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Adherence of microplastics to soft tissue of mussels: A novel way to uptake microplastics beyond ingestion☆

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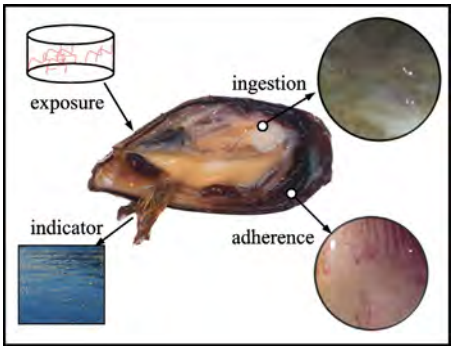
HIGHLIGHTS

- Microplastics were isolated from specific organs of mussels.
- The abundance of microplastic by weight differed in organs of field mussels.
- Microfibers were observed in foot and mantle of mussels in uptake and clearance experiments.
- Adherence contributed about 50% of the microplastic uptake in mussels.
- Adherence is a novel way for animals to uptake microplastics beyond ingestion.

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastic pollution is recognized as an emerging threat to aquatic ecosystems. One of the main environmental risks associated with microplastics is their bioavailability to marine organisms. Up to date, ingestion has been widely accepted as the sole way for the animals to uptake microplastics. Nevertheless, microplastics have also been found in some organs which are not involved in the process of ingestion. We hypothesize that the animal might uptake microplastics through adherence in addition to ingestion. To test this hypothesis, we collected mussels from the fishery farms, conducted exposure/clearance experiments and analyzed the accumulation of microplastics in specific organ of mussels. Our studies clearly showed the uptake of microplastic in multiple organs of mussels. In the field investigations, we found that the abundance of microplastic by weight but not by individual showed significant difference among organs, and the intestine contained the highest level of microplastics (9.2 items/g). In the uptake and clearance experiment, the accumulation and retention of microfibers could also be observed in all tested organs of mussels including foot and mantle. Our results strongly suggest that adherence rather than ingestion led to the accumulation of microplastics in those organs which are not involved in ingestion process. To our best knowledge, it is the first time to propose that adherence is a novel way for animals to uptake microplastics beyond ingestion. This new finding makes us rethink about the bioavailability, accumulation and toxicity of microplastics to aquatic animals.

1. Introduction

Microplastics have been recognized as emerging marine pollutants of significant concern, due to their persistence, ubiquity and toxic potential (Engler, 2012; Rochman et al., 2014; Wang et al., 2016). One of

☆ Capsule: Adherence was proved to be a novel way for animals to uptake microplastics beyond ingestion.

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the main environmental risks associated with microplastics is their bioavailability to marine organisms. Because of their small dimensions, microplastics have a similar size range to planktonic organisms and other suspended particles, making them available to an array of marine invertebrates (Wright et al., 2013; Ory et al., 2017). A lot of studies have reported that the animals can uptake microplastics through ingestion. Microplastics have been found in the intestines and stomachs in different species including fishes and birds in the field investigations such as freshwater, marine and terrestrial environments (Jabeen et al., 2017; Zhang et al., 2017). The abundances of microplastics reach 6.8×10^6 items/km^2 in freshwater and 7.6 items/individual in blue mussel (*Mytilus edulis*) in China (Li et al., 2016; Su et al., 2016).

In the laboratory exposure experiments, microbeads have also been found in other organs rather than intestine and stomach. For example, microbeads are not only found in the gills of mussels and crabs but also on the surface of foot of zooplanktons and mussels (Wegner et al., 2012; Setälä et al., 2016; Watts et al., 2016). Gill can be regarded as one of important feeding organs in many species. Foot, however, is not directly related to the feeding process. Therefore, we hypothesize that the animal might uptake and accumulate microplastics through adherence in addition to ingestion.

Mussels are the benthic extensive filter feeding organisms with a selective mechanism of suspension feeding, which leads to accumulation of microplastics, chemical pollutants and microorganisms in mussels (Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014; Paul-Pont et al., 2016; Van Cauwenberghe et al., 2015). Mussels have been widely used for biomonitoring studies in marine environments due to several advantages such as broad geographical distribution, easy accessibility and high tolerance for a considerable range of salinity. Mussels have also been used in microplastics researches including field investigations as well as laboratory exposure experiments (Von Moos et al., 2012; De Witte et al., 2014; Avio et al., 2015; Li et al., 2015, 2016).

