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Polystyrene nanoplastic exposure induces immobilization, reproduction, and stress defense in the freshwater cladoceran *Daphnia pulex*

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HIGHLIGHTS

- 75-nm nanoplastic was ingested by *Daphnia pulex*, resulting in immobilization.
- LC50 of the nanoplastic in *D. pulex* was 76.69 mg/L after 48 h.
- Adverse effects were observed on the growth, development, and reproduction of *D. pulex*.
- Nanoplastic can affect the stress-defense gene expression in *D. pulex*.

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ABSTRACT

The widespread occurrence and accumulation of plastic waste have been globally recognized as a critical issue. However, there is limited information on the adverse effects of nanoplastics on freshwater invertebrates. In this study, the effects of a polystyrene nanoplastic on physiological changes (e.g., survival, growth, and reproduction) and expression levels of stress defense genes (oxidative stress-mediated and heat shock proteins) in the freshwater flea *Daphnia pulex* were measured. The results showed that the digestive organs of *D. pulex* were strongly fluorescent after exposure to the nanoplastic particles, and the 48-h median lethal concentration (LC 50) of the nanoplastic was determined to be 76.69 mg/L. In the 21-day chronic toxicity test, dose- and time-dependent relationships were observed for body length, and the time to first eggs was significantly prolonged in the 0.5 and 1 mg/L groups. The time to clutch was delayed, and total offspring per female and number of clutches were decreased in all the treatment groups. In addition, the offspring per clutch were significantly decreased in the 0.1 mg/L group. As the nanoplastic concentration increased, expression of stress defense genes (*SOD*, *GST*, *GPx*, and *CAT*) was first induced and then inhibited. The gene expressions of heat shock proteins (*HSP70* and *HSP90*) were induced in all the treatment groups. Our results suggest that nanoplastics can be ingested by the freshwater cladoceran *D. pulex* and affect its growth and reproduction as well as induce stress defense.

1. Introduction

Plastic is widely used in agriculture, industry, construction, and other fields as well as the daily life of human beings. Consequently, worldwide production of plastic was more than 311 Tg (million metric tons) in 2014, and it is increasing by 20 Tg per year (Europe, 2015). The plastic industry has provided great conveniences to people; however, the impact of plastics on the environment cannot be ignored. Because of the extensive use of plastics and poor management, plastic waste has entered the environment and large piles of plastic waste can be found in many places (Eerkes-Medrano et al., 2015; Rezania et al., 2018). At least 5.25 trillion plastic particles which total to a weigh of 268,940 tons are currently floating in the seas (Eriksen et al., 2014). Because of ultraviolet radiation, weathering, and other factors, some plastics are converted into smaller particles in the environment. If the particle size of a plastic is 0.1 μm–5 mm, it is called a microplastic (Thompson et al., 2004),

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whereas nanoplastics are generally <100 μm in diameter (Koelmans et al., 2015). Microplastics and nanoplastics can be found in oceans and remote locations such as the Arctic, Southern Ocean, and deep-sea regions (Barnes et al., 2010; Obbard et al., 2014; Van Cauwenberghe et al., 2013; Zhao et al., 2014). Microplastics with maximum estimated concentrations of 1000–9180 items per m^3 have been recorded in surface waters (Desforges et al., 2014). Comparatively, there are fewer studies on nanoplastics, and methods to detect them in water are now required (Koelmans et al., 2015). Abrasion of plastics into particles in the nanoscale is likely to happen, and nanoplastics have been hypothesized to be abundant in aquatic environments; nanoplastics have been detected but not yet been quantified in natural systems (Koelmans et al., 2015; Setälä et al., 2016). In addition, because nanoplastics have large use potential, they enter the environment directly via the use of and emission from these products, such as drug delivery vehicles and material strengtheners (Chae and An, 2017; Salata, 2004).

Considering the unique properties of nanoplastics, such as small size and large surface area, it is urgent to pay more attention to the toxic effects of nanoplastics on environmental organisms and to elucidate the underlying mechanisms for the observed toxicity of nanoplastics. Several studies have confirmed that nanoplastics can affect the feeding, reproduction, growth, mortality, multiple molting, immune responses, and antioxidation of marine organisms (Bergami et al., 2016; Canesi et al., 2015; Cole and Galloway, 2015; Della Torre et al., 2014; Manfra et al., 2017; Sjollema et al., 2016; Ward and Kach, 2009). Freshwater is an important source of marine pollution through riverine transport (Rech et al., 2014). Many studies have shown that microplastics and nanoplastics can be widely found in the surface water and river sediment of freshwater environments (Eriksen et al., 2013; Free et al., 2014; Peng et al., 2018; Su et al., 2016). More than 160 different marine species ingest micro- or nanoplastics, while only 39 freshwater species have been found to ingest micro- or nanoplastics (Lusher, 2015; Scherer et al., 2018). Recent studies have reported the toxicity of nanoplastics in freshwater organisms, such as green algae (Nolte et al., 2017), zooplankton (Besseling et al., 2014; Nasser and Lynch, 2016; Rist et al., 2017), and fish (Greven et al., 2016; Mattsson et al., 2015). Still, knowledge on the biological impact of nanoplastics on freshwater organisms is limited and conflicting (Chae and An, 2017). Thus, further studies are required to study the effects of nanoplastics on freshwater species.

