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Ingestion and egestion of microplastic fibers in the green sea urchin *Strongylocentrotus droebachiensis*: An experimental exposure

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Abstract

Plastic is a major component of marine litter in the world's oceans and therefore it is critical we develop a better understanding of the fate of plastic in the marine environment, and the potential impacts on biota. Microplastics are the most abundant size class (<5 mm) of plastic found in the marine environment and have the potential to interact with smaller marine organisms at lower trophic levels. Microplastic fibers are a predominant type of microplastics found in the ocean, and in field studies of microplastics in benthic species. The main pathway of microplastic exposure to organisms is ingestion, which can be influenced by polymer type and biofouling state. A wide range of organisms ingest microplastic fibers, however without a better understanding of species-specific ingestion, retention, and egestion it is difficult to predict the potential ecological consequences. The current study exposed urchins to microplastic fibers, and wool as a natural fiber control, between 1 to 5 mm in length at a concentration of 128 fibers mL^{-1} for 48 hours. Fibers were exposed in two states, non-biofouled and biofouled. Post exposure urchins were kept for a depuration period of 86 hours. Intestines and faecal pellets were analyzed to investigate the influence of biofouling and fiber length on uptake, retention, and egestion of microplastic fibers. All urchins had ingested and egested microplastic fibers, but no statistically significant difference was found in ingestion or egestion between non-biofouled and biofouled fibers. Fiber length was not affected by urchin digestion. Findings about fiber retention was hampered due to low survival of urchins during the depuration period. Improvements in captivity conditions for future studies may be able to build upon understanding of retention time of microplastic fibers. This study demonstrated urchins can egested microplastic fibers in faecal pellets. As urchin faecal pellets are an important food source, microplastic fibers in faecal pellets could be an exposure pathway for other benthic species. Retained fibers in urchin intestines will be available for trophic transfer, potentially increasing the harmful impact of microplastic fibers in the environment.

Keywords: accumulation; benthic; biofouling; digestion; intestines; faecal pellets; microfibers

Abbreviations

BF	Biofouled
KOH	Potassium hydroxide
MF	Microfiber
MP	Microplastic
NaClO	Sodium hypochlorite
NBF	Non-biofouled
WWTF	Wastewater treatment facility

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1 Introduction

1.1 Marine plastic pollution

Plastics include a broad range of synthetic organic polymers with diverse applications and uses (Geyer, Jambeck, & Law, 2017). Plastic is a major component of marine litter in the world's oceans, which are considered to be the ultimate sink for mismanaged waste (Horton & Dixon, 2018). With the global production of plastics ever increasing, reaching almost 370 million tons in 2019 (PlasticsEurope, 2020), it must be considered that the rate of plastic waste input into the oceans will continue to rise (Horton & Dixon, 2018). Pathways of plastic into the marine environment can be through direct disposal from human activities on land and at sea (GESAMP, 2019), rivers, runoff, wastewater (Browne, 2015), and poorly managed waste (Jambeck et al., 2015). The abundance of plastic in the marine environment is important in understanding the potential interactions with marine organisms. A modelling study estimated that in 2010 between 4.8 to 12.7 million tons of plastic waste entered the ocean globally (Jambeck et al., 2015) amounting to 5.25 trillion plastic particles floating in the world's ocean waters (Eriksen et al., 2014). Transport of plastics in the ocean occurs due to ocean surface currents and wind, moving plastic far from its local source and causing accumulation on shorelines, sediments (Browne et al., 2011), subtropical gyers (Eriksen et al., 2014), and remote areas such as the Arctic (Halsband & Herzke, 2019; Ross et al., 2021). Sinking of plastic particles that are denser than sea water results in an accumulation in deep-sea sediments (Woodall et al., 2014). Understanding the sources, transport, and distribution of plastic in the marine environment is a critical step in improving knowledge of the behavior and fate of plastic in the oceans, and the potential impacts on biota (Henry, Laitala, & Klepp, 2019; Tang et al., 2020). The diversity of polymer characteristics influences the behavior and fate of plastics in the environment (Horton & Dixon, 2018).

1.2 Properties of plastic

Plastic debris is recognized as a substantial environmental problem, but issues remain in common categorization and definitions (Hartmann et al., 2019). The different physical-chemical properties of plastic will influence their fate in the environment, including polymer type, chemical composition, size, shape, and color (Hartmann et al., 2019; Rochman et al., 2019). The different properties of plastic polymers make them suitable to different applications. Different polymers have different densities (table 1), which is an important characteristic influencing the plastic particles position in the water column, and accordingly which organisms

will encounter it in the environment (Andrady, 2017; Botterell et al., 2019). Pelagic species are more likely to encounter plastics with density lower than sea water that are postitively buoyant in the water column, and benthic species are likely exposed to denser and biofouled (BF) plastics that sink to the sea floor (Carbery, O'Connor, & Palanisami, 2018). Size categories are a commonly used characteristic to describe plastics present in the environment and is important in understanding plastic particle interaction with biota (Lusher, 2015). The common size divisions for plastics are macro (25 – 1000mm), meso (5-25mm), micro (<5mm), and nano (<1µm; GESAMP, 2019). Negative impacts of macroplastics occur in larger marine species, such as marine mammals, turtles, seabirds, and fish, through entanglement, ingestion, and smothering (Kühn, Bravo Robelledo, & van Franeker, 2015). Micro and nanoplastics are the most abundant size class found in the ocean (Brander et al., 2020). These smaller size classes of plastic have the potential to interact with smaller marine organisms at lower trophic levels (Lusher, 2015). Microplastics (MPs) can be further classified by their source, primary or secondary. Primary MPs are manufactured at that size and secondary microplastics are created by fragmentation of larger plastic items, during use or once disposed of (GESAMP, 2015; Hale, Seeley, La Guardia, Mai, & Zeng, 2020). Primary MPs found in the environment include virgin pellets, microbeads from cosmetics, or industrial abrasives and are typically sphere shaped and symmetrical (Sundt & Syversen, 2014). Secondary MPs are morphologically diverse including fragments, films, foams, and fibers (Rochman et al., 2019). There is some confusion in the literature with primary and secondary classifications, as MP fibers are defined by some as primary (Belzagui, Crespi, Alvarez, Gutierrez-Bouzan, & Vilaseca, 2019; Boucher & Friot, 2017), and by others as secondary microplastics (Gago, Carretero, Filgueiras, & Vinas, 2018; GESAMP, 2015). This study will define MP fibers as secondary MPs, as detachment of fibers from textiles during laundry is considered here as the main pathway into the marine environment. Identifying the properties of MPs such as polymer type, density, size, and shape can help identify the source of the particle and improve understanding of the fate and impacts in the environment (Rochman et al., 2019; Tang et al., 2020).

Table 1: Applications and specific gravities of common plastic polymers. Specific gravity is the density expressed as a ratio of the materials density compared with fresh water at 4°C (note: freshwater is less dense than salt water). If specific gravity is larger than 1, the polymer is denser than freshwater (Adapted from GESAMP, 2015; Horton & Dixon, 2018)

Type of plastic	**Common applications**	**Density** (g cm^{-1})
Polyethylene (PE)	Plastic bags, storage containers	0.89-0.97
Polypropylene (PP)	Rope, bottle caps, gear, strapping	0.89-0.91
Polystyrene (PS)	Utensils, containers	1.04-1.08
Polyamide (nylon; PA)	Fishing nets, rope	1.13-1.35
Polyacrylonitrile (PAN)	Textiles, outdoor equipment	1.18
Polyethylene terephthalate (PET)	Bottles, strapping	1.29-1.40
Polyvinyl chloride (PVC)	Film, pipe, containers	1.30-1.58

1.3 Microplastic fibers

MP fibers are a predominant type of MPs present in the marine environment (Barrows, Cathey, & Petersen, 2018; Boucher & Friot, 2017) and have been found in all of the world's ocean surface and subsurface waters and marine sediments (Gago et al., 2018). Although pathways of MP and MP fibers to the Arctic are poorly understood (Halsband & Herzke, 2019) it has been hypothesized that the ocean currents flowing from the North Atlantic and North Pacific towards the Arctic transport MP fibers from the more populated southern latitudes (Lima et al., 2021; Ross et al., 2021). Despite this, there is limited understanding of MP fiber distribution and the mechanisms involved (Ross et al., 2021). A key source of MP fibers into the oceans is the release of fibers during domestic and industrial textile laundry (Browne et al., 2011; Napper & Thompson, 2016; Zambrano et al., 2019). Fibers that are released from clothing and textiles during washing enter the ocean via Wastewater Treatment Facilities (WWTFs) or are directly discharged in locations where no treatment facilities exist. There are many factors influencing the release of MP fibers through this pathway including fabric type, detergent used, age of the garment, type of washing machine, and the type of washing cycle used (De Falco et al., 2018; Napper & Thompson, 2016; Zambrano et al., 2019). In a study comparing acrylic, polyester, and cotton-polyester blend garments estimated fibers released during a "typical" 6kg washing cycle range from 130 000 to over 700 000 fibers, with acrylic garments releasing the most fibers (Napper & Thompson, 2016). The increasing production and use of synthetic textiles (figure 1)

in a range of products (including clothing, upholstery, carpets, and outdoor gear) suggests that MP fiber input into the environment will continue to rise. While some WWTFs have to ability to capture up to 99% of MPs, the released 1% potentially contributes large amounts of plastic particles to the oceans (Murphy, Ewins, Carbonnier, & Quinn, 2016). For Norway, it is estimated that 600 tonnes of MP fibers are released into the sewage systems each year (Sundt & Syversen, 2014). In more remote and low populated regions, such as the Arctic, WWTFs are often not available and there is direct input of MP fibers via wastewater into the environment (Gunnarsdóttir, Jenssen, Erland Jensen, Villumsen, & Kallenborn, 2013). Other sources of MP fibers into the marine environment can include degradation of cigarette butts and fragmentation of fishing nets and ropes that have been intentionally, or unintentionally, discarded directly into the marine environment (Cole, 2016; Welden & Cowie, 2016).

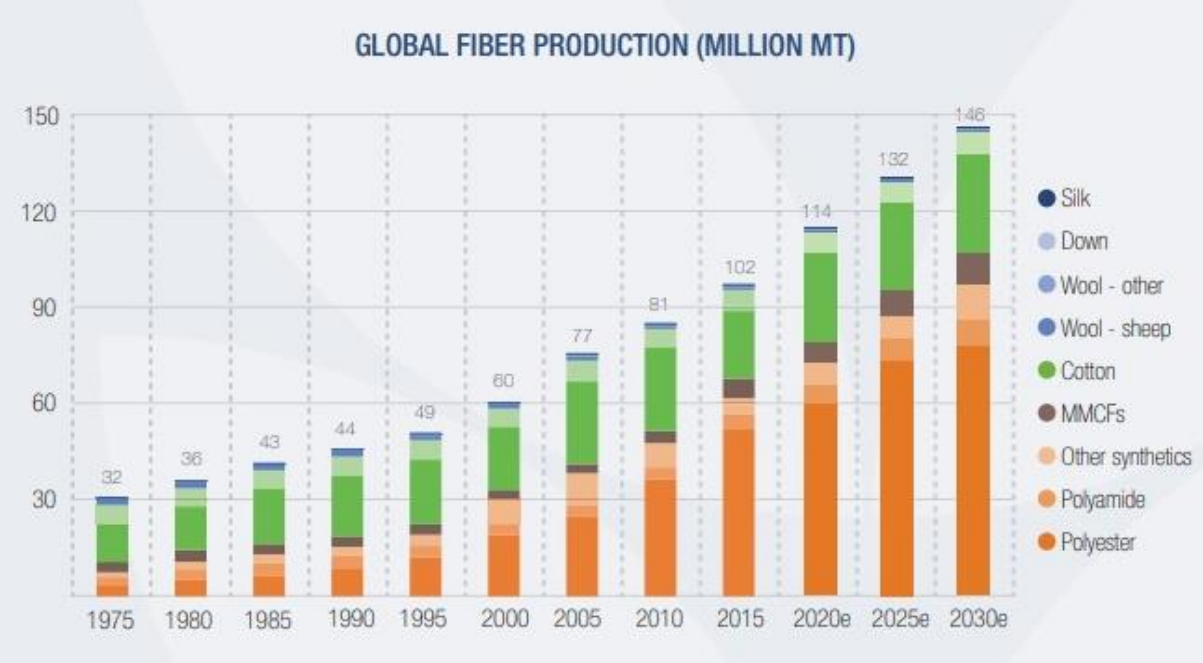


Figure 1: Global fiber production from 1975 to 2015, and estimates (e) of production until 2030. Synthetic fibers (orange) was approximately 70 million metric tonnes (MT) in 2019, making up 63% of the global fiber production. Future projections predict an increasing production of synthetic fibers (MMCFs = manmade cellulosic fibers; TextileExchange, 2020).

Studies investigating MP abundance in Arctic and Atlantic oceans and seas that specify the percentage of MP found that are fibers are presented in table 2. MP fibers are the most prevalent MP type. Studies investigating methods of measuring MP concentration in ocean waters have found that fiber concentrations may be underestimated. Manta net trawls are commonly used to measure MP concentrations (figure 2a) but allow fibers to pass through the mesh due to their narrow width (Barrows et al., 2018; Covernton et al., 2019). The studies summarized in table 2 present a large range in measured concentrations of MP indicating there is spatial heterogeneity,

which is to be expected due to variations in distribution and accumulation patterns across ocean regions (Lima et al., 2021). Different reporting units and methods used in MP abundance data collection make across study comparison difficult (Gago et al., 2018). Moreover, while field studies demonstrate that MP fibers are abundant in marine environments, laboratory studies on MPs most commonly use spherical microbeads (de Sa, Oliveira, Ribeiro, Rocha, & Futter, 2018). Microbeads are the most easily accessible MP for purchase, and methodological challenges in creating MP fibers has resulted in less laboratory studies on the MP fibers (Cole, 2016). Improvements in methods of microfiber (MF) production offer the opportunity for studies to robustly investigate MP fibers in laboratory exposure studies (Cole, 2016).

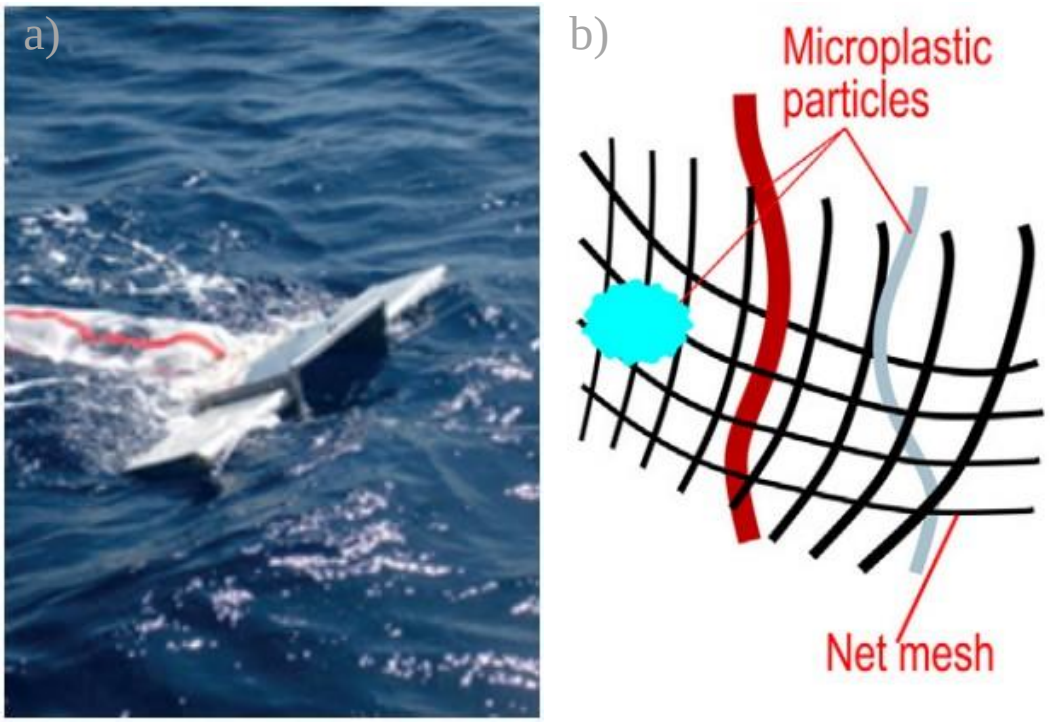


Figure 2: a) Manta net trawl collecting MPs from surface waters (GESAMP, 2015). b) Diagram showing how net trawl methods catch round shaped and fiberous MPs. The diameter of MP fibers is often smaller than the mesh size used (generally 300 – 350 µm). This allows for fibers to pass through the mesh resulting in underestimation of abundance of microplastic fibers (Covernton et al., 2019).

