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Microplastic fibers transfer from the water to the internal fluid of the sea cucumber *Apostichopus japonicus*☆

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A B S T R A C T

Microplastics (MPs) are small plastic particles less than 5 mm in diameter. MPs in the form of microfibers (MFs) are widely detected in aquatic habitats and are of high environmental concern. Despite many reports on the effects of MFs on marine animals, their effect on sea cucumbers is still unclear. In addition, our previous filed study has shown that MFs may transfer to the coelomic fluid of the sea cucumber *Apostichopus japonicus* (*A. japonicus*). Here, we show how MFs transfer to the coelomic fluid of the sea cucumber. We captured the MFs during their transfer from the water to the coelomic fluid through the respiratory tree. *A. japonicus* ingested in the MFs along with the water during respiration; the MFs got stuck in the respiratory tree or transferred to the coelomic fluid. The transferred MFs increased during 72 h of exposure and persisted for 72 h after the transfer to clean water. Among the immunity indices, lysozyme (LZM) levels increased in response to the transferred MFs, which confirms the defensive role of LZMs against strange substances. Additionally, non-significantly decreased levels of total antioxidant capacity (T-AOC), malondialdehyde (MDA), peroxidase (POD) and phenol oxidase (PPO) were observed at 24 h and 48 h post-exposure, suggesting minimal oxidative imbalance. Furthermore, there were no significant changes in the speed and the total distance moved by *A. japonicus* post MFs transfer. This study revealed that MFs transfer and accumulate in the coelomic fluid of *A. japonicus*.

1. Introduction

MPs are tiny plastic particles that can be harmful to biota (reviewed by Anbumani and Kakkar, 2018). These MPs can be of various shapes, from pellets and spheres to fragments and fibers (de Sá et al., 2018). Microfibers (MFs), which are threads that are uniformly shaped throughout their entire length (de Sá et al., 2018; Suran, 2018), are the prominent form of MPs in animal habitats (Browne et al., 2011; Van Cauwenberghe et al., 2015; Barrows et al., 2018; Henry et al., 2019), and their major source is synthetic clothing (Browne et al., 2011; de Sá et al., 2018; Barrows et al., 2018). Synthetic MFs are more hazardous than other MP particles (Au et al., 2015; Qiao et al., 2019), and they could be a vector for transferring pollutants to animals (Suran, 2018; Mohsen et al., 2019a). MFs are ingested by a wide range of animals, such as mussels (Catarino et al., 2018), fish (Compa et al., 2018), marine turtles (Duncan et al., 2019), benthic invertebrates (Courtene-Jones et al., 2019), corals (Rotjan et al., 2019) and crustaceans (Bour et al., 2018). MFs exposure decreases the filtration rate in the mussel *Mytilus edulis* (Woods et al., 2018), increases mortality in the crustacean *Daphnia magna* (Jemec et al., 2016), and reduces growth in the amphipod *Hyalella azteca* (Au et al., 2015).

Sea cucumbers are epibenthic deposit feeders that pull the sediment by the tentacles into their mouth to extract nutrients. Sea

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cucumbers breath from their anus through the respiratory tree. The respiratory tree serves as lungs that allow gas exchange between the water and the coelomic fluid of sea cucumbers (Lambert, 1997; Gao and Yang, 2015). Sea cucumbers, especially *A. japonicus* (Yang and Bai, 2015), not only have high economical and nutritional values (Toral-Granda et al., 2008; Bordbar et al., 2011) but also play a crucial role in marine ecosystems (Purcell et al., 2016). Their sediment reworking and feeding activities help to improve sediment characteristics (Pischedda et al., 2008), influence the productivity of many benthos (Uthicke, 2001), enhance water chemistry (Wolfe et al., 2018), decrease algal blooms (Michio et al., 2003) and remove aquaculture waste (Zamora et al., 2018). Sea cucumber *A. japonicus* has been harvested for centuries and its natural resources are depleted. Consequently, *A. japonicus* has been listed as an endangered species (Hamel and Mercier, 2013). Moreover, *A. japonicus* sea cucumbers ingest MFs and MFs may transfer to their coelomic fluid (Mohsen et al., 2019b). Despite many reports on the effects of MFs on marine animals, their effect on sea cucumbers is unknown. With the increasing of MFs in the ocean, especially from anthropic activities (Belzagui et al., 2019), together with their hazardous properties (Suran, 2018), their effects on such endangered species need to be clarified. To know whether these hazardous materials can influence the biological functions and the development of sea cucumbers, and whether the ingestion of these materials by sea cucumbers has implications for food web since the internal viscera of sea cucumbers can be consumed by humans (Purcell et al., 2012; Mao et al., 2015).

