

## Article

# The Influence of Polystyrene Microspheres Abundance on Development and Feeding Behavior of *Artemia salina* (Linnaeus, 1758)

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**Abstract:** In the present study, it has been evaluated how 10 µm of polyethylene microspheres can be ingested by *Artemia salina* (Linnaeus, 1758) larvae within the first 7 days of the life cycle, and the impact on their health. Twelve *A. salina* larvae (instar I) groups were exposed to different microplastics (MPs) concentrations (0–1–10–10<sup>2</sup>–10<sup>3</sup>–10<sup>4</sup> MPs/mL), with and without *Dunaliella salina* as a food source. The results highlighted that *A. salina* larvae ingest MPs in relation to the exposure times in a dose-dependent manner and are significantly influenced by food availability. The highest contamination found was 306.2 MPs/individual at 10<sup>4</sup> MPs/mL exposure without a food source. No MPs were found in the presence of the food source from 1 to 10<sup>2</sup> MPs/mL, while contamination was detected at all concentrations of MPs without a food source. The worst effect on the developmental stages was evaluated at 168 h with a food source, with a delay compared to the control of I and II instars at 10<sup>3</sup> and 10<sup>4</sup> MPs/mL, respectively. Furthermore, microalgal feeding was significantly reduced for about 50% in the presence of 10<sup>4</sup> MPs/mL. These results highlight that aquatic microplastics pollution could affect the *A. salina*'s feeding behavior and life cycle.

**Keywords:** plastics pollution; brine shrimp; crustaceans; polyethylene; aquatic pollution; developing stage

## 1. Introduction

In the last decade, microplastics (MPs) pollution in aquatic environments has become one of the most prevalent ecosystem problems [1,2]. Due to their small size, MPs debris can be easily moved by environmental factors such as coastal winds, river flows or marine currents [3,4]. Moreover, this pollution can occur through fragmentation of larger plastic debris by physical, chemical and mechanical factors, due to their long residence times in nature [5]. Once in the water, plastic debris can float or sink accordingly with their density, which can also be influenced by the biological factors such as biofouling [6,7]. For these reasons, increasingly large quantities of MPs particles are found in the marine environment in the entire water column and consequently come into contact with living organisms [8–10]. The primary or secondary ingestions of MPs by the principal taxa of aquatic organisms has been confirmed by several field studies, in gelatinous plankton [11–13], mollusks [14–16], crustaceans [17,18], bone fish [19–21], cartilaginous fish [22–24], reptiles [25,26], seabirds [27,28], cetaceans [29–31], and other aquatic organisms. Many of the

organisms in which contamination was detected, are also commercial interest species destined for human consumption, captured [32–34] or farmed [35–37], bringing this pollution to a relevant level of consumers interest.

Zooplankton has a key role in the aquatic trophic webs [38–42]. This importance is mainly related to the use of these organisms in the marine aquaculture industry, in which planktonic organisms such as rotifers and brine shrimp (*Artemia* sp.) are the primary living food source in the early stages of life of many farmed species [43–46]. Based on the ascertained biomagnification phenomena related to marine zooplankton [47–49], the deepening of the knowledge on MPs pollution in these organisms represents an important step for marine environmental and seafood-related research.

Brine shrimp, *Artemia salina*, is a small crustacean living in extreme environments such as highly saline lakes and pools [50–52] that represents an important food source for many aquatic animals. Beyond its already mentioned important role in aquaculture, due to their small size, life cycle and simplicity of its breeding, the genus *Artemia* represent a good model organism for experimental studies, mostly related to environmental toxicology [53], lately also connected in MPs toxicity research [54–56]. In natural environments, brine shrimps mainly feed on microalgae through filtration. In the same way, in aquaculture, *A. salina* previously enriched with live microalgae, yeasts or commercial enrichers is administered to the farmed organisms to transfer optimal nutrients [57,58]. Despite the known importance of *A. salina* in the aquaculture field and as an experimental model organism, few studies have been conducted on the effects of MPs on this organism, including behavioral aspects. Moreover, the interaction between microalgae, MPs and brine shrimps are far from being fully understood, and more insights are required.

In our study, we showed how during the first 7 days of the life cycle, 10 µm of Polybead® Blue Dyed Microspheres can be ingested and egested by *A. salina* larvae under *Dunaliella salina* feeding, or without a food source and the relative impacts on their behavior, health, and development.

## 2. Materials and Methods

### 2.1. Microplastics Used and Experimental Solutions

Based on the environmental feeding habits of *A. salina*, we MPs of dimensions as close as possible to the microalgae used in this study that it normally fed on. We selected the microalgae (*D. salina*) as a food source with an average size of 10 µm; therefore, our choice for the MPs size fell on the same dimension, to avoid a possible food preference based on a dimensional factors.

The MPs used in this study were 10 µm of Polybead® Blue Dyed Microspheres purchased from Polysciences in a 2 mL package in a 2.5% aqueous suspension at  $4.55 \times 10^7$  particles/mL. The blue color was chosen for a better contrast against the organisms, being clearly different from any crustacean or algal structure used in the study. According to the previous environmental studies that reported microplastic concentrations for surface coastal waters [59,60] and with the considerations made by Wang [55] on the accumulation and the real estimation of MPs dispersed in seawater, we chose to test five concentrations of MPs (1, 10,  $10^2$ ,  $10^3$  and  $10^4$  MPs/mL) for our experiments. A diluted MPs stock solution was prepared in sterilized artificial marine water and used to start the experiment and for each solutions replacement to avoid any problems related to sedimentation, aggregation or adhesion of MPs to the surfaces (REF). All solutions counts were performed in triplicate before the administration during experimental phases, using a Leica DM6B microscope at 40× magnification in a Bürker Türk counting chamber (Marienfeld, Lauda-Königshofen, Germany).

