Determining the Properties that Govern Selective Ingestion and Egestion of Microplastics
by the Blue Mussel (*Mytilus edulis*) and Eastern Oyster (*Crassostrea virginica*)

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ABSTRACT
Suspension feeding bivalve molluscs interact with different types of microplastics suspended in the water column. Most bivalves are selective suspension feeders and, thus, do not consume all particles to which they are exposed. Selection depends upon the physicochemical properties and size of the particle. Recent work has provided evidence that blue mussels, *Mytilus edulis*, and eastern oysters, *Crassostrea virginica*, ingest and egest microspheres (polystyrene) and microfibers (nylon) differently, but whether other factors, such as polymer type and shape, mediate selection have not been explored. To investigate these factors, mussels and oysters were offered similar sized nylon (Ny) and polyester (PES) microfibers or polyethylene (PE) and polystyrene (PS) microspheres, or different sized polyester microfibers during a 2-h exposure. Feces and pseudofeces were collected separately and analyzed for microplastics, and the data used to develop a linear regression model for selection. Results demonstrated clear species-specific differences in the efficiency of particle selection. Both mussels and oysters, however, exhibited size-based rejection of polyester microfibers, ingesting a higher proportion of shorter fibers than longer fibers. Polymer type did not impact selection of fibers or spheres. The relative size of particles (area, perimeter) was found to be the most important factor in predicting whether a microplastic will be rejected or ingested.

Keywords: microplastics, mollusc, bivalve, suspension feeding, selection, bioindicator

Synopsis: Bivalve molluscs selectively ingest or reject particles based on the physical properties of the particle. This study demonstrates that size and not polymer type of microplastics influence selection.
GRAPHICAL ABSTRACT
INTRODUCTION

Plastics have made major technological and medical advances possible, however, plastic debris has become a pervasive pollutant in the natural environment. Microplastics (MP, 1µm-1mm) are produced for use as primary particles in consumer products or as macroscopic plastic debris degrades. Microplastics come in a variety of polymer types, shapes, and sizes, many of which have been found in environmental samples, and are consumed by a variety of organisms.

Often, the polymers found in environmental samples align with the most commonly produced plastics worldwide (e.g., polyethylene, polyvinyl chloride, polypropylene, polystyrene, and polycarbonate). Fibers, filaments, fragments, films, foams, pellets, and spheres are the major shapes described in environmental studies, with fibers and fragments being the most common. Microplastics are a diverse collection of contaminants that can be characterized beyond size, shape, and polymer type. The dyes, additives, and associated ecotoxins (e.g., adsorbed pollutants) can also be used to describe each individual particle and contribute to the potential risk(s) posed by MP to organisms in their environment.

There has been an influx of studies in the last decade on the consumption of MP by bivalve molluscs and subsequent translocation within the viscera. As suspension feeders, bivalves can filter large volumes of water per unit time and can capture particles as small as 3 µm in diameter (> 50% efficiency depending on the species), so it is not surprising that they also ingest microplastic particles. Through their feeding activities, suspension feeders provide important ecosystem services such as nutrient cycling through the filtration of particulate nutrients from the surrounding water, excretion of inorganic metabolites, and biodeposition. Most bivalve species are sessile and inhabit near-shore environments, making them easily accessible. They have been used historically to monitor anthropogenic pollutants (e.g., metals
and petroleum hydrocarbons; U.S. Mussel Watch; Assessment and Control of Pollution in the Mediterranean region [MEDPOL] \(^{35-38}\) and are therefore being considered by some investigators as indicator species to monitor microplastic pollution. Recent work by Ward and colleagues\(^{39}\) has shown that, while bivalves meet some of the criteria for indicator species, they do not consume all particles to which they come into contact because of their particle selection capabilities.\(^{32,33,40}\) Physicochemical properties of the particles and their interactions with mucus on bivalve feeding structures influence capture and ingestion,\(^{33,41}\) thus MP of differing qualities (e.g., size, shape, polymer composition, and aspect ratio) are likely to be ingested differentially. The particle selection capabilities of bivalves, thus, would make them a poor bioindicator for MP pollution.

Little is known regarding which types of MP are consumed by bivalve molluscs. The present study was undertaken to elucidate how oysters and mussels interact with plastic microspheres and microfibers of different sizes and polymer compositions including those commonly found in the marine environment and those observed in field-collected samples (e.g., polyester, nylon-6,6, polystyrene, polyethylene). A series of exposure assays using microspheres and microfibers of different sizes and polymer types were performed with both the eastern oyster, \textit{Crassostrea virginica}, and blue mussel, \textit{Mytilus edulis}. The number of particles rejected and egested in each size and polymer category was determined to assess the uptake and elimination of MP by the bivalves. Data obtained from the selection assays were then used to construct a model to predict the likelihood that a given type of microplastic (e.g., different size, shape, polymer type) would be ingested versus rejected. The model and results are an important step in understanding the factors that govern which plastic particles will be ingested and
potentially produced harm. The model can be expanded as new data become available for a wider range of species and types of MP.

