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Microplastics Uptake and Egestion Dynamics in Pacific Oysters, *Magallana gigas* (Thunberg, 1793), Under Controlled Conditions.

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Abstract

Microplastics debris (<5 mm) are increasingly abundant in the marine environment, therefore, potentially becoming a growing threat for different marine organisms. Through aquatic animals, these can enter in the human food chain, and can be perceived as a risk for consumers' health.

Different studies report the presence of particles in marketable shellfish including the world wide commercially grown Pacific oyster *Magallana gigas* (Thunberg, 1793). The aim of this study is to examine the potential risk of microplastics entering in the human food chain through this shellfish species, investigating the dynamics of the uptake, egestion (faeces) and rejection (pseudofaeces) of microplastics in Pacific oysters under controlled conditions.

M. gigas collected from a farm in the San Teodoro lagoon (Italy), were exposed to 60 fluorescent orange polystyrene particles L^{-1} of known sizes (100, 250 and 500 µm). The uptake of each particle size was 19.4 ± 1.1%, 19.4 ± 2 % and 12.9 ± 2 % respectively. After exposure *M. gigas* were left to depurate for 72 hrs, during which 84.6 ± 2 % of the particles taken up were released whilst 15.4 ± 2 % were retained inside the shell cavity. No microplastic particles were found in the animals' soft tissues.

The results of this study, suggest that depuration is an effective method to reduce presence of large microplastic particles, in the size range 100 to 500 µm, in *M. gigas*. Importantly, the

data suggests that the burden that could theoretically be up taken by consumers from these shellfish is negligible when compared to other routes.

Capsule

Microplastic of tested sizes were not retained in the tissues but can be retained in the shell cavity; Depuration is an effective method to reduce microplastics in farmed Pacific oysters

Keywords

Microplastic; Pacific Oyster; Uptake dynamics; Depuration; Consumers risks

1. Introduction

Plastics are ubiquitously present throughout the world's oceans. In 2016 it was estimated that the production of plastics reached 335 million metric tonnes (Mt) globally (PlasticsEurope, 2018). In 2015, 6300 Mt of plastic waste was generated and, if plastic production trends and waste management will remain similar, it is expected that 12,000 Mt of plastic waste will be released to the environment by 2050 (Gündoğdu *et al.*, 2018; Jambeck *et al.*, 2015).

Plastics are believed to be one of the main contributors to ocean pollution with some areas of the ocean presenting very high concentrations, as a result in 2013 it was estimated that a minimum of 268,940 tons of plastics were present in the oceans (Eriksen *et al.*, 2014).

Microplastics are becoming ever more present in the marine environments due to human population growth. Therefore, an increase in this type of pollution is expected over the coming years and decades. Plastics and micro-plastics (particles <5mm in size) are part of everyday life and can be found in many products used daily such as packaging for food and drinks, shopping bags, toothbrushes and cosmetics (Cole *et al.*, 2011; Browne *et al.*, 2011, Hidalgo-Ruz *et al.*, 2012). Microplastics can be classified into primary microplastics which are intentionally produced at a microscopic scale (Costa *et al.*, 2010; Browne, 2015) and secondary microplastics resulting from the degradation of larger plastics into smaller pieces

by environmental processes such as weathering and photo-oxidation (Mathalon and Hill, 2014; Gewert *et al.,* 2015).

Because primary microplastics are present in cosmetics and medical applications, a major source in the sea and fresh water bodies is waste water from depuration plants (Browne *et al.,* 2011, Cole *et al.*, 2011; Duis and Coors, 2016, Carr *et al.,* 2016).

Microplastics have been considered to be dangerous for aquatic organisms' health (Alomar, 2017). Indeed, their accumulation by ingestion can lead to increased exposure to pollutants and pathogens, and effects on physiological activities linked to nutrient uptake, growth and survival (Browne *et al.,* 2011; Sussarellu *et al.*, 2016; Fendall and Sewell, 2009; Van Cauwenberghe and Janssen, 2014).

