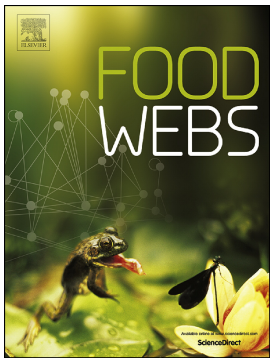

Size matters more than shape: Ingestion of primary and secondary microplastics by small predators

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PII: S2352-2496(18)30032-6
DOI: doi:10.1016/j.fooweb.2018.e00097
Article Number: e00097
Reference: FOOWEB 97

To appear in: *Food Webs*

Received date: 23 May 2018
Accepted date: 22 August 2018

Please cite this article as: Maiju Lehtiniemi, Samuel Hartikainen, Pinja Näkki, Jonna Engström-Öst, Arto Koistinen, Outi Setälä , Size matters more than shape: Ingestion of primary and secondary microplastics by small predators. Fooweb (2018), doi:10.1016/j.fooweb.2018.e00097

Size matters more than shape: Ingestion of primary and secondary microplastics by small predators

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Abstract

Experimental studies have shown how microplastics are taken up by various aquatic organisms. Most of these studies have been carried out with small (<100µm) symmetrically shaped primary microplastics (beads) which are not readily found in marine environment, and also in unnaturally high microplastic concentrations. We conducted experiments to study the ingestion of microplastics in more natural settings. We offered secondary microplastics to common planktivores, fish and mysid shrimps in their prey size categories to observe the uptake of such asymmetrically shaped fragments (PET >200 µm and ABS >100 µm) in comparison to primary microplastic beads (90 µm). Our results show that fragments of secondary plastics may end up in the food web but only in small amounts, and that the size of the fragments more than their shape is a crucial nominator influencing the numbers of plastics ingested. Future research aiming to resolve the effects of microplastics in the ecosystems should concentrate on environmentally relevant plastics and concentrations.

Key words: *secondary microplastics; feeding; entanglement; invertebrate; mysid shrimp; fish; Baltic Sea*

1. Introduction

Global plastic production has been on a constant rise since 1950's and exceeded 335 million tonnes in 2016 (PlasticsEurope 2017). Along with the increasing manufacturing and use of plastic items, plastics are accumulating in the oceans, comprising by far the largest proportion of marine debris materials worldwide (UNEP 2016). While the harm of visible litter has been acknowledged already since the 1980's (Laist 1987), interest towards the smallest fractions (micro- and nanosized particles) of plastic pollution is more recent and has constantly been growing both among scientists and the society (GESAMP 2016). Microplastics (<5 mm synthetic polymer particles, hereafter referred to as MPs) form an important component of micro-sized litter and are widespread in marine ecosystems reaching from the Arctic to the tropics (Obbard et al. 2014, Ivar do Sul et al. 2014), and from the sea surface to deep ocean floors (Moore et al. 2001, Van Cauwenberghe et al. 2013, Woodall et al. 2014, Setälä et al. 2016a). MPs are classified primary when originally manufactured to small size and secondary if fragmented from larger plastic items (GESAMP 2016). Due to the ongoing weathering of plastics in the marine environment, there is an enormous variety in size, shape, color and polymer type among the secondary marine MPs, since they can originate from the breakdown of any larger plastic item.

Due to the small size and ubiquitous distribution of MPs in the marine environment, they are available to various organisms in both pelagic and benthic habitats (Foekema et al. 2013, Mathalon & Hill 2014). Numerous studies have documented the uptake of primary MPs by zooplankton (Cole et al. 2013, Setälä et al. 2014), bivalves and other macro-sized invertebrates (Browne et al. 2008, Graham & Thompson 2009, Setälä et al. 2016b, Gray & Weinstein 2017), as well as fish (Rochman et al. 2013, Batel et al. 2016) in laboratory settings. Such experimental exposure studies have also shed light on the impacts of ingested MPs on various organisms suggesting that they can cause both physical and chemical harm (von Moos et al. 2012, Rochman et al. 2013, Au et al. 2015), and even acute mortality (Gray & Weinstein 2017).

