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Immunotoxicity of microplastics and two persistent organic pollutants alone or in combination to a bivalve species☆

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ABSTRACT

Both microplastics and persistent organic pollutants (POPs) are ubiquitously present in natural water environment, posing a potential threat to aquatic organisms. While it has been suggested that the immune responses of aquatic organisms could be hampered by exposure to microplastics and POPs, the synergistic immunotoxic impact of these two types of pollutants remain poorly understood. In addition, little is known about the mechanism behind the immunotoxic effect of microplastics. Therefore, in the present study, the immunotoxicity of microplastics and two POPs, benzo[*a*]pyrene (B[a]P) and 17β-estradiol (E2), were investigated alone or in combination in a bivalve species, *Tegillarca granosa*. Evident immunotoxicity, as indicated by alterations of haemocyte count, blood cell composition, phagocytic activity, intracellular content of ROS, concentration of Ca^{2+} and lysozyme, and lysozyme activity, was revealed for both microplastics and the two POPs examined. In addition, the expression of six immune-, Ca^{2+} signalling-, and apoptosis-related genes was significantly altered by exposure of clams to the contaminants studied. Furthermore, the toxicity of POPs was generally aggravated by smaller microplastics (500 nm) and mitigated by larger ones (30 μm). This size dependent effect on POP toxicity may result from size dependent interactions between microplastics and POPs. Data obtained in this study also indicate that similar to exposure to B[a]P and E2, exposure to microplastics may hamper the immune responses of clams through a series of interdependent physiological and molecular processes.

1. Introduction

Large quantities of plastics are widely used in a variety of consumer and industrial products, with total output reaching nearly 350 million tons per year (PlasticsEurope, 2019; Prokić et al., 2019). Both the use of plastic particles containing daily necessities (i.e., toothpastes and synthetic textiles) and the fragmentation of larger plastic products through natural weathering processes inevitably lead to the release of microplastics (MPs), fine plastic particles less than 5 mm, into the ocean environment (Andrady, 2011; Barboza and Gimenez, 2015; Cozar et al., 2014). According to estimates, there are approximately 15–51 trillion individual pieces of microplastic weighing approximately 93,000 to 236,000 tons in the world's oceans (van Sebille et al., 2015). Due to their ubiquitous presence and long turnover period, microplastics in the sea are causing increasing public concern due to their potential impacts on marine organisms and ecosystems (Alimba and Faggio, 2019; Strungaru et al., 2019; Wright et al., 2013). Specifically, polystyrene (PS) is one of the most abundant microplastics polymers found in marine environment (Enders et al., 2015), hence is widely used as a representative to assess the ecotoxicological impacts of microplastics on marine species (Ma et al., 2016; Sussarellu et al., 2016).

In recent years, growing evidence has demonstrated that exposure to microplastics may exert various adverse effects on a

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variety of aquatic animals (Avio et al., 2015; Faggio et al., 2018; Ferreira et al., 2016; Ribeiro et al., 2017). For example, ingestion of microplastics has been detected in several aquatic species, such as *Arenicola marina* (Besseling et al., 2013), *Crassostrea gigas* (Sussarellu et al., 2016), *Mytilus edulis* (von Moos et al., 2012), *Pagellus erythrinus* (Savoca et al., 2019a), *Boops boops* (Savoca et al., 2019b), and *Danio rerio* (Chen et al., 2017a), leading to digestive tract obstruction (Wright et al., 2013), insufficient food intake (Barnes et al., 2009), growth retardation (Besseling et al., 2014), and constrained reproductive capacity (Sussarellu et al., 2016). Since the distribution of microplastics *in vivo* depends on the size of plastic particle, some reported that toxicity of microplastics is size-dependent (Kashiwada, 2006). For instance, nanoscale (50 nm) polystyrene microplastics has been shown to trigger a significant stronger inhibitory effect on the growth rate, swimming ability, and visual nerve development of zebrafish larvae as compared to their larger counterparts at a size of 45 μm (Chen et al., 2017a). When particle size is small enough (i.e., nanoscale), microplastics could enter the circulatory system of aquatic organisms through intestinal tract (Browne et al., 2008), resulting in a series of *in vivo* physiological dysfunction, such as inducing inflammatory responses (Pittura et al., 2018; von Moos et al., 2012), arousing oxidative stress (Chen et al., 2017a), and causing DNA damage (O'Donovan et al., 2018). For instance, it has been suggested that PS at the size of 9.6 μm can be detected in the circulatory system of the mussel after exposure (Browne et al., 2008), however, those larger than 20 μm will be excreted by mussels without entering into the haemolymph (Van Cauwenberghe and Janssen, 2014). While it is interesting to postulate that the size-dependent difference in the penetrating ability may lead to difference in immunotoxicity, empirical data awaits in bivalve species.

More importantly, ingestion of microplastics could be a serious danger for aquatic animals due to their inherent chemical pollutants (O'Donovan et al., 2018; Pittura et al., 2018). Upon ingestion, toxic additives, such as plasticizers, antioxidants (Koelmans et al., 2014), and flame retardants (Choi et al., 2009), used in the production of plastics could be slowly released *in vivo*. In addition, due to their hydrophobicity and high specific surface area, hydrophobic persistent organic pollutants (POPs), such as 17β-estradiol (E2) and Benzo[*a*]pyrene (B[*a*]P), from the surrounding water environment may attach to the surface of microplastics (Rochman, 2015). In this circumstance, microplastics could act as vectors to facilitate internalization of condensed doses of these pollutants through ingestion, subsequently causing serious physiological damage to aquatic animals (Cole et al., 2011).

Due to this potential "Trojan horse" effect and the fact that microplastics are often present in the water environment along with other contaminants, the synergistic impacts of microplastics and other pollutants on aquatic organisms have become one of the most discussed topics in the research field of aquatic ecotoxicology (Ivar do Sul and Costa, 2014). In recent years, a growing number of studies suggest that both the bioaccumulation and toxicity of some pollutants could be significantly affected by the co-presence of microplastics (Chen et al., 2017a,b). For example, the bioaccumulation of phenanthrene in *Daphina magna* was significantly enhanced by 50 nm polystyrene microplastics (Ma et al., 2016). In addition, co-exposure to 125–250 μm low density polyethylene microplastics along with polybrominated biphenyl ethers (PBDEs) or polychlorinated biphenyl (PCB) has significantly greater toxicity to liver tissue of zebrafish compared to exposure without microplastics (Rainieri et al., 2018). Furthermore, it has been shown that excretion of fluoranthene in marine mussels, *Mytilus* spp., was significantly inhibited by the co-presence of 2 and 6 μm polystyrene microplastics (Paul-Pont et al., 2016). However, to date, the combined immunotoxicity of microplastics and chemical pollutants such as POPs remains poorly described in bivalve molluscs. Harnessing robust immunity is crucial for a species survival in the complex ocean environment (Gobi et al., 2018). Therefore, alterations in immunotoxicity have profound impacts on organisms, highlighting the need for further investigation.

In addition to microplastics, POPs, such as endocrine disrupting chemicals (EDCs) and polycyclic aromatic hydrocarbons (PAHs), are ubiquitously detected in the ocean environment. For example, the presence of various types of EDCs ranging from ng/L to μg/L concentrations has been identified in marine environments (Kim et al., 2007; Klamerth et al., 2010). Similarly, though the oceanic contamination level of PAHs remains unclear on a global scale, PAHs ranging from 103 to 29,325 ng/L have been detected in seawater along the coasts of China (Su et al., 2017). The 17β-estradiol (E2) and benzo[*a*]pyrene (B[*a*]P) are representative compounds of EDCs and PAHs, respectively. According to previous report, E2 accounts for as high as 50–90% of the total estrogenic activity in some water bodies, making it a typical EDC threat to aquatic organisms (Yan et al., 2012). Similarly, B[a]P, one of the most toxic PAHs, is widely used as a model of PAHs for exploring the ecotoxicological impacts of PAHs (Di Giulio et al., 1993). Both E2 and B[a]P have been suggested to be immunotoxic to marine animals, including bivalve molluscs (Shi et al., 2018; Su et al., 2017); however, whether the immunotoxicity of these pollutants is altered by the co-presence of microplastics has yet to be elucidated.

