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Exposure to 2,3,7,8-tetrachlorodibenzo-paradioxin (TCDD) hampers the host defense capability of a bivalve species, *Tegillarca granosa*

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ABSTRACT

Though increasing reports of deleterious impacts of dioxins and polychlorinated biphenyls (PCBs) on a variety of marine organisms have been described, their effects on the host defense capability of marine bivalve mollusks remain poorly understood. In the present study we used 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) as a representative of dioxins and PCBs to investigate its impacts on the host defense capability of the blood clam, *Tegillarca granosa*. After exposure of clams to a range (0, 0.01, 0.1, 1, and 10 μg/L) of TCDD for 96 h, the total count, cell type composition, and phagocytic rate of haemocytes were analyzed. In addition, alkaline phosphatase (ALP) activity, cell viability, and the extent of DNA damage of haemocytes were also investigated. Our results showed that exposure to relatively high TCDD concentrations led to significant reductions in the total count and phagocytic activity of haemocytes, which could be accounted by aggravated DNA damage and reduced cell viability. In addition, the percentage of red granulocyte was significantly decreased whereas that of basophil granulocyte was significantly increased upon high doses TCDD exposure (effective concentrations are 1 μg/L and 10 μg/L for red and basophil granulocytes, respectively). Moreover, clams exposed to TCDD had a significant higher activity of ALP, may also indicate an enhanced ability to eliminate pathogens through direct dephosphorylation process whereas a suppressed inflammatory response through indirect regulating of downstream molecular cascade reaction. These findings suggest that TCDD may hamper the host defense capability and therefore render bivalve mollusks more vulnerable to pathogen infections.

1. Introduction

Dioxins and polychlorinated biphenyls (PCBs), produced as unwanted byproducts of chemical manufacturing, waste incineration, and utility of fossil fuel, are considered to be one of the most hazardous man-made compounds [1,2]. Due to the intrinsic characteristics of high lipophilicity and low biodegradability, dioxins and PCBs are persistent in the marine environment and can be bioaccumulated in the food chains (up to pg/g in fish and shellfish), posing threats to a variety of marine species and seafood consumers [3–8]. Therefore in recent years, great concerns have been drawn from the public over the deleterious impacts of dioxin and PCB contaminations on marine organisms [2,3,5,7]. To date, a series of toxic effects, such as sex ratio bias [9–11], skeletal abnormality [12,13], and reproductive defects [14,15], exerted by dioxin and PCB exposures have been detected in both terrestrial and aquatic model species. While extensive efforts have been made to reveal dioxins and PCBs contaminations in bivalve species, especially in the edible groups, little is known about their toxicity on bivalve mollusks to date [16–18].

The 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic members of the family of dioxins and PCBs [7,19]. Due to its high toxicity and ubiquitous presence in a variety of environments including the ocean system, TCDD has been used as a representative to study the toxicological impacts of dioxins and PCBs on marine species [7]. In recent decades, several actions of TCDD toxicity have been reported in model animals such as rats [20–22]. Firstly, TCDD binds to the specific aryl hydrocarbon receptor (AhR), a highly conserved transcription factor involved in the expression of a wide variety of genes [20–22]. Secondly, it has been suggested that TCDD can provoke oxidative stress and subsequently lead to DNA damage, resulting in severe toxicity [23]. In addition, endocrine disruption and/or signal transduction alteration have also been reported [24,25]. However, the question on whether TCDD exert any toxicity, on bivalve mollusks through any of these pathways has yet to be explored.

In addition, though the toxicity of dioxins and PCBs such as TCDD on host defense capability has been well elaborated in terrestrial

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mammal species [10–12,15,26], data in marine bivalve mollusks is lacking. To the best of our knowledge while conducting our experiments, only one study has reported the toxicity of TCDD on host defense parameters of bivalve species thus far [27]. During an investigation on the synergic effects of TCDD and TiO_2 nanoparticle on Mediterranean mussel *Mytilus galloprovincialis*, it was revealed by chance that the phagocytic activity of haemocytes was dramatically hampered (~50% with respect to the control) by *in vitro* exposure to TCDD at a dosage of 2 μg/L for 30 or 60 min, suggesting a significant toxicity of TCDD to the host defense capability in bivalve species [27]. However, *in vivo* exposure assays (0.25 μg/L TCDD for 96 h) conducted in the same study failed to detect any significant alteration in the phagocytic activity of haemocytes [27]. Therefore, further studies are urgently needed to provide more confirmative conclusions. Furthermore, the physiological mechanism underlying the toxicity of TCDD on host defense, if any, requires elucidation in bivalve mollusks likewise.