Nonetheless, when environmental toxicity tests were performed in different marine invertebrates, for example in larvae of *Tripneustes gratilla* (Linnaeus, 1758) exposed to 10 - 45 µm microspheres and *Mytilus edulis* (Linnaeus, 1758) exposed to microspheres with diameters between 3 and 90 µm, it became apparent that only very high concentrations of microplastics (10,000 times higher than the maximum concentration of microplastic particles currently found in the sea water) generated significant adverse physiological effects (Duis and Coors, 2016). Still, some considerations would warrant caution since very high concentrations of microplastics have already been observed at some sites; plastics are extremely persistent in the environment and, due to further fragmentation, their presence is expected to further increase (Auta, 2017).

Von Moos *et al.,* (2012) studied the effect of exposure and ingestion of microplastics (≤80µm) in Blue mussel (*Mytilus edulis,* Linnaeus, 1758). These authors reported that the smallest particle sizes were accumulated in gills and digestive gland with a consequent strong inflammatory response and a lysosomal membrane destabilization. Unfortunately, no information on excretion was provided by these authors and conclusions on the fate of the larger particles cannot be made. Cole *et al.,* (2011) investigated the presence of microplastics

(between 1 and 10µm) and their effect on food intake and growth of Pacific oyster larvae. They found that microplastics were ingested with only limited impact on feed intake and no consequences on growth rates being observed. Van Cauwenberghe and Janessen, (2014), investigated the presence of different microplastics particles (size class 5-10, 11-15, 16-20, 21-25, >25 µm) in farmed blue mussel and Pacific oyster, showing that these were present in both species at concentration of 0.36 ± 0.07 particles g^{-1} and 0.47 ± 0.16 particles g^{-1} soft tissue, respectively. The same authors also depurated animals from the same batches for 72 hrs observing a significant reduction in the abundance of microplastics, concluding that although depuration was an effective procedure, the consumption of farmed bivalves could potentially represent a risk to consumers' health. Nonetheless, Wright and Kelly (2017), in their review, report that there is still no clear evidence that the absorption of microplastics has a direct impact on human health, but that their accumulation could exert dose-dependent toxicity, due to the leaching of other pollutants or the presence of pathogens on their surface, therefore suggesting that the assessment of exposure levels is of fundamental importance.

Still, the concomitant evidence of microplastics being accumulated in bivalve soft tissue and the presence of wastewater effluent (one of the major sources of microplastics in the environment) in the same water catchment areas as shellfish farming activities deserves further studies (Rochman *et al.,* 2015). Indeed, Sussarellu *et al.,* (2016) studied possible influence of microplastics (2 and 6 µm) on the physiology of Pacific oysters, finding that individuals exposed to microplastics showed lower fecundity, possibly linked to the substances leached by the microplastics during digestion process if not directly caused by their accumulation. This study also indicated that although microplastics were observed in the digestive system, no tissue accumulation was observed, therefore suggesting an efficient egestion process.

The presence of microplastics in commercially relevant bivalves, including Pacific oysters, has been reported by different studies (Van Cauwenberghe and Janessen, 2014, Li *et al.,*

2015, Cole and Galloway, 2015, Phuong *et al.*, 2018, Sussarellu *et al.*, 2016, Fernández *et al.*, 2018, Von Moos *et al.*, 2012, Pont *et al.*, 2016, Silva *et al.*, 2016). Two main objectives have been pursued by previous investigations: 1) determination of the presence and the abundance of microplastics in individuals collected from the wild, farms and retailers to establish potential risks for consumers; 2) the determination of the potential adverse effects to animals' physiology caused by the exposure to plastics under controlled conditions.

However to date, there is still limited knowledge on the relationship between plastics uptake and egestion (Van Cauwenberghe and Janessen, 2014). Therefore, the first aim of this present study was to investigate the adult oysters' egestion dynamics after exposure to known concentration of microplastics under controlled conditions. Moreover, previous studies have so far used microplastics of sizes comparable to phytoplankton cells. However, in the marine environment, microplastics are present in sizes often larger than microalgae cells and there are evidence suggesting that bivalves could potentially up-take particles as large as 500 µm (O'Donohe and McDeromtt, 2014). Still, no information on the ability of oysters to uptake, retain and egest larger particles is currently available. Consequently, the second aim of this study was to determine whether larger particles had the potential to remain in the marketable product post depuration by employing sizes larger than those commonly used in previous microplastics absorption studies. The size classes of 100 ± 7.42, 250 ± 23.2 and 500 ± 52,34 µm were chosen because Van Cauwenberghe and Janssen (2014), found that *Crassostrea gigas* reared in the Atlantic Ocean (average shell length of 9.0 ± 5.0 cm), showed a prevalence of microplastics size > 25 µm, and because studies on mussels and Pacific oysters so far were focused only on microplastics of a size comparable to phytoplankton or in general at size between 0.5 and 90 µm (Sussarellu *et al.*, 2016, Cole and Galloway, 2015, Van Cauwenberghe *et al.*, 2015, Farrell and Nelson, 2013, Browne *et al.*, 2008, Von Moos *et al.*, 2012), without taking in to account that in the marine environment microplastics are present

