Selective ingestion and egestion of plastic particles by the blue mussel (*Mytilus edulis*) and eastern oyster (*Crassostrea virginica*): implications for using bivalves as bioindicators of microplastic pollution

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ABSTRACT

Microplastics (MP; 1 μm–1 mm) of various shapes and compositions are ingested by numerous marine animals. Recently, proposals have been made to adopt bivalve molluscs as bioindicators of MP pollution. To serve as indicators of MP pollution, however, the proposed organisms should ingest, without bias, the majority of plastic particles to which they are exposed. To test this premise, eastern oysters, *Crassostrea virginica*, and blue mussels, *Mytilus edulis*, were offered variously sized polystyrene microspheres (diameters 19-1000 μm) and nylon microfibers (lengths 75-1075 x 30 μm), and the proportion of each rejected in pseudofeces and egested in feces determined. For both species, the proportion of microspheres rejected increased from ca. 10-30% for the smallest spheres to 98% for the largest spheres. A higher proportion of the largest microsphere was rejected compared with the longest microfiber, but similar proportions of microfibers were ingested regardless of length. Differential egestion of MP also occurred. As a result of particle selection, the number and types of MP found in the bivalve gut will depend upon the physical characteristics of the particles. Thus, bivalves will be poor bioindicators of MP pollution in the environment, and it is advised that other marine species be explored.

Keywords: Microplastic; Bioindicators; Rejection; Ingestion; Egestion; Pseudofeces; Feces; Bivalve; *Crassostrea virginica*; *Mytilus edulis*

48 **GRAPHICAL ABSTRACT**

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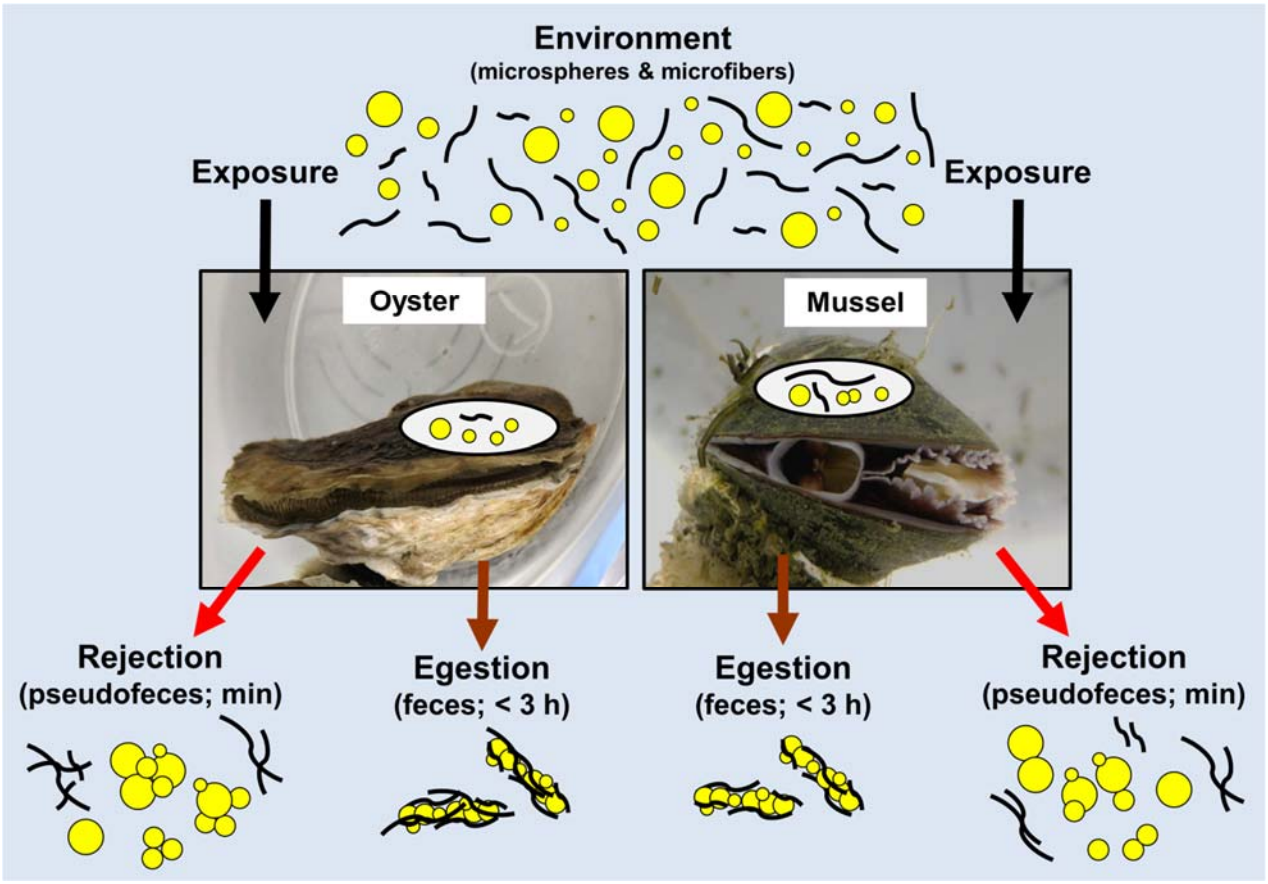

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INTRODUCTION

Plastic debris in the marine environment is a wide-spread pollutant interacting with, and affecting a range of organisms from larvae to vertebrates.[1,2] Equally problematic are the myriad of microplastic (MP) particles (1 μm – 1 mm)[3] that are manufactured for consumer products or are produced as a result of macroplastic degradation.[1,2,4] Marine waters globally are contaminated with a mixture of MP of various shapes (e.g., spherical, angular, fibers) and compositions (e.g., polystyrene, polypropylene, nylon, low- and high-density polyethylene). A large portion of MP particles are suspended in the water column, and are available for capture and ingestion by planktonic and benthic suspension feeders. Ingested MP can produce deleterious effects under certain laboratory conditions.[5-12]

Recently, many studies have focused on the uptake of MP by suspension-feeding bivalve molluscs because they process large volumes of water per unit time, and capture particles as small as 3 μm with high efficiency (e.g., >50% depending on species[13-15]). Studies have shown that bivalves ingest MP under ambient environmental conditions,[16-21] and as such, it is assumed that these species will be one of the most impacted groups. Additionally, because bivalves are broadly distributed, abundant, easily accessible, and sessile organisms, they have been used to monitor numerous environmental contaminants worldwide (e.g., U.S. Mussel Watch; Assessment and Control of Pollution in the Mediterranean region [MEDPOL]; North East Atlantic Oslo and Paris Commission (OSPAR)).[22-25] Recently, several workers have proposed that bivalves could also be used to assess the load of MP in different environments.[18-20, 26-32] These proposals are based largely upon correlations between the types and abundance of MP in the environment and those found in the soft tissues of several bivalve species. Criteria required for taxa to be indicators of environmental impacts have been outlined previously.[25] Based upon these criteria, it is recommended that species proposed as bioindicators of MP pollution in the

environment should have the following characteristics: 1) be ubiquitous and relatively easy to collect; 2) interact significantly with the surrounding environment through particle-feeding processes; and 3) ingest, without bias, the majority of plastic particles to which it is exposed. With respect to bivalves, a large body of research demonstrates that bivalves feed selectively on a range of particles, i.e., they do not simply ingest all particles that are captured by the gills [13,15] Thus, bivalves would fail to meet the third criterion.

