First evidence of ingestion and retention of microplastics in seahorses (*Hippocampus reidi*) using copepods (*Acartia tonsa*) as transfer vectors

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1. Introduction

Microplastics (MPs) are defined as synthetic solid particles or polymeric matrices insoluble in water with sizes ranging from 1 µm to 5 mm (Frias & Nash, 2019). MPs are characterized by being persistent in the environment due to their low biodegradation, representing a significant hazard to marine ecosystems. These synthetic particles can be formed from a wide range of components but the most widely used plastic material is polyethylene (PE) (Arthur et al., 2009; 2018; Demirors, 2011). According to their origin, MPs can be classified as primary, when they are manufactured for a particular application, and as secondary, when they originate from the weathering of larger plastics (Napper & Thompson, 2020). Global concern has increased over the last years on the detrimental effects of MPs on the environment and organisms but their effects and long-term consequences remain largely unknown (Díaz-Mendoza, 2020; Farrell & Nelson, 2013; Moore, 2008). MPs are a major concern for marine ecosystems since they can be ingested by a wide range of marine species and transmitted through the food web (Cole et al., 2013; Beiras et al., 2018; Bellas et al., 2016, Ugwu et al., 2021). Aquatic organisms can ingest MPs either directly due to confusion with their food or through indiscriminate feeding behavior, or indirectly by trophic transfer (Derraik, 2002; Cole et al., 2013; Kühn et al., 2015; Nelms et al., 2018, Bai et al., 2021). In fish, the incorporation of MPs can produce diverse effects such as feeding disorders, behavior alterations or mortality (Chae & An, 2017; Horton et al., 2018).

Acartia tonsa (Dana, 1849) is an ecologically relevant copepod species. As for many copepod species, *A. tonsa* is widely distributed and located at the base of the food web, being part of the diet of many species (Kwok et al., 2015, Bai et al., 2021). Therefore, the accumulation of MPs by this species may pose a risk to its predators (Carbery et al., 2018).

The long-snouted seahorse *Hippocampus reidi* (Ginsburg, 1933) is a tropical species presenting small home ranges and inhabiting shallow coastal habitats (Rosa et al., 2007). Currently, this species is classified as near-threatened by the IUCN (Oliverira & Pollom, 2017). Seahorses are very sedentary fishes since they usually remain holdfasted to the vegetation or on the substrate because due to their limited swimming capability (Rosa et al., 2007; Delunardo et al., 2013, 2015, 2020). This attribute increases the risk of exposure to pollutants (Delunardo et al., 2013, 2015, 2020). A healthy marine habitat is related to the thrive of seahorses (Vincent et al., 2011), since they are bioindicators of contamination (Delunardo et al., 2013, 2015; Cohen, 2017).

The incorporation, retention, and impact of MPs on seahorses have never been investigated. Therefore, this study aimed to assess the ingestion and retention of MPs by the copepod *A. tonsa* and their further incorporation into *H. reidi* juveniles. The specific objectives of the study were: a) to assess the ingestion of MPs in the prey of seahorses, *A. tonsa*, and b) to evaluate the ingestion and retention of MPs in seahorses using *A. tonsa* as a transfer vector.

2. Materials and Methods

2.1. Seahorses broodstock

Adult seahorses *Hippocampus reidi* were maintained in *ad hoc* aquaria (Planas et al., 2021) at the rearing facilities of Instituto de Investigaciones Marinas (IIM-CSIC) in Vigo (Spain). We selected *H. reidi* as target species due to two main reasons: (1) The species is one of the most traded seahorse in aquariology, and (2) the species is easily cultivated, providing high and rather reliable survival rates, which is very important in experimental challenges requiring reduced variability regarding factors other than those tested. Seawater temperature was maintained constant within an annual temperature

regime of 26 ± 0.5ºC. A natural-like photoperiod regime for the species was applied (16L:8D). Pumped seawater was filtered (5 µm), UV treated, and 10-15% daily exchanged. Water quality was checked periodically for NO_2^-, NO_3^- and NH_4^+/NH_3 content (0 mg L^{-1}). Salinity and pH levels were maintained constant at 38 ± 1 and 8.1 ± 0.1, respectively. Further details are provided by Planas (2021).