The physical ingestion of microplastic by organism leads to blockage of the intestinal tract, inhibition of gastric enzyme secretion, reduction of feeding stimuli, decrease in steroid hormone levels, delay in ovulation and lack of reproduction (Wright et al., 2013; Canesi et al., 2015). Notable histological changes and a strong inflammatory response are observed in mussels after exposure to 2.5 g/L high-density polyethylene (Von Moos et al., 2012). *M. edulis* reduces its filtering activity after exposure to 0.1 g/L polystyrene microbeads (Wegner et al., 2012). Micropolystyrene at 32 μg/L leads to an increase in hemocyte mortality and triggered substantial modulation of cellular oxidative balance in *M. spp.* (Paul-Pont et al., 2016).

The accumulation and potential risks of microplastic are closely related the pathways for the microplastics entering the body of organisms. Therefore, it is critical to clarify the uptake pathways of microplastics in organisms. In the present study, we collected mussels from the fishery farm, conducted exposure/clearance experiments in the laboratory and analyzed the accumulation of microplastics in specific organ of mussels. The aim of the present study was to determine if there was a way for aquatic organisms to uptake microplastics beyond ingestion.

2. Materials and methods

2.1. Sample collection

The blue mussel (*M. edulis*) was collected from a fishery farm area in Zhoushan, Zhejiang, East China Sea. Some specimen were kept in −20 °C immediately for microplastic analysis, the others were cultured for exposure experiments. The total length (cm) and whole body weight (g) of the mussels were measured (Supplementary Table 1). One hundred and twenty-six mussels were totally used throughout the study.

2.2. Laboratory uptake and elimination experiment

The mussels were acclimatized for 5 days in laboratory conditions with aerated artificial seawater at 18 ± 1 °C, 28‰ salinity and a 12 h light-dark illumination regime. The water was filtered through 0.45 μm filter paper and maintained at 18 °C for the exposure experiments. Four mussels were randomly put into a 5 L glass tank with 4 L seawater. Four tanks were set for each group. Two control groups and two exposure groups were used for the exposure experiment. The same experiment was repeated thrice. The man-made microfibers were prepared manually using scissors. The plastics materials were cut into tiny pieces and then mixed with filtered water. The glass bottles were shaked well until the fibers were mixed thoroughly. The solution was mixed well and then filtered through nylon filters. The filtered fibers were transferred to clean bottles to prepare the stock solution 100 mL. Form the stock solution, 5 mL solution with microfibers was filtered using nylon filter. The microfibers were picked up from the filters under a stereomicroscope, and the size ranges of microfibers were measured. The abundance of microplastics was 2000 microfibers/L in the exposure experiment.

Forty-eight hours after exposure, mussels were collected from 2 control tanks and 2 treatment tanks for microplastic analysis. Mussels in the rest 2 control tanks and 2 treatment tanks were rinsed with filtered water three times and transferred into the tanks with clean water and aeration for elimination experiment. Forty-eight hours after elimination, the mussels were collected.

2.3. Dissection of mussel organs

The mussels from the fishery farm and laboratory experiments were washed with filtered water to remove the associated debris and byssal threads. Six replicates with 30 mussels were used for the field samples, and three replicates with 30 mussels were used in laboratory experiments. The organs were dissected according to the method of Avio et al. (2015) with slight modifications. In brief, a small knife was inserted between two valves on the dorsal side, and the anterior adductor muscle was cut to open the valves. The organs were divided based on their functions. Some of them (i.e., gills, intestine, stomach) were closely related to the ingestion process, and the others (i.e., mantle, gonad, adductor and visceral tissue) were not involved in the ingestion process (Fig. 1). The organs were kept in separate clean petri dishes and covered with aluminum foil to avoid contamination. The same organs in each five mussels were pooled together as one replicate.