### 2.2. *A. Salina* Hatching and Feeding

*A. salina* larvae were obtained from Blue Line Artemia Cyst (Acquariomania, Macerata, Italy) and hatched in a 1 L glass beaker in sterilized artificial marine water (Dissolved oxygen-DO 6.6 mg/L; salinity 37 ppt; pH  $8.2 \pm 0.1$ ) made with Caledonia Reef Salt

(ReeFlowers Aquarium Solutions, Başakşehir/İstanbul, Turkey) and ultrapure water (Milli-Q; Merck, Darmstadt, Germany). Strong aeration was provided in the hatching phase, with a temperature of  $26 \pm 0.5$  °C. After the hatching and for all the experimental time, a pure strain culture of live *D. salina* was provided as a food source. Microalgae were cultured at  $20 \pm 1$  °C, salinity 30 ppt, with a 4500 lux 24-h light photoperiod in f/2 medium. The microalgae concentration per ml was counted in triplicate for all solutions before the administration as verification during experimental phases of feeding rate, under a Leica DM6B microscope at  $40\times$  magnification in a Bürker Türk counting chamber. DO, salinity, temperature and pH were measured using a multiparameter probe as reported by Sanfilippo [61].

### 2.3. Experimental Design

The experimental procedures were performed at Center of Experimental Fishpathology of Sicily, Messina, Italy (CISS) [62–64]. After 24 h of incubation, the obtained *A. salina* nauplii (instar I) were collected and counted under a Leica M205 C (Leica Microsystems, Milan, Italy) stereoscope in a S50 Sedgewick Rafter counting chamber (Marienfeld, Lauda-Königshofen, Germany). Twelve experimental groups, in triplicate, were transferred with an automatic pipette in 10 mL glass test tubes with different concentration of MPs (0-1-10-10<sup>2</sup>-10<sup>3</sup>-10<sup>4</sup> MPs/mL), and treated as two experimental groups with the *D. salina* food source (Group A) and without the food source (Group B). Experimental treatments are reported in Table 1. A number of 60 *A. salina* nauplii (10 nauplii/mL, total solution volume 6 mL) was placed in each replicate. For the entire experiment, the glass tubes were placed in a rack and maintained in laboratory for 168 h at  $24 \pm 1$  °C, with a 12D:12N photoperiod. Seven time points were set at 0-6-12-24-48-96-168 h. For every time point, five specimens per each replicate were sampled, clarified in 4% buffered formalin, and photographed under a Leica DM6B (Leica Microsystems, Milan, Italy) microscope equipped with a Leica DFC7000T (Leica Microsystems, Milan, Italy) Imaging system and LAS X software for further analysis. The total length (from the naupliar eye to the anus) of sampled specimens was recorded for each treatment at each time point. Mortality was recorded daily and collected with a glass pipette and transferred to a well slide under microscope to be analysed and photographed. The experimental solutions were replaced by 90% daily for each treatment to provide constant feeding to brine shrimps and to avoid MPs sedimentation [55].

**Table 1.** Experimental Treatments Summary.

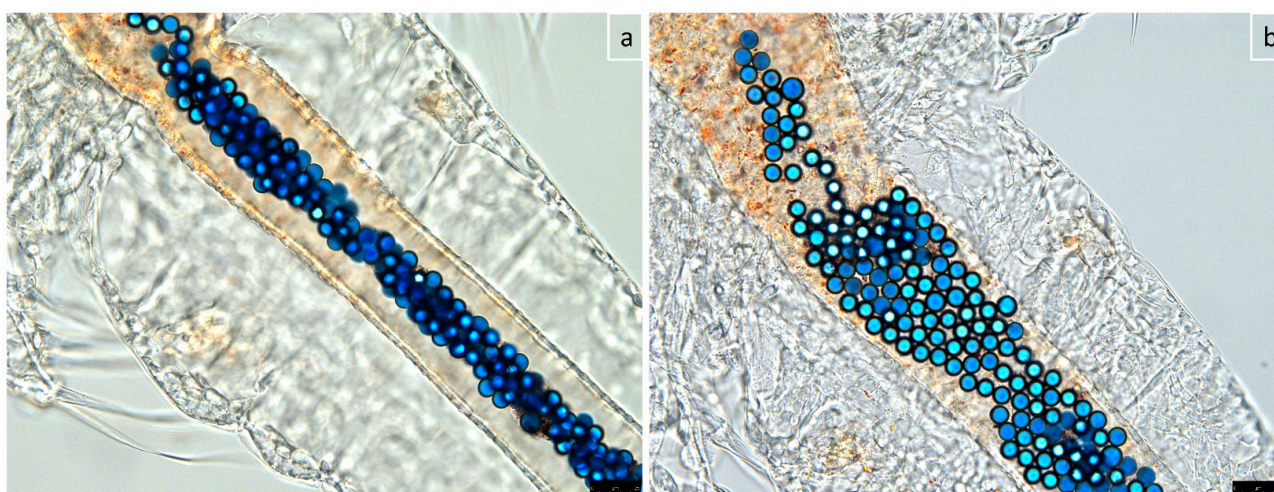
Experimental Groups	Concentration
	N°MP/mL
A0	0
A1	1
A2	10
A3	100
A4	1000
A5	10,000
B0	0
B1	1
B2	10
B3	100
B4	1000
B5	10,000

From our pre-experimental tests, the sedimentation of MPs started after 48 h from administration in the presence of *A. salina* nauplii activity that provides constant motion to the solution. Before starting the experiment, we performed a 24-h ingestion/egestion trial using the method previously described by Bergami et al. [65]. In this trial, we found that brine shrimp nauplii egested about 90% of MPs after 3 h post-ingestion. Based on this data, the daily replacement of the solutions was considered necessary to avoid the

decrease of the MPs concentration in the replicates due to the egestion rate through fecal pellets elimination.

#### 2.4. Microplastic Uptake Assessment

To assess and quantify the different MPs uptake in *A. salina*, a direct counting and identification under a Leica DM6B microscope were performed [66,67]. In addition, several images of the analyzed specimens, from each group and time point sampled, were captured at different magnification with Leica DM6B equipped with a Leica DFC7000T Imaging system and LAS X software, for further analysis [68] with ImageJ software [69]. The identification of MPs was favored in some rare cases by gently crushing the organisms with the cover slide, to better spread and highlight the MPs on the slide (Figure 1).