2.2. Seahorse juveniles and copepods

A total of 600 newborn juveniles from a single batch were transferred to a 30 L filtered (1 µm) seawater aquarium, maintained at 26ºC and 32 salinity, and fed twice daily on live copepods *Acartia tonsa*. The copepods were cultivated in 500 L tanks at 26ºC and fed on an algal mixture (algal mix) consisting of *Rhodomonas lens* and *Isochysis galbana* (2:1 ratio). Only copepods retained by a 125 µm mesh were offered to seahorses. Further details on seahorse juveniles and copepods cultivation are provided by Randazzo et al. (2018).

2.3. Microplastics

The microplastics (MPs) used for the experiments consisted of polyethylene microspheres of 1-5 µm in diameter (Cospheric, California, EEUU) stained with a red fluorophore (575 mm excitation and 607 mm emission). MPs diameter fell within the optimum particle size for *A. tonsa* (Bartram et al., 1981). The stock suspension of MPs was prepared by adding 1 mg microspheres to 1 L of 1-µm filtered seawater. The resulting suspension was treated with UV light (40,000 µWs/cm^2). The dispersion of the microspheres and the homogeneity of the suspension were guaranteed by adding 3 µL of the surfactant Tween 20.

2.4. Experiment 1: Retention of MPs in fed and fasted copepods.

Acartia tonsa copepods from the cultivation units were transferred into two 4-L beakers (>180 µm; 1 copepod ml^{-1}). Copepods from one of the beakers (FE) were fed on the

algal mix described above, whereas those from the other beaker (FA) were fasted for 6 hours to allow gut evacuation. The content of each beaker was further distributed into two 2-L beakers and the copepods exposed for 60 minutes to two different MPs concentrations (10 and 100 μg L^{-1}; fed copepods FE-10 and FE-100, and fasted copepods FA-10 and FA-100, respectively), at 26ºC and soft aeration.

After the exposure to MPs, 1 L from each treatment (ca. 1,000 copepods) was filtered (180 μm mesh size). The retained copepods were resuspended in clear seawater and maintained deprived of food for 120 minutes at 26ºC. Samples of copepods (>50 copepods per sample) were sequentially taken at different times (1, 15, 30, 60, 90, 120 minutes), fixed in absolute ethanol, and stored in Eppendorf tubes for further observation under the fluorescence microscope.

2.5. Experiment 2: Ingestion of MPs by seahorse juveniles

Copepods resulting from FE and FA treatments in experiment 1 were carefully rinsed and filtered (180 μm mesh size) to avoid the presence of non-ingested MPs in the water. Then, the copepods were offered to *H. reidi* juveniles previously fasted for 24 hours. Twenty-four 10-days old seahorses (17.84 ± 1.96 mm in length) were distributed in four 2-L beakers (6 juveniles per beaker). Each beaker received one of the following types of copepods (1 cop mL^{-1}): FA-10, FA-100, FE-10, and FE-100 μg MPs L^{-1}. Subsequently, six seahorses were collected from each beaker after 20 and 60 minutes of exposure to MPs charged copepods (3 seahorses at each time), fixed in paraformaldehyde (PFA) and observed under a fluorescence stereoscope.

Pellets were not collected and the abundance of microplastics in control seawater was not verified. However, the number of microspheres can be inferred from the manufacturer's data and our own fluorescence calibrations. We regularly check the

amount of particles in the seawater for quality control. The amount of particles in control seawater, filtered through 1 µm and treated with UV light, is usually below 100 particles mL^{-1}, including mainly natural particles.