Table 2: Field studies of MP concentrations from Arctic and Atlantic ocean regions. All concentrations are number of particles per unit of measurement. There is a range of sampling locations and reporting units. Studies included have provided information of the percentage of MP particles found that were fibers. Standard deviation (sd) or standard error (se) has been included if provided in the study.

Region	Sample	Microplastic particle concentration	% fibers	Reference
Arctic	Surface water	49 m^{-3} ± 15.4 se	91.4%	(Ross et al., 2021)
Arctic	Surface water	31.3 L^{-1} ± 6.5 se	91 %	(Barrows et al., 2018)
Atlantic		13.4 L^{-1} ± 0.9 se		
Arctic	Surface water	0.34 m^{-3} ± 0.31 sd	95 %	(Lusher, Tirelli, O'Connor, & Officer, 2015)
	Sub surface water	2.68 m^{-3} ± 2.95 sd		
Arctic	Surface water*	20,000 m^{-3} ± 20,000 se	100 %	(Lima et al., 2021)
North Atlantic	Surface water*	1,800 m^{-3} ± 1,720 se	100%	
Southern European seas	Surface sediment (deep sea)	6,965 m^{-2} ± 3,669	100%	(Sanchez-Vidal, Thompson, Canals, & de Haan, 2018)
North Atlantic, Mediterranean Sea, Indian Ocean	Surface sediment (deep sea)	13.4 50 mL^{-1} ± 3.5 se	100%	(Woodall et al., 2014)
Tromsø	Beach sediment	72 kg^{-1} (dry weight)	98.7%	(Lots, Behrens, Vijver, Horton, & Bosker, 2017)

*Modelling study

1.4 Biofouling

Plastics are by design durable and once in the environment they are a persistent pollutant (Sait et al., 2021). Over its lifetime in the environment plastic is subject to weathering and degradation which will alter the physical and chemical properties (Vroom, Koelmans, Besseling, & Halsband, 2017). The changes in surface properties of MPs will influence the behavior of the particle, its interaction with biota, and the potential ecological impacts (Galloway, Cole, & Lewis, 2017; Lambert, Scherer, & Wagner, 2017). The level of degradation and transformation of MPs may be an important factor influencing the potential for negative consequences in the environment (Sørensen et al., 2021). Once in the environment

microorganisms colonize the surface of plastic, a process known as biofouling (figure 3; Lambert et al., 2017). Biofouling is the formation of a biofilm and begins with absorption of organic nutrients, which then attracts diverse communities of microbes (Oberbeckmann, Löder, & Labrenz, 2015) including algae, bacteria, fungi and protozoans (Rummel, Jahnke, Gorokhova, Kühnel, & Schmitt-Jansen, 2017). The composition of the microbial community varies depending on spatial and temporal factors, as well as the polymer composition and surface texture (Oberbeckmann et al., 2015). Under laboratory conditions biofilm formation on plastic has been found to occur within 1 week, and substantially increased over 3 weeks (Lobelle & Cunliffe, 2011). When the MP surface is covered with a biofilm, it may slow the degradation of plastic by reducing UV exposure (Rummel et al., 2017), which is a significant driver of plastic degradation (Sørensen et al., 2021). There has been evidence of preferential feeding on BF MP over 'pristine' plastics, which has been attributed to infochemicals emitted from the biofilm acting as a feeding cue to many organisms (Vroom et al., 2017). The formation of biofilms will influence the vertical transport of a MP particle in the water column, causing positively buoyant MP particles to become negatively buoyant (Rummel et al., 2017) and increase its sinking velocity (Kaiser, Kowalski, & Waniek, 2017). The intrinsic density of plastic polymers may leave less dense MP fibers in surface waters until other mechanisms such as biofouling cause sinking (Bagaev, Mizyuk, Khatmullina, Isachenko, & Chubarenko, 2017). Orientation and shape of the fiber will also influence the sinking velocity, as will the hydrodynamics of the specific location (Bagaev et al., 2017).

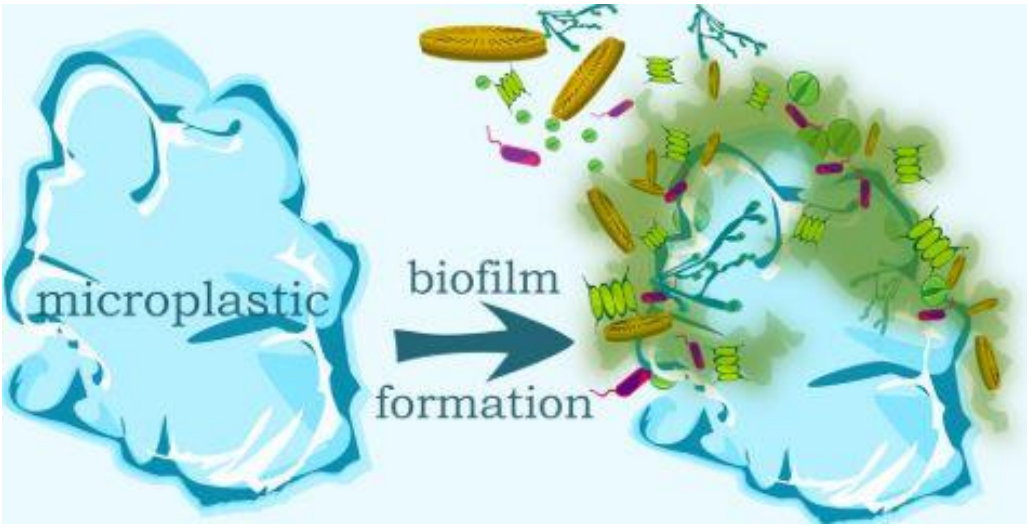


Figure 3: Biofilm formation is the process of colonization of microbial communities on the surface of MPs. Composition of the microbial community depends on the oceanic conditions and polymer characteristics, and changes over time (Rummel et al., 2017).

1.5 Bioavailability of microplastic fibers

The previously discussed characteristics of MPs (abundance, density, size, shape, and age) influence the bioavailability of MP in the marine environment (Rummel et al., 2017). The main pathway of MP exposure to organisms is ingestion (Lusher, 2015) which can be active, if MP resembles natural prey, or passive via particle filtration (Foley, Feiner, Malinich, & Hook, 2018). Ingestion of MP particles has the potential to impact organisms through mechanical processes, such as blockage or damage to intestines and decreased energy uptake (Capone, Petrillo, & Misic, 2020; Watts, Urbina, Corr, Lewis, & Galloway, 2015). There is also potential toxicological effects from MPs due to additives and chemicals used during production, or pollutants sorbed to plastics from the environment (Rochman, 2015). Uptake pathways of MP other than ingestion have been demonstrated in laboratory studies, including through inspiration across gills in crabs (Watts et al., 2014) and respiration in sea cucumbers (Mohsen et al., 2020; Watts et al., 2014). Studies investigating differences in ingestion of microbeads compared with microfibers have found varying results depending on organisms and feeding behavior (Bour, Avio, Gorbi, Regoli, & Hylland, 2018). A better understanding and quantification of uptake, retention times, and ability to remove MP fibers in marine organisms is required (Au, Lee, Weinstein, van den Hurk, & Klaine, 2017; Gouin, 2020). Increased residence time of MP particles in the digestive tract may lead to accumulation and trophic transfer (Au et al., 2017). There is evidence to show that MP fibers have longer residence times (Au et al., 2017), but not in all organisms (Bour, Hossain, Taylor, Sumner, & Almroth, 2020). Bioaccumulation is when there is a greater concentration of MP found in the organisms than the environment (Gouin, 2020) and will occur when the ingestion of MPs is higher than the amount egested (Au et al., 2017). While bioaccumulation and trophic transfer of MPs has been hypothesized, there is mixed findings in the literature. Studies have shown there is trophic transfer of MPs (Watts et al., 2014), but with little evidence of biomagnification, meaning higher trophic levels are not more exposed to MPs than lower trophic levels (Bour et al., 2018; Gouin, 2020). It is known that a wide range of organisms ingest MP fibers, but without better understanding of species specific ingestion, retention, and egestion it is difficult to predict the potential ecological consequences of MP fiber ingestion (Gouin, 2020; Lusher, 2015).

1.6 Synthetic microfiber ingestion by benthic organisms

Field studies investigating the occurrence of MPs in benthic organisms found fibers to be the predominant type, however, variability is found across taxa (Awour et al., 2020; Courtene-Jones et al., 2017; Fang et al., 2018; Fang et al., 2021). Studies in Arctic and sub-Arctic regions

have found MP fibers in a range of benthic groups including polychaetes, starfish, crabs, brittle stars, bivalves, whelks, fish, and shrimp (Bour et al., 2018; Fang et al., 2021; Fang et al., 2018). The findings of MP fibers in benthic organisms at higher rates than other types of MPs has three possible explanations: fibers are a predominant type of MP found in the oceans; fibers may be more likely to sink to the benthos due to biofouling and aggregation into balls; finally, there is a knowledge gap in the uptake, retention, and egestion dynamics of MP fibers in the marine environment (Fang et al., 2018). Benthic biota may play a crucial role in the fate and distribution of MP and MP fibers not only through ingestion, but also other processes such as bioturbation and burrowing (figure 4; Galloway et al., 2017; Pinheiro, Ivar do Sul, & Costa, 2020). Benthic habitats may act as a sink for negatively buoyant MP fibers, but also a source through resuspension of MP via bottom currents (Pinheiro et al., 2020). More research is needed to understand the biological interactions between benthic organisms and MP fibers. Bivalves are a taxonomic group that are commonly used to monitor coastal pollution, and have been used widely in studies investigating MP (Kazour & Amara, 2020). However, it has been suggested that due to their selective feeding strategy and ability to efficiently reject MP particles, bivalves are poor biomonitors for MP pollution (Ward et al., 2019; Weis, 2020; Woods, Stack, Fields, Shaw, & Matrai, 2018). This along with the field study findings that filter feeders are less exposed to MP than deposit feeders or predators (Bour et al., 2018) suggests other taxa groups should be investigated to monitor MP fiber pollution in benthic habitats (Ward et al., 2019). Echinodermata: Echinoidea is a benthic group in which interaction with MP fibers have been investigated very little (Suckling & Richard, 2020) and will be investigated in this study with the species *Strongylocentrotus droebachiensis*, the green sea urchin.

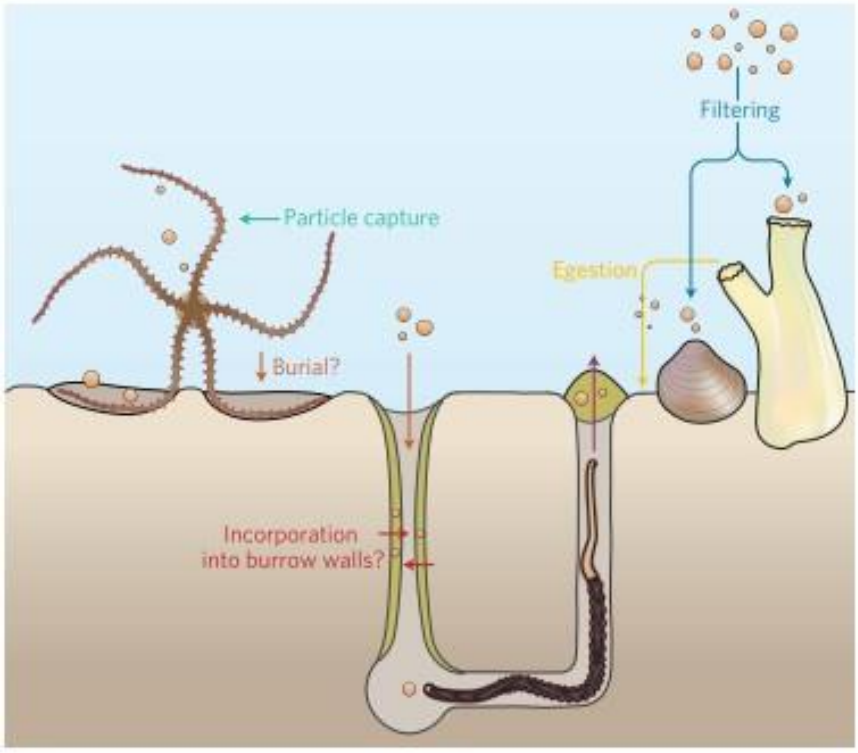


Figure 4: Mechanisms of benthic organisms that may influence microplastic fate and distribution. Filter feeding mussels and sea squirts may increase bioavailability of microplastics to benthos by uptake from the water column and excretion in the benthic boundary layer. Bioturbation of sediments by organisms like sea stars, and burrowing of worms may lead to burying of microplastics deeper in the ocean sediments (Galloway et al., 2017)

1.7 Microplastics interactions with sea urchins

Sea urchins play a pivotal role in shallow coastal food webs as well as ecosystem structure and function (Steneck, 2013). When urchin populations boom, urchin grazing can influence phase shifts from highly productive kelp forest to barren ground (Scheibling & Hatcher, 2013). This destruction of kelp forest due to large aggregations of urchins is accompanied by large shifts in other populations, including decreases in epifaunal invertebrates, other herbivores, and predatory fish populations (Scheibling & Hatcher, 2013). Urchins provide an important energetic link between macroalgae and benthic communities (Mamelona & Pelletier, 2005). They feed on macroalgae and through shredding and digestion make it available to smaller benthic organisms (Porter, Smith, & Lewis, 2019). Urchin faecal pellets are an important food source for benthic deposit and suspension feeders (Dethier et al., 2019; Mamelona & Pelletier, 2005; Scheibling & Hatcher, 2013). There are few studies that have investigated the interaction of MP with this key benthic organism. Investigating MP ingestion, whether MPs are accumulating in urchins or egested in faecal pellets, is important in understanding the behavior, fate, and biological interactions of MPs. Ingestion of MPs has been found in wild urchins, with fibers being the predominant type observed (Bour et al., 2018; Feng et al., 2020). The urchin digestive system has an inverted-funnel shape with a large mouth close to the sediment and a

small anus on top, which may contribute to accumulation of MPs (Feng et al., 2020). In a study of sea urchins off the northern-China coast, it was found that the gut was their main organ of MP accumulation (Feng et al., 2020). The average size of MPs in the gut was 1299 ± 969 µm, with smaller particles found in the gonads and coelomic fluid (Feng et al., 2020). A laboratory study exposing the urchin species *Paracentrotus lividus* to 10 and 45 µm polystyrene microbeads found the larger MPs in the digestive system (Murano et al., 2020). More than 70% of the smaller microbeads were found in the water vascular system, which may have lower retention time than the digestive system due to continuous inflow and outflow of seawater (Murano et al., 2020). These findings led to MP fiber uptake in the size range chosen for this study (1 to 5mm in length) to be investigated through intestinal tract and faecal pellet analysis. Fibers are the dominant shape of MPs found in the oceans and in field studies of benthic organisms, so there is a need for studies exploring MP fiber dynamics within benthic communities. To date no published laboratory studies on adult sea urchins and MP fiber interactions have been conducted. While laboratory studies cannot reflect the complexity of the environment (Bour et al., 2018) it is an important baseline for future studies investigating the dynamic interaction of MPs with benthic biota (Pinheiro et al., 2020). This study aims to contribute to knowledge of ingestion, retention, and egestion of MP fibers in a widely distributed benthic species, *S. droebachiensis.*

1.8 Study species

S. droebachiensis (figure 5) is a long lived, slow growing species found widely across Arctic-boreal habitats and is the most broadly distributed member of its family, Stronylocentrotidae (Scheibling & Hatcher, 2013). It is found commonly in shallow subtidal zones from 0 to 50 meters in depth, but also at depths up to 300 meters (Scheibling & Hatcher, 2013). *S. droebachiensis* prefers rocky substrata, although is known to be found on sediment (Scheibling & Hatcher, 2013). It is a species that shows low genetic divergence but large variety in phenotypic plasticity, feeding behavior, and reproductive timing within and between populations due to endogenous and exogenous factors (Scheibling & Hatcher, 2013). *S. droebachiensis* is prey to a wide range of fish species, invertebrates such as crabs, lobsters and sea stars, sea birds and otters (Scheibling & Hatcher, 2013). *S. droebachiensis* is also an important aquaculture species cultivated for human consumption of its roe (Scheibling & Hatcher, 2013).