In this study, differently sized polystyrene microspheres and microfibers were delivered directly to the inhalant margin of the eastern oyster, *Crassostrea virginica*, and blue mussel, *Mytilus edulis*. Uptake and elimination of MP were assessed by determining the number of plastic particles rejected and egested in each size and shape category, and by examining the way in which particles were handled by the gill (*in vivo*) and eliminated at the pseudofeces-discharge site (aka, principal-discharge area[33]). These data were then used to test the following null hypotheses: 1) the number of MP particles rejected in each size class equals the number of particles ingested (spheres or fibers); 2) the proportion of MP rejected in pseudofeces and egested in < 3 h is independent of size (spheres or fibers); 3) the proportion of large MP rejected is independent of shape (1000-µm spheres vs 1075-µm fibers).

METHODS

Collection and maintenance of animals. Oysters, *Crassostrea virginica*, and mussels, *Mytilus edulis*, were collected from natural populations in Long Island Sound and cleaned of fouling organisms. A strip of Velcro® was secured to one shell of each animal using a two-part marine epoxy.[34] Bivalves were placed in lantern nets and suspended from a dock adjacent to the University of Connecticut at Avery Point. They were held in the natural environment for several days before use in the experiments. Approximately 24 h before the start of an experiment,

oysters and mussels were secured to craft sticks by means of the attached Velcro®, placed in a large holding tray filled with aerated, natural seawater (hereafter termed seawater), and transferred to an environmental chamber at 20º C under a 12 h light, 12 h dark cycle. They were fed the microalga *Tetraselmis* sp.[35] and allowed to acclimate to experimental conditions.

Preparation of plastic particles. Fluorescent polystyrene microspheres with a median diameter of 19 µm, and non-fluorescent polystyrene microspheres with median diameters of 113, 287, 510 and 1000 µm (density = 1.04 g/cm^3, Table S1) were obtained from Polysciences, Inc. and Cospheric, Inc. The diameter of each microsphere size class was verified by light microscopy. Black nylon fibers (Nylon 6.6; ~30 µm width) were obtained from A.C. Moore, Inc., and cut to median lengths of 75, 587, and 1075 µm (density = 1.14 g/cm^3; Table S1). The 75 µm fibers were cut using a cryogenic microtome following previously published methods,[36] and the 587 and 1075 µm fibers were cut by hand with a razor blade under a stereomicroscope. The polymer compositions of microspheres and microfibers were verified with Raman (Renishaw System 2000, Renishaw plc) and FTIR (Nicolet Magna 560, Thermo Fisher Scientific) microspectroscopy. Recorded spectra were compared against commercial Raman and FTIR spectral libraries (KnowItAll® Software, Bio-Rad Laboratories, Inc; Fig. S1).

Concentrated stock suspensions of each particle type were prepared in Milli-Q water. Working suspensions were prepared by diluting the stock suspensions with filtered seawater (GF/C filter, nominal pore size of 1.2 µm) and then aging the suspensions at ca. 20º C for three days.[34,37] Aging MP in seawater better mimicked conditions in the natural environment. After aging, particles were used in experiments described below.

Selection experiments. All experiments were conducted in an environmental chamber (20º C, 12 h:12 h light:dark cycle) following the general procedures used in previous experiments.[38] Oysters (5.2-7.9 cm shell height) and mussels (4.6-7.2 cm shell length) were offered MP in

round plastic containers filled with 700 mL of filtered seawater (cartridge filtered, nominal pore size = 0.2 μm; hereafter referred to as FSW). Containers were thoroughly cleaned and rinsed with deionized water prior to use. One bivalve was positioned in each container by securing the craft stick to which it was attached, to the container rim by means of a wooden clip.[34] Each container was supplied with gentle aeration and an initial concentration of microalgal food (*Tetraselmis* sp.) at 5000 cells/mL. Three different groups of oysters and mussels were used in the experiments, with each group receiving one of three MP suspensions. In Experiment 1, bivalves were offered a mixed microsphere suspension (four different sizes, median diameters of 19, 113, 287, and 510 μm); in Experiment 2, oysters and mussels were offered a mixed microfiber suspension (two different sizes, median lengths of 75 and 587 μm); and in Experiment 3, bivalves were offered a mixture of spheres and fibers (median diameter of 1000 μm and median length of 1075 μm). The number of particles in each size class offered to bivalves decreased with increasing sphere diameter or fiber length (Table S1).

Bivalves were offered MP by slowly delivering a small volume of one of the working suspensions near the inhalant aperture of an actively feeding animal using a micropipette.[39,40] Three, 200-μL aliquots were offered sequentially to each animal over 5 to 10 min during a single dosing period, with delivery of doses separated by 20 min. With each dose, bivalves were offered (nominal number) 735 microspheres (Experiment 1, all sizes), 495 microfibers (Experiment 2, all sizes), or 34 spheres and fibers (Experiment 3, both sizes). Not all MP particles offered to the bivalves entered the mantle cavity as a result of the minute and instantaneous adjustments bivalves made in the position of the inhalant mantle margin and in pumping rate. Those that were drawn into the mantle cavity were captured and represent the actual number of plastic particles to which the bivalves were exposed. In total, animals were offered six doses over a 2-h time period. After the first, third, and fifth dose, microalgal food

(*Tetraselmis* sp.) was added to each container (concentration ca. 5000 cells/mL). The total concentration of particles to which bivalves were exposed (microalgal cells, MP) was below the threshold that stimulates excessive production of pseudofeces.[41-43] During the 2-h selection experiments, bivalves were continuously monitored and visible pseudofeces produced by the animals were collected. Any bivalve that closed before receiving at least five doses of MP was not used in the final analyses.

At the end of the 2-h exposure period, bivalves were held for an additional 1 h in their original containers so that they could purge residual pseudofeces (total of 3 h after initial exposure). Microalgal food was delivered at the same intervals as during the exposure period. Bivalves were then transferred to clean, aerated containers filled with filtered seawater and microalgal food (*Tetraselmis* sp.) at a concentration of 10000 cells/mL and allowed to depurate MP. All discernable pseudofeces and feces in the original containers were identified under a stereomicroscope and collected in separate centrifuge tubes (15 mL). Importantly, identifying pseudofeces with the aid of a microscope was essential for two reasons: 1) at the low particle concentrations used, MP were often rejected as individual particles or clumps containing several particles (verified by endoscopic examination, see below) which were not visible with the unaided eye; and 2) some MP particles were not captured by the bivalves and instead settled to the bottom of the container. Therefore, to distinguish between particles rejected as pseudofeces and those that settled to the bottom before entering the mantle cavity and being captured, only particles with a mucus corona (Figure S2) were collected as pseudofeces. This approach ensured that estimates of the number of particles rejected were conservative values. Feces that were produced during the first 3 h were considered intestinal in origin, and were analyzed separately from glandular feces produced later in time[44]. After 24 h, animals were again transferred to clean containers with seawater and microalgal food, and biodeposits collected as described above.