However, the results of experimental laboratory studies should not be directly applied to natural conditions. When conducting environmentally relevant experimental research on the effects of MP ingestion to marine organisms and food webs, there are four primary variables that should be addressed: the concentration, size, shape and polymer type of the particles. There seems to be a mismatch between the MPs present in the environment and MPs used in laboratory experiments since most experiments have been run with MP concentrations higher than those commonly found in the environment (Phuong et al. 2016). Most studies have also used virgin particles of uniform size and shape that fail to accurately represent the conditions in the field. This inconsistency is likely to influence our understanding of the marine MP problem.

To fully address the potential effects of MPs in the marine environment, environmentally relevant concentrations, size, shape and type of the particles should be met. With our experimental setup we firstly aim to monitor the ingestion of secondary MPs of different polymer materials, sizes and shapes, and secondly, to observe other mechanical harm (entanglement) of MPs to the animals. We demonstrate that secondary MPs within the feeding range of the experimental animals, common small predatory species in the Baltic Sea, do not represent a significant proportion of their diet even when the animals are exposed to elevated MP concentrations. The outcome of this study is discussed in a wider perspective taking into account the present knowledge gaps that relate to the concentration and distribution of MPs in different marine environments with emphasis on the Baltic Sea.

2. Material and Methods

To study the ingestion of and entanglement to secondary MPs, small-scale mesocosm experiments were carried out in June 2016 and 2017. Two sets of experiments were performed. First trials were conducted with littoral mysids (*Praunus* sp.) and three-spined sticklebacks (*Gasterosteus aculeatus*) to study the potential ingestion of small fragments of used ketchup bottles made of polyethylene terephthalate (PET). Further experiments were conducted with pelagic mysid shrimps (*Mysis relicta*) to compare if the ingestion of secondary fragments made of polyethylene terephthalate (PET) bottles and toy bricks made

of acrylonitrile butadiene styrene (ABS) differ from the ingestion of primary microplastic beads made of polystyrene (PS).

2.1 Secondary plastic fragments

Two types of secondary plastic fragments made of PET were produced from red used ketchup bottles and green used soft drink bottles. The targeted size range of the fragments (200–500 µm) corresponds well to the size of local mesozooplankton, which are fed upon by higher trophic level invertebrates and fish of the northern Baltic Sea (e.g. Viherluoto & Viitasalo 2001a). Both types of PET bottles were cleaned with tap water, rinsed with Milli-Q water and grinded. Although the post-consumer PET is already aged during use, the PET was further weathered with mild heating (30–40 °C) and hydrated with Milli-Q water during the grinding process (1–1.5 h) to mimic their weathering state in the environment. The material was sieved to the size fraction of 200–500 µm, although later inspection of the produced fractions revealed that there were also longer particles, up to 1500 µm that went through the sieves. To estimate the number of particles for the experimental additions, the mass corresponding to approximately 1000 particles was estimated by weighing 20 replicates with a known number of particles. The average estimated mass for the 1000 PET particles was 11.21 mg (± 0.03) and 12.15 (± 0.02), respectively for red and green particles (Mettler Toledo MX5 scale; d = 1 µg). For each mesocosm experiment, adjusted weight of MPs was added to correspond to concentration of 250 particles L^{-1}.

The third type of secondary MPs was produced by grinding toy bricks with a metallic kitchen grater. The produced irregular orange ABS fragments were gently washed with tap water and sieved to obtain a size fraction of 100–200 µm, although later inspection of the produced fragments revealed that also with ABS there were longer particles, up to 1000 µm that went through the sieves. The MPs were stored in a glass bottle with MQ water. The concentration of MPs in the solution was determined by counting the fragments in five subsamples with a total volume of 50 µL (Leica MZ7.5, 0.63–5.0× magnification).

2.2 Collection of study animals and experimental setup

The animals used in the study were common macrozooplankton (mysid shrimps) and fish (three-spined stickleback) species of the study region. For the first set of trials littoral mysids and sticklebacks were collected in the vicinity of Tvärminne Zoological Station (University of Helsinki, Finland) (59º 49' N, 23º 17' E), western Gulf of Finland, northern Baltic Sea one day before the start of the experiment. Mysid shrimps (*Praunus* spp.) were collected with hand-nets, and three-spined sticklebacks (*Gasterosteus aculeatus*) were caught using a beach seine. Zooplankton prey was collected with a 100 µm plankton net with a cod end. For the second set of trials mysids (*Mysis relicta*) and their zooplankton prey were collected from the Bothnian Sea, northern Baltic Sea (station SR5, 61°05.00', 19°34.78', depth 126 m) with a 100 µm zooplankton net with a closed cod end with net hauls from the bottom to the surface. After capture, all animals were transferred to a temperature controlled room, and allowed to acclimate overnight to experimental conditions mimicking *in situ* conditions (for the first set littoral animals: 15℃, 16:8h light:dark regime, gentle aeration, ambient zooplankton community, for the second set pelagic mysids: 5℃, darkness, gentle aeration, ambient zooplankton community).