The blood clam, *Tegillarca granosa*, is a traditional aquaculture bivalve species with a wide distribution in the Indo-Pacific region [28–30]. Inhabiting in the intertidal mudflats, where persistent organic pollutants such as TCDD are normally precipitated and concentrated [31,32], blood clams are highly likely to be challenged by environmental TCDD contamination. Since retaining a robust host defense capability is crucial for bivalve mollusks survival, additional toxicity on the host defense capability exerted by TCDD exposure, if any, may render blood clams more susceptible to pathogen infections and subsequently pose potential threats to the aquaculture industry.

Therefore, in order to improve our current understanding of the toxicity and the mechanism of effects of dioxins and PCBs on host defense capability of bivalve mollusks, the present study was conducted using TCDD as a representative to answer the following questions: (1) How and to what extent will host defense of blood clam, indicated by the total haemocyte counts (THC), cell type composition, and phagocytic activity of haemocytes, be affected by TCDD exposure? (2) Could the toxicity of TCDD on host defense capability of blood clam, if detected, be attributed to alterations in alkaline phosphatase (ALP) activity, DNA damage, and cell viability of haemolymph and/or haemocytes?

2. Materials and methods

2.1. Experimental animals

Adult *T. granosa* of similar size with shell heights at 18.73 ± 1.24 mm were collected in July 2017 from Qingjiang (28°28′ N and 121°11′ E), Wenzhou, Zhejiang province of China, where waterborne TCDD was found to be under the detection limit. Prior to experiment, the animals were acclimated for one week in a 1000 L plastic tank filled with 800 L sand filtered 24 h aerated seawater (obtained from the sampling site, temperature at 24.9 ± 0.2 °C, pH at 8.10 ± 0.01, and salinity at 21.38 ± 0.20‰). As described by Shi [28] and Su [29], blood clams were fed with microalgae *Platymonas subcordiformis* and the seawater in the tank was replaced once daily during the acclimation period.

2.2. Exposure of blood clams to TCDD

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, > 99% pure) was purchased from Jianglai Industrial Limited (Shanghai, China). According to preliminary study and previous reports [3,33], a series of exposure concentrations, 0 (control), 0.01, 0.1, 1, and 10 μg/L, were used in the present study. After one week acclimation, the blood calms (N = 75) were randomly assigned into 15 plastic containers (5 exposure concentrations × 3 replicates) with 1 L seawater. According to the method of Guerreiro [3], a stock solution of TCDD (1 g/L) was prepared by dissolving TCDD in dimethylsulfoxide (DMSO, Beyotime, China) and the desired exposure concentrations were achieved by adding corresponding volumes of stock solution into the seawater. Blood clams were exposed to experimental concentrations of TCDD for up to 96 h in this study. During the experiment, seawater was changed daily and the clams were fed with microalgae *P. subcordiformis* before water renewal. After 96 h TCDD exposure, three blood clams were randomly picked from each experimental group for each analysis.

2.3. Analysis of THC and cell type composition of haemocytes

Haemocyte samples were collected following the methods described previously [26,34]. Briefly, after rinsing with 0.1 M phosphate buffer saline (PBS, pH 7.4), 50 μL haemolymph was extracted from the cavity of individuals using a 1 mL syringe. The haemolymph was immediately transferred into a 1.5 mL eppendorf tube with 50 μL 2.5% glutaraldehyde, prechilled on ice. A volume of 900 μL PBS was then added to make up a final haemocyte suspension. The total haemocyte counts (THC, indicated by haemocytes count per mL) were determined with a Neubauer's haemocytometer (XB-K-25, Anxin Optical Instrument) under Nikon eclipse E600 microscopy at 200 × magnification [28–30].

Similarly, after mixing freshly extracted haemocytes with 2.5% glutaraldehyde at a volume ratio of 7:3 [28,30], the haemocyte suspensions were centrifuged at 4000 rpm for 4 min. Blood smears were made with blood precipitates. Once air dried, the smears were stained with Wright-Giemsa stain (G1020, Solarbio) for cell type composition analysis. The counts of various cell types were determined under a Nikon eclipse E600 light microscope at a magnification of 1000 × . According to previous studies [29], three major cell types of haemocytes, including red granulocyte, basophil granulocyte, and hyalinocyte, were identified and counted in the present study. At least 100 haemocyte cells were scored for each sample to ensure accuracy.