2.4. Hydrogen peroxide treatment

The isolation of microplastics from mussels followed our previous methods for bivalves (Li et al., 2015). In brief, blank extraction group without tissue was performed simultaneously to correct the potential procedural contamination. All of the liquid (freshwater, saltwater and hydrogen peroxide) was filtered with 1 μm filter paper prior to use. All containers and beakers were rinsed three times with filter water before use to avoid contamination. The organs of mussels were emptied

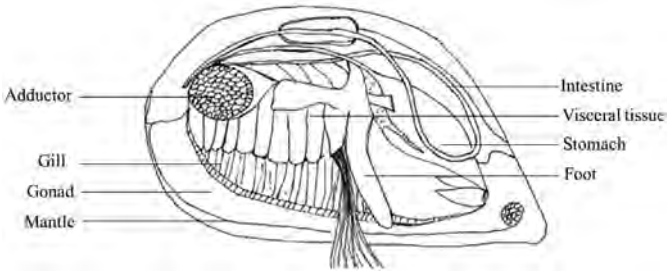

Fig. 1. The specific organs in blue mussel (*Mytilus edulis*).

into a 1 L glass bottle with 35 cm height. About 250 mL 30% H_2O_2 was added to each bottle to digest the organic matter depending on the weight of the soft tissues in each bottle. The bottles were covered and placed in an oscillation incubator at 65 °C with 80 rpm for 48 h.

2.5. Saline solution floatation and filtration

A prefiltered saturated saline solution (1.2 g/mL) was used to separate the microplastics from dissolved liquid of the soft tissue via floatation (Li et al., 2015). Approximately 800 mL filtered saturated saline solution was added to each bottle. The overlying water was directly filtered over a 5 μm pore size, 47 mm diameter cellulose nitrate membrane filter (Whatman AE98) using a vacuum with a pump. Next, the filter was placed into clean petri dishes with a cover for further analysis. All of the experimental procedures were finished as soon as possible.

2.6. Observation and validation of microplastic

The filters were observed under a Carl Zeiss Discovery V8 Stereo microscope (Micro Imaging GmbH, Göttingen, Germany), and images (25–80 magnification) were taken with an AxioCam digital camera. A visual assessment was applied to identify the types of microplastics according to the physical characteristics of particles. The identification was further validated with μ-FT-IR microscope (Thermo Nicolet iN10 MX) following the methods of Li et al. (2015).

2.7. Statistics analysis

Statistical analyses were performed using SPSS 22.0. The quantities of microplastic for more than two groups were determined using one way analysis of variance (ANOVA) followed by Tukey's HSD (homogenous variances) or Tamhane-Dunnett (heterogeneous variance) post-hoc test was performed to compare the abundance of microplastics in mussel organs. A significance level of 0.05 was chosen, and significant difference between two groups was analyzed using the Student's *t*-test.

3. Results

3.1. Accumulation of microplastics in specific organs of mussel in field

The abundance of microplastic by weight but not by individual showed significant difference among organs ($p < 0.05$), and the intestine contained the highest level of microplastics (9.2 items/g) (Fig. 2A, B). The proportion of fiber ranged from 51% in stomach to 71% in gill, dominating in all types of microplastic in mussels ($p < 0.01$) (Fig. 2C). The size of the microplastic ranged from 0.05 to 5 mm in mussels (Fig. 2D).

Proportion of microplastics in small size (0.05–0.25 mm) dominated in all size categories. In contrast, the highest proportion of large size (1–5 mm) microplastic in all organs was <30%, which means the lowest level in all size categories ($p < 0.05$) (Fig. 2D). The largest microplastics in size (4–5 mm) were only found in foot and adductor tissue.

3.2. Accumulation of microplastics in specific organs of mussels in the uptake experiment

In the uptake experiment, the accumulation of microfibers could be observed in all tested organs of mussels. In comparison with control group (Fig. 3A and B), the abundance of fibers in mussel organs was significantly increased after exposure by both weight and individual ($p < 0.01$) (Fig. 3C, D). The accumulation of fiber showed significant difference among organs in terms of items per gram ($p < 0.05$). The