**Figure 1.** Ten  $\mu\text{m}$  of Polybead® Blue Dyed Microspheres ingested by a Group B *Artemia salina* specimens at T96 treatment B5 ( $10^4$  MPs/mL). (a) Normal view; (b) view of the same specimen gently crushed with the cover slide. Scale bars: 25  $\mu\text{m}$ .

#### 2.5. Estimation of Feeding Rate

To better evaluate *A. salina* feeding behavior, a parallel test with all the same conditions was performed, except for the quantity of brine shrimps. One *A. salina* specimen was added in each 10 mL glass test tube for each treatment ( $0$ – $1$ – $10$ – $10^2$ – $10^3$ – $10^4$  MPs/mL) in triplicate. To assess the ingestion rate and the influence of MPs presence on its trend, counts after 24–48–96 and 168 h were made for each replicate under a Leica DM6B microscope at  $40\times$  magnification in a Bürker Türk chamber (hemocytometer). To obtain the ingestion rate as microalgae, the cells/larvae/h method was used as previously described by Frost [70]. The provided pure culture of *D. salina* was counted daily before the solutions replacement. An additional positive control was used to evaluate the growth of *D. salina* without brine shrimps feeding. Moreover, based on intestinal and MPs surfaces measurement, an estimation was carried out on the images obtained at each time point to calculate through the use of ImageJ software [69], the percentage of intestinal tract occupied by the MPs, and consequently subtracted from *D. salina* ingestion.

#### 2.6. Data Analysis and Elaboration

For the *A. salina*, the total length and the intestinal tract measurement was used by ImageJ software [69]. The evaluation of the developing stages were performed as suggested by Wang [56] for *Artemia parthenogenetica* and relied on our control treatment. All data are expressed as means  $\pm$  standard deviation (SD). One-way Analysis of Variance (ANOVA) or non-parametric Kruskal–Wallis tests, followed by Fisher's Least Significant Differences (LSD) post-hoc test, were used to evaluate significant differences in microplastic abundance, total length and mortality rate between the experimental groups. Differences were considered significant at  $p < 0.05$ .

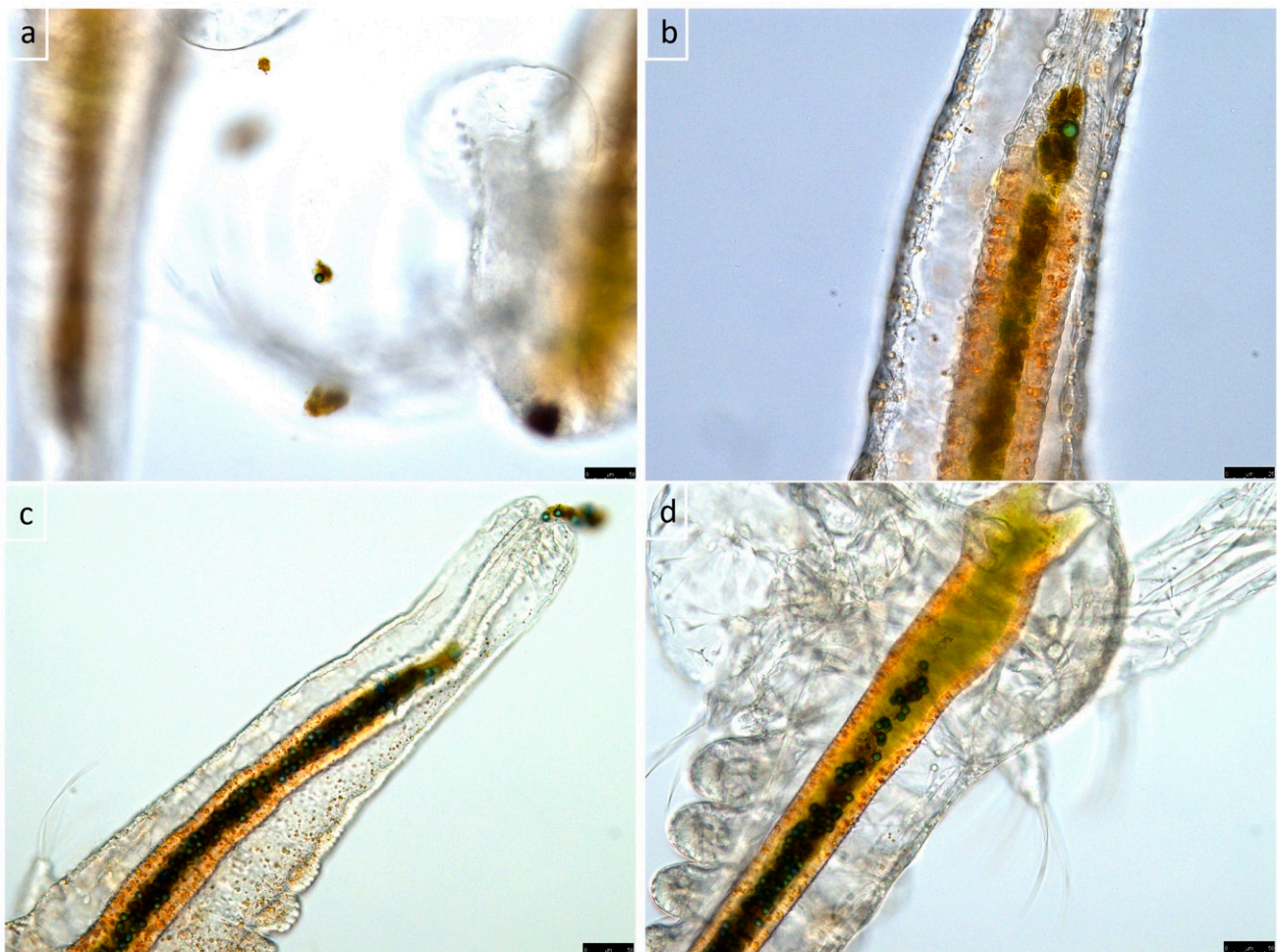


### 3. Results

Our results showed significant differences in MPs ingestion, growth and mortality rate ( $p < 0.05$ ) dependent on different treatment, MPs concentration and time of exposure.