in different sizes and adults' Pacific oysters can uptake larger size microplastics from the environment.

2. Materials and Methods

2.1. Pacific Oyster source and experimental set-up

Pacific oysters (20 oysters 85 ± 2.3g/ind.) were collected from a farm in the San Teodoro Lagoon (Italy) (40°48'39.18''N, 9°40'24.42''E), and kept in a cold box until arrival to the laboratory. Oysters were then transferred to an aerated rectangular tank and left to acclimatize for 48hrs at 22°C temperature and 36 ppm salinity (Choi *et al.*, 2008). For the purpose of this study, oysters were individually deployed in individually deployed in 20 glass spherical aquariums of 1.5 L, filled with filtered sea water.

With the aim to keep the water in movement each aquarium was supplied with an air-stone connected to a valve and an air pump. Water temperature, salinity and dissolved oxygen were monitored and maintained (by daily water exchange) respectively at 22°C, 36 ppm and 8.5 mg/L.

Preliminary trials were performed to determine both the level of aeration required and the most suitable type of microplastics polymer. For this purpose, three polymers of the following densities were tested: polystyrene 1.04-1.1 g/cm^3; polyamide 1.12-1.15 g/cm^3; polycarbonate 1.20-1.22 g/cm^3 (Avio *et al.*, 2016, Enders *et al.*, 2015). With the aim to keep the microplastics beads suspended in the water column to maximise their chances to be filtered by the oysters, batches of 30 microplastics per polymer were deployed to an experimental tank and aeration was adjusted by a valve. Once the appropriate aeration was identified by observing the microplastics distribution on the water column, the ability of the chosen polymer to withstand the tissue digestion procedure (Li *et al.*, 2015) was tested. This was conducted using a sterile container containing soft tissues of 3 Pacific oysters (80 ± 3.5 g/ind.) plus 9 plastic beds per size class (100 ± 7.42, 250 ± 23.2 and 500 ± 52,34 µm) of the

157 microplastics chosen for the study (3 replicates). The soft tissue was covered with hydrogen
158 peroxide 15%, this was added until the oyster was completely digested (Avio *et al.*, 2015).
159 Once the oysters were digested the remaining solution was filtered using 47 mm Whatman
160 GF/F filters (0.6 – 0.8 µm) and then analysed under the dissecting microscope (Leica Mz8).
161
162 2.2. Microplastics
163 The selected microplastics were fluorescent polystyrene microspheres purchased from
164 Degradex Hopkinton (MA 01748). These particular beads were selected because of their
165 colour (fluorescent orange with Excitation/Emission 530/582 nm) and because their density
166 was similar to seawater (UNESCO,1981, Capolupo *et al.*, 2018).
167 Three microplastics sizes were used: 100 ± 7.42, 250 ± 23.2 and 500 ± 52,34 µm (Fig. 1A)
168 and 600 microplastics of each size, were individually counted under a stereo microscope,
169 using an UV lamp (Surenhap 100 LED) to enhance fluorescence (Fig. 1B), and micro-
170 dissecting tweezers (World Precision Instruments, FL 34240-9258 USA).
171 Beads were then allocated (thirty beads per size) to twenty 1.5 ml Eppendorf tubes, (Fig. 1C).
172
173 2.3. Exposure and Microplastics uptake
174 The experiment was carried out in 2 parts: 24hrs exposure (Cole and Galloway, 2015) and
175 72hrs depuration (Van Cauwenberghe and Janessen, 2014). During the first 24hrs
176 experimental individuals (n=20) were individually exposed to 30 Microplastic particles of
177 each size (100, 250 and 500 µm) with a density of 60 particles per litre. This particles density
178 despite being higher than the ones commonly reported in sea water (De Lucia *et al*., 2014)
179 was chosen for analytical and practical reasons.
180 At the end of the exposure period the aeration was stopped and each oyster was collected
181 using long tweezers, oysters and tools were carefully observed using a UV lamp to increase
182 beads fluorescence and washed taking care that no microplastics adhered to the oysters' shell