METHODS

Animal collection and maintenance

Eastern oysters, *Crassostrea virginica* (5.3-8 cm shell height), were collected from an aquaculture facility (Mystic River, Noank, Connecticut). Blue mussels, *Mytilus edulis* (4.5-7.07 cm shell length), were collected from docks at the University of Connecticut Avery Point campus (Groton, Connecticut). Velcro was attached to one shell of each bivalve with a marine epoxy, and the animals were then placed in lantern nets suspended off the docks until they were used in experiments (within a week). For an experiment, bivalves were brought to the laboratory and acclimated to the experimental conditions (20 °C, 12 h:12 h light/dark cycle, Salinity of ~30, density of ~1.021 g cm⁻³) in an aquarium filled with aerated seawater (8-12 animals in approximately 12 L of water). Twenty-four hours before an experiment each oyster and mussel was attached to a craft stick by means of Velcro. Each day during acclimation (≤ 2 days), biodeposits were removed, seawater was replaced, and animals were fed twice with a diet of cultured microalgae (*Tetraselmis* sp.) sufficient to bring the concentration in the aquarium to approximately 10,000 cells mL⁻¹.

Particle description

Plastic particles used in the experiments were chosen according to the following criteria: 1) their polymer types had been found in coastal waters of Long Island Sound and elsewhere,⁶,¹²,¹⁹,⁴² 2) their density allowed them to be mixed into suspension upon agitation, and 3) their size was within the range of consumption for both oysters and mussels.³²,⁴³ Fluorescent
Nile red polystyrene microspheres (PS; 31-µm diameter, density = 1.04 g/cm³) were purchased from Spherotech and fluorescent green polyethylene spheres (PE; 26-µm diameter, density = 1.026 g/cm³) were purchased from Cospheric. Black nylon upholstery string (Nylon-6,6, Ny, 30-µm diameter, density = 1.14 g/cm³; Coats Extra Strong ®) was purchased from A.C. Moore. Polyester thread (PES, 15-µm diameter, density = 1.38 g/cm³; Coats and Clark All Purpose ®) was purchased from Walmart. Microfibers were created by cutting the purchased thread to the desired length using a cryogenic microtome at (fibers < 500 µm),44 or by hand under a stereomicroscope using a razor blade (fibers ≥ 500 µm). Nylon microfibers were cut to median lengths of 75, 587, and 1075 µm. Polyester microfibers were cut to median lengths of 65, 500, and 970 µm.

Concentrated stock suspensions of each type of microplastic (spheres and fibers) were prepared in Type 1 laboratory water (Milli-Q Integral A10 system; MQ). Working suspensions were prepared by diluting stocks in 1.2-µm filtered seawater (GF/C filter). The working suspensions were aged at room temperature (~20 °C) for 3 days prior to being used in selection experiments. Aging allowed a biofilm to form, better mimicking particles in the natural environment.45,46 Working suspensions were used in exposure assays and extra working suspensions were counted to confirm the particle concentrations delivered to mussels and oysters.

Particle characterization

Ten particles of each microplastic type (e.g., PS, PE, nylon, polyester) were used to characterize size and shape. Particles were viewed under a microscope and measurements made by means of image-analysis software (ImageJ) to calculate shape characteristics.47 These characteristics included area, aspect ratio, circularity, convexity, convex-hull area, convex-hull
perimeter, diameter, major axis, minor axis, perimeter, roundness, and solidity (Table S1).

Aspect ratio was calculated as the minor axis divided by the major axis; the lower the aspect ratio, the more elongated the particle. Scanning electron microscopy (SEM) was used to produce high resolution images of particles (Figure S1). The spherical diameters of spheres were verified using an electronic particle counter (Coulter Multisizer IIe). Fiber diameter and length were measured using a light microscope. The polymer compositions of the micro-spheres and fibers were confirmed with attenuated total reflectance µFTIR (micro-Fourier-transform infrared spectroscopy; Nicolet™ iN10™ MX Infrared Microscope, Thermo Fisher Scientific) in combination with OMNIC software and the Polymer Kit 1.0 library (Figure S2, S3).

