Biological effects of tris (1-chloro-2-propyl) phosphate (TCPP) on immunity in mussel *Mytilus galloprovincialis*

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Organophosphate flame retardants (OPFRs), TCPP, Biological effects, immunity

**ABSTRACT**

Organophosphate flame retardants (OPFRs) are increasingly produced and used as alternatives of brominated flame-retardants (BFRs) and have become emerging marine environmental contaminants. So far, however, little is known regarding the biological effects of OPFRs in marine organisms. In this study, the biological effects of one of the most abundant OPFRs, tris (1-chloro-2-propyl) phosphate (TCPP), on the immunity of mussel *Mytilus galloprovincialis* were characterized by testing the reactive oxygen species, apoptosis, antioxidant system and immunity-related gene expressions. Results indicated that both TCPP exposures (10 and 100 nmol L\(^{-1}\)) significantly \(p < 0.01\) enhanced reactive oxygen species production and the high dose of TCPP induced more apoptosis and oxidative stress in mussel hemocytes. TCPP also induced an obvious hormesis phenomenon (low dose inhibition and high dose stimulation) in mussel hemocytes, as indicated by the gene expression profiles of *caspase 8* and *mytimacin*. The down-regulated gene expression levels of lysosomes suggested that both TCPP exposures inhibited the innate immunity in mussel *M. galloprovincialis*. The significantly \(p < 0.01\) increased gene expression levels of *TLK, galectin, PGRP* and *LITAF* demonstrated that TCPP induced dose-dependent immune stress in mussels. Overall, this work suggested that TCPP could influence the immune system in marine mussel *M. galloprovincialis*.

**1. Introduction**

Organophosphate flame retardants (OPFRs) are increasingly produced and used as alternatives of brominated flame-retardants (BFRs), such as the polybrominated diphenyl ethers (PBDEs) (Wang et al., 2015). Nowadays, the OPFRs can be frequently detected in seawaters and sediments from the Bohai Sea (Zhong et al., 2018; Zheng et al., 2017), which has posed a risk on marine organisms and subsequent human health via food chains. Among the OPFRs, the tris (1-chloro-2-propyl) phosphate (TCPP) is one of the most frequently detected halogenated OPFRs in both waters and sediments due to its wide usage as flame retardants in polyurethane foam and epoxy resin, with a concentration up to 31.4 ng L\(^{-1}\) in the seawater from the Bohai Sea in China (Zhong et al., 2018; Zheng et al., 2017; Andresen et al., 2004; Bacceloni et al., 2008; Martinez-Carballo et al., 2007; Regnery and Puttmann, 2010). Although the adverse effects to human health and ecosystem of OPFRs have been reported in a few researches (Reemtsma et al., 2008; Ren et al., 2008; Pillai et al., 2014), there is a lack of studies on the biological effects of OPFRs in marine organisms. Compared with brominated flame retardants, such as PBDEs and tetrabromobisphenol A (TBBPA), however, OPFRs have received little attention with regard to the adverse biological effects and ecological risk. Therefore, it is necessary to characterize the biological effects of OPFRs in marine organisms.

The marine bivalve, mussel *Mytilus galloprovincialis*, distributes widely in the Bohai Sea and is consumed as popular seafood by local residents. Therefore mussel *M. galloprovincialis* has been an important species in marine aquaculture industry in China. In addition, as a filter-feeder, mussel *M. galloprovincialis* plays an important role in coastal ecosystem. Due to its high capacity to accumulate environmental contaminants, this bivalve is also a preferable bioindicator used in marine biology and ecotoxicology, as well as in ‘Mussel Watch Program’ (Goldberg et al., 1983; Jernelov, 1996). However, there was a lack of investigations on the toxicological effects of OPFRs in marine mussels.

As an emerging class of marine environmental contaminants in the Bohai Sea, the OPFRs have been of great concern to researchers. In this work, the mussel *M. galloprovincialis* was used as experimental animal to study the chronic biological effects of one of the most frequently...
detected OPFRs, TCPP. The immune system of *M. galloprovincialis* is innate and consists of an open and vascular system including hemocytes and humoral components (Tanguy et al., 2018). The hemocytes provide the first line of defense against immune stressors by phagocytosis and encapsulation. Then, the humoral components including lysosomal enzymes, aminopeptidases, lectins and antimicrobial molecules may be produced to destroy immune stressors (Tanguy et al., 2018). In this study, therefore, the reactive oxygen species, apoptosis, antioxidant system and immunity-related gene expressions in hemocytes were tested to elucidate the biological effects of TCCP on the immunity in mussel *M. galloprovincialis*.

