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Immunotoxicity and neurotoxicity of bisphenol A and microplastics alone or in combination to a bivalve species, *Tegillarca granosa*☆

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ABSTRACT

Though invertebrates are one of the largest groups of animal species in the sea and exhibit robust immune and neural responses that are crucial for their health and survival, the potential immunotoxicity and neurotoxicity of the most produced chemical bisphenol A (BPA), especially in conjunction with microplastics (MPs), still remain poorly understood in marine invertebrate species. Therefore, the impacts of exposure to BPA and MPs alone or in combination on a series of immune and neural biomarkers were investigated in the invertebrate bivalve species blood clam (*Tegillarca granosa*). Evident immunotoxicity as indicated by alterations in hematic indexes was observed after two weeks of exposure to BPA and MPs at environmentally realistic concentrations. The expression of four immune-related genes from the NFκB signaling pathway was also found to be significantly suppressed by the BPA and MP treatment. In addition, exposure to BPA and MPs led to an increase in the *in vivo* contents of three key neurotransmitters (GABA, DA, and ACh) but a decrease in the expression of genes encoding modulatory enzymes and receptors for these neurotransmitters, implying the evident neurotoxicity of BPA and MPs to blood clam. Furthermore, the results demonstrated that the toxic impacts exerted by BPA were significantly aggravated by the co-presence of MPs, which may be due to interactions between BPA and MPs as well as those between MPs and clam individuals.

1. Introduction

As an important industrial chemical widely used primarily for the production of epoxy resins and polycarbonate plastics, bisphenol A (BPA) is one of the most produced chemicals worldwide (Belfroid et al., 2002; Vandenberg et al., 2009). With an annual production of 2,721,554 tons, more than 100 tons of BPA are unintentionally released into the environment, which poses potential threats to organisms and ecosystems (Michałowicz, 2014; vom Saal et al., 2012; Vandenberg et al., 2009). Through effluents from wastewater treatment plants and landfill sites as well as leaching from BPA-containing wastes, BPA is continuously discharged into aquatic environments, resulting in a ubiquitous presence of BPA in various aquatic environments all over the world (Huang et al., 2012). For example, approximately 10–1000 ng/L BPA has been detected in estuarine and/or coastal seawaters in Europe (Arditsoglou and Voutsa, 2012; Staples et al., 2018; Tran et al., 2015), Asia (Duong et al., 2010; Fu et al., 2007; Hashimoto et al., 2005; Huang et al., 2012), and North America (Klečka et al., 2009; Staples et al., 2018). In recent years, it has been demonstrated that exposure of aquatic species to BPA may exert toxic impacts on a series of physiological processes such as reproduction (Aarab et al., 2006; Laing et al., 2016), growth (Li et al., 2017), development (Balbi et al., 2016), immune response (Juhel et al., 2017), and endocrine regulation (Birceanu et al., 2015). In addition, increasing evidence indicates that the immune and neural systems of aquatic animals could be the main targets for waterborne BPA (Saili et al., 2012; Kim et al., 2020; Canesi et al., 2007; Juhel et al., 2017). Although invertebrates are the largest group of marine animals and play crucial roles in marine ecosystems, the potential immunotoxic and neurotoxic impacts of BPA still remain poorly understood in marine invertebrate species (Canesi et al., 2007; Juhel et al., 2017).

Approximately 7000 million tons of plastic waste has been produced since the 1950s (about 4.8–12.7 million tons of which are released into the ocean every year), and thus represents a global environmental issue (Andrady, 2011; Geyer et al., 2017; Jembeck

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et al., 2015; Mincer et al., 2016; Rochman et al., 2013a). Microplastics (MP) are defined as plastic particles have diameters smaller than 5 mm (Andrady, 2011; Arthur et al., 2008), which can be derived from microplastics containing daily necessities and the fragmentation of larger plastic wastes (Andrady, 2011; Barboza and Gimenez, 2015; Cozar et al., 2015). Approximately 93,000 to 236,000 tons of MPs are estimated to be present in the sea, and the concentration of MPs can reach as high as 9180 particles/m^3 (Desforges et al., 2014; van Sebille et al., 2015). For example, MPs at the concentration more than 2 kg/km^2 have been detected in the five major ocean gyres (Cozar et al., 2015). Recently, adverse impacts on various physiological processes, including hampering immune responses, exerted by exposure to waterborne MPs are increasingly documented in aquatic species (Besseling et al., 2014; Sussarellu et al., 2016; Chen et al., 2017a; O'Donovan et al., 2018; Tang et al., 2020). For instance, evident immunotoxicity, as indicated by significant altered hematic indexes were detected in blood clam, *Tegillarca granosa*, upon polystyrene MP exposure (Tang et al., 2020).

Due to the intrinsic characteristics of MPs including their large specific surface area and hydrophobicity, MPs can act as vectors for other environmental contaminants such as metals, pharmaceuticals, and polycyclic aromatic hydrocarbons (Barboza et al., 2018; Hirai et al., 2011; O'Donovan et al., 2018; Rochman et al., 2013b; Wu et al., 2016). In addition, leaching of toxic additives, such as plasticizers, antioxidants, and flame retardants, used in the production of plastics into the ocean environment also puts a threat to various marine organisms (Choi et al., 2009; Koelmans et al., 2014). In recent years, growing evidences suggests co-presence of MPs may exert significant impacts on the bioaccumulation and toxicity of other pollutants in aquatic species (Oliveira et al., 2013; Pittura et al., 2018; Zhang et al., 2019; Tang et al., 2020). For example, the bioaccumulation of cadmium in *Danio rerio* (Oliveira et al., 2013), genotoxicity of polycyclic aromatic hydrocarbons to *Mytilus galloprovincialis* (Pittura et al., 2018), neurotoxicity of roxithromycin to *Oreochromis niloticus* (Zhang et al., 2019), and immunotoxicity of 17β-estradiol and benzo[*a*]pyrene to *T. granosa* (Tang et al., 2020) were all significantly affected by the co-presence of MPs, which highlights the need for further investigation of the interaction between MPs and other pollutants.