#### 3.1. MPs Ingestion Evaluation and Mortality

MPs ingestion was detected in both experimental Groups A and B (Figures 2 and 3, Table 2). Microspheres were found in treatments A4–A5 and B1–B5. Significant differences were observed in MPs ingestion ( $p = 0.003$ ) when exposed to a concentration of  $10^4$  MP/mL compared to other concentrations. The main variations in MPs ingestion between treatment A and B were observed between the exposure time T0, T6, T12 and T24 vs. T48 in the Group A and between T0, T6, T12 and T24 vs. T96 in the Group B.

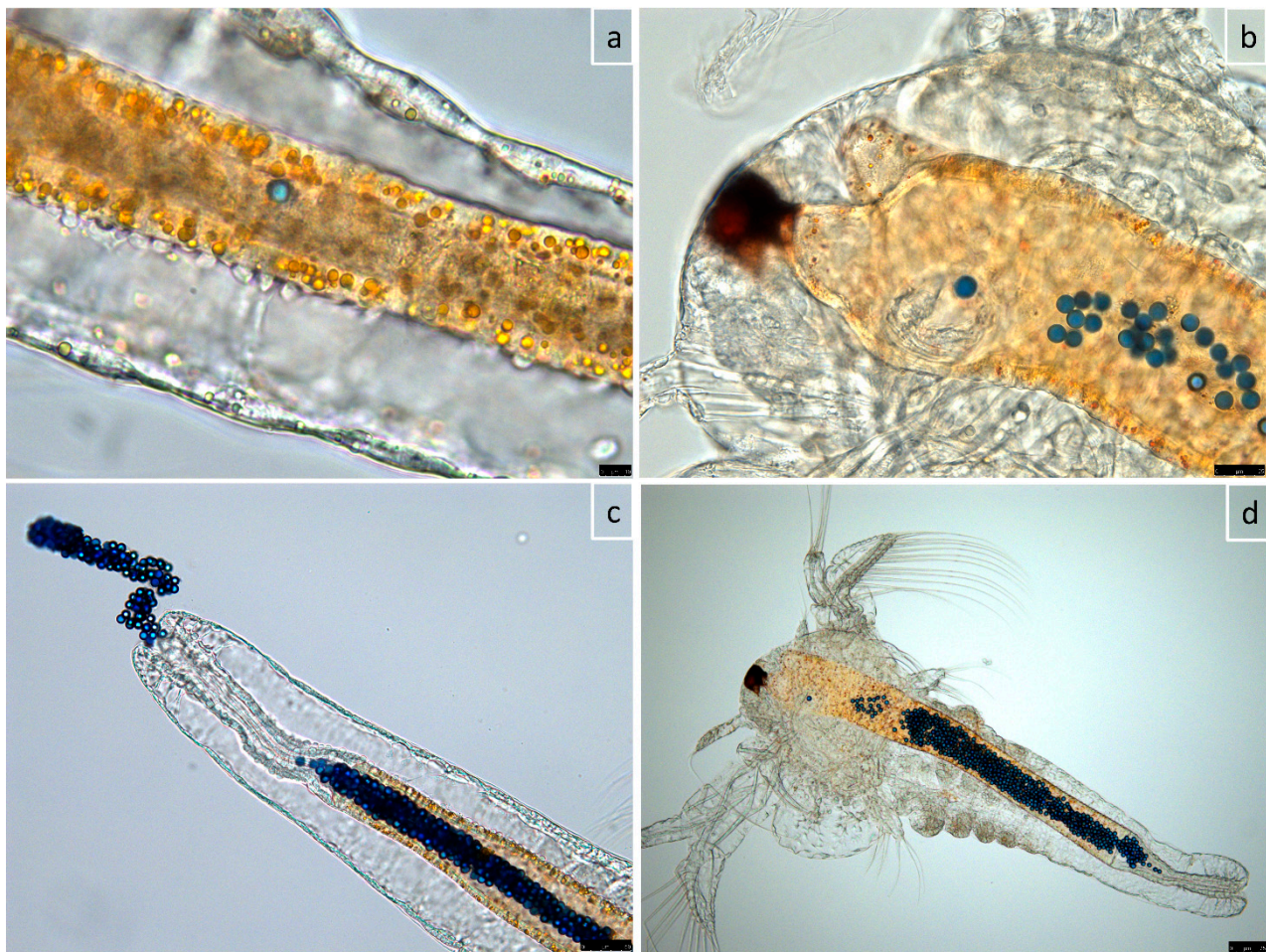


**Figure 2.** Ten  $\mu\text{m}$  of Polybead® Blue Dyed Microspheres detected in the gut and feces of Group A specimens at T48. (a) Treatment A3 ( $10^2$  MPs/mL); (b) treatment A4 ( $10^3$  MPs/mL); (c,d) treatment A5 ( $10^4$  MPs/mL). Scale bars: 25  $\mu\text{m}$  (a); 50  $\mu\text{m}$  (b–d).

Group A showed significant variation in MPs ingestion ( $p < 0.001$ ) depending on exposure concentration. In particular, the main differences were detected between  $10^4$  MPs/mL and all other concentration investigated.

Mortality rates varied according to exposure time ( $p < 0.001$ ) (Table 3), with the main differences observed between T168 vs. T0, T168 vs. T12 ( $p = 0.014$ ). Similar results were observed in the Group B, where MPs abundance resulted to be dependent on exposure concentration ( $p = 0.001$ ). Even in this case, the mortality rate followed an exposure time-dependent trend ( $p < 0.001$ ), with a peak of mortality at T96 and T168.





**Figure 3.** Ten  $\mu\text{m}$  of Polybead® Blue Dyed Microspheres detected in the gut and feces of Group B specimens at T48. (a) Treatment B1 (1 MPs/mL); (b) treatment B4 ( $10^3$  MPs/mL); (c,d): treatment B5 ( $10^4$  MPs/mL). Scale bars: 10  $\mu\text{m}$  (a); 25  $\mu\text{m}$  (b); 50  $\mu\text{m}$  (c); 75  $\mu\text{m}$  (d).

