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Effects of Microplastics on Reproduction and Growth of Freshwater Live Feeds *Daphnia magna*

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Abstract: In recent years, much research has focused on studying the damage caused by microplastics to the ecological environment and human health. Indeed, MPs are often consumed by shellfishes and zooplanktons due to their similarity in size to POM (particular organic matter). Especially in zooplankton, the accumulation of MPs in the body affects the reproductive system and the growth rate of juveniles. Moreover, toxins derived from MPs are continuously accumulated in predators of zooplankton and impact the whole ecosystem across the food chain. In this work, we found that even though MPs were internalized by and adherent around *Daphnia magna*, there were no significant differences in the survival rate of their adults and offspring. However, the population of ovigerous adults under high MPs exposure for 7 days decreased significantly, suggesting an extension of the days of sexual maturity in *D. magna*. The removal of MPs after 7 days' MPs treatment resulted in an increase in *D. magna* juveniles and neonates which indicated their growth was reduced or inhibited in the MPs environment. Overall, the uptake of MPs led to negative effects on population reproduction and the growth of offspring in *D. magna*.

Keywords: microplastics; *Daphnia magna*; survival; reproduction

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1. Introduction

In 2008, the NAOO held a meeting on marine plastic pollution and defined MPs (microplastics) as plastic particles found in oceans with sizes of less than 5 mm or 1 µm, and NPs (nanoplastics) as plastic particles with a size range of 1 µm to 100 nm [1–5]. There are two major sources of plastic particles [6], including primary MPs and secondary MPs. Primary MPs are plastic particles directly or indirectly released into the ocean, which may derive from the laundry process, cosmetic pearls (Microbeads), factory manufacturing processes, or particles generated by the tires of vehicles when braking, etc. Secondary MPs in oceans are derived from plastic material degraded and broken down by UV rays of the sun and other physical processes [7–9]. About 90% of the world's plastic materials are made up of polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), or polyethylene terephthalate (PET) [10]. These materials are widely used around the world because they are cheap, easy to manufacture, and have alternative properties to natural products including wood, stone, and glass [3]. However, concerns of the acute and chronic effects of environmental microplastics (MPs) on aquatic organisms are rapidly increasing,

especially in the absorption of heavy metals, dioxin and other highly toxic compounds. According to SAPEA (2019) [11], the risk of environmental MPs is not currently high but could become widespread across the whole ocean in future.

The use of plastic materials increased dramatically over the past few years, with a total global production of 1.7 million tons of plastic materials in 1950 [10]. From 1950 to 2014, annual plastic production increased at least 20-fold to 300 million metric tons [10]. Large amounts of plastic materials enter water environments from land, with an estimated 4.8–12.7 million tons of plastic materials entering the ocean each year [12]. In addition, the potential degradation of large plastic pellets increases the risk posed to marine life and the environment [13–18]. A growing number of studies focus on the behavior and effects on marine life under the exposure of MPs [19,20]. MPs also enter freshwater ecosystems through land-based wastewater [21–24]. Therefore, in recent years, an increasing number of studies revealed the effects of plastic particles on freshwater organisms [25–27], providing data on an increasingly important environmental issue in freshwater ecosystems [25,26,28].

Zooplankton organisms are the primary consumers in the food chain and are important bait for aquaculture and fisheries. Some studies revealed the possible hazards of plastic particles in the food chain, such as plankton acting as potential carriers of toxic substances derived from the ingestion of plastic particles [29]. Additionally, the toxic substances are accumulated in higher order consumers through transfer in the food chain, leading to harmful impacts on mammals and birds [30]. Similarly, MPs are also ingested by live feed organisms in aquaculture and are transferred and accumulated in fish bodies, resulting in delayed growth and decreased production or harmful effects on human health when toxic seafood is consumed.