In reality, due to the ubiquitous presence of BPA and MPs in the sea, marine organisms could be threatened by these two pollutants simultaneously. In addition, it has been suggested that waterborne BPA can be absorbed by MPs (Chen et al., 2017b), which may promote the BPA uptake by aquatic species. Therefore, the toxicity of BPA to marine species is likely affected by the co-presence of MPs. However, to the best of our knowledge, this speculation has not been verified with empirical data in marine invertebrate species.

Invertebrates account for approximately 95% of all animal species and play essential roles in various ecosystems including the sea (Han et al., 2019). The ocean environment is complex and presents numerous biotic and abiotic challenges; thus, robust immune and neural responses are essential for the health and survival of marine invertebrates (Zha et al., 2019; Guan et al., 2018). Marine invertebrates such as bivalve mollusks have well-developed nervous systems (Brenneis and Richter, 2010) and are able to regulate their immune response and maintain homeostasis in a manner similar to that of vertebrates through the coordination of neural, endocrine, and immune systems *via* neurotransmitters, cytokines, and transcription factors (Harbuz, 2003; Steinman, 2004; Liu et al., 2016b). However, the combined impacts of BPA and MPs on immune and neural responses still await investigation.

Many marine bivalve mollusks, such as blood clam, live in coastal areas where various land-sourced contaminants including BPA and MPs converge in the sea and therefore are more susceptible to these pollutants (Gallagher et al., 2016; Isobe et al., 2015; Peters and Bratton, 2016). In addition, bivalve mollusks have a limited ability to escape from pollutants and readily ingest pollutants through their filter feeding behavior (Jin et al., 2008; Kim et al., 2002; Li et al., 2016a; Shi et al., 2014). Blood clam, *T. granosa*, is a typical sessile filter feeding bivalve species with a wide distribution along coasts of Indian-Pacific Ocean (Peng et al., 2015; Shao et al., 2016). Although it has been adopted as a model species to address ecotoxicological impacts of many pollutants (Guan et al., 2019; Han et al., 2019; Zha et al., 2019), the immunotoxicity and neurotoxicity of BPA in blood clam has not been elucidated, especially with the co-presence of MPs.

Therefore, to improve our present understanding of the toxicities of BPA and MPs, the immunotoxic and neurotoxic effects of BPA with and/or without the co-presence of MPs on blood clam were investigated in the present study. Due to the extensive distribution in marine environment (Enders et al., 2015; Ma et al., 2016; Sussarellu et al., 2016) and observed synergistic effect on the toxicity of pollutants in aquatic organisms (Chen et al., 2017a; Shi et al., 2020; Sun et al., 2020; Tang et al., 2020), polystyrene (PS) particles were selected as representative MPs in this study. In addition, according to previous studies (Fu et al., 2007; Hashimoto et al., 2005; Basheer and Lee, 2004; Arditsoglou and Voutsa, 2012; Lechner et al., 2014), BPA at the doses of 10 ng/L and 100 ng/L and MPs at the concentration of 1 mg/L were adopted to mimic environmentally realistic scenarios. Hematic indexes including the total count (THC), cell type composition, and phagocytic activity of hemocytes as well as the expression of genes from the immune-related NFκB signaling pathway were examined. Due to their significant implication in neurotoxicity (Guan et al., 2018; Liu et al., 2016c; Zha et al., 2019), *in vivo* concentrations of three major neurotransmitters, namely, γ-aminobutyric acid (GABA), dopamine (DA), and acetylcholine (ACh), and the expression of corresponding regulators and receptors for these neurotransmitters were evaluated as well.

2. Materials and methods

2.1. Chemicals

PS MPs (25 mg/mL in pure water solution) were purchased from the Regal Nano-plastic Engineering Research Institute (Jiangsu, China), the size of which was verified microscopically (TEM, JEM-1230, JEOL, Tokyo, Japan) to be 490 ± 11 nm (physiochemical properties has been reported by Zhou et al., 2020). Bisphenol A (BPA, ≥99.0%, CAS80-05-7) was obtained from the Ziqibio Co. Ltd. (Shanghai, China) and a stock solution at a concentration of 1 mg/L was prepared by dissolving the drug in dimethyl sulfoxide (DMSO), which was stored at 4 °C before use.

2.2. Experimental animals and acclimation

Blood clams (shell length at 29.2 ± 2.2 mm, mean ± SD) were collected in July 2019 in Yueqing Bay, Wenzhou, China (28°28′ N and 121°11′ E). After collection, clam individuals were immediately transported to the Zhejiang Mariculture Research Institute and acclimated for two weeks (aerated sand-filtered seawater, temperature at 27.5 ± 0.3 °C, pH at 8.06 ± 0.02, and salinity at 21.90 ± 0.01‰). During the acclimation period, blood clams were fed the microalgae *Tetraselmis chuii* at the satiation feed rate twice a day and the seawater was changed daily to maintain the clams in decent condition (Han et al., 2019; Shi et al., 2019a).