Therefore, this work was designed to answer the following questions: Do MFs transfer to the coelomic fluid of *A. japonicus*? If so, what are the biological consequences concerning that transfer, and do MFs persist in the coelomic fluid after transferring *A. japonicus* to clean water?

2. Materials and methods

2.1. Experimental animals

Sea cucumbers (n = 150) were obtained from a local farm in Rushan, China (36°46′40.7″N 121°34′34.7″E). As the animals were obtained from a local farm, no license was needed to collect them. The animals were acclimated in the laboratory using sand-filtered seawater with 30 ppt salinity at 16 ± 1 °C for one week. After two days of starvation, the animals were selected randomly and separated into groups; there were no significant differences between the groups ($P < 0.05$).

2.2. Synthetic MFs preparation

MFs are mainly detected from the habitat of *A. japonicus* (Mohsen et al., 2019b). Therefore, MPs in the form of MFs were prepared from polyester threads that are commonly used in clothing manufacturing. These threads were separated into small MFs (length: 1–5 mm) using a cutting machine and scissors ((Supporting information (S) Fig. S1) to simulate a washing machine process. The MFs were red in color, and thus, it was easy to distinguish them under the microscope. Observation of the MFs in the coelomic fluid of *A. japonicus* was performed by making an opening on the ventral surface that let the coelomic fluid flow freely into a clean Petri dish (Mohsen et al., 2019b). A dissecting microscope (SMZ-161-BLED, Motic, China) or a fluorescence microscope (OLYMPUS IX51) was used to detect and visualize the MFs. A hand tally counter was used to count the MFs.

2.3. A. japonicus exposure to MFs from the water

To investigate the possibility of MFs transfer from the water, an initial experiment was conducted. The individuals were placed in 4.8 L circular covered tanks with a diameter of 27 cm with three replicates; oxygen was supplied by air stones. The average sea cucumber weight was 25 ± 0.76 g (Mean ± SE). Firstly, the animals were exposed to a concentration of 0.023 MF mL^{-1}, which is reported from the Bohai Sea in China (Dai et al., 2018). However, using this concentration, a homogenous suspension of the MFs could not be achieved, because some of the MFs were aggregated, suspended or settled down on the bottom of the tank. MFs in the habitat may lose buoyancy or move with the current, and then, approach the anus of the sea cucumber. Therefore, to investigate the possibility of MFs transfer, we used concentrations of 0.003 gL^{-1} and 0.006 gL^{-1}, approximately 25 MF mL^{-1} and 40 MF mL^{-1}. The animals were sampled 1 h, 3 h, and 12 h postexposure to examine the possibility of MF transfer. Consequently, we observed that MFs transfer from the water to the coelomic fluid of *A. japonicus* and their transfer depends on their location within the anus opening possibly during the first breath. The closer the MFs were to the anus, the greater the chance of transfer was. This is likely because of an increased possibility of reaching the walls of the respiratory tree and thus transferring. Hence, experimentally, an increase in the MFs exposure rate can reveal the MFs pathway into the organism. Subsequently, an experiment was conducted to examine the enzymatic responses associated with the MFs transfer. The animals were exposed to the same MF concentrations and sampled 24 h, 48 h and 72 h postexposure. Then, we examined the persistence of the MFs after transferring the animals to clean water. The animals were transferred to sand-filtered water at 72 h postexposure. The water was changed daily, and the animals were sampled at 72 h posttransfer.

