Microplastic Ingestion by a Benthic Amphipod in Different Feeding Modes

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ABSTRACT
Microplastics adversely affect organisms through physical damage, inhibition of food assimilation, and/or toxicity of chemical leachates. We investigated the influence of feeding mode on microplastic ingestion by using polystyrene microbeads (diameter: 4.1 and 20.6 μm) and the estuarine benthic amphipod Grandidierella japonica, which can switch between filter-feeding and deposit-feeding modes. When provided with sediment, amphipods burrowed and were in the filter-feeding mode; they ingested 4.1 and 20.6 μm beads in the ratio at which the two sizes were suspended in the water. Without sediment, however, the amphipods were mainly in the deposit-feeding mode and ingested more 20.6 μm beads, which tended to be deposited on the bottom, compared with 4.1 μm beads. In addition, the number of microbeads ingested by the amphipods in sediment increased as the amount of food provided (i.e., fish food TetraMin) increased, whereas no such increase was observed for the amphipods without sediment. These results indicate that the microbead ingestion was dependent on feeding mode (i.e., presence/absence of sediment), amount of food, and distribution of microbeads (i.e., sizes of microbeads). To better understand the ingestion, accumulation, and toxicity of microplastics in aquatic environments, we recommend that more attention be paid to behavioral changes in benthic organisms.

Keywords: plastic pollution, feeding type, microplastic ingestion, benthos, Grandidierella japonica

INTRODUCTION
Plastic pollution is of increasing scientific, public, and regulatory concern due to its ubiquity and persistence. Microplastics, generally defined as plastic particles less than 5 mm [1], are released from intentionally manufactured products (i.e., primary sources) and from the breakdown of larger plastic items (i.e., secondary sources) [1]. Ultimately, these particles are transferred to various aquatic environments, including surface water, marginal sea, polar water, and deep sea [2–4]. Although most virgin microplastics (e.g., polyethylene and polypropylene) are less dense than seawater, they lose buoyancy after floating in the water column likely due to biofouling [5] or are incorporated into fecal pellets [6,7], thereby sinking to the seafloor. Consequently, microplastics are accumulated in benthic environments [8,9] and ingested by benthic organisms [10,11]. Ingestion or contact with microplastics may result in adverse effects on organisms through physical damage, inhibition of food assimilation [12], and/or toxicity of chemical leachates [13,14].

The abundance of microplastics in benthic organisms varies depending on several factors, such as the size of microplastics [15,16] and the habitat, size, and trophic level of organisms [15–17]. Among these factors, the effects of feeding behavior and feeding mode (e.g., filter-feeding, deposit-feeding, or predation) are still unclear. While laboratory experiments reported that filter-feeders (or suspension-feeders) ingested more microplastics compared with deposit-feeders (or detritus-feeders) [15,18], field studies reported that filter-feeders ingested less microplastics than species with other
feeding modes [19] or that the microplastic abundance ingested was independent of feeding mode [10,11,20].

In previous studies [15,18–20], feeding modes were classified by species. As seen in polychaetes, bivalves, and amphipods, however, a single benthic species can use more than one feeding mode and switch between them according to water current and availability of suspended food particles [21]. Thus, classifying feeding modes at the species level might not be suitable for benthic organisms, and the resulting assessments about microplastic ingestion might be biased. For a better mechanistic understanding of microplastic toxicity to benthic organisms, we need to investigate the relationship between feeding behavior at the organism level and microplastic ingestion, accumulation, and toxicity.

In this study, we performed microplastic ingestion experiments using polystyrene microbeads of two different sizes (4.1 and 20.6 μm in diameter) and the estuarine epibenthic amphipod *Grandidierella japonica*. This species has been used in toxicity assessments of environmental contamination, including sediment [22–25] and urban road dust [26,27]. Furthermore, our observations indicate that this species can switch feeding modes between filter- and deposit-feeding, depending on whether it is dwelling in sediment or present on the sediment surface, which fits well with the purpose of this study. First, we performed sinking experiments of microbeads and compared the results with theoretical sinking velocity, confirming the difference in sinking velocity between microbead sizes. Then, to investigate microbead ingestion by *G. japonica* in different feeding modes, we performed 90-min ingestion experiments in the absence and presence of sediment, with different amounts of food. The results of ingestion experiments included some of the data reported in our previous study [28]. However, the previous study used microbead ingestion as an endpoint of road dust toxicity, which was conducted for a different purpose than this study.