2.3. Exposure experiments

In this study, seven experimental groups were set up in triplicate including (1) a control in which the clams were reared in seawater without BPA or MPs, (2) a solvent control (S-control) in which the clams were exposed to seawater containing 0.01% (v:v) DMSO, (3) two BPA exposure groups, which were treated with 10 ng/L or 100 ng/L BPA, (4) an MP exposure group, which was exposed to 1 mg/L PS MPs and (5) two BPA-MP coexposure groups, which were reared in seawater containing 10 ng/L or 100 ng/L BPA along with 1 mg/L MPs. The MP solution purchased was homogenized *via* ultrasonication at 70% amplitude for 5 min. Subsequently, 1.2 mL of homogenized commercial MP solution was added to corresponding MP exposure tanks containing 30 L filtered seawater to achieve the desired exposure concentration of 1 mg/L (approximately 1.45×10^7 particles/mL).After acclimation, 630 blood clams were randomly assigned to 21 experimental tanks filled with 30 L of sand-filtered seawater containing corresponding concentrations of BPA and/or MPs. The exposure lasted for 14 days, during which clams were fed *T. chuii* daily. Two hours after feeding, the experimental seawater was replaced with sand-filtered seawater containing BPA and/or MPs at designed experimental concentrations. The exposure concentrations of MPs and BPA in seawater were determined for each experimental group following the methods of Lindholst et al. (2003), and presented in supplementary materials (Table S1 and Table S2). In addition, following reported methods (Chen et al., 2017b; Lindholst et al., 2003; Lu et al., 2016), the contents of MPs and BPA accumulated in the soft tissue of clams were verified after 14-day experimental treatment as well (provided in supplementary material as Table S3).

2.4. Analysis of hematic parameters

Six clams from each experimental group (n = 6) were used for the analysis of hematic indexes after two weeks of corresponding treatment following the methods described by Shi et al. (2019a) and Su et al. (2017, 2019). Briefly, a wet mount was prepared with hemocytes extracted from the cavity of each clam individual and used to estimate the THC under an Olympus BX53 microscope (magnification of 400 ×). The counts of different types of hemocytes were also determined microscopically after Wright's staining and subsequently used to estimate the cell type composition. After mixing with Alsever's solution (volume ratio 1:1) and a quick determination of hemocyte concentration, the phagocytosis assay was conducted by incubating hemocytes with a yeast suspension ($1.35 \pm 0.7 \times 10^8$ cells/mL) at a yeast:hemocyte ratio of 10:1 at 25 °C for 30 min. The phagocytic rate of each sample was then estimated microscopically after fixation and Wright's staining.

2.5. Determination of the in vivo contents of neurotransmitters

Following the methods of Guan et al. (2018) and Shi et al. (2019a), six clams from each experimental group (n = 6) were used to estimate the *in vivo* contents of GABA, DA, and ACh using commercial ELISA kits (ML086216, ML090244, and ML095412, respectively; MLBIO biotechnology Co. Ltd., Shanghai, China). Briefly, clam specimens were dissected on ice, and the gill tissue, where hemocytes are often concentrated (Guan et al., 2018; Zha et al., 2019), was carefully peeled off and homogenized individually with an electric homogenizer (ART, MICCRA D-1, Germany) followed by a 20-min centrifugation at 2000 rpm at 4 °C. The supernatant was subsequently used to determine the *in vivo* contents of GABA, DA, and ACh with a microplate reader (Thermo Multiskan Go, USA) at the absorption wavelength of 450 nm following the manufacturer's instructions.

2.6. Gene expression analysis

After 14 days of corresponding exposure, six clams (n = 6) from each experimental group were used for the gene expression analysis. Following the methods of Guan et al. (2019) and Zha et al. (2019), total RNA was extracted from gill tissue with an EASYspin Plus RNA extraction kit (Aidlab, RN2802) and reverse transcribed into first-strand cDNA using the PrimeScript™ RT reagent kit (TaKaRa, RR037Q). Real-time PCR was subsequently performed using a CFX96 Real-Time System (Bio-Rad, USA) following our previously reported protocols (Shi et al., 2019b). In this study, the expression of genes encoding TNF receptor associated factor 6 (TRAF6), TAK1-binding protein 2 (TAB2), inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα), and nuclear factor kappa B subunit 1 (NFκB1) from the NFκB signal pathway were investigated. In addition, the expression of genes encoding the modulatory enzymes gamma-aminobutyric acid transaminase (GABAT), monoamine oxidase (MAO), acetyl cholinesterase (AChE) and the receptors gamma-aminobutyric acid type A receptor subunit delta (GABAD), dopamine receptor D3 (DRD3), and muscarinic acetylcholine receptor M3 (mAChR3) for the three neurotransmitters investigated were also analyzed. 18S rRNA was employed as an internal reference and all primers (Table 1) were synthesized by TsingKe Biotech (Hangzhou, China).

2.7. Statistics

Differences in the THC, cell type composition, and phagocytic activity of hemocytes as well as the *in vivo* contents of the three neurotransmitters tested were compared among experimental groups by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. For all analyses, data normality and variance homogeneity were verified with Shapiro-Wilk's and Levene's tests, respectively. The arcsine square root transformation was performed prior to analysis for raw data that does not fulfill the pre-assumptions of ANOVA. Gene expression levels were compared using the Duncan multiple range test. The Origin-Pro 8 software package was used for all analyses and a *p*-value less than 0.05 was accepted as statistically significant.

Table 1
Primer sequences used for real-time PCR in the present study.