and to the tools used. The water used for the exposure was, at this point, filtered through a 47 mm GF/F filter using a filtration unit Millipore and a vacuum pump. Again, all filtration equipment was checked for the presence of adhered beads. Post filtration each filter was individually stored inside labelled 50 mm petri dishes. Uptake was measured subtracting the final number of beads recovered onto the filters from the initial number used for exposure.

2.4. Depuration and egestion

The oysters collected after exposure were transferred to a new tank, again filled with 1.5 L of filtered sea water. Aeration was not supplied in order to avoid faeces and pseudo-faeces mixing.

At 24hrs intervals over a total of 72hrs, each oyster was removed from each tank using the same procedure described earlier, and transferred to a new tank under the same environmental conditions.

The water left in the original tank during the 24, 48 and 72 hrs after exposition, was filtered and beads counted using the same procedure described before.

Finally, at the end of the trial (72 hrs after exposure) oysters were collected from the experimental tanks and externally washed and dissected taking care that the water contained in the shell cavity was stored in a plastic tray.

The Digestive gland, gills and mantle of each oyster were dissected, washed and placed in labelled sterile containers. The water contained in the shell and the water used to wash the tissues was collected and filtered as described previously.

All dissected tissues of each individual were digested using hydrogen peroxide 15%, at room temperature of 22°C for 7 days, and the resulting digestate was filtered as described previously.

2.5. Statistical Analysis

Prior to analyses, percentage data were arc-sine transformed, and all data were checked for normality and homogeneity of variance. Uptake and residual microplastics post depuration data were analysed by one-way ANOVA followed by post-hoc Tukey's Multiple Comparison tests where significant differences occurred. Egestion over time for particles of all sizes was analysed by general linear model followed by a Tukey post-hoc test where significant differences occurred.

Statistical analyses were performed using Minitab v.18 with a significance level of 5 % ($p < 0.05$). All results are presented as mean ± SE.

3. Results

3.1. Microplastics uptake

At the end of the 24 hrs exposure, the uptake (% of missing beads) of the different sizes (100, 250 and 500 µm), was 19.4 ± 1.1%, 19.4 ±2% and 12.9 ± 2% respectively. No significant difference in uptake between the microplastics of 100 and 250 µm was observed, however beads of 500 µm in size had a significant lower uptake when compared with the others sizes ($P = 0.009$) (Figure 2).

3.2. Depuration and egestion

Table 1 illustrates the percentage of microplastics recovered from the depuration water, and tissues at the different time points over the depuration period. A significant effect of time ($p < 0.001$) and a significant interaction between time and treatment ($p < 0.02$) was observed. The excretion of microplastics beads of all sizes was significantly higher during the first 24 hrs in comparison with the later time points. Furthermore, no significant difference was recorded in the excretion of microplastic particles of 100µm and 500µm between 48 and 72 hrs of depuration, whilst significantly more beads of 250 were released after 48hrs in comparison to 72hrs of exposure. (Fig. 3).

Although the vast majority of ingested microplastic particles were released during the 72hrs of depuration, 17.7 ± 3.8, 16.7 ± 2.4 and 5.4 ± 2.7 % of microplastic particles of 100, 250 and 500 µm respectively were still present in the water contained inside the shell cavity. At this location a significant difference in the abundance of each particle size class was observed, with the largest size class being significantly less abundant than the other two ($p = 0.007$) (Fig. 4). Importantly, no microplastic particles were found in the digestive gland and in the other tissues post digestion.

Taking into account each time step there was a decreasing egestion of microplastic particles during the depuration time: 63.9 ± 3%, 17 ± 2.2% and 3.7 ± 0.9 % in 24, 48 and 72 hrs, respectively. Only 15.4 ± 2% of the microplastic particles were retained within the oysters after 72 hrs of depuration (Tab. 1).