2. Materials and methods

2.1. Animal culture and experiment design

Adult mussels *Mytilus galloprovincialis* (shell length: 4–6 cm) were purchased from a local culturing farm (Yantai, China). After transported to laboratory, the mussels were acclimatized in filtered and aerated seawater in glass containers for 7 days and then were randomly divided into four groups (seawater control, solvent control, low and high doses of TCCP treatments). For each group, there were two replicate containers each containing 20 individual mussels. The mussels cultured in the filtered seawater and filtered seawater containing 0.00025% DMSO (v/v) were used as seawater control and solvent control groups, respectively. TCCP (CAS No. 13,674-84-5) was purchased from J&K Chemical Co., Ltd. The TCCP-treated mussels were exposed to two concentrations (10 and 100 nmol L\(^{-1}\)) of TCCP, respectively. The concentrations of TCCP stock solutions were 1 and 10 ng L\(^{-1}\) in DMSO to ensure the same DMSO concentrations in the TCPP treatments. For each group, there were two replicate containers each containing 20 individual mussels. The mussels cultured in the filtered seawater and filtered seawater containing 0.00025% DMSO (v/v) were used as seawater control and solvent control groups, respectively. TCCP (CAS No. 13,674-84-5) was purchased from J&K Chemical Co., Ltd. The TCCP-treated mussels were exposed to two concentrations (10 and 100 nmol L\(^{-1}\)) of TCCP, respectively. The concentrations of TCCP stock solutions were 1 and 10 ng L\(^{-1}\) in DMSO to ensure the same DMSO concentrations in the TCPP-exposed groups to that of solvent control group. During the period of acclimation and exposure, the mortalities for all groups were recorded and found to be less than 5% without significant differences among the groups. All the animals were kept at 18–20 °C under a photoperiod of 12 h light and 12 h dark, and fed with 1.5 g *Chlorella vulgaris* per day. After a chronic exposure for 42 days, the mussels were taken out of the exposure tanks and opened by using scalpels to cut off the adductor muscle carefully. Then, the hemolymph was collected from the adductor by using sterile injection syringes and mixed immediately with equivoluminal anticoagulant (20.80 g of glucose, 8.00 g of sodium citrate, 3.36 g of ethylene diamine tetraacetic acid (EDTA), and 22.50 g of sodium chloride in 1 L of pure water). Then the hemolymph samples were filtrated by screen spun silks of 300 meshes to exclude impurities and used in subsequent experiments. For all the experiments, 4 biological replicates were used in each group and each biological replicate consisted of blood from four individual mussels.

2.2. Hemocytes RNA extraction, cDNA synthesis and gene expression analysis

Approximately, 10 mL of mussel blood was centrifuged (5000 rpm for 5 min) to enrich hemocytes and TRIzol Reagent (Invitrogen) was used to isolate total RNA. The extracted RNA was purified with RNeasy mini kit (Qiagen) and resuspended in purified water (RNase free). Total RNA quality and concentrations were evaluated by the NanoDrop 1000 spectrophotometer (Thermo Scientific). By employing M-MLV reverse transcriptase (Promega) and Oligo dT primers, 500 ng of total RNA was used to synthesize cDNA. All the processes were followed the manufacturer’s instructions.

For gene expression analysis, 12 immunity related genes were selected and the corresponding primers were referred to the publications (Wang et al., 2013, 2012; Martins et al., 2014; Gerdol et al., 2012; You, 2013) and listed in Table 1. The 7300 Real Time PCR System (Applied Biosystems) was employed to conduct qPCR analysis. Briefly, 6 μL of 50 times diluted cDNA, 10 μL of SYBR green PCR master mix (Applied Biosystems), 0.8 μL (10 mM) of forward and reverse primer and 3.2 μL of nuclelease-free water (Qiagen) were added to 20 μL of reaction volume. The qPCR program was designed as 95 °C for 7 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s and 60 °C for 10 min. The *M. galloprovincialis* 18S rRNA gene was chosen as reference gene and the comparative CT method (2\(^{-\Delta\Delta CT}\) method) was used to analyze the expression level of the genes (Livak and Schmittgen, 2001).