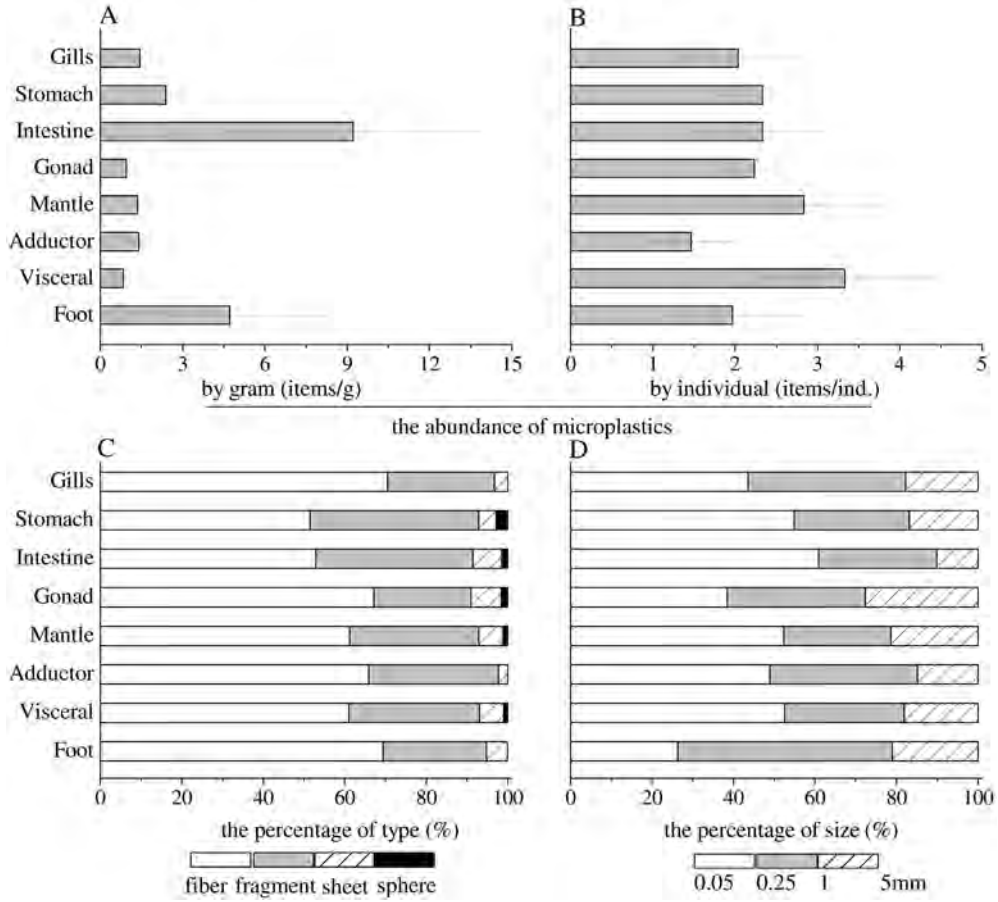

Fig. 2. The abundances (A, B), types (C) and sizes (D) of microplastics in specific mussel organs from the field investigations.

intestine showed the highest level of fiber by weight (171 items/g) (Fig. 3C).

3.3. Retention of microplastics in specific organs of mussels in elimination experiments

In the elimination experiment, the remnant fibers could still be found in all of the organs in the mussels, but the level of concentrations were significantly decreased in comparison with the exposure groups ($p < 0.05$) (Fig. 4A–B). The elimination of fiber showed significant differences among organs in terms of items per gram ($p < 0.05$). The retention of fiber showed significant difference among organs by weight ($p < 0.05$), and the intestine represented the highest level of fiber retention among all organs (91 items/g) (Fig. 4C).

4. Discussion

In previous studies, uptake and tissue distribution of microplastics has already been described in mussels after exposure to microplastics in the laboratory (Browne et al., 2008; Von Moos et al., 2012) (Supplementary Table 2). The first site of particle uptake is on the gill surface, mediated by microvilli activity and endocytosis, and the microplastics are further uptaken into the stomach, intestine and digestive tubules via ciliae movement (Von Moos et al., 2012). The particles with small size (e.g. <3.0 or 9.6 μm) can even translocate from the gut cavity to the haemolymph and inside the haemocytes (Browne et al., 2008).

In the present study, most microfibers were >100 μm in the laboratory experiments, which could not enter the circulatory system and be transferred to those non-ingestion organs. These results suggested that it was impossible for those microfibers to be uptaken into the non-ingestion organs via ingestion process. In the natural conditions, mussels have a great chance to get access to microplastic by direct contact instead of ingestion. For example, as filter feeders, the production of pseudofeces is very common in mussels' lifespan (Beninger et al., 1999). This process will lead to the relocation of microplastic in mussels via foot and mantle. This might be the reason why we found high accumulation of microplastics in foot. Therefore, we deduced that these microplastics could accumulate in these organs through adherence to the surface of the tissue rather than through ingestion process.