After 48 h, bivalves were removed from the containers, and final biodeposits collected. Twice each day during the depuration period, animals were delivered a volume of microalgal food (*Tetraselmis* sp.) to bring the final concentration in the containers to ca. 10000 cells/mL. Previous studies have demonstrated that > 90% of anthropogenic particles are egested by oysters and mussels within the first 48 h post-exposure.[37,45-49] Thus, the quantity of MP found in feces is representative of the quantity of plastic particles ingested.

To release microspheres and microfibers from collected biodeposits for numeration, samples were subjected to a digestion protocol. Each sample was first centrifuged for 5 min at 1500 rcf (g). The seawater supernatant was decanted, the pellet resuspended in 5 ml of DI water, spun for another 5 minutes, and again decanted. This washing process was repeated two additional times to remove salts which react with sodium hydroxide (NaOH) to form a precipitate. After preparation, 2 mL of 1 N NaOH were added to each centrifuge tube.[34] Samples were then resuspended by means of a Vortex Genie® and allowed to digest for at least three days. After digestion, samples were diluted with 2 mL of DI water to bring the total volume of each to ca. 4.0 ml. Sub-samples (1 mL) were added to a rafter cell and the number of microspheres and fibers in each size class counted under a stereo or compound microscope (depending upon size). For the 19-µm spheres, counts were performed by means of fluorescent microscopy. Three to four replicate counts were performed for each sample. The number of particles per mL was then multiplied by the volume of sample to obtain the total number of plastic particles of each size class that were rejected or ingested. When analyzing samples of pseudofeces and feces from bivalves exposed to 75- and 587-µm fibers, tightly bound agglomerates often were observed. As there was no way to determine when the agglomerates formed (i.e., during production of biodeposits, prior to, or after treatment with NaOH), individual particles in agglomerates with five or more fibers were not counted. Instead the agglomerates were quantified. No significant

differences were found between the number of agglomerates in pseudofeces and feces produced by either oysters or mussels ($p>0.1$, paired t-test).

Data analysis. Separate tests were conducted for each species of bivalve. Two-way mixed model analysis of variance (ANOVA, GLM) for repeated measures procedures were used to compare the number of particles rejected (pseudofeces) to that ingested (total in all feces) using particle size and biodeposit type (pseudofeces, feces) as fixed effects and individual bivalves as the random effect. Separate models were run for Experiment 1 (mixed microspheres) and Experiment 2 (mixed microfibers). For microsphere data, both oyster and mussel models demonstrated a significant interaction effect between size and biodeposit type ($p < 0.001$). Therefore, each model was divided, and paired t-tests used to examine differences in the number of particles rejected versus ingested in each size class. For microfiber data, only the model for mussels showed significant treatment effects. Differences in the mean number of particles rejected versus ingested for each size class were determined using a multi-comparison test (Tukey's HSD). Paired t-tests were also used to compare the number of 1075-µm fibers and 1000-µm beads rejected and ingested by oysters and mussels (Experiment 3).

One-way mixed-model ANOVA (GLM) for repeated measures procedures were used to compare the proportion of particles rejected and proportion of particles egested in <3 h using particle size as the fixed effect and individual bivalves (oysters or mussels) as the random effect. The proportion of microplastics rejected (spheres or fibers) was calculated as number rejected ÷ total number of captured particles (number in pseudofeces, intestinal feces, glandular feces). The proportion of microplastics egested in < 3 h was calculated as number in intestinal feces ÷ total number in both intestinal and glandular feces. Separate models were run for Experiment 1 (microspheres) and Experiment 2 (microfibers). If significant differences were found, a multi-comparison test (Tukey's HSD) was used to determine differences between means. Paired t-tests

were used to compare the proportion of the largest microspheres (1000 μm) and microfibers (1075 μm) rejected in pseudofeces (same group of oysters or mussels, experiment 3). Two-sample t-tests were used to compare the proportion of 1000-μm and 510-μm spheres, and proportion of 1075-μm and 587-μm fibers rejected in pseudofeces (two different groups of oysters, or two different groups of mussels, comparison of selected data from Experiments 1, 2 and 3). Prior to analyses, data were tested for normality and homoscedasticity, and transformed (square root) if required. Statistical analyses were performed using Systat 13, and for all tests an alpha level of 0.05 was used.

Endoscopic examination. Detailed observations of the production of pseudofeces and the handling of plastic particles on the gills and labial palps of oysters and mussels were accomplished by means of video endoscopy.[50,51] The endoscope, optical adapter, and attached CCD camera (Cohu, Inc.) were mounted onto a micromanipulator to enable fine positioning around the pseudofeces-discharge site and within the mantle cavity. This site is the region of the mantle at which pseudofeces are rejected and varies with species of bivalve. For oysters, the site is located at the anteroventral region of the mantle, adjacent to the labial palps. In contrast, for mussels, the site is located at the most posterior region of the mantle, near the junction between the inhalant aperture and exhalant siphon. Digital video was recorded onto 8-mm videocassettes (Hi-8, Sony) for archival purposes. Representative video sequences were captured and saved to a computer hard drive using Movie Maker (Microsoft). Still images were captured from video segments using VideoPad Editor (NCH Software), and minor adjustments to brightness and contrast were made to improve clarity.

Oysters (7.4-11.5 cm shell height) and mussels (6.8-8.0 cm shell length) were acclimated to laboratory conditions in a 38-L aquarium filled with aerated, filtered seawater (20-22 ºC). Animals were delivered microalgal food *ad libitum*, consisting of a mixture of the microalga

247 *Tetraselmis* sp. and Shellfish Diet (Reed Mariculture), and 50% of the water in the aquarium was
248 changed daily. Prior to internal observations, a small portion of the ventral region of the shell of
249 each oyster and mussel was trimmed to accommodate the optical insertion tube (OIT) of the
250 endoscope and prevent damage to the tube when the animal adducted its valves. Shell material
251 was carefully removed without damaging the underlying mantle, and animals were allowed to
252 recover for one day before being examined.[52-54] Prior to endoscopic observation, each bivalve
253 was placed in a 1-L aerated chamber filled with filtered seawater (ca. 21 ºC), delivered several
254 mL of microalgal food (*Tetraselmis* sp.), and allowed to acclimate to experimental conditions.
255 Observations were made after the animal opened its valves and showed signs of feeding (i.e.
256 shells open, mantles extended).
257 Two different observational assays were performed. In the first, the endoscope was oriented
258 near the pseudofeces-discharge site, and the relative form and amount of pseudofeces produced
259 was assessed (individual particles, small particle clumps, large particle bolus). In the second
260 assay, the OIT was inserted between the valves of the bivalve and observations made of the
261 capture and transport of plastic particles on the gills and labial palps. As in the selection
262 experiments, mixed microspheres, mixed microfibers, and a mixture of large microspheres and
263 microfibers were offered to the bivalves. Three, 200-µL aliquots were offered sequentially to
264 each animal over 5 to 10 min during a single dosing period, with delivery of doses separated by
265 20 min. For the second assay, occasionally it was necessary to deliver near the inhalant aperture
266 more than three aliquots of MP suspension in order to observe particle capture in the small area
267 of the gill that was being examined.
268
269 **RESULTS**

Selection experiments. The number of microspheres rejected versus ingested by oysters and mussels depended upon particle size (Table 1). For oysters, a significantly lower number of 19-μm spheres was rejected in pseudofeces compared to that ingested, whereas for the larger diameter spheres (287, 510, 1000 μm), significantly higher numbers were rejected ($P<0.01$). Equal numbers of 113-μm spheres were rejected and ingested. Mussels showed a similar trend, but rejected significantly lower numbers of 19- and 113-μm spheres and rejected a significantly higher number of 1000-μm spheres compared to that ingested ($P<0.01$; Table 1). Equal numbers of 287- and 510-μm spheres were rejected and ingested. Notably, no 1000-μm spheres were ingested by either oysters or mussels. The rejection and ingestion of microfibers by the bivalves showed a different trend (Table 1). Oysters rejected and ingested equal numbers of fibers regardless of size. In contrast, mussels rejected a significantly lower number of 587- and 1075-μm fibers ($P<0.05$ and $P<0.01$, respectively), and rejected and ingested equal numbers of 75-μm fibers.

