CRediT author Statement

According to the CRediT (Contributor Roles Taxonomy) the authors' contributions are as follows:

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Phytoplankton Cell
Microfiber
Particles Entering
Accepted Particles
Rejected Particles

Effects of long-term exposure to microfibers on ecosystem services provided by coastal mussels

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Abstract

The biofiltration capacity of bivalve populations is known to alleviate the effects of coastal eutrophication. However, this important ecosystem service could potentially be impaired by the increasing microplastic abundance in near shore environments. It is known that relatively large microplastics (~500μm) impair the filtration capacity of bivalves, however, the effect of smaller microplastics, and specifically microfibers, is not known even though they are more common in many natural systems and similar in size to phytoplankton, the main food source of mussels. Here, we investigated the effects of long-term exposure to microfibers (MFs), which are smaller than 100μm, on the biofiltration capacity of the blue mussel, *Mytilus edulis*. Our findings show that long-term exposure (here 39 days) to microfibers significantly reduced (21%) the clearance of phytoplankton (*Tetraselmis* sp). While previous studies have shown that larger microplastics can decrease the filtration capacity of mussels after short-term exposure, our findings suggest that, for smaller MFs, mussel's clearance capacity is significantly affected after long-term exposure (39 days in this study). This may be due to the accumulation of MFs in the digestive system. In addition, the most efficient phytoplankton consumers were more susceptible to MF accumulation in the digestive system. This suggests that prolonged exposure to MF of coastal mussels could

negatively impact the biofiltration of more potent individuals, thus decreasing the ecosystem service potential of the population as a whole.

Capsule

We found that long-term exposure to small microfibers can impair the phytoplankton clearance by coastal mussels.

Key Words

Ecosystem services, particle selection, coastal ecosystems, microfibers, phytoplankton clearance

Introduction

The intensification of anthropogenic activities along the coastline poses critical environmental pressures on coastal ecosystems. Specifically, coastal eutrophication and Harmful Algal Blooms (HABs) are currently ranked as the most critical stressors of marine ecosystems (Anderson et al., 2002; Kellogg et al., 2014; van der Schatte Olivier et al., 2018), with important implications on both ecosystem and public health (Landsberg, 2002). These effects can be remediated by the ecosystem services provided by filter-feeding organisms, such as bivalves, that remove excess microalgal biomass from the water column (Prins et al., 1998; Tantanasarit et al., 2013; van der Schatte Olivier et al., 2018) and make nutrients available to bottom feeders by biodeposition on the sediment (Kellogg et al., 2014; van der Schatte Olivier et al., 2018). However, coastal ecosystems are also subject to a variety of environmental stressors, such as plastic pollution, which could impact the ability of bivalves to perform these services. Investigating the potential effect of such stressors on the ability of bivalves to perform ecosystem services is thus of fundamental importance for our understanding of coastal ecosystems (Fisher et al., 2008) and is necessary for informing evidence-based environmental policies (Rochman, 2016).

Microplastic (<5mm) pollution has been recently identified as a major environmental stressor in coastal systems and associated biological communities (Mathalon and Hill, 2014; Ryan and Turra, 2019). Previous studies have shown that the ingestion of microplastics by bivalves can result in reduced filtration rates (Rist et al., 2016; Woods et al., 2018; Xu et al., 2016), decreased respiration (Rist et al., 2016), lower energy intake (Xu et al., 2016), inflammation of cell tissue (Von Moos et al., 2012), damaged gills (Cheung and Shin, 2005) and reduced fecundity (Gardon et al., 2018; Sussarellu et al., 2016). While most of these studies have focused on microplastic fragments and beads (Gardon et al., 2018; Rist et al., 2016; Sussarellu et al., 2016; Von Moos et al., 2012; Xu et al., 2016), very little is known about the effect of microfibers (MFs), which is the dominant form of microplastics in the marine environment (Covernton et al., 2019; Davidson and Dudas, 2016; Qu et al., 2018; Railo et al., 2018). The underrepresentation of MFs in studies is mainly due to fact that MFs are not available for commercial purchase and their preparation in the lab is tedious, thus experimentations especially with specific size ranges are scarce (Wagner et al., 2017). As MFs within the 10-40µm size range are both within the preferred feeding size range of mussels (Fernández and Albentosa, 2019; Ruppert et al., 2004; Strohmeier et al., 2012; Van Cauwenberghe et al., 2015; Willer and Aldridge, 2017) and represent the majority of MFs in the water column (Covernton et al., 2019; Doyle et al., 2011; Thompson et al., 2004) more information about their ecosystem effects is urgently needed.