Primers	Sequences (5′ to 3′)	Accession no.
a. Internal reference 18S rRNA		
18S−F	CTTTCAAATGTCTGCCCTATCAACT	JN974506.1
18S-R	TCCCGTATTGTTATTTTTCGTCACT	
b. Immune-related genes		
TRAF6-F	CATGAAGGCACCTCCTGGAA	MH507321
TRAF6-R	CAATCACACCAACAGAGTCAGT	
TAB2-F	CCACCAAGAATCCACCAT	JZ898321
TAB2-R	TCGCAGCATTCACACTTA	
IKKα-F	ATATTGTGCTGGTGGAGATT	JZ898319
IKKα-R	GCTTCAGATCACGGTGTATA	
NF-kB1-F	AATCAAGCAGGTGTAGTAGAC	MH507319
NF-kB1-R	CAGACAGGACAGCCAGAT	
c. Neurotransmitter-related genes		
GABAT-F	GGCACCTGACAACAGAGGCTAT	MH156847
GABAT-R	GGGAGCTTCGGGATAACCTGTT	
MAO-F	GGTCCTGAACTGTGAGTGTCCTTC	MH156850
MAO-R	GGATGTCATCGTTATTGGAGGAGGT	
AChE-F	CCTCACTAGGAGTTGTATTTGGGTT	MH156845
AChE-R	CTTGGGAAGATGTGCTTGATGCTA	
GABBD-F	CAACTAAGCCGTCAGGATAA	MH156848
GABBD-R	CACTTGAGCTAGATACCAGAG	
DRD3-F	GATGGCGATAATCATGGCGTGTT	MH156846
DRD3-R	ATAATGGCTGCTTGAACATGGACAT	
mAChR-F	GCCCGTGAGTAACTTCCCATAAACA	MH156849
mAChR-R	CCAGACAACATCGTTCTTCGCAAAT	

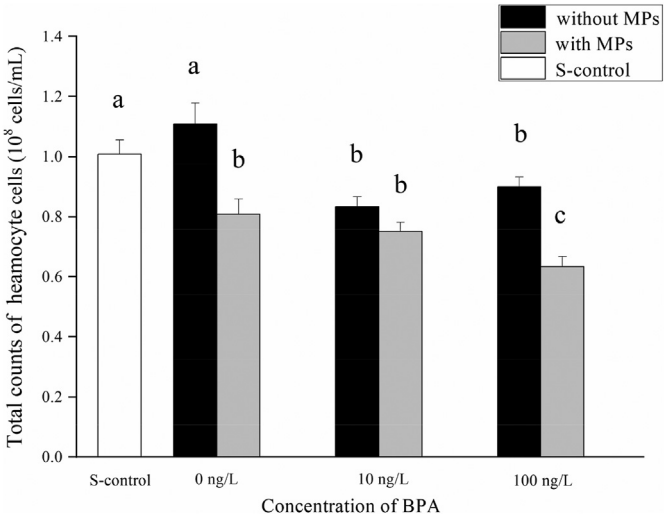

Fig. 1. Total count of hemocytes (THC) in blood clams after 2 weeks of exposure to BPA and MPs alone or in combination (n = 6, mean ± SEM). S-control: solvent control. Mean values that do not share the same superscript were significantly different at $p < 0.05$.

3. Results

3.1. Impacts of exposure to BPA and MPs alone or in combination on hematic indexes

Exposure to BPA and MPs alone or in combination led to significant reductions in THC (Fig. 1, $F_{6,35} = 12.74$, $p = 1.41E-7$), indicating the evident immunotoxicity of these two pollutants to blood clams. In addition, although no significant difference was detected among clams exposed to a low dose of BPA (10 ng/L) with and without MPs, coexposure to 100 ng/L BPA and MPs had a significantly smaller THC value than exposure to BPA alone (Fig. 1, $p < 0.05$), suggesting an aggravation of immunotoxicity caused by the co-presence of MPs. The results also showed that THC was not affected by the solvent DMSO.

Except for clams exposed to the low dose (10 ng/L) of BPA alone, the cell type composition of hemocytes were significantly altered in clams exposed to the high dose of BPA and coexposed to BPA and MPs (Table 2, $p < 0.05$). Specifically, a significant decrease in the percentage of red granulocyte ($F_{6,35} = 16.85$, $p = 4.94E-9$) and evident increases in the proportions of basophil granulocyte ($F_{6,35} = 17.01$, $p = 4.39E-9$) and hyalinocyte ($F_{6,35} = 3.41$, $p = 9.33E-3$) were detected under these exposures. In addition, the toxic impacts of BPA on the cell type composition were significantly aggravated by the co-presence of MPs. In this study, the cell type composition was not affected by the solvent DMSO.

The phagocytic activities of hemocytes were significantly constrained by the exposure of clams to BPA and MPs alone or in combination (Fig. 2, $F_{6,35} = 39.88$, $p = 3.14E-14$), indicating that these two pollutants present evident immunotoxicity to blood clam. Coexposure of clams to BPA and MPs resulted in significantly lower phagocytic activities compared with the exposure of clams to corresponding doses of BPA alone. In addition, the phagocytic activity was found to decease with the increase in dose of BPA tested, implying that this adverse impact induced by BPA may occur in a dose-dependent manner.

3.2. Impacts of exposure to BPA and MPs alone or in combination on in vivo contents of GABA, DA, and ACh

Although the *in vivo* content of GABA was inhibited by the solvent DMSO but not affected by exposure to BPA or MPs alone, clams exposed to 1 mg/L MPs along with 10 and 100 ng/L BPA (Fig. 3A, $F_{6,35} = 16.73$, $p = 5.37E-9$) had a significantly higher *in vivo* GABA content, with values approximately 1.22 and 1.40 times of that of the control, respectively. The results demonstrated that the *in vivo* content of DA was significantly induced upon BPA and MP exposure alone or in combination (Fig. 3B, $F_{6,35} = 21.90$, $p = 1.63E-10$), indicating the evident neurotoxicity of the two pollutants to blood clam. Moreover, the toxic impact of BPA on the *in vivo* content of DA occurred in a dose-dependent manner and was aggravated by the co-presence of MPs (Fig. 3B). Similar to GABA, the *in vivo* content of ACh was not affected by exposure to BPA and MPs alone while coexposure of clams to 100 ng/L BPA and 1 mg/L MPs resulted in a significantly higher level of *in vivo* ACh, which was approximately 1.52 times of that of the control (Fig. 3C, $F_{6,35} = 10.54$, $p = 1.14E-6$). In this study, the *in vivo* contents of both DA and ACh were not affected by the solvent.