Most bivalve molluscs are sessile benthic filter feeders inhabiting coastal areas, where pollutants are often concentrated (Capillo et al., 2018; Freitas et al., 2019; Pagano et al., 2016; Pagano et al., 2017; Torre et al., 2013). The existence of pollutants such as microplastics and POPs in proximity may pose a significant threat to these bivalve species. The blood clam, *Tegillarca granosa* Linnaeus 1758, is widely distributed along Indian and West Pacific coasts and is not only a traditional commercial species but also plays crucial ecological roles in the coastal ecosystem (Peng et al., 2015). Similar to other bivalve species, blood clam lacks antigen-antibody mediated immune responses and mainly relies on the phagocytosis of haemocytes to fight against pathogens (Liu et al., 2016). Among all three types of haemocytes of blood clam, the red granulocyte has the highest phagocytic activity and therefore been widely used as an indicator to detect immunotoxicity (Burgos-Aceves and Faggio, 2017; Liu et al., 2016). In the process of phagocytosis, pattern recognition receptors such as TLRs (toll like receptors) located on the membrane of haemocytes recognize pathogen materials and trigger the engulfment of foreign particles by haemocytes (Barton and Medzhitov, 2003). Once engulfed, pathogen particles will be degraded *in vivo* by LZM (lysozymes) or ROS (reactive oxygen species) through the oxygen-independent or -dependent degradation pathways (Finkel, 2011; Pollard and Cooper, 2009; Song et al., 2010; Tomanek et al., 2011). Though ROS plays crucial role in the degradation of engulfed particles, excessive ROS production may hamper the process of phagocytosis both directly and indirectly (Pollard and Cooper, 2009; Su et al., 2018). On one hand, overproduction of ROS could inhibit the phagocytic activity of haemocytes through imposing oxidative stress (Pollard and Cooper, 2009; Tomanek et al., 2011). On the other hand, excessive ROS may trigger the apoptosis process of haemocytes and result in less numbers of haemocytes available for phagocytosis (Circu and Aw, 2010).

In the processes of phagocytosis and apoptosis, several cross-linked molecular pathways play crucial modulatory roles (Elmore, 2007; Stuart and Ezekowitz, 2005). For instance, IKKα (inhibitor of nuclear factor kappa-B kinase subunit alpha) and NFκB (nuclear factor NF-kappa-B p105 subunit) of the NFκB signalling pathway will be activated sequentially upon the recognition of pathogen by TLR4 (toll-like receptor-4) via up-regulating TRAF6 (TNF receptor-

associated factor 6) (Wu and Arron, 2003). As a cellular transcription factor, NFκB activation will not only enhance immune responses such as phagocytosis, but also counteract with haemocytes apoptosis via activating Bcl-2 (B-cell lymphoma-2), which inhibits the activation of apoptosis executor, the caspases (Elmore, 2007; Vallabhapurapu and Karin, 2009). In addition, NFκB signalling pathway and apoptosis pathway are also interplayed by Ca^{2+} signalling pathway (Edlich et al., 2005; Lilienbaum and Israel, 2003). On one hand, an increase in intracellular Ca^{2+} will trigger the apoptosis process via activating caspase-3 (Tantral et al., 2004). On the other hand, the elevation of Ca^{2+} can activate PKC (protein kinase C) and CaM (calmodulin), which upregulates IKKα and downstream NFκB and therefore downregulates apoptosis through the inhibitor Bcl-2 (Edlich et al., 2005).

Though it has been demonstrated that many other pollutants may exert immunotoxic impacts on marine bivalve species through affecting haemocyte characteristics, altering intracellular ROS, LZM, and Ca^{2+}, and interrupting related molecular pathways (Pittura et al., 2018; Shi et al., 2017; Zha et al., 2019), it remains questionable whether these parameters of the blood clam will be affected by microplastics. Therefore, in the present study, the impacts of microplastic exposure alone or in combination with B[a]P or E2 on a series of immune-related traits, including total haemocyte count, blood cell composition, phagocytic activity, *in vivo* content of ROS, concentrations of Ca^{2+} and LZM, and LZM activity, were investigated in the blood clam. In addition, microplastics of two different sizes (30 μm vs. 500 nm) and pollutants at two different doses (5 or 50 μg/L for B[a]P and 0.1 or 1 μg/L for E2) were tested to examine any size- and/or dose-dependent effects as well as interactions between microplastics and pollutants. Furthermore, expression of genes encoding immune-, Ca^{2+}-signalling-, and apoptosis-related proteins was also analysed to look into the mechanism underlying the immunotoxic effects detected.

2. Materials and methods

2.1. Experimental animals and acclimation

Adult blood clams (18.95 ± 2.03 mm) were obtained from Yueqing Bay (28° 280′ N and 121°110′ E, Wenzhou, China) in June 2018. Prior to experiment, clams were acclimatized for 7 days in a 2000-L tank filled with filtered natural seawater (temperature of 28.0 ± 0.5 °C, pH of 8.10 ± 0.05, and salinity of 21.0 ± 0.5‰). During acclimation, seawater was continuously aerated and renewed daily. *Platymonas subcordiformis* microalgae was provided at satiation rate every day to maintain decent body condition of the clams prior to exposure experiments (Han et al., 2016; Zhao et al., 2017).

2.2. Exposure experiments

Polystyrene microplastics at nominal sizes of 30 μm and 500 nm were purchased from Regal Nano-plastic Engineering Research Institute (Jiangsu, China). Prior to exposure experiments, sizes of the microplastics were verified by electron microscopy (TEM, JEM-1230, JEOL, Tokyo, Japan). E2 (purity≥98.0%, CAS50-28-2) and B[a]P (purity≥98.0%, CAS 50-32-8) were purchased from Solarbio Science & Technology Co. Ltd (Beijing, China). According to previous studies (Shi et al., 2018; Su et al., 2019), stock solutions of E2 and B[a]P at concentrations of 1 g/L and 150 μg/mL, respectively, were prepared by dissolving corresponding amounts of E2 or B[a]P in dimethyl sulfoxide (DMSO).

Two controls and fourteen experimental exposure groups were prepared in triplicates for the present study (Table 1). Clams reared in seawater without microplastics or pollutants were used as common controls (C-control). To rule out any effects brought about by the solvent DMSO, solvent controls (S-control) were performed with seawater containing 0.03% (v:v) and 0.01% (v:v) DMSO for B[a]P and E2, respectively. Two microplastics exposure groups were created with experimental seawater containing 1 mg/L microplastics at sizes of 30 μm or 500 nm. Similarly, two B[a]P exposure groups and two E2 exposure groups were created by exposing clams to seawater containing 5 or 50 μg/L B[a]P or 0.1 or 1 μg/L E2. In addition, eight co-exposure groups were set up to investigate synergistic impacts of microplastics and POPs tested (Table 1). For simplicity, the following abbreviations are used. M and N indicate exposure of clams to microplastics at 30 μm or 500 nm, respectively. B and E indicate exposure of individuals to B[a]P or E2, respectively. L and H indicate low or high exposure concentrations of the corresponding pollutants, respectively. For example, MBL represents the experimental group exposed to microplastics at a size of 30 μm along with the lower dose (5 μg/L) of B[a]P. Since microplastics, E2, and B[a]P have been previously detected in polluted areas at as high as 0.697 mg/L, 92 ng/L, and 4.79 μg/L (Kostich et al., 2013; Lechner et al., 2014; Zhou and Maskaoui, 2003), the exposure concentrations of microplastics (1 mg/L), E2 (0.1 or 1 μg/L) and B[a]P (5 or 50 μg/L) were selected to simulate realistic severe pollution scenarios. Exposures were conducted in 40 L experimental tanks containing corresponding doses and/or types of microplastics, B[a]P, and/or E2 with 30 individual blood clams. An exposure duration of 4 days was adopted, and blood clams were fed *P. subcordiformis* microalgae every day 2 h before seawater replacement. Zero individual mortality was observed throughout the exposure period.