Figure 5: Green sea urchin Strongylocentrotus droebachiensis. Photo: Claudia Halsband

1.8.1 Feeding and digestion

Urchin ingestion and egestion is highly dependent on the species, population, quality, and quantity of available food, as well as the surrounding physical and biotic environment (Holland, 2013). *S. droebachiensis* is a generalist feeder known to ingest algae, invertebrates, microbes, and detritus, though brown macroalgae is the primary diet of most populations (Scheibling & Hatcher, 2013). The digestive system of urchins is an example of a "continuous-flow stirred-tank reactor", that is ingested food gets continuously mixed in the stomach and intestine (Holland, 2013). Due to the mixing that occurs, one meal will not appear in faeces at the same time (Lawrence, Lawrence, & Watts, 2013). Gut transit time is affected by the frequency of feeding and the amount of food that is consumed (Lawrence et al., 2013) as the urchin digestive system is low in musculature and requires input through feeding to create pressure to remove the gut contents (Porter et al., 2019). *S. droebachiensis* gut transit time is approximately two days, but can be up to 20 days and is not affected by food quality as seen in some other species (Lawrence et al., 2013). The feeding rate of this species varies primarily due to food availability and to a lesser extent due to urchin density, body size, reproductive state, and season (Scheibling & Hatcher, 2013). Despite much research on sea urchin feeding, there is still a lack of thorough understanding of extrinsic or intrinsic factors affecting consumption rates

(Suskiewicz & Johnson, 2017). *S. droebachiensis* is known to be able to survive long periods of low food supply and starvation, but growth and reproductive output are reduced under such conditions (Scheibling & Hatcher, 2013). Adult *S. droebachiensis* show a linear increase in feeding rate between the sizes 30 to 60 mm in diameter, but when translated into feeding rate per unit of urchin mass, the relationship shows a decline (Scheibling & Hatcher, 2013).

1.9 Objectives of this study

Studying and quantifying MP fibers in the environment will help to understand their temporal and spatial distribution. Accumulation and effects of MP fibers at the individual level is currently poorly understood. With improved understanding at an individual and population level, a clearer picture of the ecological impacts of MP fibers can be developed. This experimental study exposed adult green sea urchins (*Strongylocentrotus droebachiensis*) to the most encountered MP type, fibers. The aims of this study were to determine the ingestion, retention in the intestinal tract, and egestion in faecal pellets of two types of MFs: synthetic blue polyacrylonitrile fibers and natural white wool fibers. The urchins were exposed to the MFs in two states: biofouled (BF) and non-biofouled (NBF). We hypothesize that:

1. Intestines and faecal pellets can be used to quantify fiber ingestion.
2. Urchins ingest both synthetic and natural fibers (acrylic and wool fibers).
3. Urchins ingest higher proportions of BF fibers than NBF fibers, regardless of fiber type.
4. Urchins will egest fibers in faecal pellets.
5. Retention of fibers in the intestines depends on fiber length; longer microfibers will be retained in the intestinal tract for a longer time than shorter microfibers.

2 Methods

2.1 Sample collection

S. droebachiensis were collected during the summer in northern Norway near Tromsø, on 24 July 2020, location coordinates 69°39'49.1"N 18°45'28.6"E. The collection site is located on the south side of the bridge from Kvaløya to Håkøya (figure 6). Collection took place from the shore at less than 1-meter depth, and adult individuals were selected to be of similar size. The urchins were transported 30 minutes by car in buckets with sea water from the collection site to the Akvaplan-niva experimental facility in Kraknes, Kvaløya.

Figure 6: Collection site near Tromsø on south side of Håkøya bridge. Collection process from rocky bottom shore at approximately knee height.

2.2 Fiber preparation

Fibers were prepared at the SINTEF laboratory in Trondheim following the protocol of Cole (2016), with some minor modifications (Sait et al., 2021; Sørensen et al., 2021). Fibers were cut to lengths 1, 2, 3, and 5 mm to be representative of lengths observed from domestic washing machines (Belzagui et al., 2019; Napper & Thompson, 2016). Two fiber types were used for exposure: blue polyacrylonitrile (acrylic) fiber and white wool, as a natural control fiber (table 3). Acrylic and wool fibers were chosen because they have a higher density than seawater, therefore increased likelihood of sinking to benthic habitats. The blue color was chosen so that fibers would be visible within the organic matrix, and blue is a common color of fibers found

in the environment (Barrows et al., 2018; Feng et al., 2020; Gago et al., 2018). When studying MFs the length to diameter ratio must be considered, and which dimension is used to define the size (Hartmann et al., 2019). This study uses the length for size categorization of the MFs.

Table 3: Study fiber characteristics

Fiber	**Color**	**Density** ($g\ mL^{-1}$)	**Length** (mm)	**Width** (mm)	**Weight per fiber** (µg)
Polyacrylonitrile (acrylic)	Blue	1.18	1 – 5	0.20	1.13
Wool	White	1.31	1 – 5	0.22	1.00

2.2.1 Preparation of stock solutions

The two BF treatment stock solutions were prepared by adding the fibers with 65µm mesh filtered seawater on 6th July 2021. The acrylic and wool fibers were placed into glass bottles, shaken for 10 minutes, and incubated for 3 weeks at 12.3˚C to allow for biofilm development on the fibers (Lobelle & Cunliffe, 2011). The stock solutions for the non-biofouled (NBF) treatments were prepared adding the fibers to filtered seawater on the day the exposure experiment began.

2.2.2 Stock solution concentration

The concentration of fibers was estimated by weighing the fibers to aim for an exposure concentration of approximately 150 fibers mL^{-1} (table 4). There was a limitation of the amount of prepared fibers that were available, so the final value of the target concentration for all four treatments was set to approximately 128 fibers mL^{-1}. Based on results from a previous study (Sathananthan, 2020) we assumed that the actual number of fibers suspended in the seawater would be approximately 3 times less than expected when applying the weight of fibers to prepare the stock solutions. This was confirmed by visual count of stock solutions (table 4). For the manual count of stock solution concentration 5mL was analyzed under the microscope and total the number of fibers counted. A plastic pipette was used to measure out the amount and to reduce cross contamination it was rinsed between samples three times with MilliQ water. All instruments used were washed three times with MilliQ water between uses. There was some variation in the fiber concentration among the different treatment stock solutions although all fiber treatment groups appear within the same order of magnitude. This concentration is much higher than environmental measurements of MP fibers (see table 2) and was chosen to increase interaction between urchins and fibers.

Table 4: Fiber weight and particle concentration (target, estimated, and observed) for each treatment. Four groups were weighed, one for each treatment (non-biofouled (NBF) acrylic, biofouled (BF) acrylic, NBF wool, BF wool). Target fiber concentration was calculated using weight per fiber (see table 3) and was estimated to be 3 times less, based on a previous study (Sathananthan, 2020). Observed fiber concentration was measured from visual count of 5mL of stock solutions.

Treatment group	**Total weight of fibers for stock solution** (mg L^{-1})	**Target fiber concentration** (# mL^{-1})	**Estimated fiber concentration** (# mL^{-1})	**Observed fiber concentration** (# mL^{-1})
Acrylic (BF)	153.2	128.8	42.9	38.9
Acrylic (NBF)	135.0	128.6	42.9	45.8
Wool (BF)	153.2	128.8	42.9	49.2
Wool (NBF)	135.0	128.6	42.9	69.1

2.2.3 Stock solution fiber lengths

Fiber length data from stock solutions were collected (figure 7), as it was hypothesized that increased fiber length may increase retention time in the intestinal tract. Fibers were cut with even distribution of lengths 1, 2, 3 and 5 mm. The size categorization grouping used (<1mm, 1-3mm, >3mm) would result in the largest percentage of fibers found in the group 1 to 3 mm, as this includes three of the four size groups fibers were cut to. Predictably approximately 75% of fibers will be in this length category. There was occurrence of fibers smaller than 1mm in all stock solutions, which is expected due to challenges in precision of cutting at this length. NBF acrylic fibers had the highest percentage (18%) with length less than 1mm. BF acrylic had the highest percentage (21%) of fibers longer than 3mm. Both acrylic groups showed the predicted percentage of fibers in the 1 to 3mm size range. NBF and BF wool showed similar size distribution, with a larger than predicted percentage of fibers in the 1 to 3mm range. Both wool treatments show a very low representation of fibers larger than 3mm.

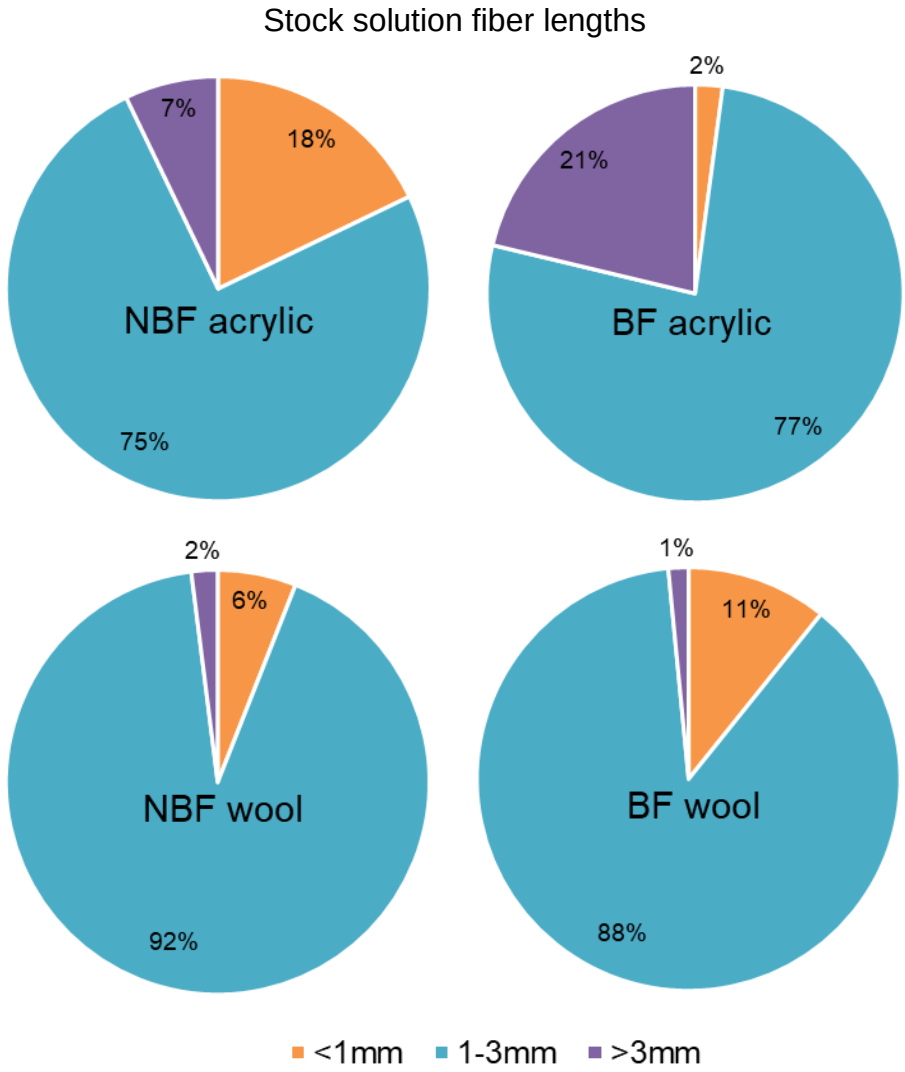


Figure 7: Fiber length distribution of stock solutions for each treatment (non-biofouled (NBF) acylic, biofouled (BF) acrylic, NBF wool, BF wool). Fiber length was measured categorically into 3 groups: <1mm (orange), 1-3mm (blue), and >3mm (purple). Due to fibers being cut at lengths 1, 2, 3, and 5 mm 75% of fibers were expected to be found in the 1-3mm category.

2.3 Laboratory exposure

After collection of the urchins, they were placed together into an open tank for 48 hours to allow adjustment to the laboratory conditions and evacuation of gut contents (figure 8). They were provided with brown kelp (*Saccharina latissima*) for grazing. This gut evacuation period allowed for excretion of any environmental MP ingested in the field prior to the experiment. As the seawater used at the facility is filtered to 65µm a low potential for MP contamination (within the study size range) was assumed.

Figure 8: Urchin adjustment and gut evacuation of 48 hours in open tank.

Treatments were prepared by diluting 170mL of stock solution with 180mL of seawater (figure 9a) to get an approximate fiber concentration of 128 fibers mL^{-1}. Four treatments were prepared, NBF and BF fibers of each type, acrylic and wool. There was one control group not exposed to fibers, resulting in a total of five treatment groups (figure 10). Each group had six individual urchins in separate beakers, a total of 30 urchins. The beakers used where 565mL in size and made of clear plastic. Brown kelp was given to all individuals. To minimize contamination container lids were placed on top, but not sealed. Beakers were placed in random order into the open tank (figure 9b) with water pumped and filtered directly from the adjacent ocean running through the tank to allow for environmentally relevant temperature regulation.

Figure 9:a) Measurement and dilution of stock solutions into beakers. b) Beakers placed randomly into the open tank with running water for temperature regulation.

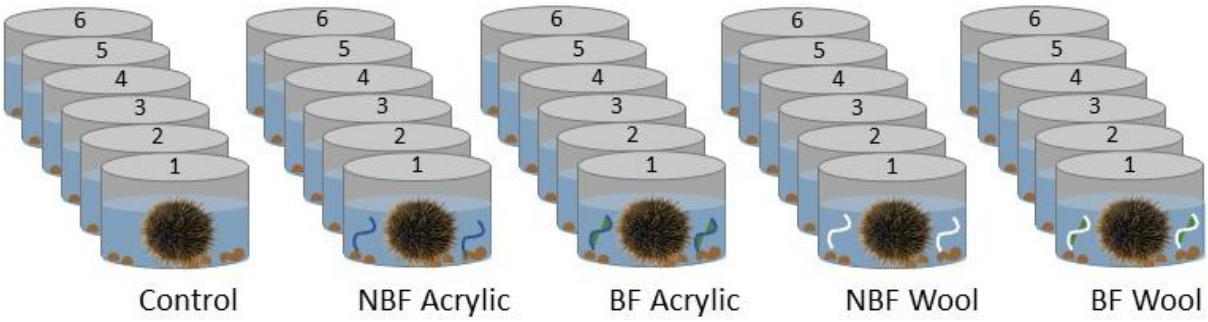


Figure 10: Exposure experiment had five treatment groups: Control, non-biofouled (NBF) acrylic, biofouled (BF) acrylic, NBF wool, BF wool. Each group included 6 urchins. Urchins were kept individually in 565mL clear plastic beakers.

After 48 hours faecal pellets were collected from all beakers and MF exposure stopped. From each group three individuals were placed into a new beaker and with fresh seawater and kelp to allow for a depuration period of 86 hours. The other three individuals were dissected, and the intestines collected and stored in ethanol. There was a second collection of faecal pellets at 72 hours after experiment start time. At 134 hours after experiment start there was a third collection of faecal pellets, and all remaining urchins were dissected. Before dissection sea urchin diameter was measured with a vernier caliper (figure 11a). All collected faecal pellets from different time points were stored in separate plastic containers with ethanol (figure 11b). The collection of samples from each time point is visually depicted in figure 12.