3.3. Impact of exposure to BPA and MPs alone or in combination on the expression of tested genes

The results of this study (Fig. 4 and Fig. 5, $p > 0.05$) showed that the expression of all genes tested was not affected by the solvent DMSO. Except for *TRAF6* in clams exposed to a low dose (10 ng/L) of BPA alone, the expression of *TRAF6*, *TAB2*, *IKKα*, and *NFκB*, which are in the immune-related NFκB signaling pathway, were all significantly suppressed by BPA and MPs alone as well as in combination (Fig. 4). Although it was not affected by BPA and MPs alone, the expression of *GABAT* was significantly downregulated by the coexposure of clams to BPA and MPs (Fig. 5A). Similarly, the expression level of *AChE* was only inhibited by a high dose of BPA and the coexposure of BPA and MPs but not by exposure to a low dose of BPA and MPs alone (Fig. 5A). Compared to that of control, the expression of *MAO* was significantly suppressed by exposure to BPA and MPs alone and in combination (Fig. 5A). In addition, except

Table 2
Percentages of three major types of hemocytes after 2 weeks of exposure of blood clams to BPA and MPs alone or in combination (n = 6, mean ± SEM). Control: clams reared in seawater without BPA, MPs, and solvent; and S-control: solvent control. Mean values that do not share the same superscript were significantly different at $p < 0.05$.

Trails	Percentage (%) of the		
	Red granulocyte	Basophil granulocyte	Hyalinocyte
S-control	82.6 ± 0.6^{a}	14.5 ± 0.8^{a}	2.9 ± 0.3^{ab}
Control	84.1 ± 0.5^{a}	13.4 ± 0.4^{a}	2.5 ± 0.2^{a}
1 mg/L MPs	80.0 ± 0.5^{b}	17.7 ± 0.4^{b}	2.4 ± 0.3^{a}
10 ng/L BPA	82.9 ± 0.9^{a}	13.7 ± 0.8^{a}	3.1 ± 0.7^{ab}
10 ng/L BPA +1 mg/L MPs	77.4 ± 0.5^{b}	18.7 ± 0.5^{b}	3.9 ± 0.1^{b}
100 ng/L BPA	80.3 ± 0.6^{b}	15.8 ± 0.6^{b}	3.9 ± 0.3^{b}
100 ng/L BPA +1 mg/L MPs	75.9 ± 0.6^{c}	20.6 ± 0.5^{c}	3.6 ± 0.2^{b}

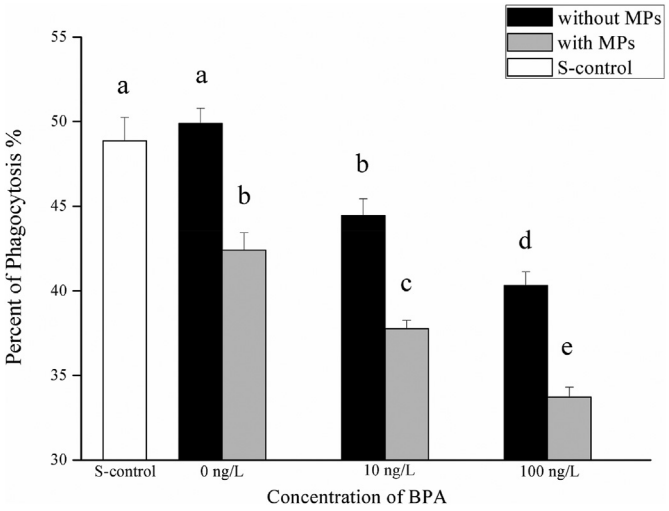

Fig. 2. Phagocytosis of hemocytes after 2 weeks of exposure of blood clams to BPA and MPs alone or in combination (n = 6, mean ± SEM). S-control: solvent control. Mean values that do not share the same superscript were significantly different at $p < 0.05$.

for *mAChR* in clams exposed to 1 mg/L MPs alone, the expression of genes encoding receptors for GABA, DA, and ACh was all significantly downregulated by the 2-week treatment of BPA, MPs, and their combinations (Fig. 5B).

4. Discussion

In the environment, sessile filter feeding bivalve mollusks such as blood clam may be simultaneously exposed to BPA and MPs (Arditsoglou and Voutsa, 2012; Isobe et al., 2015; Peters and Bratton, 2016). Although both immune and neural systems have been suggested to be the main targets for pollutants such as BPA (Juhel et al., 2017; Guo et al., 2019), to the best of our knowledge, the immunotoxicity and neurotoxicity of BPA with the co-presence of MPs still remain poorly understood in marine bivalve species. The results of the present study demonstrated that both BPA and MPs can exert immunotoxic and neurotoxic impacts on blood clam at environmentally realistic concentrations. Furthermore, the co-presence of MPs can aggravate the immunotoxicity and neurotoxicity of BPA to blood clam.

Phagocytosis by hemocytes is the first line to fight against environmental pathogens in bivalve mollusks (Loker et al., 2004). In this study, exposure to both BPA and MPs resulted in reductions in THC, alterations in the cell type composition, and suppression of the phagocytic activity of hemocytes, indicating the evident immunotoxicity of BPA and MPs to blood clam. These adverse impacts exerted by BPA and MPs could render blood clam more susceptible to environmental challenges and therefore pose a substantial threat to this species. According to the results obtained in this and previous studies, the alterations in the hematic parameters upon BPA and MP exposure may be due to following reasons.