2.4. Phagocytosis assays

Following the methods described by Shi [28] and Su [29], Yeast (*Saccharomyces cerevisiae*, instant dry yeast, Angel Yeast) suspension containing (1.45 ± 0.05) × 10^8 yeast cells per ml was prepared by dissolving 7 mg yeast powder in 1 mL 0.1 M PBS. A volume of 100 μL haemolymph was exacted from each clam as described above and transferred into a 1.5 mL eppendorf tube with 100 μL Alsever's solution (ALS, Noble Ryder). After spinning at 1000 rpm for 15 s, supernatant was removed and yeast suspension was added at a yeast-haemocytes ratio of 10:1 [28,35]. The mixture was incubated at room temperature (25 °C) for 30 min and fixed with 100 μL 2.5% glutaraldehyde. Haemolymph smears were subsequently prepared and stained with Wright's stain. The phagocytic rate for each individual was determined microscopically under 1000 × magnification with a Nikon eclipse E600 light microscope. Three individuals from each experimental group and more than 100 haemocyte cells for each individual were scored.

2.5. Measurement of alkaline phosphatase (ALP) activity

ALP activity test was carried out using the alkaline phosphatase assay kit (Jiancheng, Nanjing, China) following manufacturer's instructions. Briefly, 50 μL haemocytes was extracted from the cavity of individuals using a 1 mL sterile syringe after 96 h post treatment. After the addition of 50 μL substrates and 50 μL buffer (supplied by the kit), mixture was incubated at 37 °C for 15 min followed by termination of the reaction via adding 150 μL chromogenic agent. Absorbance values were determined at the wavelength of 520 nm using a microplate reader (Thermo Scientific, THM#51119200) and ALP activities were calculated by putting the obtained absorption values into a standard curve. One unit of ALP enzymatic activity was defined as 1 mg of phenol liberated from the samples by ALP per 100 μL haemocytes [36].

2.6. Cell viability analysis

Following manufacturer's instructions cell viability was measured with MTT cell proliferation and cytotoxicity assay kit (Beyotime, Hangzhou, China). In brief, after seeding 100 μL haemocytes into a 96-well plate at a concentration of 2×10^3 cells/well, 10 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was added to each well followed by an incubation in dark at 37 °C for 4 h. Next, 100 μL formazan solvent was added and the plate was reincubated in dark at 37 °C till a complete dissolution of the crystal of formazan dye is achieved. Absorbance was determined at 570 nm using a microplate reader. Following the method described by Ong [37], relative percentages of cell viability were obtained by comparing against the blank control.

2.7. Evaluation of DNA damage through comet assays

Following the method of Gong [38], the comet assays were performed with an OxiSelectTM comet assay kit (Cell Biolabs, San Diego, CA, USA). Briefly, after extraction 15 μL of haemolymph were mixed with 75 μL of 0.5% low melting point agarose and then transferred onto the CometSlide™. Upon solidifying, the cell-agarose mixture was incubated in a freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 10% DMSO and 1% Triton X-100) in dark at 4 °C for 1 h, followed by 30 min incubation in alkaline solution at 4 °C to allow DNA unwinding. Electrophoresis was performed in alkaline solution at 30 V for 15 min. After two runs of wash (5 min each) with precooled deionized water at 4 °C and dehydration with 70% ethanol, slide was stained with Vista Green DNA dye. Fluorescence images were captured using an Olympus BH-2 fluorescence microscope (Olympus, Melville, NY) at the excitation and emission wavelengths of 520 and 590 nm, respectively. Following the method of Gong [38], the degree of DNA damage was analyzed with Photoshop CS6. For all the samples tested, at least 100 randomly selected cells were analyzed.

2.8. Statistical analysis

One-way ANOVAs followed by Tukey's post hoc tests were conducted to compare THC, phagocytic rate, cell type composition, ALP activity, cell viability, and the extent of DNA damage of haemocytes subjected to TCDD exposure at various experimental concentrations. For all analyses, Levene's and Shapiro-Wilk's tests were used to verify the homogeneity and normality of variance, respectively. In cases where assumptions were not satisfied by the raw data, i.e. the percentage data for phagocytic rate and cell type composition, data were arcsine square root transformed prior to the analysis following the method described by Brosnan [39]. All statistical analyses were performed using OriginPro 2017 and a p value less than 0.05 was accepted as significant difference.