The skin of seahorses was not translucent enough to allow the direct visualization of ingested MPs under the fluorescence stereoscope. Therefore, the juveniles were depigmented by treating the fish for 2 hours with a solution consisting of 166 µL H_2O_2 at 5% and 100 µL KOH at 0.1% in 750 µL distilled water. Previous assays demonstrated that the treatment did not affect MPs fluorescence.

2.6. Image acquisition and data analysis

Copepods were observed under an upright microscope (DM5500B Leica, Germany) with a HC PL Fluotar 20x/0.5 objective. Seahorse juvenile's images were acquired with a stereoscope (M165FC Leica Germany). All images were captured with a CCD camera (DFC310FX Leica, Germany) with transmitted light and red filter fluorescence illumination modes. FIJI software (Schindelin et al., 2012) was used to estimate the number of MPs in the images. The microspheres that formed aggregates were counted as a function of the fluorescence intensity emitted by the aggregate. The fluorescence area of each aggregate was measured and then divided by the mean fluorescence area of all individually identified MPs, obtaining an estimate of the total number of microspheres in each specimen. Combinations of transmitted light and fluorescence images were made with FIJI software. Merged images allowed us to locate red fluorescence signal from MPs within the body of copepods or seahorses.

A three-way ANOVA was conducted in experiment 1 to determine the effects of MPs evacuation time, MPs concentration, and the feeding condition of copepods (pre-feeding or fasting) on the number of MPs particles counted in copepods. Another three-way ANOVA was conducted in experiment 2 to examine the effects of exposure time, MPs

concentration, and the feeding condition of copepods on the number of MPs present in seahorses. In both cases, residuals analysis was performed to test for the assumptions of ANOVA. Normality was assessed using Shapiro-Wilk's normality test and homogeneity of variances was assessed by Levene's test. Residuals were normally distributed ($p > 0.05$) and variances were homogenous ($p > 0.05$).

2.7. Ethics statement

Fish handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (R.D. 53/2013, 1st February 2013) and the Regional Government Xunta de Galicia (Reference REGA ES360570202001/16/EDU-FOR07/MPO01 and REGA ES360570202001 /15/FUN/BIOL.AN/MPO01).

3. Results

3.1. Retention of MPs in copepods

After 1 minute exposure, the presence of MPs was higher in previously fasted copepods (FA) than in those fed on the algal mix (FE). At that time, MPs were visible at 10 and 100 μg L^{-1} in both FA (1.1 ± 0.7 MPs and 5.4 ± 1.2 MPs, respectively and FE (0.4 ± 0.5 MPs and 2.4 ± 1.1 MPs, respectively) copepods (Figure 1). After 15 minutes of exposure, MPs were only visible at 100 μg L^{-1} in FA copepods (1.8 ± 0.8 MPs). All MPs observed in copepods were located in the mouth, digestive tract and anal segment.

3.2. Retention of MPs in seahorses

After 20 minutes exposure of seahorses to fed (FE) and fasted (FA) copepods submitted to the lower concentration of MPs (10 μg L^{-1}), the juveniles showed the presence of 3 ± 1 and 16.3 ± 4.5 MPs, respectively. Those densities were lower than in juveniles fed on

copepods pre-exposed to the higher concentration (100 μg L^{-1}) (29.3 ± 5.7 and 56.3 ± 3.5 MPs in FE- and FA-copepods, respectively) ($p < 0.05$) (Figures 2 and 3). For both MPs concentrations used in the copepods exposure (10 and 100 μg L^{-1}), the quantity of MPs present in seahorses was higher when fed on FA-copepods ($p < 0.05$) (Figure 3). Regardless of the concentration of MPs used in the copepods exposure, the particles were distributed in the anterior and middle part of the digestive tract (Figure 2).