Previous studies on microplastics in animals mainly focus on the ingestion process. Microplastic pollution have been found in mussels in field investigations and even from fishery markets (De Witte et al., 2014; Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014; Li et al., 2015, 2016). The translocation and accumulation of microplastics particles have also been observed in various organs of mussels in several laboratory experiments (Browne et al., 2008;

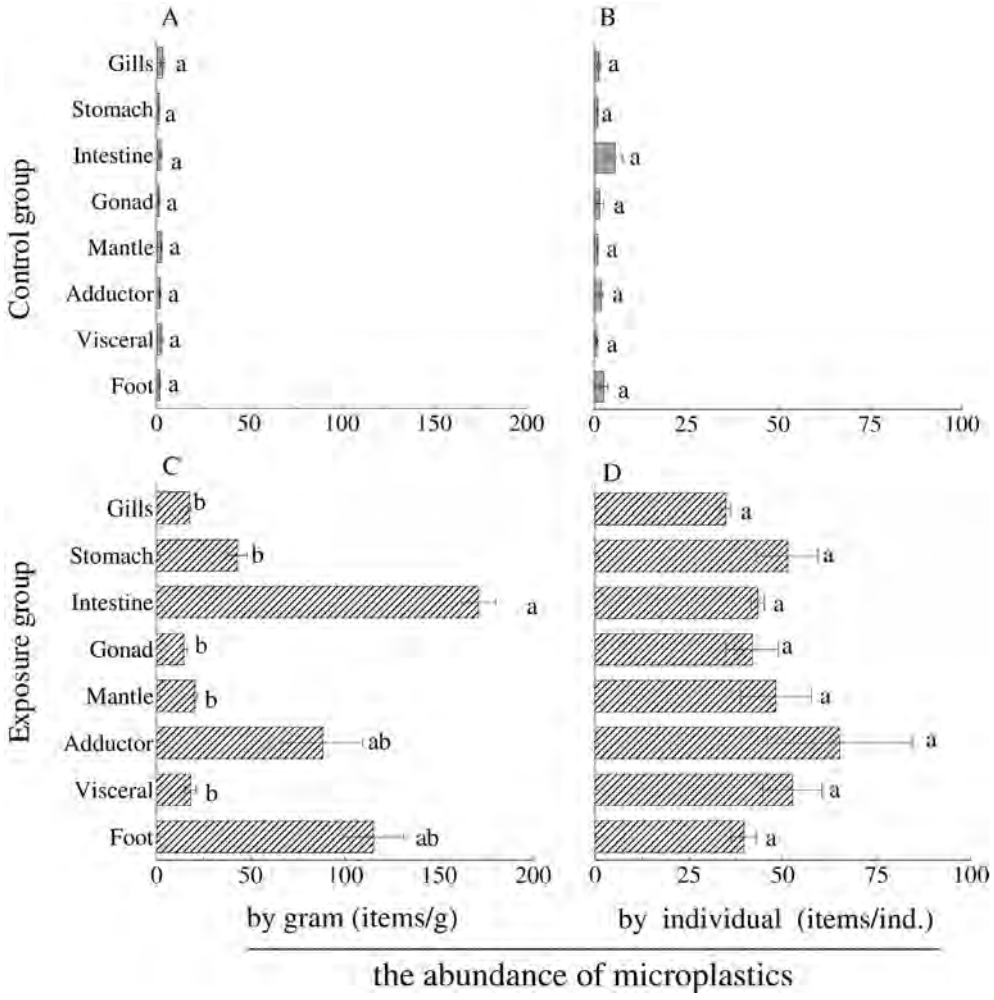

Fig. 3. The accumulation of polyester microfibers in specific mussel organs after 48 h exposure. Each value represents the mean ± SD of three replicates ($n = 3$). Each value represents the mean ± SD of six replicates ($n = 6$). One way analysis of variance (ANOVA) were used to calculate the significant differences among the microplastic abundance in different organs, Student's *t*-tests were used to compare the difference between control and experimental groups. The letters above the bars indicate significant differences ($p < 0.05$). If two arbitrary groups have the same letter, then they are not significantly different.

