017). However, it

does support the suggestion that *C. fluminea* are highly efficient at selectively filtering food and avoiding non-food particulates.

These results also suggest that the use of clean water in laboratory ecotoxicology studies of MFs may give comparable results to environmentally relevant water given a lack of difference in the ingestion and retention of fibres. The turbidity of the water used was not measured; therefore, further research investigating the impact of different turbidity levels on MF ingestion should be considered.

5.7 Biofilmed vs. Clean Particles

5.7.1 Gammarus pulex

G. pulex ingested significantly greater numbers of both cotton ($p < .001$), and polyester fibres ($p = .043$), when biofilms were present (figure 23). This was expected, as *G. pulex* has a known preference for biofilm materials (Bärlocher and Kendrick, 1975; Lange et al., 2005; Bloor, 2011). As well as the attraction of *G. pulex* to biofilms themselves, biofilms contain microorganisms that release chemical cues, which can disguise the nature of plastic particles and increase the likelihood of ingestion (Savoca et al., 2017; Vroom et al., 2017; Procter et al., 2019) particularly by organisms such as *G. pulex* that can detect chemical cues. The formation of biofilms is also likely to cause an increase in fibre settling rates (Auta et al., 2017; Semcesen and Wells, 2021), which could make the fibres more accessible to *G. pulex*, a benthic feeder.

G. pulex retained a significantly greater number of cotton fibres when biofilmed ($p < .001$); however, this is likely caused by their significantly higher ingestion rate in comparison to non-biofilmed equivalents (discussed in section 5.5.1). This was not the case for polyester, where no significant difference in retention was observed between biofilmed and clean fibres.

The different ingestion and retention of cotton and polyester when biofilms are present may be due to the distinct microbial communities that form on their surfaces. Fibre morphology and hydrophobicity influence microorganism adherence, with synthetic polymers like polyester being less colonized than natural fibres like cotton (Harrison et al., 2018; Čuk et al., 2024; Zambrano et al., 2019; Zambrano et al., 2020; Royer et al., 2021).

Despite the higher average ingestion of biofilmed fibres, not all *G. pulex* ingested comparable amounts of fibres, with a large range in the numbers of both polyester and cotton fibres ingested between individuals (figure 23). This variability is likely caused by the aggregation of fibres, which tends to occur when a biofilm is present. Aggregation can result in pockets of high or low fibre concentrations, leading to varied ingestion rates across individuals, even within the same exposure scenario (Jemec et al., 2016).

These results suggest that biofilms enhance MF ingestion, particularly for cotton, potentially mimicking the natural food sources of *G. pulex* and disguising chemical cues.

5.7.2 Corbicula fluminea

In contrast to *G. pulex*, *C. fluminea* showed no significant differences in the ingestion or retention of biofilmed versus clean fibres, regardless of whether they were cotton or polyester. This may be due to the filter-feeding mechanism of *C. fluminea*, filtering algae and other suspended particles rather than feeding directly on biofilms. However, this contrasts with previous research where biofilming increased MP ingestion by filter feeding oysters (Fabra et al., 2021), suggested to be caused by the nutritional value of the biofilm disguising the toxicity of the plastic.

Unlike for *G. pulex*, the increased settling of biofilmed fibres may have reduced their availability in the water column for *C. fluminea* to filter. However, although regarded as filter feeders for the purpose of this study, *C. fluminea* can adjust their feeding mechanism when required. Many bivalves are known to also deposit feed, particularly during juvenility when the individual is small, to supplement filter feeding (Cummings and Graf, 2015), including *C. fluminea* (Way et al., 1990; Hakenkamp et al., 2001). During the experimental exposures, a number of *C. fluminea* were observed sweeping their pedal along the bottom of the beakers, likely in an attempt to deposit feed. However, very few particles were ingested by *C. fluminea* when fibres were biofilmed, despite the potentially higher settling rate.

The disparity between the effects of biofilms on MF ingestion in *G. pulex* and *C. fluminea* underscores the importance of species-specific investigations of MF ingestion due to the influence of feeding mechanisms. As shredders, *G. pulex* feed on biofilm-associated materials, making biofilmed fibres more similar to their natural

food sources. In contrast, *C. fluminea* both filter particles from the water column and deposit feed, yet consistently low numbers of fibres were ingested both with and without a biofilm. These differences further highlight the complexity of MF ingestion and retention across species and feeding strategies.