After 60 minutes of exposure, fluorescent MPs were observed in seahorses fed on FE- or FA-copepods at both initial MPs concentrations (10 and 100 μg L^{-1}). Seahorses fed with copepods pre-exposed to 100 μg MPs L^{-1} presented a higher amount of MPs (29.3 ± 4.9 MPs and 43.3 ± 10.7 MPs with FE- and FA-copepods, respectively) than those exposed to 10 μg MPs L^{-1} (6.7 ± 3.8 and 7.3 ± 3.2 MPs in FE- and FA-copepods, respectively) ($p < 0.05$) (Figure 3). Fluorescent MPs densities found in seahorses fed on FA-copepods for 60 minutes were lower than in those exposed to FA-copepods for 20 minutes ($p < 0.05$) (Figure 3). After 60 minutes, MPs were mainly accumulated close to the anus of seahorses, where discrete spots and aggregates were observed (Figure 4).

4. Discussion

Our results indicate that MPs accumulated along the entire digestive tract of *Acartia tonsa*, from mouth to anal segment. The large variability in the accumulation observed after 1 minute of treatment indicates a different adaptation of copepods to MPs ingestion, suggesting that those results must be taken with caution. Ideally, a longer sampling period (e.g., 5 min) would have been more appropriate. However, we also observed that MPs were shortly retained (< 15 minutes) by the copepods, suggesting fast evacuation under the experimental conditions tested. It is feasible that other types of MPs, concentrations, and shapes will provide different results. Although the ingestion

of MPs by marine organisms, and specifically by zooplanktonic species, has been well documented (Cole et al., 2013; Beiras et al., 2018), much less attention has been paid to retention of MPs in organisms. This is a matter of concern since the harmful effects (i.e., mechanical or biological) of MPs rely on their retention times within the digestive tracts of the organisms (Ory et al., 2018; Yu et al., 2021).

Fasted copepods (FA) showed higher accumulation of MPs than those fed with the algal mix (FE). This might be due to the fact that many copepods are selective suspension feeders, preferring algal cells over inert particles when fed on mixed diets (DeMott, 1988). Also, as reported by Cheng et al. (2020), a low availability of microalgae increases the ingestion of small-size MPs (0.5-2 μm) and the retention time of MPs in the calanoid copepod *Pseudodiaptomus annandalei*, because it facilitates the encounter between copepods and MPs. Other findings also reported that previous fastening (i.e., empty guts) enhanced the accumulation and retention of inert particles in the gut of some filter feeders (Planas & Cunha, 1999). A mixture of inert particles and microalgae would increase gut content fluidity resulting in faster evacuation rates.

Copepods in general and *A. tonsa* in particular are preferred prey for seahorse juveniles and adults both in nature (Planas et al., 2020) and *ex situ* (Blanco & Planas, 2015). Our study reveals the potential trophic transfer of MPs from zooplankton to seahorses when fed on copepods pre-exposed to small-size MPs. The results also suggest that copepods, previously exposed to polyethylene microspheres for more than 15 minutes and subsequently transferred to clear seawater, would evacuate progressively the ingested MPs. It is likely (but further assessment is needed) that the concentration of MPs in copepods will remain more or less stable when maintained in an environment permanently affected by MPs. Hence, seahorse juveniles exposed to those hazardous conditions would be capable to continuously incorporate MPs via prey organisms.

198 However, our findings should not be extrapolated to other types of MPs differing in
199 shape, sizes, and polymeric composition.
200 Seahorse juveniles incorporated and accumulated MPs in some parts or along the whole
201 digestive tract, depending on the experimental conditions. The juveniles exposed for 20
202 minutes to MPs-charged copepods preferentially accumulated MPs from mouth to anus,
203 while those exposed for 60 minutes accumulated the plastic particles near the anus. The
204 last finding is likely due to fast evacuation rates in both juveniles and copepods. Jinhui
205 et al. (2019) suggested that the growth in *Hippocampus kuda* was affected by the trace
206 metals present or adsorbed by MPs but did not observe any significant mechanical
207 ºobstruction of the gut due to MPs accumulation. However, direct comparisons with the
208 present study are not straightforward due to differences in experimental design and MPs
209 characteristics.
210 After long exposure periods (60 min), the seahorses showed lower presence of MPs
211 than in the short (20 min) exposure condition. It might be due to a fast evacuation of
212 plastic particles in the copepods. MPs charged copepods would start evacuating the gut
213 content from their transference to clear seawater. Hence, the accumulation of MPs
214 inside the copepods would be progressively reduced. Consequently, seahorse juveniles
215 ingesting more freshly charged copepods would show a higher accumulation of
216 copepods along the whole gut. Contrarily, the concentration of juveniles ingesting less
217 fresh copepods (i.e., lower MPs charge) would decrease, accumulating the plastic
218 particles on the posterior part of the gut (e.g., near the anus).
219 The presence of free MPs near the anus suggests that seahorse juveniles are not capable
220 to excrete polyethylene microspheres efficiently. It could be due to the strong
221 aggregation and adherence properties of polyethylene particles (Kolandhasamy et al.,
222 2018; Wang et al., 2021). A potential consequence of MPs ingestion by juvenile