For both oysters and mussels, the proportion of microspheres rejected in pseudofeces increased with sphere size, whereas rejection of fibers was variable and showed no trend with size (Figure 1A, B). Significantly different proportions of spheres were rejected by oysters across the 19-, 113-, 287- and 510-μm size classes ($P < 0.01$). No difference was found in the proportions of 287- and 510-μm spheres rejected. Mussels also rejected significantly different proportions of spheres across the four size classes ($P<0.05$), but no differences were found between 113-μm spheres and the 19- and 287-μm spheres (Figure 1A). For both species, a significantly higher proportion of 1000-μm spheres was rejected compared to the proportion of 510-μm spheres rejected ($P<0.05$). In contrast, there was no significant difference in the proportion of 75- and 587-μm fibers, or between the proportion of 587- and 1075-μm fibers rejected by either species (Figure 1B).

Additionally, for both oysters and mussels, the proportions of ingested 510-µm spheres that were egested in < 3 h was significantly higher than those of the other three size classes ($P<0.01$; Figure 1C). No differences were found between the 19-, 113-, and 287-µm size classes for either species. The proportions of 75- and 587-µm fibers egested by oysters in < 3 h were not significantly different, nor were the proportions of egested 587- and 1075-µm fibers (Figure 1D). In contrast, although the proportions of 75- and 587-µm fibers egested by mussels in < 3 h were not significantly different, there was a significant difference in the proportions of egested 587- and 1075-µm fibers ($P<0.01$). A lower proportion of the longer fibers was egested by mussels in < 3 h (Figure 1D).

When microspheres and microfibers were delivered simultaneously, both oysters and mussels rejected a significantly higher proportion of 1000-µm diameter spheres than 1075-µm long fibers (oysters $P<0.05$, mussels $P<0.01$; Figure 2).

Endoscopic examination. Examinations *in vivo* indicated that the gills of oysters and mussels could capture and transport all sizes of microspheres and microfibers (Figure 3A, B). The heterorhabdic gills of oysters generally carried larger spheres (diameter > 19 µm) and fibers (length > 75 µm) to the ventral (aka, marginal) grooves, and smaller particles to the dorsal (aka, basal) tracts. Upon entering the grooves and tracts, MP were transported anteriorly towards the labial palps. The homorhabdic gills of mussels carried all MP to the ventral grooves. In both species, large spheres (e.g., 510 µm) rotated on the frontal surface during ciliary transport, and large fibers (587 and 1075 µm) were oriented parallel to the anterior-posterior axis before entering the ventral grooves. Examination of the pseudofeces-discharge sites on the mantle provided information on the process by which plastic particles are rejected. Oysters accumulated MP destined for rejection in mucous boluses of various sizes. Periodically, oysters adducted their valves and ejected the material from the mantle cavity which often caused the boluses to

fragment into smaller masses. Plastic particles of all sizes and shapes were rejected (Figure 3C, E), and the process of accumulation and ejection often took 20 min or longer. Mussels also rejected MP of all sizes and shapes. Generally, microspheres were ejected as singlets, doublets, or in small boluses (Figure 3D). Large fibers (587 µm) were released individually or in mucous boluses containing smaller fibers (75 µm; Figure 3F). Typically, spheres and fibers began to be rejected within 20 min of exposure. Importantly, most of the pseudofeces rejected by oysters and mussels, including the small boluses, were too small to be seen by the unaided eye.

DISCUSSION

The quantitative data provided here falsify all three null hypotheses. Oysters and mussels did not ingest all encountered MP indiscriminately. Rather, they rejected a higher proportion of large spheres and ingested a higher proportion of small spheres. Although there were no similar relationships with fibers, on average oysters rejected >50% and mussels >20% of all fibers to which they were exposed. Differences between the two species may reflect the more complex heterorhabdic gill structure of oysters, which perform bidirectional transport and particle selection.[55-58] The homorhabdic gill structure of mussels perform predominately unidirectional transport and cannot carry out particle selection.[51,59] As a result, oysters have two sites for particles selection (gills and labial palps), whereas mussels have only one (labial palps). The rejection of all microspheres with a diameter of 1000 µm by both species of bivalves demonstrates that there is an upper limit to the size of plastic particles that can be handled and ingested. In this study, the limit for ingestion was 1000 µm for low aspect-ratio particles (e.g. spheres, fragments). For particles with a high aspect ratio, such as fibers, handling and ingestion is less constrained provided that one dimension is within the size that can be ingested. Although the current study did not test the selection of MP < 19 µm in size, previous studies have

342 demonstrated that synthetic particles with a diameter of 10 μm (e.g., alumina, silica, polystyrene)
343 can be preferentially ingested or rejected based on their surface properties (i.e., surface charge,
344 wettability, organic coating) [45,60] Therefore, even plastic particles smaller than 19 μm can be
345 subjected to the selection process and potentially be rejected or ingested depending on their
346 surface characteristics. Results of the current study are congruent with those of previous research
347 that has examined the selection of plastic particles by bivalves.[46, 60-62] For example, using
348 different diameters of glass and polystyrene microspheres (10, 40, 150, 275, 370, 410 μm),
349 Tamburri and Zimmer-Faust[46] found that oysters (*C. virginica*) rejected 30-40% of the smallest
350 spheres and ca. 100% of the largest spheres, regardless of sphere type. Woods et al.[62] examined
351 the rejection and ingestion of polyethylene terephthalate fibers (ca. 460 μm in length) by mussels
352 (*M. edulis*). They found that at a concentration of 30 x 10^3 microfibers/L, mussels rejected 71%
353 of the fibers in pseudofeces, with only ca. 9% of the particles being ingested.

354 The residence time of MP within the gut of bivalves also can be affected by microsphere size,
355 with particles <500 μm being retained longer. For oysters, no relationship was found between
356 length of microfiber and proportion egested in < 3 h; however, ca. 70% of all fibers were egested
357 within this time period. For mussels, a lower proportion of the longest microfibers were egested
358 in < 3 h compared to the shorter fibers, suggesting that the gut residence time for these fibers is
359 higher. Post-ingestive selection of MP by bivalves has been demonstrated previously.[63,64] In a
360 study on the sea scallop, *Placopecten magellanicus*, Brillant and MacDonald[64] found that 20-μm
361 polystyrene spheres were retained in the gut longer than 5-μm spheres. They also reported that
362 residence time of 9-μm polystyrene spheres was longer than that of similar-sized glass spheres (8
363 μm) with a higher density. None of the spheres, however, were observed in histological sections
364 of the digestive gland, suggesting that the differential treatment of spheres occurred in the
365 stomach. Taken together, these data demonstrate that the selection of plastic particles in the gut

366 of bivalves occurs, and the time course over which MP are egested will depend on particle size
367 and shape.