Browne et al. (2008) showed that ingested polystyrene microspheres (3 and 10µm) were translocated to the circulatory system of the marine bivalve *M. edulis* and remain there for more than 48 days. Furthermore, Von Moos et al. (2012) demonstrated that small plastic particles (0-80µm) were taken up into epithelial cells of the digestive system of mussels, where they induced a strong inflammatory response. Hence, smaller particles are more likely to be ingested and seem to undergo translocation more readily than particles larger than 100µm (Kolandhasamy et al., 2018; Ward et al., 2019). However, most of these studies have focused on short-term exposure and the subsequent acute effects, while little is known about chronic consequences of continuous long-term exposure to MFs. Here, we hypothesize that the translocation and long-term presence of particles <100µm into the digestive system and tissue of

organisms, will negatively affect the ecosystem service of phytoplankton clearance by coastal mussel populations.

To test this hypothesis, we investigated, in a lab experiment, the impact of long-term exposure to MFs on the ability of mussels to remove excess biomass of microalgae from the water column. The main objectives of this study were (a) to investigate the phytoplankton removal capacity of individual mussels throughout a period of continuous exposure under pristine and MF-polluted conditions and (b) to identify any relationship between phytoplankton removal capacity and amount of MFs accumulated in the digestive system of mussels.

Materials and Methods

Microfiber preparation

We used nylon as the material for our MFs as this is one of the most common materials of MFs found in the environment. The abundance of these MFs can be attributed to nylon's extensive use in aquaculture and fisheries (e.g., nets and ropes) (Cole et al., 2011; Davidson and Dudas, 2016; Ryan and Turra, 2019) as well as the clothing industry (e.g., synthetic textile fibers released in effluent water from washing machines) (Browne et al., 2011; Li et al., 2015; Magnusson and Norén, 2014; Salvador Cesa et al., 2017). Additionally, nylon, having neutral buoyancy, can be widely distributed within the water column (Cole et al., 2011) thus being highly bioavailable to filter-feeding organisms.

The microfibers were prepared as per Cole (2016). In summary, nylon (polyamide-6) threads (10μm diameter) were encapsulated within a freezing agent, solidified in dry ice and a cryotome machine was used to cut them in 30μm length. The freezing agent was then melted, and the cylindrical MFs were retrieved. The resulting length was 35.20μm (± 12.9S.D) with only 8% of the MF being >100μm long (see Supplementary material: Microfiber size distribution). Although this method is not widely used due to the increased requirement in time and effort, the MFs produced are highly appropriate for experimentation purposes as they have specific shape and structure. This renders

them easily distinguishable from other types of MFs potentially encountered in samples due to airborne inputs.

Mussel collection and acclimation

For this experiment, rope-grown, juvenile mussels (33.9mm; ±1.6S.D.) were collected from Loch Sunart (56°41'15.7"N, 5°36'55.0"W) in May 2019. *M. edulis* was selected as a model organism due to its (a) global coastal distribution (MacDonald and Ward, 2009), (b) low position at the trophic chain (Rist et al., 2016), (c) great abundance, particularly near polluted and eutrophic sites (Beyer et al., 2017; Li et al., 2019), (d) greater water clearance rate in comparison to other bivalves (MacDonald and Ward, 2009), and (e) economic importance e.g., in shellfish aquaculture (van der Schatte Olivier et al., 2018; Willer and Aldridge, 2017).