Wettability and zeta potential are important physicochemical properties that can influence particle selection in bivalves. Wettability was determined by measuring contact angle between a drop of MQ water and the surface of each polymer type. Contact angles of PS spheres were determined using methods described previously. Briefly, a seawater suspension of the microspheres (aged 3 days) was vacuum filtered onto polycarbonate filters (3-µm pore size) to form a uniform layer of microspheres on the filter. The filters were rinsed with ammonium formate to remove salt from aging in seawater. Control filters were prepared with filtered seawater (0.2 µm) and all filters were dried at 70°C. This method could not be used for the microfibers or polyethylene spheres because a uniform pad could not be obtained on the filters. Instead of pads, solid sheets of nylon, polyethylene terephthalate (representative of polyester), and polyethylene were used. The sheets were aged for 3 days and then rinsed with deionized water to remove salts after aging. Sheets were dried at 70°C before performing measurement. Contact angles were measured by placing a drop of MQ water (4 µL) on top of the dry pad or sheet and photographs taken immediately with a digital camera attached to a side-
mounted dissecting microscope (i.e., goniometer). The angle at the interface between the drop of MQ water and the filter or sheet was measured using ImageJ and the software plug-in Contact angle. Materials with a contact angle > 90° are classified as non-wettable (hydrophobic) and those with an angle < 90° are considered wettable (hydrophilic; Table S1). Surface charge of the MP was analyzed with a Zetasizer Nano ZS© (Malvern Instruments Inc.) based on previous methods. Seawater suspensions of each microplastic (aged 3 days) were pipetted into capillary cells and placed into the Zetasizer. Zeta potential was calculated using the Smoluchowski equation, the measured electrophoretic mobility, and known values of viscosity and dielectric constant of the suspension. Zeta potential is a representative measure for surface charge of MP used in selection assays.

Selection assays

All experiments were conducted in the environmental chamber in which the bivalves were acclimated (20°C, 12 h:12 h light/dark cycle, salinity of ~30). Bivalves were placed in individual, aerated containers with 700 mL of filtered seawater (0.2-µm cartridge filtered, salinity of 31) and microalgal food (Tetraselmis sp., 5000 cells mL⁻¹). The animals were positioned in the containers by securing the craft stick to which the bivalves were attached to the rim of the container with a wooden clip. Separate groups of oysters and mussels were used in each of three exposure experiments which examined how different attributes of the MP affected feeding (see below; Figure 1). During exposure, three 200-µL aliquots of the designated microplastic suspensions were offered to the animal over a 5- to 10-minute dosing period. Each aliquot of the working suspension was slowly delivered near the inhalant aperture using a micropipette. This delivery method allowed the particles to become entrained within the inhalant current. Bivalves were dosed 6 times over ca. 2 h with 20-minute intervals between each dosing.
An additional aliquot of microalgal food was dispensed after every other dose to bring the concentration in each container to ca. 5000 cells mL$^{-1}$ and promote active feeding. After the final dose, bivalves were delivered another aliquot of microalgal food (final concentration in container ca. 5000 cells mL$^{-1}$) of microalgal food and left in the exposure containers for an additional hour to purge residual pseudofeces (initial exposure time = 3 h). After this period, bivalves were rinsed with filtered seawater (0.2 µm) over the containers to remove any remaining biodeposits stuck to their shells. Each animal was moved to clean aerated container without MP to begin a 48-h depuration period, during which they were fed twice a day with microalgal food (final concentration in container ca. 10,000 cells mL$^{-1}$). After 24 h, each animal was again rinsed with filtered seawater (0.2 µm) over their container to remove any biodeposits stuck to their shells and moved into clean, aerated containers containing 10,000 cells mL$^{-1}$ of microalgal food for the remaining 24 h of depuration. After the 48-h depuration period animals were removed from the containers, labeled, and frozen.
Figure 1. The experimental design explained. Three experimental groups of mussels and oysters were exposed to one of three microplastic combinations: a mixture of 3 fiber sizes, 2 fiber types, or 2 sphere types. After exposure and throughout depuration, pseudofeces and feces (intestinal and glandular) were collected under a stereomicroscope and the number of particles were counted to determine how many particles were rejected (pseudofeces) or egested (intestinal and glandular feces).