seahorses for a long period might lead to mechanical obstructions in the last part of the gut, although this hypothesis needs further investigation.

Currently, the concentration of MPs in the seas range from < 0.0001 to 1.89 mg L^{-1} (Beiras et al., 2020). The concentrations of MPs used in our study were higher but fell within the range of the maximum reported concentrations (2-670 μg L^{-1}). This suggests that wild seahorse juveniles would potentially accumulate a considerable amount of MPs in polluted environments both by direct suction of seawater and by indirect transfer via live prey. Furthermore, MPs abundance in the marine environment is probably being underestimated, with very little information available for particles < 100 μm and with almost no information for particles < 10 μm, indicating that exposure concentrations of MPs in the environment and the resulting potential harmful effects may be even higher than currently estimated (Beiras et al., 2020; Lindeque et al., 2020). MPs shape may influence MPs retention, whereas particle size influence the translocation ability of MPs inside an organism (i.e., from the digestive tract to other tissues) (Santos et al., 2021). MPs may act as carriers of contaminants and potentially expose to risks aquatic organisms.

5. Conclusion

This study provides the first evidence on the indirect incorporation of MPs in seahorse *Hippocampus reidi* juveniles using copepods (*Acartia tonsa*) as vectors. Our results revealed a direct proportional relationship between the concentrations of MPs in prey and in the digestive tract of seahorses. The demonstration of the accumulation of MPs on different parts (depending on the experimental conditions) along the digestive tract of seahorses, either directly due to their ingestion or indirectly by drinking water, is of great concern. However, more research should be performed on the effect of other types

of MPs and on the impact of these pollutants in seahorses as potential bioindicators of pollution and contamination.

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388 **Figure 1.** Copepod previously fasted (FA) exposed to 100 μg MPs L^{-1} after 1 minute
389 was observed under transmitted light (A) and red filter fluorescence (B). Merge image
390 shows MPs signal accumulated in the digestive tract (C). Scale bars represent 100μm.