The first set of experiments was carried out in 4 L aquaria in the same climate controlled facility. In total 36 aquaria were prepared, consisting of 18 experimental units and 18 control units (Table 1). Before the addition of the study organisms, all aquaria were filled with 2 L of <10 µm filtered seawater. All units received the same concentration of mesozooplankton community (final concentration: 50 adult copepods L^{-1}). After 1 h of acclimation, the experiment was started by adding one type of secondary plastic fragments (ketchup bottle, PET) to the experimental units. For each treatment unit, approximately 1000 plastic particles was added, thus providing an average concentration of 250 particles L^{-1}. Although, it should be noted that the actual experimental MP concentrations in the aquaria were slightly lower as some of the particles remained floating due to the surface tension.

For the second set of trials three different types of plastic particles of different size and shape were used: green secondary post-consumer PET (200–1500 µm), secondary orange ABS fragments (>100µm), and primary microplastics; fluorescent PS microbeads (90 µm) purchased from the manufacturer (Polysciences). Four experimental treatment waters were prepared mixing particles with filtered (0.8 µm)

seawater: one for each particle type and one mixture containing all particle types. The total plastic particle concentration in each treatment was adjusted to a concentration of 250 particles L^{-1} as in the first experiment. Four replicates of each treatment were prepared and four controls. Experimental glass bottles (volume 1.17 L) were filled to 1 L volume, and a mixture of the on-site collected zooplankton (final concentration 25 ind. L^{-1}) and ambient phytoplankton community (dominated by centric diatoms and dinoflagellates) was added as prey for the mysids. One mysid shrimp was gently added to each bottle, which were after that filled with filtered seawater and attached to a plankton wheel (0.5 rpm) to keep the plastic particles suspended in water.

2.3 Sample processing and microscopy

After the incubation (1.5 h for fish and 3 h for mysids), animals were collected from the experimental units. The fish were immediately terminated by decapitation, and all specimens were measured to the nearest mm under a stereomicroscope and carefully checked for visible plastic fragments on their surfaces (Wild M4, 5–20× magnification). If fragments were observed, they were removed with tweezers. Mysids and three-spined sticklebacks were dissected under a stereomicroscope on clean petri dishes, their digestive tracts and stomachs removed and opened under a stereomicroscope (Leica M125, 8–100× magnification). The ingestion of plastic particles was visually verified from the dissected mysid guts and stomachs (Viherluoto et al. 2000, Setälä et al. 2014, 2016b). Red and green PET and orange ABS particles were clearly visible and counted under the stereomicroscope, whereas the fluorescent microbeads were counted by using an inverted epifluorescence microscope (Leica DMIRB, 100–200x). A melting test was done for red PET particles found from the fish stomachs to verify that the particle was plastic, simply by touching the particle with a hot needle.

2.4 Statistical analyses

A Linear Mixed-effects Model (LMM), fitted by REML (Restricted Maximum Likelihood) estimation, was used to test differences in the number of plastic fragments between the predator units (mysid or three-spined stickleback), and treatment vs. control (presence vs absence of plastic). Predator and treatment were used as fixed effects. To test for differences in ingestion between different MPs (green soft drink

bottle, toy brick, microbead, mixture and control), and to compare ingestion rates between two experiments (ketchup bottles vs. others, please see above) with each other, we applied univariate ANOVAs using logged data ($\log_{10}$ x+1), followed by pairwise comparisons. To check for differences in particle entanglement (in appendages), we used a non-parametric Kruskal-Wallis ANOVA. All data were checked for normality and heterogeneity of variances. The analyses were performed using SPSS 21.0.