**MATERIALS AND METHODS**

**Test organisms**

*Grandidierella japonica* was obtained from a brood stock that has been maintained since 2009 at the Department of Urban Engineering, the University of Tokyo [22,28]. The amphipods were maintained in aquaria containing river sediment and reconstituted seawater called GP2 medium [29] at 20 practical salinity units (PSUs). The river sediment was collected from the Komatsugawa tidal flats (35°41′16″N, 139°51′06″E), sieved through a stainless sieve (mesh size: 2.0 mm), and stored at 4°C until use. The amphipods in the aquaria were maintained at 25°C under a photoperiod of 16 h light/8 h dark and provided with continuous aeration and ground fish food (TetraMin).

**Microbead stock solution**

Fluorescent polystyrene spherical microbeads were purchased from Spherotech (Lake Forest, USA) in two different sizes: 2.5–4.5 μm in diameter (mean: 4.1 μm, FP-4052–2) and 18.0–24.9 μm (mean: 20.6 μm, FP-20052–5). These sizes were selected because larger polymethylmethacrylate microbeads (mean: 60.8 μm, FPMA-60062–5) were not ingested by *G. japonica* in a preliminary experiment (data not shown), and because the growth of this species might be inhibited by exposure to natural sediment with an average particle size of 3.5 μm [22]. Both 4.1 and 20.6 μm beads were labelled with yellow (excitation wavelength: ~460 nm, emission wavelength: ~480 nm) and supplied as 1.0% w/v suspensions in water containing 0.02% sodium azide.

**Microbead sinking experiments**

Microbead solution was prepared in a glass beaker (2.6 cm internal diameter) by adding the 1.0% stock suspension of both 4.1 and 20.6 μm beads to 10 mL (i.e., about 2.1 cm depth) of reconstituted seawater at 20 PSU to achieve 5 mg beads/L, which is equivalent to 1.3 × 10^8 and 1.0 × 10^6 particles/L for the 4.1 and 20.6 μm beads, respectively. To investigate whether bead sinking velocity was affected by the presence of fish food, TetraMin (< 250 μm) was also added to the solution to achieve 500 mg TetraMin/L (control: 0 mg/L). After mixing the solution with a stainless-steel spoon, the beaker was kept at room temperature. At 0.5, 1.0, 1.5, 2.0, 4.0, and 6.0 h after mixing, 5 μL of the solution was taken from 1 cm below the water surface, with six replicates for each treatment and time, and filtered with a glass fiber filter (pore size: 0.6 μm, GS-25, Advantec, Tokyo, Japan). The number of microbeads retained on the filter was counted using a fluorescent microscope (BX51, Olympus, Tokyo, Japan).

The measured sinking velocity was compared with that predicted by using Stokes’ law:

\[
v_s = \frac{D_p^2 (\rho_f - \rho) g}{18 \eta}
\]

where \(v_s\) is the settling velocity (cm/s), \(D_p\) is the microbead diameter (= 4.1 × 10^-4 or 20.6 × 10^-4 cm), \(\rho_f\) is the density of a polystyrene micro bead (= 1.05 g/cm^3), \(\rho\) is the density of seawater at 20 PSU (= 1.0236 g/cm^3), \(g\) represents the gravi-
tational acceleration (= 980 cm/s²), and η is the viscosity of seawater (= 1.0 × 10⁻² g/cm/s). Stokes’ law holds true when the Reynolds number (Re) is < 1, where

\[
Re = \frac{v \cdot D_p \cdot \rho}{\eta}
\]  
(2)

The estimated Re values for 4.1 and 20.6 μm microbeads were < 0.01, indicating that the sizes of microbeads in this study were within the applicable domain of Stokes’ law.