4. Discussion

The aim of this study was to investigate the uptake and egestion dynamics of known sizes (100, 250 and 500 µm diameter) of microplastic particles in Pacific oysters, during a 24hrs exposure and a subsequent 72hrs depuration period. Depuration is a common practice in bivalve aquaculture whereby bacteria are egested to comply with European food safety legislation (regulation 853/2004, 852/2004 and 2073/2005) (Who, 2019, Martínez *et al.*, 2009, Doré and Lees, 1995). In this study, Pacific oysters showed an efficient egestion rate, egesting 84.6 ± 2 % of the microplastic particles taken up, while only the 15.4 ± 2 % of beads taken up were retained within the shell cavity, post depuration.

To date, studies on microplastic uptake have been conducted mainly to investigate their potential negative physiological effects on marine live, including bivalves, or to establish whether animals entering the human food chain could be a carrier of these particles and therefore represent a risk for consumers (Sussarellu *et al.*, 2016, Fernández *et al.*, 2018, Von Moos *et al.*, 2012, Pont *et al.*, 2016, Silva *et al.*, 2016, Van Cauwenberghe and Janessen,

2014). The main difference between these approaches has been the controlled nature of the studies. The former employed controlled conditions (known density, type and size of the microplastics employed), whilst the latter focused on the abundance of plastics in marketable products without considering levels of exposure, uptake or the nature of the polymers.

In contrast, our study investigated both the uptake and egestion dynamics under controlled conditions to more robustly describe the fate of microplastic particles of 100 to 500 µm diameters during exposure and depuration therefore contributing to the collective knowledge on these dynamics in shellfish produced for human consumption.

Amongst the studies focused on the risks for consumers, the one conducted by Van Cauwenberghe and Janessen (2014) provides the only comparable platform for the interpretation of the results presented here. Comparison of the studies shows a slight difference in egestion rate post-depuration (74.5 % vs 84.6 ± 2 %), this can be attributed to the difference in materials and diameters of the particle used and by the food sorting mechanisms of the Pacific oysters which discriminates not only based on size but also based on chemical cues present on the surface of the particles (Kiørboe, *et al.,* 2012, Ward *et al.,* 1997).

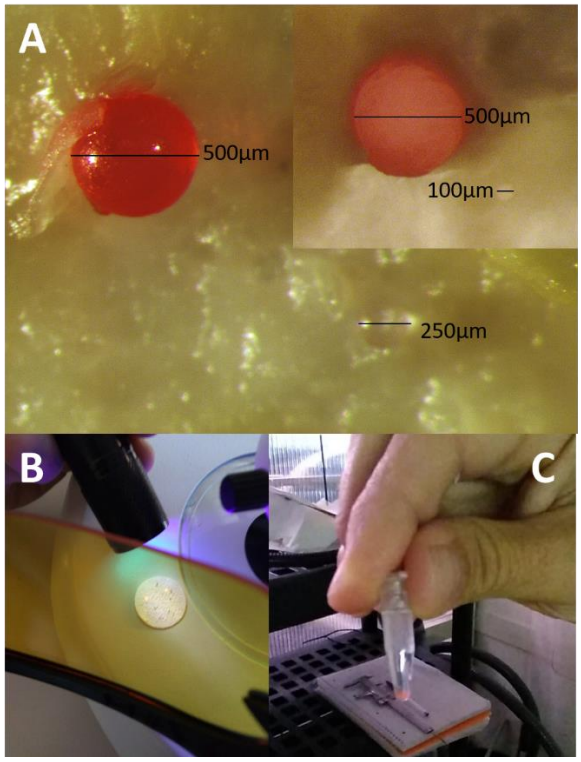
In this study no microplastic particles were observed within the oysters' tissues, while in the Sussarellu *et al.,* (2016) study, microplastic particles were found in the stomach and the intestine of Pacific oysters. This can be attributed to the difference in the particle size used (100, 250 and 500 vs 2-6 µm), and it is possible that the *C. gigas* food sorting mechanisms recognise only the smaller size as a food source due to similarity in size with phytoplankton (Ward and Shumway, 2004). However, different studies point out that bivalve can ingest larger particle size. For instance, blue mussels can ingest early larval stages of sea lice, *Lepeoptheirus salmonis* (Kröyer 1837), with an average size of roughly 500 µm. Furthermore, during a microplastics survey conducted in the Dutch North Sea, the presence of large plastics (up to 5mm in size) was also observed in Pacific oysters (Molloy et al., 2011, O'Donohe and