To address this gap in information, we investigated the uptake as well as effects of nanoplastics in the freshwater cladoceran *Daphnia pulex*. The species has a small body size (1.1–3.5 mm in female), wide geographical distribution, genetic homogeneity, high reproduction rate, and can be easily cultured in the laboratory, making it a model organism for aging, evolutionary, and ecotoxicological studies (Ebert, 2005; Lampert, 2006). *D. pulex* is a non-selective filter-feeder, and it floats along waves to efficiently graze on small particles, such as nanoplastics. Polystyrene is one of the most commonly used plastic polymers worldwide (Browne et al., 2011; Mathalon and Hill, 2014). Thus, polystyrene nanoplastics were selected as the research material for this study. We investigated the ingestion of polystyrene nanoplastics by *D. pulex* over a period of 24 h and studied the physiological changes (e.g., survival, growth, and reproduction) caused by and toxic effects of the nanoplastics on the expression of 6 stress defense genes (*Mn-SOD*, *CAT*, *GPx*, *GSTD*, *HSP70*, and *HSP90*) by performing a 21-day chronic toxicity test. The objective of this study was to provide insights into the relationship between changes in the growth and reproduction of *D. pulex* and the responses of its stress defense genes to nanoplastic exposure. The results of this study can improve our understanding of the mechanisms underlying nanoplastic toxicity in aquatic invertebrates and provide a scientific basis for the development of bio-indicators for the early identification of nanoplastics.

2. Material and methods

2.1. Culture of D. pulex

Daphnia pulex specimens were supplied by the Laboratory of Zooplankton Adaptation and Evolution, East China Normal University, and fed with the single-cell green alga *Chlorella pyrenoidosa*. The green alga was obtained from Guangyu Biological Technology Co., Ltd., Shanghai, China. The *D. pulex* specimens were continuously cultured in 4-L glass beakers, which were filled with approximately 3 L of medium, at 20 ± 0.5 °C and a light:dark (L:D) cycle of 16:8 h. The dissolved oxygen concentration in the culture was maintained at 5 mg/L or above. Before the exposure experiments, gravid female *D. pulex* specimens were collected and cultured individually in 50-mL glass beakers until they oviposited. Then, healthy neonates (<24 h) from their third brood were collected and used for the tests.

2.2. Polystyrene nanoplastic

Unlabeled and green fluorescence-labeled polystyrene nanoplastics were purchased from BaseLine Chromtech Research Centre, Tianjin, China. The nanoplastics were monodisperse polystyrene microspheres 75 nm in nominal diameter and supplied in 10-mL aqueous suspensions of 25 mg/mL (1.06×10^{13} particles/ml) and 10 mg/mL (5.32×10^{12} particles/ml) concentrations. The excitation and emission wavelengths of the fluorescently labeled nanoplastics were 488 nm and 518 nm, respectively. The composition of the virgin PS beads was confirmed by Fourier-transform infrared spectroscopy (FTIR) and the aggregation of nanoplastic in water was checked by dynamic light scattering (Liu et al., 2018; Lu et al., 2016).

2.3. Ingestion of the polystyrene nanoplastics

The *D. pulex* specimens were exposed to 2 mg/L (1.06×10^9 particles/ml) of the fluorescently labeled polystyrene nanoplastic and incubated at 20 °C in the dark for 24 h. The specimens were then washed with double-distilled water to remove the nanoplastic-containing water from the skin. The nanoplastics in *D. pulex* were observed with a fluorescence microscope (Olympus BX53F; Olympus Corporation; Tokyo, Japan). A bright-field image and a dark-field image were obtained, and the 2 images were stacked.