In the laboratory, mussels were placed in 5L aquariums containing artificial saltwater (salinity 32ppm) and were allowed to purify for 2 days with continuous monitoring of water chemistry indicators such as ammonia and nitrates. For acclimation to the experimental conditions, forty-four mussels were individually placed in 800ml glass vessels equipped with cylindrical mesh stands to support the mussels at a standard height of 4cm from the bottom across all vessels. The experimental vessels were under diurnal photoperiod (12:12) and the water temperature was maintained between 12-13°C and was constantly aerated via air pumps to also ensure sufficient mixing of the water column. Mussels were fed $3x10^6$cells/L of *Tetraselmis* sp. monoculture (Riisgard, 1991) once per day for another 2 days (Browne et al., 2008; Defossez and Hawkins, 1997). On the 5^{th} day the mussels were starved for 24 hours prior to the initiation of the experiment.

Experimental Design

The experiment consisted of a MF exposure treatment and a control treatment that was lacking MFs, and each treatment consisted of 22 replicates (i.e., 22 glass vessels containing a single mussel each). Mussels in both treatments were fed daily, with a single dose of *Tetraselmis* sp. at a concentration of $3x10^6$cells/L (see Supplementary

Material: *Tetraselmis* sp. culture). A concentration of 24,000MF/L of MFs was also added only to the MF treatment at the time of feeding. The continuous aeration of the water ensured the constant resuspension of MFs and *Tetraselmis* cells in the water column. To avoid airborne microplastics contamination, cotton lab-coat and vinyl gloves were worn at all stages of the study. Furthermore, the experimental vessels were located in a wooden enclosed box minimising the settlement of airborne fibers in our vessels. The ambient conditions were maintained as detailed above and artificial salt-water was changed every second day. The water changes and daily feeding was performed at the end of the light period. The mussels' shell (length) was measured at the beginning and end of the experiment to determine any effect of microfibers on their growth.

The total duration of the experiment was 52 days and water samples for the quantification of phytoplankton consumption were taken every 13 days after day 1, for a total of 5 sampling points (Days 1, 13, 26, 39 and 52). For each sampling point, the glass vessels were drained and cleaned, then 870ml of water per vessel were prepared with the addition of *Tetraselmis* sp. and MFs, as per treatment requirements. Water samples (70ml) were collected from each experimental vessel at 0 and 24 hours to measure the concentration of algae and MFs. The phytoplankton and MF percentage consumption at each time point was thus estimated as:

$$\frac{\text{Concentration 0h} - \text{Concentration 24 h}}{\text{Concentration 0h}} \times 100.$$

There is no literature, at the moment, focusing on concentrations of <100μm microplastics, probably due to the challenging methodologies involved in the sampling and quantification of such small microplastic fractions in the marine environment. Reports of current marine concentrations of microplastics >100μm are of limited guidance here as different studies have suggested that the ambient concentrations of smaller microplastics are underestimated (Barrows et al., 2017; Covernton et al., 2019; Lindeque et al., in press; Phuong et al., 2016). Therefore, due to the lack of available published data, we used a concentration of 24,000MF/L, in accordance with other mussel exposure studies. Woods et al. (2018) and Wang et al. (2020) used concentrations in the range of 3,000-30,000particles/L and 10-1,000,000particles/L

195 respectively while higher concentration of 42,000 particles/L and 110,000particles/L
196 were used by Van Cauwenberghe et al. (2015) and Browne et al. (2008) respectively.
197
198 Long-term experiments with mussels are subject to contamination with periphyton
199 diatom species that are attached as biofilm to the inner and outer shell of the mussels
200 collected from the field (Pérès et al., 1996; Sweat, 2016). To minimise the impact of
201 these opportunistic diatoms, the surfaces inside the experimental containers (glass and
202 mussels) were cleaned every second day. Moreover, to account for potential effects of
203 diatom contamination in our statistical inference, water samples (50ml) were analysed
204 spectrophotometrically (Parsons et al., 1984) and chlorophyll-c, a proxy for diatom
205 biomass, was accounted for in our models.
206
207 ***Sample Analysis***
208
209 For the quantification of phytoplankton removal capacity of mussels, 20ml of water,
210 from samples preserved with lugol, were filtered using Sartorius™ Cellulose Nitrate
211 Membrane Filters (0.45μm pore size, 25mm diameter). The filters were then dried for
212 one hour in an incubator at 40^{o}C. Each filter was then made transparent using
213 immersion oil and examined under a light microscope. The phytoplankton cells were
214 counted in 15 randomly selected fields of view (coefficient of variation <0.7), on the
215 surface of the filter paper (MFs were also estimated for our records). Manual counting
216 was preferred to an automated technique to ensure sensitivity of counting at low
217 phytoplankton concentrations (i.e., after 24h of feeding), to enable the distinction of
218 phytoplankton from MFs, and to avoid the overestimation of counts due to the potential
219 presence of other particles such as airborne fibers, mussel faeces, pseudofaeces and
220 gametes.
221
222 For the quantification of MFs in the digestive track of mussels, the organisms were
223 individually wrapped and preserved in a -20^{o}C freezer upon the termination of the
224 experiment. Each mussel was defrosted for 30 minutes in room temperature before the
225 soft tissue was removed from the shell and washed under running Milli-Q water for 30
226 seconds to eliminate any MF possibly attached to the surface of the mussel's tissue
227 (Kolandhasamy et al., 2018). The digestive gland, which surrounds the stomach (Morton