368 Qualitative results from *in vivo* examinations demonstrate that MP of different sizes and
369 shapes are captured and handled by the feeding organs in the same manner as natural particles.[53,
370 65-67] Additionally, examination of the pseudofeces-discharge sites of oysters and mussels
371 provided information that has implications for previous and future studies on interactions
372 between MP and bivalves. The observations presented here demonstrate that at low
373 concentrations, bivalves can reject individual plastic particles or small particle masses that
374 cannot be seen with the unaided eye. This fact has not been appreciated by many previous
375 workers who have collected biodeposits without the aid of a microscope. By doing so, they have
376 likely underestimated the number of plastic particles rejected, because not all of the rejected
377 pseudofecal material was collected, and over-estimated the number of plastic particles that were
378 ingested, because the feces were contaminated with pseudofeces.[12,62,68] Such errors have led
379 some researchers to suggest, incorrectly, that the quantity and types of MP ingested by bivalves
380 accurately represent those suspended in the natural environment. Future studies that aim to
381 examine selection of MP by bivalves under environmentally-relevant concentrations should
382 differentiate and collect biodeposits with the aid of a microscope.

383 The presented data clearly demonstrate that MP size and shape affects the rejection, ingestion,
384 and egestion of plastic particles by oysters and mussels. These results are congruent with
385 previous laboratory studies on particle feeding in bivalves, and support the results of field studies
386 that examined uptake of MP by mussels.[21,49] For example, in a recent study Zhao et al.[21]
387 quantified the number and type of MP in mussels (*M. edulis*) and suspended marine aggregates
388 in samples collected during two different months of the year. Calculations of the number of
389 plastic particles that mussels encountered per day, based on known clearance rates and the

390 measured abundance of microplastics in aggregates, demonstrated that mussels contained only ca.
391 1% of the available MP in their digestive gland and gut. Therefore, a large portion of plastic
392 particles were likely rejected or rapidly egested in feces. Although MP abundances in marine
393 aggregates varied significantly over time, no temporal differences in the abundance of plastic
394 particles ingested by mussels were observed. These data demonstrate the consistency of particle-
395 feeding processes of mussels (e.g., capture efficiency, particle selection).

396 Results of the current study and the rich body of literature on particle-selection capabilities of
397 many bivalve species[13,15,69] clearly demonstrate that bivalves are not robust indicators of MP
398 pollution, and explain why the number of MP identified in bivalves is typically low compared to
399 that in the environment.[21, 29,49] The quantity and quality of MP identified in bivalves collected *in*
400 *situ* will not be a good proxy for the concentration and type suspended in the water, and will be
401 biased toward small, low aspect-ratio particles (e.g., spheres) and high aspect-ratio particles (e.g.,
402 fibers). If the loads of MP to which bivalves are exposed in the environment are episodic rather
403 than constant (e.g., higher concentrations after a wind-induced resuspension event), the time
404 course over which plastics of different size and shape are egested will further complicate
405 attempts to extract environmental information. It is strongly advised that other marine species be
406 explored as sentinel organisms of MP pollution.
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Table 1. Number of microspheres (A) and microfibers (B) rejected and ingested by oysters and mussels. Outcomes of statistical comparisons (paired t-test) are also shown. Note that not all particles delivered to the bivalves were actually drawn into the mantle cavity and captured as a result of minute and instantaneous adjustments in the position of the inhalant mantle margin and pumping rate. Data are means ± standard deviation in parentheses; n = 11 oysters and 8 mussels for mixed spheres (19-510 μm), 7 oysters and 8 mussels for mixed fibers (75 and 587 μm), and 8 oysters and 10 mussels for the largest spheres (1000 μm) and fibers (1075 μm); * = P<0.05, ** = P<0.01, ns = not significant.

A. Microspheres			
Species Median diameter, μm	Rejected (mean ± SD)	Ingested (mean ± SD)	Significance
Oyster			
19	171.1 (164.1)	550.1 (377.1)	**
113	402.1 (276.1)	315.5 (151.8)	ns
287	215.1 (100.9)	55.2 (35.0)	**
510	6.7 (3.3)	2.6 (3.6)	**
1000	15.4 (8.6)	0	**
Mussel			
19	143.7 (170.8)	1073.1 (319.5)	**
113	268.9 (205.3)	1065.2 (327.9)	**
287	113.4 (105.4)	198.3 (96.1)	ns
510	5.6 (3.9)	4.0 (4.1)	ns
1000	14.2 (8.4)	0	**
B. Microfibers			
Species Median length, μm	Rejected (mean ± SD)	Ingested (mean ± SD)	Significance
Oyster			
75	232.4 (79.9)	241.3 (193.6)	ns
587	156.7 (108.1)	51.4 (57.4)	ns
1075	48.8 (44.6)	26.1 (26.6)	ns
Mussel			
75	607.2 (789.1)	1302.5 (485.1)	ns
587	67.7 (50.8)	220.2 (77.3)	*
1075	23.6 (13.0)	92.7 (36.3)	**

