Journal of Oceanology and Limnology
Vol. 39 No. 3, P. 979-988, 2021
https://doi.org/10.1007/s00343-020-0118-2

Effect of polystyrene microplastics and temperature on growth, intestinal histology and immune responses of brine shrimp *Artemia franciscana**

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Received Mar. 24, 2020; accepted in principle May 18, 2020; accepted for publication Jun. 28, 2020

Abstract Microplastics pollution and seawater temperature rise have been the major environmental issues, threatening the survival and biodiversity of marine organisms. This study evaluated the combined effect of temperature and polystyrene microplastics (MP) on *Artemia*, a filter-feeding crustacean that is widely used for environmental toxicology studies. Brine shrimp *Artemia franciscana* were exposed to three MP concentrations (0, 0.2, and 2.0 mg/L) and three temperatures (22, 26, and 30 °C) for 14 d. In general, higher MP concentration and temperature led to a decreased survival rate and growth. Two-way ANOVA analysis indicated that the survival rate of *Artemia* was significantly impacted by both MP concentration and temperature (P<0.05), but there was no significant interaction between two factors (P>0.05). Growth of *Artemia* was significantly impacted by temperature (P<0.05), and with a significant interaction between two factors (P<0.05). Furthermore, the enzymatic activity, intestinal histological analyses, and immune gene expression were determined for *Artemia* reared at 30 °C with three MP concentrations (0, 0.2, and 2.0 mg/L). The results showed that 2.0-mg/L MP resulted in reduced *Artemia* intestinal microvilli and exfoliated epithelia cells, significantly increased acid phosphatase (ACP) activity (P<0.05) and immune-related gene *ADRA1B* and *CREB3* expression, revealing that higher MP concentration could induce oxidative and immunological stress on *Artemia* at 30 °C. Overall, our study suggests that MP and temperature have combined adverse effect on *Artemia*, especially at relatively high temperature and polystyrene MP concentration. These findings are important to understand the potential ecological risks posed by these two factors on the organisms in marine environment.

Keyword: *Artemia franciscana*; combined effect; microplastics; temperature

1 INTRODUCTION

Nowadays, plastic pollution has become one of the serious environmental problems worldwide (Andrady, 2011; PlasticsEurope, 2017). It has been reported that 10% of the world's plastic waste eventually enters the ocean (Secretariat of the Convention on Biological Diversity and Scientific and Technical Advisory Panel GEF, 2012), and that up to 90% of the waste in ocean are small fragments (Eriksen et al., 2014). Microplastics (MP), usually referring particles less than 5 mm, has become an emerging threat to marine environment and biodiversity due to its toxic and bio-accumulative effects (Jambeck et al., 2015; Bakir et al., 2016). MP can easily get into aquatic food web and result negative impact on marine organisms, and thus threaten the food safety of fisheries and aquaculture

* Supported by the Science and Technology Project of Tianjin Municipal (No. 17ZXZYNC00060), the Yangtze Scholars and Innovative Research Team in University of Ministry of Education of China (No. IRT_17R81), the National Science Foundation of Tianjin (No. 18JCQNJC78500), and the Foundation of Tianjin Key Laboratory of Marine Resources and Chemistry (Tianjin University of Science & Technology), China (No. 201704)
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products (Wright et al., 2013; Lusher, 2015; Jabeen et al., 2017; Barboza et al., 2018b; Li et al., 2018). Being as a main planktonic consumer of the marine food web, zooplankton plays an important role in energy transfer and greatly contributes to the diversity of marine ecosystem. Excess intake of MP causes a series of adverse impacts on zooplankton, including intake disorders, acute oxidative stress, reduced reproduction, and survivals (Lee et al., 2013; Cole et al., 2015; Jeong et al., 2016). In addition, the retention of MP in the body may be transferred and accumulated along marine trophic webs, further affecting the stability of the entire marine ecosystem (Wang et al., 2016; Martins and Guilhermino, 2018).

Although MP has proven many negative impacts on zooplankton, few studies have investigated the combined effects of MP and other environmental factors on marine species. The interaction of environmental factors are not only the sum of the independent responses but mostly be synergistic or antagonistic (Crain et al., 2008). Temperature is one of the key drivers of biological response, which determine the physiological fitness and distribution range of a species by influencing its growth, feeding, metabolism, reproduction, and behavior (Place et al., 2008). In recent years anthropogenic environmental changes likely cause global warming and result in the rise of ocean temperatures (Brierley and Kingsford, 2009). Therefore, marine organisms are facing threats posed by the simultaneous presence of MP and elevated temperature.