5.8 Fibre Length and Its Impact on Ingestion

5.8.1 Reservoir Fibre Length

The average fibre lengths used in this study for both biofilmed (Cotton = 320.54 µm, Polyester = 408.95 µm) and non-biofilmed fibres (Cotton = 548.12 µm, Polyester = 395.95 µm) fit within the average sizes of MFs commonly found in the environment (200-700 µm, Allen et al., 2019; Dris et al., 2015; Wright et al., 2020, see figures 25 & 26). Therefore, the results found here are likely to be broadly representative of the interactions between MFs and invertebrates in the environment.

One key observation is that when biofilmed, the length of cotton fibres decreases, with the average cotton fibre reducing in length by 227 µm when a biofilm is present. There was no comparable reduction in the length of polyester fibres. The shorter fibre length of cotton led to a higher concentration of fibres in the biofilmed cotton reservoir (Table 1). The mechanism for this is not known, but it is hypothesized to be related to the physical and biological conditions in the garden pond, which could have contributed to fibre breakdown during their residence in the environment, as well as the characteristics of the fibres. Cotton fibres, like most natural fibres, are staple fibres, short fibres with irregular surface structures, while polyester is a filament fibre, a long continuous length of fibre which is often smoother and stronger than staple fibres (Lawrence, 2015). Polyester fibres are known have as much as twice the tenacity of cotton (Lawrence, 2015), therefore when exposed to the same stressors, cotton fibres are more likely to break and shorten than polyester.

The ingestion of MPs by organisms is often proportional to their concentration in their environment, with a number of invertebrates including *G. pulex, Centropages typicus (copepod), Daphnia magna (water flea),* and *Gammarus fossarum (freshwater shrimp),* exhibiting MP and MF ingestion proportional to concentration levels (Scherer et al., 2017; Redondo-Hasslerham et al., 2018; Blarer & Burkhardt-Holm, 2016). *C. fluminea* have also been shown to ingest fibres in proportion to the concentration in the water (Su et al., 2018; Li et al., 2019). Therefore, the higher

concentration of cotton fibres in biofilmed treatments associated with their shorter length and breakdown, may have made them more available for ingestion. This effect was not observed with polyester fibres, which retained their original length even when biofilms were present. This suggests that biofilms did not significantly affect the structural integrity of polyester fibres. The greater availability of cotton fibres in the water column after biofilm formation points to a higher environmental risk of NMF ingestion, as smaller MPs and MFs are more widely available for ingestion throughout food webs.

The consistency of length in polyester fibres highlights a critical difference between natural and synthetic fibres. Cotton fibres fragment more easily than polyester under mechanical stress, leading to a greater number of shorter cotton fibres. The fibre concentration was not adjusted according to the effect of biofilm formation, as this reflects the environmental reality, as NMFs like cotton are more prone to fragmentation, leading to their higher concentrations in aquatic environments compared to MPFs. The increased fragmentation and shorter length of cotton fibres when biofilmed may also explain why greater amounts of NMFs are frequently reported in environmental studies compared to MPFs (e.g. Dris et al., 2017; Stanton et al., 2019).

5.8.2 Ingestion Trends in Corbicula fluminea

C. fluminea ingested significantly more short biofilmed polyester ($p = .028$) and non-biofilmed cotton fibres ($p < .001$) than those present in the reservoirs (figure 27). However, this pattern was not seen for biofilmed cotton or non-biofilmed polyester fibres. Previous studies have shown that *C. fluminea* are more likely to ingest polyester fibres less than 250 µm in length (Li et al., 2019), with bivalves known to reject long MFs (Ward et al., 2019). This accounts for the greater ingestion of short fibres; however, this finding is not consistent for non-biofilmed polyester and biofilmed cotton. This may be due to the differing retention times of fibres of different lengths. Previous research has shown that the time for egestion of MPs by bivalves varies depending on the size of particles, with larger MPs retained for longer (Brilliant and MacDonald, 2000). This could therefore indicate that the non-significant results may have been influenced by the egestion mechanisms of *C. fluminea*, as well as the ingestion mechanism, as they may have retained larger fibres for longer than short fibres, increasing the average length of fibres in the gut of *C. fluminea*.