2.3. Analysis of total counts, cell type composition, and phagocytic activity of haemocytes

Total counts and cell type composition of haemocytes were determined according to reported methods with a few modifications (Shi et al., 2017; Su et al., 2017). In brief, three individuals were randomly chosen from each experimental group after corresponding treatment, and haemolymph was subsequently extracted from the cavity of each individual using a 2-mL syringe. One hundred microliters of haemolymph was transferred into a 1.5 ml centrifuge tube pre-filled with 100 μL 2.5% glutaraldehyde and 800 μL PBS. A wet mount was prepared with the fixed haemocytes

Table 1
Experimental exposure groups in the present study. C-control: seawater without microplastics and pollutants; S-control: seawater with solvent DMSO while no pollutants; MPs: microplastics; M: MPs at a size of 30 μm; N: MPs at a size of 500 nm; B: B[a]P; E: E2; L: lower exposure dose of corresponding POPs; H: higher exposure dose of corresponding POPs.

Microplastics	Without POPs	Concentration of B[a]P		Concentration of E2	
		5 μg/L	50 μg/L	0.1 μg/L	1 μg/L
Without MPs	C-control S-control	BL	BH	EL	EH
With MPs at the size of 30 μm	M	MBL	MBH	MEL	MEH
With MPs at the size of 500 nm	N	NBL	NBH	NEL	NEH

and used to estimate the total counts of haemocytes with a Neubauer's haemocytometer (XB-K-25, Anxin Optical Instrument) under Nikon eclipse E600 microscopy at a magnification of 400 × .

After extraction, 700 μL haemolymph were mixed with 300 μL of 2.5% glutaraldehyde followed by a 4-min centrifugation at 4000 rpm. Blood smears were subsequently made with 50 μL blood precipitate. Once air dried, the blood smear was stained with Wright's stain (G1020, Solarbio, China) and used for cell type composition analysis. According to previous studies (Liu et al., 2016), three types of haemocyte cells, red granulocytes, basophil granulocytes, and hyalinocytes, were quantified under a Nikon eclipse E600 microscope at a magnification of 400×. To assure accuracy of the data, more than 100 cells were scored for each sample.

Phagocytic activity assays were performed *in vitro* with extracted haemocytes according to methods described by Liu et al. (2016) and Su et al. (2018). Briefly, three blood clams were randomly chosen from each experimental group after corresponding treatment for 4 days. After extraction, haemolymph was immediately mixed with pre-cooled Alsever's solution (R1016, Solarbio, China) at a volume ratio of 1:1. Once the haemocyte concentration was determined microscopically as described above, haemocytes collected from 100 μL of haemolymph through centrifugation (1000 rpm for 15 s) were mixed with pre-prepared yeast suspension (Instant dry yeast, Angel Yeast, China) at a yeast: haemocyte ratio of 10:1. After incubation at 25 °C for 30 min, the haemocyte-yeast sample was fixed with 100 ml 2.5% glutaraldehyde and used to prepare blood smears followed by Wright's Gimesa staining. Phagocytic rate was subsequently estimated under an Olympus BX53 light microscope at a magnification of 400×. More than 200 haemocytes were scored for each sample to ensure data accuracy.

2.4. Determination of intracellular concentrations of ROS and Ca^{2+}

Following methods described by Guo et al. (2017) and Su et al. (2018), concentrations of ROS and Ca^{2+} from haemocytes were estimated through quantification of fluorescent intensity using the ROS specific fluorescent stain 2, 7-dichlorofuorescin diacetate (DCFH-DA, E004, Nanjing Jiancheng Bioengineering Institute, China) and the Ca^{2+} specific fluorescent stain Fluo-4 AM (S1060, Beyotime, China), respectively.

After 4 days of corresponding treatment exposure, six individuals were randomly chosen from each experimental group for analysis of intracellular ROS and Ca^{2+}. After centrifugation at 1000 rpm for 15 s, haemocytes were washed with PBS for 30 s and incubated in 1 ml PBS containing 2 μL/mL DCFH-DA at 20 °C in the dark for 45 min. Haemocytes were next washed for 30 s with PBS twice and used to estimate fluorescent intensity. ROS specific fluorescent intensity was measured using a Nikon Eclipse E600 microscope at excitation and emission wavelengths of 500 nm and 525 nm, respectively. Image Pro-Plus (IPP) was subsequently used to quantify fluorescent intensities using the following equation: fluorescent intensity = the total optical density of the stained haemocyte area/the stained area of haemocytes (Su et al., 2019). Similar methods as those described above were adopted to determine intracellular Ca^{2+} in haemocytes, except that staining was conducted with 2.6 μL/mL Fluo-4 AM for 1 h, and 494 and 516 nm were used as excitation and emission wavelengths, respectively.

2.5. Measurement of the concentration and activity of LZM in haemocytes

Following the method of Su et al. (2019), after extraction and counting, haemocytes collected from 6 clams per experimental group were used to determine the content and activity of LZM using commercial ELISA kits (FK-97441 and FK-97442, respectively, FKBIO, Shanghai). Briefly, 10 μL diluted haemocytes (volume ratio of haemocytes to PBS at 1:9) were incubated with 40 μL diluent from the corresponding kit provided in a microwell plate at 37 °C in the dark for 30 min. After rinsing with the provided wash buffer for 5 times, haemocyte samples were incubated with 50 μL conjugate reagent for another 30 min. Samples were next rinsed with wash buffer and incubated with corresponding chromogenic reagents A and B (50 μL of each) in the microwell plate for 15 min, after which the chromogenic reaction was terminated with the provided stop buffer. A microplate reader (Thermo Multiskan Go, USA) was subsequently used to determine absorbance values for samples at a wavelength of 450 nm. LZM concentration and activity of the samples were determined by comparing the obtained absorption values to corresponding standard curves calibrated with the haemocyte counts.

2.6. Gene expression analysis

Expressions of three major types of genes were assessed in this study. Immune related genes tested include *TRAF6*, *IKKα*, and *NF-κB* from the NF-κB signalling pathway, and *TLR4* from the toll-like receptor pathway. Expression of *CaM* from Ca^{2+} signalling, and *Bcl-2* and *Caspase-3* from the haemocyte apoptosis pathway were also investigated.

After 4 days of corresponding treatment exposure, six individuals were randomly chosen from each experimental group for gene expression analysis. Total RNA was extracted from haemocytes following a previous method using an EASYspin Plus tissue/cell rapid RNA exaction kit (Aidlab, RN2802) (Peng et al., 2016). Before reverse transcription into first strand cDNA using the PrimeScript™ RT reagent kit (TaKaRa, RR037Q), gel electrophoresis and NanoDrop 1000 spectrophotometer (Thermo Scientific) were used to assure sample quality and to determine the concentration of RNA, respectively. Real-time PCR was performed using a CFX96 Real-Time System (Bio-Rad, USA) with the following amplification cycles: denaturation at 95 °C for 5 min, 39 amplification cycles at 95 °C for 20 s, 61 °C for 20 s, and 72 °C for 20 s. The melting curve analysis and $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) were applied to verify the reliability of qPCR products and to determine relative expression of the genes tested, respectively. Expression of the 18S rRNA was used as an internal reference (Brosnan et al., 2011; Su et al., 2018). All primers used in the present study were synthesized by TsingKe Biotech (Hangzhou, China) and are listed in Supplement 1.

2.7. Statistical analysis

Two-way ANOVAs were employed to detect the impacts of microplastic particle sizes, exposure concentrations of B[a]P or E2, and the interactions between microplastics and POPs on immune-related parameters. One-way ANOVA followed by Tukey's post hoc test was performed to detect difference in immune-related parameters among experimental groups. For all analyses, Shapiro-Wilk's and Levene's tests were used to assess assumptions of normality and homogeneity of variances, respectively. In cases where these assumptions were not satisfied by raw data, the data were arcsine square root transformed prior to analysis (Brosnan et al., 2011). The Duncan multiple range test was performed to compare expression levels of genes (Tallarida and Murray, 1987). All statistical analyses were performed using OriginPro 8.0, and a *p*-value less than 0.05 was accepted as a statistically significant difference.