In freshwater aquaculture, *Daphnia* spp. plays an important role as a kind of live feed. *D. magna* constitutes a major component of food webs in the freshwater ecosystem, being not only the main food item of fish but also the main herbivore of algae [31]. The MPs ingested by *Daphnia magna* depend on the type, size and shape of the particles [30,32,33]. Jemec et al. [29] found that *D. magna* ingests the fibers of MPs, which are up to 1400 µm in length and up to 528 µm or 106 µm in width. MPs have a high chance of being eaten by large zooplankton, such as juvenile fish [33,34]. MP intake depends on the ability of zooplankton to ingest food and their preservation. Studies have shown that spiky particles (e.g., fragments, fibers) present a greater threat as they are more difficult to digest than smooth particles (i.e., spherical beads) [35]. In *D. magna*, MPs and NPs were found to cause a wide range of adverse effects, such as mortality, feeding inhibition, and a decrease in the reproduction rate, among others [24,30,33,36–40]. Here, *D. magna* were immersed in an MP-enriched environment to evaluate potential effects on their physiological responses and understand the potential impacts on their survival, reproduction, body size and production of offspring after long-term immersion with MPs, which may provide important information for future human aquaculture of feed organisms.

2. Materials and Methods

2.1. Animals

D. magna is a small filter feeder freshwater crustacean with cyclic solitary reproduction and the population is usually dominated by females [41]. *D. magna* were collected from a co-aquaculture pond with milkfish and white shrimp in Maito, Taiwan. *D. magna* with a body length of about 0.5–1.5 mm were collected using a 100-mesh plankton net. The water condition was as follows: 6‰ salinity, pH 7.8, 5.6 ppm DO, 1 ppm NH_3, and 0.5 ppm NO_2^- and the water was refreshed once per 2 days. *D. magna* were collected, with 200 individuals/per treatment, kept in a 200-L FRP tank for 7 days, and disinfected with 20 ppm formalin and 10% florfenicol for 3 days. The water quality in a 200-L tank was maintained by using freshwater within the specific range of pH 7.18~7.90, water temperature of 27~28 °C, 7 ppm DO, less than 0.5 ppm NH_3 and 0.1 ppm NO_2^- under 5000 lux of light intensity at a photoperiod of 16:8 light/darkness. Additionally, they were

fed *Chlorella* sp. 20 mL per day (concentration was 10^6 cell/mL). The *D. magna* population were selected without considering male and female individuals.

2.2. Microplastics (MPs, Composed of Polystyrene)

The MPs purchased from Sigma-Aldrich (cat#59769) were carboxylate-modified polystyrene particles with a latex bead-like shape and an average particle size of 0.5 μm, and shared a yellow-green fluorescence at 470~505 nm. During the period of MP treatment, all the freshwater was filtered using 0.1 μm filters to completely remove residue plastic particles that ensured all responses of *D. magna* resulted from MPs provided from the study. The stock of MPs solution (1.05 g/mL with 10% solids) were diluted to filtered water to prepare the working reagents of MPs (1.5 mg/mL). Additionally, all treatments including 0 (control), 0.01, 0.02, 0.05 and 0.1 mg/L MPs were derived from the working reagents of MPs under 3 repetitions. The MPs (ingested or not) were determined by fluorescent signals observed using a fluorescence microscope (Euromex). The images of MPs internalization were verified using a digital camera (SCMOS CCD) equipped on the microscope.

2.3. Determining Sexual Maturation and Offspring Survival

During 7 or 30 days incubation with MPs or MP-free freshwater, the ovigerous behavior of *D. magna* females was determined as an indicator of first sexual maturation. The number of *D. magna* eggs per 20 females and the survival rate of their offspring were recorded after 7 days MPs treatment. After 7 days MPs treatment, all of the *D. magna* including adults, juveniles and neonates produced from 20 females were harvested to examine the residual MPs uptake inside their body. Additionally, the *D. magna* parents were removed and the offspring were transferred to MP-free freshwater for 30 days cultivation to evaluate their sexual maturation. After 30 days of MP-free freshwater incubation, 500 mL of water containing well-mixed *D. magna* offspring was harvested to evaluate body length and the population ratio of adults (with clutched eggs), juveniles (without clutched eggs and over 2.5 mm in length) and neonates (less than 2.5 mm in length). All the tests were performed in triplicate.

2.4. Statistics

The microplastic measurement method was based on the fluorescence signal observed via a Fluorescence microscope (Euromex, Mataró, Spain). The images of MPs internalization were verified using a digital camera (SCMOS CCD) equipped on the microscope. The number of MPs in *D. magna*, the number of eggs, survival rate and survival rate of offspring in the 7 days immersion were calculated and the differences among treatment groups were tested using One Way ANOVA. Tukey's post hoc tests were performed with stocking and the ratio of adults, juveniles and neonates in the total number of *D. magna* after 30 days was calculated.