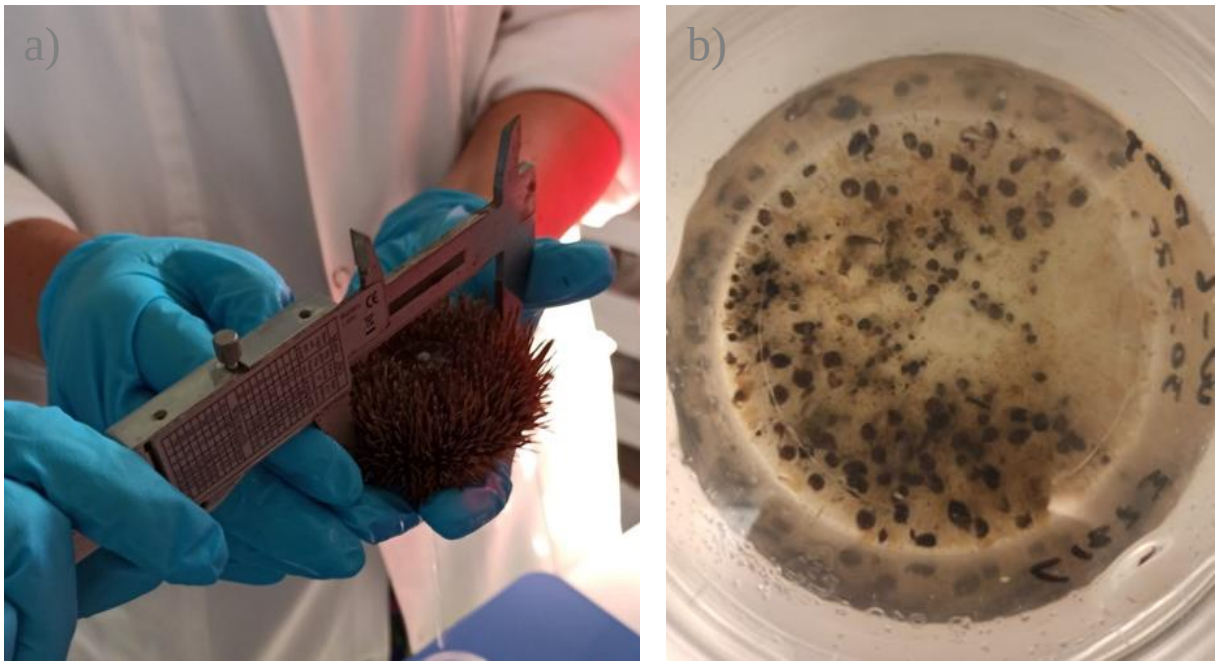


Figure 11: a) Measurement of urchin diameter with vernier caliper. b) Plastic container storing faecal pellets in ethanol.

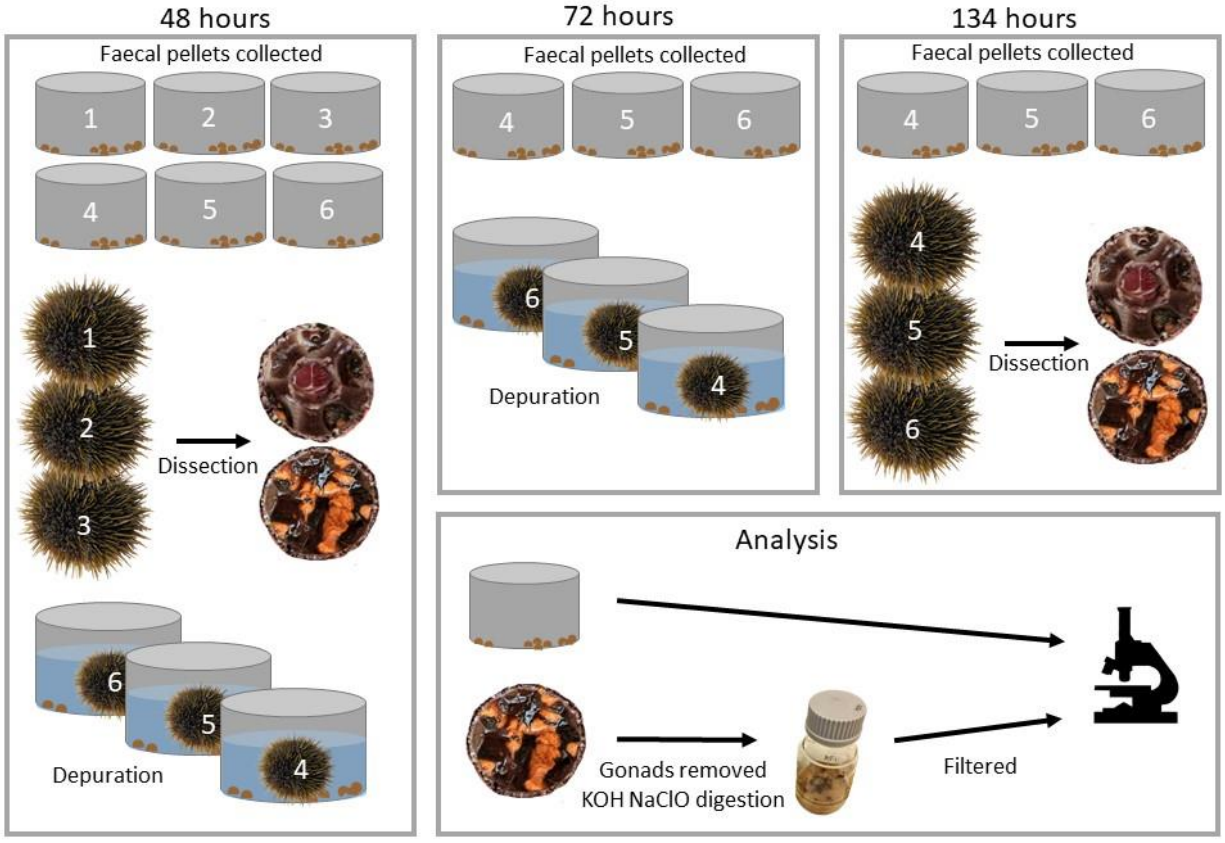


Figure 12: Visual explanation of collected samples from one treatment group (6 urchins) for each time point (48, 72 and 134 hours after experiment start time). At 48 hours: faecal pellets collected from 6 urchins, 3 urchins dissected, 3 urchins placed in fresh seawater for depuration. 72 hours: faecal pellets collected from 3 urchins, 3 urchins placed in fresh seawater for depuration. 134 hours faecal pellets collected from 3 urchins, 3 urchins dissected. Analysis: Faecal pellets stored in ethanol until visual sorting and analysis with microscope. Intestines separated from gonads at dissection. Intestines were stored in ethanol, then digestion of organic matter with potassium hydroxide (KOH) and sodium hypochlorite (NaClO). After digestion intestine contents was filtered over glass microfiber filter then analyzed under microscope.

2.4 Dissection procedure

All dissections took place over a large plastic container. A scalpel was used to cut around the circle of the mouth parts to loosen and remove the mouth and Aristotle's lantern (figure 13a). The scalpel was used to pierce the shell at the widest point, and scissors were used to carefully cut around the diameter of the urchin (figure 13b). The gonads were removed as they were not to be analyzed in this experiment. The intestinal tissue was collected and placed into a clear plastic beaker with ethanol. The urchin shell, scalpel, other tools, and container were all flushed thoroughly with MilliQ water which was added into the intestine collection beaker to collect any tissue or fibers that may have stuck to the equipment. Between each dissection equipment was thoroughly washed with tap water and then MilliQ water.

Figure 13: a) Insides showing the urchin intestinal tract and gonads. Bottom left of the image shows the removed Aristotle's lantern and mouth parts. b) Careful separation of the urchin body after cutting around the diameter with scissors.

2.5 Sample analysis

2.5.1 Digestion pilot study

An alkaline digestion protocol was chosen to be tested due to the tendency of other digestion methods to result in color leaching and destruction of some polymer types (Enders, Lenz, Beer, Stedmon, & Browman, 2017). In brief the chosen method used a mix of saturated potassium hydroxide (KOH) solution and sodium hypochlorite (NaClO) at a ratio of 1:1 at 15% dilution (Enders et al., 2017). The saturated KOH solution was prepared with pellets diluted in microfiltrated water at 1120 gL^{-1}. A pilot study was run to test if the two fibers used in the study would be affected by this process. Visual inspection under the microscope showed acrylic fibers had no visible damage at the magnification required for counting and measuring fibers. The wool fibers however were very negatively affected. Most of the wool fibers were unable to be identified and those that were visible had obvious damage. Very few fibers were recognizable

and those that were had distinct changes in texture, diameter, and length (figure 14a). Most fibers were not visible or turned into a white clot (figure 14b).

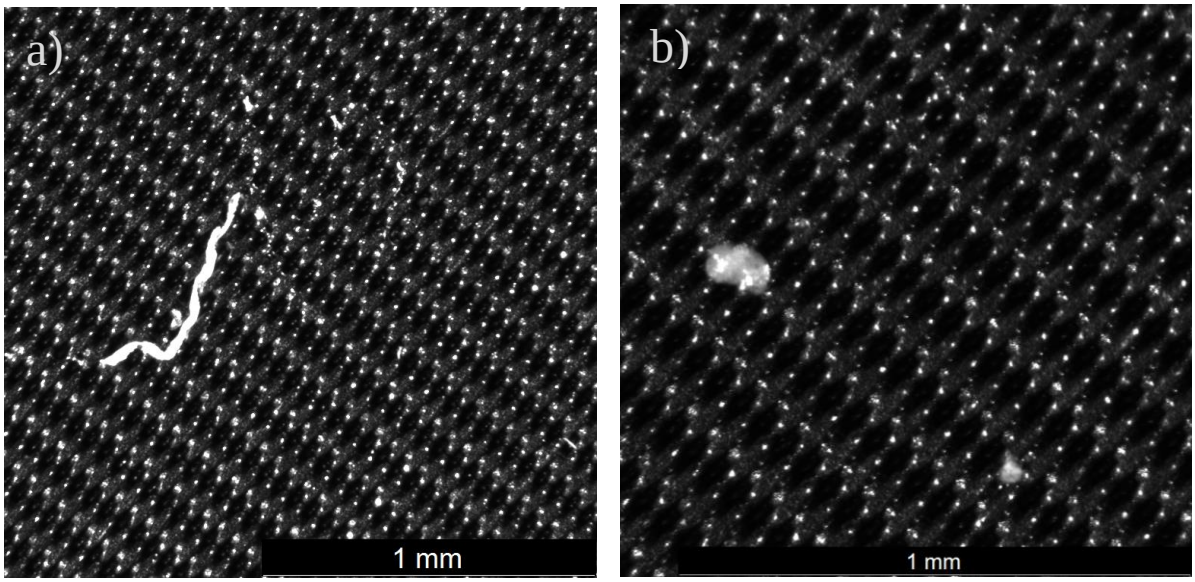


Figure 14: a) Wool fiber showing physical changes in texture, length, and diameter after digestion with potassium hydroxide (KOH) and sodium hypochlorite (NaClO). b) The remains of disintegrated wool fibers seen as two white clots on the filter.

2.5.2 Intestines

The intestine contents required use of organic matter digestion method due to the large amount of tissue present in the samples (figure 15a). Digestion solution was prepared fresh on the day of use. Ethanol was syphoned from intestine samples using a plastic pipette, and then weighed. For each gram of tissue 5mL of digestion solution were mixed in a glass bottle (figure 15b). All samples were placed in an ultrasonic bath for 15 minutes, shaken thoroughly for 2 hours then left at room temperature for 24 hours.

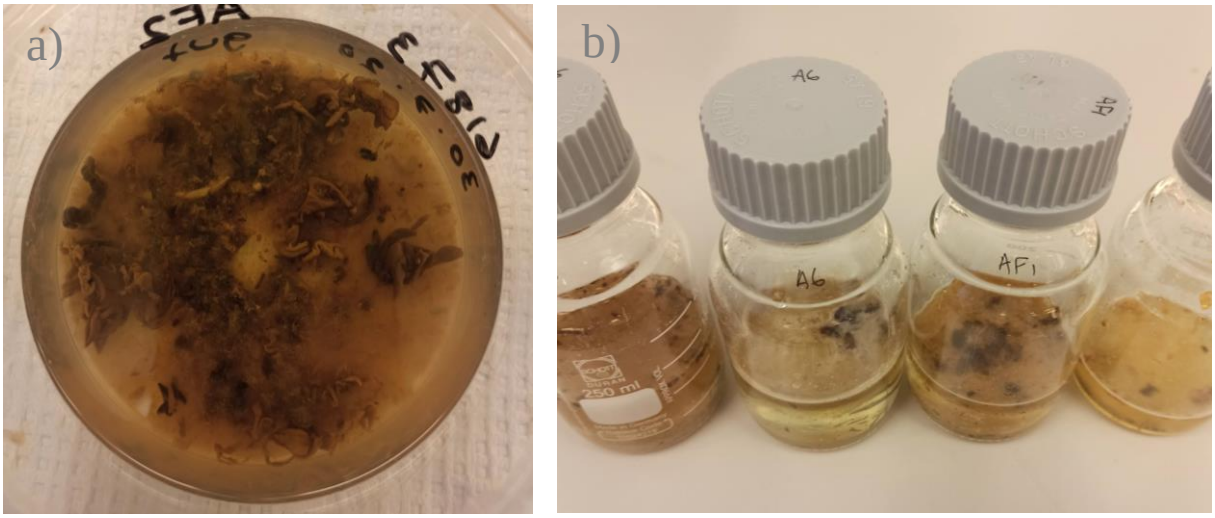


Figure 15: a) Intestine sample stored in ethanol prior to digestion and analysis. The large amount of organic matter would make visual analysis of intestine contents difficult and time consuming. b) Intestine digestion in glass bottles for 24 hours.

The digested liquid was filtered over glass microfiber filters in a ceramic Büchner funnel (figure 16a). Due to inefficiency of the digestion method the filtration process was very slow, so an air pressure pump was required. The urchin spines remining in the samples on occasion pierced the filter paper, resulting in some escape of liquid through the hole. For microscopy the filter was divided into sections to improve accuracy of counting method. Sections were drawn onto a petri dish that was placed on top of sample (figure 16b). Fibers were counted under the microscope using a hand tally counter.

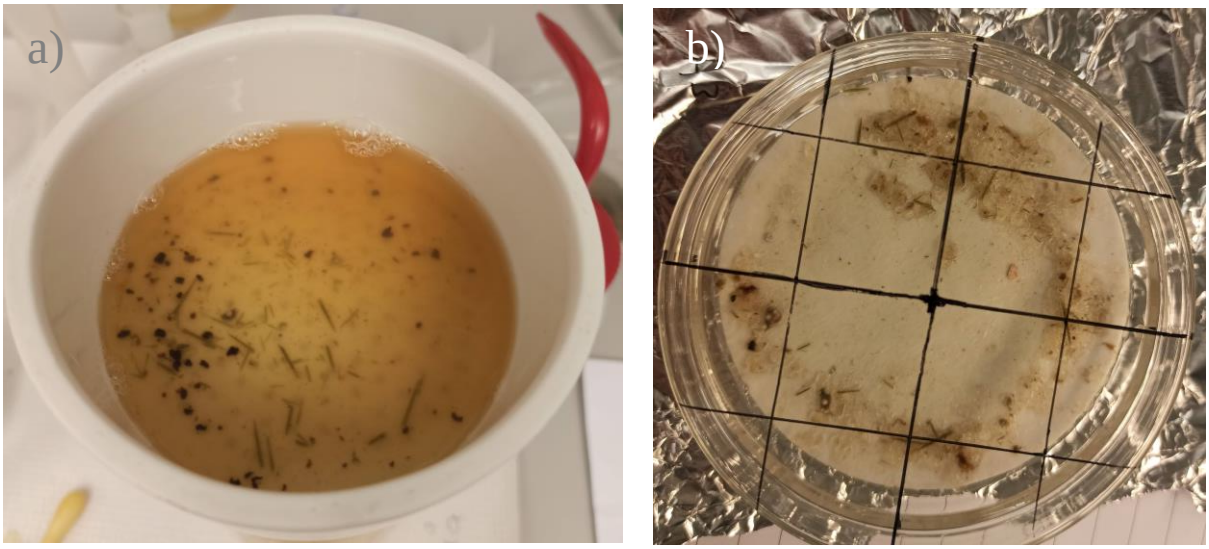


Figure 16: a) Filtration of digested intestine contents over glass microfiber filter using ceramic Büchner funnel. b) Filter analysis showing method of petri dish placed on top with drawn on division into sections

Images were taken of one quarter of the filter to later analyze fiber lengths. Overlapping images were pasted to make one image (figure 17), and to reduce double counting measured fibers were marked. This process combining images was done manually and there was some difficulty in overlapping each image perfectly. Fiber length analysis was conducted with the same method as described in section 2.2.3. A method involving computer analysis with the program ImageJ for fiber lengths analysis was tested, but proved not to be feasible due to poor digestion and background noise on the filter. Even with better digestion, the huge number of fibers resulted in many overlapping and intertwined fibers that would have complicated automated analysis.