2.4. Acute toxicity test

The acute toxicity test was performed on the basis of the Organization for Economic Cooperation and Development (OECD) guideline for *Daphnia* sp. Acute Immobilization Test (OECD, 2004). Five replicates of 5 neonates (a total of 25 neonates that were less than 24 h of age) were exposed nanoplastic concentration of 10 (5.32×10^9 particles/ml), 50 (2.66×10^{10} particles/ml), 100 (5.32×10^{10} particles/ml), 150 (7.77×10^{10} particles/ml), 200 (1.06×10^{11} particles/ml), or 400 mg/L (2.12×10^{11} particles/ml) in 50-mL glass beakers containing 40 mL of the test media for 48 h and 16:8 h (L:D) photoperiod. On the basis of the guideline, the *D. pulex* specimens were not fed during the exposure to the nanoplastics. After 48 h, each test vessel was checked for immobilization; individuals that were non-responsive within 15 s after gentle agitation of the test beakers were considered immobilized. The test is valid if the total immobilized control specimens do not

exceed 10%. The number of immobilized specimens was counted, and the median lethal concentration (48 h LC50) and confidence limits were calculated using the 'Probit' analyses in SPSS v19.0.

2.5. Reproductive tests

To examine the effects of nanoplastic exposure on the growth and reproduction of *D. pulex*, 21-day reproductive tests were performed according to standardized protocols. The *D. pulex* specimens were randomly divided into 5 groups: control and four treatment groups. The treatment groups were exposed to 0.1 (5.32×10^7 particles/ml), 0.5 (2.66×10^8 particles/ml), 1 (5.32×10^8 particles/ml), and 2 mg/L (1.06×10^9 particles/ml) of the nanoplastics, corresponding to 1/500, 1/100, 1/50, and 1/25 of the 48 h LC50. Ten replicates of individual neonates (<24 h) from the third brood were tested for each concentration (OECD, 2004). The specimens were maintained in 50-mL glass beakers with 40 mL of the medium in a semi-static test setup; the medium was changed every 48 h. All daphnids were fed with the green alga *C. pyrenoidosa* at a total carbon concentration of 400 μg C/L and maintained at 20 °C and 16:8 h (L:D) photoperiod, according to Jiang (2013b) and Liu et al. (2018). During the experimental incubations, several response variables related to growth and reproduction, including the time to first offspring, total number of neonates per daphnia, and size of the mother daphnias at the end of the test, were measured. The number of neonates was counted daily. The size of the mother daphnias was measured from above the eye to the base of the abdomen at 7, 14, and 21 days by using a microscope (BX43F; Olympus Corporation, Tokyo, Japan) with a connected camera (DP21; Olympus Corporation, Tokyo, Japan) and the growth rate was calculated. To visualize the variations between the treatment groups, growth and reproduction were expressed as z-scores, namely, normalized to the mean of the respective control as: $Z = (\alpha\text{-}\overline{X}_{control})/S.D.$, where α is the observed value in the treatment group, $\overline{X}_{control}$ is the mean value of the control, and S.D. is the standard deviation of the sample population (Ogonowski et al., 2016).

2.6. RNA isolation and quantitative RT-PCR

To examine whether the polystyrene nanoplastic induces oxidative stress, neonates younger than 24 h (third brood) were placed in 1000-mL glass beakers (20 neonates per beaker) containing 500 mL of solution. Four replicates were tested for each concentration. Tubes with 20 neonates were snap frozen in liquid nitrogen and stored at −80 °C. When all sample replicates were collected, they were thawed on ice and used for isolation of RNA. The specimens were homogenized in a 2-mL centrifuge tube containing 1 mL of TRIzol reagent, according to the manufacturer's protocol. The RNA quantity and quality were examined using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA) and 1% agarose gel electrophoresis. cDNA was generated using the total RNA with the PrimeScript ™ II 1st Strand cDNA Synthesis Kit 6210A (TaKaRa, Japan), according to the manufacturer's instructions.

The primer sequences and product sizes are listed in Table 1. Quantitative RT-PCR was conducted using CFX96™ RT-PCR (Bio-Rad, Hercules, CA, USA) and SuperReal PreMix Plus (SYBR Green) FP205 (Tiangen, Beijing, China). The PCR volume contained 1.2 μL of the cDNA template, 10 μL of SYBR, and 7.6 μL of ddH_2O with 0.6 μL of each 10 μM primer. The 18S gene was used as the internal reference. All the RT-qPCRs were completed in quadruplicate and normalized to the control gene. The thermal profile for PCR was 95 °C for 15 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 30 s. At the end of PCR, the samples were processed for melting curve analysis at 65 °C–95 °C with 0.5 °C increment.

Primers were adopted from previous studies (Liu et al., 2018; Tang et al., 2015).

2.7. Data analysis

Relative changes in the transcript abundance of the genes were normalized to 18S and calculated using the $2^{-\triangle\triangle Ct}$ method. All statistical analyses were performed using SPSS Statistics 19.0, and the graphs were created with GraphPad 7.0. One-way analysis of variance and Duncan's test were used to determine whether the data were statistically significant ($p < 0.05$) between the control and treatment groups. When the data distribution was skewed, the analysis methods described by Liu et al. (2016) were used.