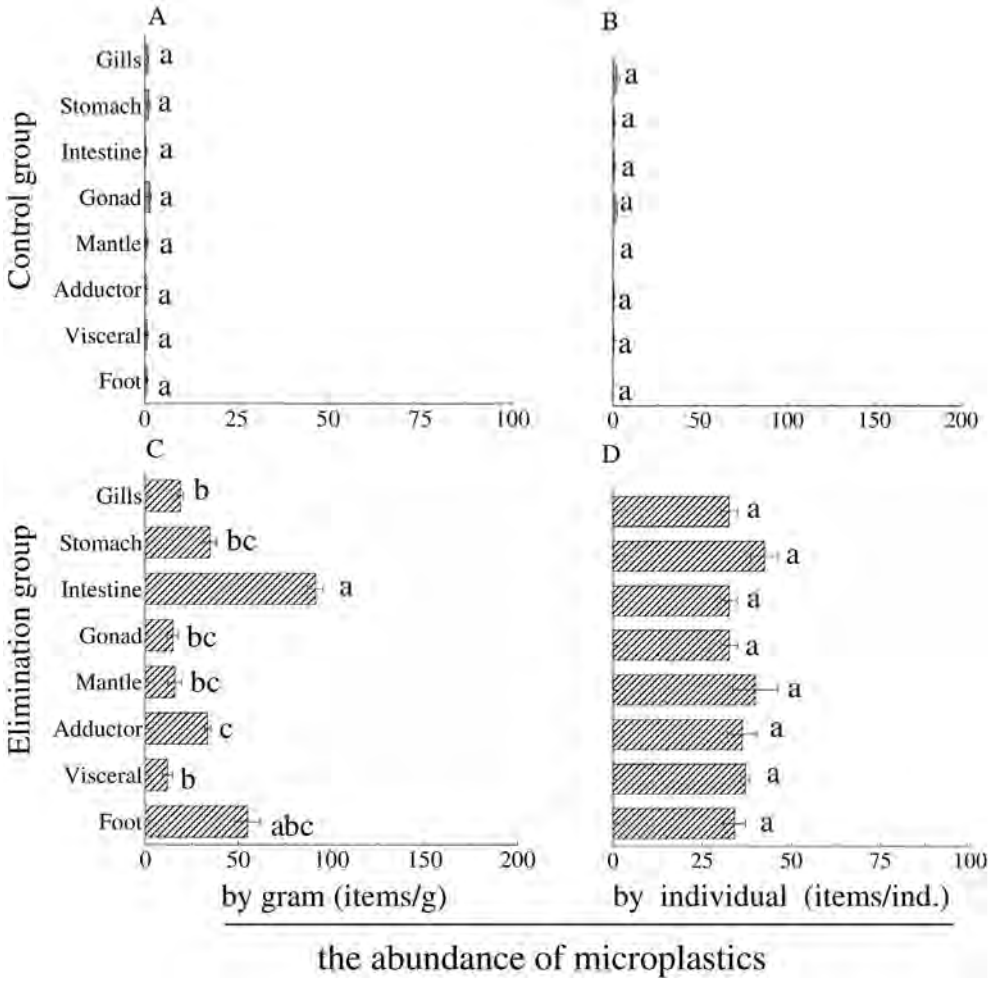

Fig. 4. The retention of polyester microfibers in specific mussel organs after 48 h exposure. Each value represents the mean ± SD of three replicates ($n = 3$). Each value represents the mean ± SD of six replicates ($n = 6$). One way analysis of variance (ANOVA) were used to calculate the significant differences among the microplastic abundance in different organs, Student's *t*-tests were used to compare the difference between control and experimental groups. The letters above the bars indicate significant differences ($p < 0.05$). If two arbitrary groups have the same letter, then they are not significantly different.

Von Moos et al., 2012). However, the uptake way of microplastics to mussels beyond ingestion receives little attention in previous studies.

To our best knowledge, it is the first time to propose that adherence is a novel way for animal to uptake microplastics beyond ingestion. In addition, we found that the proportion of microplastics through adherence accounted for 42–59% of the total microplastics in the whole tissue of mussels. There were no significant differences in the numbers of microplastics through adherence and ingestion ($p > 0.05$) (Fig. 5). This analysis suggested that the accumulation of microplastics through adherence cannot be neglected.

This new finding makes us rethink about the bioavailability, accumulation and toxicity of microplastics to animals. First of all, the consideration of adherence way will increase the estimation of bioavailability of microplastics to organisms, especially to those non-filtering feeders. In addition, when we regard ingestion is the sole way for animals to uptake microplastics, we always consider feeding habit, the size of mouth and the structure of intestine of animals as the main factors to affect the ability of uptaking and accumulating microplastics. In the adherence process, however, the surface area and sticking ability might play more important role in gathering microplastics. Some non-filter feeders also might have high ability of accumulating microplastics from the environments. Gutow et al. (2016) found that the adherence of microplastics to seaweeds would provide a pathway for microplastic from the water to marine benthic herbivores. Similarly, the adherence of microplastics to animals would be a novel way for microplastics to be transferred in food web.