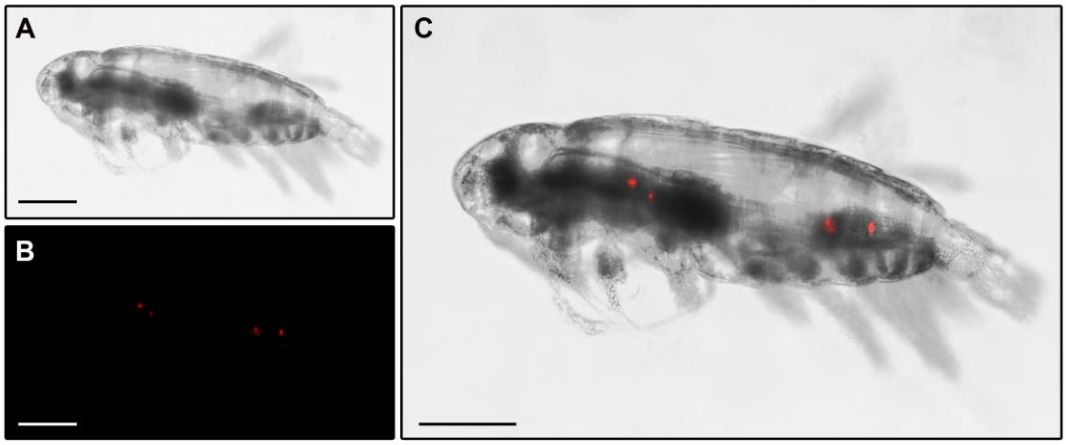

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Figure 2. Seahorse juveniles (*H. reidi*) fed for 20 min on FA copepods previously exposed to 10 (A, B) and 100 μg MPs L^{-1} (C, D). Left panels show seahorses observed under transmitted light and right panels show red light emitted from MPs in the same field of view. Scale bars represent 1mm.

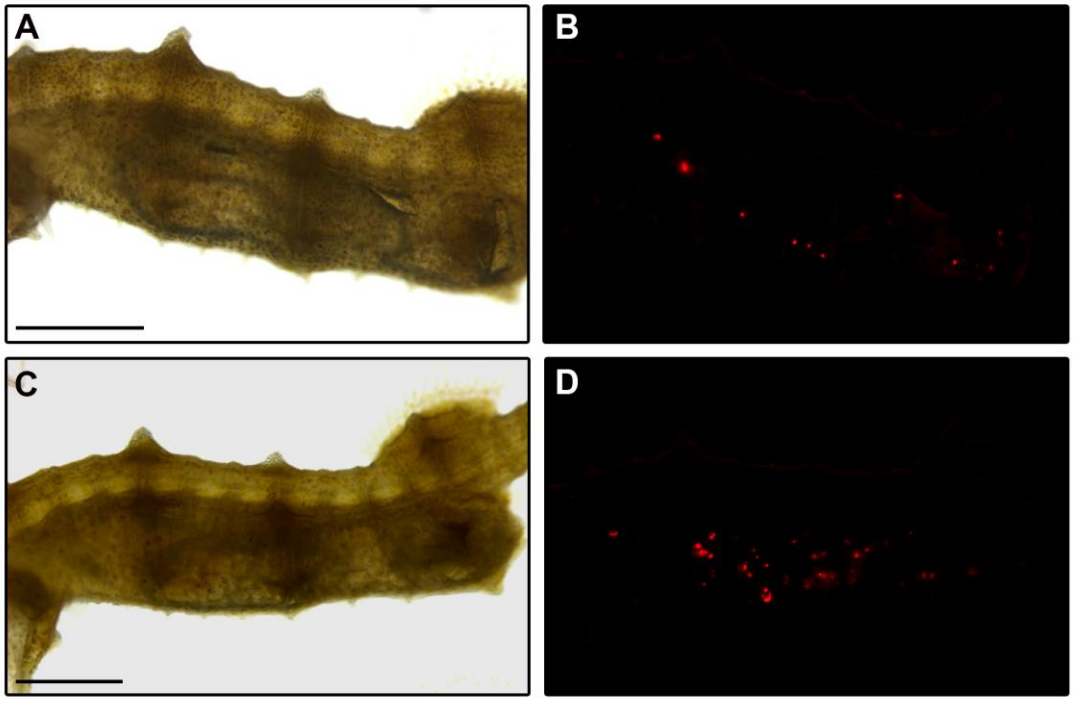

Figure 3. Microplastics present in seahorse juveniles (*H. reidi*) after 20 and 60 minutes of exposure to FA (fasted) or FE (fed with microalgae) copepods maintained with two initial concentrations of MPs (10 and 100 µg L^{-1}. Statistical significance ($p < 0.05$) is shown with asterisks: black asterisk denotes significant differences between groups FA and FE belonging to the same group of initial MPs concentration; red and green asterisks denote differences between the groups of 10 and 100 µg L^{-1} belonging to the same group of copepods FA (red) and FE (green).