3. Results

3.1. No Effluences on the Survival Rate of D. magna Even with Higher MPs Accumulation

The survival rate of *D. magna* exposed to MPs for 7 days was not significantly different in all groups ($p = 0.984$, Figure 1). After 7 days of exposure to plastic particles, the amount of residual MPs in *D. magna* bodies increased significantly followed by an increasing intensity of MPs incubation ($p < 0.001$). The amount of residual MPs in *D. magna* bodies was in the range of 0, 12.82 ± 0.28, 13.07 ± 0.21, 18.28 ± 0.38 and 23.82 ± 0.38 particles in the groups of control, 0.01, 0.02, 0.05 and 0.1 mg/L MPs, respectively (Figure 2). The Tukey test revealed significant differences between groups ($p < 0.001$), except for the groups of 0.01 vs. 0.02 mg/L ($p = 0.82$).

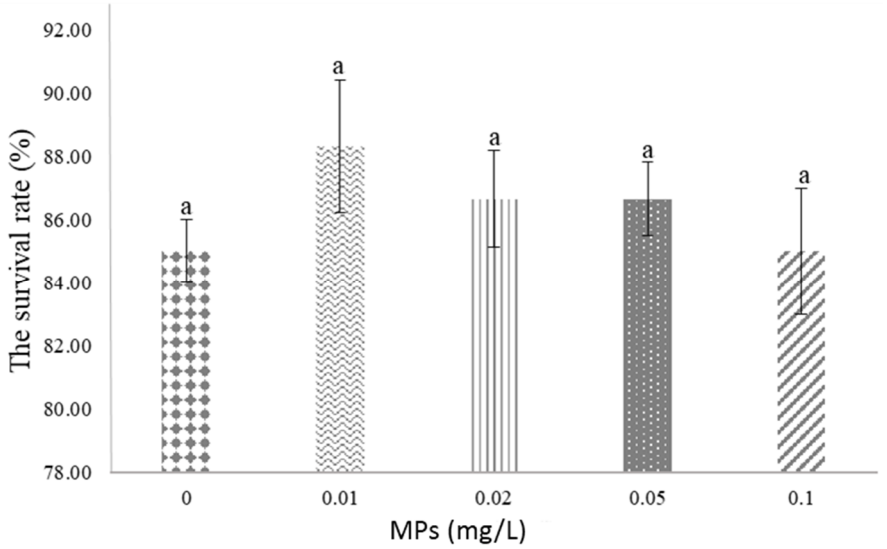

Figure 1. The survival rate of *D. magna* exposed to MPs for 7 days. A total of 20 *D. magna* females were incubated with different MP treatments for 7 days. All treatments showed no significant differences on the survival rates ($p = 0.984$). All the tests included 3 independent replicates. Lowercase letters indicate significant differences between treatments.