Immediately after transferring bivalves from one container to another, the original containers were examined under the stereomicroscope, and all pseudofeces and feces in the containers collected (Figure 1). Biodeposit samples were collected at 3, 24, and 48 h. Collection under a microscope is important to avoid sampling of MP not incorporated in biodeposits and to differentiate pseudofeces from feces accurately. The three types of biodeposits (pseudofeces,
intestinal feces, and glandular feces) are voided on different time scales and represent important feeding endpoints. Pseudofeces are mucus-bound particles rejected by bivalves prior to ingestion. Microplastics are typically rejected within minutes to up to an hour after exposure. Microplastics that are ingested are later egested as intestinal or glandular feces. Intestinal feces are composed of material that was subjected to extracellular digestion in the stomach and is egested typically in <3 h. Glandular feces contain particles that are processed through intracellular digestion within the sacs of the digestive diverticula and are egested over 2-9 h.

Therefore, the feces collected within the initial 3 h exposure is considered intestinal, and the feces collected during depuration (24 h, 48 h) glandular. Feces were washed with MQ water, centrifuged (1500 rfc), and digested with 2 mL sodium hydroxide (NaOH; was tested prior to ensure it does not damage the MP of interest) at room temperature. Subsamples (1 mL, x 3 replicates) of each pseudofecal and fecal sample were transferred to a Rafter Chamber and the number of MP in each counted and recorded. The number of MP rejected as pseudofeces, egested as intestinal feces, or egested as glandular feces, was quantified. Fluorescent spheres were counted under the fluorescent microscope (Olympus BX51) and all the fibers were counted using light microscopy.

Each of the three experiments were designed to examine how different attributes of the MP mediated feeding processes. In the Fiber-Size experiment, animals were offered a mixed polyester fiber suspension (3 size classes, median lengths of 65, 500, and 950 µm) to test the effect of fiber length on feeding. For the Fiber-Type experiment, oysters and mussels were offered both nylon and polyester fibers (median lengths of 70 and 65 µm respectively) to test the effect of polymer type on ingestion and egestion of fibers. In the Sphere-Type experiment, animals were offered polyethylene and polystyrene microspheres (median diameters of 31 and 26
µm respectively) to test the effect of polymer type on ingestion and egestion of spheres. The first group of bivalves (Fiber-Size experiment) was offered 440 fibers of 65-µm length, 55 fibers of 500-µm length, and 30 fibers of 950-µm length per dose (Table S2). The second group (Fiber-Type experiment) was offered 440 nylon and 440 polyester fibers (both ca. 70 µm in length) per dose; and the final group (Sphere-Type experiment) was offered 405 polyethylene and 405 polystyrene microspheres (both ca. 30 µm in length) per dose (Table S2). The size of particles was chosen to be within the range that bivalves can efficiently capture and ingest, and a size that allowed accurate enumeration in biodeposits. The total concentration of particles (algae food and MP) remained below the threshold for excessive pseudofeces production.

**Data analyses**

A Welch’s ANOVA was used to compare the number of particles ingested and rejected for each polymer type and size to account for unequal variances and non-normal distribution. Separate models were run for each bivalve species and experiment. To determine the proportion of MP rejected, the number of particles rejected was divided by the total number captured (the sum of the number of particles in pseudofeces, intestinal feces, and glandular feces). The proportion of MP egested in < 3 h was calculated as the number of particles in intestinal feces divided by the number of particles in both the glandular feces and intestinal feces. The calculated proportions were used in the mixed model analysis for each set of exposures. Two-way mixed model analysis of variance (ANOVA, GLM) for repeated measures procedures were used to compare the proportion of particles rejected (pseudofeces) and the proportion of particles egested in < 3 h using particle size or type and species (oyster, mussel) as fixed effects and individual bivalves as the random effect. Data were tested for normality and homoscedasticity before
analysis and transformed (square root) if necessary. Separate models were run for each set of exposures. Statistical analyses were performed using R with an alpha level of 0.05 for all tests.

**Model development**

Data presented here and in Ward et al.\textsuperscript{39} were used to develop a linear regression model. The purpose of the model was to predict, using selection assay data and particle characteristics, the likelihood that a given type of microplastic will be ingested using selection assay data and particle characteristics. The characteristics considered (denoted in italics to emphasize these are all formal predictors in the models) were *area*, *aspect ratio*, *circularity*, *convexity*, *convex-hull area*, *convex-hull perimeter*, *diameter*, *major axis*, *minor axis*, *perimeter*, *roundness*, *solidity*, *wettability*, and *zeta potential* (Table S1). The determination of all these characteristics was described previously in this section. Discovering which physicochemical predictors are the controlling variables in the feeding experiments is challenging for two reasons. First, there is a high degree of collinearity between many of the predictors. For example, *area* and *minor axis* have a Pearson correlation of 0.97, *perimeter* and *major axis* 0.98, and *circularity* and *roundness* 0.96. Collinearity in the predictors can lead to large uncertainties around estimated regression coefficients. Secondly, there are more predictors than samples (13 predictors vs. 12 samples for each of oysters and mussels). These challenges make standard regression models unusable, thus a hybrid approach was taken.