3. Results

3.1. Impacts of exposure to microplastics and B[a]P or E2 alone or in combination on total count, cell type composition, and phagocytic activity of haemocytes

All the haemocyte parameters measured were unaffected by exposure to solvent DMSO alone (Table 2 and Table 3). Compared to controls, blood clams exposed to microplastics, B[a]P, or E2 alone at all tested sizes and/or doses yielded significantly reduced total haemocyte counts (THC), decreased proportions of red granulocytes, and reduced phagocytic activity (Tables 2 and 3). Results also suggest that the adverse effects on haemocyte traits occurred in dose- and particle size-dependent manners for each pollutant, which increase with elevated concentrations of B[a]P or E2 and decrease with increasing particle size of microplastics (Tables 2 and 3). In addition, the THC, proportion of red granulocytes, and phagocytic activity of haemocytes were significantly affected by not only the concentration of pollutants and the particle size of microplastics but also by the interactions between the two (Table 4 and Table 5). In general, the results obtained indicate that the toxic impacts of B[a]P and E2 on haemocyte parameters were significantly enhanced by microplastics at nano-scale, whereas mitigated by those at micro-scale (Tables 2 and 3). For instance, compared to those exposed to B[a]P or E2 alone at the corresponding exposure doses, blood clams co-exposed to microplastics at micro-scale have significant higher total numbers of haemocytes and phagocytic activities, whereas those co-exposed to microplastics at nano-scale have evident lower THC and phagocytosis (Tables 2 and 3).

3.2. Impacts of exposure to microplastics and B[a]P or E2 alone or in combination on concentrations of ROS and Ca^{2+} in haemocytes

The concentrations of ROS and Ca^{2+} in haemocytes were unaffected by the exposure of calms to solvent DMSO alone (Fig. 1). Compared to controls, ROS content in haemocytes, measured by specific fluorescent intensities, was significantly induced by exposure of blood clams to microplastics, B[a]P, or E2 alone at all tested sizes and/or doses (Fig. 1A and B). Except for those individuals exposed to microplastics at a size of 30 μm, haemocytes of blood clams exposed to smaller size microplastics (500 nm), B[a]P, or E2 alone exhibited significantly reduced intracellular concentrations of Ca^{2+} (Fig. 1C and D). Though no significant difference was detected between BL and BH groups, the impact of microplastics, B[a]P, and E2 on ROS and Ca^{2+} concentrations generally occurred in size- and dose-dependent manners (Fig. 1). In addition, except for Ca^{2+} from clams exposed to high doses of E2 (1 μg/L), the impacts of B[a]P and E2 on ROS and Ca^{2+} were mitigated by the co-presence of microplastics at a size of 30 μm and were aggravated by those at a size of 500 nm.

3.3. Impacts of exposure to microplastics and B[a]P or E2 alone or in combination on the concentration and activity of LZM from haemocytes

No significant impact on concentration and activity of LZM was detected for clams exposed to solvent control (Fig. 2). Compared to controls, both the concentration and activity of LZM from haemocytes were significantly decreased in response to exposure of blood

Table 2
THCs, percentages of three haemocytes, and phagocytic activities of blood clams exposed to microplastics, B[a]P, and the combination of microplastics and B[a]P. C-control: seawater without any tested pollutant; S-control: seawater with solvent DMSO but no tested pollutant; M: microplastics at a size of 30 μm. N: microplastics at a size of 500 nm; B: B[a]P; L: exposure dose of 5 μg/L; and H: exposure dose of 50 μg/L. All data was presented as means ± SEM. Mean values that do not share the same superscript were significantly different.

Trails	THC	Percentage (%) of the			Phagocytosis
	(× 10^8mL-1)	Red granulocyte	Basophil granulocyte	Hyalinocyte	(%)
S-control	1.667 ± 0.093^{ae}	82.6 ± 0.9^{a}	15.9 ± 1.1^{ab}	1.6 ± 0.1^{a}	50.4 ± 1.0^{a}
C-control	1.733 ± 0.060^{a}	84.3 ± 0.1^{a}	14.2 ± 0.2^{a}	1.5 ± 0.2^{a}	52.1 ± 0.7^{a}
M	1.467 ± 0.033^{b}	82.1 ± 0.2^{b}	16.6 ± 0.3^{b}	1.3 ± 0.2^{a}	47.1 ± 0.4^{b}
N	1.133 ± 0.017^{cd}	77.9 ± 0.1^{c}	20.5 ± 0.1^{c}	1.6 ± 0.1^{ab}	32.4 ± 0.8^{c}
BL	1.167 ± 0.088^{c}	78.5 ± 0.4^{c}	20.1 ± 0.3^{c}	1.4 ± 0.1^{ab}	43.2 ± 0.8^{d}
MBL	1.517 ± 0.081^{be}	82.1 ± 0.2^{b}	16.9 ± 0.3^{b}	1.0 ± 0.1^{a}	47.4 ± 0.8^{b}
NBL	0.900 ± 0.050^{d}	75.6 ± 0.3^{d}	22.3 ± 0.3^{d}	2.1 ± 0.1^{b}	24.2 ± 1.1^{e}
BH	0.967 ± 0.044^{d}	75.9 ± 0.5^{d}	22.6 ± 0.8^{d}	1.5 ± 0.1^{ab}	38.7 ± 1.0^{f}
MBH	1.233 ± 0.044^{bc}	79.0 ± 0.3^{e}	19.9 ± 0.5^{c}	1.1 ± 0.2^{a}	43.5 ± 0.5^{d}
NBH	0.750 ± 0.029^{f}	72.6 ± 0.3^{d}	25.3 ± 0.3^{e}	2.0 ± 0.2^{b}	21.5 ± 0.9^{e}

Table 3
THCs, percentages of three haemocytes, and phagocytic activities of blood clams exposed to microplastics, E2, and a combination of microplastics and E2. C-control: seawater without any tested pollutant; S-control: seawater with solvent DMSO but no tested pollutant; M: microplastics at a size of 30 μm; N: microplastics at a size of 500 nm; E: E2; L: exposure dose of 0.1 μg/L; and H: exposure dose of 1 μg/L. All data was presented as means ± SEM. Mean values that do not share the same superscript were significantly different.

Trails	THC	Percentage (%) of the			Phagocytosis
	(× 10^8mL-1)	Red granulocyte	Basophil granulocyte	Hyalinocyte	(%)
S-control	1.667 ± 0.093^{a}	82.6 ± 0.9^{ab}	15.9 ± 1.1^{ab}	1.6 ± 0.1^{a}	50.4 ± 1.0^{a}
C-control	1.733 ± 0.060^{a}	84.5 ± 0.1^{a}	14.2 ± 0.1^{a}	1.3 ± 0.1^{a}	52.1 ± 0.7^{a}
M	1.467 ± 0.033^{b}	82.1 ± 0.2^{b}	16.7 ± 0.3^{b}	1.3 ± 0.2^{a}	47.1 ± 0.2^{b}
N	1.133 ± 0.017^{cd}	77.9 ± 0.1^{c}	20.5 ± 0.1^{c}	1.6 ± 0.1^{ac}	32.4 ± 0.8^{c}
EL	1.383 ± 0.067^{b}	79.2 ± 0.7^{be}	18.9 ± 0.6^{b}	1.9 ± 0.1^{ab}	45.7 ± 0.8^{b}
MEL	1.667 ± 0.044^{a}	82.5 ± 0.4^{d}	16.1 ± 0.4^{d}	1.4 ± 0.2^{a}	48.2 ± 0.7^{d}
NEL	1.067 ± 0.060^{d}	75.0 ± 0.5^{f}	22.9 ± 0.3^{e}	2.2 ± 0.1^{bc}	27.5 ± 0.8^{e}
EH	1.017 ± 0.012^{d}	78.7 ± 0.4^{e}	20.1 ± 0.4^{c}	1.2 ± 0.3^{a}	43.7 ± 0.6^{f}
MEH	1.317 ± 0.044^{bc}	80.6 ± 0.6^{b}	17.8 ± 0.4^{b}	1.7 ± 0.2^{a}	47.4 ± 0.5^{b}
NEH	0.767 ± 0.017^{e}	73.0 ± 0.2^{g}	25.0 ± 0.2^{f}	2.0 ± 0.1^{c}	26.2 ± 0.8^{e}

Table 4
Results of two-way ANOVAs showing the impacts of microplastic particle size (30 μm and 500 nm in diameter), concentration of B[a]P (0, 5, and 50 μg/L), and their interaction on THC, cell type composition, and phagocytic activities of haemocytes from blood clams. In total, five linear models using THC, percentages of the three types of haemocytes, and phagocytosis as response (Response = α·microplastic particle size + i·concentration of B[a]P + ɤ·interaction + error) were constructed and used for the two-way ANOVA analysis, respectively. Result obtained for each response was listed in the corresponding column below.