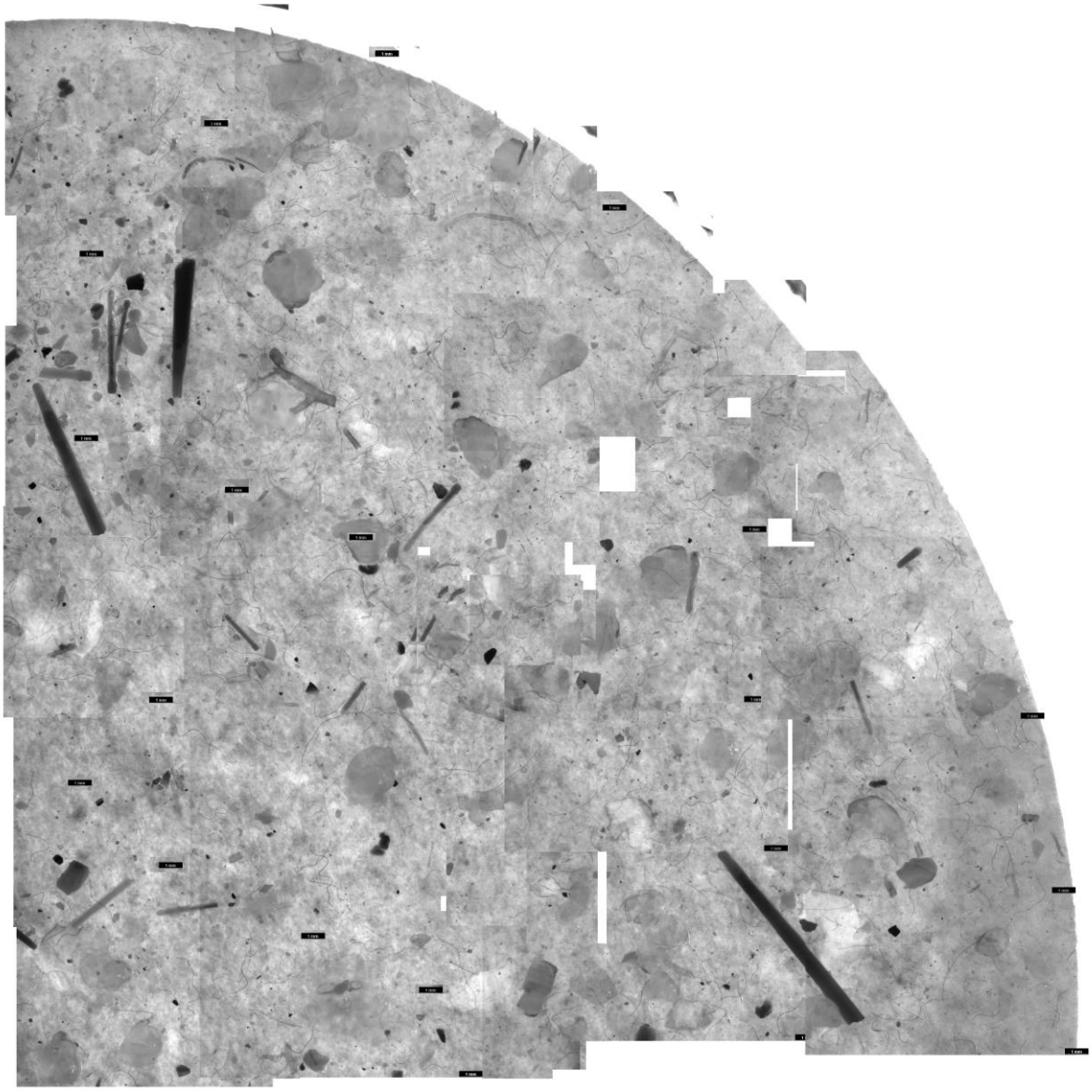


Figure 17: Overlapping images were merged to analyze fiber lengths from a quarter of the filter with digested intestine contents. Poor digestion of urchin spines and other organic matter is visible. The light grey areas are from poorly digested material (light brown in color) that clogged the filter resulting in very slow filtering.

2.5.3 Faecal pellets

Faecal pellets were analyzed under the microscope by a visual sorting process, which would allow for analysis of wool fibers that would have been destroyed if the digestion method was used. Due to the high number of pellets available for analysis from the 48 hour time, 25 percent of the total was analyzed as a representative sample. A minimum of 10 faecal pellets were analyzed, which was more than 25 percent for some individuals. All data was extrapolated and is presented in results to equal the predicted number of fibers if all faecal pellets were analyzed. For microscopy analysis faecal pellets were taken from ethanol using a plastic pipette and placed onto a glass petri dish. Pellets were inspected for any fibers that may have been attached

to the outside and not actually encapsulated (figure 18a). Pellets were then carefully dissected using pincers and tweezers (figure 18b). Fibers from each pellet were counted, and photos taken to determine fiber lengths. The faecal pellets showed diverse contents, suggesting that complete gut evacuation was not achieved from the 48 hour open tank period prior to exposure (figure 19).

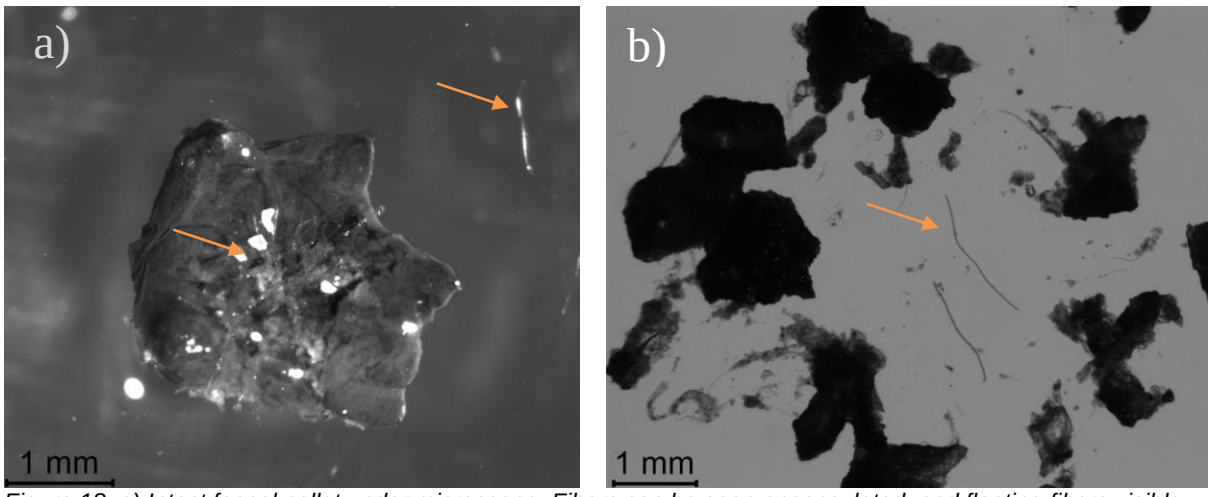


Figure 18: a) Intact faecal pellet under microscope. Fibers can be seen encapsulated, and floating fibers visible around outside the pellet. Care was taken to ensure no confusion between irrelevant fibers. b) Dissected faecal pellet, central in image are fibers that were inside the pellet

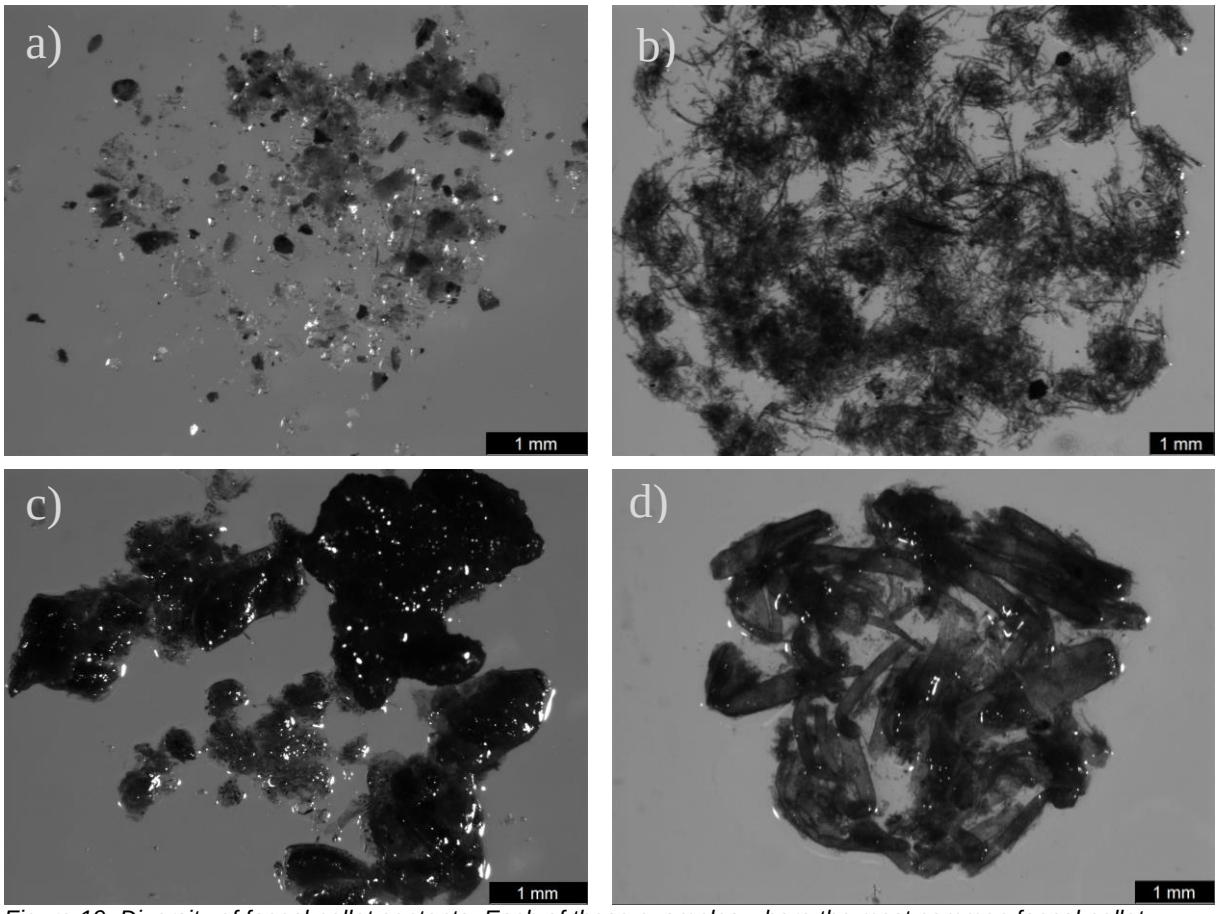


Figure 19: Diversity of faecal pellet contents. Each of these examples where the most common faecal pellet contents. a) sand and shell b) brown fiberous plant matter c) hard black lumps d) tube like brown plant matter.

2.6 Software and statistics

The stereomicroscope used for microscopy was a Leica M205 FA, and images where taken using Leica camera DFC 3000 G. Leica imaging software LAS X (version 3.6.0.20104) was used for image analysis. Microsoft Paint was used for merging and marking intestine filter images. Microsoft Excel (version 2008 for Microsoft) was used for raw data organization, initial calculations, and initial data visualization. RStudio (version 1.2.5033 for Microsoft) was used for data visualization and statistical analysis. Data was tested for normality using visual evaluation of QQplots and Shapiro-wilks tests. When normality was met, parametric tests ANOVA and Students t-test where used. Non-parametric distribution-free permutation tests were used where data was not normal. The permutation test was chosen also as a more robust statistical test for small sample size (Hayes, 1996). Significance level was set to $p < 0.05$.

3 Results

3.1 Body size and faecal pellet production

Urchin body size, measured using diameter, ranged from 45 to 64 mm (figure 20) with a mean of 54.62 ± 3.96mm (mean ± standard deviation, sd). Despite variations in body size across treatment groups, mean diameters of urchins were not significantly different among groups (ANOVA, p=0.24). When comparing only the urchin groups fed acrylic fibers, body size showed a significant difference between mean diameters (Student t-test, p=0.02). The NBF acrylic group had a significantly smaller mean diameter than the BF acrylic group. There was no significant difference found when comparing NBF and BF wool groups (Student t-test, p=0.18).

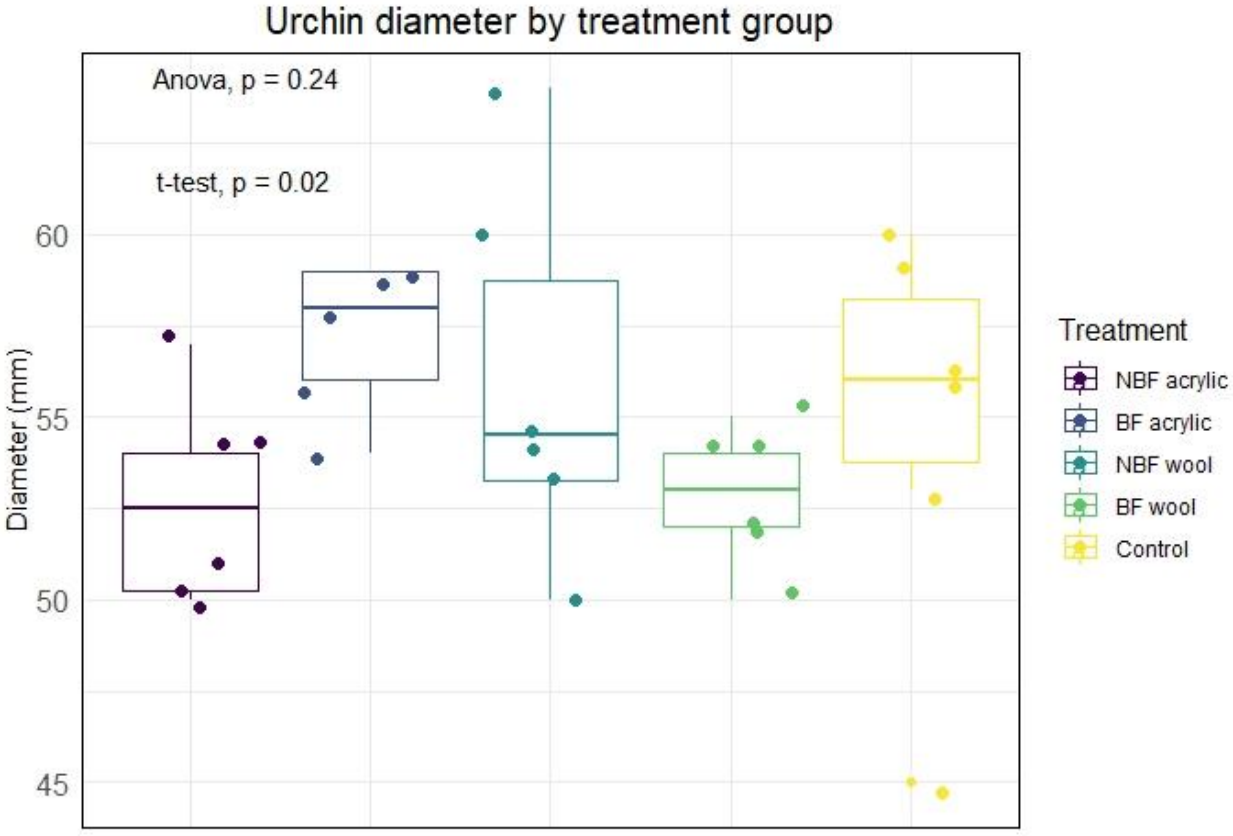


Figure 20: Urchin body size was measured by diameter, displayed here separated by the treatment groups non-biofouled (NBF) acrylic, biofouled (BF acrylic), NBF wool, BF wool, control. The variation in diameter across groups was analyzed to investigate if body size plays a role in experiment findings. ANOVA test showed no significant difference between all groups body size (p=0.24). Statistically significant difference was found between the two acrylic fiber exposed groups (t-test, p=0.02). There was no significant difference in body size between the wool exposed treatments (t-test, p=0.18).

At 48 hours all individuals showed faecal pellet production and the faecal pellet count ranged from a minimum of 4 to a maximum of 174, with a mean of 72.43 ± 45.91 (sd). Considerable variation in faecal pellet production was also observed across groups (figure 21). At 134 hours minimum faecal pellet count was zero and maximum was 38, with a mean of 5.± 11.77 (sd).

While some individuals that continued to produce faecal pellets at 72 and 134 hours were dead on dissection, other individuals that where alive at dissection had not produced any faecal pellets after 48 hours.