Tissue lesion caused by exposure to pollutants such as MPs may recruit and consume a proportion of hemocytes (von Moos et al., 2012; Tang et al., 2020). Therefore, the reduction in THC in the circulatory system upon BPA and MP exposure may be caused by the reallocation of hemocytes among tissues. In addition, since NFκB plays a crucial role in hematopoiesis as a transcript factor to trigger proliferation (Gonzalez-Murillo et al., 2015), the suppression of *NFκB* upon BPA and MP exposure detected in the present

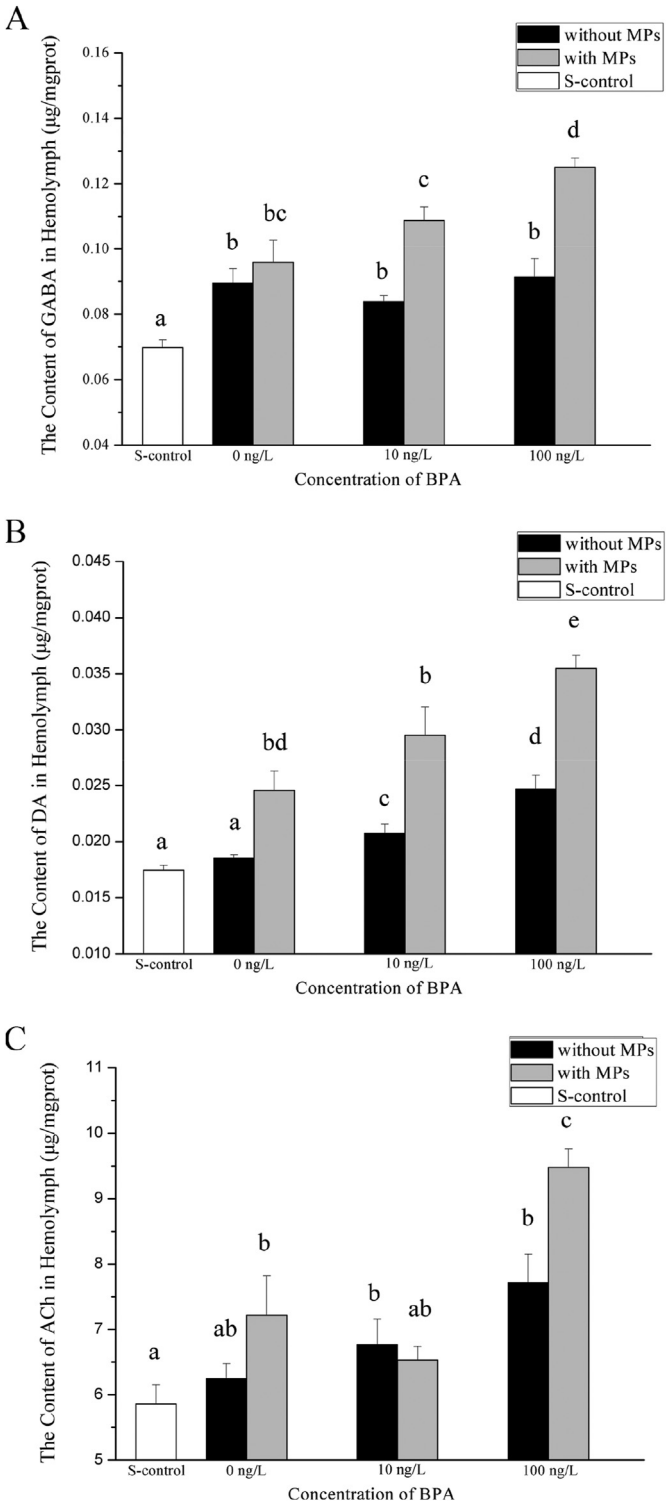

Fig. 3. *In vivo* contents of GABA (Fig. 3A), DA (Fig. 3B), and ACh (Fig. 3C) in blood clams after 2 weeks of exposure to BPA and MPs alone or in combination (n = 6, mean ± SEM). S-control: solvent control. Mean values that do not share the same superscript were significantly different at $p < 0.05$.

study may constrain hemocyte renewal and thereby result in reduced THC as well as cell type composition alterations. In this study, exposure to BPA and MPs led to significantly fewer red granulocytes in the hemocyte population of blood clam. Since red granulocytes are the most phagocytic active hemocytes (Liu et al.,