Brine shrimp *Artemia* is a filter-feeding zooplankton that can adapt to a wide salinity range. It has some virtues such as shorter life cycle, bigger offspring number per brood, and easy to handle in the laboratory (Lavens and Sorgeloos, 1996), and thus has been widely used as a test organism for ecotoxicology study (Manfra et al., 2015; Vannuccini et al., 2015; Ekonomou et al., 2019). Nowadays, *Artemia* are increasingly used as test organisms for marine ecotoxicology study, with special focus on the toxicity of micro- and nano-particles on *Artemia* growth, morphology and physiology properties (Rodd et al., 2014; Bergami et al., 2017; Minetto et al., 2017; Rotini et al., 2018; Sarkheil et al., 2018; Varó et al., 2019). These studies mostly focused on short-term toxicity test with *Artemia* nauplii. In fact, the increase of MP concentration may lead to increased mortality of some marine species after a long-term exposure. Thereby, it is necessary to provide evidence of the chronic effects by exposing *Artemia* to MP for long time course.

In this study, the 14-d culture was conducted to evaluate the effects of MP and temperature on *Artemia* in the laboratory condition. Our hypothesis is that MP and temperature have combined effects on *Artemia* for a long-term exposure, and cause a series of responses at physiological, biochemical, molecular and histological level. To test this hypothesis, the survival rate was determined in time course. After 14-d exposure, *Artemia* growth was determined (Bergami et al., 2017). The immune-related oxidative enzymes acid phosphatase (ACP) and catalase (CAT) were used as indicators of biochemical responses (Hou et al., 2000; Bhuvaneshwari et al., 2018). Two immune genes adrenergic receptor alpha-1B (*ADRA1B*) and cyclic AMP-responsive element-binding protein 3 (*CREB3*) were used as indicators of apoptosis pathways (Zhang et al., 2018). Moreover, intestinal histology was further examined to assess possible damage of epithelial cells lining the digestive tract (Wang et al., 2019). The outcome of this study may provide insight into the combined effect of MP and temperature on marine organism.

2 MATERIAL AND METHOD

2.1 Experimental design

The cysts of *Artemia franciscana* from the Great Salt Lake, USA were hatched following the general procedure. Exact 0.100-g cysts were placed in a glass cone containing 200-mL artificial seawater (30 salinity, ASW, Instant Ocean, USA), and with continuous aeration and illumination (1 000 lx) at 28 °C. Polystyrene microplastic beads with particle size of 4–6 μm were purchased from Tianjin BaseLine ChromTech Research Centre (Tianjin, China). The MP suspension (25 mg/mL) were prepared using deionized water and were sonicated prior to use.

Experiment was conducted in combination of three temperature (22, 26, and 30 °C) and three MP concentrations (0, 0.2, and 2.0 mg/L) with nine treatments. Each treatment was run in three replicates. The temperature degrees were selected according to surface temperature variability (20–30 °C) of the ocean (Shen et al., 2007). For the MP levels, 0.2 mg/L was considered ecologically relevant MP concentration in marine environments (Barboza et al., 2018a), and 2.0 mg/L was selected considering a severe scenario where MP pollution occurs.

One hundred newly hatched nauplii were randomly transferred into a 250-mL glass bottle containing

Table 1 Primers used for real time-PCR

Primer name	Forward primer (5′→3′)	Reverse primer (5′→3′)
CREB3	TCTATCCCCTGAGTCGAACA	CATCCTCAAATTCCCTTATCC
ADRA1B	GATGGAGATGCTTGGGAACA	ACCGTGCGTCTTGGGATG
gapdh	GTTGATGGCAAACTCGTCATA	CCACCTTCCAAGTGAGCATTA

200-mL artificial seawater (ASW) (salinity 30). *Artemia* were fed twice a day with microalgae *Dunaliella salina* at a final density of 100 000 cells/mL. To evaluate the effect of MP on *Artemia* during long-term exposure, the survival rate was determined after 1-, 3-, 6-, 10-, and 14-d culture. *Artemia* were put back in the bottles after counting the survivals. The survival rate was calculated as follows:

Survival rate (SR, %)=$N/N_0\times100$,

where N is the survived *Artemia* number, and N_0 is the initial *Artemia* number.