McDeromtt, 2014, Leslie *et al.,* 2013). Our results suggest that these larger particles could probably be filtered by the oysters but, instead of being ingested, they are retained within the shell cavity by adhesion. Therefore, with the assumption that in the marine environment microplastics of different size have the potential to be accumulated in marketable bivalves (Andardy 2011, Koelmans *et al.,* 2015), the present study further clarifies the uptake and egestion dynamics of larger particles and the associated potential risks for consumers. Indeed, microplastic may not necessarily have to be ingested to represent a potential exposure risk to consumers as adhesion to external tissue may still be considered as a vehicle for trophic transfer.

Importantly, during the depuration period, microplastic particles were observed in faeces and pseudo-faeces, but it is not possible to conclude here that the beads have been ingested, because these were not observed within the digestive system. Further work focused on the ingestion and excretion of microplastic particles of different sizes class, including particles larger than microalgae cells, should be conducted to estimate gut transit time of these particles.

In conclusion our data, taken together with results from other studies, strongly indicate that *M. gigas* could be a carrier of different microplastic sizes in the human food chain, not only through the absorption and inclusion in tissues (Bricker *et al.,* 2014, Van Cauwenberghe and Janessen, 2014, Li *et al.*, 2015, Rochman, *et al.,* 2015, Wright and Kelly, 2017, Bouwmeester *et al.,* 2015), but also through the adhesion of these particles in different parts of the internal cavity of the oysters shell. Nonetheless, the exposure density of 60 microplastics L^{-1} used in this study, is higher than the density of microplastic particles (<5 mm) commonly reported in coastal Mediterranean Sea areas 5 $*10^{-4}$ microplastic particles L^{-1} (De Lucia *et al*., 2014). Assuming that the uptake for all sizes observed in this study (16.2 ± 1.2 %) is applicable to the wider farming context, the number of particles filtered by each individual would be 1.2 $*10^{-4}$, which would become 4.3 $*10^{-5}$ per individual after 24 hrs depuration. This final

313 microplastic burden can be considered lower if compared with the number of microplastic
314 particles found by Schymanski *et al.*, (2018) contained in drinking water (from 11 ± 8 to 118
315 ± 8 particles L^{-1} depending on the type of package). Therefore, the risks for consumers can be
316 considered negligible for the particle size tested if compared to the amount of microplastic
317 particles that can be uptaken in everyday life.
318 Pacific oysters are farmed world-wide for human consumption, and microplastic particles are
319 widely distributed in the environment and therefore available to filter feeders. However, after
320 depuration the number of microplastic particles decreased significantly suggesting that this
321 standard procedure is an effective method to reduce the presence of larger microplastic
322 particles in marketable Pacific oysters even when no depuration would be compulsory due to
323 sanitary reasons such in the case of class A waters.
324



325

326 *Figure 1* **A.** Different Microplastic particle sizes used during this study. Picture was taken on a 47mm GF/F filter B. 500 µm
327 Microplastics on a 25mm GF/F filter with fluorescence enhanced by a UV light. **B**. Microplastics with fluorescence enhanced

using an UV lamp **C**. Microplastics mix composed by 30 Microplastics per size class (100, 250 and 500 µm) ready to be deployed for the exposure trial.