**Table 2.** Ingested microplastics counted for each experimental group (A and B) and exposure time. Data are shown as means  $\pm$  SD ( $n = 3$ ).

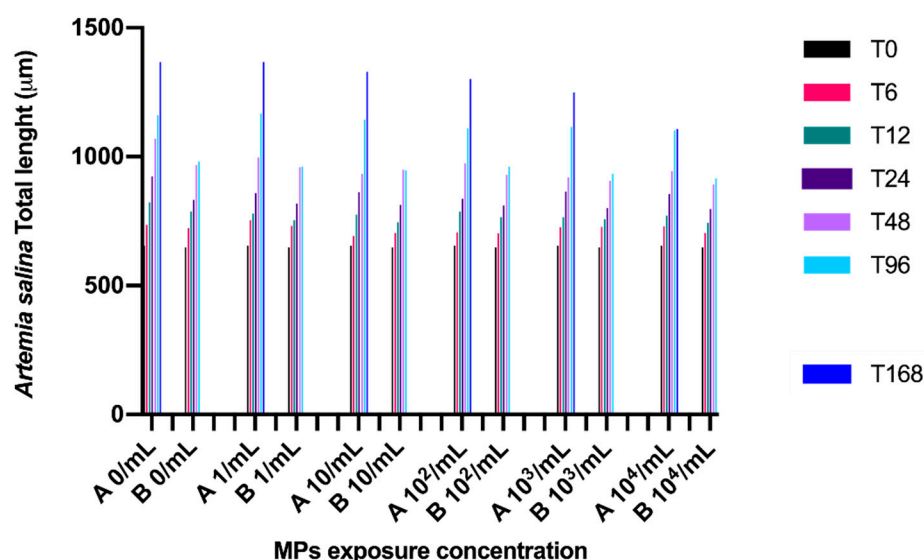
Experimental Groups	Exposure Time						
	T0	T6	T12	T24	T48	T96	T168
A0	0	0	0	0	0	0	0
A1	0	0	0	0	0	0	0
A2	0	0	0	0	0	0	0
A3	0	0	0	0	0	0	0
A4	0	0	$0.4 \pm 0.38$	$2.2 \pm 1.57$	$2.4 \pm 1.46$	$4.4 \pm 1.44$	$4.4 \pm 1.32$
A5	0	$1.4 \pm 1.16$	$13.2 \pm 12.4$	$43.6 \pm 28.7$	$122 \pm 51.8$	$108.2 \pm 38.3$	$110.2 \pm 31.4$
B0	0	0	0	0	0	0	0
B1	0	0	0	$0.2 \pm 0.44$	$0.6 \pm 0.54$	$0.8 \pm 0.83$	
B2	0	0	$1 \pm 1$	$1.4 \pm 0.54$	$2.8 \pm 1.92$	$2.2 \pm 1.48$	
B3	0	$1.4 \pm 1.94$	$7.4 \pm 7.66$	$6.4 \pm 4.39$	$8 \pm 9.46$	$5.4 \pm 4.15$	
B4	0	$22.4 \pm 29.3$	$23.8 \pm 14.9$	$72.8 \pm 28.1$	$85.6 \pm 9.81$	$75 \pm 49.4$	
B5	0	$37.6 \pm 51.5$	$157.4 \pm 68.6$	$169.4 \pm 65.6$	$300.4 \pm 42$	$306.2 \pm 49.3$	

**Table 3.** *Artemia salina* mortality rate (%) calculated for the experimental Group A and B.

Experimental Groups	Exposure Time						
	T0	T6	T12	T24	T48	T96	T168
A0	0	0	0	0.55	0	0.55	1.11
A1	0	0.55	0	0	0.55	1.11	1.66
A2	0	0	0.55	0	0.55	1.11	1.66
A3	0	0	0	0.55	1.11	2.22	2.22
A4	0	0	0	0	1.11	3.33	1.66
A5	0	0	0	0.55	0.55	2.22	2.77
B0	0	0	0	0	1.11	6.66	92.22
B1	0	0	0	0.55	2.22	8.33	88.88
B2	0	0.55	0	0	3.33	11.11	85
B3	0	0	0	0	2.22	12.22	85.55
B4	0	0	0	0.55	3.88	14.44	81.11
B5	0	0	1.11	0	3.33	13.33	82.22

### 3.2. Influence of MPs Ingestion on Growth and Development of *A. salina*

Total body length showed significant differences between the Group A and B ( $p = 0.028$ ) (Figure 4). Indeed, analysis of variance highlighted significant variations also within both experimental groups. Group A showed significant variation between A0 and all others treatments ( $p < 0.001$ ), except with A1 ( $p > 0.05$ ). The highest differences of 258  $\mu\text{m}$  in total body length were detected at T168 between A0 (1366.824  $\mu\text{m}$ ) and A5 (1108.746  $\mu\text{m}$ ) and of 116  $\mu\text{m}$  between A0 (1366.824  $\mu\text{m}$ ) and A4 (1249.606  $\mu\text{m}$ ). At T96, a difference of 61  $\mu\text{m}$  was detected between A0 (1162.024  $\mu\text{m}$ ) and A5 (1101.746  $\mu\text{m}$ ).

**Figure 4.** *Artemia salina* total body length at each concentration and experimental time.

MPs concentration less influenced total body length within the Group B, showing at T96 a difference of 66  $\mu\text{m}$  comparing B0 (981.26  $\mu\text{m}$ ) with B5 (915.164  $\mu\text{m}$ ), but not statistically relevant.

Qualitative anatomic features analysis performed from images of analyzed specimens showed significant differences between the two groups. Group B showed a significant delay in the development as compared to the Group A specimens. Indeed, the maximum reported development stage for the Group B (B0) ranged between instars II and III at T96 (before the total mortality due to starvation), where the naupliar eye was still a simple eye, and the antennae were the only active appendages because of the total absence of thoracopods. However, Group A (A0) reached at the same time point with the instar IV,



where the naupliar eye was in differentiation towards the compound structures and an evident primordium of thoracopods were present. Moreover, at T168 in Group A replicas, the instar V was reached with the complete differentiation of the eye in median and compounds and an evident formation of thoracopods that become swimming appendages (Figure 5).