**Microbead ingestion experiments**

Microbead ingestion experiments were performed following the method described in our previous study [28] and the results were partially published in that report [28]. The juvenile amphipods, defined as individuals retained on a 500-μm nylon mesh after being separated from the adults with a 710-μm mesh, were retrieved from the culture aquaria. The body length of the amphipods, measured along the mid-line between the tip of the rostrum and the end of the telson, was on average 3.1 ± 0.5 mm. One juvenile was transferred to a glass beaker (2.6 cm internal diameter, the same as used in the sinking experiment) containing quartz sand (WS-10BR, HARIO, Tokyo, Japan) at > 1 cm depth (Fig. 1A; seven replicates), TetraMin (< 250 µm), and 10 mL of reconstituted seawater at 20 PSU. The particle size distribution of the quartz sand was 0.5% < 63 µm, 6.2% 63–106 µm, 42.9% 106–250 µm, and 50.4% 250–2000 µm fraction [22]. Three amounts of TetraMin (0, 5, and 20 mg per beaker) were tested to examine the effects of co-existing food on microbead ingestion. Microbeads of two different sizes (i.e., 4.1 and 20.6 µm) were added to the seawater to achieve a concentration of 25 mg/L, which is equivalent to 6.6 × 10⁸ and 5.2 × 10⁶ particles/L for 4.1 and 20.6 µm beads, respectively. The bead concentrations were determined as high as possible so that more 20.6 µm beads would be ingested by the amphipods, and were considered as not lethally toxic in the short 90-min exposure based on 10-day 50% lethal concentrations (LC50) of polyethylene particles with 10–27 µm in diameter to an amphipod *Hyalella azteca* (4.6 × 10⁷ particles/L) [6]. To investigate the effects of burrowing behavior on microbead ingestion, juvenile amphipods were exposed to microbeads in the same way except that quartz sand was not added to the test beaker (13 replicates).

During 90 min of exposure to microbeads in the sand treatment, all individuals constructed U-shaped tubes and buried themselves in the quartz sand (Fig. 1A). In our preliminary observation, 90 min was enough time for all amphipods to ingest 4.1 µm beads and was less than the time it takes for all ingested beads to be excreted (> 3 h). After the exposure period, all the tested individuals were alive and were retrieved and fixed in isopropanol. The microbeads attached to the body surface were removed by gently washing the amphipods with 5% Tween 80 and Milli-Q water, with the effectiveness of washing checked using a fluorescence microscope. Not all of the microbeads on the body surface

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**Fig. 1** Images of fluorescent microbead ingestion experiments. A) *Grandidierella japonica* burrowing in the U-shaped tube in quartz sand in a glass vial with an outer diameter of 3.5 cm. Red-colored powder is ground fish food, TetraMin. B) Fluorescent micrograph of *G. japonica* that ingested 4.1 and 20.6 µm microbeads.
could be washed away, but there were clearly fewer microbeads retained on the body surface than inside the body (Fig. 1B). Each washed amphipod was homogenized in 5 mL of Milli-Q water by ultrasonication (Sonifier 450, Branson, St. Louis, MO, USA). A portion (1–2 mL) of resulting liquid was then filtered with a glass fiber filter (0.6-μm pore size), and the number of microbeads retained on the filter was counted using a fluorescent microscope (BX51, Olympus). This counting process was repeated at least twice for the same amphipod individual, and the arithmetic mean was used for subsequent analysis. The coefficients of variation of number of counted beads for the identical amphipod homogenate were 2 ± 2% (mean ± SD) for 4.1 μm beads and 13 ± 11% for 20.6 μm beads, indicating high precision of the counting procedure.

Data analysis

All data were analyzed using R software (ver. 4.1.3) and visualized with the R package “ggplot2” (ver. 3.3.5) [30]. The R codes and datasets are available at https://github.com/kyohiki/mp_g_japonica.