The Elastic Net regularized regression model\textsuperscript{58} was used as a prefilter to identify important predictors and thereby reduce the dimensionality of the feature space. The Elastic Net consists of both *ridge*\textsuperscript{59} and *lasso*\textsuperscript{60} penalties on the parameters. The ridge penalty is effective at properly splitting importance among correlated predictors, as it will tend to shrink the corresponding parameter estimates (\(\beta\) values) toward one another. The lasso is much better at
shrinking parameter estimates toward zero, thus eliminating them from the problem entirely rather than just making them small. The Elastic Net is a compromise that combines the good features of both penalty terms. Because there is not yet an agreed upon method for calculating Elastic Net p-values, the important predictors identified by the Elastic Net (i.e., those with nonzero coefficients) were then used in standard linear regressions for each bivalve species. All regression models (Elastic Net and standard) used standardized predictors and were fit to (natural) log-transformed ingestion probabilities.

RESULTS

Number of Microplastics Rejected and Ingested

The number of MP rejected versus ingested by oysters and mussels was significantly different for almost all particle types used in the three experiments (Welch’s ANOVA, p <0.05; Table 1). Typically, the number of plastic particles ingested was higher than the number rejected. The exception was for oysters delivered 500-µm and 970-µm polyester fibers, which were not rejected and ingested differently (Welch’s ANOVA, p>0.05; Table 1). The length of the polyester fibers also significantly affected the number ingested (Welch’s ANOVA, p<0.05). Both species ingested a higher number of 65-µm fibers compared to the longer fibers.

Table 1. Number of MP rejected in pseudofeces (captured but not ingested) and ingested by oysters and mussels. The statistical comparisons of each polymer type for (A) polyester microfibers from Fiber-Size experiment, (B) nylon and polyester microfibers from Fiber-Type experiment, and (C) polyethylene and polystyrene microspheres from Sphere-Type experiment. The bivalves did not interact with every particle offered; some particles were not drawn into the mantle cavity as the
bivalves adjusted the mantle margin and pumping rate during the exposure. Mean +/- SD are shown; n=10 mussels and 9-10 oysters for all comparisons; ** = p <0.01, * = p <0.05, ns = not significant.

### A. Polyester fibers (Fiber-Size)

<table>
<thead>
<tr>
<th>Species</th>
<th>Median length, µm</th>
<th>Rejected mean (SD)</th>
<th>Ingested mean (SD)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>30.5 (25.4)</td>
<td>152.1 (62.7)</td>
<td>152.1 (62.7)</td>
<td>*</td>
</tr>
<tr>
<td>500</td>
<td>32.4 (31.7)</td>
<td>30.8 (20.3)</td>
<td>30.8 (20.3)</td>
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</tr>
<tr>
<td>970</td>
<td>34.0 (24.1)</td>
<td>18.4 (10.8)</td>
<td>18.4 (10.8)</td>
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<tr>
<td>Mussel</td>
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<td></td>
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</tr>
<tr>
<td>65</td>
<td>14.1 (8.9)</td>
<td>363.07 (58.2)</td>
<td>363.07 (58.2)</td>
<td>*</td>
</tr>
<tr>
<td>500</td>
<td>10.9 (7.0)</td>
<td>106.1 (125.4)</td>
<td>106.1 (125.4)</td>
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<tr>
<td>970</td>
<td>18.27 (12.5)</td>
<td>43.93 (20.1)</td>
<td>43.93 (20.1)</td>
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### B. Nylon & polyester fibers (Fiber-Type)

<table>
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<th>Species</th>
<th>Median length, µm</th>
<th>Rejected mean (SD)</th>
<th>Ingested mean (SD)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nylon (70 µm)</td>
<td>18.8 (19.4)</td>
<td>153.2 (112.0)</td>
<td>153.2 (112.0)</td>
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<tr>
<td>Polyester (65 µm)</td>
<td>9.8 (7.3)</td>
<td>115.3 (91.7)</td>
<td>115.3 (91.7)</td>
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<tr>
<td>Mussel</td>
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</tr>
<tr>
<td>Nylon (70 µm)</td>
<td>4.5 (3.81)</td>
<td>355.4 (135.1)</td>
<td>355.4 (135.1)</td>
<td>*</td>
</tr>
<tr>
<td>Polyester (65 µm)</td>
<td>5.4 (3.81)</td>
<td>426.7 (265.9)</td>
<td>426.7 (265.9)</td>
<td>*</td>
</tr>
</tbody>
</table>