3. Results

3.1. Effects of the nanoplastics on the mortality rate

At the end of the bioassays (48 h), no total immobilization exceeding 10% was observed in the control (Fig. 1). For 400 mg/L

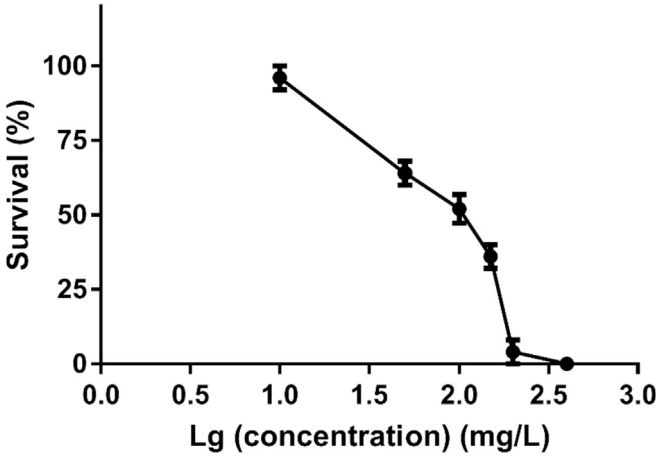

Fig. 1. Acute toxicity analysis on the effects of nanoplastic exposed to *Daphnia pulex* at various concentrations (between 10 and 400 mg/L) using neonates (<24 h post-hatch) for 48 h.

Table 1
Primer sequences, amplicon sizes, and accession numbers used in qPCR reaction.

Gene	Accession number	Forward sequence (5'-3')	Reverse sequence (5'-3')	Amplicon size (bp)
CAT	NCBI_GNO_227174	ACCAACGAGATGGTCCAATG	TCATCGGCAGTGTTGTATCG	154
GSTD	NCBI_GNO_144203	CCAGAGCACTTTGACCCATT	CGATCTATCGCTGATTGCCA	176
GPx	NCBI_GNO_500033	AAAATGCGGTTACACTCACG	ATTTCCAGAGAGGATGAGCG	230
HSP70	NCBI_GNO_306193	CATCTTGAACGTGACTGCTG	ATTCTTGGCCGATACTCGTT	169
HSP90	KC845247	GAACTTTTCGAGGAGTTGGC	TCCTTCATGCGAGAAACGTA	194
Mn-SOD	NCBI_GNO_297184	ATTGCTACTGTCGCTGATGA	ATGCTTCGATTTAATGGCGG	164
18S	AF014011	CAAAGCCCGACTCTCTTCAC	CGTTGGGATACACCTGCTTT	238

and 10 mg/L of the nanoplastics, 100% and 4% mortalities, respectively, were observed in the exposed specimens (Fig. 1). The LC50 values of the nanoplastics on the freshwater cladoceran *D. pulex* at 48 h was determined to be approximately 76.69 mg/L (95 CI, lower: 32.41 mg/L, upper: 127.35 mg/L).

3.2. Effects of the nanoplastics on growth and reproduction

During 24 h of exposure, the nanoplastics were readily ingested by the freshwater cladoceran, as exhibited by the fluorescence in the digestive organs of *D. pulex* (Fig. 2).

The results demonstrated that the nanoplastics significantly inhibited the growth of *D. pulex* (Fig. 3A). When compared with the control group, the body length was significantly decreased in only the 0.5 mg/L and 0.1 mg/L treatment groups after 7 and 14 days, respectively, and in all the treatment groups after 21 days ($p < 0.05$, Fig. 3A). These results showed that the toxic effects of the nanoplastics on body length were time- and dose-dependent (Table 2).

GR of the specimens was higher in 0–7 days than in 7–14 and 14–21 days; in particular, for the controls, $GR_{0\text{-}7}$ was about a fifth of $GR_{7\text{-}14}$ (Fig. 3B). The nanoplastic reduced the GR of the specimens more pronouncedly in 0–7 days. Indeed, significant changes in $GR_{0\text{-}7}$ were recorded for 0.1, 0.5, and 1 mg/L of the nanoplastic ($p < 0.05$). No significant differences in $GR_{7\text{-}14}$ were observed among the treatment groups ($p > 0.05$), but a significant decrease in $GR_{14\text{-}21}$ was detected in the 2 mg/L group ($p < 0.05$).