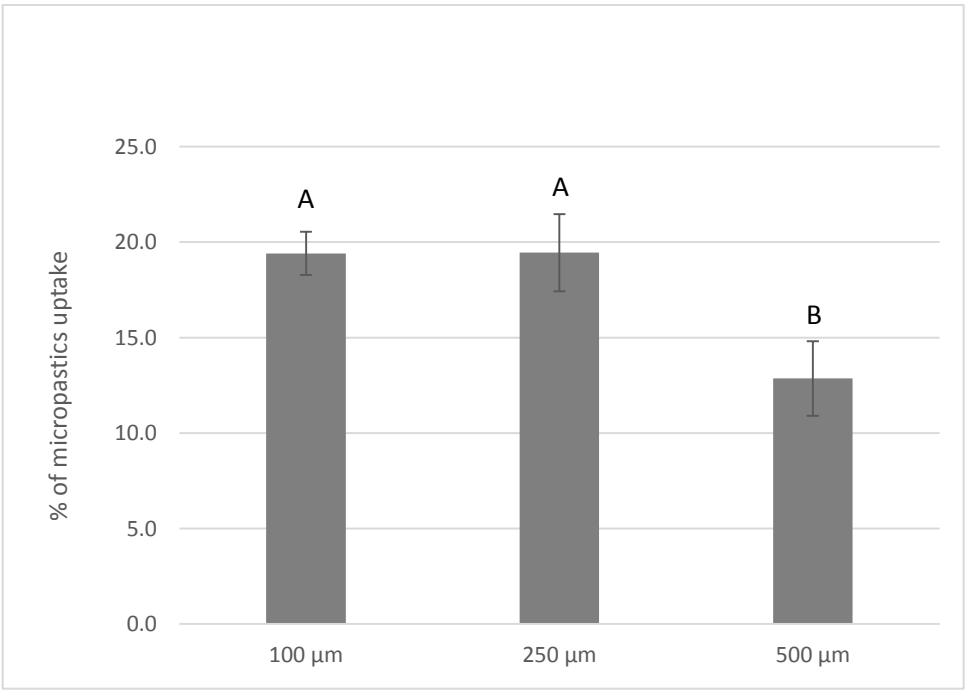
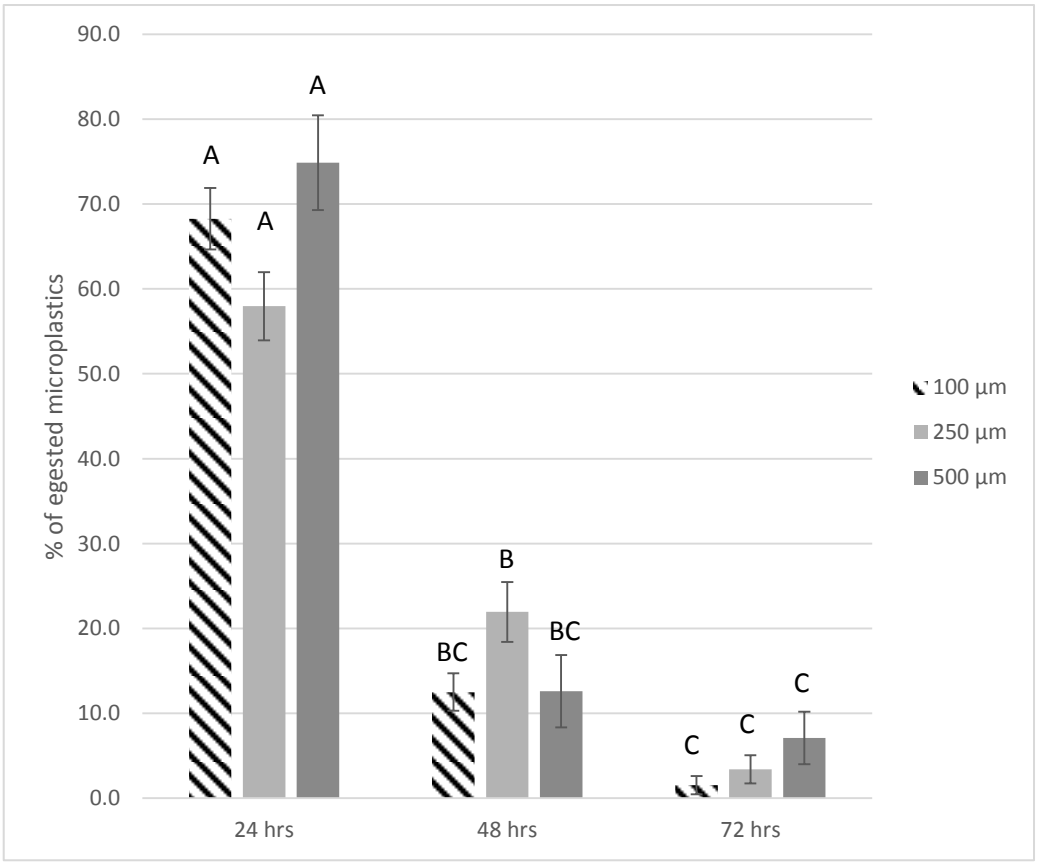


Figure 2 Uptake of the different Microplastic particle size classes from ambient water. Significant differences (P value > 0.05) are showed by different letters, results are presented as mean ± SE; n=20.