**Figure 5.** Development of Group A *Artemia salina* nauplii six treatments (A0–A5) in time (0–168 h). Scale bars: 75  $\mu$ m for time points T0. 250  $\mu$ m for time points T24–T168.

No evident anatomical differences were detected between the different treatments of Group B (Figure 6).

Within Group A, A0 at T168 reached instar V; A4 and A5 replicates reached instars IV and III respectively, showing different average developmental stages (Figure 5).

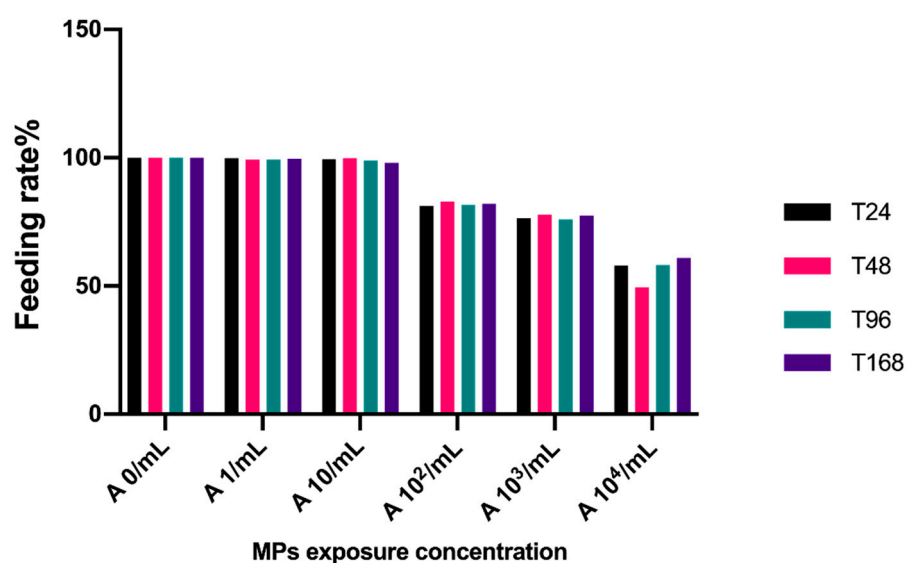
### 3.3. Influence of MPs Ingestion on Feeding Behavior

The results of a parallel experiment (see Section 2.5) are shown in Figure 7. The maximum microalgal ingestion rate reduction was 50.58% at T48 when exposed to  $10^4$  MPs/mL, of 24.08% at T96 when exposed to  $10^3$  MPs/mL and of 18.70% at T24 when exposed to  $10^2$  MPs/mL. It was only for the  $10^4$  MPs/mL exposure that registered a remarkable, increasing time-dependent trend of the microalgal ingestion rate, reaching 39.09% at T168. The data obtained from the positive control of *D. salina*, showed that the growth of algal concentration in absence of brine shrimps cannot be considered negligible. For this reason, we did not consider the influence of the algal growth concentration on the ingestion rate data.





**Figure 6.** Development of Group B *Artemia salina* nauplii six treatments (B0–B5) in time (0–96 h). Scale bars: 75 µm for time points T0–T48; 250 µm for time point T96.



**Figure 7.** *Artemia salina* feeding rate (%) at each concentration and experimental time.

The results of ingestion area measurement reported in Figure 8 showed a strong reduction of unoccupied MPs area in both A5 and B5, as well as in B4. The maximum values of MPs filling reached in A5 and B5 treatments was of 44.43% and 90.82%, respectively.

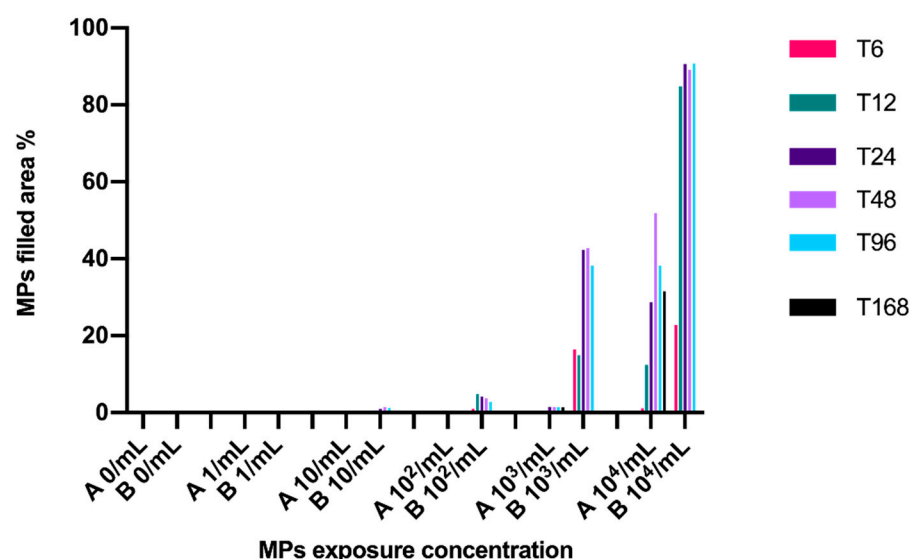


Figure 8. *Artemia salina* MPs filled area (%) at each concentration and experimental time.

Additionally, the microalgae ingestion rate (%) showed a negative correlation with the MPs-filled area ( $r = -0.9$ ;  $p < 0.001$ ) and with MPs ingestion ( $r = -0.7$ ;  $p < 0.001$ ).

#### 4. Discussion

The ingestion of various sizes of plastic debris by different aquatic organisms has been assessed in recent years. In fact, it is how these pollutants can affect biological systems and living organisms is the major issue under investigation of this topic [71,72]. It is known that plastic pollution can affect the natural environments and biota at all levels, in particular in terms of MPs and nanoplastics (NPs) [73,74]. Moreover, the influence of MPs on the biological, immunological and cellular functions is far from being completely understood in aquatic living organisms [75]. Microplastics ingestion was reported in many zooplanktonic organisms used as a model, such as *Daphnia magna* for larger MPs debris [68,76], copepods [77,78] and *Artemia* sp. [54,56] for smaller fractions (5–10  $\mu\text{m}$ ). These results, in addition those obtained in this study, can help to comprehend the fate and behavior of MPs in natural environments and effects on biota.