To examine the toxic effects of the nanoplastics on reproduction, we measured the reproduction time (time to first eggs and time to first clutch) and number (total offspring per female, number of clutches, and offspring per clutch). The results show that the nanoplastics significantly prolonged the reproduction time and decreases the reproduction number of *D. pulex*. The time to first clutch was significantly prolonged in the exposed specimens when compared with the control ($p < 0.05$, Fig. 3D). No significant differences in the time to first eggs were observed in the specimens exposed to 0.1 and 2 mg/L of the nanoplastics ($p > 0.05$); however, the time to first eggs was significantly prolonged in the specimens exposed to 0.5 and 1 mg/L of the nanoplastics ($p < 0.05$, Fig. 3C). A significant decrease in total offspring per female and number of clutches was observed among the treatment groups when compared with the control ($p < 0.05$, Fig. 3E and F). A significant decrease in offspring per clutch was observed at the low concentration of 0.1 mg/L ($p < 0.05$); however, no significant effects were observed in the other treatment groups ($p > 0.05$, Fig. 3G).

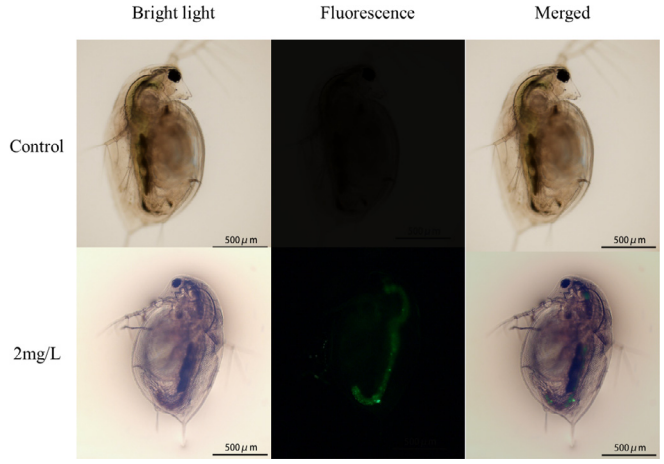

Fig. 2. Images of the fluorescence-labeled polystyrene nanoplastics ingested by *Daphnia pulex*. Images of the 2 mg/L fluorescence-labeled nanoplastics were obtained at 24 h.

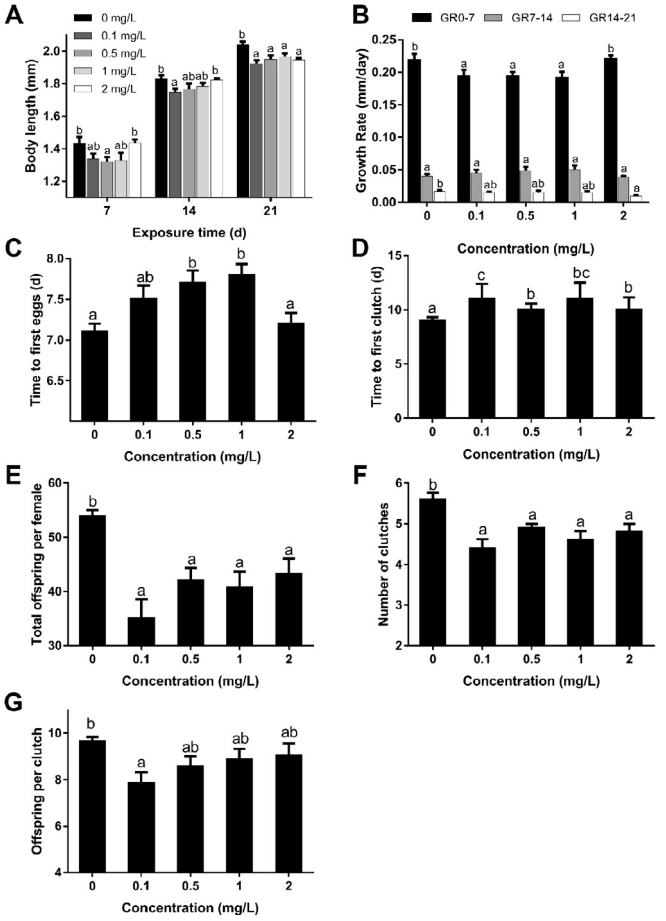

Fig. 3. Effects of exposure to different concentrations of the nanoplastics on the growth and reproduction of *Daphnia pulex*. A, Body length; B, Growth rate; C, Time to first eggs; D, Time to first clutch; E, Total offspring per female; F, Number of Clutches; G, Offspring per clutch. The data are presented as means ± SE.

Table 2
Two-way ANOVA of the interaction of exposure time and nanoplastic concentration with the body length of *Daphnia pulex*.

Parameter	Source of variation	Df	F	*p*
Body length	Nanoplastic concentration	4	662.995	<0001
	Time	2	7.245	<0001
	Nanoplastic concentration × Time	8	1.097	0.369

Radar plots were used to analyze the sensitivity of the growth and reproduction indexes to the nanoplastic. The results confirmed that the nanoplastics affected the growth and fecundity of *D. pulex* (Fig. 4).