and Puljas, 2018), was separated from the rest of the mantle and organs. The digestive gland and stomach were then immerged in a 25ml, 0.31% trypsin solution (Courtene-Jones et al., 2017), and gently stirred for 30mins at 45°C. The solution was then centrifuged at 3,500rpm, 15°C for 15 minutes resulting in the settlement of organic matter and MFs at the bottom of the tube as precipitate. Most of the supernatant was removed, leaving about 1ml to prevent any disturbance to the precipitate layer, which was then homogenised using a pipette. The homogenised mixture was inspected under an optical microscope (x10/0.25) and all laboratory-produced MFs were quantified (smallest MF size detected was 13.8μm).

Data analysis

To test the effect of treatment, sampling day and diatom fouling (chlorophyll-c) on the phytoplankton percentage consumption by mussels, we used a generalised linear mixed model (GLMM). The response variable, comprising of proportions bounded between 0 and 1, was not normally distributed (Shapiro-Wilk normality test, p-value<0.05), thus the beta distribution was used to model the data. Since the beta distribution does not accept exact values of zero and one, data were transformed using the following equation:

$$Y_{transformed} = \frac{[Y*(N-1)+0.5]}{N},$$

where $Y_{transformed}$ is the transformed value of the phytoplankton consumption proportion, Y, and N is the sample size (Smithson and Verkuilen, 2006). Sampling day was included as a continuous variable to account for the long-term effects of the MFs. Mussel ID was included as random effect to account for repeated, non-independent measures taken from the same animal. The possible models were fitted using the R glmmTMB package (Brooks et al., 2017) and model selection was performed based on Likelihood Ratio Tests (LRT). Independent t-tests were used to compare treatment effects within the same sampling day. To determine the effect of MFs on the growth of mussels we used a linear model with the treatment as an explanatory variable.

A linear model was used to test for the effect of phytoplankton consumption, MF consumption and diatom fouling on the MFs accumulated in the digestive gland and stomach. Prior to the analysis, data were log-transformed to eliminate

heteroscedasticity in MF counts (see Fig. S2) across the values of phytoplankton consumption.

Results

Effect of long-term MF exposure on phytoplankton removal capacity

The average consumption of *Tetraselmis* cells across the treatments and sampling points was 85.9% (± 18.8S.D.). Across sampling points, the average *Tetraselmis* consumption in the MF exposure treatment was 83.1% (± 20.6S.D.) whereas in the control treatment was 88.7% (± 16.5S.D.). There was a significant interaction between treatment and sampling day (Table 1), which suggests that the effect of MFs on the phytoplankton removal capacity by mussels varied in time. After 26 days of exposure, mussels exposed to MFs showed a greater variation in the phytoplankton consumption i.e., clearance capacity. On day 39, mussels exposed to MFs had a significantly lower phytoplankton removal capacity by 21.3% compared to the mussels in the control treatment (t-test, p=0.0014, N=44) (Fig. 1). On the last sampling day (day52) there was no significant difference between the two treatments (t-test, p=0.17, N=44). This coincided with a spark of opportunistic diatoms across all replicates (Fig. S3), which had a significant negative effect on *Tetraselmis* consumption by mussels (Table 1). No significant difference was observed between the growth of mussels in the control (0.50mm; ± 0.22S.D.) and microfiber (0.59mm; ± 0.22S.D.) treatments ($F_{1,42}$=1.5728, p=0.2167) upon termination of the experiment.