2.4. A. japonicus exposure to MFs from the ingested sediment

To examine the probability of MFs transfer from the ingested sediment through the gastrointestinal epithelium of *A. japonicus*, a diet containing 40% algae powder and 60% sea mud that mixed with MFs was prepared. *A. japonicus* does not differentiate between MFs and sediment particles (Mohsen et al., 2019b), thus the MFs were expected to pass through the intestines and reveal the probability of MFs transferring. Therefore, the individuals were exposed to the MFs in the feed at concentrations of 0.6 MF g^{-1} and 1.2 MF g^{-1}, which are reported from the field (Mohsen et al., 2019b), or at a concentration of 10 MF g^{-1}. The individuals were placed in tanks (45.33.25 cm) with three replicates. The oxygen was supplied by air stones, the feed was introduced once a day and the water in each tank was changed daily. The average sea cucumber weight was 83.23 ± 0.48 g (Mean ± SE). The animals were sampled 1 h, 3 h, 12 h, 72 h and 168 h postexposure to examine the possibility of MF uptake by the gastrointestinal epithelium. The MFs were not found to transfer to the coelomic fluid and this investigation was continued for 60 d for further confirmation.

2.5. Enzyme analysis of the coelomic fluid due to MF transfer

The coelomic fluid of sea cucumbers is somewhat like blood in the vertebrates. The coelomic fluid transports nutrients and contains phagocytic coelomocytes, which have immunity function (Lambert, 1997; Gao and Yang, 2015). Therefore, to examine the physiological status of *A. japonicus*, the activities of thirteen enzymes that represent immunity and oxidative responses were analyzed. The coelomic fluid was collected in test tubes and stored in liquid nitrogen for preservation at - 80 °C until analysis. The specific tests used in this study were performed with commercial kits from Nanjing Jianchen Biological Institute (Nanjing, China). Further details are described in Table S1.

2.5.1. Immune defense indices

The immunity indices, including lysozyme (LZM), myeloperoxidase (MPO), acid phosphatase (ACP) and alkaline phosphatase (AKP) activities were measured.

2.5.2. Oxidative stress indices

The oxidative stress indices, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), succinate dehydrogenase (SDH), lactate dehydrogenase (LDH), total antioxidant capacity (T-AOC), malondialdehyde (MDA), peroxidase (POD) and phenol oxidase (PPO) activities were measured.

2.6. Behavior recording post MF transfer

Sea cucumber activity was filmed at 72 h postexposure for 12 h in clean water using an overhead camera. The film was analyzed using Ethovision XT 10.1 (Noldus Inc., the Netherlands) software and evaluated to determine the velocity and total distance moved by the individuals.

2.7. Statistical analysis

The Shapiro-Wilk normality test with a 95% confidence level was used to examine the normality of the data. To detect significant differences between the treatments, a one-way ANOVA followed by Tukey's post hoc test was used for normally distributed data; otherwise, the Kruskal-Wallis H test followed by the Mann-Whitney test was used ($P < 0.05$). SPSS Statistics 20.0 statistical software (SPSS Inc., Chicago, IL) was used to conduct all statistical analyses.