Secondly, the consideration of adherence way will make us pay attention to the accumulation of microplastics in some organs rather than intestine and stomach in organisms. In previous studies, we mainly measure the microplastics in gills, stomach and intestine, especially in fish (Jabeen et al., 2017). Our present study suggest that microplastics might be present in all main organs. It will underestimate microplastics if we just use the number of microplastics in stomach and intestine to stand for the total number of the whole tissue. We also might underestimate the risk of microplastics when we suppose that we will not uptake microplastic if we get rid of stomach and intestine of animals in the diet.

Finally, the consideration of adherence way will make us reevaluate the toxicity of microplastics. A plenty of studies have proved that microplastics have the potential to cause a lot of adverse effects, including endocrine disruption, energy disturbance, oxidative stress, immunity and neurotransmission dysfunction, and even genotoxicity (Lee et al., 2013; Rochman et al., 2014; Avio et al., 2015). In the laboratory experiments, microbeads are the most commonly used microplastics. Microbeads are likely to be eliminated out of the intestine and maintain in the bodies of organisms in a relatively short time, which is supposed to bring little toxicity to the targeted organisms. In fact, microfibers are more popular in the real environments (De Witte et al., 2014; Mathalon and Hill, 2014; Su et al., 2016; Li et al., 2016). Our results suggest that

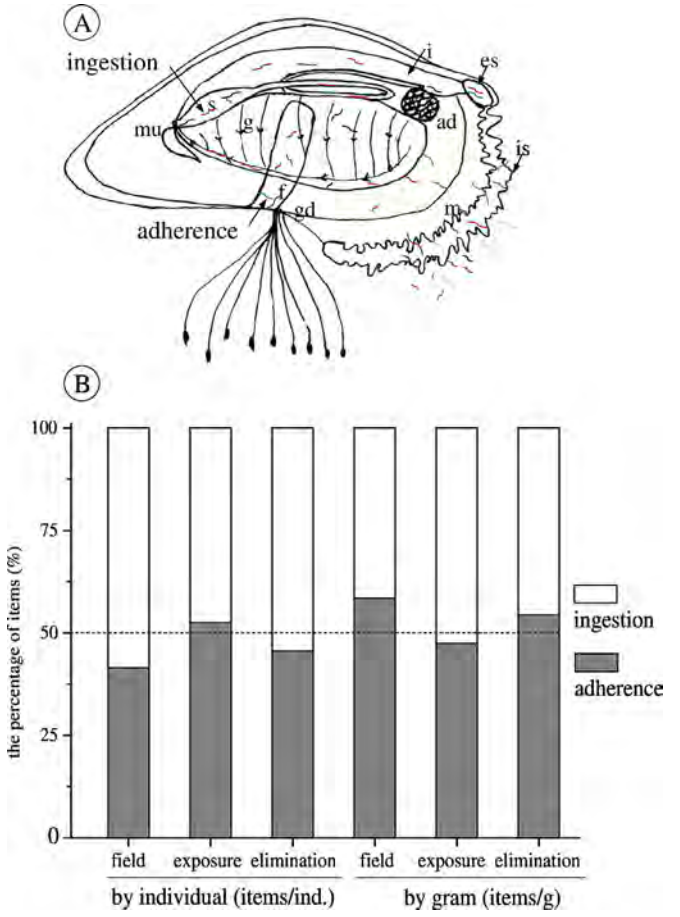

Fig. 5. Ingestion and adherence of microplastics in the mussels. A, outline of the pathway of microplastic ingestion and clearance; B–C, the of proportion of ingestion and adherence of microplastics by items/g (B) and by items/individual (C). Gill, intestine and stomach were regarded as being involved in ingestion process, and the rest organs were supposed to be only involved in the adherence process. Abbreviations: ad, adductor tissue; es, exhalant siphon; f, foot; g, gills; gd, gonad; m, mantle skirt; mu, mouth; i, intestine; is, inhalant siphon; s, stomach.

microfibers are likely to adhere to the surface of the tissue and might maintain for a longer period. Therefore, the toxicity of microplastics might be stronger than the results got from the experiments in which only microbeads are used and ingestion is considered.

In conclusion, our results strongly suggest that adherence is a novel way for animals to accumulate microplastics beyond ingestion. This new finding makes us rethink about the bioavailability, accumulation and toxicity of microplastics to aquatic animals.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.08.053.

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