3.3. Effects of the nanoplastics on the expression of the stress defense genes

To examine the effects of the nanoplastics on the stress defense of *D. pulex*, we tested the gene expressions of antioxidant enzymes and heat shock proteins (HSPs).

With an increase in the concentration of the nanoplastic, the effect of the nanoplastic on the expression of oxidative stress-mediated genes (*SOD, GST, GPx*, and *CAT*) first increased and then decreased; however, the changes in gene expression were different (Fig. 5). No significant difference in the expression of *SOD* was

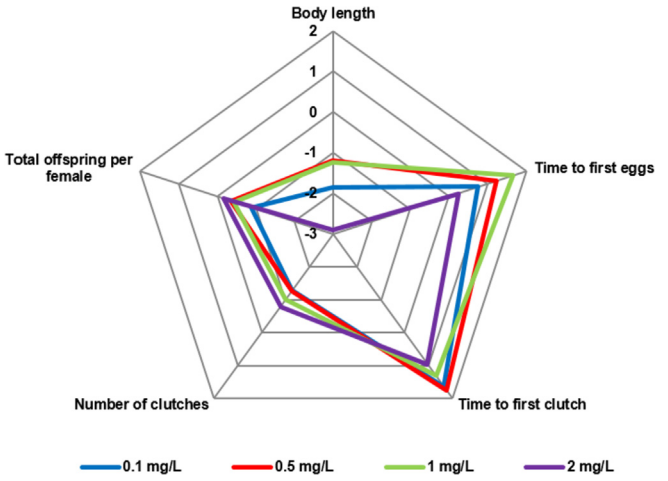

Fig. 4. Integrated fitness responses. Radar plot of growth and fecundity across all test concentrations and Z-score normalized to the control. $Z = (\alpha\text{-}\overline{X}_{control})/S.D.$, where α is the observed value in the treatment group, $\overline{X}_{control}$ is the mean value of the control, and S.D. is the standard deviation of the sample population. Positive values denote a positive response on fitness.

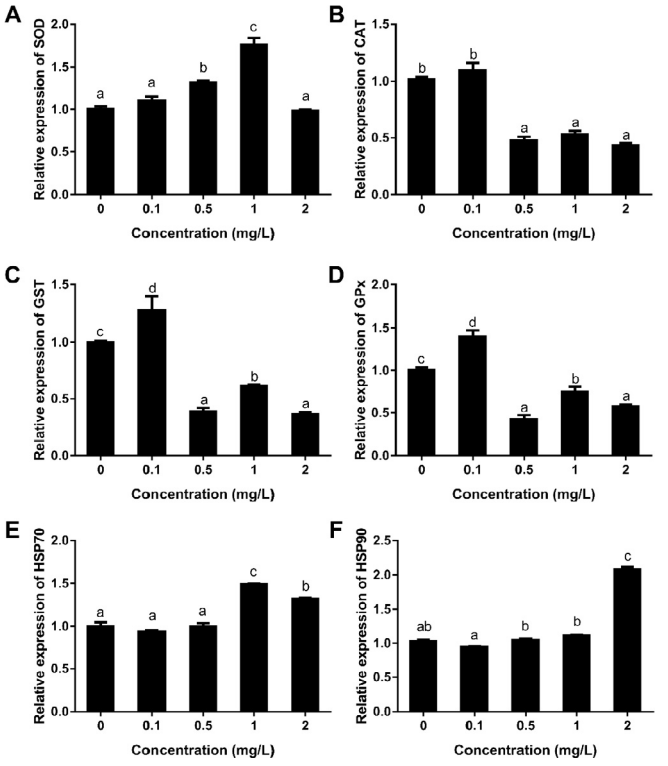

Fig. 5. Effects of exposure to different concentrations of the nanoplastic on expression of the stress defense genes in *Daphnia pulex* (A: *SOD*; B: *CAT*; C: *GST*; D: *GPx*; E: *HSP70*; F: *HSP90*). The results represent the mean ± S.E. values of 4 replicate samples.

observed in the 0.1 and 2 mg/L groups ($p > 0.05$), whereas a significant increase in expression was found in the 0.5 and 1 mg/L groups ($p < 0.05$, Fig. 5A). A significant increase in the expression of *GST* and *GPx* was recorded in the 0.1 mg/L group ($p < 0.05$), and a significant decrease was observed in the 0.5, 1, and 2 mg/L groups ($p < 0.05$, Fig. 5C and D). In addition, the expression of *CAT* in the 0.1 mg/L group increased, but not significantly higher than that in the control group ($p > 0.05$); the expression of *CAT* significantly decreased in the 0.5, 1, and 2 mg/L groups ($p < 0.05$, Fig. 5 B).