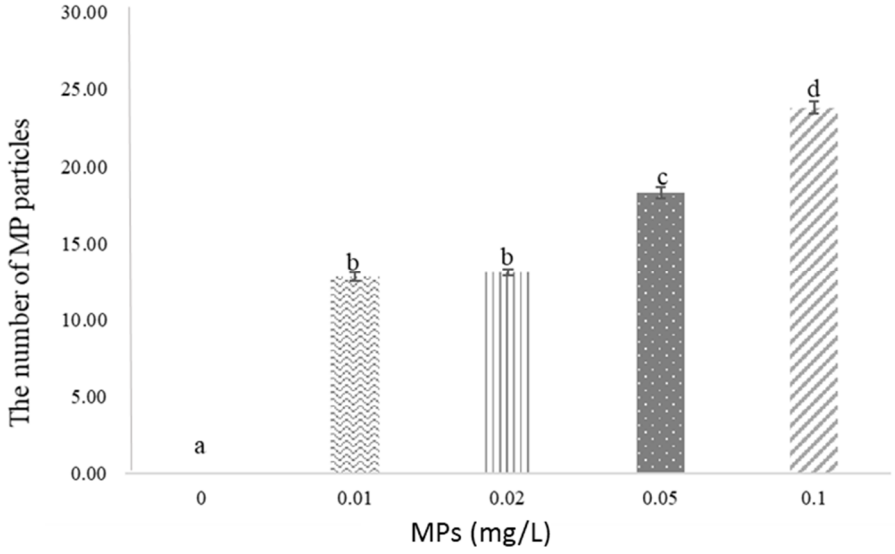

Figure 2. Quantification of residual MPs in *D. magna* after 7 days exposure to MPs. After 7 days MPs treatment, the residual MPs uptake inside the body of the *D. magna* containing adults, juveniles and neonates derived from 20 females were evaluated. The MPs in body of *D. magna* significantly increased following an increase in MPs ($p < 0.001$). The highest accumulation of MPs in *D. magna* appeared under 0.1 mg/L MPs treatment. All the tests included 3 independent replicates. Lowercase letters indicate significant differences between treatments.

3.2. MPs Ingestion Induced Delayed Sexual Maturity in D. magna

The effect of exposure to MPs led to an increase in the number of days of birth to clutching of eggs in *D. magna* ($p < 0.05$) compared to the control group. In the control group, the occurrence of egg clutches was found on the third day, but on the fourth day in the 0.01, 0.02 and 0.05 mg/L groups and the fifth day in the group of 0.1 mg/L (Figure 3). During MP incubation, the number of *D. magna* with clutched eggs in the control group was 0.67 ± 0.58, 1.00 ± 1.00, 4.00 ± 1.00, 1.67 ± 1.53 and 1.00 ± 1.00 from the 3rd day to 7th day, and 0.33 ± 0.58, 3.33 ± 1.53 and 3.33 ± 0.58 in the 0.01 mg/L group from the 4th day to 6th day, respectively. From the 4th day to 7th day, an average number of

ovigerous *D. magna* (1.00 ± 1.00, 3.67 ± 0.58, 4.67 ± 4.62 and 0.67 ± 0.58) was observed in the 0.02 mg/L group and 0.33 ± 0.58, 4.33 ± 4.93, 2.00 ± 1.00 and 3.00 ± 1.00 in the 0.05 mg/L group, respectively. The 0.1 mg/L 0.1 mg/L treatment of MPs possessed an average of 0.33 ± 0.58, 2.67 ± 0.58 and 2.00 ± 1.00 of *D. magna* bearing eggs from the 5th day to 7th day (Figure 3). After 7 days of MP exposure, the eggs held by *D. magna* decreased significantly with an increasing intensity of MPs ($p < 0.05$). After 7 days of MPs incubation, the results showed that the number of eggs in 0.1 and 0.05 mg/L groups (2.07 ± 0.06 and 4.70 ± 0.60) was less than in the 0.02, 0.01 mg/L and control groups (6.43 ± 0.08, 7.431 ± 0.81 and 7.93 ± 0.47) (Figure 4).