Accumulation of MFs in the digestive gland and stomach

The number of MFs accumulated in the digestive gland and stomach of the 22 mussels that were subject to the MF treatment, had a high variation ranging between 24 and 3,170 with a mean of 475MF per mussel (± 651S.D.) (Fig. S2). The MF accumulation varied positively with *Tetraselmis* consumption ($F_{1,18}$=9.90, p=0.0056) (Fig. 2) whereas the MF consumption ($F_{1,18}$=0.52, p>0.1) or the presence of diatoms ($F_{1,18}$=0.8027, p>0.1) had no influence on the MFs accumulated.

Discussion

Findings from this long-term experiment indicate that the capacity of mussels to remove phytoplankton biomass from the water column can be negatively impacted by long-term exposure to MFs of 10-100μm size range. Specifically, mussels exposed to MFs showed an average decrease of 21.3% in their phytoplankton removal capacity after 39 days of exposure to ambient concentrations of MFs. This finding is important as it indicates that the ecosystem service of mitigating eutrophication and HABs in coastal systems can be impaired by the presence of another dominant stressor such as MFs. Another long-term exposure experiment (44 days) that used PVS particles (1-50μm), at a higher concentration than those used in our study, also showed a decrease in the clearance rate of mussels by 79% (Rist et al., 2016). These findings stress the importance of prolonging experimental duration, a suggestion also stressed by Qu et al. (2018) and Von Moos et al. (2012).

Despite the fact that short-term effect of MFs was not observed in our study, mussels exposed to MFs showed higher unpredictability in their clearance capacity from earlier on (day 26), as indicated by the higher variation in the phytoplankton removal percentages. A short-term study exposing mussels to 3μm and 9.6μm of polystyrene microspheres (15,000particles/treatment) for 3 hours reported a lower clearance rate 48 days after the exposure than after 6 days. This suggests that the effect on the mussel clearance capacity was exacerbated even after the termination of a short-term exposure. Thus, we can only expect that the continuous long-term exposure to microplastics, which is an environmentally plausible condition, will have a more deleterious effect on the ecosystem services provided by coastal mussels. For more realistic and representative measures of future laboratory exposure studies, there is a need for accurate estimates of environmental concentration of microplastics <100μm.

Another important finding of this study was that higher amounts of MFs in the digestive gland of mussels were associated with higher microalgae consumption. This can be explained by considering the physiological feeding mechanism of a mussel, as illustrated in figure 3. After uptake, through the inhalant siphon of the mussel (Fig. 3, step 1), food particles are sorted by size at the lamellae filaments of the gills (Fig. 3, step

2). At this pre-ingestion level, particles smaller than 1-6µm pass through the interfilamental gaps and are immediately expelled along with water. Thereafter, the larger particles that have been retained, will be led by the frontal cilia of the gills to the food grooves from where they reach the labial palps for further sorting (Fig. 3, step 3). There is a literature gap regarding the exact sizes being sorted at the labial palps; however, rejected large and excess particles are released in the mantle cavity to be ejected as pseudofaeces (Ren et al., 2006; Rouillon and Navarro, 2003; Ruppert et al., 2004). The remaining smaller particles are directed to the mouth, oesophagus and stomach (Fig. 3, step 4) where extracellular digestion is initiated by the rotation of the crystalline style (Morton, 1983; Ward et al., 2019), and the sorting fields will direct particles >100µm (Kolandhasamy et al., 2018) to the rejection truck to be excreted as faeces. Particles <100µm, either enter the digestive ducts or remain suspended in the stomach (Ruppert et al., 2004). Due to the similarity in size of MFs investigated in this study with the food particles (i.e., microalgae) consumed, MFs passed the pre-ingestion sorting and reached the stomach and digestive gland. This was also observed by Fernández and Albentosa (2019) where mussels showed no difference in the clearance of microalgae and microplastics of similar size and explains our finding that mussels that were high-consumers were more susceptible in accumulating MFs in their gut. This also suggests that the presence of MFs in the water column may specifically impair individuals with the highest clearance capacity, which, in the long-term, can impact the ecosystem service of microalgal removal by mussel populations.