### C. Polyethylene & polystyrene spheres (Sphere-Type)

<table>
<thead>
<tr>
<th>Species</th>
<th>Median length, µm</th>
<th>Rejected mean (SD)</th>
<th>Ingested mean (SD)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polystyrene (31 µm)</td>
<td>24.9 (23.8)</td>
<td>252.1 (127.7)</td>
<td>252.1 (127.7)</td>
<td>*</td>
</tr>
<tr>
<td>Polyethylene (26 µm)</td>
<td>43.3 (37.3)</td>
<td>375.9 (179.5)</td>
<td>375.9 (179.5)</td>
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<tr>
<td>Mussel</td>
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</tr>
<tr>
<td>Polystyrene (31 µm)</td>
<td>19.2 (15.6)</td>
<td>385.5 (237.3)</td>
<td>385.5 (237.3)</td>
<td>*</td>
</tr>
<tr>
<td>Polyethylene (26 µm)</td>
<td>31.7 (31.7)</td>
<td>591.6 (93.7)</td>
<td>591.6 (93.7)</td>
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</table>
Proportion of Microplastic Rejected and Ingested

When exposed to a mixture of polyester microfibers (lengths of 65-, 500-, or 950-µm; Fiber-Size), length significantly affected the proportions of fibers rejected by both mussels and oysters (mixed model ANOVA, p<<0.0001, Figure 2A). Significant differences in the proportion of microfibers rejected in pseudofeces were found between the three size classes (Tukey’s HSD, p<0.02). Both mussels and oysters rejected a higher proportion of 950-µm fibers than 500-µm fibers, and more 500-µm fibers than 65-µm fibers. Additionally, there were significant differences in the proportion of polyester fibers rejected by mussels and oysters in each size class (Tukey’s HSD, p<0.003, Figure 2A). Oysters rejected 13.86% more 65-µm fibers, 34.41% more 500-µm fibers, and 32.82% more 970-µm fibers than mussels. No differences were found in the proportion of 65-, 500-, or 950-µm polyester microfibers egested in < 3 h within or between species (mixed model ANOVA, p > 0.05, Figure 2B).

Figure 2. Proportion (%) of polyester microfibers rejected in pseudofeces (A) and egested in feces in <3 h (B) by mussels and oysters. Fibers were composed of the same polymer (polyester), but were of different size. For each species (mussel, oyster), mean values that are significantly different are designated by different letters (p at least < 0.05). Bars designated by asterisks indicate
significant differences in the means between mussels and oysters (* indicates p < 0.05). Data are means ± standard error of the mean; n = 10 for both mussels and oysters.

When exposed to nylon and polyester microfibers of the same size (Fiber-Type), there was no significant difference in the proportion of fibers rejected by either mussels or oysters (mixed model ANOVA, p > 0.05, Figure 3A). There was, however, a significant difference between the proportions of each fiber type rejected by mussels and by oysters (mixed model ANOVA, p<0.0001, Figure 3A). Oysters rejected 9.97% more nylon fibers and 7.79% more polyester microfibers than mussels. No differences were found in the proportion of nylon and polyester fibers egested in < 3 h within or between species (mixed model ANOVA, p > 0.05, Figure 3B).

Figure 3. Proportion (%) of nylon and polyester microfibers rejected in pseudofeces (A) and egested in feces in <3 h (B) by mussels and oysters. Fibers were composed of different polymers (nylon, polyester) but were of about the same size (65-70 µm). For both species (mussel, oyster), no significant differences were found between polymer type. Bars designated by asterisks.
indicate significant differences in the mean values between mussels and oysters (* indicates p < 0.05). Data are means ± standard error of the mean; n = 10 (mussels) and 9 (oysters).

When exposed to PE and PS microspheres of the same size (Sphere-Type), no significant differences were found in the proportion of spheres rejected or egested in < 3h within or between species (mixed model ANOVA, p > 0.05, Figure 4).

**Figure 4.** Proportion (%) of PE and PS microspheres rejected in pseudofeces (A) and egested in feces in <3 h (B) by mussels and oysters. Spheres were composed of different polymers (PE, PS) but were of about the same size (26-31 µm). For both species (mussel, oyster), no significant differences were found between polymer type, or between mussels and oysters. Data are means ± standard error of the mean; n = 10 for both mussels and oysters.