In microbead sinking experiments, to assess the number of microbeads suspended in water over time we used a generalized linear model with a Poisson error distribution and a log link function. To test whether microbead sinking was affected by the presence of TetraMin, we compared the fit of the models with and without the categorical term of TetraMin using Akaike’s information criterion (AIC).

Due to non-normality of the ingestion data, the non-parametric Kruskal–Wallis test was first applied to examine if there were differences in the number of ingested beads between food amounts, using the “kruskal.test” function. In the post hoc analyses, a Steel–Dwass test was applied using the “pSDCFlig” function in the R package “NSM3” (ver. 1.16) [31]. The non-parametric Wilcoxon test was also applied to test differences between sand treatments, using the “wilcox.test” function.

RESULTS AND DISCUSSION

Sinking velocity of microbeads

In sinking experiments, the number of 4.1 and 20.6 μm beads retrieved from 1 cm below the water surface was close to the nominal value (i.e., 130 particles and 1 particle per 5 µL) and decreased over time (Fig. 2). The number of retrieved microbeads was not different between TetraMin treatments, as indicated by the small differences in AIC values (i.e., ΔAIC < 1) between models with and without TetraMin terms. Also, microscopic observation indicated that no microbeads were attached to TetraMin flakes. The regression lines (Nagelkerke’s pseudo R-squared: 0.74 and 0.43 for 4.1 and 20.6 μm, respectively) indicated that the time to reach half the initial bead concentration was 11 and 3 h for 4.1 and 20.6 μm, respectively. This estimation was roughly consistent with the theoretical time (11.5 and 0.5 h) for 4.1 and 20.6 μm beads to sink 1 cm as predicted by Stokes’ law. Both theoretical and experimental estimations showed faster settling of 20.6 μm beads compared with 4.1

![Fig. 2](image-url)
μm beads, which indicates that more 20.6 μm beads were available on the sediment surface or on the bottom of a test beaker in 90-min microbead ingestion experiments (see the next section).

Microbead ingestion in different food and sediment conditions

All tested G. japonica individuals ingested 4.1 μm polystyrene beads (median: 1235 particles per amphipod, range: 33–10,505). The number of 4.1 μm beads ingested by G. japonica in the presence of quartz sand increased as the amount of TetraMin increased (Kruskal–Wallis test, $p = 0.039$), while there was no difference among TetraMin treatments without quartz sand (Fig. 3). Also, when TetraMin was not added, there was no difference in the number of 4.1 μm beads between quartz sand treatments (Wilcoxon rank sum test, $p = 0.54$). The number of 4.1 μm beads ingested by G. japonica was comparable with those of 1 and 10 μm beads by water flea Daphnia magna exposed at the bead concentrations of $3.0 \times 10^5$ particles/L but higher than those by an amphipod Gammarus pulex and a midge larva Chironomus riparius at the same concentrations [15].

These results can be explained by the ability of G. japonica to switch between filter- and deposit-feeding modes. When an individual built a U-shaped tube and dwelled inside the tube (Fig. 1A), the amphipod actively irrigated overlying water and captured the suspended particles that were introduced into the tube (i.e., filter-feeding). In contrast, when G. japonica was outside the tube and on the sediment surface, it ingested food particles by picking them up with its gnathopods (i.e., deposit-feeding) as well as by filtering. Since G. japonica was in the filter-feeding mode in the presence of quartz sand, increasing the amount of food would enhance irrigating activity and the resulting microbead ingestion. Without quartz sand, the amphipod was mainly in the deposit-feeding mode and could selectively ingest TetraMin on the bottom of the beaker, not microbeads, thereby leading to the absence of TetraMin addition effects.

The number of ingested 20.6 μm beads was fewer than that of 4.1 μm beads, with median of 6 particles per amphipod (range: 0–423). Twenty-six percent of tested individuals did not ingest 20.6 μm beads. The number of 20.6 μm beads ingested was not significantly different between food amounts, whether with or without quartz sand (Fig. 3, Kruskal–Wallis test, $p > 0.05$), although there was an increasing trend with food amounts when quartz sand was added (median: 3, 40, 21 particles per amphipod for 0, 5, 20 mg TetraMin, respectively). The number of 20.6 μm beads ingested by G.