2.3. Analysis of reactive oxygen species (ROS) and apoptosis

Approximately, 600 μL of mussel blood was centrifuged (5000 rpm for 5 min) to enrich hemocytes. Following the manufacturer’s instructions, the ROS Assay Kit (the method of 2′, 7′-dichlorofluorescin (DCF) labeling, Beyotime) and the One Step TUNEL Apoptosis Assay Kit (the method of fluorescein isothiocyanate (FITC) labeling, Beyotime) were used in pretreatment of the ROS and apoptosis analysis, respectively. Then the treated hemocytes (10,000 individuals for one sample) were screened and sorted by the flow cytometer (FACS Aria) at excitation wavelength of 488 nm and emission wavelength of 525 nm.

2.4. Analysis of superoxide dismutase (SOD) activity and malondialdehyde (MDA) content

About 600 μL mussel blood was centrifuged (5000 rpm for 5 min) to enrich hemocytes. Following the manufacturer’s instructions, SOD Assay Kit (Nanjing Jiansheng Bioengineering Institute) and MDA Assay Kit (Nanjing Jiansheng Bioengineering Institute) were used in pretreatment of the SOD and MDA analysis, respectively. Then the treated samples were analyzed by using Infinite M200 microplate spectrophotometer (Tecan Infinite).

2.5. Statistical analysis

The statistical analysis of all data was performed by the one-way analysis of variance (one-way ANOVA) using the statistical software, SPSS 13.0. All the data analyzed followed normal distributions and variances. The p values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Effects of TCCP on ROS production, apoptosis and antioxidant system

The mussels *M. galloprovincialis* were exposed to two concentrations of TCCP (10 and 100 nmol L\(^{-1}\)) for 42 days. The reactive oxygen species (ROS) and apoptosis in mussel hemocytes were measured by flow cytometry. Basically, both ROS and apoptosis levels were not significantly different between seawater control and solvent control groups. Therefore, only the mussel samples from solvent control group were used for comparisons in subsequent analysis.

As shown in Fig. 1, ROS productions were highly significantly (p < 0.01) elevated in both TCCP-treated mussel samples. In addition, the ROS level in the high dose (100 nmol L\(^{-1}\)) of TCCP treatment was significantly higher (p < 0.01) than that in the low dose (10 nmol L\(^{-1}\)) of TCCP treatment. Obviously, both TCCP exposures enhanced ROS production in mussel hemocytes. As it is known, excessive ROS production in mussel hemocytes. As it is known, excessive ROS production in mussel hemocytes.
TCPP treatments were indicated with asterisks (**p < 0.01). It was interestingly found that the expression level of MyD88 was significantly down-regulated. However, this gene expression was significantly up-regulated in the high dose (100 nmol L⁻¹) of TCPP treatment. It seemed that there was a phenomenon of low dose inhibition and high dose stimulation related to the effects of TCPP on immunity in mussel M. galloprovincialis. The genes include two lysozymes (G-type and C-type lysozymes), two caspases (caspase 8 and caspase 2), two antimicrobial peptides (mytimacin and BD6), myeloid differentiation factor 88 (MyD88), and peptidoglycan recognition receptors (PGRP: peptidoglycan recognition receptors; TLR2: toll-like receptor 2; LITAF: lipopolysaccharide-induced tumor necrosis factor-alpha factor; Jun-like: transcription factor effecting in MAPK pathway).

3.2. Effects of TCPP on the expressions of immunity related genes

The hemocytes in mussels are the main immune effector cells involved in multiple intracellular defense mechanisms, including phagocytosis, encapsulation, respiratory burst induction (e.g., generation of superoxide anions) and release of humoral factors (lysosomal enzymes, agglutinins or lectins, cytokine-like molecules, bioactive peptides and antimicrobial peptides) (Canesi et al., 2002; Venier et al., 2011; Gerdol and Venier, 2015). In this work, twelve representative immunity related genes were selected for gene expression quantification to elucidate the effects of TCPP on immunity in mussel M. galloprovincialis. The genes were classified as initiators or effectors of innate immunity against bacterial pathogens (Wang et al., 2013; Wang et al., 2012). As shown in Fig. 4, the expression levels of G-type and C-type lysozymes (GLYZ and CLYZ) in mussel hemocytes were down-regulated in both TCPP-exposed groups, without or with statistical significances. The down-regulated gene expression levels of GLYZ and CLYZ suggested that both TCPP exposures inhibited the innate immunity in mussel M. galloprovincialis.