3. Results

3.1. *A. japonicus* exposure to MFs from the water

The MFs were captured during their transfer from the water through the branch of the respiratory tree under a fluorescence microscope (Fig. 1). Also, we observed that when MFs were present, *A. japonicus* ingested in the MFs along with water during respiration. The MFs entered the anus with the water, and expulsion with the water was attempted, but the MFs got stuck in the branches of the respiratory tree (Fig. S2) or transferred to the coelomic fluid (Fig. S3). Ingesting MFs with the first respiratory intake of water by *A. japonicus* may increase the probability of MFs being transferred to the coelomic fluid. Furthermore, the number of MFs transferred

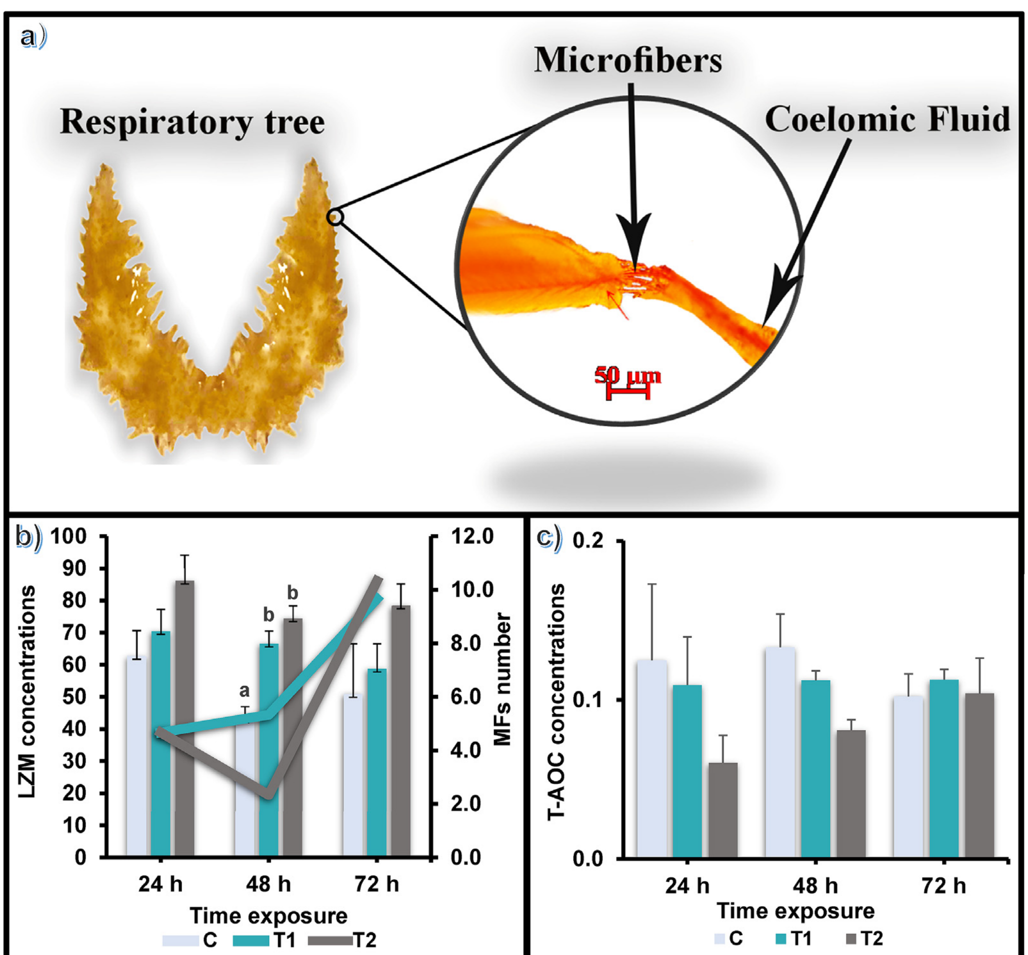

Fig. 1. **a)** Microfibers (MFs) crossing a branch of the respiratory tree, **b)** average MFs number in the coelomic fluid (line chart) and the lysozyme (LZM) responses of the coelomic fluid (U/ml) (column chart) at 24 h, 48 h and 72 h postexposure (Mean ± SE), **c)** total antioxidant capacity (T-AOC) (mmol/L) at 24 h, 48 h and 72 h postexposure. Different letters indicate a significant difference and columns without letters indicate no significant difference (Mean ± SE) ($P < 0.05$).

increased over time, except for one case in T2 at 48 h postexposure, without a significant difference ($P < 0.05$) (Fig. 1). In addition, at 72 h post-transfer to the sand-filtered water, the MFs were present in the coelomic fluid, and their number was comparable with those sampled at 72 h postexposure in both treatments (Fig. S4) ($P < 0.05$). No animals were died or eviscerated during the experiment.