Figures

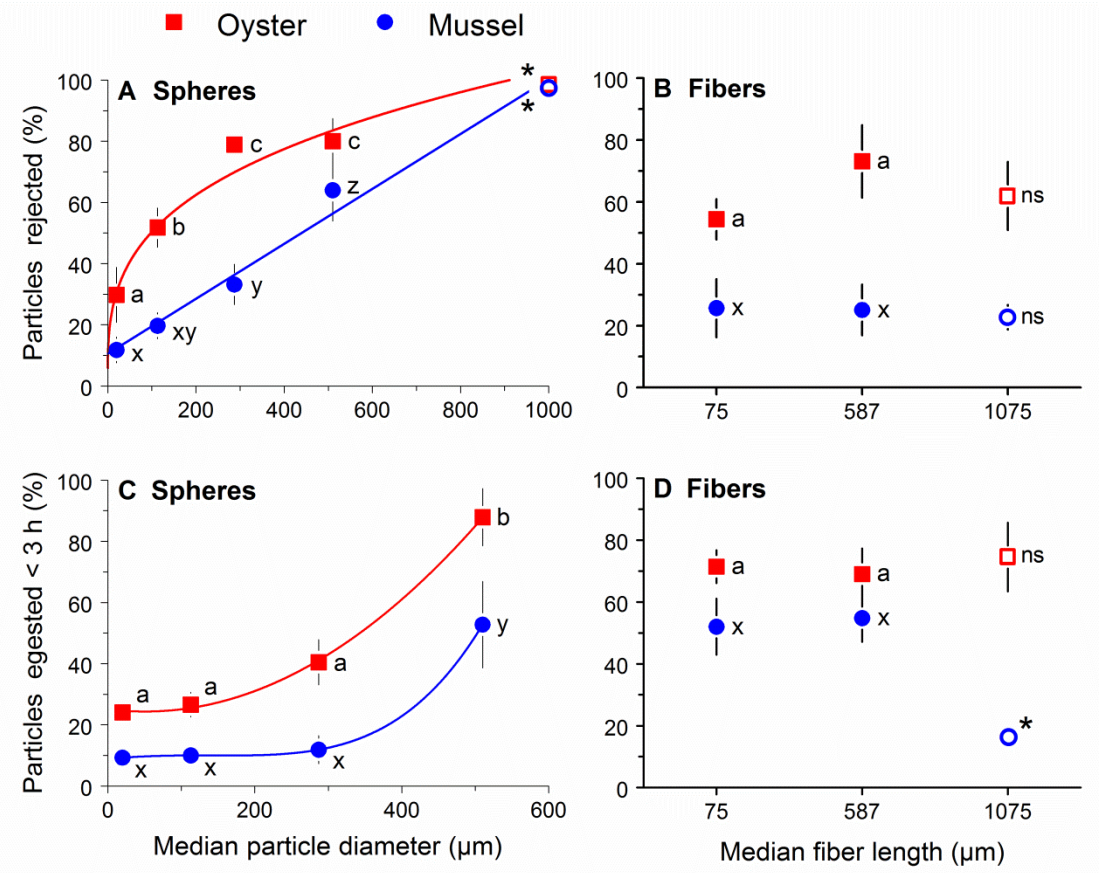

Figure 1. The proportion (%) of microspheres and microfibers rejected in pseudofeces and egested in feces in < 3 h (see text for determination of proportions). Closed symbols indicate data from bivalves that were delivered a mixture of microspheres of different diameters (A, C) or microfibers of different lengths (B, D). Open symbols indicate data from a separate group of bivalves delivered a mixture of large microspheres (1000-µm diameter) and microfibers (1075-µm length; A, B, D). For each species (oyster, mussel), means that are significantly different are designated by different letters (repeated-measures tests; P at least <0.05). Trends based on lines of best fit (regression) are provided for data that show a relationship with particle size. Asterisks and ns indicate significant and non-significant differences, respectively, between means of largest and second largest size classes (two-sample tests; P<0.05). Data are means ± standard error of the mean; n = 7-11 (oysters) and 8-10 (mussels).

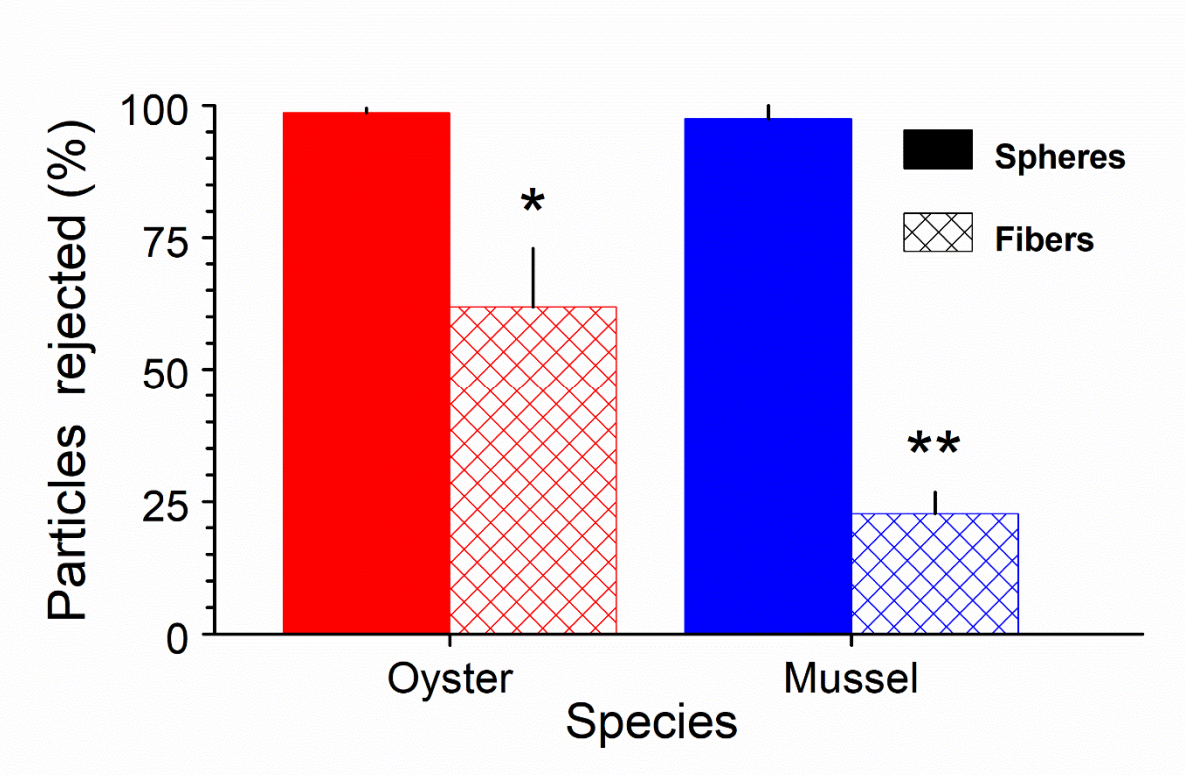

Figure 2. The proportion of large microspheres (1000-µm diameter) and microfibers (1075-µm length) rejected by oysters and mussels (see text for determination of proportions). Asterisks indicate significant differences between rejection of spheres and fibers for oysters ($P<0.05$) and mussels ($P<0.01$). Data are means ± standard error of the mean; n = 8 (oysters) and 10 (mussels).