The gene expression levels in the *D. pulex* specimens exposed to the nanoplastics for 21 days are shown in Fig. 5. Significant changes were observed in the case of *HSP70* and *HSP90*. Namely, the nanoplastic caused a significant increase in the expression of *HSP70* at high concentrations (1 and 2 mg/L; $p < 0.05$), whereas no significant differences were observed at low concentrations (0.1 and 0.5 mg/L; $p > 0.05$; Fig. 5E). In addition, no significant differences were observed in the expression of *HSP90* at low concentrations (0.1, 0.5, and 1 mg/L; $p > 0.05$), but significant increases in the expression of *HSP90* were detected in the 2 mg/L group when compared with the control ($p < 0.05$, Fig. 5F).

4. Discussion

Daphnia spp., as primary consumers and a major food source for higher trophic organisms, are one of the most important species in freshwater ecosystems. Therefore, it is important to evaluate the effects of nanoplastics on *Daphnia* spp. for performing ecological risk assessments in aquatic environments and understanding the critical impacts of nanoplastics on aquatic ecosystems as a whole. *Daphnia* spp. have poor feeding selectivity, which can result in the ingestion of microplastics and nanoplastics (Besseling et al., 2014; Nasser and Lynch, 2016; Rehse et al., 2016; Rosenkranz et al., 2009). In this study, *D. pulex* specimens ingested 75-nm nanoplastics. The digestive organs of the *D. pulex* specimens were strongly fluorescent after exposure to the nanoplastics, which were also found in the thoracic appendices (data not shown). Since *D. pulex* is a non-selective filter-feeder, we speculated that the nanoplastics were mainly filtered through the body. However, previous studies have shown that, in addition to filtration, nanoplastics can enter the body by other means, such as penetration into the brood chamber through caudal appendices and absorption through the body surface (Cui et al., 2017). However, in this study, we did not detect the nanoplastics entering the fleas through these 2 pathways, especially absorption through the body surface. Therefore, the intake pathways of nanoplastics in *Daphnia* spp. and zooplankton need to be studied further.

The LC50 values of the nanoplastic on the freshwater cladoceran *D. pulex* at 48 h was determined to be approximately 76.69 mg/L. Currently, acute toxicity tests are critical for evaluating the physiological health of test species exposed to toxicants. On the basis of the guidelines for the hazard evaluation of new chemical substances, the toxicity of nanoplastics should be classified as intermediate when compared with other nanomaterials, such as nano-TiO_2 (48 h LC 50, 9.2 mg/L) and nano-CeO_2 (48 h LC50, 91.79 mg/L) (Artells et al., 2013; Hall et al., 2009). Mortality is a reliable ecotoxicological endpoint. However, a high level of exposure rarely occurs in the natural environment; In general, an environmental dose of nanoplastics may not cause acute toxicity during a short period of exposure. Thus, it is necessary to have other indicators for nanoplastics.

Sensitive indicators such as growth and reproduction have been used as endpoints for ecological stress or chemical toxicity in *Daphnia* (Liang et al., 2017; Steinkey et al., 2018). Thus, we examined the body length, GR, and other indicators of growth and the time to first eggs, time to first clutch, total offspring per female, number of clutches, offspring per clutch, and other reproductive indicators. The effects of toxicants on the growth and reproduction of the test species are broadly accepted parameters and have been found to be more sensitive indicators than immobilization (Roh et al., 2007), as observed in this study. We found that exposure of the freshwater cladoceran *D. pulex* to the nanoplastic induced physiological alterations that led to significant repercussions on growth and reproduction. Exposure of the cladoceran *Daphnia magna*, rotifer *Brachionus koreanus*, copepod *Paracyclopina nana*,

and *Parvocalanus crassirostris* to microplastics or nanoplastics had negative effects on growth and reproduction (Besseling et al., 2014; Heindler et al., 2017; Jeong et al., 2016, 2017), which is consistent with our findings. Interestingly, the time to first eggs was significantly prolonged at the lower concentrations of 0.5 and 1 mg/L, whereas no significant prolongation was observed at 2 mg/L. We speculate that, when exposed to a high concentration of the nanoplastic, freshwater cladoceran *D. pulex* can enhance tolerance to the nanoplastic to survive, which is similar to the findings of a study performed using Cd (Klerks and Weis, 1987; Muyssen and Janssen, 2004). In addition, at 0.1 mg/L, no significant difference was observed in the time to first eggs, whereas the time to first clutch was significantly prolonged. The reason may be that low concentrations of nanoplastics activate embryonic lethal genes in vivo (Della Torre et al., 2014). The time to first eggs was prolonged, so the number of clutches and total offspring per female were also affected. In addition, the lowest value for total offspring per female was detected at the lowest concentration (0.1 mg/L); however, as the concentration increased, the total offspring per female increased. The reason may be that at a high concentration of the nanoplastic, *D. pulex* adjusts its breeding strategy, giving birth to more progeny to cope with the stress (Jiang et al., 2013a; Lyu et al., 2016).