3.2. A. japonicus exposure to MFs from the ingested sediment

A. japonicus ingested the feed that mixed with MFs, however, the MFs were not found to transfer from the water to the coelomic fluid through the gastrointestinal epithelium even up to 60 d of exposure.

3.3. Enzymatic responses of the coelomic fluid due to MF transfer

3.3.1. Immune defense indices

The activity of the LZMs in the treated animals was higher than that in the control animals at 24 h, 48 h and 72 h postexposure in both treatments. The activity in the treated animals showed a significant difference from that in the control animals at 48 h postexposure in both treatments ($P < 0.05$) (Fig. 1). The activities of AKP, ACP, and MPO in the treated animals were comparable with those in the controls (Figures S5- S7).

3.3.2. Oxidative stress indices

The concentrations of T-AOC, MDA, PPO and POD showed nonsignificant decreased levels at 24 h and 48 h but not at 72 h postexposure in both treatments (Fig. 1 and S8-10). Also, the concentrations of SOD, GSH, CAT, LDH, and SDH in the treated animals were not significantly different from those in the control animals in either treatment and their levels did not show a regular tendency (Figs. S11−S15).

3.4. Behavior recording after MFs transfer

After 72 h of exposure, the total distance moved by treated *A. japonicus* was not significantly different from that of the controls during the 12 h of observation in clean water (Fig. S16) ($P < 0.05$). Also, the average velocity of treated *A. japonicus* was not significantly different from the controls (Fig. S17) ($P < 0.05$). The average velocity of *A. japonicus* was 0.7 mm s^{-1}.

4. Discussion

4.1. A. japonicus exposure to MFs from the water

MFs are widely detected in aquatic habitats and are of high environmental concern, likely because of their size (Henry et al., 2019). In our previous field study, we speculated that MFs may transfer to the coelomic fluid of *A. japonicus*. MFs were found in the coelomic fluid of *A. japonicus* specimens that collected from eight sites along the Bohai Sea and the Yellow Sea in China (Mohsen et al., 2019b). Therefore, here we investigated the probability of MFs transfer in the laboratory. We detected MFs that transferred to the coelomic fluid of *A. japonicus* from the water through the respiratory tree; this process has not been previously shown. The respiratory tree serves as lungs that are responsible for gas exchange between the water and the coelomic fluid. *A. japonicus* breathes by drawing water through the anus and then expelling it (Gao and Yang, 2015). Therefore, the MFs entered the respiratory tree through the process of breathing. The MFs increased by time exposure except in one case in T2 at 48 h postexposure, because the MFs got stuck in the respiratory tree (Fig. S2). Thus, MFs can physically harm the respiratory tree if they fit to enter the anus. The shape of the MFs has an important role in penetrating the walls of the respiratory tree since a single MF can pass through (Fig. S18). This process is likely to happen in nature over the lifetime of *A. japonicus* because MFs were detected from the coelomic fluid of *A. japonicus* in farms, with an average weight of 53 g−144 g (Mohsen et al., 2019b). In addition, the MFs may accumulate in the coelomic fluid over time. After transfer the sea cucumber to clean water for 72 h, the number of the transferred MFs was comparable with those sampled at 72 h postexposure, suggesting that the transferred MFs had no way out of the coelomic fluid. This accumulation is different from that in mussels. The mussel *Mytilus edulis* accumulate the MFs during the filter-feeding but these MFs can finally be ejected. The mussel *Mytilus edulis* can decrease an average of 63% of the accumulated MFs within 6 h posttransfer to clean water (Woods et al., 2018). Increasing the MFs accumulation in the coelomic fluid of *A. japonicus* will not likely result in mortality but might disrupt the biological functions and the development of *A. japonicus*, which worth further investigation. Additionally, MFs accumulation in the coelomic fluid of *A. japonicus* might provide a route for subsequent transport of contaminants to the internal viscera of sea cucumbers, since MFs adsorb contaminants from the surrounding environment (Suran, 2018; Mohsen et al., 2019a). The internal viscera of sea cucumbers can be consumed by humans. In Japan, the intestine is processed into salted product and the gonad is used to make soup or tea (Mao et al., 2015). In Malaysia, the coelomic fluid of the sea cucumber *Stichopus horrens* is used to prepare medicinal products (Purcell et al., 2012).