**Model**

**Table 2.** Regression model results for the oyster and mussel ingestion data, after parameter filtering via the Elastic Net.
Applying the ElasticNet to the oyster and mussel data resulted in only two particle variables having nonzero coefficients: area and perimeter; the other eleven variables had coefficients of zero. These predictors were the same for both species of bivalves and arose independently, i.e., a separate model was fitted to data for each bivalve. These two variables were then used in standard regression models for oysters and mussels separately. These results are summarized in Table 2. For the oyster data, both area and perimeter had negative coefficients (-0.24 and -0.29, respectively) and both were significant (p-values of 0.05 and 0.02, respectively). The adjusted R² of the model was 0.71 and the model was significant (p = 0.003).

For the mussel data, again both area and perimeter had negative coefficients (-0.26 and -0.05), but only area was significant (p = 0.0008), the model had an adjusted R² of 0.81 and was also significant (p = 0.0006). Thus, for both species, ingestion probability decreased as area and perimeter increased. No other particle characteristics – area, aspect ratio (minor/major), circularity, convexity, convex-hull area, convex-hull perimeter, diameter, major axis, minor axis, perimeter, roundness, solidity, wettability, or zeta potential - played a role in the ingestion of spheres and fibers of these size ranges. Major and minor axes did not appear in the final models because they were highly correlated with the variables area and perimeter (area and minor axis correlate at 0.97, perimeter and major axis at 0.98), further indicating that fiber and sphere shape

<table>
<thead>
<tr>
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<th>Oysters</th>
<th>Mussels</th>
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<tr>
<td><strong>Area</strong></td>
<td>-0.24* (p = 0.05)</td>
<td>-0.26* (p = 0.0008)</td>
</tr>
<tr>
<td><strong>Perimeter</strong></td>
<td>-0.29* (p = 0.02)</td>
<td>-0.05 (p &gt; 0.3)</td>
</tr>
<tr>
<td><strong>Adjusted R²</strong></td>
<td>0.71</td>
<td>0.81</td>
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<tr>
<td><strong>Model p value</strong></td>
<td>0.003</td>
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were the drivers of ingestion dynamics in these bivalves. Various other ad hoc feature reduction schemes (removing features based on correlation with other features or prior intuition) were tested and the scientific results were similar in those cases - area and/or perimeter (or other variables highly correlated with them) were the only significant variables.

**DISCUSSION**

The results presented here clearly demonstrate a definable impact of microplastic size on particle selection mechanisms in two species of bivalve molluscs. Oysters and mussels did not ingest indiscriminately all plastic particles that were captured on the gills, but rather exhibited size-based rejection of microfibers and microspheres. When delivered polyester microfibers, both species rejected a higher proportion of longer fibers compared to shorter fibers. On average, oysters rejected >45% of 500-µm fibers and >60% of 970-µm fibers, whereas mussels rejected >10% of 500-µm fibers and >25% of 970-µm fibers. Polymer type had no influence on the selective ingestion of similar sized microfibers (nylon vs polyester) or microspheres (PE vs PS). Oysters rejected a higher percentage of all particle types than did mussels and significantly more in the cases of microfibers. The differences between species are likely a result of different gill structures. Mussels have homorhabdic gills which perform mainly unidirectional transport and are not capable of particle selection. Oysters have a more complex heterorhabdic gill structure which performs bidirectional transport of particles and allows for particle selection on the gills. This means the oysters have two sites for potential particle selection (the gills and labial palps), whereas all potential particle selection in mussels happens on the labial palps.

Previous studies by the authors showed that the upper size limit for ingestion of microspheres was a diameter of 1000-µm, but particles with a low aspect ratio such as
microfibers can be ingested at 1000-µm lengths. Ingestion of microfibers is not as limited as spheres if one dimension of the fiber is within the size range of what these bivalves can ingest. The orientation of the microfiber on the gills or at the labial palps likely influences how the particle is handled and transported. For instance, the authors have previously observed 1-mm microfibers being rejected in similar proportions to 500 µm microfibers in blue mussels. The orientation of the longer fibers on the gills and at the labial palps likely played a role and allowed the mussels to ingest the longer fibers in similar proportions to the shorter fibers. The prior and present results align with those of previous studies that have examined the selection of plastic particles by bivalves. For example, Tamburri and Zimmer-Faust demonstrated that the eastern oyster rejected 30–40% of the smallest spheres (10 µm) to which they were exposed, and 100% of the largest spheres to which they were exposed (410 µm) no matter the sphere type (glass or polystyrene). In the current study, it was clear that when there was no size difference between microfibers or microspheres, no preferential ingestion was observed, regardless of polymer type. Both oysters and mussels rejected PE (26 µm) and PS (31 µm) microspheres in similar proportions, as well as the nylon (70 µm) and polyester (65 µm) fibers. While this study did not explore the selection of particles below 20 µm, previous works have shown that bivalves can selectively reject or ingest particles as small as 10 µm (e.g., alumina, silt, polystyrene) based upon particle surface properties such as charge and hydrophobicity.