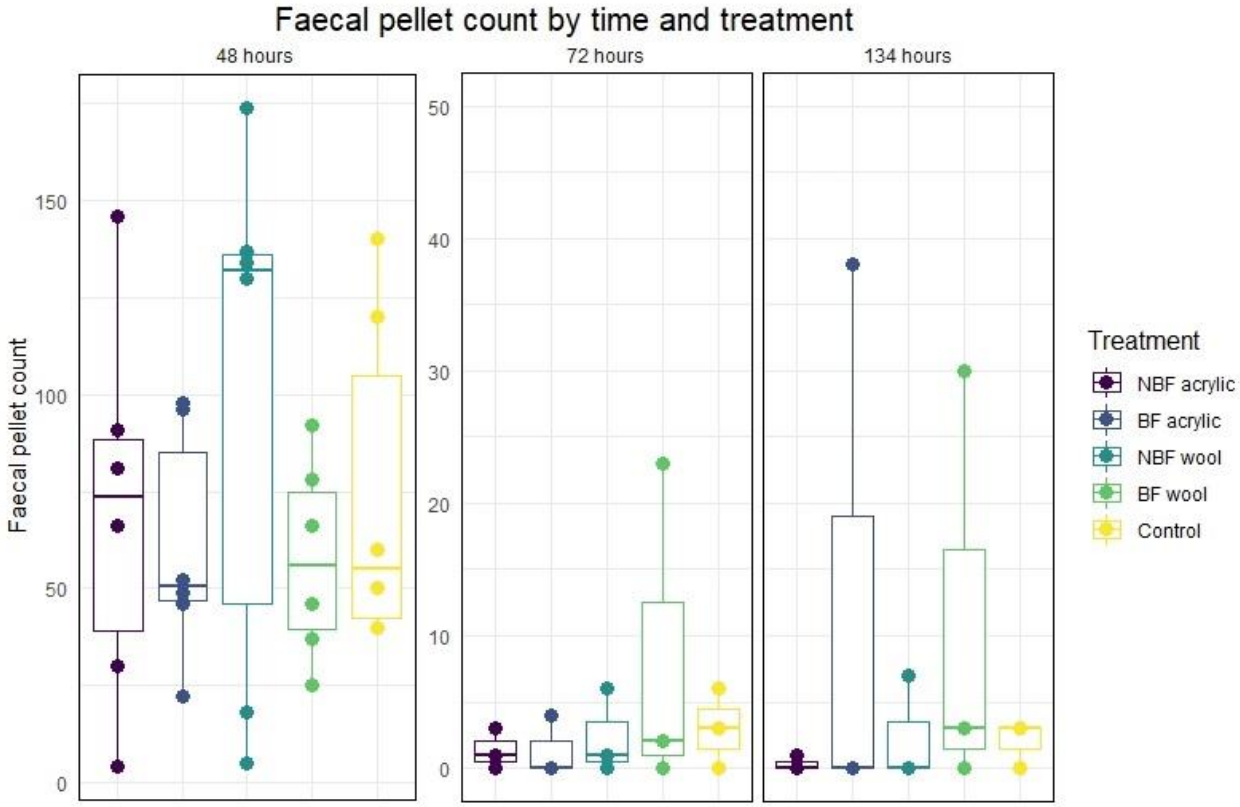


Figure 21: Faecal pellet count displayed by treatment groups (non-biofouled (NBF) acrylic, biofouled (BF acrylic), NBF wool, BF wool, control) and faecal pellet collection times (48, 72, and 134 hours). Note different Y-axis values. Large variability can be seen in faecal pellet production in all treatment groups.48 hours includes all individuals (n=30). At 48 hours half the individuals where dissected, at 72 and 134 hours total of 15 individuals remain. Lower faecal pellet production is seen at 72 and 134 hours in all treatment groups. The highest faecal pellet count at 72 hours is an individual in the BF wool treatment group. At 134 hours the highest counts include the same BF wool individual and an individual from the BF acrylic group.

There was no significant relationship between the faecal pellet count and body size at any time point (permutation test, 48 hours p=0.24, 72 hours p=0.74, 134 hours p=0.32). These findings allowed body size to be disregarded as an additional variable influencing feeding activity. Due to the potential influence of the fiber exposure on feeding activity, the same tests were run only on the control group and showed similar results (permutation test, 48 hours p=0.51, 72 hours p=0.37, 134 hours p=0.37). In addition, there was no influence of BF state or fiber type on production of faecal pellets (permutation test; 48 hours p=0.18 and p=0.49, respectively). At 72 and 134 hours, the individuals producing the most faecal pellets were from the BF exposed groups, but the difference across all groups was not significant (permutation test, 72 hours p=0.42, 134 hours p=0.17).

3.2 Survival

Upon dissection at 48 and 134 hours, urchins were categorized into alive, almost dead, and dead. Alive urchins showed healthy spine and tube feet movement. Urchins that were almost dead showed small movement of spines. Urchins with no movement in spines or tube feet were recorded dead. Figure 22 shows the survival of urchin individuals at dissection. At 48 hours all dissected individuals were alive. At 134 hours, urchins showed a high death rate. This poor survival rate was an important consideration when choosing what data could be used and analyzed. It is noteworthy that we do not believe that the treatments were the cause of death, as the control group had similar results, and instead can be attributed to the captivity conditions.

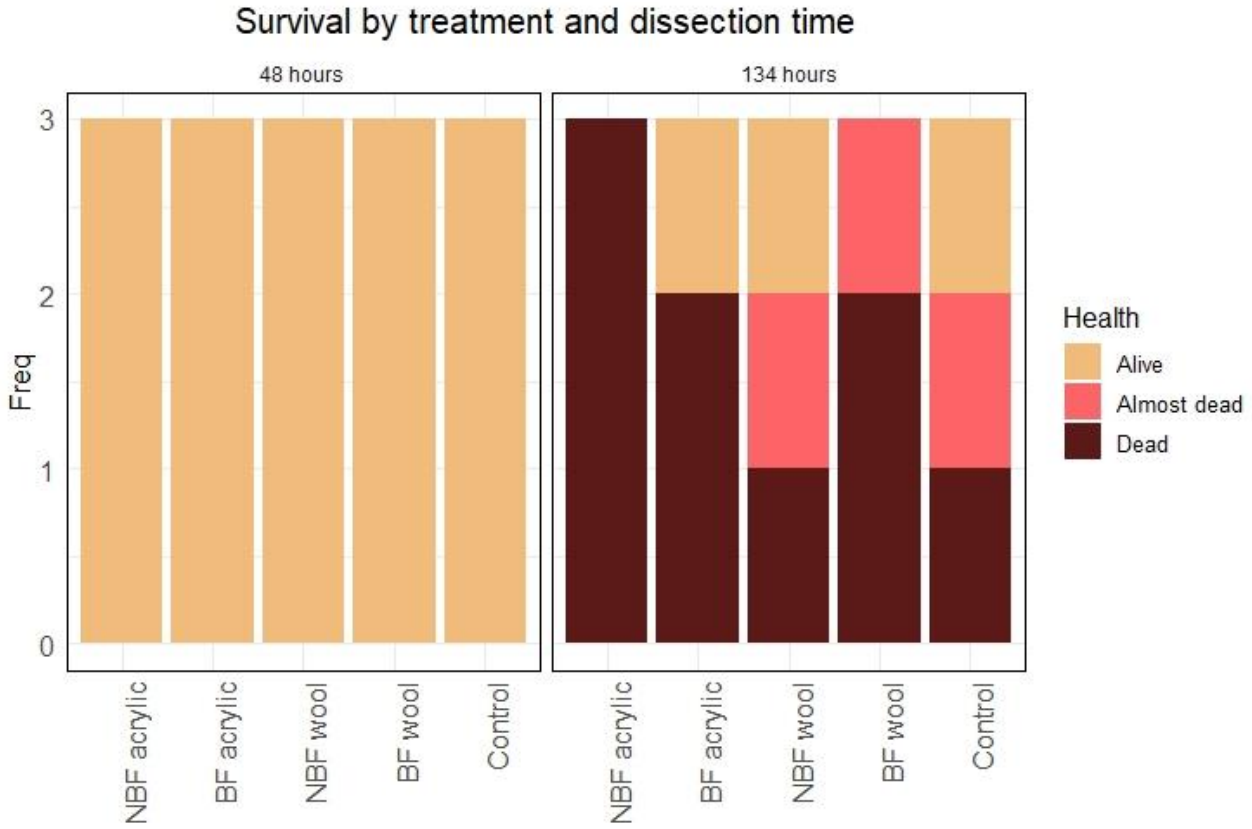


Figure 22: Survival of urchins recorded at each dissection time, 48 and 134 hours after experiment start time. Number of individuals in each treatment for each time is 3 (total n=15). 100% survival at 48 hours. At 134 hours 20% (3 individuals) were alive, 20% (3 individuals) were almost dead and 60% (9 individuals) had died.

3.3 Retention of microplastic fibers

As our results revealed the digestion methods with KOH and NaClO damage the wool fibers, the individuals fed wool were not included in the intestine analyses. Fiber counts for intestines were thus only available for the two acrylic treatment groups, NBF and BF. To increase our sample size for statistical analysis individuals from both 48 and 134 hour dissection times were grouped. The assumption was made that there was no change in the intestine fiber counts between 48 and 134 hours as no fibers were found in faecal pellets between 48 and 134 hours

in both groups. The intestine fiber count for the NBF acrylic fiber group had a higher mean of 1147 ± 616 (sd; figure 23). The BF group mean fiber count was 560 ± 443 (sd). Yet, there was no significant difference in number of fibers found in the intestines between the NBF and BF treatment groups (permutation test, p=0.09).

Figure 23: Fiber count in intestines from NBF and BF acrylic treatments. The NBF acrylic group has a higher mean than the BF treatment, but no statistical significance was found (permutation test, p=0.09). The smaller point size indicates dissection at 48 hours and larger point size dissection at 134 hours. The health of the individual at dissection is indicated by the shape of the point, circle they were alive and triangle they were dead.

The total amount of fibers ingested by each urchin was calculated by adding the number of fibers in intestines and faecal pellets for each individual urchin (figure 24). The diameter was not significantly related the total number of ingested fibers (permutation test, p=0.45). Likewise, the total number of ingested fibers did not significantly differ between NBF and BF acrylic fiber exposed groups (permutation test, p=0.10). To explore fiber retention, the percentage of the total ingested fibers remaining in intestines was calculated (figure 25). The fibers found in the intestines was converted to a percentage of the total fibers ingested. The mean percentage of fibers retained in intestines were not significantly different between the NBF or BF fiber treatments, with mean of 81.3% and 75.9%, respectively (permutation test, p=0.17). There were two outliers; one individual from the NBF group that ingested 882 fibers but did not egest any fibers, and one individual from the BF group that egested all of the 4 ingested fibers in faecal pellets. After removing the two outliers, the difference between

treatments became significant (permutation test, p=0.04) with the NBF acrylic treatment group having higher retention of fibers in intestines than the BF treatment.

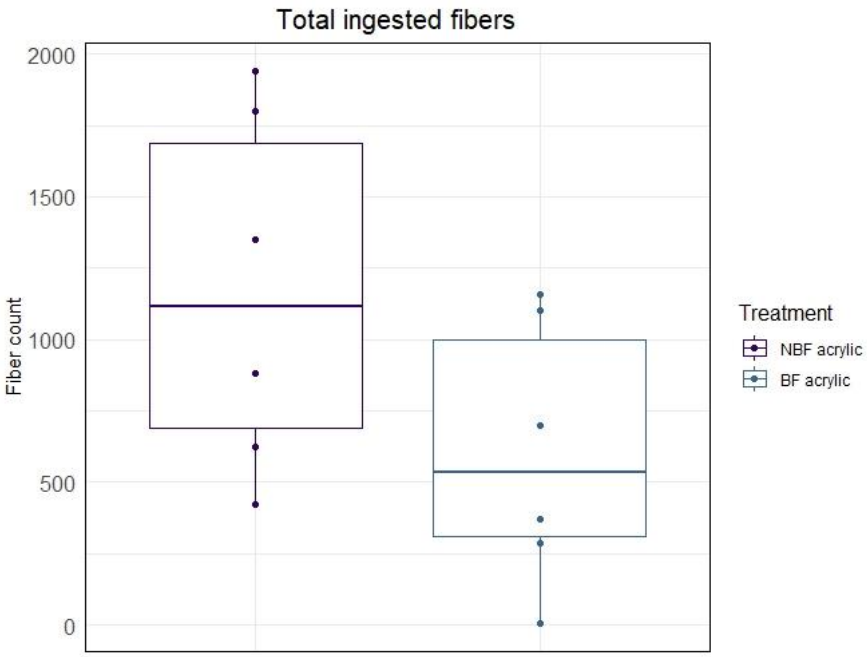
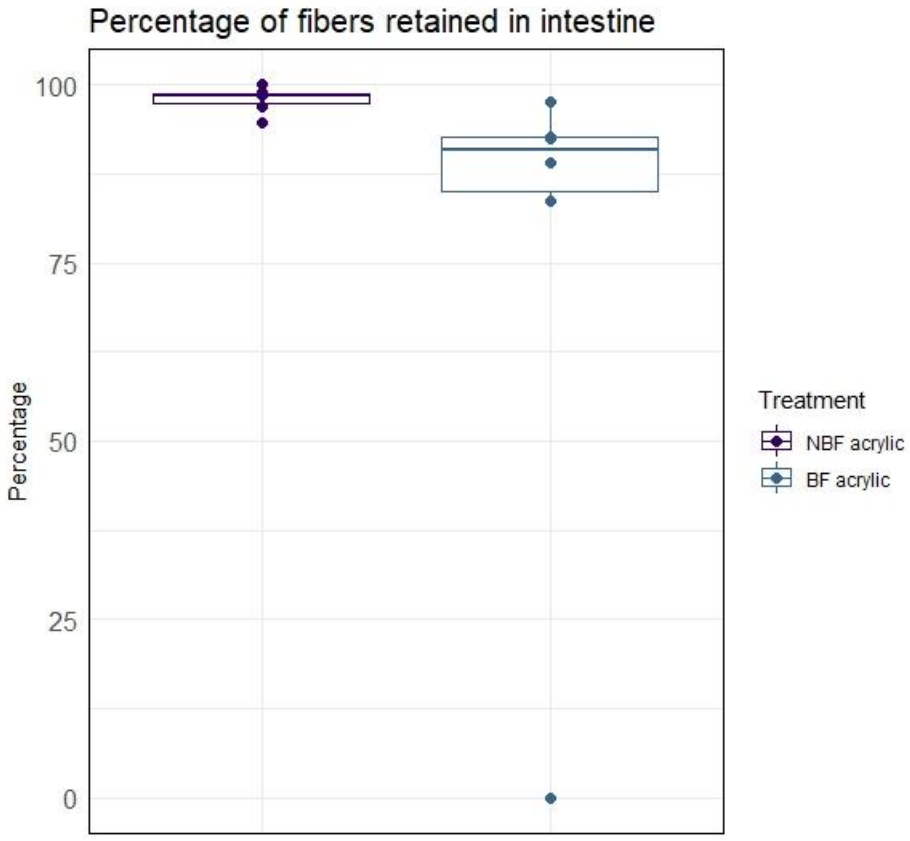


Figure 24: The total number of fibers ingested was calculated by adding together the fiber count from intestines



and faecal pellets. The NBF acrylic group had a higher mean number of fibers than the BF group but this was not statistically significant (permutation test, p=0.10).

Figure 25: Percentage of total ingested fibers retained in intestines. Including outliers, no statistically significant difference between NBF and BF treatment groups (permutation test, p=0.80). With two outliers removed there is statistically significant difference between groups, with higher retention in NBF group (permutation test, p=0.04).

3.4 Fiber concentration in faecal pellets

Acrylic and wool fibers from both NBF and BF exposures were found in faecal pellets. The number of fibers found in faecal pellets was highest at 48 hours, with very few fibers found in faecal pellets at 72 and 134 hours (figure 26). There was no significant relationship found between diameter and the number of fibers found in faecal pellets (permutation test, p=0.30). Data from the 48 hour faecal pellet collection was used for statistical analysis to investigate if fiber type or BF state influenced the number of fibers found in faecal pellets. For this analysis data for both fiber types were grouped, regardless of biofouling state. Wool fibers had a higher mean and larger variance of fibers counted in faecal pellets after 48 hours than acrylic fibers, although this difference was not significantly different (figure 27; permutation test, p=0.07). When grouping biofouling state and ignoring fiber type, the fiber count in faecal pellets at 48 hours showed a higher variance in the NBF fiber group, but no statistically significant difference was found (figure 28; permutation test, p=0.55).