On day 14, thirty *Artemia* from each replicate were randomly collected, the average individual body length was measured under a stereomicroscope.

The effect of three MP concentrations (0, 0.2, and 2.0 mg/L) combined with 30 °C were studied to evaluate the immune-related enzyme activity, immune gene expression and intestine histology of *Artemia* in bigger scale setup. Newly hatched *Artemia* nauplii were randomly distributed into 9 plastic cones containing 5-L ASW. The initial density of *Artemia* was 100 ind./L. Each treatment was run in three replicates. *Artemia* were reared at 30 °C with 14 h L∶10 h D photoperiod for 14 d, and were covered to prevent water evaporation. The nauplii were fed *D. salina* twice a day at a final density of 100 000 cells/mL.

2.2 Enzyme activity assays

On day 14, *Artemia* were collected and rinsed with deionized water. The excess water was blotted using tissue paper. Exact 0.100-g *Artemia* were weighed and homogenized in physiological saline (1∶9, w∶v). The suspension was centrifuged at 2 500 r/min for 10 min at 4 °C, and supernatant was collected. The enzyme activity of acid phosphatase (ACP) and catalase (CAT) assays were performed using analytical kits (Nanjing Jiancheng, China) and expressed as unit per milligram of soluble protein (U/mg protein). The soluble protein content was determined using the Coomassie Brilliant Blue (G-250) method.

2.3 Intestinal histology assays

On day 14, the intestine of *Artemia* in the group of 0 and 2.0-mg/L MP were dissected, and fixed in 4% paraformaldehyde for 48 h. After rinsed with phosphate buffer and dehydrated subsequently with ethanol dilutions (70%, 80%, 90%, and 100%), the tissues were made transparent with xylene, embedded in paraffin and cut in a microtome at 4-μm thickness. After hematoxylin-eosin staining, pictures were taken under Inverted Phase Contrast Microscope (Nikon-TiE, Japan).

2.4 RNA extraction, cDNA synthesis and real time-PCR

Gene expression analysis was performed according to Vannuccini et al. (2015). RNA concentrations were measured using a traycell spectrophotometer (Eppendorf, Germany) and RNA quality was detected on 1.2% agarose gel. Two milligram of total RNA was transcribed to cDNA using PrimeScript™ RT reagent Kit (Code No. RR037A). Two genes of adrenergic receptor alpha-1B (*ADRA1B*) and cyclic AMP-responsive element-binding protein 3 (*CREB3*) were chosen to perform real time-PCR, and glyceraldehyde 3-phosphatase dehydrogenase (*gapdh*) was selected as housekeeping gene (Chen et al., 2009).

Real time-PCR was performed using a CFX Connect thermal cycler. Amplification was performed in triplicate in a total volume of 25 μL containing 2-μL cDNA, 100 nmol/L of each primer and 12.5 μL of TB Green® Premix Ex Taq (RR820A, TaKaRa). The cycling conditions were 95 °C for 30 s for polymerase activation, followed by 40 PCR cycles of 95 °C of 10 s, at 50–60 °C of 30. The calculated relative expression ratio of each gene was based on the PCR efficiency (E) and C_t of sample compared with control, and expressed in comparison to the reference genes. Primers of these genes are listed in Table 1.

2.5 Statistical analysis

Differences in enzymatic activity and gene expression between treatments were analyzed using one-way ANOVA. The effects of different temperatures and MP concentrations on survival and growth of *Artemia* were analyzed by two-way ANOVA. All data were tested for normality and homogeneity of variance before ANOVA was done.