2.9. Safety measurements

Due to high toxicity grade and carcinogenic risk, thorough safety measurements were adopted to minimize human and environmental exposure to TCDD during the experiment. Protective gloves, filter-type respirator, and goggles were adopted throughout the handling of TCDD. Previous studies [40,41] have shown that ultrasound rapidly removes TCDD in aqueous solution especially in the presence of Fe(III) under UV irradiation, which catalyzes the sonochemical degradation of TCDD. Therefore, to prevent undesirable TCDD release into the environment, all possible sources of TCDD contaminations in this study, including treatment seawater, containers, biological specimens, and protective wears, were treated with ultrasound (15 W, 20 kHz) with Fe $(NO_3)_3 \cdot 9H_2O$ and UV irradiation for 4 h.

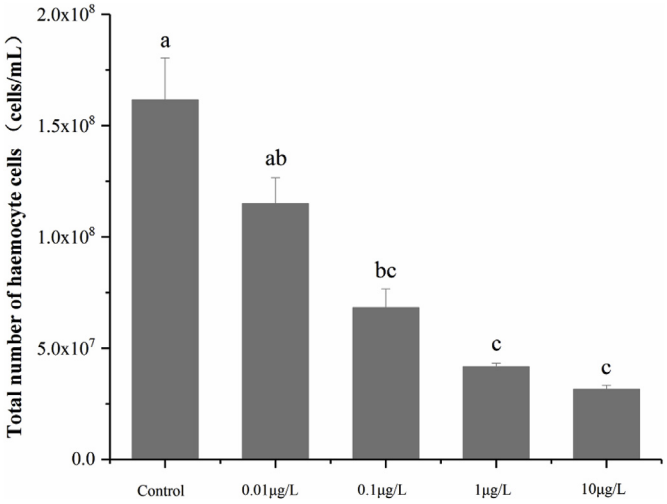

Fig. 1. The THCs (in terms of haemocytes count per mL) of *T. granosa* after 96 h exposure to 0 (control), 0.01, 0.1, 1, and 10 μg/L of TCDD. All data were presented as mean ± SEM and the mean values that do not share the same superscript were significantly different at $p < 0.05$.

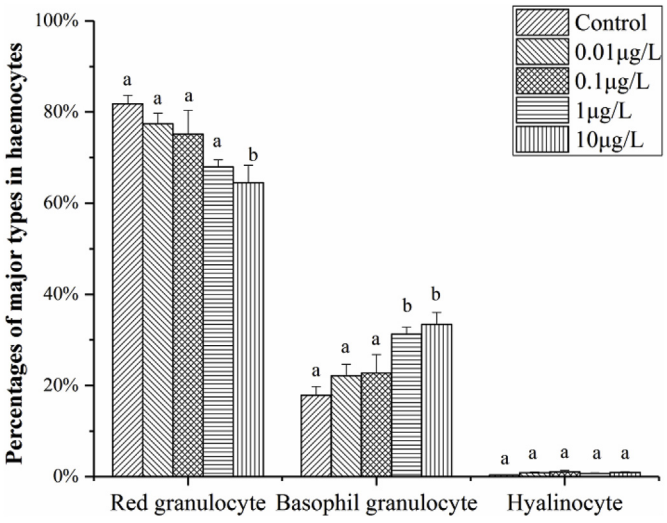

Fig. 2. The percentages of three major types of haemocytes (red granulocyte, basophil granulocyte, and hyalinocyte) of *T. granosa* after 96 h exposure to 0 (control), 0.01, 0.1, 1, and 10 μg/L of TCDD. All data were presented as mean ± SEM and the mean values that do not share the same superscript were significantly different at $p < 0.05$.