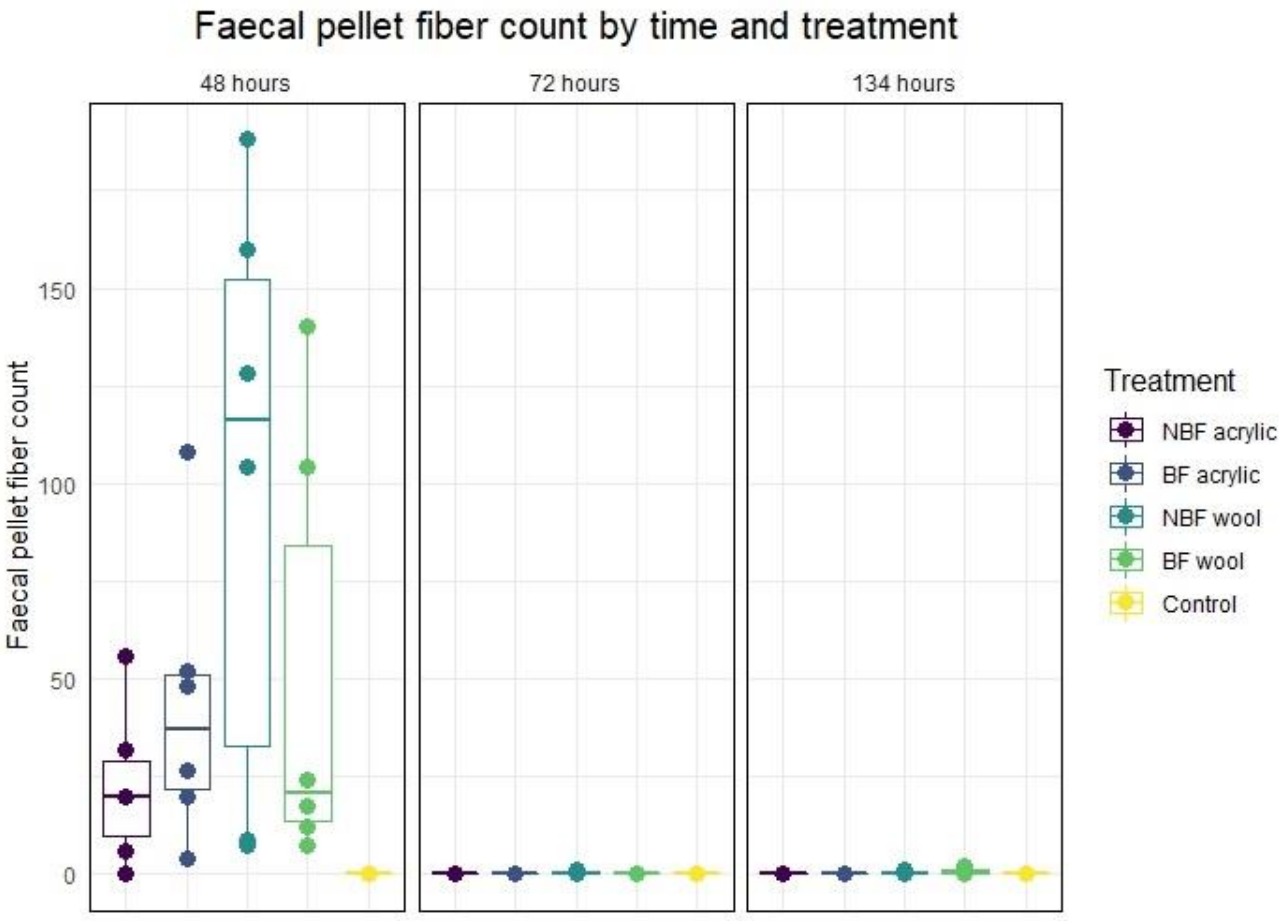


Figure 26: Fiber egestion as determined by counting fibers in faecal pellet at three collection times: 48, 72, and 134 hours. Variability across treatments was found at 48 hours, with very low fiber egestion at 72 and 134 hours.

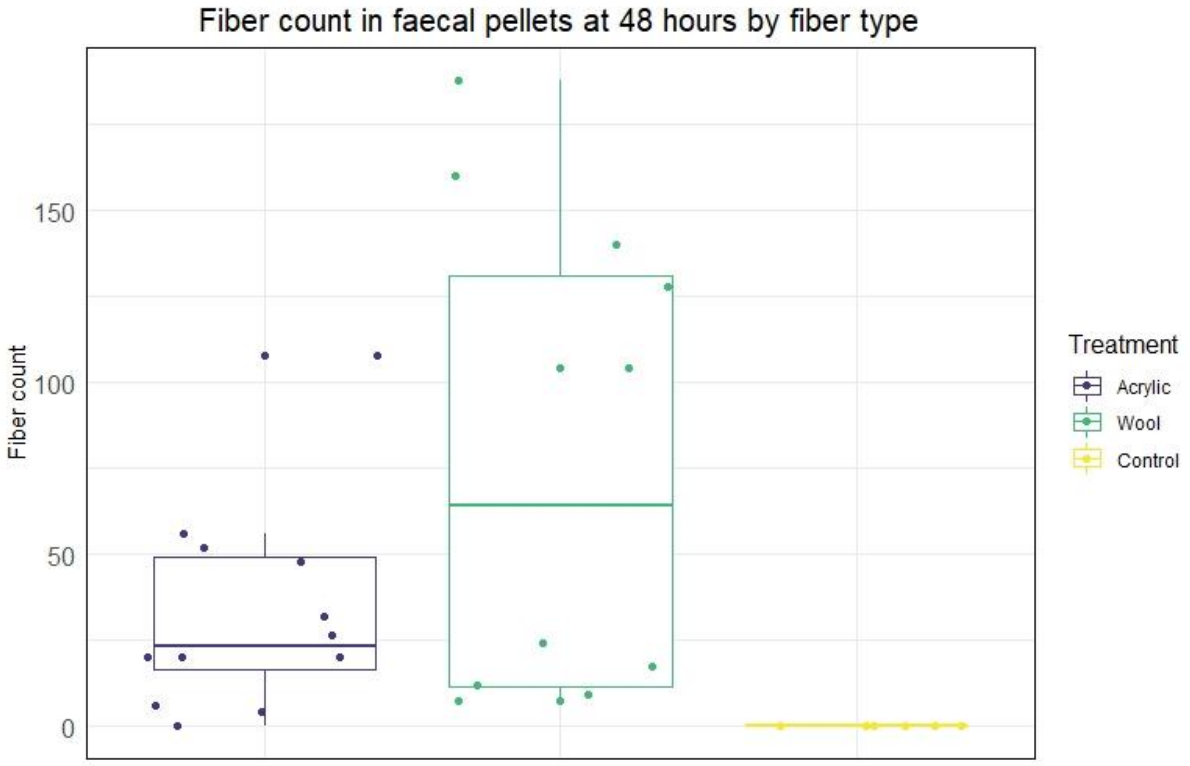


Figure 27: Egestion of fibers in faecal pellets at 48 hours with acrylic and wool fibers grouped (ignoring biofouling state). Wool fiber egestion had higher mean and larger variability than acrylic fibers. No significant difference was found (permutation test, p=0.07).

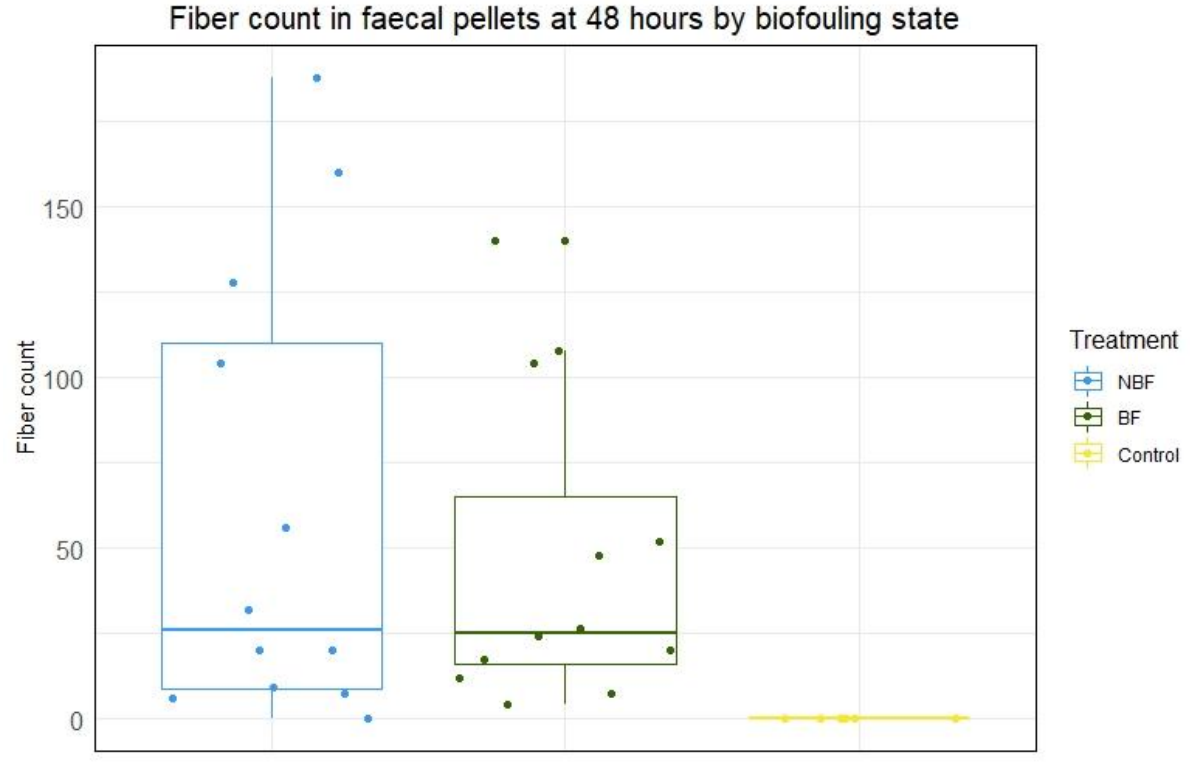


Figure 28: Egestion of fibers in faecal pellets at 48 hours with non-biofouled (NBF) and biofouled (BF) fibers grouped (ignoring fiber type). NBF and BF groups had the same mean egestion of fibers, with higher variability in the NBF group. No significant difference was found (permutation test, p=0.55).

3.5 Fiber lengths after exposure

3.5.1 Intestine fiber lengths

Intestine data showed similar fiber length distributions between NBF and BF acrylic fiber treatments (figure 29). The percentage of fibers present that are less than 1mm in length is consistent with findings of fibers in this size range in the stock solutions. The percentage of fibers longer than 3mm was low in both NBF and BF treatments.

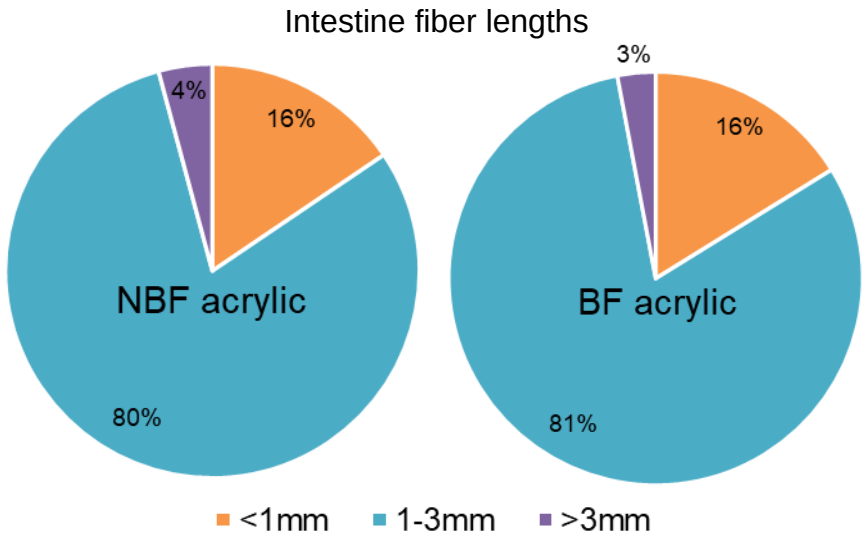


Figure 29: Fiber length distribution in intestines by percentage of total fibers measured, for acylic treatments (non-biofouled (NBF) acylic, biofouled (BF) acrylic). Fiber length was measured categorically into 3 groups: <1mm (orange), 1-3mm (blue), and >3mm (purple).

3.5.2 Faecal pellet fiber lengths

Length of fibers in faecal pellets (figure 30) did not differ greatly from the expected distribution, when compared with the stock solution. There was a low percentage of fibers longer than 3mm in all treatments, with no fibers of this length category found in the NBF acrylic group faecal pellets. While the NBF acrylic group had higher retention of fibers in intestine, there was not more fibers >3mm in the intestines in this group.

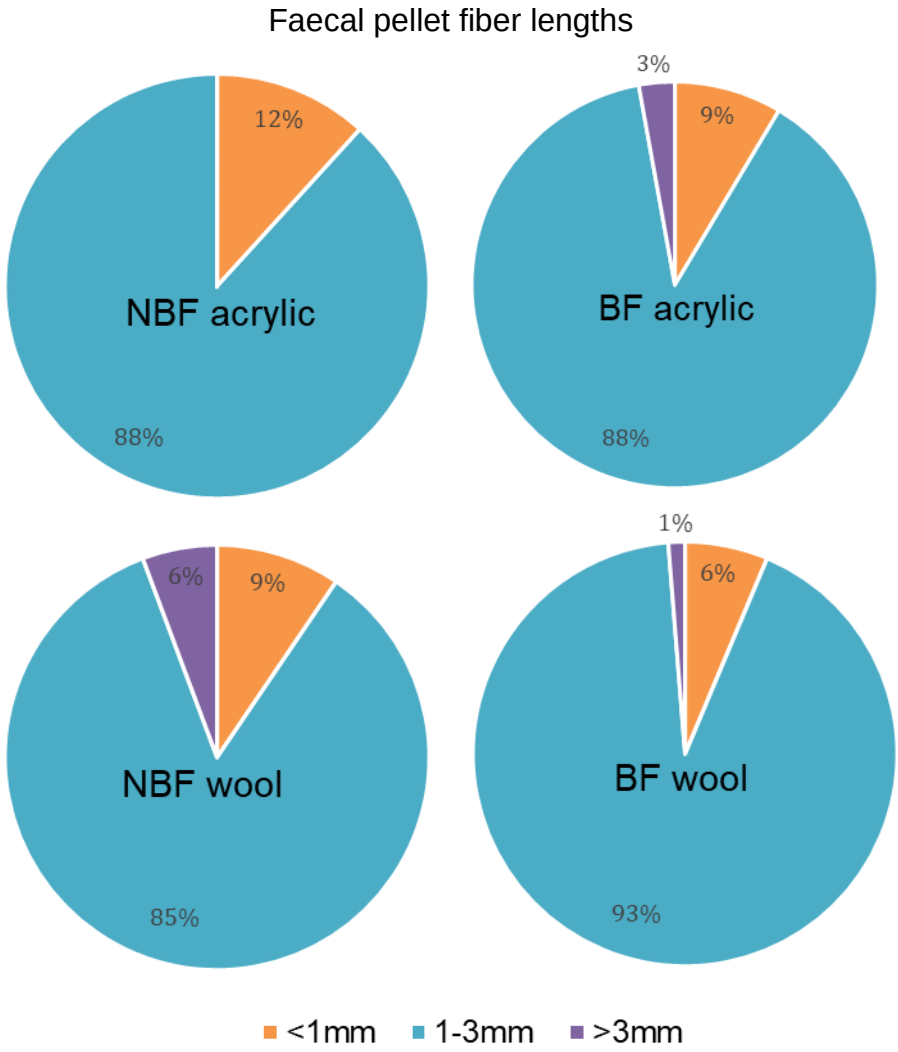


Figure 30: Fiber length distribution in faecal pellets by percentage of total fibers measured for all treatments (non-biofouled (NBF) acylic, biofouled (BF) acrylic, NBF wool, BF wool). Fiber length was measured categorically into 3 groups: <1mm (orange), 1-3mm (blue), and >3mm (purple).

4 Discussion

The aim of this study was to determine the microfiber ingestion, retention in the intestinal tract, and egestion ability in the species *Strongylocentrotus droebachiensis*. The hypotheses tested were:

1. Intestines and faecal pellets can be used to quantify fiber ingestion.
2. Urchins ingest both synthetic and natural fibers (acrylic and wool fibers).
3. Urchins ingest higher proportions of BF fibers than NBF fibers, regardless of fiber type.
4. Urchins will egest fibers in faecal pellets.
5. Retention of fibers in the intestines depends on fiber length; longer microfibers will be retained in the intestinal tract for a longer time than shorter microfibers.