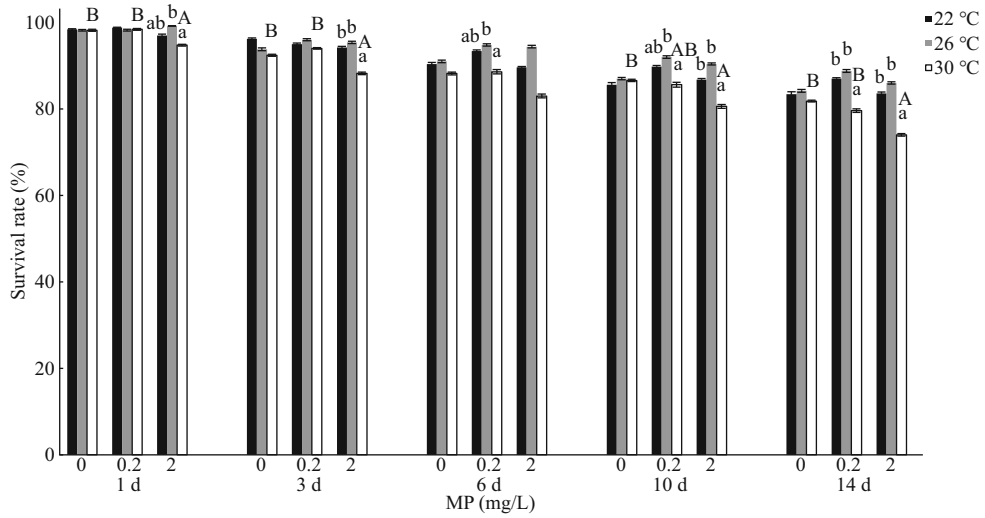

Fig.1 One-way ANOVA analysis on survival rate of *Artemia* exposed to different temperatures and MP concentrations for 1, 3, 6, 10, and 14 d

Lower-case letters indicate significant differences between temperatures for each MP concentration (P<0.05). Capital letters indicate significant differences between MP concentrations for each temperature (P<0.05).

Table 2 Two-way ANOVA analysis on the effects of temperature (22, 26, and 30 °C) and MP concentration (0, 0.2, and 2.0 mg/L) on *Artemia* survival rate

Exposure period (d)	Survival rate			
	df	*T*	MP	*T*×MP
1	MS	0.001	0.002	0.001
	F	2.776	3.325	2.867
	P	0.076	0.047	0.037
3	MS	0.006	0.002	0.002
	F	8.251	2.873	2.220
	P	0.001	0.070	0.086
6	MS	0.018	0.004	0.002
	F	12.555	2.999	1.666
	P	<0.001	0.062	0.179
10	MS	0.012	0.004	0.003
	F	6.911	2.682	1.880
	P	0.003	0.082	0.135
14	MS	0.026	0.006	0.004
	F	16.871	3.798	2.297
	P	<0.001	0.032	0.078

MS: mean square; *T*: temperature; *T*×MP: interaction between MP and temperature; *F*: sum of mean square of main effect/sum of mean square error.

Tukey's honest significant difference test was performed to identify differences at P<0.05.

3 RESULT

3.1 Survival and growth

One-way ANOVA analysis showed higher MP concentration and temperature led to a decrease in survival rate of *Artemia* (Fig.1). Throughout the experiment, *Artemia* exposed to 30 °C and 2.0 mg/L showed the lowest survival rate compared to other groups. By using two-way ANOVA analysis, temperature showed significantly influence on the survival rate of *Artemia* throughout the experiment (P<0.05), with the exception after 1-d exposure (Table 2). In addition, survival rate was significantly affected by MP after 1-d exposure and day 14 (P<0.05). There was no interaction between MP and temperature with the exception after 1-day exposure.

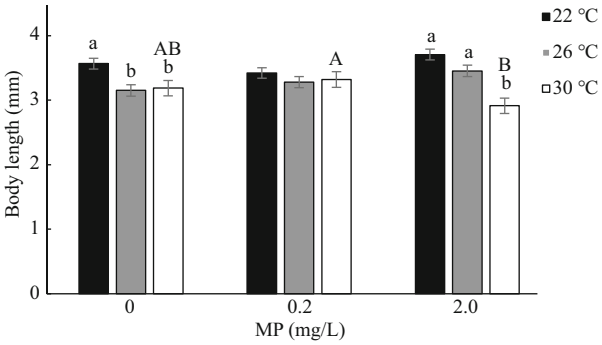

Fig.2 One-way ANOVA analysis on body length of *Artemia* exposed to different temperatures and MP concentrations for 14 d

Lower-case letters indicate significant difference between temperatures for each MP concentration (P<0.05). Capital letters indicate significant differences between MP concentrations at each temperature (P<0.05).