3. Results

All animals were in good condition at the end of the experiments. All mysids and fish did feed on the offered zooplankton during the experiment, as prey were visible in their stomachs. Experimental MPs were found inside animals of all tested taxa (22 % of the sticklebacks and 23 % of mysids). The secondary plastics most commonly present in the stomachs were orange >100 µm ABS fragments: they were found in 75% of the mysids when offered as the only MPs and in all mysid individuals when provided in mixture with green PET fragments and fluorescent PS beads. The average numbers of ingested ABS particles in these treatments were 5.5 ± 6.6 and 3.25 ± 0.6 pieces per individual, respectively (Fig. 1). Also red PET fragments from the ketchup bottle (>200 µm) were ingested by 22% of the sticklebacks and 8% of the mysids, and the PS beads were ingested by 50 % of the mysids. There was a significantly higher number of the ketchup bottle fragments inside mysids and sticklebacks (Linear Mixed Model: $F_{1,32}$=.507 p=0.042) as well as other tested materials in pelagic mysids, compared with the control (ANOVA: F_4=7.161, p=0.002), but there was no significant difference in the number of ingested MPs between the predators (p>0.05). Significantly more MPs were ingested in the treatment where three types of MPs were offered to mysids with the ambient zooplankton community compared with the other treatments with single MP types (Tamhane Post Hoc test: p=0.013). The highest numbers of ingested MP fragments were found from mysids; 15 ABS toy brick fragments were found inside the digestive tract of one mysid and six PET ketchup bottle particles inside another individual. 8% of mysids had also ingested some fibers, although these were not intentionally offered in the experiments. No soft drink bottle fragments (200-1500 µm) were eaten.

Plastic fragments were entangled to the swimming appendages of six mysids. The highest number of entangled fragments per individual was 3 pieces of green PET fragments. Significantly more of the green PET fragments (size on average 400-1000 µm) were found entangled to mysids compared to other fragment types (Kruskall-Wallis test: p=0.032). None of the beads (90 µm) were observed attached to the swimming appendages.

4. Discussion

Conducting environmentally relevant experimental work on ingestion and effects of MPs is challenging (Phuong et al. 2016). Evidence of microplastic ingestion by marine organisms comes mostly from simplified laboratory experiments which cannot be directly applied to natural conditions. At the moment there is still a mismatch between "reality" and laboratory experiments. So far most experiments are run with microplastic concentrations higher than those commonly found in the environment, and with virgin particles of uniform size and shape that do not represent the actual environmental conditions. This inconsistency is likely to affect our interpretations of the study results as there are indications of secondary microplastics causing more negative effects on e.g. zooplankton feeding compared to primary microplastics (Ogonowski et al. 2016). Our study was able to meet three of the four primary requirements we had set for environmentally relevant experiments: size, shape, and polymer type of the used MPs. Firstly, we used MPs of various size within the range of 90–1500 µm, which are in the the prey size category of our study organisms. Secondly, the shape of these particles was irregular, as secondary fragments usually are. Thirdly, we used polymer types which are commonly found in marine environments. In addition, the ingestion of these secondary post-consumer MPs and polystyrene beads was compared. The secondary plastics used were weathered PET fragments from post-consumer bottles, and ABS fragments from used toy bricks. Of these, PET is one of the most common consumer plastics used and recycled, and thus environmentally relevant; 3.3 million tonnes of PET plastics were used in Europe during the year 2014 (PlasticsEurope 2015). The crushing and shredding processes of recycled plastic waste, such as PET, yield to high amounts of secondary MPs that can be distributed into the surrounding environment.

However, adjusting our fourth requirement, concentration of the experimental MPs was difficult. First of all, we realized that the present environmental data do not provide enough information on the concentrations of MPs – at least not in all environmentally relevant size fractions (Phuong et al. 2016). In overall, recent studies carried out worldwide and in the study area show the heterogeneity of the data on MP distribution and abundance: for example the measured MP concentrations in the Baltic Sea water vary from 0.1 to over 100 000 particles m^{-3} depending on the study, sub-region and mesh size used (e.g. Norén 2007, Gorokhova 2015, Setälä et al. 2016a, Gewert et al. 2017, Railo et al. 2018). Another problem that experimental exposure studies have to deal with is the dilemma between exposure level (in this case particle concentration) and incubation time. Most likely using low concentrations of MPs requires longer exposure time for detecting ingestion. On the other hand, the longer the incubation time, the more likely also becomes the egestion of potentially ingested particles. The problems can be overcome by increasing the concentration of MPs, but then again it drives the experimental conditions further away from natural conditions. We mimiced natural conditions in our experiments as well as possible, however, the problem with the concentration partly remained.