Factors	THC	Percentage (%) of the			Phagocytosis
		Red granulocyte	Basophil granulocyte	Hyalinocyte	(%)
Diameter of plastics particle	$F_{1,17} = 142.23$ $p = 5.18 \times 10^{-8}$	$F_{1,17} = 834.06$ $p = 1.85 \times 10^{-12}$	$F_{1,17} = 362.03$ $p = 2.49 \times 10^{-10}$	$F_{1,17} = 40.48$ $p = 3.60 \times 10^{-5}$	$F_{1,17} = 2230.30$ $p = 5.33 \times 10^{-15}$
Concentration of B[a]P	$F_{2,17} = 20.83$ $p = 1.25 \times 10^{-4}$	$F_{2,17} = 163.48$ $p = 1.97 \times 10^{-9}$	$F_{2,17} = 91.14$ $p = 5.55 \times 10^{-8}$	$F_{2,17} = 0.53$ $p = 0.61$	$F_{2,17} = 93.78$ $p = 4.73 \times 10^{-8}$
Interaction (Diameter × Concentration of B[a]P)	$F_{2,17} = 4.17$ $p = 0.042$	$F_{2,17} = 13.39$ $p = 8.79 \times 10^{-4}$	$F_{2,17} = 3.88$ $p = 0.05$	$F_{2,17} = 5.44$ $p = 0.01$	$F_{2,17} = 39.32$ $p = 5.38 \times 10^{-6}$

Table 5
Results of two-way ANOVAs showing the impacts of microplastic particle size (30 μm and 500 nm in diameter), concentration of E2 (0, 0.1, and 1 μg/L), and their interaction on THC, cell type composition, and phagocytic activities of haemocytes from blood clams. In total, five linear models using THC, percentages of the three types of haemocytes, and phagocytosis as response (Response = α·microplastic particle size + ß·concentration of E2 + ɤ·interaction + error) were constructed and used for the two-way ANOVA analysis, respectively. Result obtained for each response was listed in the corresponding column below.

Factors or interaction	THC	Percentage (%) of the			Phagocytosis
		Red granulocyte	Basophil granulocyte	Hyalinocyte	(%)
Diameter of plastics particle	$F_{1,17} = 240.03$ $p = 2.68 \times 10^{-9}$	$F_{1,17} = 449.51$ $p = 7.05 \times 10^{-11}$	$F_{1,17} = 553.09$ $p = 2.09 \times 10^{-11}$	$F_{1,17} = 28.70$ $p = 1.72 \times 10^{-4}$	$F_{1,17} = 1197.86$ $p = 2.16 \times 10^{-13}$
Concentration of E2	$F_{2,17} = 38.58$ $p = 5.95 \times 10^{-6}$	$F_{2,17} = 38.34$ $p = 6.14 \times 10^{-6}$	$F_{2,17} = 43.19$ $p = 3.29 \times 10^{-6}$	$F_{2,17} = 7.20$ $p = 8.84 \times 10^{-3}$	$F_{2,17} = 9.94$ $p = 0$
Interaction (Diameter × Concentration of E2)	$F_{2,17} = 6.58$ $p = 0.012$	$F_{2,17} = 13.09$ $p = 9.63 \times 10^{-4}$	$F_{2,17} = 16.65$ $p = 4.15 \times 10^{-10}$	$F_{2,17} = 2.24$ $p = 0.15$	$F_{2,17} = 14.64$ $p = 1.09 \times 10^{-11}$

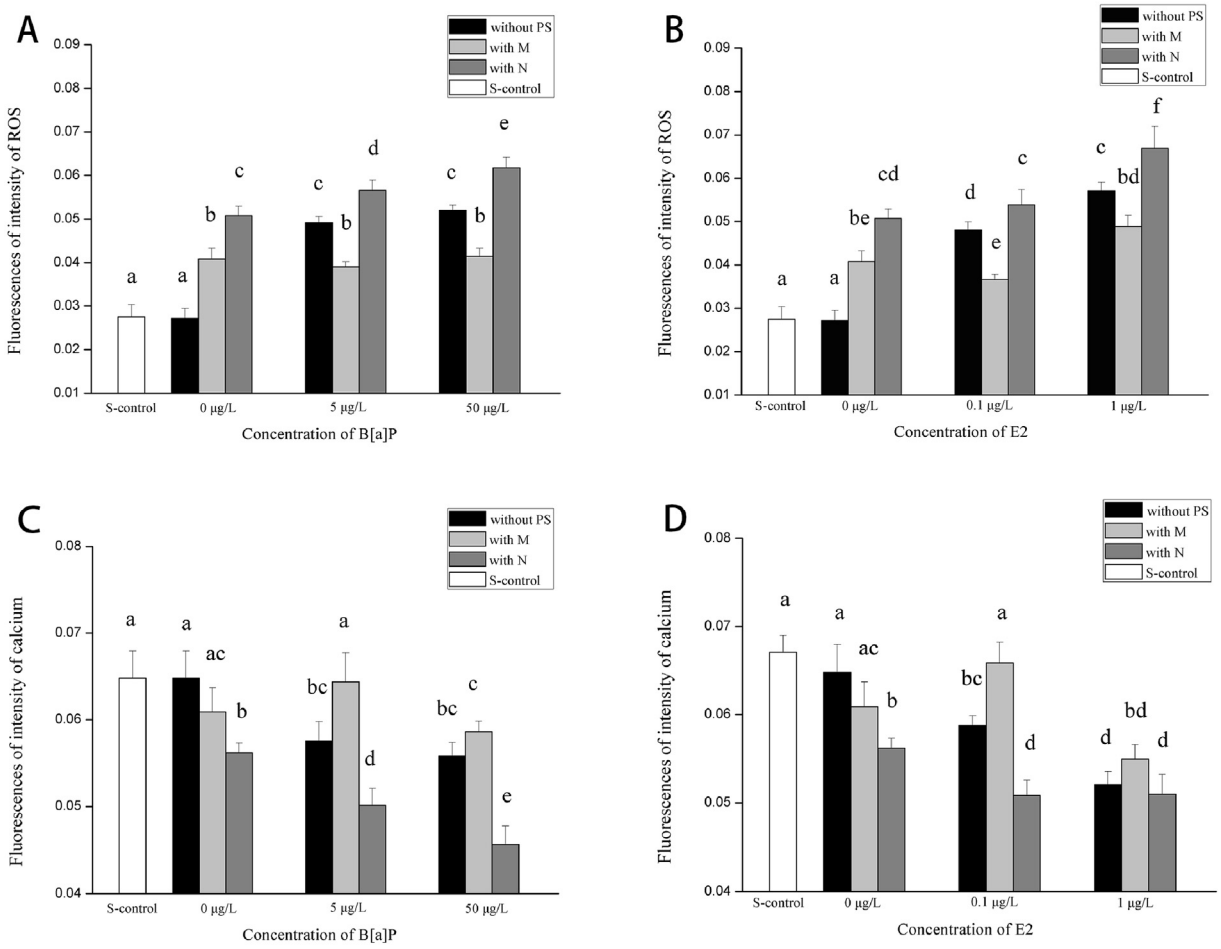

Fig. 1. Concentrations of ROS and Ca^{2+} from haemocytes in response to exposure of blood clams to microplastics, B[a]P, and E2 alone or in combination. A & C: ROS and Ca^{2+} contents of clam haemocytes exposed to microplastics or B[a]P alone and in combination. B & D: ROS and Ca^{2+} contents of haemocytes from clams exposed to microplastics or E2 alone and in combination. PS:polystyrene microplastics; C-control: seawater without any tested pollutant; S-control: seawater with solvent DMSO but no tested pollutant. All data was presented as means ± SEM. Different letters on bars represent significant differences between groups with $p < 0.05$.