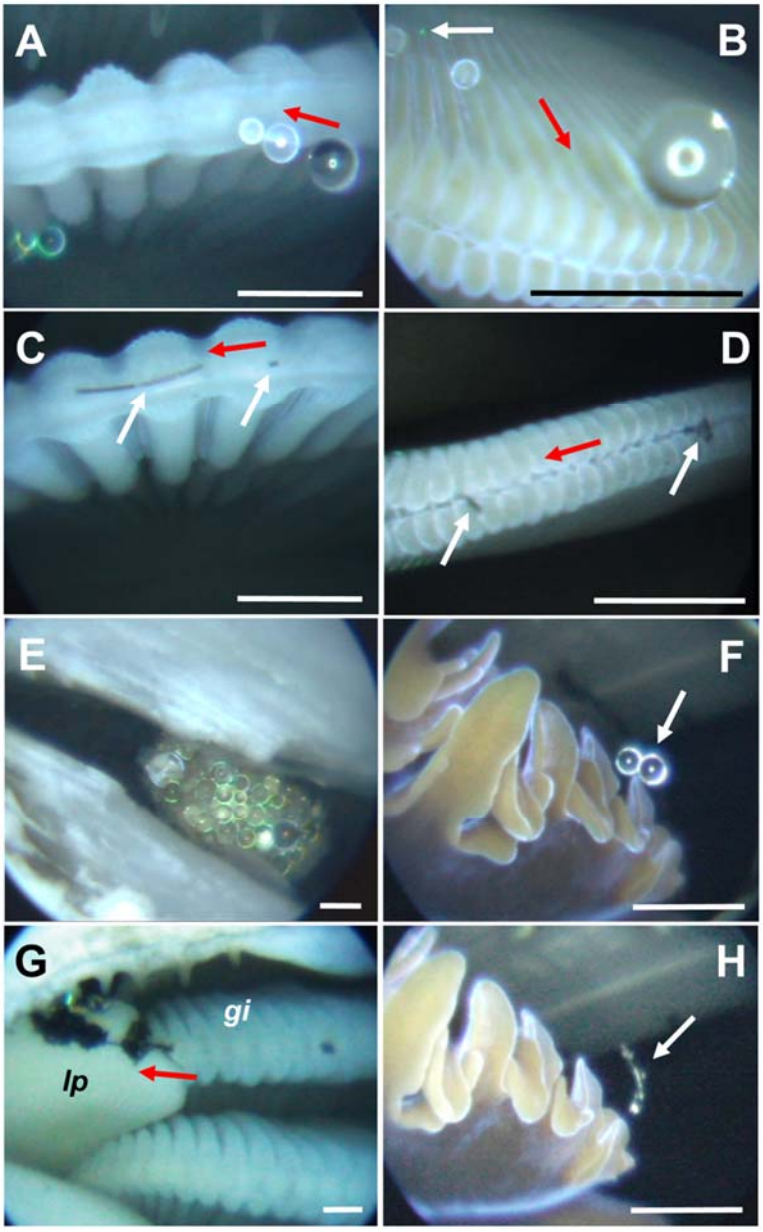

Figure 3. Endoscopic examination of feeding structures of oysters and mussels at low particle concentrations. After capture, microspheres of all sizes were transported to the margin of the gill of oysters (A) and mussels (B). Note the transport of a 19-µm sphere (white arrow in B) alongside larger spheres. Fibers of different sizes (white arrows) were also transported anteriorly in the ventral groove of the gill of oysters (C) and mussels (D). At the pseudofeces-discharge site of the oyster, spheres (E) and fibers accumulated in mucus boluses and were rejected. At the pseudofeces-discharge site of the mussel, one or two spheres (F) or fibers (H, white arrows) at a time were often rejected. Within the mantle cavity of the oyster (G), small fiber boluses were transported from the gills (*gi*) to the smooth side of the labial palps (*lp*), and then to the pseudofeces-discharge site for rejection. In many instances, the rejected microplastics could not be seen by the unaided eye. Magnification ca. 150x; Red arrows indicate direction of movement of material on the gills and palps. Scale bars ca. 500 µm for foreground images.

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Notes

The authors declare no competing financial interest.

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Supporting Information Available

Size and number of microspheres and microfibers offered to oysters and mussels in the three experimental treatments (Table S1). Raman and FTIR spectra of microspheres and microfibers showing concordance with polystyrene and nylon 66 polymers, respectively (Figure S1). Examples of microplastics in mussel pseudofeces and feces (Figure S2). Descriptions of video sequences from endoscopic observations corresponding to uploaded videos 1-7 also provided. This information is available free of charge via the Internet at http://pubs.acs.org.

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Supporting information

Selective Ingestion and Egestion of Plastic Particles by the Blue Mussel (*Mytilus edulis*) and Eastern Oyster (*Crassostrea virginica*): Implications for Using Bivalves as Bioindicators of Microplastic Pollution

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Number of Tables: 1

Number of Figure: 2

Number of Videos: 7 (descriptions below)

Table S1. Size and Number of Microspheres and Microfibers Offered to Oysters and Mussels in the Three Experimental Treatments[a]

Treatment	Size Range (μm)	Number Offered Per Dose	Total Number Offered*	Internal Exposure[t] (i.e., number captured)	
				Oyster	Mussel
Mixed spheres (median diameter, μm)					
19	18-20	405	2430	721 (467)	1217 (301)
113	106-120	225	1350	718 (344)	1334 (365)
287	200-375	90	540	270 (120)	312 (152)
510	420-600	15	90	9 (5)	10 (6)
Mixed fibers (median length, μm)					
75	50-100	440	2640	474 (211)	1910 (659)
587	375-725	55	330	208 (87)	288 (40)
Mixed fibers & spheres					
1000 μm spheres (median diameter)	975-1060	7	42	15 (9)	14 (8)
1075 μm fibers (median length)	875-1250	27	162	75 (36)	117 (36)

[a]Note that number of spheres and fibers offered per dose and total number offered should be considered nominal. Not all particles offered were drawn into the mantle cavity and captured by the bivalves so the actual internal exposure was lower. The number of particles captured in each size class is the total identified in pseudofeces and feces (intestinal and glandular). *In most cases, 6 doses were offered to each bivalve; [t]data are means ± standard deviation in parentheses (n = 7-11 oysters; 8-10 mussels).

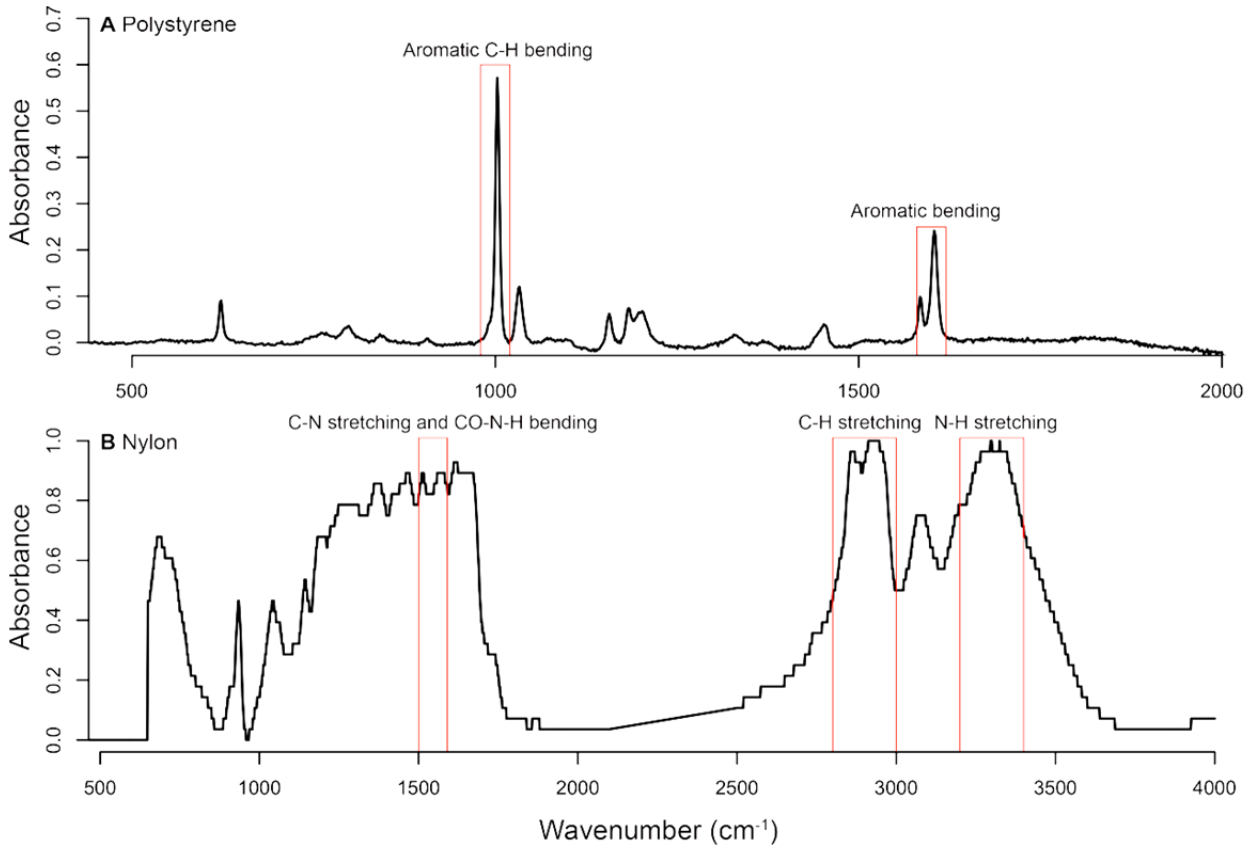

Figure S1. Raman spectrum of microspheres (A) showing concordance with reported polystyrene spectra. Peaks at 1580–1640 cm^{-1}, indicating aromatic bending, and at ca. 1000 cm^{-1}, indicating aromatic C-H-bending, are clearly present and consistent with polystyrene.[1] FTIR spectrum of microfibers (B) showing concordance with reported nylon 66 spectra. Peaks at 3317 cm^{-1}, indicating N-H stretching vibration, 2939 cm^{-1}, indicating C-H stretching vibration, and at 1554 cm^{-1}, indicating both C-N stretching and CO-N-H bending modes (amide II), are all apparent and consistent with nylon.[2]