We decided to use a concentration, which can be relevant for hot spot areas (Norén 2007), but knew also that the commonly observed concentrations for these size fractions are lower in the Baltic Sea (e.g., Magnusson & Norén 2011, Magnusson 2014, Setälä et al. 2016a). When exposed to our experimental concentration, on average 0.2 ± 0.4 particles were found from three-spined sticklebacks and 0.8 ± 2.3 from mysid shrimps. These results demonstrate that even when animals are exposed to relatively high MP concentrations, the ingestion of an environmentally relevant type of MPs may not be significant. On average, the numbers of ingested MPs were relatively low in both studied small predators even though the particles corresponded to the size of their natural prey. Compared to previous studies carried out with the commonly used small and symmetrical virgin plastic pellets (Browne et al. 2008, Graham & Thompson 2009, Cole et al. 2013), and even when using the same study organisms (Setälä et al. 2014, Setälä et al. 2016b), the ingestion of polystyrene beads in this study can be considered negligible. In the case of mysid shrimps their feeding mode affects the ingestion efficiency of MPs. Mysids are able to switch between raptorial feeding on selected prey of larger size and passive filtration of small particles, such as

phytoplankton (Viitasalo & Rautio 1990). This selective raptorial feeding explains the negligible uptake of larger sized MPs and even the beads compared to the previously observed efficient MP filtration of 10 µm PS beads (Setälä et al. 2016b). As the experiments were conducted in filtered seawater, small prey were absent; hence, mysids were only feeding raptorially. In relatively low MP concentration this resulted in a low number of ingested particles.

Mysid shrimps and three-spined sticklebacks are known to actively select their prey (Visser 1982, Ibrahim & Huntingford 1989, Viherluoto & Viitasalo 2001a) and were probably able to select mesozooplankton over MP fragments. Size-selective feeding may also explain why ABS fragments of a certain size fraction (100–200 µm) were selected over other sizes (90 and over 500 µm) of MPs in the given mixtures. The mysids utilised in the experiments were of the size (~1.5 cm) which feeds readily on zooplankton prey of over 150 µm, mostly cladocerans and copepods (Viherluoto & Viitasalo 2001a). The color of the offered particles (orange ABS, green and red PET, yellow PS) did not play a significant role in the prey selection, as mysids are shown to locate their prey by mechano-reception and not based on visual signals (Viherluoto & Viitasalo 2001b). Most probably mysids have encountered the particles in the experimental water by accident, captured them and decided based on the size of the particle whether to ingest or to reject it.

Besides affecting the feeding mode and therefore ingestion probability of a particle, the size and shape of the MPs seem also to affect the entanglement to these particles. Our results show that MPs can be trapped in the appendages of mysids, and that most of the entangled particles were the largest filamentous fragments made of a soft drink bottle (200–1500 µm, PET). It is possible that mysids tried to reject these particles after capture as too large for ingestion without success. These results indicate that different-sized particles in the environment represent different pathways for MP exposure and impacts even in just one organism: in case of mysid shrimps, smaller particles are more probable to get ingested whereas larger, filamentous particles may more easily lead to entanglement. Entanglement to MPs may cause nuisance for the animals via hampered swimming, filtering, or prey capture as shown earlier for copepods (Cole et al. 2011).

The MP content of 333 three-spined sticklebacks collected from different open sea areas in the northern Baltic Sea was recently studied (Budimir et al. 2018), with no evidence of MP ingestion. However in another study where three-spined sticklebacks (120 individuals) were collected from coastal sites of the northern Baltic Sea, MPs were found from 12.5% of the fish (Zidbeck, 2018). Such observations are supported by our experimental results (22% of the sticklebacks ingested MPs). Field studies from other regions with various fish species have shown similar results with 2.4–36.5% of the studied fishes having ingested plastic particles (Boerger et al. 2010, Foekema et al. 2013, Lusher et al. 2013, Avio et al. 2015, Liboiron et al. 2016, Rummel et al. 2016). However, little is known on the consequences of ingestion; degraded polymer structures can leach endocrine disrupting, plastic derived chemicals that have a potential to disturb the energy metabolism shown with three-spined sticklebacks (Katzenberger 2015), but it remains unknown how low numbers of ingested MPs can generate visible negative effects.