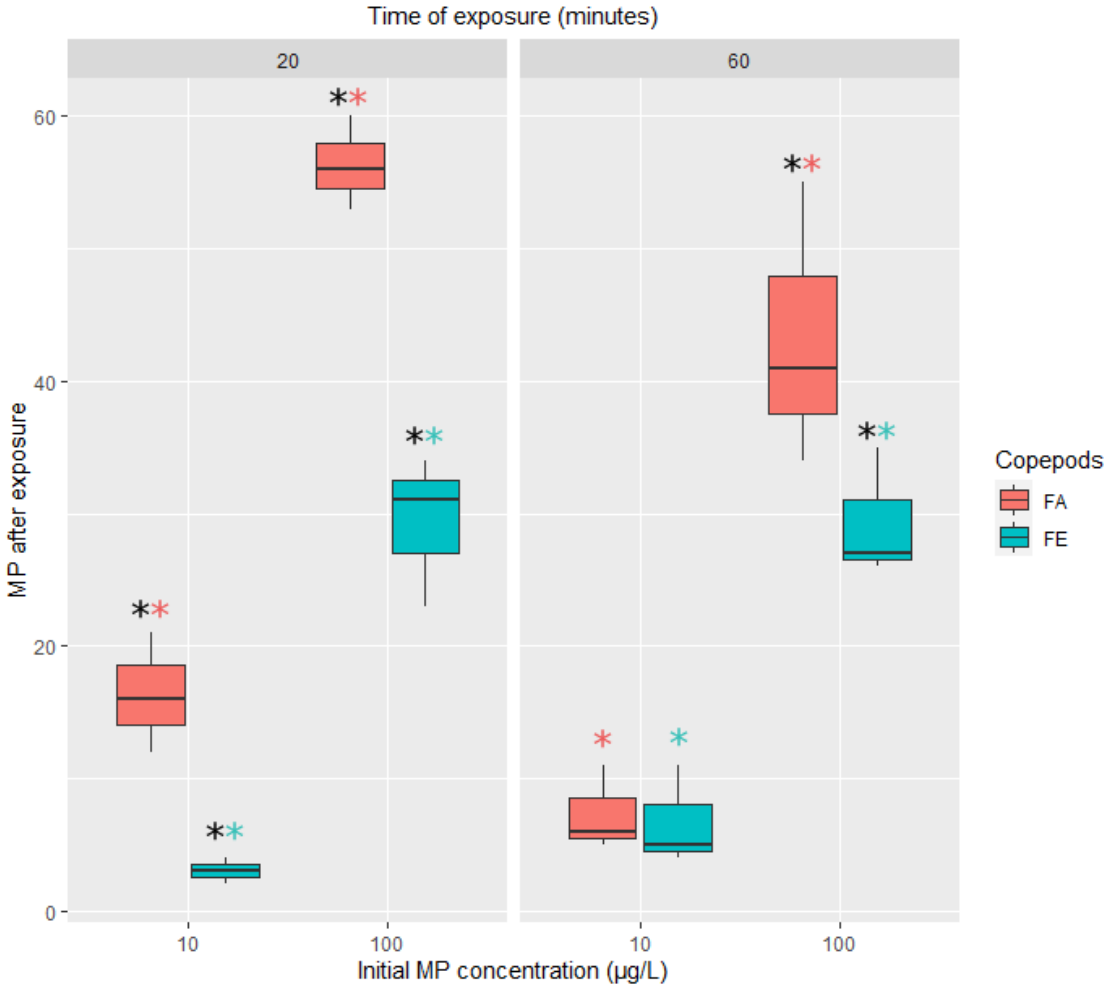

Figure 4. *H. reidi* fed for 60 minutes on FA copepods, previously exposed to 100 μg L^{-1} of MPs. Seahorse was observed under transmitted light (A) and under red filter fluorescence (B). Merge image shows MPs signal accumulated in the last part of the digestive tract, close to the anus (C). Scale bars represent 1μm.