Many investigators have been concerned about the residence time of MP within the viscera of bivalves because a longer residence time would allow for more chemical leaching. The residence time of the MP used in this study, and in others, indicate that most MP do not linger in the gut. Fiber length had no effect on the residence time of the ingested microfibers. Approximately 30–40% of all polyester fibers were egested by oysters in < 3 h, regardless of
length. The same was observed in mussels, which egested 20-30% of the ingested polyester fibers in < 3h regardless of length. The residence time of microfibers and microspheres within the gut of oysters and mussels was not affected by polymer type. When PE and PS microspheres (26-31 µm diameter) were delivered together, the same proportions of both types were egested in < 3 h by oysters (ca. 30%) and by mussels (ca. 18%). Interestingly, the proportion of nylon and polyester fibers egested in < 3 h by both bivalve species was lower when the microfibers were delivered together compared to the previous study when the 1075 µm microfibers were delivered separately from the 75 and 589 µm microfibers. It is possible that the two polymer types physically interacted in the gut causing a longer residence time. Alternatively, because the two sets of experiments were conducted in different years with different groups of bivalves, intrinsic factors (e.g., nutritional status, physiological state) could have been different between the two groups and caused the slightly longer gut-residence time when nylon and polyester fibers were delivered together. Brillant and MacDonald previously demonstrated post-ingestive selection in the sea scallop (*Placopecten magellanicus*) showing that density influenced residence time. Polystyrene spheres were retained in the gut longer than denser glass spheres suggesting that differing densities could affect the digestion or handling of particles within the stomach. The MP compared in this study had similar densities indicating that polymer types of similar densities would not be handled differently in the gut. Post-ingestive selection can depend upon the particle shape, size, and potentially polymers of different densities.

Model results provided insight into the factors that influence the rejection and ingestion of plastic particles by oysters and mussel. In line with the results of the selection assays, the relative size of the particles is the most important factor in predicting whether a microplastic will be rejected or ingested. Specifically, of the 13 physicochemical characteristics measured for the
plastic particles used in the selection assays – area, aspect ratio (minor/major), circularity, convexity, convex-hull area, convex-hull perimeter, diameter, major axis, minor axis, perimeter, roundness, solidity, wettability, or zeta potential – area and perimeter were the only significant predictors of selection. As the relative size of a particle increased, the probability of it being ingested decreased and the probability of it being rejected increased. Although the model developed in this study was a good first step, the robustness of the model predictions was limited by 1) the narrow range of physicochemical characteristics of the plastic particles, and 2) the low number of bivalve replicates used in each selection assay. Performing selection assays with bivalves is labor intensive and can be carried out with a statistically appropriate number of replicates but that sample size is considered low when developing a numeric model. In the future, data from studies that use other types of plastic particles, with different physicochemical characteristics, and other bivalve species could be added to the model to expand its robustness.

This study demonstrated the selection of microspheres and microfibers by oysters and mussels and indicated that size influences the rejection and ingestion of MP. A large proportion of MP were rejected, or quickly egested, in feces by both oysters and mussels, thus explaining the very low concentrations repeatedly observed in bivalves collected in the field. Microplastics have been used historically to track bivalve feeding behavior because particles can be obtained with a size and density similar to natural phytoplankton and can easily be identified in the gut and biodeposits. The results of this study demonstrate size-based selection of plastic particles by oysters and mussels with clear differences between species. A large portion of plastic particles were rejected prior to ingestion or egested in < 3 h. This work furthers the understanding of how different microplastic shapes, sizes, and polymer types are handled by bivalves, with results consistent with previous works on particle-feeding processes in
bivalves. These animals do not consume particles indiscriminately. Such consistent particle selection capabilities reinforce the contention that oysters and mussels are poor bioindicator species for MP pollution in coastal waters. Particle selection is complex and can be influenced by particle density, surface characteristics, or potentially, plastic additives. Alternative comparisons investigating particles of different characteristics are essential for determining which types of MP are ingested and possibly accumulated in bivalves.

ASSOCIATED CONTENT

Supporting information
Shape and physicochemical characteristics of each polymer and particle type used in the exposure assays (Table S1); Number and size of microfibers and microspheres offered to oysters and mussels for each experimental treatment (Table S2); SEM images of microfibers and microspheres used in the different experimental treatments (Figure S1); µFTIR spectra of fibers used and the associated library matches, confirming polymer type (Figure S2); and µFTIR spectra of spheres used and the associated library matches, confirming polymer type (Figure S3).

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