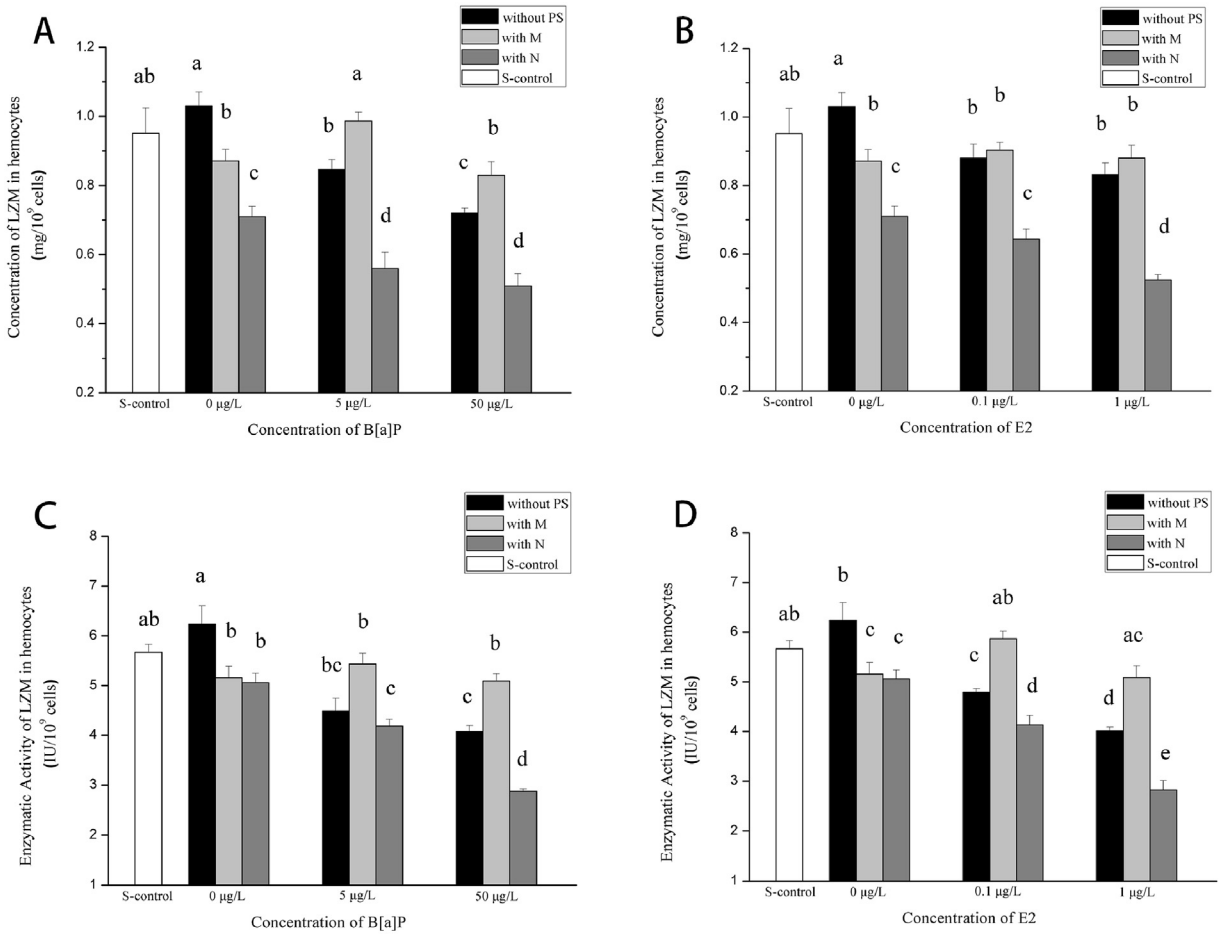

Fig. 2. Concentrations and activities of LZM from haemocytes after exposure of blood clams to microplastics, B[a]P, and E2 alone or in combination. A & C: Concentrations and activities of LZM from haemocytes of clams exposed to microplastics and B[a]P alone and in combination. B & D: Concentrations and activities of LZM from haemocytes of clams exposed to microplastics and E2 alone and in combination. PS:polystyrene microplastics; C-control: seawater without any tested pollutant; S-control: seawater with solvent DMSO while no tested pollutant. All data was presented as means ± SEM. Different letters on bars represent significant differences between groups with $p < 0.05$.

clams to microplastics, B[a]P, or E2 alone at all tested sizes and/or doses (Fig. 2). Though the impacts of microplastics and B[a]P exposure alone on the activities of LZM were not significantly affected by the size of microplastics or the concentrations of B[a]P tested (Fig. 2C), the attenuating effects on LZM concentrations were shown to be particle size- and dose-dependent (Fig. 2A). In addition, except for LZM activities from haemocytes of clams exposed to lower dose (5 μg/L) B[a]P, the impacts of B[a]P exposure on the contents and activity of LZM were mitigated by the co-presence of microplastics at a size of 30 μm and were aggravated by those at a size of 500 nm (Fig. 2A and C). Similarly, the impacts of microplastics on the content of LZM were significantly affected by the particle size of microplastics (Fig. 2B); however, these size dependent impacts were not detected for LZM activity (Fig. 2D). Though the impacts of E2 on LZM content were not significantly affected by exposure doses, these impacts were aggravated by the co-presence of microplastics at a size of 500 nm (Fig. 2B). In addition, the co-presence of microplastics exerted similar size-dependent effects on the toxicity of E2 on LZM activity as well (Fig. 2D). Among all experimental groups, co-exposure of clams to smaller size (500 nm) microplastics and higher doses of pollutants (50 and 1 μg/L for B[a]P and E2, respectively) led to the lowest concentrations and activities of LZM (Fig. 2).

3.4. Impacts of exposure to microplastics and B[a]P or E2 alone or in combination on the expression of the tested genes

Except for *TLR4* in clams exposed to microplastics at a size of 30 μm, expression levels of immune related genes (*IKKα*, *NFκB*, *TRAF6*, and *TLR4*) were significantly suppressed by exposure of clams to microplastics, B[a]P, or E2 alone at all tested sizes and/or doses (Fig. 3). In addition, with only a few exceptions, the inhibitory impacts of B[a]P and E2 exposure on the expression of immune related genes were generally mitigated by the co-presence of microplastics at a size of 30 μm and were aggravated by those at a size of 500 nm (Fig. 3). Co-exposure to larger size (30 μm) microplastics along with high doses (1 μg/L) of E2 brought expression levels of *NFκB* and *TLR4* back to normal levels compared to controls (Fig. 3B). Similar compromised expression in response to B[a]P or E2 exposure and mitigation or aggravating effects exerted by the co-presence of microplastics at different sizes were also detected for *CaM* (Fig. 4). In addition, except for *Bcl-2* of clams exposed to microplastics at a size of 30 μm, expression levels of the negative regulator *Bcl-2* and the positive executor *Caspase-3* in the apoptosis pathway were significantly up-regulated and down-regulated, respectively (Fig. 4). Furthermore, alterations of the expression of *Bcl-2* and *Caspase-3* in response to B[a]P or E2 were generally mitigated by the co-presence of microplastics at a size of 30 μm and aggravated by those at a size of 500 nm.