In this study, we investigated how the ingestion of 10  $\mu\text{m}$  of polystyrene microspheres in the first phases of life cycle can influence *A. salina* larvae vitality, development and feeding behavior. The results obtained during the experimental phases highlighted that MPs can be easily internalized by brine shrimps through filtration. This feeding strategy is common in zooplankton organisms. This and other features make *A. salina* an excellent model organism for the study of MPs pollution influence on zooplanktonic trophic webs, as already suggested by other authors [79,80]. Moreover, these organisms are involved in biomagnification phenomena [29,48].

In our study, we found that MPs are already ingested by *A. salina* larvae 6 h post hatching in a dose-dependent manner, with the influence of exposure time reaching up to a maximum ingestible value after 48 h of exposure. These results are in accordance with other studies on MPs ingestion by zooplanktonic organisms that reported comparable ingestion times [55,81]. These values were maintained in the subsequent time points, highlighting that a threshold value for the ingestion of MPs under this condition was likely reached.

The data reported in Table 2 show the differences found between the replicates with and without a food source. It is evident that in the presence of microalgae, the ingestion of MPs is lower, probably because during their non-selective filtration activity, brine shrimps encounter rarely and randomly the plastic debris in the experimental solutions filled of microalgal cells [55]. It is also interesting to note that in the presence of microalgae, no MPs was found at the lower tested concentrations (1, 10, 10<sup>2</sup> MPs/mL) during the

sampling steps. This represents important data if compared to the common environmental MPs concentrations reported by other authors [59,60,82,83], which are usually not far from 1 MPs/mL, whose ingestion could hence be unusual by these organisms. However, as reported in Figure 2, we found evident traces of a few egested MPs at 10 and 100 MPs/mL concentrations in fecal pellets. This proves that the ingestion of MPs occurs even at these concentrations, although in a minimal percentage not detected inside the organisms. In accordance with Wang [55,56], this detection problem was probably due to the disproportion between MPs debris and microalgal cells in water.

On the contrary, MPs contamination was detected at all the tested concentrations in absence of *D. salina* food source from T24. This feature confirmed that in the absence of other suspended particles, brine shrimps encounter and actively filter 10 µm of polystyrene microspheres, maintaining it inside the intestinal tract for a long time, as reported for other zooplanktonic organisms [84–86]. This also demonstrated the non-selective filtration activity of *A. salina*, in accordance with Wang [55] and Bergami [65] that reported respectively for *A. parthenogenetica* and *A. franciscana* the same feeding behavior. Indeed, despite it is not deriving any nourishment from MPs, brine shrimps filter all the debris of their available feeding range suspended in water without preference, in a dose-dependent manner. It could be very interesting in the future to investigate if in the presence of environmental high value of MPs pollution, grazing animals are physically attracted by non-nutritive suspended matter comprised in their filtrations range.

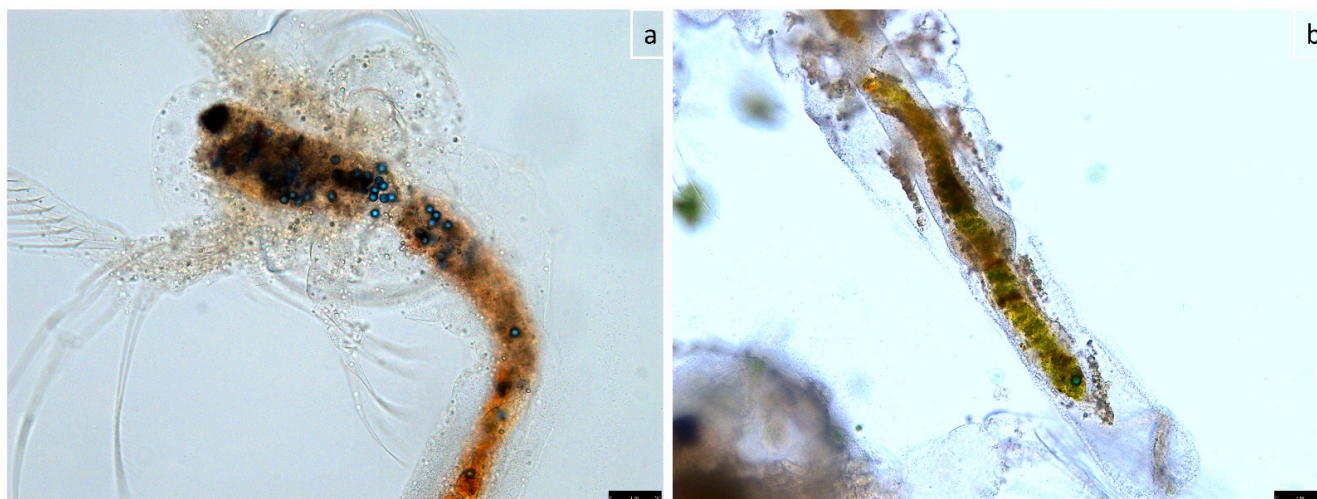
Mortality was observed constantly during the experiment. This data was chosen as outcome by some previous toxicity studies regarding *A. salina* [87,88]. Massive mortality event occurred in our experiment between 96 h and 120 h for all the specimens of Group B, due clearly to starvation. This led to an absence of specimens at T168, which made all the results of the two groups comparable until T96. This time represents the life limit found in our study for non-fed *A. salina* in the presence of MPs. As a consequence, the mortality data showed a strong time-dependent trend in Group B. Compared with the mortality trend reported for *A. parthenogenetica* exposed to 10 µm MPs by Wang [55,56], little difference was found in Group A (only between T168 and T0/T12) demonstrating the absence of an acute toxic effect of MPs exposure. As a generalized effect, the developmental delay caused by the higher tested concentration of MPs ( $10^2$ ,  $10^3$  and  $10^4$  MPs/mL), had little plausible contribution to mortality along the timeline for both Group A and B. These data are in accordance with the major development delay detected for A3, A4, and A5 when compared to A0 (control), which reasonably led to a higher mortality [89]. As evident from Figure 9, in almost all cases, the observed dead specimens showed exposure to MPs contamination, confirming their ingestion and retention capacities until the end of vital activity, in agreement with the MPs retention demonstrated for copepods by Cole [84].