3. Results

3.1. The impacts of TCDD exposure on THC, phagocytic rate, and cell composition of haemocytes

The THC (Fig. 1), cell type composition (Fig. 2), and phagocytic rate (Fig. 3) of haemocytes were all significantly ($p < 0.05$) affected by TCDD at relatively high exposure concentrations. While the effect of the lowest dose (0.01 μg/L) of TCDD was not significant, the THC of clams decreased significantly to about only 42.27%, 25.77%, and 19.59% of that of the control when treated with 0.1, 1, and 10 μg/L TCDD, respectively (Fig. 1). In addition, the percentage of red granulocyte decreased whereas that of basophil granulocyte increased, both significantly, upon exposure to high doses of TCDD (10 μg/L for red granulocyte; 1 and 10 μg/L for basophil granulocyte) (Fig. 2). After a 96 h exposure to 10 μg/L TCDD, the percentages of red and basophil granulocyte were about 78.87% and 1.87 times of those of controls,

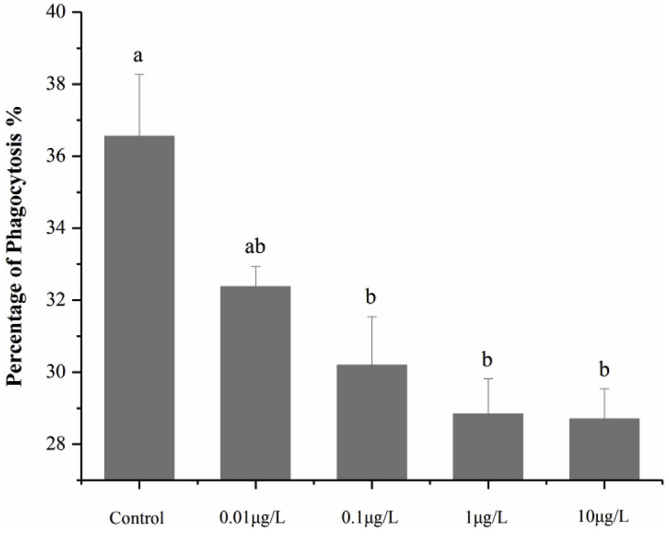

Fig. 3. The phagocytic rates of haemocytes of *T. granosa* after 96 h exposure to 0 (control), 0.01, 0.1, 1, and 10 μg/L of TCDD. All data were presented as mean ± SEM and the mean values that do not share the same superscript were significantly different at $p < 0.05$.

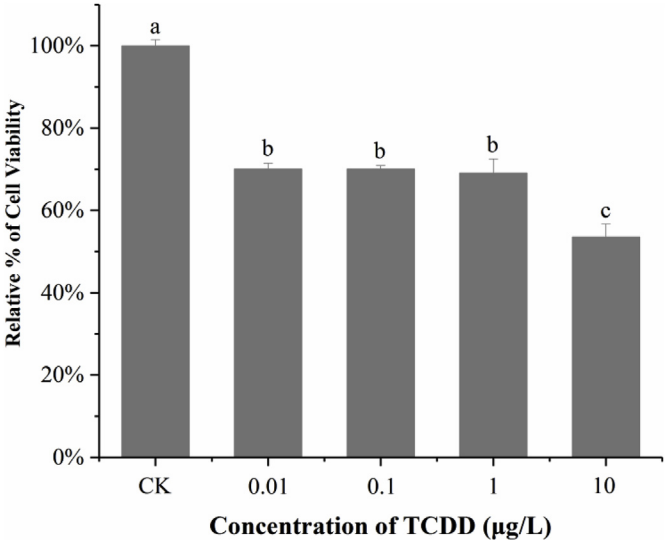

Fig. 5. The Relative cell viabilities of haemocytes after 96 h exposure to 0 (control), 0.01, 0.1, 1, and 10 μg/L of TCDD. All data were presented as mean ± SEM and the mean values that do not share the same superscript were significantly different at $p < 0.05$.

respectively (Fig. 2). Similarly, while unaffected at lowest dosage exposure (0.01 μg/L), the phagocytic activity of haemocytes were significantly hampered by 96 h TCDD exposure at higher dosages, at 82.61%, 78.91% and 78.53% of that of the control for 0.1, 1, and 10 μg/L TCDD exposure groups, respectively (Fig. 3). The data indicated a significant hampered host defense capability of blood calms upon exposure to TCDD.

3.2. Impacts of TCDD exposure on the ALP activity, cell viability, and DNA damage

In the present study, ALP activities were found to be significantly ($p < 0.05$) affected by TCDD exposure (Fig. 4). After 96 h exposure of blood clams to 0.01, 0.1, 1, and 10 μg/L TCDD, the ALP activities were found to be about 2.52–4.70 times higher than that of control (Fig. 4). In addition, TCDD exposure led to significant reductions in haemocytes viabilities, suffering a decline to about 70.08%, 70.11%, 69.09%, and 53.55% of that of control for 0.01, 0.1, 1, and 10 μg/L TCDD exposure groups, respectively (Fig. 5). In addition, the extent of DNA damage were significantly aggravated by exposure of individuals to high dose TCDD, which were about 7.00 and 8.45 times higher than that of control for 1 and 10 μg/L TCDD treatment groups, respectively (Fig. 6).