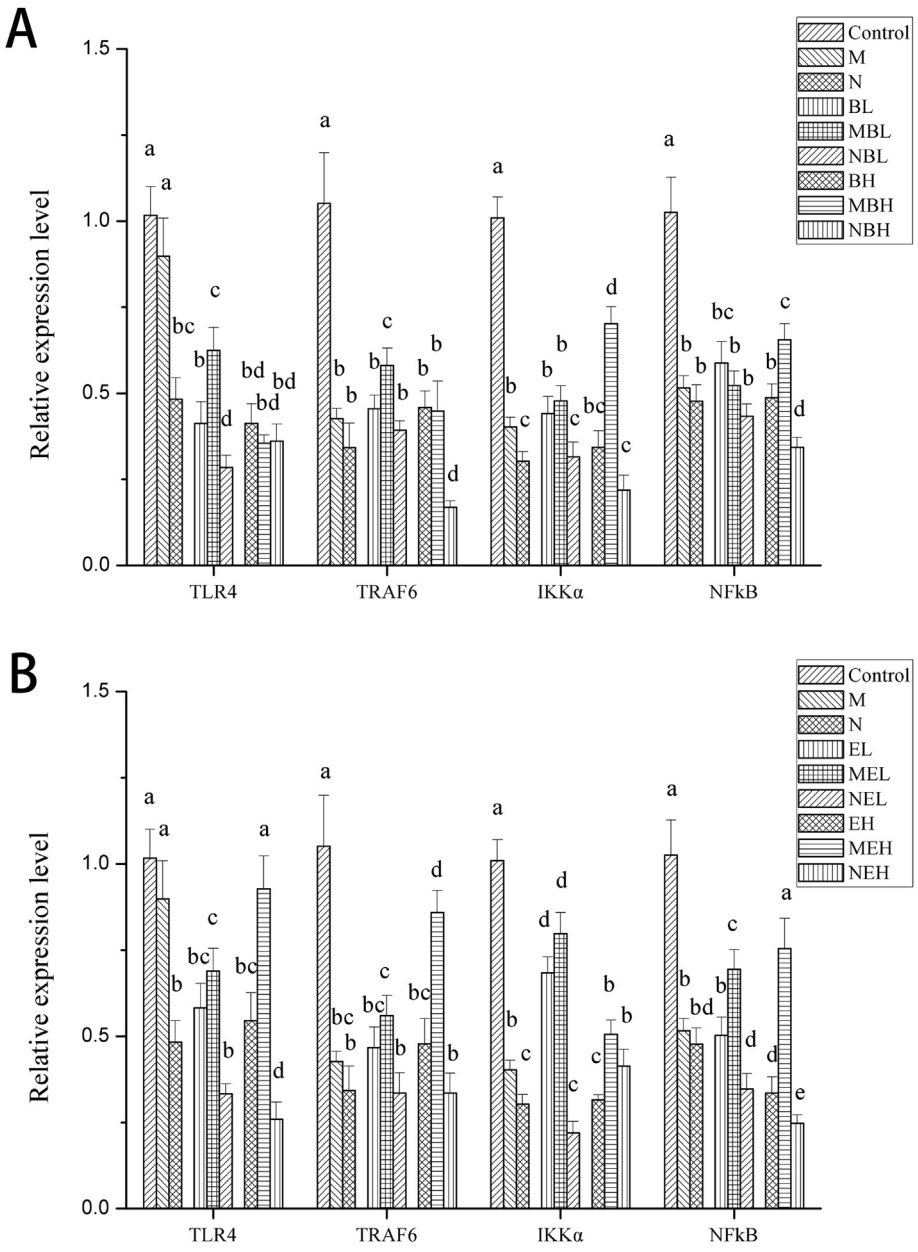

Fig. 3. Impacts of exposure of blood clams to microplastics, B[a]P, and E2 alone or in combination on expression of *TRAF6*, *IKKα*, *NFκB*, and *TLR4*. A: Exposure of clams to microplastics and B[a]P alone or in combination. B: Exposure of clams to microplastics and E2 alone or in combination. All data was presented as means ± SEM. Different letters on bars represent significant differences between groups with $p < 0.05$.

4. Discussion

Since bivalve molluscs primarily rely on haemocytes to fight against pathogen infections (Canesi and Procházková, 2014; Loker et al., 2004), altered hematic parameters in response to exposure of blood clams to microplastics, B[a]P, and E2 described in this study demonstrate an evident immunotoxicity of these pollutants to the species. Furthermore, results obtained in this study indicate that microplastics, B[a]P, and E2, may hamper the immune responses of blood clam similarly through (1) reducing the total number and the proportion of most immune-active cell type of haemocytes, (2) constraining the recognition, engulfment, and degradation of foreign materials by haemocytes, and (3) interrupting immune, Ca^{2+} signalling, and apoptosis-related molecular pathways.

In this study, it was shown that exposure to the tested pollutants led to THC reduction, rendering reduced haemocytes available to fight against pathogen infections. In addition, since the red granulocytes possess the highest phagocytic activity among the different types of haemocytes in the blood clam (Burgos-Aceves and Faggio, 2017; Liu et al., 2016; Matozzo et al., 2016), the reduction in the proportion of red granulocytes along with that of THC could result in an overall reduced phagocytic capacity and therefore hamper the immune response. The reduction in THC induced by the tested pollutants may result from both heightened depletion and constrained renewal of haemocytes. It has been shown that a proportion of haemocytes will infiltrate into the digestive gland upon exposure to both POPs and microplastics in the mussel *Mytilus edulis* (von Moos et al., 2012). In this circumstance, the recruitment of haemocytes into other tissues may

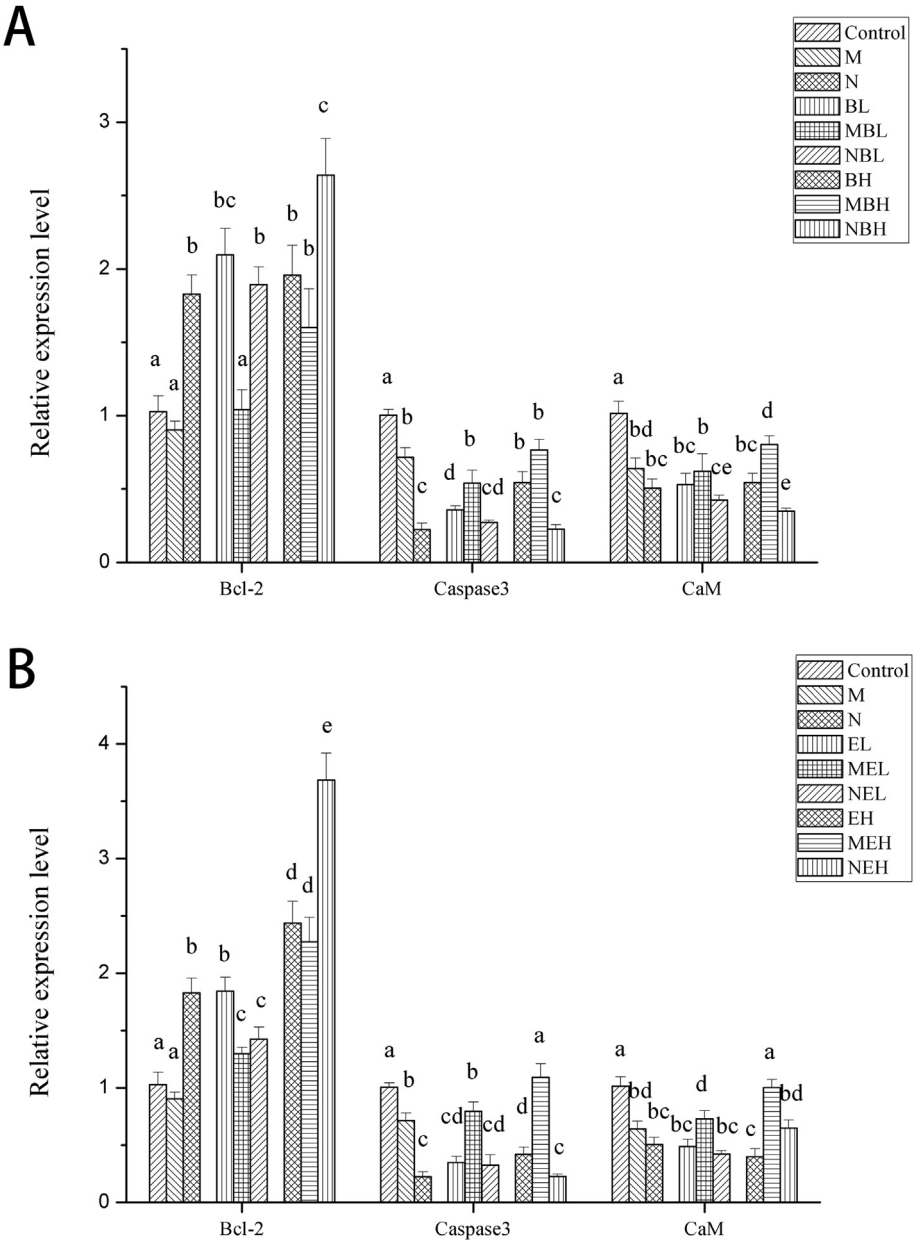

Fig. 4. Impacts of exposure of blood clams to microplastics, B[a]P, and E2 alone or in combination on the expression of *CaM*, *Bcl-2*, and *Caspase-3*. A: Exposure of clams to microplastics and B[a]P alone or in combination. B: Exposure of clams to microplastics and E2 alone or in combination. All data was presented as means ± SEM. Different letters on bars represent significant differences between groups with $p < 0.05$.