The MP load is usually less than two particles on average per fish that have ingested plastic (e.g. Foekema et al. 2013, Lusher et al. 2013, Rummel et al. 2016, Budimir et al. 2018). As observed in our study, exposure to relatively high concentrations of secondary MPs does not automatically lead to high MP ingestion in fish. This is an important take-home message as the majority of experiments which have indicated marked ingestion of MPs have worked with primary microplastics only (e.g. Cole et al. 2013, Setälä et al. 2014, Batel et al. 2016). Also the importance of fibers, that seem to be the most dominant particle shape found from the environment (Browne et al. 2011, Ivar do Sul & Costa, 2014, Waite et al. 2018), should be acknowledged. In our study, the ingestion of fibers by mysids was as common as the ingestion of PET fragments from the ketchup bottles, even though PET fragments were intentionally given in relatively high concentrations and the fibers were unintentionally in the experimental units. This observation highlights the ubiquitous presence of fibers around us, and the importance of contamination-preventing practices when handling environmental samples. Our results clearly indicate the need for further studies using secondary MPs of environmentally relevant sizes, shapes and types, in concentrations as close to natural conditions as possible.

5. Acknowledgements

We want to thank the personnel of the Tvärminne Zoological Station for the great facilities and help, and the students on the field course of the experimental plankton ecology for the effective field and laboratory work during the first set of trials. Erasmus+ exchange students Tine Bizjak and Tamara Gajst at University of Eastern Finland are thanked for post-consumer microplastic production. The experiment was performed according to current Finnish legislation on animal welfare, approved by the ethical committee of animal experiments according to the §497/2013 legal act and degree. This study (0-class experiment) meets the terms of the Animal Care Committee at the County Board.

6. Funding sources

This work was supported by the Academy of Finland (no 296169, 276947). Supporting funds were also received from the Ministry of Environment (MERIROSKA), the University of Helsinki, and the Walter and Andrée de Nottbeck Foundation. Funding sources had no role in preparation of the article, in study design in the collection, analysis or interpretation of data, or in the decision to submit the article for publication.

7. Declarations of interest: none

There are no conflicts of interest concerning our article.

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Table 1. Experimental set-ups. The studied organisms (numbers and size) and incubation times. In the first (1.) set of trials the tested MPs were fragmented ketchup bottles (PET), in the second (2.) set of trials they were fragmented soft drink bottles (PET), toy bricks (ABS) and primary beads (PS). All MPs were offered in 250 particle L^{-1} concentration. In the mixture treatment the total MP concentration was 250 particle L^{-1}. The number of controls was 9 in the 1. set of trials per tested animal and 4 in the 2. set of trials.

	Set of trials and treatments	Number of replicates	Number of animals per unit	Mean size of the animals (cm) ± SD	Incubation time (h)
Praunus spp.	1. Ketchup bottles (PET)	9	4	1.6 ± 0.2	3
Gasterosteus aculeatus	1. Ketchup bottles (PET)	9	1	5.6 ± 0.5	1.5
Mysis relicta	2. Soft drink bottles (PET)	4	1	1.7 ± 0.2	3
Mysis relicta	2. Toy bricks (ABS)	4	1	1.7 ± 0.1	3
Mysis relicta	2. Beads (PS) -Mixture of all plastic types	4	1	1.7 ± 0.1	3
Mysis relicta	2. Mixture of all plastic types (soft drink bottle, toy brick, bead)	4	1	1.6 ± 0.2	3

Figure legends

Figure 1. The number (average + standard deviation) of primary (PS beads, 90 µm) and secondary microplastics (ABS fragments of toy bricks >100 µm, ketchup bottle PET >200 µm) in the intestines of three-spined sticklebacks and mysid shrimps after the experimental incubations. The numbers above the bars show the percentage of animals with MPs. Soft drink bottle fragments were not eaten thus they are not included in the figure.

Figure 2. The number (average + standard deviation) of secondary microplastics (ABS fragments of toy bricks >100 µm, ketchup bottle PET >200 µm, soft drink bottle PET >200 µm) entangled in the appendages of mysid shrimps. The numbers above the bars show the percentage of animals with MPs.