Capsases constitute the core of the apoptotic machinery and are classified as initiators or effectors of apoptosis that is an essential biological process in the maintenance and development of homeostasis in immune system. Among the caspase family, caspase 8 is an initiator caspase playing critical roles in diverse apoptotic pathways (Romero et al., 2011). It was interestingly found that the expression level of caspase 8 in the low dose (10 nmol L⁻¹) of TCPP treatment was significantly down-regulated. However, this gene expression was significantly up-regulated in the high dose (100 nmol L⁻¹) of TCPP treatment. It seemed that there was a phenomenon of low dose inhibition and high dose stimulation related to caspase 8 expressions induced by TCPP exposure in mussel hemocytes. This phenomenon of low dose inhibition and high dose stimulation (or low dose stimulation and high dose inhibition) has been defined as hormesis, which can be frequently found in toxicology studies on the organic toxic chemicals (Vom Saal et al., 1997; Wetherill et al., 2002). Apparently, the significant oxidative stress was observed in the mussel samples from the low dose of TCPP treatment. It seemed that the increase of ROS produced by the low dose (10 nmol L⁻¹) of TCPP was not up to the threshold to induce oxidative stress in mussel hemocytes.

Table 1
The list of primers used for the determination of internal control and quantification of gene expressions by qPCR.

| Gene name | Accession No. | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|-----------|---------------|------------------------|------------------------|
| 18s rRNA  | L33452        | AGAAAAGGCTACACACATCC   | TGCCCTCTACATTGTCACTC   |
| GLYZ      | JQ863366      | GCACTCTATGACTACTGGG    | TGAAAACCGGACATTTGGAC   |
| GLYZ      | JQ244770      | ATCCAAAGGCTATGGTCCT    | TAACTGACAGCCGGGATACCA  |
| Caspase 2 | H0244499      | GATATAGCAAGGCTGGCAATG  | GACCTTACAGGCTACGGACATC |
| Caspase 8 | H024450       | CCAACCCATGAAACACCAGAC  | GTAATACCAACGCCCTATATCA |
| Mytimacin | FR873274      | CTCTGCAAATTCCTCACAT    | ATCCTTGCCCAGCAAGAGA   |
| BD6       | FR873266      | AGCATCCACAGGATTGTC     | TAGCCTACACATCTCTG     |
| MyD88    | RY12712       | GTGTAGGTGGTGGTGGTGGT   | TTTACGTGGTCTTCAGGAGGATA |
| Galactin  | KP125914      | GTGACCACCCACTGGAGA     | CCAGCTTACGGTCTGCA     |
| PGRP      | AJQ21541      | AGGGTGGTGGTGGTGGTGA    | CGACATGTCGGTGGTCCTG   |
| TLR2      | RY173687      | AAGGCCCTGGAATTACAGG    | TCTGCCGGTCTACCGAGCA   |
| LITAF     | KF110677      | TTTACACCATGAGACAGAATT  | GCCAAAATGATGCTTGTCT    |
| Jun-like  | AJQ21551      | AGCATTGCCAGATTGAGAA    | GCGAAAAGATCCTTGTTGCT    |

Abbreviations: GLYZ, G-type lysozyme; CLYZ, C-type lysozyme; BD6: big defensin 6; MyD88: myeloid differentiation factor 88; PGRP: peptidoglycan recognition receptors; TLR2: toll-like receptor 2; LITAF: lipopolysaccharide-induced tumor necrosis factor-alpha factor; Jun-like: transcription factor effecting in MAPK pathway.

Fig. 1. Reactive oxygen species (ROS) levels in the hemocytes of mussels M. galloprovincialis exposed to TCPP for 42 days. DCF: 2′,7′-dichlorofluorescin. Significant differences between control and each TCPP treatment and between TCPP treatments were indicated with asterisks (**p < 0.01).