reduce the total number of haemocytes in the circulatory system. In addition, according to the data obtained, constrained haemocyte renewal through disrupting proliferation may also contribute to the THC decline detected. Since intracellular Ca^{2+} initiates cell proliferation by regulating transcription factors, such as NF-κB (Berridge et al., 2000; Gonzalez-Murillo et al., 2015; Pinto et al., 2015), the reduction in intracellular Ca^{2+} content and the down-regulation of *NF-κB* in response to pollutant exposure may disrupt the process of proliferation and led to THC decline.

Phagocytosis, including a series of sequential events including recognition, engulfment, and degradation of exogenous substances by haemocytes, is the key cellular immune response of bivalve molluscs (Song et al., 2010). However, the results obtained in this study indicate that all the crucial processes could be hampered by exposure to the pollutants tested. It's well known that foreign substances are recognized by haemocytes via pattern recognition receptors such as TLRs (Barton and Medzhitov, 2003). In the present study, *TLR4* expression was found to be suppressed by pollutants exposure, which could in turn render haemocytes less efficient in recognizing exogenous particles. In addition, since actin filaments, which mediate the process of engulfment, are sensitive to ROS (Pollard and Cooper, 2009; Tomanek et al., 2011), induced intracellular ROS upon exposure to microplastics, B[a]P, and E2, may interfere the engulfment process via damaging actin filaments (Xu et al., 2013; Su et al., 2018). The engulfed pathogen particles will be degraded *in vivo* by LZM or ROS (Finkel, 2011; Pollard and Cooper, 2009; Song et al., 2010; Tomanek et al., 2011). Therefore, the deceases in the contents and activity of LZM detected in pollutant treatment groups could impair the degradation ability of haemocytes, leading to hampered phagocytic activity. Though the

induction of ROS upon pollutant exposure could theoretically enhance oxygen-dependent degradation of exogenous substance (Dupre-Crochet et al., 2013), excessive intracellular ROS may inhibit the phagocytic activity not only through impairing normal function of haemocytes via imposing oxidative stress (Pollard and Cooper, 2009; Tomanek et al., 2011), but also triggering apoptosis and subsequently constraining the number of haemocytes available for phagocytic reaction (Circu and Aw, 2010; Faggio et al., 2016; Savorelli et al., 2017; Sehonova et al., 2018).

In addition, the results obtained in this study suggest that microplastics, B[a]P, and E2 may also exert immunotoxic impacts on blood clam through interrupting immune, Ca^{2+}, and apoptosis related molecular pathways. The activation of immune related molecular pathways, such as the toll-like receptor and NF-κB signalling pathway, is crucial to trigger immune responses in bivalve molluscs (Seth et al., 2006). In the present study, expression of *TLR4* in the toll-like receptor pathway and *IKKα*, *TRAF6*, and *NFκB* in the NF-κB signalling pathway was significantly inhibited, which could partially explain the impaired immune response detected (Seth et al., 2006). In addition, it is well known that intracellular Ca^{2+} plays an indispensable role in the onset of phagocytosis, especially in the maturation of phagosomes (Nunes and Demaurex, 2010). Moreover, the intracellular Ca^{2+} regulator calmodulin (*CaM*) is also a key molecule triggering activation of the NF-κB signalling pathway (Lilienbaum and Israel, 2003). In this circumstance, lower intracellular contents of Ca^{2+} and suppressed expression of *CaM* detected in response to pollutant exposure offers another mechanism for the immunotoxicity observed. Data obtained in this study also demonstrated that *Bcl-2* and *Caspase-3* from apoptosis pathway were significantly up-regulated and down-regulated in response to pollutant exposure, respectively. This alteration could be a feedback molecular response to aggravated apoptosis induced by elevated intracellular ROS and indicate an interruption of apoptosis process, which may hamper the normal function of haemocytes and result in reduced immune ability.

The decline in intracellular concentrations of Ca^{2+} upon pollutant exposure observed may result from suppressed activity of Ca^{2+} channels and impaired regulatory capability of intracellular Ca^{2+}. It has been suggested that ROS can hamper the activity of several calcium transporters anchored in the plasma membrane, such as L-type Ca^{2+} channels (Gorlach et al., 2015; Hengartner, 2000). Since the intracellular contents of ROS were induced by pollutant exposure in this study, influx of Ca^{2+} into the haemocytes could be inhibited by induction of ROS. In addition, expression of *CaM*, a key intracellular Ca^{2+} regulator, was significantly down-regulated upon pollutant exposure, which may also contribute to reduced intracellular Ca^{2+}.

For the first time, the present study showed that the immunotoxicity of B[a]P and E2 were affected by the co-presence of microplastics in a particle size dependent manner in bivalve molluscs. It was demonstrated that the deleterious impacts of B[a]P or E2 on the immune responses of blood clams were mitigated by the presence of larger sized microplastics and aggravated by those of smaller size, which may be due to following size-dependent differences.

First, compared to microplastics of larger size, smaller microplastics have a larger surface area: volume ratio and greater penetrating capability (Fotopoulou and Karapanagioti, 2012; Kashyap et al., 2007), which may facilitate the internalization of organic pollutants into clams. Since the toxicity of many pollutants, including B[a]P and E2, are dose-dependent (Canesi et al., 2004; Santacroce et al., 2015), microplastics at a smaller size may aggravate immunotoxicity of these pollutants through this "Trojan horse" effect. Second, as shown in the present study, microplastics are immunotoxic in a particle size dependent manner as well. No matter whether this difference is due to the penetrating ability or surface area: volume ratio (i.e., smaller microplastics have relatively larger area surface compared to larger one and therefore may be more effective in releasing toxins it carries), co-exposure to smaller microplastics and organic pollutants may be more toxic simply due to the add-on effect of these two types of pollutants. Third, exerting a series of deleterious impacts on aquatic organisms (Besseling et al., 2014; Sussarellu et al., 2016; von Moos et al., 2012; Wright et al., 2013), exposure to microplastics may render the species in a stressful condition and subsequently constrain the energy available for detoxification (Paul-Pont et al., 2016). Since detoxification is often an energy costing process (Oliveira et al., 2013), smaller microplastics that were shown to be more toxic in this study may significantly hamper detoxification of B[a]P or E2, resulting in reinforced toxicity. Finally, it has been suggested that it is unlikely for microplastics larger than 20 μm in diameter to enter the circulatory system of bivalve species, such as *Mytilus edulis* (Van Cauwenberghe and Janssen, 2014). In addition, accelerated clearance rate, which may shorten the *in vivo* residence time of microplastics, was detected in bivalve species *Crassostrea gigas* upon microplastics exposure (Sussarellu et al., 2016). In this circumstance, B[a]P or E2 absorbed on the larger microplastics may pass through the intestine of blood clam quickly without extensive discharge and causing severe damages. All these factors may result in mitigation exerted by large size microplastics on the toxicity of B[a]P and E2.

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.2019.113845.

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