4. Discussion

With growing concerns over the deleterious impacts of dioxin and PCB contaminations on marine organisms in recent years, their toxicity on host defense capability of marine bivalve mollusks however remains poorly understood [27]. Our data showed that host defense responses, as indicated by THC, cell composition, and phagocytic rate of haemocytes, were significantly hampered by the exposure of blood clams to TCDD, a representative member of dioxins and PCBs. As compared to

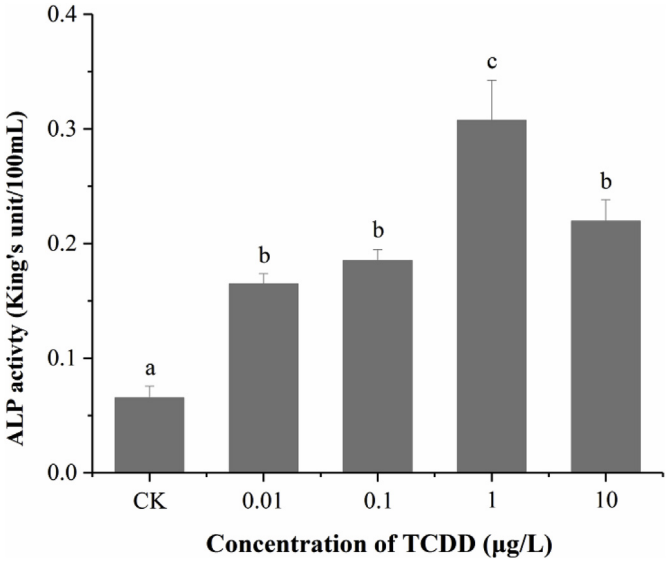

Fig. 4. The alkaline phosphatase (ALP) activities of haemocytes after 96 h exposure to 0 (control), 0.01, 0.1, 1, and 10 μg/L of TCDD. All data were presented as mean ± SEM and the mean values that do not share the same superscript were significantly different at $p < 0.05$.

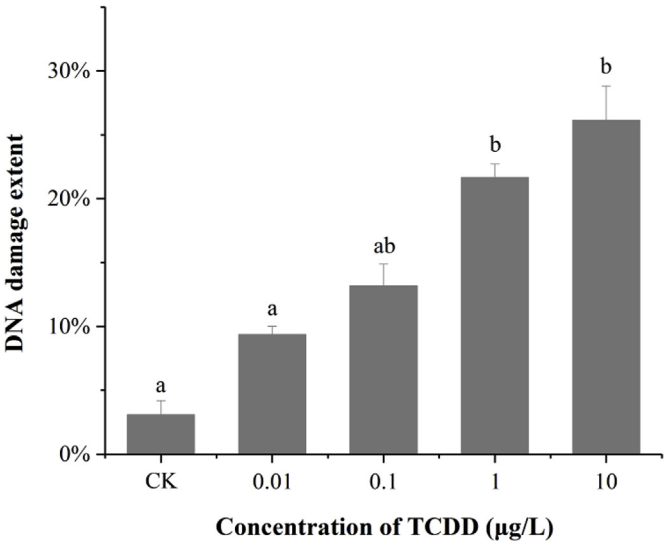

Fig. 6. The extents of DNA damage of haemocytes evaluated via comet assays after 96 h exposure to 0 (control), 0.01, 0.1, 1, and 10 μg/L of TCDD. All data were presented as mean ± SEM and the mean values that do not share the same superscript were significantly different at $p < 0.05$.

the controversial results previously reported between *in vitro* and *in vivo* phagocytosis assays in *M. galloprovincialis* using a fixed exposure concentration [27], evident toxicities of TCDD to blood clams on host defense capability were detected in our study in a dose dependent manner. Since phagocytic rate of haemocytes of blood clams was both significantly reduced by TCDD, albeit at different exposure concentrations (0.1 μg/L of the present study and 0.25 μg/L for that reported in *M. galloprovincialis*) for the same exposure duration (96 h), the apparent discrepancy in effective concentrations suggests a possible species specific tolerance level. On the other hand, the previous *in vivo* assays conducted in *M. galloprovincialis* missed detecting any alteration in phagocytosis might be attributed to the different sensitivity of *M. galloprovincialis* to TCDD combined with a lower exposure concentration tested.