4.2. A. japonicus exposure to MFs from the ingested sediment

Sea cucumbers are deposit feeders that ingest the sediment to extract nutrients (Gao and Yang, 2015). Sea cucumbers ingest sediment that mixed with MFs in laboratory experiments (Graham and Thompson, 2009), in deep water (Taylor et al., 2016), in the wild (Renzi et al., 2018) and in farms (Mohsen et al., 2019b). In the current investigation, the ingested MFs passed through the intestines without transferring to the coelomic fluid through the gastrointestinal epithelium. This is likely because of that the synthetic MFs cannot be taken up by the gastrointestinal epithelium or penetrate the thick walls of the intestines.

4.3. Enzymatic responses of the coelomic fluid due to MF transfer

4.3.1. Immune defense indices

The immune system of the sea cucumber is an innate system that includes a nonspecific defense mechanism to attack foreign cells (Xue et al., 2015). The activities of typical enzymes, including LZM, ACP, AKP and MPO, which are commonly used as indicators for evaluating the immunity status of *A. japonicus*, were measured (Wang et al., 2008; Chi et al., 2014; Xue et al., 2015; Liu et al., 2016; Jiang et al., 2016; Huo et al., 2018). Among the immunity indices, LZM showed a response to the transferred MFs and appeared to have a defensive role against the transferred MFs in the coelomic fluid of *A. japonicus*. Significantly increased levels of LZM were observed at 48 h postexposure, while LZM levels were also higher in the treated animals than in the control animals at 24 h and 72 h postexposure, but without significant difference. This might be ascribed to destabilization in the LZMs of cells due to different growth phases (Von Moos et al., 2012). LZMs are extremely common enzymes that play a defensive role against foreign substances in the sea cucumber (Canicatti and Roch, 1989). In *A. japonicus*, LZMs respond to stress challenges such as low oxygen levels ($P < 0.05$) (Huo et al., 2018), salinity changes ($P < 0.05$) (Bai et al., 2015), temperature fluctuations ($P < 0.05$) (Wang et al., 2008; Bai et al., 2018), and the injection of saccharides ($P < 0.05$) (Li et al., 2009). Thus, MFs transfer to the coelomic fluid caused physiological stress to *A. japonicus* that induced LZM activities. High LZM activities were also noticed in the bivalve *Mytilus galloprovincialis* that exposed to cationic polystyrene nanoparticles (Canesi et al., 2015). On the other hand, MP exposure decreases LZM levels in the crab *Eriocheir sinensis* ($P < 0.05$) (Liu et al., 2019). MPO enzyme is also involved in the immunity response (Klebanoff, 2005), but did not show a significant difference from the control. This might suggest that *A. japonicus* follows a specific mechanism to defend against the transferred MFs, elevating LZM levels. For ACP and AKP enzymes, they are two types of phosphatase enzymes that cause dephosphorylation, which can be correlated with inflammatory conditions (Sacco et al., 2012); they have been used in the assessment of the immunity status of *A. japonicus* (Chi et al., 2014; Liu et al., 2016; Jiang et al., 2016; Huo et al., 2018). Neither ACP nor AKP levels in the treated animals were significantly different from those in the control animals ($P < 0.05$). However, ACP and AKP levels significantly change in *A. japonicus* under low oxygen conditions ($P < 0.05$) (Huo et al., 2018) and under dietary probiotic supplementation conditions ($P < 0.05$) (Chi et al., 2014; Liu et al., 2016). In addition, previous studies reported a significant difference in AKP and ACP levels between treated and control animals after MP exposure in the discus fish *Symphysodon aequifasciatus* ($P < 0.05$) (Wen et al., 2018) and in the crab *Eriocheir sinensis* ($P < 0.05$) (Liu et al., 2019).Thus, this suggests that *A. japonicus* responds differently to the effect of the transferred MFs. Similarly, the exposure to MPs does not significantly alter ACP levels in the worm *Hediste diversicolor* ($P < 0.01$) (Revel et al., 2018).