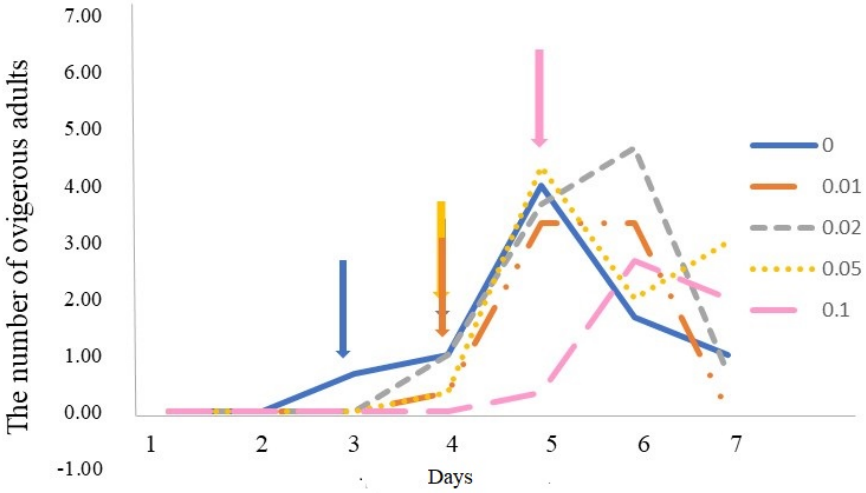

Figure 3. Effects on the population and the days of sexual maturity in *D. magna* under exposure of MPs. After MP treatment of *D. magna* females, 20 newborn neonates were transferred to and incubated in MP-free freshwater for 30 days. The *D. magna* offspring showed fast sexual maturity with 3 days under freshwater (no MPs) and released their eggs at 5th day. However, the other offspring derived from MP-treated *D. magna* presented delayed sexual maturity (over 4 days) and offspring production (6th day). All the tests included 3 independent replicates.

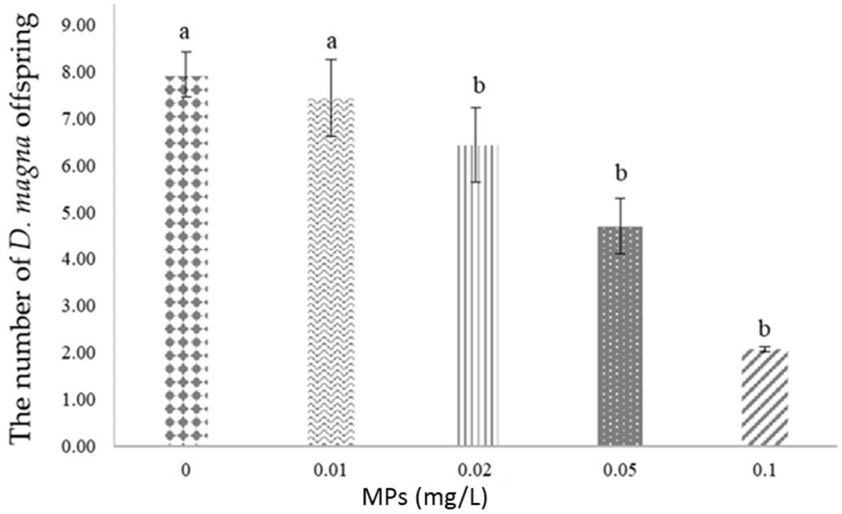

Figure 4. The effects of MP treatments on the offspring population of *D. magna*. Each group of 7 days MP treatment contained 20 individuals of *D. magna* females. The number of offspring produced from different groups was calculated to determine the effects of MP treatments on the offspring population. Apparently, except for 0.01 mg/L MP treatment, all offspring produced by the *D. magna* females showed a significant decreased tendency after 7 days incubation of MPs ($p < 0.05$). All tests included 3 independent replicates. Lowercase letters indicate significant differences between treatments.

3.3. Greater MPs Enhanced the Number of Neonates in *D. magna* Offspring

After 7 days MP treatment, the *D. magna* offspring were transferred to MP-free tanks for 30days. After 30 days, the results indicated that the body size of the newborn offspring had no significant differences. The body length of the offspring in groups of control, 0.01, 0.02, 0.05 and 0.1 mg/L were 3.11 ± 0.07, 3.02 ± 0.02, 3.07 ± 0.01, 2.92 ± 0.06 and 3.03 ± 0.05 mm, respectively (Table 1). However, the population number of *D. magna* offspring increased. There were 32.02 ± 3.31, 33.99 ± 02.83, 33.40 ± 0.71, 29.55 ± 14.06 and 45.68 ± 11.00 individuals in the control and other groups with increased MPs.

Table 1. The average (± SE) body length and number of *D. magna* after MPs exposure *.

MP (mg/L)	Body Length (mm)	Number of *D. magna*
control	3.11 ± 0.07	32.02 ± 3.31
0.01	3.02 ± 0.02	33.99 ± 2.83
0.02	3.07 ± 0.01	33.40 ± 0.71
0.05	2.92 ± 0.06	29.55 ± 14.06
0.1	2.94 ± 0.05	45.68 ± 11.00

* *D. magna* were incubated in MP-containing water for 7 days and then all of them were transferred to the MPs-free tanks for 30 days. All tests included 3 independent replicates.