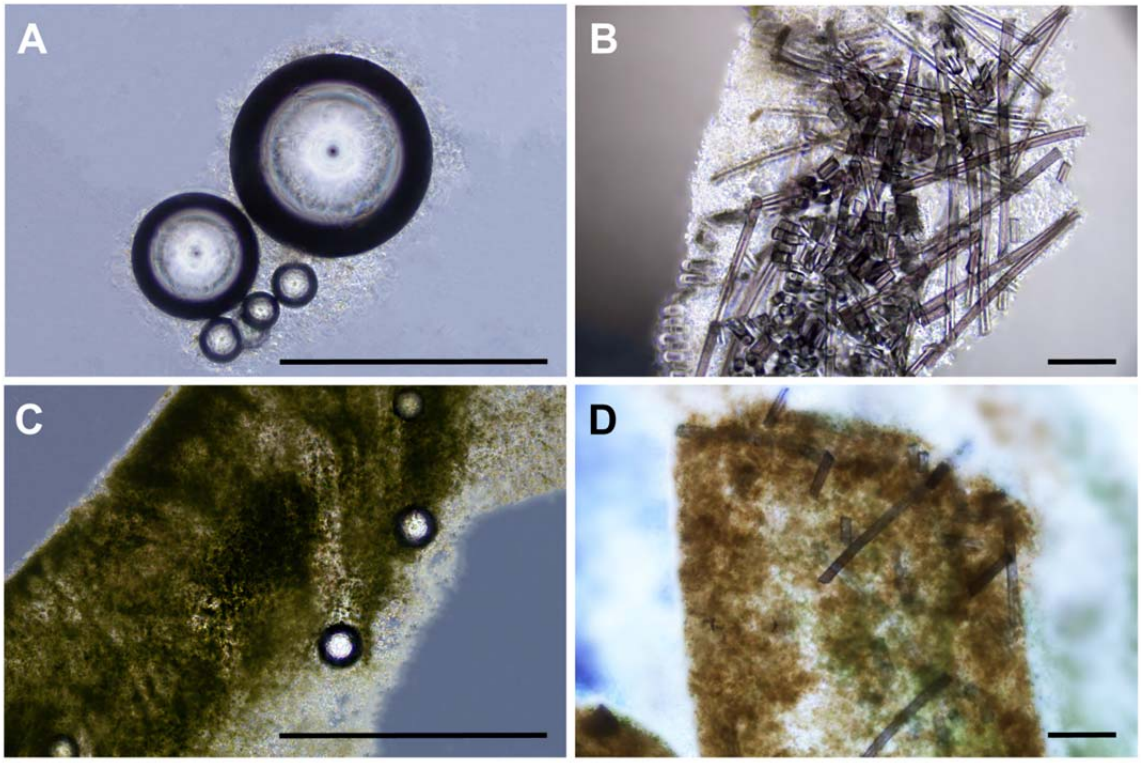

Figure S2. Examples of microplastics in the biodeposits of mussels. Microspheres and microfibers were observed in pseudofeces (A**,** B). Note the mucus halo surrounding the particles. Microspheres and microfibers also were observed in feces (C**,** D). Scale bars = 200 µm.

Video Descriptions (see uploaded video)

1) Mussel frontal-ventral fibers - (*Mytilus edulis*) In vivo, real-time video of the transport of 75 μm fibers from the frontal surface to the ventral margin of the gill. Note fibers entering the groove proper. Particles are being transported anteriorly (left) to the labial palps and mouth. Magnification ca. 150x.

2) Mussel frontal-ventral spheres - (*Mytilus edulis*) In vivo, real-time video of the transport of different size spheres from the frontal surface to the ventral margin of the gill. Note that larger spheres are transported anteriorly on the margin (more likely rejected) whereas smaller spheres (19 μm, yellow) are transported in the groove proper (more likely ingested). Particles are being transported anteriorly (left) to the labial palps and mouth. Magnification ca. 150x.

3) Mussel pseudofeces small and large spheres - (*Mytilus edulis*) Real-time video of the rejection of two large (ca. 287 μm) and small (ca. 113 μm) spheres at the pseudofeces-discharge site. This site is located at the most posterior region of the mantle, near the junction between the inhalant aperture and exhalant siphon. The rejected microplastics could not be seen by the unaided eye. Magnification ca. 150x.

4) Mussel pseudofeces small fibers - (*Mytilus edulis*) Real-time video of the rejection of a bolus of fibers (ca. 75 μm and 587 μm) at the pseudofeces-discharge site (for location, see description of video #2). The rejected bolus (ca. 250 μm wide) could not be seen by the unaided eye. Magnification ca. 150x.

5) Oyster frontal-ventral-dorsal fibers - (*Crassostrea virginica*) In vivo, real-time video of the transport of fibers (75 μm, 587 μm) from the frontal surface to the ventral margin of the gill.

Note that fibers of both sizes are transported in the groove proper. Second half of video shows fibers in the dorsal tract of the gill. Note that only small fibers appear in the dorsal tract. Particles are being transported anteriorly (left) to the labial palps and mouth. Magnification ca. 150x.

6) Oyster frontal-ventral-dorsal spheres - (*Crassostrea virginica*) In vivo, real-time video of the transport of different size spheres from the frontal surface to the ventral margin of the gill. Note that spheres of all sizes are transported in the groove proper. Second half of video shows spheres in the dorsal tract of the gill. Note that only small spheres (ca. 113 μm) appear in the dorsal tract. Particles are being transported anteriorly (left) to the labial palps and mouth. Magnification ca. 150x.

7) Oyster pseudofeces spheres - (*Crassostrea virginica*) Real-time video of the rejection of a bolus of spheres of different sizes at the pseudofeces-discharge site. This site is located at the anteroventral region of the mantle, adjacent to the labial palps. Magnification ca. 150x.

References for Supporting Information

(1) Zhao, S.; Danley, M.; Ward, J. E.; Li, D.; Mincer, T. J. An approach for extraction, characterization and quantitation of microplastic in natural marine snow using Raman microscopy. *Analytical Methods* **2017**, *9*, 1470-1478.

(2) Du, Y.; George, S. Molecular layer deposition of nylon 66 films examined using *in situ* FTIR spectroscopy. *The Journal of Physical Chemistry* C **2007**, *111*, 8509-8517.