After 14-d exposure, *Artemia* length increased from 0.35–0.40 mm to 3–4 mm. High temperature and MP concentration significantly decreased *Artemia* growth in a long-term exposure, which is consistent with the survival rate (Fig.2). Two-way ANOVA analysis showed that *Artemia* length was significantly

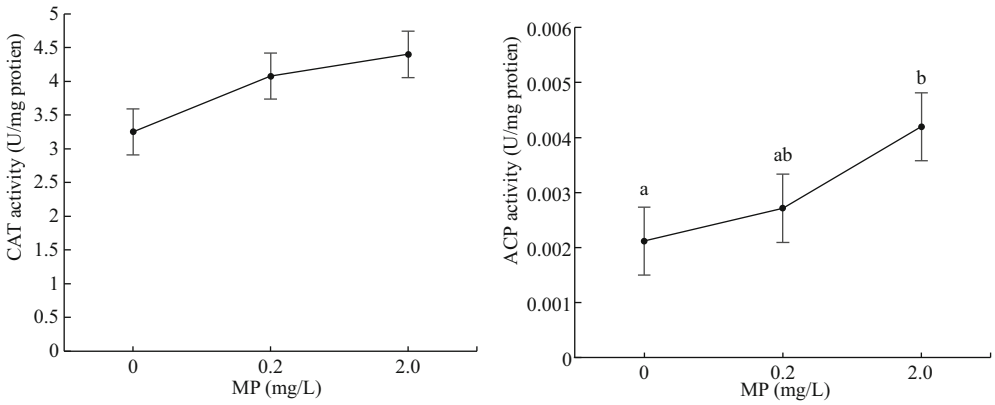

Fig.3 CAT and ACP activity of *Artemia* exposed to different MP concentrations at 30 °C for 14 d

Lower-case letters indicate significant differences at $P<0.05$.

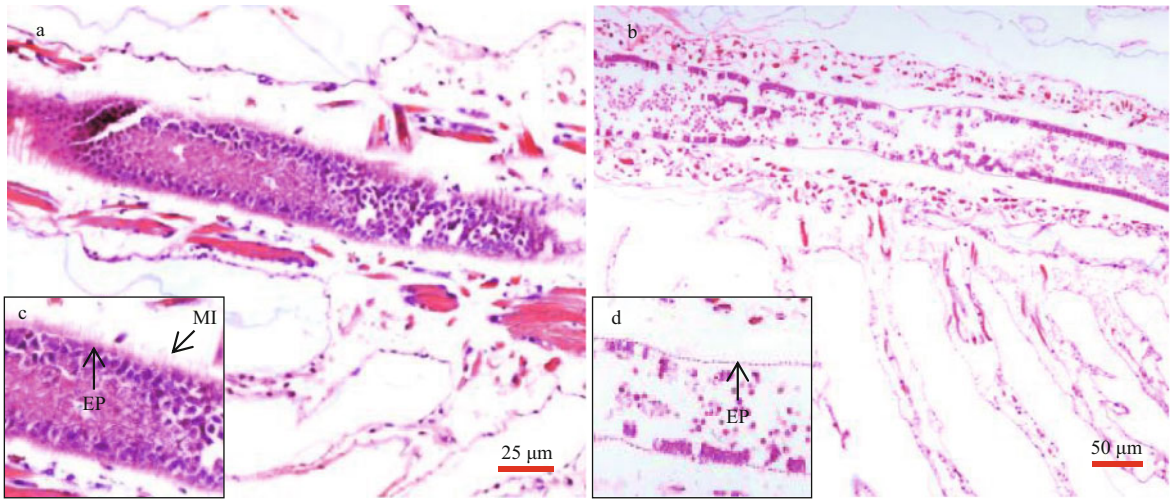

Fig.4 Intestinal tissue of *Artemia* exposure to 0 (a) and 2.0-mg/L (b) MP at 30 °C for 14 d

c and d are enlarged images for intestinal tissue in a and b, respectively. Scale bar was 25 and 50 μm for a and b, respectively. The abbreviation EP stands for epithelial cell layers and MI stands for microvilli.

affected by temperature ($P<0.05$), and there was a significant interaction between MP and temperature ($P<0.05$) (Table 3).

3.2 Enzymatic activity

Both CAT and ACP activity increased with the increasing MP concentrations (Fig.3). There was no significant difference in CAT activity in different groups ($P>0.05$), while ACP activity showed significant difference between 0 and 2.0-mg/L MP ($P<0.05$).