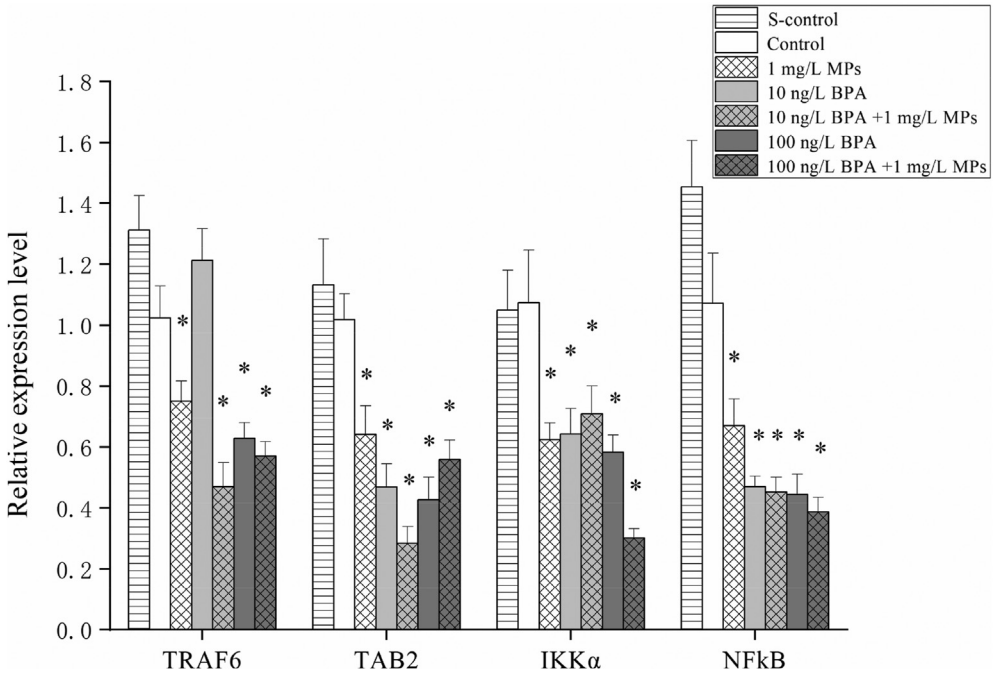

Fig. 4. Relative expression levels of four genes from the NFκB signaling pathway after 2 weeks of exposure of blood clams to BPA and MPs alone or in combination (n = 6, mean ± SEM). S-control: solvent control. "*" indicated significant difference compared to that of control at $p < 0.05$.

2016a), a reduction in the proportion of red granulocyte along with the decline in THC available offer an explanation for the inhibited phagocytosis observed after two weeks of the BPA and MP treatment. The NFκB signaling pathway plays essential regulatory roles in immune response such as phagocytosis (Zhu et al., 2014); therefore, the suppression of phagocytosis may also be caused by the downregulation of *TRAF6*, *TAB2*, *IKKα*, and *NFκB* from the NFκB signaling pathway. Moreover, BPA and MPs may exert adverse impacts on these key immune parameters through their neurotoxicity as well.

In this study, the BPA and MP treatment led to evident neurotoxicity, which was indicated by alterations in the *in vivo* contents of GABA, DA, and ACh as well as the suppression of corresponding regulatory enzymes and receptors. In recent years, increasing evidence suggests that neurotransmitters such as GABA, DA, and ACh play crucial roles in regulating immune responses such as phagocytosis in invertebrate species (Liu et al., 2016b, 2018). For instance, it has been demonstrated that the upregulation of phagocytosis, nitric oxide synthase activity, and hemocyte apoptosis induced by LPS can be significant suppressed by GABA in Pacific oyster *C. gigas* (Li et al., 2016b). Similar immunosuppression effects were also documented for DA, which was shown to suppress the phagocytic activity of hemocytes and promote susceptibility to the pathogen *Lactococcus garvieae* in giant freshwater prawn *Macrobrachium rosenbergii* (Li et al., 2005). In addition, by binding to its specific receptor on the membrane of hemocytes, ACh can downregulate the transcription factors NFκB and the second messenger cAMP, thereby inhibiting the immune response such as phagocytosis (Gallowitsch-Puerta and Tracey, 2005; Liu et al., 2016c). Since exposure to BPA and MPs generally led to increases in the *in vivo* contents of GABA, DA, and ACh, alterations in hematic parameters may partially be attributed to the elevated immunosuppression effects of these neurotransmitters. Similarly, the disruption of the NFκB signaling pathway upon BPA and MP exposure, which was indicated by the suppressed expression of *TRAF6*, *TAB2*, *IKKα*, and *NFκB*, may also result from the neurotoxicity of these pollutants such as the induction of ACh.

The *in vivo* contents of neurotransmitters are strictly modulated by corresponding regulatory enzymes (Hermida-Ameijeiras et al., 2004; Jebali et al., 2013; Rajalakshmi et al., 1965). Therefore, the induction of the *in vivo* contents of GABA, DA, and ACh observed in this study may be due to the downregulation of their modulatory enzymes (GABAT, MAO, and AChE). In addition, it is well known that neurotransmitters exert physiological modulatory functions by binding to corresponding specific receptors on target cells (Amara and Kuhar, 1993). Since *GABAD*, *DRD3*, and *mAChR* encode the corresponding receptors for GABA, DA, and ACh, respectively, and were significantly downregulated by BPA and MP exposure, the induction of the *in vivo* contents of these neurotransmitters may also represent compensatory feedback to the suppressed receptors.

The results also revealed that the immunotoxicity and neurotoxicity of BPA were significantly aggravated by the co-presence of MPs, which may be caused by interactions between BPA and MPs as well as that between MPs and clams. First, due to the absorption of BPA on MPs (indicated by the significant lower BPA concentration detected for BPA-MPs co-exposure group compared to that exposed to BPA alone, Table S2), co-presence of MPs may facilitate the entry of BPA into the body of blood clam through the "Trojan horse" effect and therefore resulted in aggravated accumulation of BPA in the clams (Table S3). Since the toxicity of BPA was shown to be dose-dependent (i.e., the toxicity of BPA on phagocytic activity and *in vivo* contents of DA in this study), when more BPA is carried into the clam with the assistance of MPs, the toxic impacts will be more severe. In addition, the inhibition of detoxification by MPs may be another reason for the enhanced toxicity of BPA. It has been suggested that exposure to MPs may suppress P-glycoprotein, a member of the subfamily of ATP-binding cassette (ABC) transporters that plays an important role in detoxification (Paul-Pont et al., 2016). Since ABC transporters are also key molecules that can exclude BPA and its metabolites (Mazur et al., 2012), the co-presence of MPs may constrain BPA exclusion and thereby exert aggravated toxic effects. Finally, insufficient energy available for