Fig. 2. Apoptosis levels in the hemocytes of mussels M. galloprovincialis exposed to TCPP for 42 days. FITC: fluorescein isothiocyanate. Significant differences between control and each TCPP treatment and between TCPP treatments were indicated with asterisks (**p < 0.01).
apoptosis ratio in both TCPP treatments revealed the similar profiles compared to that of caspase 8 expression profile (Fig. 2). As an apoptotic initiator, the mRNA levels of caspase 8 can be increased in mussels M. galloprovincialis under stress conditions, such as heat, cold and toxic chemicals which can induce obvious apoptosis (Chiang et al., 2001). Clearly, the gene expression levels of caspase 8 confirmed that TCPP exposures induced hormesis and the high dose of TCPP exposure cause significant apoptosis in mussel hemocytes. Caspase 2 is another initiator caspase that may induce cytochrome-c release and formation of an active complex of cytochrome-c with other apoptotic proteases (Wang et al., 2008). However, the expression level of caspase 2 presented a contrary profile to that of caspase 8 in high dose of TCPP-exposed mussel hemocytes. It might suggest that the gene expression of caspase 2 was down-regulated to compensate for the up-regulation of caspase 8 to maintain the homeostasis of caspasers in immune system in mussel hemocytes from the high dose of TCPP treatment.

Big defensin (BD) and mytimacin are two antimicrobial peptides that are humoral components of the innate immunity, playing a fundamental role in the innate immunity in invertebrates lacking adaptive immunity, to prevent the invasion of pathogens (Gerdol et al., 2012). The significant (p < 0.01) down-regulation of BD6 suggested that the high dose of TCPP exposure inhibited the innate immunity in mussel M. galloprovincialis, as similarly shown by the expression profile of CLYZ. In TCPP-exposed mussel hemocytes, the expression profiles of mytimacin revealed a phenomenon of low dose inhibition and high dose stimulation consistent with that of caspase 8, which confirmed the hormesis effect of TCPP to mussel M. galloprovincialis. In addition, the contrary gene expression profiles of mytimacin also showed a compensation mechanism to maintain the homeostasis of BD6 in immune system in mussel hemocytes treated with TCPP. The transcription factor effecting
in MAPK pathway (Jun-like) is responsive to bacterial infections, which has been described in oyster Crassostreain dornongensis (Gerold and Venier, 2015). The Jun-like protein is also involved in immune and stress responses. In this study, the Jun-like protein was not significantly altered to TCP exposure in mussel hemocytes.

Toll-like receptors (TLRs) belong to an ancient family of pattern recognition receptors (PRRs) which play a crucial role in initiating and activating the innate immune system, while myeloid differentiation factor 88 (MyD88) is the universal and essential adapter molecule that participate in the signal transduction of TLR signalling pathways under bacterial stimulation (Toubiana et al., 2013). The gene expression levels of TLR and MyD88 were significantly up-regulated in M. galloprovincialis challenged with Vibrio anguillarum, Vibrio splendidus and Microccus luteus (Toubiana et al., 2013), which was also observed in the marine bivalve Cyclina sinensis challenged by V. anguillarum and M. luteus (Ren et al., 2016). Galectins belong to a class of soluble animal lectins and have been indicated as PRRs for bacteria in bivalves specifically bind β-galactoside sugars (Sato et al., 2009). Peptidoglycan recognition receptors (PGRPs) are also important PRRs and able to recognize bacteria by specifically binding peptidoglycan, the major component of bacterial cell walls (Gerold and Venier, 2015). Lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF) is a transcription factor responsible for lipopolysaccharide-induced transduction of tumor necrosis factor-alpha. LITAF is basically involved in immune response against viruses and gram-negative bacteria (Venier et al., 2011). In both TCP-exposed mussel hemocyte samples, the gene expression levels of TLR, galecin, PGRP and LITAF were significantly (p < 0.01) increased in a dose-dependent manner. However, MyD88 was only significantly (p < 0.01) overexpressed in the high dose (100 nmol L⁻¹) of TCP treatment. These findings demonstrated that TCP induced dose-dependent immune stress in mussels.

4. Conclusions

Organophosphate flame retardants (OPFRs) are emerging marine environmental contaminants. Among the OPFRs, the tris (1-chloro-2-propyl) phosphate (TCP) is one of the most frequently detected organophosphate esters in the aquatic environment of Austria. Sci. Total Environ. 332, 155–161. The effects of Silver Nanoparticles on Mytilus galloprovincialis: possible function diversification and adaptive evolution. Plos One 7, e45148.

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