Our findings suggest that long-term exposure to even small MFs can negatively impact the ability of mussels to perform ecosystem services. This impairment did not occur as a result of potential disruption of the filtration process due to larger particles damaging the cilia of the gills (Cheung and Shin, 2005), or filtered out at the pre-ingestion phase and the pseudofaeces production (Woods et al., 2018), but rather as a result of accumulation of MFs in the digestive gland. The exact mechanism by which MF accumulation might have affected the clearance capacity of microalgae in this experiment is unknown. However, earlier work has shown that microbeads of 10µm diameter can penetrate the biological membranes of the digestive gland and translocate into the mussel's tissue (Rist et al., 2016), 3µm and 9.6µm polystyrene particles can reach the circulatory system of mussels (Browne et al., 2008), and that the presence of

polyethylene particles can trigger an inflammatory response (Von Moos et al., 2012). Additionally, the presence of MFs in the stomach and digestive gland of mussels could trigger a feeling of satiation making the uptake of more food less likely and eventually leading to the mussel's starvation (Gall and Thompson, 2015). Further research is required to determine the specific mechanism underlying the effect of ingested small MFs on the physiology of the organisms.

An interesting observation from our experiment was that the fouling of our experimental vessels by opportunistic diatoms had a negative impact on the clearance of *Tetraselmis* cells, comparable to that of MFs. More research is required on whether this is linked to the fouling diatom in our experiment showing a structural resemblance in size and shape to our MFs (Fig. S5) or e.g., to preferential grazing by the mussels of the fouling diatoms compared to the flagellate *Tetraselmis* cells (Cucci et al., 1985; Ren et al., 2006; Rouillon and Navarro, 2003; Safi and Hayden, 2010; Shumway et al., 1985).

Conclusion

Our findings show that long-term exposure to MF (<100μm) can significantly decrease the clearance capacity of mussels, and thus the ecosystem services they provide. MFs accumulated in the digestive gland and stomach of mussels which was linked to the intensity of phytoplankton consumption. This suggests that individuals with high clearance capacity would be more susceptible to microfiber ingestion. These effects may vary in the presence of different phytoplankton species, thus we stress that further research is required on this topic.

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their constructive comments. Lastly, we would like to thank Emma Teeluckingh, Hannah Crook, Phoebe Kaiser-Wilks and Rosalie Bailie for their help in implementing the experiment.

Figures and Tables

Table 1: Summary of the best-supported model explaining the variation in phytoplankton removal capacity by mussels. ΔAIC and LRT indicate the increase in AIC and difference in the log likelihood of the model given the data, respectively, if the variable was dropped.

	Estimate	Std. Error	Z value	P value	Δ AIC if dropped	LRT if dropped
Intercept	1.95	0.229	8.49	<0.001		
Chlorophyll-C	-2.62	0.723	-3.61	<0.001	10.4	Df=1, <0.001
Day-treatment interaction	-0.018	0.007	-2.36	<0.018	8.3	Df=3, <0.002