In *D. magna* offspring, the number of adults was 25.77 ± 1.39, 25.73 ± 2.74, 26.32 ± 1.82, 21.27 ± 2.79 and 20.94 ± 5.22 and numbers were not significantly different in all groups. The population of juveniles in the groups of 0.05 and 0.1 mg/L MPs accounted for 49.17 ± 12.90 and 33.36 ± 5.82, which was significantly different from the other groups ($p < 0.05$, Figure 5). Similarly, the treatment with 0.1 mg/L MPs showed 45.68 ± 11.00 individuals of *D. magna* in the neonate stage which was significantly different compared with other treatments ($p < 0.05$, Figure 5). It may be suggested that the removal of MPs after 7 days MPs treatment resulted in an increasing number of *D. magna* juveniles and neonates that indicated their growth was initially reduced or inhibited in an MPs environment.

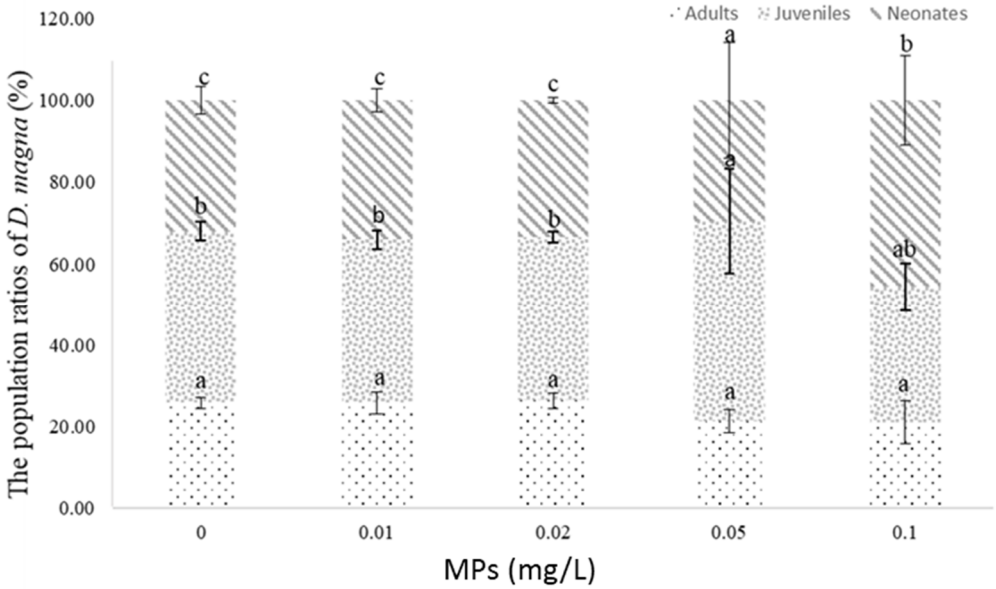

Figure 5. The population of adults, juveniles and neonates in MP-treated *D. magna* after 30 days of freshwater incubation. After 7-day exposure to MPs of female parents, all the newborn *D. magna* were transferred to the freshwater without MPs for 30 days. Additionally, the 500 mL of water volume containing well-mixed adults, juveniles and newborn neonates of *D. magna* was harvested and different ratios in whole population were found in 0.05 and 0.01 mg/L MP-treated groups. All the tests included 3 independent replicates. Lowercase letters indicate significant differences between treatments.

4. Discussion

Plankton are an important source of protein, lipid and carbohydrates in marine and freshwater ecosystems [42–44]. MPs may induce the death of zooplankton populations due to a delay in sexual maturity [45], the nutrient depletion of juvenile and neonates [46] and toxin accumulation [47], leading to harmful impacts for higher consumers through the food chain of the ecosystem and human aquaculture [48]. Elgarahy et al. [49] pointed out that MPs present a high potential of adsorption of a variety of pollutants on their surfaces. In this study, it was found that MPs easily attached to the surface of plankton and ingested into their body. This is important for *D. magna*, as they have complex life cycles [50,51]. The current result is similar to that of the study of Wang et al. [52]. MPs are able to pose not only direct harm to organisms but also act as vectors to transport an indirect hazard, leading to implications in the function and resilience of aquatic ecosystems. Adult *D. magna* attach themselves to rocks on the bottom of the water near the shore but their juveniles are planktonic [39,53,54]. The absorption of a large amount of MPs may result in harmful effluence on their juveniles, thereby reducing the population.