This study aimed to fill knowledge gaps in the interactions between a widely distributed benthic organism and MP fibers. Ingestion studies such as this are an important baseline for future studies investigating biological and ecological effects of MP fibers (Pinheiro et al., 2020). Species-specific ingestion and egestion rates are important to quantify to improve understanding of the biological uptake of MP fibers and their fate in marine food webs (Gouin, 2020). This is the first laboratory exposure experiment that demonstrates the urchin species *S. droebachiensis* ingests and egests anthropogenic particles in the form of synthetic and wool fibers. All exposed fiber lengths from 1 to 5 mm were ingested in both BF and NBF state.

4.1 Urchin body size

Urchin body size was represented in this study by the diameter. The diameter of the urchin was not significantly related to the total ingestion of acrylic fibers. Although no significant difference was found, the group exposed to NBF acrylic fibers ingested a higher mean number of fibers. In wild populations smaller sea urchins have been found with higher MP concentrations than larger urchins (Feng et al., 2020). In the present study, the group exposed to NBF acrylic fibers was significantly smaller in body size than the group exposed to BF acrylic fibers. While not significant, the higher concentration of fibers in the NBF group are in accordance with the fact that smaller urchins ingest higher numbers of MP fibers. Feng et al. (2020) had a much wider range of urchin diameters (between 28 to 76 mm) and multiple urchin species were included. The lack of significant findings in the relationship between body size and total fiber ingestion may be due to small sample size and/or a small range of body sizes

represented in this study. Urchins were deliberately selected from the field to be of similar size as the preliminary goal of this study was not to investigate the influence of body size on MP fiber ingestion.

4.2 Faecal pellet production

Faecal pellet production can be used as an indicator of feeding rate of sea urchins, as continued food input is required to egest intestinal contents (Porter et al., 2019). In the present study the faecal pellet count is therefore considered to be a proxy for feeding activity. No statistically significant relationship was found between faecal pellet production and body size, fiber type or BF treatment. Despite this, after 48 hours the highest average faecal pellet production occurred in urchins exposed to NBF fibers, in both wool and acrylic fibers. This contradicts previous findings from Porter et al. (2019), where the urchin species *Paracentrotus lividus* showed increased feeding rates when exposed to BF macroplastic (polyethylene trays) when compared with NBF plastic. This discrepancy may be due to differences in size and polymer type of plastic used in both studies. However, during the depuration period, the present study showed that the faecal pellet count was higher in individuals exposed to BF fibers. This indicates feeding activity was higher in the BF fiber exposed individuals post exposure. The survival rate during the depuration period does question this trend and will be discussed in more detail in section 4.7.1.

The presence of natural food has been found to strongly influence feeding of urchins exposed to plastic under laboratory conditions and must therefore be included to reflect exposure conditions in the environment (Porter et al., 2019). However, starvation periods are known to occur in urchin barrens or due to seasonal unavailability of food. *S. droebachiensis* feeding rate under laboratory conditions has also been shown to depend on the ration (high or low) and the frequency of feeding (intermittent or continuous; Minor & Scheibling, 1997). Under low nutrient conditions urchins are known to enter a maintenance state (Lawrence, Plank, & Lawrence, 2003), leading to increased gut retention time to increase nutrition acquisition (Minor & Scheibling, 1997). Food was continuously supplied during this experiment suggesting reasons other than food availability caused decreased feeding activity. If urchins are exposed to MP fibers in an environment with low food availability, the increased intestine retention times may result in accumulation and trophic transfer of MP fibers (Porter et al., 2019). Due to uncertainties of the intrinsic and extrinsic influences on feeding rate in sea urchins (Suskiewicz & Johnson, 2017) a longer depuration period, consideration of feeding

regime, and larger sample size may improve future studies of this kind. Exposure to MP fibers under different feeding regimes of low or high food availability, to represent environmental conditions of urchin barren or kelp forest, could demonstrate retention time of fibers under these different scenarios.

4.3 Microfiber ingestion and egestion

4.3.1 Fiber concentrations in intestines

Fiber concentrations in the urchin intestines showed high variability among individuals with a higher mean count of NBF fibers than BF, but was not statistically significant. It must be considered that while no significant relationship was found between the urchin body size and intestine fiber count, there was a significant different in diameter between the NBF and BF groups. It is possible the difference in body size between NBF and BF groups influenced the higher number of fibers found in the intestines of the NBF treatment, if smaller urchins ingest higher amounts of MP as found in previous studies (Feng et al., 2020). The lack of statistical significance may be due to small sample size and individual difference in feeding activity. Further research with larger sample size is needed to investigate the relationship between biofouling and MP fiber ingestion.

4.3.2 Fiber concentrations in faecal pellets

S. droebachiensis egested both acrylic and wool fibers in faecal pellets, in both NBF and BF states. Urchin diameter had no relationship with the number of fibers found in faecal pellets at 48 hours, showing that body size does not influence the urchin's ability to egest fibers. No relationship was found between faecal pellet count and the total number of fibers egested, suggesting that fiber egestion is not related to feeding activity. The large number of faecal pellets (individual faecal pellet count: mean 56, minimum 0, maximum 145) available for analysis resulted in the decision to analyze 25 percent of the total faecal pellets. Due to the mixing of stomach contents during digestion, which was visible in variability of faecal pellet contents, it is possible that the chosen percentage may not be representative of fiber egestion. Instead of the time-consuming visual dissection of faecal pellets, the same digestion method used for intestine MP fiber extraction could be applied to the faecal pellets from acrylic treatments. Visual dissection was chosen so results could be obtained and compared with the findings from wool fiber exposure.

Results from 48 hour fiber egestion grouped together fiber type and BF state, to increase the sample size in the statistical analyses. Although no findings were significant, fiber type had a greater influence on fiber egestion than BF state. When grouping fiber egestion by BF state, ignoring fiber type, the mean fiber count was equal between groups but there was higher variation for NBF fibers. The four highest fiber counts in the NBF group are from the wool fiber exposed groups, and pooling data in this way may be misleading. The higher mean egestion of wool fibers after 48 hours could suggest there is higher total ingestion, or the urchins excrete wool fibers more readily. Methods allowing for digestion of intestines containing wool fibers will need to be tested to allow for improving understanding of the processes at work with ingestion and egestion of wool fibers.

4.4 Retention of microplastic fibers

Acrylic fibers were examined in both intestines and faecal pellets, which was required to analyze the retention of fibers. NBF acrylic fibers were found at higher concentration in the intestines but at lower concentration in faecal pellets, suggesting that NBF plastic fibers remained in the intestines longer than BF plastic fibers. No significant difference between NBF or BF acrylic fibers was found for the percentage of total ingested fibers retained in the intestines. However, after removal of the two outliers a significant difference was found for higher retention of NBF fibers. Removal of outliers with a small sample size must be interpreted cautiously. There is a low chance of discovering an effect that is true in a small sample size (increasing type 1 error; Button et al., 2013). The statistical significance with removal of the outliers is an interesting finding that promotes further exploration to determine if BF fibers have lower retention time, or are egested more readily. The outlier from the NBF acrylic exposure that did not egest any fibers was dissected after the 48 hour exposure and was not given any time for depuration. The outlier in the BF group that egested all fibers had ingested far less fibers than any other individual. The egestion of all the 4 ingested fibers may suggest that if *S. droebachiensis* are exposed to MP fibers at low concentrations, egestion of particles may occur in a short period of time. How this individual had such a low uptake of fibers under the here applied MP fiber concentration is not clear. This individual had the highest faecal pellet count at 134 hours, suggesting continued feeding activity, and was alive at dissection. Large individual variability in consumption rates and gut retention times has been reported in previous studies of sea urchins exposed to plastics (Porter et al., 2019) and may explain these outlying individuals. Previous studies have noted a higher ingestion of plastics when biofouled (Hodgson, 2018; Vroom et al., 2017) which was not found in this study. A future study with a

larger sample size would be recommended to investigate if higher intestine concentration from the NBF fibers was due to urchin body size.

Due to the high mortality at the last time point this study presents limited understanding of the retention time of MP fibers in *S. droebachiensis* post exposure. A previous study on the amphipod *Hyalella azteca* found that MP fibers have a longer retention time than other MP particles, and also resulted in an increased gut retention of natural food items (Au, Bruce, Bridges, & Klaine, 2015). The increased retention time of MP fibers may increase the potential for trophic transfer (Au et al., 2017). While the depuration data was not used due to high mortality, given adequate length of time urchins may, or may not, egest all fibers. The gut retention time of *S. droenachiensis* is approximately 2 days (Lawrence et al., 2013), but for future studies on this species a depuration period of up to 20 days would be ideal to see if all MP fibers are egested. Better understanding the retention of MP fibers in urchins is important in understanding the potential accumulation of MP fibers, and if urchins act as a sink for MP fibers.

4.5 Fiber length

The distribution of fiber lengths ingested by *S. droebachiensis* showed little change from the length distribution in the stock solutions. While some variation was found in length distribution between ingested fibers and the stock solution, there was no evidence of breaking or deterioration of ingested fibers as a result of the digestion process in the urchin intestines. The urchin digestive system does not appear to physically alter the acrylic or wool fibers used in this study. Physical alterations of ingested MP fibers have been demonstrated in a 4 week chronic exposure in the crab species *Carcinus maenas,* including decreased fiber size and fibers forming balls (Watts et al., 2015). While the digestive system of the crab influences this biotransformation, the longer exposure time may allow for retention or accumulation that could influence biotransformation of the MP fibers. A chronic exposure study in *S. droebachiensis* could help to demonstrate if biotransformation of MP fibers occurs in this species, and if length of fibers plays a role in retention time. For future studies a more precise method of fiber length measurement would be recommended. It was chosen to categorically assign fibers into three size groups due to the large number of fibers from intestinal contents requiring quantification. An improved digestion method may allow for automated fiber length measurement rather than manual, which may reduce the possibility of human error (Lusher 2020) and the time needed

for analysis. However, the issue of overlapping and entangled fibers will continue to present a challenge even to computer analysis (Rebelein, Int-Veen, Kammann, & Scharsack, 2021).

4.6 Ecological impact

As biofouling is known to occur quickly in marine waters and can lead to MP particles becoming negatively buoyant, it is to be expected that benthic taxa such as *S. droebachiensis* will encounter BF MPs in the environment. MPs and MFs with density greater than seawater, such as the acrylic and wool fibers used in this study, will sink to benthic environments. Understanding the processes and dynamics of ingestion, retention, and egestion is critical in understanding fate and impacts of MP fibers in the marine environment. The movement of plastics in the ocean is not only driven by physical oceanographic processes, interactions with biota can also alter the fate of plastics in the ocean (Porter et al., 2019). This study has demonstrated for the first time *S. droebachiensis* ingest MFs, and egest these fibers packaged in faecal pellets. As urchin faecal pellets are an important food source for other benthic invertebrates, the ability to egest MP fibers in faecal pellets could increase exposure of other benthic detritivores, scavengers, and suspension feeders (Scheibling & Hatcher, 2013; Watts et al., 2015). Retained fibers will be available for trophic transfer, potentially increasing the harmful impact of MP fibers in the environment. Understanding of the distribution of MPs in marine food webs will help to predict the possible ecological effects (Bour et al., 2018). This study contributes the knowledge that *S. droebachiensis* will ingest MFs present in the environment, but have the ability to egest MFs. The harmful impact of fibers while passing through the intestinal tract is still unclear as well as the impact of retained fibers. To what degree *S. droebachiensis* can remove ingested microfibers via egestion remains uncertain and requires further investigation. The exposure of MPs must be considered in the context of the multiple stressors that marine organisms are facing, including changes in ocean temperatures and ocean acidification which are known to negatively impact *S. droebachiensis* (Scheibling & Hatcher, 2013). This species plays a key role in the transition between states from rich kelp forest to urchin barrens, which has large impacts on the broader ecology and food webs in these locations. Future studies should measure the physiological response of sea urchins to MP fiber ingestion, including chemical additive effects, to improve understanding of population and ecosystem effects.

4.7 Limitations and future recommendations

4.7.1 Survival

The low survival rate of urchins along the duration of this study was a limiting factor to analysis of MF and urchin interactions. The survival of only three individuals across the depuration period restricts the possibility of understanding the retention and egestion dynamics of MP fibers in *S. droebachiensis*. As the control group demonstrated a similar death rate to treatment groups, it is assumed that death was not due to fiber exposure. Previous studies have successfully kept urchins in laboratory conditions for up to 24 months (Meidel & Scheibling, 1999), including 2 week periods of starvation (Lawrence et al., 2003; Minor & Scheibling, 1997). In a previous study on the urchin species *Arbacia punctulata* after MP exposure for 24 hours under storm-like conditions (decreased salinity), there was no significant impact on adult sea urchin physiology (Suckling & Richard, 2020). Suckling and Richard (2020) used 9µm polystyrene spheres, which would predictably have different effects than MP fibers, but reinforces the assumption that urchin death in this study was due to captivity conditions. For future replication of this study it is recommended that urchins are kept under aerated water conditions, or with daily changing of seawater in the beakers. Consideration of using a larger beaker size would be recommended. Dethier et al. (2019) demonstrated an interesting design allowing for individual urchin captivity with water exchange and faecal pellet collection. Construction of a similar system may be worthwhile and could be used for future studies of sea urchin and MP interactions.

4.7.2 Experimental design

Finding the balance of the smallest number of organisms required to provide robust and reliable findings is an important scientific question (Button et al., 2013). Low statistical power is an issue presented with small sample sizes and will reduce the likelihood of finding a statistically significant effect (Button et al., 2013). Low power will also increase the probability of a type 1 error (rejecting the null hypothesis when it is true) and if a true effect is found the magnitude is exaggerated (Button et al., 2013). The small sample size and low statistical power may explain the lack of significant findings in this study. The distribution-free permutation test was chosen as it is a more robust statistical test to use for a small sample size (Hayes, 1996). Due to the individual variability in feeding, future studies investigating sea urchin and MP interactions would benefit from a higher number of individuals than used in this study.

4.7.3 Data collection

For replication or similar studies, extra data collection would be recommended. Weighing urchins before dissection could contribute information to understanding size related feeding rate differences. As urchin diameter increases feeding rate increases linearly, but when body mass is considered the feeding rate of small urchins is the same, or higher than, large urchins (Scheibling & Hatcher, 2013; Suskiewicz & Johnson, 2017). It was considered to back calculate the body mass of the urchins using the diameter data but it was concluded insufficient as height of urchin shell would be needed to accurately do this (Ebert, 1988). More thorough recording of feeding regime would be informative to understand and calculate specific feeding rates during the experiment. Consideration of intermittent feeding would be suggested if increased feeding rate would be desirable (Minor & Scheibling, 1997). However, starvation periods due to seasonal food availability or presence of urchins on barren grounds is an environmentally realistic condition. A future study could compare the difference in MP fiber retention under different food availability conditions. Weighing of the intestinal tract before storage in ethanol could also provide valuable information, as it was visually noticed that some intestines had more contents than others, however this was not recorded systematically prior to intestine digestion.

4.7.4 Digestion protocol

The poor efficiency of digestion of intestine contents was a hindering factor to accurate analysis of acrylic fibers. For future improvements different protocols should be tested. A higher concentration of chlorine should be explored, as could a longer digestion time or increased temperature during digestion (Dehaut et al., 2016). Enzyme digestion methods are known to be gentler on MPs (Cole et al., 2014) and may be an option to explore that could also include the digestion of wool fibers. This was not tested in this study due to costs, availability, and time constraints.

5 Conclusion

Strongylocentrotus droebachiensis ingested and egested both synthetic and natural fibers (acrylic and wool fibers) in both NBF and BF states. Biofouling did not impact the ingestion and egestion of MP fibers. Higher ingestion was found in the NBF acrylic exposed group, but is likely due to the smaller body size of this group. Fiber length was not found to be affected by urchin digestion. *S. droebachiensis* shows promise for future studies regarding MP ingestion and egestion studies. Improvements in urchin captivity conditions will contribute better understanding of retention times and the ability of this species to egest MFs. Urchin faecal pellets are an important food source in benthic communities and their ability to egest MP fibers may increase exposure of other benthic species. Retained fibers in urchin intestines will be available for trophic transfer, potentially increasing the harmful impact of microplastic fibers in the environment. This study adds understanding of the biological uptake of MP fibers and their fate in marine food webs.

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