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**Fig. 3** Number of 4.1 and 20.6 μm beads ingested by the amphipod Granddidierella japonica in conditions with different food amounts and sediment (yellow circles: with sediment, blue triangles: without sediment). Bars represent 25, 50, and 75 percentiles of the number of ingested microbeads. An asterisk indicates a statistically significant difference from the no-feeding condition (Steel–Dwass test, $p < 0.05$). The data of 4.1 μm beads with sediment were reported in supplementary materials of our previous report [28].
*G. japonica* was comparable with that of 10–27 μm beads by the amphipod *H. azteca* at the bead concentrations from $1.0 \times 10^5$ to $1.0 \times 10^8$ particles/L [6].

When the amphipods burrowed into sand, they ingested more 4.1 μm beads than 20.6 μm beads by a factor of 126 ± 16 (mean ± SD, based on linear regression), which was comparable to the theoretical ratio (i.e., $130 = 20.6^{3/4.1^3}$) of the number of beads suspended in water at the same weight-basis concentration (Fig. 4). This corroborates the passive ingestion of *G. japonica* in the filter-feeding mode. The amphipods ingested more 20.6 μm beads in the absence of sand compared with the sand treatment (Fig. 4), likely because they ingested particles through deposit-feeding as well as filter-feeding and more 20.6 μm beads were deposited on the bottom of the glass beaker compared with 4.1 μm beads. The ratio of 4.1 to 20.6 μm beads ingested for most individuals fell between 130 and 5.0 ($= 130 \times 4.1^2/20.6^2$; the theoretical ratio of the number of beads settled on the bottom), which supports the idea of the amphipods using both feeding modes in the absence of sediment.

**Implications for microplastic effect research**

The influence of feeding mode on microplastic ingestion by organisms is still being debated [15,18,19]. This study using *G. japonica* as the model benthic species showed that such influences were dependent on the distribution of microbeads (i.e., suspended in water or deposited on the sediment surface) and amount of co-existing food. The amphipods in filter-feeding mode ingested more suspended microbeads than those in the deposit-feeding mode, but this difference was only apparent when fish food (i.e., TetraMin) was added and thus enhanced the irrigation activity by the amphipods burrowing in sediment. In contrast, the amphipods in the deposit-feeding mode ingested more deposited microbeads than those in the filter-feeding mode, as expected. Switching feeding modes by a single benthic species, as illustrated here, has ecological relevance and should be considered in microplastic effects studies, which have been criticized for lacking ecological and environmental relevance, including the use of extremely high microplastic concentrations, short exposure duration, and lack of microplastic aging [12].

The experimental approach used in this study can be applied to not only microplastic ingestion research but also the study of accumulation kinetics in benthic organisms. Such investigations may be more toxicologically relevant, because some plastics are egested slower and such microplastics retained in digestive tracts may induce higher toxic effects [6]. The residence time in digestive tracts appears to be longer for smaller microplastics [32], suggesting the size-specific ingestion capacity found in this study should be investigated in further accumulation and toxicity research.

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**Fig. 4** Relationship between the number of 4.1 and 20.6 μm beads ingested by the amphipod *Grandidierella japonica*. Different colors and shapes represent different sediment and food (TetraMin) conditions, respectively. Solid and dotted lines indicate theoretical ratios of 4.1 to 20.6 μm beads deposited on the bottom and suspended in water, respectively.
CONCLUSIONS

To examine the effects of feeding modes on microplastic ingestion by benthic organisms, we investigated the ingestion of polystyrene spherical microbeads by the estuarine amphipod *G. japonica* in several different conditions: with and without sediment and with different amounts of food. We found that the number of ingested microbeads varied depending on feeding mode (i.e., presence of sediment), amount of food, and distribution of microbeads (i.e., sizes of microbeads). To better understand the ingestion, accumulation, and toxicity of microplastics, we recommend that more attention be paid to behavioral changes in benthic organisms.

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REFERENCES