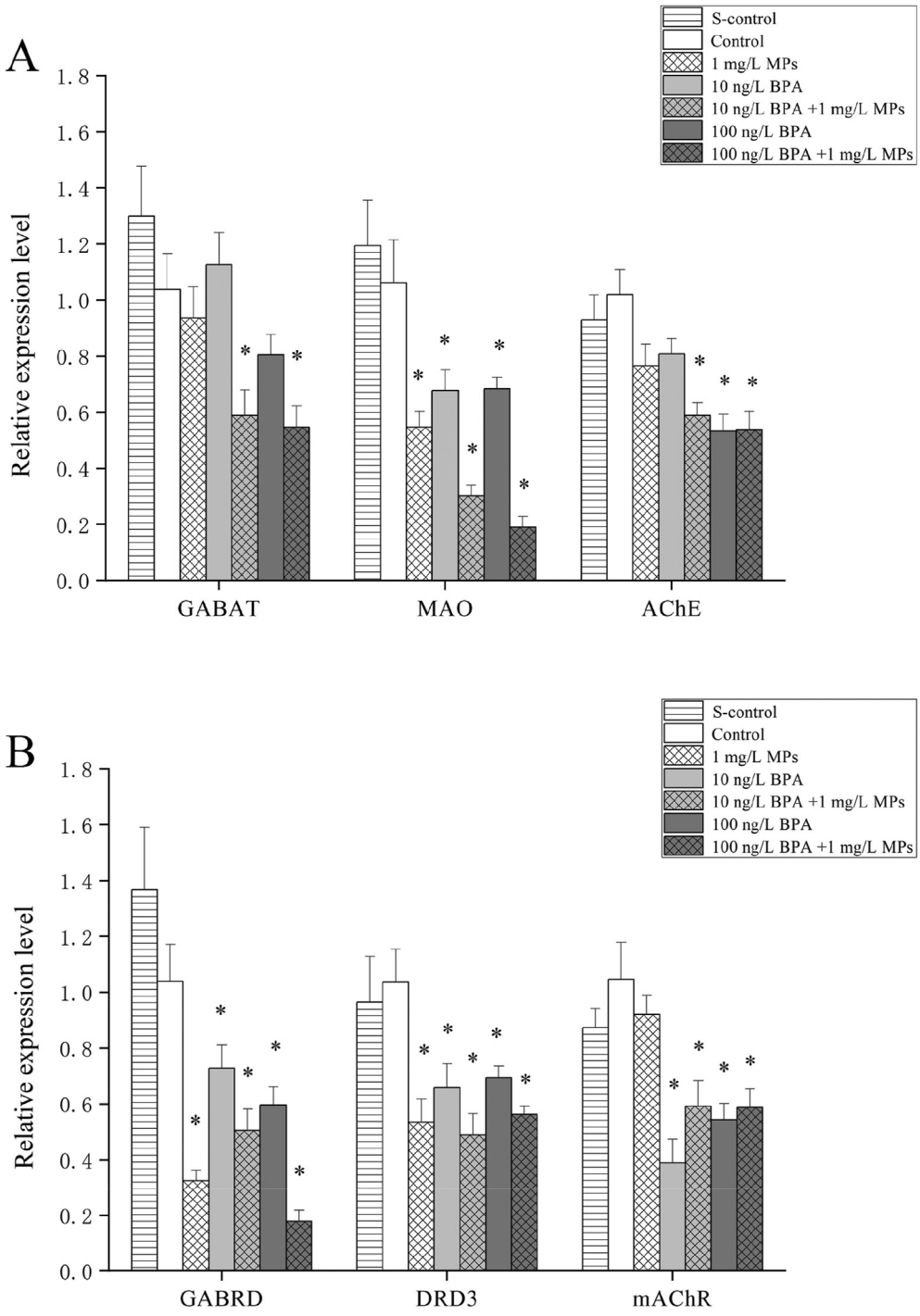

Fig. 5. Relative expression levels of genes encoding modulatory enzymes (Fig. 5A) and receptors (Fig. 5B) for GABA, DA, and ACh after 2 weeks of exposure to BPA and MPs alone or in combination (n = 6, mean ± SEM). S-control: solvent control. "*" indicated significant difference compared to that of control at $p < 0.05$.

immune response and detoxification upon MP exposure may contribute to the enhanced BPA toxicity with the co-presence of MPs as well. It has been suggested that exposure to MPs can significantly constrain the energy supply of an organism (Watts et al., 2015; Wright et al., 2013; Gardon et al., 2018). Since energy is required for both detoxification and immune responses (Oliveira et al., 2013; Wu et al., 2019; Borregaard and Herlin, 1982), the energy deficit caused by MPs may promote the toxic impacts of BPA.

5. Conclusion

The results of this study demonstrated that exposure to BPA and MPs alone or in combination at environmentally realistic concentrations could exert significant immunotoxic and neurotoxic impacts on the blood clam, which may be caused by alterations in a series of physiological and molecular processes. In addition, the toxicity of BPA could be significantly aggravated by the co-presence of MPs, which suggests the presences of MPs with other pollutants may exert more severe adverse impacts on marine species and

therefore should be further addressed in future work.

CRediT authorship contribution statement

Yu Tang: Conceptualization, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Weishang Zhou:** Software, Validation, Investigation. **Shuge Sun:** Visualization, Investigation. **Xueying Du:** Data curation, Validation, Investigation. **Yu Han:** Visualization, Data curation, Formal analysis. **Wei Shi:** Resources, Investigation. **Guangxu Liu:** Conceptualization, Project administration, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115115.

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