3.3 Intestinal histopathological analyses

Longitudinal sections of *Artemia* intestines in the group of 0 and 2.0-mg/L MP were shown in Fig.4. After 14-d exposure, the intestinal epithelial cells were closely connected and microvilli were regularly-ordered in control group. By contrast, the number of intestinal microvilli decreased and epithelia cells exfoliated in 2.0-mg/L MP treatment, which implied that 2.0-mg/L MP could damage the intestinal tissue of *Artemia*.

Table 3 Two-way ANOVA analysis on the effects of temperatures (22, 26, and 30 °C) and MP (0, 0.2, and 2.0 mg/L) on *Artemia* growth on day 14

Parameter	*Artemia* length		
df	*T*	MP	*T*×MP
MS	4.211	0.065	1.287
F	8.200	0.127	2.505
P	<0.001	0.880	0.043

MS: mean square; *T*: temperature; *T*×MP: Interaction between MP and temperature; *F*: sum of mean square of main effect/sum of mean square error.

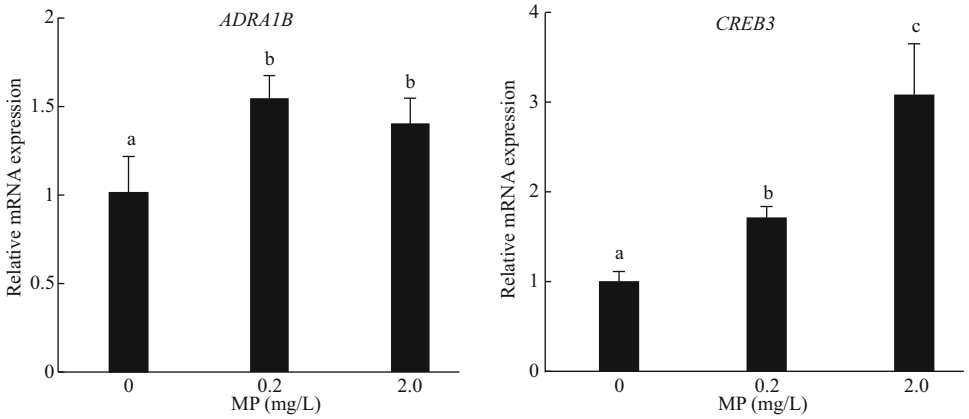

Fig.5 Genes expression of *ADRA1B* and *CREB3* in *Artemia* after 14-d exposure to different MP concentrations at 30 °C

Lower-case letters indicate significant differences (P<0.05).

3.4 Immune gene expression

A further confirmation of the defense mechanism is given by the gene expression of both *ADRA1B* and *CREB3*, which was significantly upregulated in *Artemia* exposed to 0.2- and 2.0-mg/L MP for 14 d. After long-term exposure, a dose-dependent modulation of *CREB3* was observed, and the gene expression increased with the increase of MP concentration (P<0.05). Compared to the control, 0.2- and 2.0-mg/L MP exposure resulted in significant up-regulation of *ADRA1B* (P<0.05) (Fig.5).

4 DISCUSSION

To the best of our knowledge, the present study is the first to investigate the long-term effects of both MP and temperature on juvenile and adult *A. franciscana*, using multi-effect criteria, such as survival rate, growth, intestinal histology, and immune responses. Researchers believed that increases in temperature beyond the marine organism's suitable temperature range directly affect the metabolism and cause growth and physiological stress (Barber and Blake, 2006). Our results showed that temperature had significant effects on survival and growth of *Artemia* after a long-term exposure, this is consistent with the funding of Browne and Wanigasekera (2000). Given that the temperature was the main factor affecting oxygen consumption (Irwin et al., 2007), it is a reasonable inference that survival rate and growth relate to the fact that rising temperatures impose additional developmental stress by speeding metabolism.