Highlights

- Naturally occurring microplastics were used in the experiments
- Planktivores were exposed to secondary microplastics (PET, ABS)
- Apparent ingestion of plastic fragments was low
- Entanglement to mysids was observed

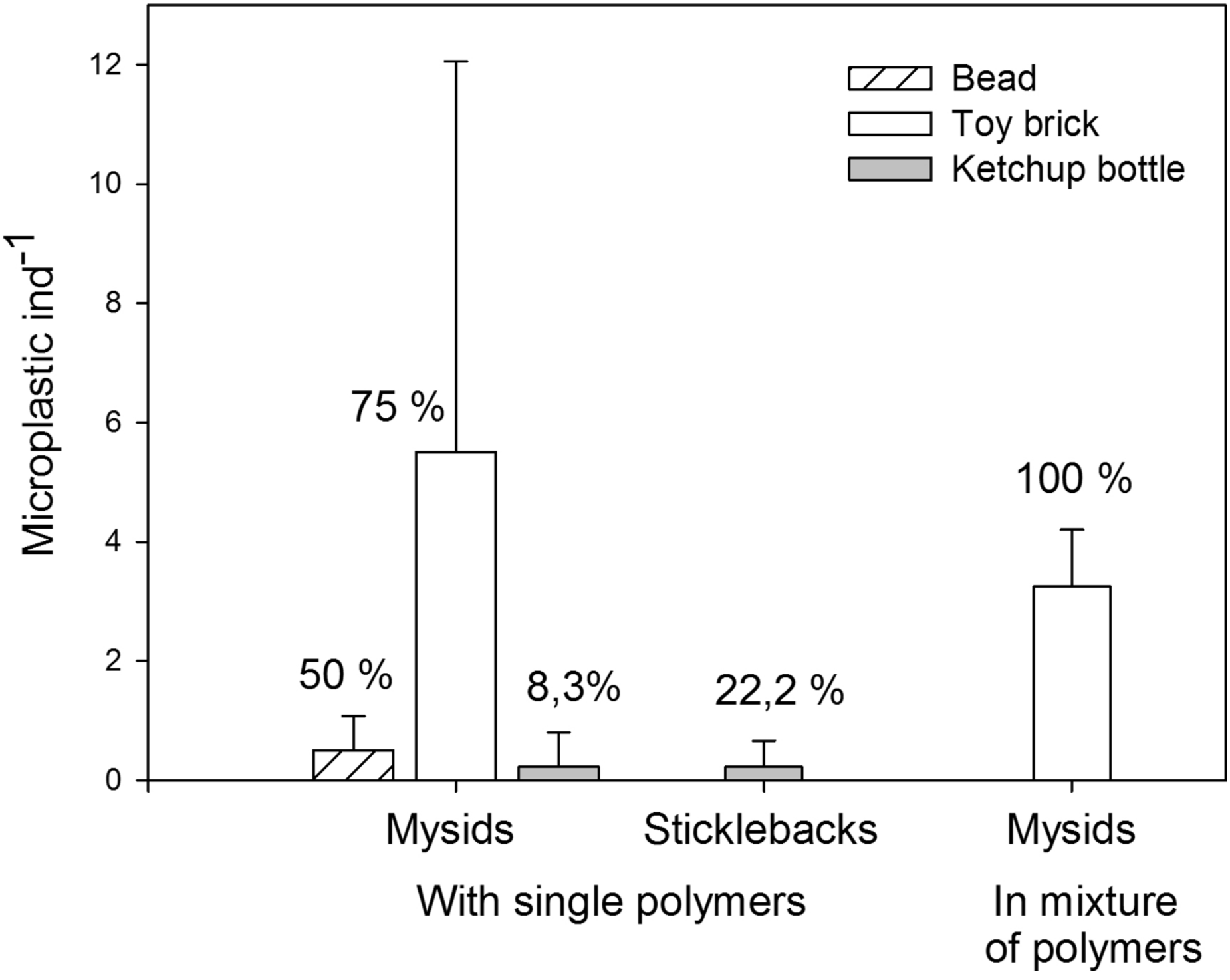
Bead
Toy brick
Ketchup bottle
Microplastic ind-1
12
10
8
6
4
2
0
75 %
50 %
8,3%
22,2 %
100 %
Mysids
Sticklebacks
Mysids
With single polymers
In mixture of polymers