In this study, nanoplastic exposure affected fecundity in all the treatment groups (significant prolonged time to first eggs was observed at 0.5 and 1 mg/L; time to first clutch and total offspring per female were significantly decreased at 2 and 0.1 mg/L, respectively), which suggests that development was more susceptible than fecundity to nanoplastic exposure; the radar plots confirmed this result. Exposure of *D. magna* to environmentally relevant (65 ± 7.1 and 550 ± 23 ng/L) tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) significantly decreased body length, but fecundity was only affected by exposure to high concentrations (6500 ± 1400 ng/L) (Li et al., 2015); these results were consistent with our findings.

Environmental pollutants can induce the generation of reactive oxygen species (ROS) and subsequent oxidative stress (Lushchak, 2011; Wu et al., 2018). The metabolic activity of organisms can be adjusted to overcome the ROS derived from exposure to toxicants, especially the defense system related to detoxification. SOD is a key factor that can convert the superoxide to H_2O_2. CAT, GST, and GPx are also important antioxidants that convert H_2O_2 into water and other harmless substances (such as oxygen and GSSG). Therefore, the expression of stress defense genes such as *SOD*, *CAT*, *GST*, and *GPx* was determined to reflect the effects of the nanoplastic on ROS. The results showed that the expression levels of *SOD*, *CAT*, *GST*, and *GPx* in the low-concentration group were significantly upregulated when compared with the control group. This indicates that the low concentration of the nanoplastic causes overproduction of the superoxide anion and, consequently, H_2O_2, the final product of O_2^- dismutation. However, with the increase of nanoplastic concentration, the expression of antioxidant enzyme genes (*CAT*, *GST*, and *GPx*) showed a downward trend, probably because the antioxidant system was damaged by high concentrations of the nanoplastic. Oxidative damage to organisms by microplastics and nanoplastics has been reported in organisms such as the lugworm *Arenicola marina*, algae *Chlorella* and *Scenedesmus*, Crab *Eriocheir sinensis* and zebrafish *Danio rerio* by measuring ROS and total antioxidant capacity (Bhattacharya et al., 2010; Browne et al., 2013; Chen et al., 2017; Yu et al., 2018). The results indicated that the nanoplastics had an impact on the antioxidant system and caused damage to the organism.

In addition to the defense of antioxidant systems, organisms also synthesize specific proteins to cope with environmental pollution and other stressful environments. Heat-shock proteins (HSPs) play an important role in maintaining the stability of the protein structure and are often considered important markers of environmental pollution stress (Hamer et al., 2004; Rhee et al., 2009). Therefore, we examined the effects of the nanoplastic on the expression of *HSP70* and *HSP90* in the freshwater cladoceran *D. pulex*. The results showed that, *HSP70* and *HSP90* expression levels increased significantly in the nanoplastic groups when compared with the control group, showing that the nanoplastic affected the protein structure and induced the expression of HSPs involved in intracellular protein denaturation overlap, and prevented protein degradation or further aggregation (Kiang and Tsokos, 1998). The expression of *HSP70* in the 1 mg/L group was significantly higher than that in the control group, whereas the expression of *HSP90* in the 2 mg/L group was significantly higher than that in the control group. Previous studies have demonstrated that *HSP70* is more suitable than *HSP90* as a molecular marker for the environmental stress response in *D. pulex*. Gene transcription, translation, and function of HSPs are energy-consuming processes. Thus, long-term stress conditions will inevitably affect the growth of *D. pulex*.

In summary, nanoplastics can be ingested by the freshwater cladoceran *D. pulex* and affect its growth and reproduction as well as induce stress defense. To date, the effects of nanoplastics on aquatic invertebrates, especially freshwater organisms, have not been investigated in detail. Therefore, this study provides a better understanding of the effects of nanoplastics on the freshwater model species *D. pulex* at molecular and individual levels.

5. Conclusions

There is limited information on nanoplastic exposure and toxicity, and we need to understand the consequences of this threat to the environment. Our findings showed that the exposure of environmentally relevant or higher concentrations of a polystyrene nanoplastic may induce notably adverse effects on the freshwater cladoceran *D. pulex* at different levels of biological organization. Exposure to the nanoplastic induced stress defense in *D. pulex*, and had effects on growth and reproduction, which may be particularly worrisome because they can negatively affect the population dynamics of *D. pulex* and its food web interactions. Aquatic organisms are exposed to nanoplastics as well as other environmental pollutants, resulting in possibly higher toxic effects than those pointed out in this study. Thus, further studies on the effects of nanoplastics on aquatic organisms should be prioritized to understand the ecological hazards for freshwater ecosystems.

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