The results indicated that the survival, reproduction and body size of offspring were defective, which may indirectly lead to the production of live feeds, *D. magna*, and provide important implications in future aquaculture. Here, we found that a high dose of MPs did not have any significant effect on the survival rate of *D. magna*, and the same result was shown for their offspring population, which is consistent with the study of Canniff and Hoang [55]. Schür et al. [56] also proposed that exposure to wastewater-incubated MPs resulted in a low mortality of zooplankton. However, this result is not consistent with that of Bosker et al. [57], who observed a significant decline in *D. magna* population biomass due to microplastics exposure. However, the results revealed MPs had a significant effect in delaying the days to sexual maturity and the number of ovigerous *D. magna*, which is not consistent with the findings of Canniff and Hoang [58], which presented that the reproduction of *D. magna* was not affected by MPs. However, the study of Ogonowski et al. [24] proposed that exposure to SMPs (secondary MPs) caused elevated mortality, an increased inter-brood period and decreased reproduction [24]. Furthermore, for long-term effluences, Eltemsah and Bøhn [59] found that MPs caused increasing mortality to *D. magna* within 120 h but not within 48 h. Additionally, the juveniles demonstrated higher sensitivity to MPs exposure than the adults. Under MPs exposure, the *D. magna* juveniles showed higher sensitivity (>50%) which slightly increased their mortality, and reduced the growth and stimulation of early reproduction [59]. Moreover, our results showed that the number of newborn offspring continued to increase followed by an increase in MPs in water. Bosker et al. [56] obtained a similar result, in that no significant impact was observed on the number and body length of newborn *D. magna* offspring following treatment with high MPs. The damage caused to microvilli by NPs aggregates provides an example to explain the negative effluences of food consumption and growth in *Chironomus riparius* larvae [60]. The same result regarding the correlation of *D. magna* body length with MP content was found in the current study.

MPs reportedly accelerate the aggregation of MPs. MPs stimulate marine phytoplankton to increase sticky protein secretions, which accelerates the aggregation of MPs [61–66] that are commonly referred to as "marine plastic snowflakes". MPs are more likely to adhere to extracellular polymers and affect the normal interaction of marine ecosystems. A large amount of MPs in the ocean leads to the death of phytoplankton due to a change in the composition of their extracellular secretions [67]. In this study, we found that *D. magna* was able to adhere to and adsorb MPs on its body surface after 7 days incubation with abundant MPs. However, MPs agglomeration, occurring at higher MPs concentrations, yielded a lower ingestion rate [24,33,38]. When a predator ingests *D. magna* in this situation, a high number of MPs enter into the food chain, which increases the bioaccumulation and risk posed to the ecosystem. These planktonic organisms are considered to be carriers and transboundary transporters of MPs, which may disrupt community structure and ecological functions [68–70]. The ingestion of these nano and microplastic particles by a variety of

organisms, including bivalves, mussels, shrimp, oysters, radiopods, and silkworm, affects their fertility, metabolism, and mortality [71–73].

5. Conclusions

Overall, the incubation of *D. magna* in the presence of MPs (0.01 mg/L, 0.02 mg/L, 0.05 mg/L and 0.1 mg/L) for 7 days indicated that MPs lead to offspring with delayed physiological development in terms of egg-breeding behavior, growth in body length, and morphology, which may result in potential impacts on survival in large fleas. Fortunately, the removal of MPs was found to be an effective way to recover their delayed physiological and morphological development, which provides important clues to further understand the interactions between MPs and the survival, sexual maturity, and offspring of *D. magna*. Furthermore, these findings contribute to risk evaluations of marine and freshwater MPs.

Author Contributions: B.C. conceived of the idea and developed the theory and experimental design. C.-H.H. and T.-W.C. co-contributed to the measurements and planning and supervised the work. B.C. processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. C.-H.K. and Y.-Y.C. manufactured the samples and characterized them with statistics. M.-C.H. aided in interpreting the results and worked on the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Studies with invertebrates (such as *Daphnia magma*) do not currently require ethical approval.

Data Availability Statement: All data were presented in this manuscript.

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