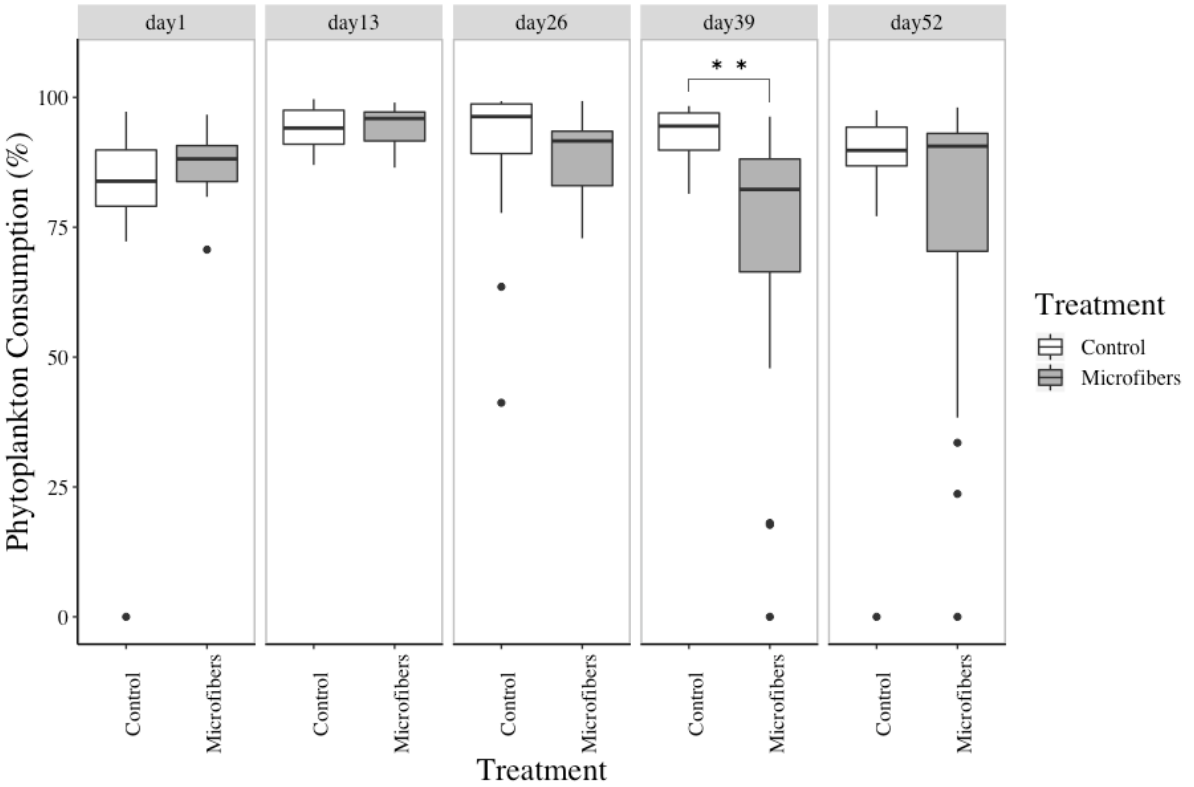
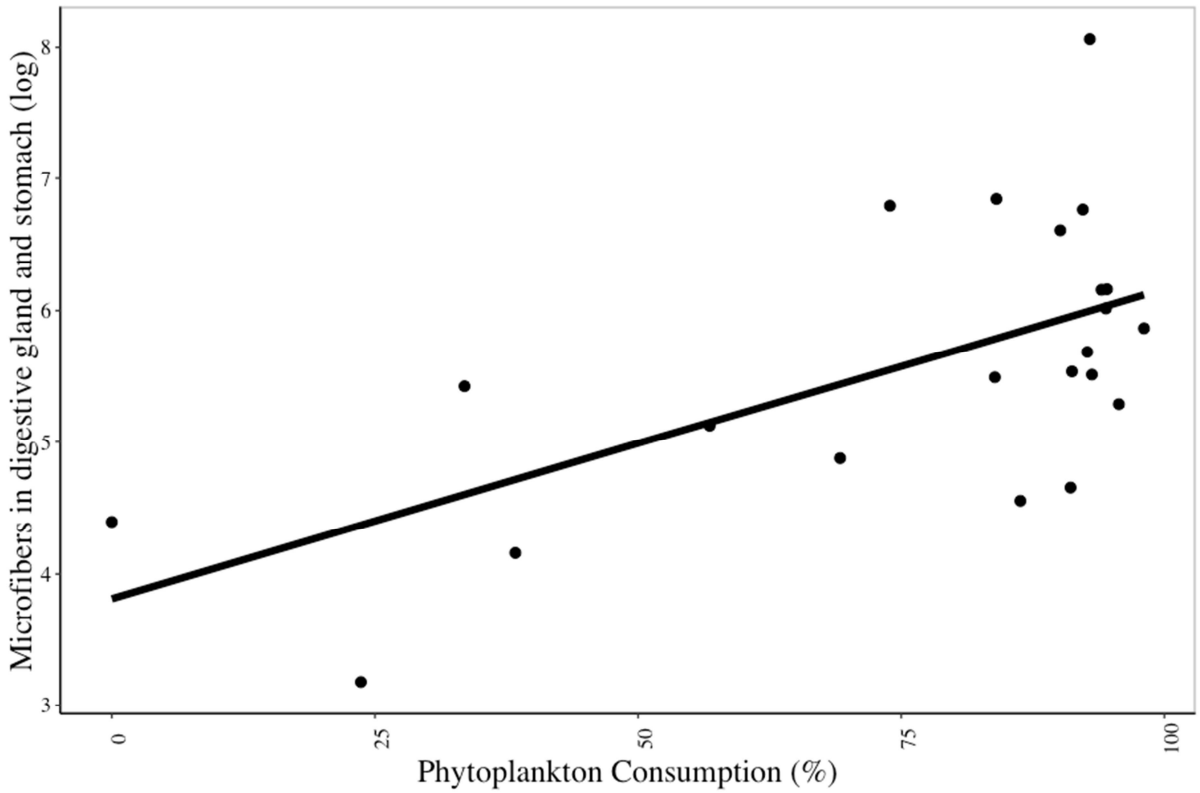