4.3.2. Oxidative stress indices

Oxidative stress is an imbalance between antioxidants and oxidants in favor of oxidants that can cause damage to cells (Sies, 1997). Antioxidant enzymes such as SOD, MDH, GSH, CAT, T-AOC, LDH, SDH, PPO, and POD evaluate the oxidative response of *A. japonicus* (Yu et al., 2016; Bai et al., 2017; Huo et al., 2018).

Non significantly decreased levels of MDH, PPO, POD, and T-AOC were observed at 24 h and 48 h post-exposure but not at 72 h post-exposure, which might indicate that the oxidative stress caused by the transferred MFs is minimal and might be recovered. The oxidative status of *A. japonicus* changes significantly under the conditions of salinity challenge ($P < 0.05$) (Bai et al., 2015), ascorbic acid intake ($P < 0.05$) (Bai et al., 2017), temperature fluctuation ($P < 0.05$) (Bai et al., 2018), hypoxia ($P < 0.05$) (Huo et al., 2018) and after saccharide injection ($P < 0.05$) (Li et al., 2009), which indicates that the oxidative stress caused by the transferred MFs is minimal in comparison to the effect of other stressors on *A. japonicus*. Similarly, the translocated MPs to the gut cavity of the mussel *Mytilus edulis* do not disturb the oxidative status (Browne et al., 2008).

4.4. Behavior recording

Behavioral assessment is used to characterize the effect of pesticides, metals, drugs, and gases. Also, assessing motor activity can be suitable to examine neurotoxicity (Kulig et al., 1996). In *A. japonicus*, locomotor behavior is affected by melatonin exposure (Ding et al., 2019), reproduction in females (Ru et al., 2017), substrate type (Qiu et al., 2014) and water velocity (Pan et al., 2015). However, in the current study, there was no effect of the transferred MFs during 72 h of exposure on the locomotor behavior of *A. japonicus*, including the velocity and total distance moved ($P < 0.05$). Similarly, the swimming speed and total distance moved were not affected by MP exposure in the tadpole *Xenopus laevis* (De Felice et al., 2018). On the other hand, nano plastics affect the locomotion behavior of zebrafish *Danio rerio* (Chen et al., 2017).

In conclusion, here we show that synthetic MFs can cause physical harm and accumulate in the internal fluid of an endangered and commercially important species. The transferred MFs altered LZM levels, but neither the oxidative stress indices nor the locomotion behavior of *A. japonicus* were significantly affected. Given the observed fragility of the respiratory tree, other shapes of MPs may transfer to the coelomic fluid as well. This process might disrupt the physiological processes and the development of *A. japonicus*, or it might be a route for the transport of pollutants to the internal viscera of sea cucumbers, which worth further investigation. Also, a long-term experiment will be required to determine whether *A. japonicus* can adapt to MFs accumulation over time.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113606.

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