The anatomical features, such as the total body length, were usually used for toxicity evaluations [90–92]. Data reported in the present study showed a decreasing trend in average total body length compared to the increase of MPs exposure concentrations in Group A. These results are in contrast with Wang [56] which did not report any differences in growth rate of *A. parthenogenetica* depending on concentration exposure, but in accordance with the results obtained by Kokalj [91] for *Artemia franciscana*. In our case, the biggest differences were detected at T96 between control and A4, A5, B4 and B5, with a higher value in Group A, highlighting a more significant effect of MPs ingestion in the presence of a food source. Moreover, for Group A, this effect was more marked at T168, demonstrating a correlation of growth also with the exposure time, as expected following a normal developing rate. Hence, an evaluable trend on this data was detected during the experiment and seems to be comparable between Group A and B with the same negative effect on growth proportionally to an increase in MPs ingestion. Moreover, as expected, a significant difference in total body length was detected comparing Group A with Group B, due certainly to the absence of food source.

Focusing the attention on the developmental stage, our results, as expected, highlighted the major differences between Groups A and B. The maximum reached instar for



the control of Group B was comprised between instars II and III at the end of exposure (96 h); indeed, after this time a mass mortality event happened. This was attributable to the absence of a food source. Group A reached the instar IV at T96, showing anatomical differentiations in line with the normal development of the species [93,94]. After all, following normal development, instar V was reached at the end of exposure time (168 h) with the complete differentiation of the eye in median and compounds and an evident formation of thoracopods, which later become swimming appendages. As expected, the shared nutrients deficiency had a leveling effect between different experimental treatment. Group A developmental stages were probably attributable to the presence of a nutrient source that allowed a normal anatomical development. This highlighted that differences in developmental stages were attributable to the presence of MPs. In detail, while the control reached instar V, the replicates A4 and A5 reached instars IV and III, respectively, showing a different average development. These results are in contrast with Wang [56], who did not report any differences in developmental stages of *A. parthenogenetica* depending on the MPs concentration exposure.



**Figure 9.** 10 µm Polybead® Blue Dyed Microspheres detected in dead specimens. (a) Group B specimen; (b) Group A specimen. Scale bars: 50 µm.

We assume that the delay in development is due to the physical filling of intestinal tract by MPs, which reduced the ingestion of microalgae.

The filtration rate is one of the main focused targets in the toxicological study of organisms with this food strategy [24,81,90,95]. As reported in previous studies, the ingestion of plastic debris led to a reduction of the feeding rate in a dose-dependent manner in *A. parthenogenetica* [55], *A. franciscana* [65], *D. magna* [91] and *Calanus helgolandicus* [96]. Our results obtained from Group A are in accordance with these findings and showed a strong negative correlation between the MPs uptake and the microalgal ingestion rate in *A. salina*. During the experiment, the average maximum reduction of 50.58% of ingestion rate was found at T48 for the B5. This value is related with the maximum value of ingested MPs and the maximum reduction of intestinal area due to MPs ingestion; both were detected at the same time point. Moreover, the ingestion rate has risen to 39.09% at T168, with a similar trend to MPs ingestion rate, demonstrating the capacity of *A. salina* to filter debris in a non-selective and in a dose-dependent manner.

Furthermore, our results showed that the ingestion rate data were not influenced by microalgal concentration growth during the experiment. Indeed, *D. salina* concentration growth in absence of brine shrimps did not show a significant variation during the experimental period. This was mainly due to our algal cultivation conditions in which *D. salina* does not reach (even with 24D photoperiod) concentration in the pure culture, which is much higher than those used in the study. Hence, this is the maximum level of

maturation of our pure culture. Moreover, the photoperiod used during the experimental phases (12D:12N) probably did not promote the algal concentration growth.

The intestinal tract is one the most studied targets in zooplankton toxicity studies [55,65,91]. For this reason, in our study, we focused attention to evaluate the feeding behavior of *A. salina* mainly on this anatomical structure of the larvae. The results of the estimation of the ingestion area show that a strong negative correlation between the MPs occupied area and microalgae ingestion rate was found. The absence of food source determined a maximum filling MPs value at T96 of 90.82% for B5. In Group A, the maximum intestinal MPs filling of 44.43% was reached in A5 at T96, demonstrating that in the presence of a similar size food source, the intestinal area available for the MPs uptake was strongly reduced and vice versa. These findings are strictly connected to differences found in total body length and developmental stages. As expected, the lower ingestion of nutritional food source due to MPs uptake, led to a developmental delay in a dose-dependent manner.

## 5. Conclusions

In this study, the ingestion and the influence of 10 µm polystyrene microspheres on growth and feeding behavior of *A. salina* in the presence or absence of microalgae *D. salina* food source were evaluated. Our results showed that the uptake of MPs in this organism is strongly influenced by the simultaneous presence of plastic debris and microalgae cells in water. MPs ingestion, in a dose-dependent manner, led to a developmental delay and a reduction in the total body length. Moreover, negative correlation between MPs ingestion and microalgal feeding rate was found, mainly due to a physical reduction of the available intestinal area for microalgal ingestion and subsequent nutrient assimilation. These anatomical variations probably led to a minor functional deficiency. Future studies will be needed to confirm these correlations. It will be essential to intensify the research on the biological and physiological effects of plastic pollutants in zooplankton model organisms to better understand how these mechanisms could influence the aquatic trophic webs and the effects on human health due to biomagnification phenomena.

The results of the present study can help build understanding on the fate and behavior of MPs in aquatic environments and how these pollutants can be transferred through the trophic chain.

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**Institutional Review Board Statement:** Ethical review and approval were waived for this study, due to the absence of a specific regulation on the use of crustacean species for research [97].

**Informed Consent Statement:** Not applicable.

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