Artemia nauplii is capable of ingesting MP beads ranging from 1 to 20 μm, and even bigger (Batel et al., 2016; Cole et al., 2016). A recent report suggested that there were no significant detrimental effects on growth and survival after a 14-d exposure to MP with 0.4, 0.8, and 1.6 mg/L at 25 °C, and *Artemia* can ingest and egest MPs when continuously exposed to these concentrations (Peixoto et al., 2019). However, our study found that MP had significant effects on the survival rate of *Artemia* after 14-d exposure, and survival rate and body length were significantly decreased at 30 °C and 2.0-mg/L MP concentration. Bergami et al. (2017) reported that naonoplastics PS-NH_2 were able to disrupt the physiology and the energy flow in developing *A. franciscana*, and we can certainly deduce that the ingestion and accumulation of polystyrene MP particles (4–6 μm) in the digestive tract might limit food intake and significantly affect growth and development of *Artemia* (Besseling et al., 2014; Cole et al., 2016; Wang et al., 2019). Moreover, although temperature had significant effects on survival and growth of *Artemia* after a long-term exposure, MP and temperature showed a combined stress on *Artemia*, especially at relatively higher level. Prior research has suggested that temperature was the dominant factor affecting oxygen consumption rates in *Artemia*, which showed a significant increase with increasing temperature from 0 to 30 °C (Irwin et al., 2007). Therefore, it can be hypothesized that the higher temperature could increase MP ingestion, metabolism and energy consumption of *Artemia*, and thus aggravating the toxic effect of MP.

Oxidative stress occurs when the antioxidant defenses are overwhelmed by the production of reactive oxygen species (ROS). ACP is often used as a marker for intracellular lysosomal detection and as a reliable tool for environmental pollution assessment (Rajalakshmi and Mohandas, 2005). CAT is well known as an antioxidant enzyme that catalyzes

hydrogen peroxide to water and oxygen, and plays an important role against hydroxyl radical toxicity (Bagnyukova et al., 2005; Ighodaro and Akinloye, 2018). Our data showed that the enzyme activity of CAT and ACP increased with the increasing of MP concentration after 14 d. In particular, a significant increase in ACP activity was observed in *Artemia* when exposed to 30 °C and 2-mg/L MP concentration, revealing that high temperature and MP concentration could induced oxidative stress on *Artemia* more easily. This may be due to environmental stimuli that lead to lysosomal membrane instability and therefore to increased ACP activity (Pampanin et al., 2002).

For most of marine organisms, especially zooplankton, their alimentary canal (gut) is the most important target organ where MP accumulates (Browne et al., 2008; Jemec et al., 2016). Wang et al. (2019) reported that after 24 h of exposure to 10-μm polystyrene at 10 and 100 particles/mL, the decreased and disordered microvilli, increased mitochondria and the presence of autophagosomes were observed in *Artemia* intestine tissue. Similarly, we found that the intestinal microvilli and epithelia cells of *Artemia* decreased significantly and arranged disorderly when exposed to 2.0-mg/L MP at 30 °C, suggesting the relatively high MP concentration and temperature can cause an adverse impact on intestinal tissue. Since intestinal microvilli and epithelia are involved in a series of physiological processes, we can speculate that the damage of intestinal tissue could affect the nutrient absorption and energy metabolism, and ultimately pose a threat to *Artemia* growth and health (Gunasekara et al., 2011).

Apoptosis is a programmed cell death that is regulated by genes, which is essential for normal development and homeostasis in plants and throughout the animal kingdom (Kiss, 2010). *CREB3* and *ADRA1B* have been identified as apoptosis-related genes in *Artemia* (Zhang et al., 2018). Our study showed that *ADRA1B* and *CREB3* genes had high expressions in response to high MP concentration and temperature, indicating that the two genes may play an important role in regulating apoptosis, development and metabolism (Ping et al., 2011; Tressel et al., 2011). The strong induction of *CREB3* and *ADRA1B* genes indicates that high MP concentration and temperature may cause immunological stress, and affect the energy flow as well as the normal physiological processes in *Artemia* over prolonged exposure.

5 CONCLUSION

MP and temperature have a combined adverse effect on survival and growth of *Artemia*, especially at relatively high temperature and MP concentration. Temperature had significant effect on *Artemia* growth, and had significant interaction with MP concentrations on growth at 14 d. Exposure of *Artemia* at 30 °C and 2.0-mg/L MP significantly decreased the growth and survival rate of *Artemia*, reduced the number of intestinal microvilli and epithelia cell, as well as induced oxidative stress and immunological stress. Our research provides a scientific basis for the risk assessment of MP pollution and elevated temperature on zooplankton in the marine environment. Further research is required to evaluate the combined effects of MP and temperature on intestinal absorptive function and intestinal microflora, and their possible impacts on the immune function of *Artemia*.

6 CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

7 DATA AVAILABILITY STATEMENT

The research data used in this study can be shared upon request.

8 ETHICS STATEMENT

The study protocol was approved by the Committee on the Ethics of Animal Experiments of Tianjin University of Science and Technology.

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