Selective particle ingestion likely explains the limited ingestion of the fibres used in this study, many of which exceeded the upper size limit for particle filtration by *C. fluminea* of 16um suggested by Way et al. (1990).. Despite this, MFs were ingested by *C. fluminea*. This finding correlates with research by Jemec et al., (2016) which found MPFs greater than the upper limit of ingestion were ingested by *D. magna* therefore suggesting that MFs have the potential to be ingested regardless of the preferred size limit of organisms who ingest them. This may be attributable to the high length-to-width ratio of the fibres which may have allowed for ingestion when correctly aligned, however these results suggest that MFs of the lengths used in this study are more likely to be rejected than ingested by *C. fluminea*.

5.8.3 Fibre Length and Significance in *Gammarus pulex*

No significant difference in length was found for the fibres ingested by *G. pulex* contrasting with previous research suggesting that organisms ingesting MPs and MFs might contribute to their fragmentation (Mateos-Cárdenas et al., 2020; Khedre et al., 2023). However, the p-value of 0.051 for the difference in the length of non-biofilmed cotton fibres ingested by *G. pulex* does suggest that with a greater sample size a significant result is possible, and that this is an important avenue for future research to pursue, and that additional research into fibre fragmentation dynamics in aquatic ecosystems would be valuable.

This result also suggests that the greater ingestion of biofilmed particles by *G. pulex* was not influenced by the length of the particles. Instead, for cotton, the greater ingestion was likely influenced by the increased fibre concentration, while for polyester biofilms likely disguised the chemical cues of the fibres.

6. Conclusion

The results presented here show that both NMFs and MPFs (cotton and polyester) are ingested by the freshwater invertebrates *G. pulex* and *C. fluminea*, and that the time taken to egests these particles may be longer than would be expected from typical, natural foods. The ingestion and retention of MFs varies between species, and is influenced by the fibre type and the presence of biofilms. The water turbidity did not influence results.

The variation between species is likely due to differences in feeding mechanisms, which also impacted the organisms' interaction with fibres when biofilmed. These findings suggest that the ecological characteristics of invertebrates play a major role in their interactions with MFs. Filter feeders, like *C. fluminea*, are typically considered to be highly susceptible to MF ingestion due to their unselective feeding strategies. However, this study suggests that these organisms are capable of selectively rejecting MFs, thus limiting their retention and potential harmful impacts. In contrast, deposit feeders like *G. pulex* appear to be at greater risk of prolonged MF retention, as they are less capable of rejecting particles based on their characteristics.

The retention of MFs and difficulty in egesting both NMFs and MPFs could lead to biomagnification across trophic levels, posing risks to the broader food web. Although MPFs may be retained longer than NMFs, both fibre types have the potential to negatively impact ecosystems through their ingestion by invertebrates. The study highlights the need for further research on the impacts of NMFs to enhance our understanding of their environmental risks and to ensure accurate sustainability assessments, underscoring the ecological risks posed by both plastic and natural MFs in freshwater environments, and contributing to the broader discussion on environmental pollution and sustainability.

The push from the fashion industry towards increased use of NMFs such as cotton may be misleading the consumer about the impact of the products they are buying. This research highlights the potential risks of both MPFs and NMFs to invertebrates, with both *G. pulex* and *C. fluminea* ingesting and retaining both types of fibres. These risks are often overlooked due to the perception that NMFs are less harmful and more biodegradable than plastic alternatives, which have been the central focus of environmental concern throughout public and political discourse. Not only are organisms likely to be directly impacted by their ingestion, but the significant burden on water supplies to grow NMFs and the high CO2 emissions involved in their production (Niinimaki et al., 2020) suggests that NMFs are unlikely to be more sustainable or less harmful than MPFs.

Future studies should further investigate how fibre length, fibre type, and biofilm formation influence the ingestion and retention of both natural and synthetic MFs across a wider range of species. Special attention should be given to the different

feeding strategies of invertebrates and how these influence their interactions with MF pollutants. Longer periods of exposure may also provide insights into the capacity of freshwater invertebrates to egest MFs, particularly given the finding of continual MF ingestion by *G. pulex* over time.

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