Figure 1

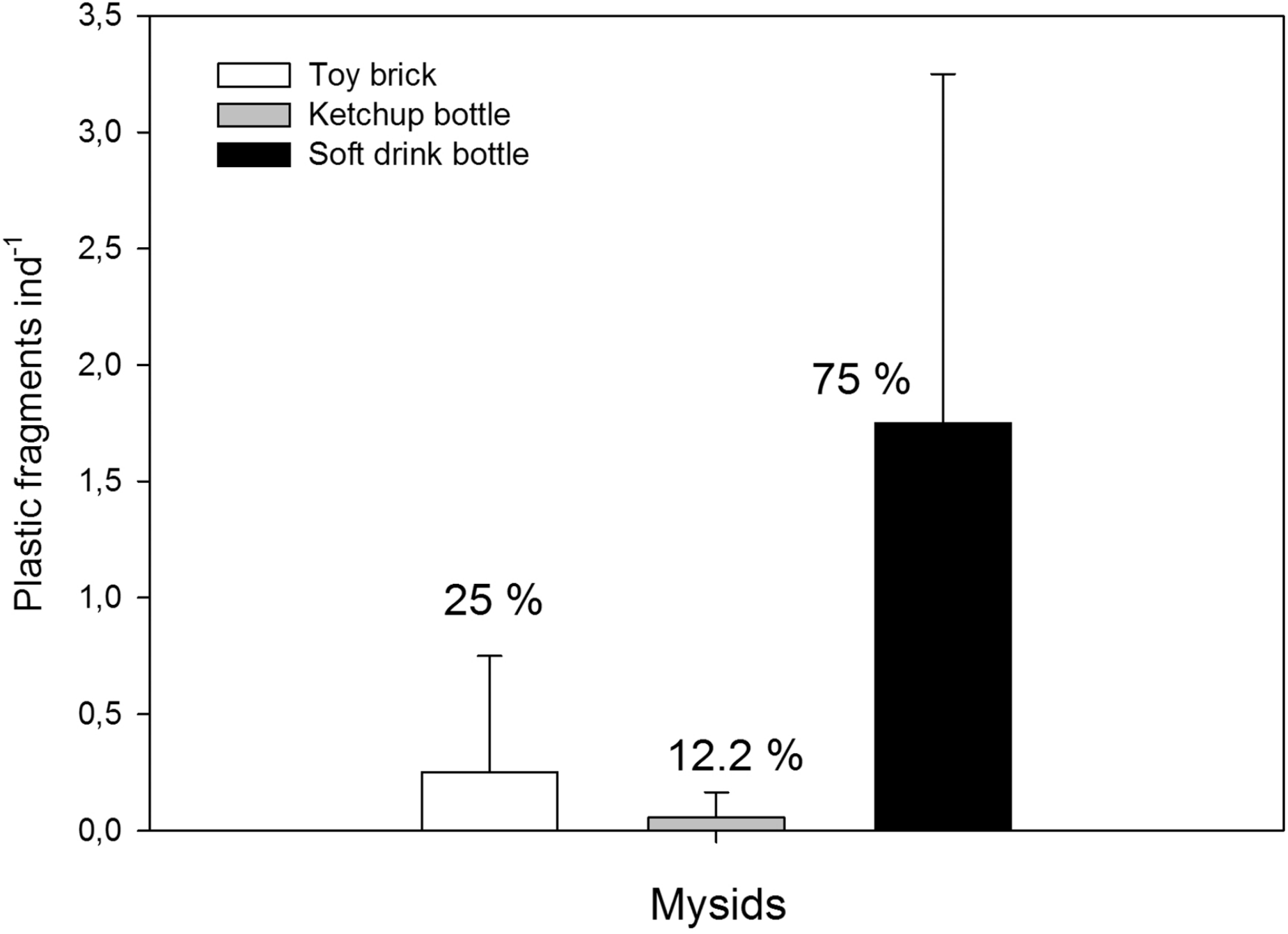
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
Plastic fragments ind-1
Toy brick
Ketchup bottle
Soft drink bottle
25 %
12.2 %
75 %
Mysids

Figure 2