Figure 1: The percentage of *Tetraselmis* sp. consumed by mussels in the Control (white) and Microfiber (gray) Treatments at each sampling day (1, 13, 26, 39 and 52). A significant difference ($p<0.01$) between the two treatments at day 39 is indicated with asterisks (**). Please note that "Day" was included as a continuous variable in the corresponding statistical model.



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411

412 **Figure 2:** Relationship between the microfibers retained in the digestive gland and
413 stomach of mussels, and consumption of *Tetraselmis* cells at the end of the experiment
414 (day52).

415

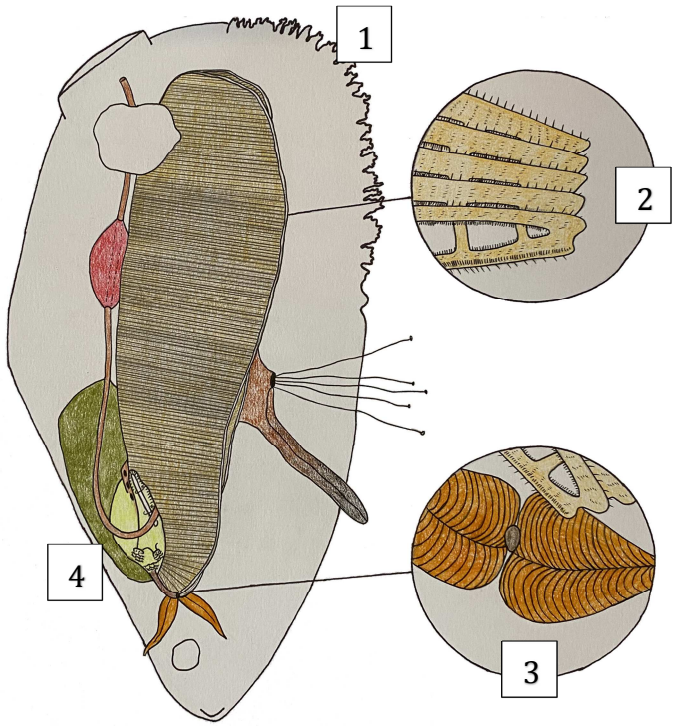

Figure 3: Internal anatomical diagram of a mussel displaying the 4 main particle-sorting areas: (1) In the inhalant syphon, particles <5000µm long & <50µm wide enter the mussel (Kolandhasamy et al., 2018; Newell, Shumway, Cucci, & Selvin, 1989; Rosa, Ward, & Shumway, 2018)). (2) At the gills, particles >1-6µm are retained (Dral, 1967; Rosa et al., 2018; Ruppert et al., 2004) and transported to the food grooves from where they enter (3) the labial palps for further sorting (rejected particles form pseudofaeces). Accepted particles are lead into (4) the stomach, where particles <100µm can enter the digestive system (Kolandhasamy et al., 2018) (for more details see Fig. S4).

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Highlights

- Our experiment showed that small microfibers can impact clearance in the long term
- A wide range of microfiber quantities was found in the digestive system of mussels
- Efficient phytoplankton consumers were more susceptible to microfiber accumulation
- Increasing microfiber pollution can impact coastal ecosystem services by mussels