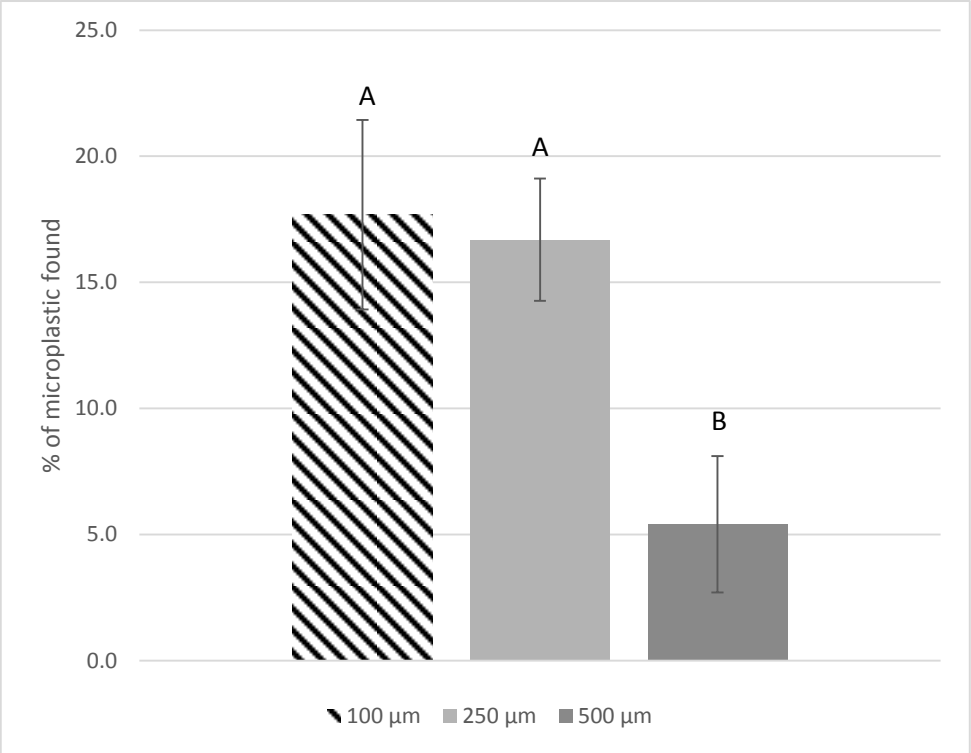


335

336 *Figure 3* Egestion dynamics of the different microplastic particle sizes. Significant differences (P value > 0.05) are showed

337 by different letters, results are presented as mean ± SE; n=20.

338



339

340 *Figure 4* Residual microplastic particles of the different sizes post depuration. Significant differences (P value > 0.05) are

341 showed by different letters, results are presented as mean ± SE; n=20.

342

343 *Table 1* Summary of the percentages of egested during 72 hrs depuration, and non-egested post depuration,
344 Microplastics, both divided by sizes and as a mix of beads (100, 250 and 500 µm).

Microplastics beads egested and non-egested in:	***100µm*** **%**	***250µm*** **%**	***500µm*** **%**	***Mix*** **%**	***Mix*** **%**
24 hrs	*68.3 ± 3.6*	*58 ± 4.0*	*74.9 ± 5.6*	*63.9 ± 3.0*	
48 hrs	*12.5 ± 2.2*	*21.9 ± 3.5*	*12.6 ± 4.3*	*17 ± 2.2*	*84.6 ± 2*
72 hrs	*1.5 ± 1.1*	*3.4 ± 1.7*	*7.1 ± 3.1*	*3.7 ± 0.9*	
Internal cavity	*17.7 ± 3.8*	*16.7 ± 2.4*	*5.4 ± 2.7*	*15.4 ± 2*	*15.4 ± 2*
Digestive gland	*0*	*0*	*0*	*0*	
Other soft tissues	*0*	*0*	*0*	*0*	

345

346

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353

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