In addition, regardless of the limited studies on the toxicity of dioxins and PCBs to bivalve mollusks on host defense capability and the types, doses, and durations of exposures varied among these investigations, similar deleterious impacts of other types of dioxins and PCBs on the defense responses were also detected in other marine bivalve species [36,42]. For example, exposure of scallop, *Chlamys farreri*, to organic pollutant Aroclor1254 (a highly chlorinated PCB mixture) at concentrations ranging from 0.05 to 50 μg/L also led to significant dose-dependent reductions in THC and phagocytosis and cell type composition alterations [42]. Similarly, evident toxicity of palmitoleic acid, an effective algicide, to the defense responses, indicated by reductions in THC, was revealed in the bay scallop, *Argopecten irradians* [36]. The consistency of the present study with those reported previously indicates the toxicity to marine bivalves on host defense capability could be a universal intrinsic characteristic of dioxins and PCBs.

Cell viability and DNA damage analyses conducted in the present study showed that TCDD exposure brought about significant reduction in cell viability and aggravation in DNA damage of the haemocytes, possibly contributing to the reductions in THC and phagocytosis observed. It is known that DNA damage triggers cell apoptosis [43]. Essentially, the aggravation in haemocyte DNA damage induced by TCDD exposure could lead to a decrease in the number of haemocytes through accelerating the process of apoptosis. In addition, since DNA integrity is crucial for the maintenance of normal cellular functions [3], a heightened DNA damage is postulated to play a role in the reduction in cell viability upon TCDD exposure. In this circumstance, it is not surprising to observe suppressed phagocytic rate in conjunction with less viable haemocytes after exposure of clams to TCDD. Causatives of DNA damage seem to be prevalent for different types of dioxins and PCBs [43–45], which offers an explanation to why toxicity on host defense responses could be a universal characteristic of various dioxins and PCBs.

In the present study, clams exposed to TCDD produced higher activities of ALP, a polyfunctional phosphomonoester hydrolase enzyme participating in the degradation of invading foreign material [36,42–44]. Similar alterations in ALP activity have been detected in bivalve species exposed to a wide variety of contaminations including the other types of dioxins and PCBs [41–43]. For instance, bay scallop, *A. irradians*, exposed to 80 mg/L palmitoleic acid for 3 h exerted significant higher ALP activity [36]. Given that ALP activity alterations are prevalent in animals under different kinds of stress [41–43], the increase in ALP activity upon TCDD exposure detected in the present study could be a common stress response. Since ALP plays important roles in host defense against pathogens [46], the alteration of ALP activity detected may consequently reflect host defense capability of blood clams against pathogen in both direct and indirect manners. On one hand, ALP can directly fight against pathogen microbial through dephosphorylating inflammatory microbial ligands like lipopolysaccharides, unmethylated cytosine-guanine dinucleotides, flagellin, and extracellular nucleotides [46,47]. On the other hand, it has been suggested that ALP could mediate inflammation via repression of the downstream Toll-like receptor (TLR)-4-dependent and MyD88-dependent inflammatory cascade [46,47]. Therefore, the higher level of ALP activity detected in TCDD exposure group may indicate an enhanced ability to eliminate pathogens through direct dephosphorylation process along with a suppressed inflammatory response through indirect regulation of cascading molecular reactions.

Encompassing a robust host defense capability is crucial for bivalve species survival in the complicated marine environments, where the animals are frequently challenged by various pathogens and multiple environmental contaminations [45,48,49]. Our findings revealed evident toxic effects of TCDD on the host defense capability of marine bivalve species, which may render bivalves more vulnerable to pathogen infections. However, since the presence of TCDD may affect the community composition and pathogenicity of pathogens as well as host-pathogen interactions, further investigation is needed to evaluate the potential impacts of TCDD contamination on disease outbreak in bivalve species.

Acknowledgments

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References

[1] P.S. Kulkarni, J.G. Crespo, C.A.M. Afonso, Dioxins sources and current remediation technologies - a review, Environ. Int. 34 (2008) 139–153.
[2] T.L. Wade, S.T. Sweet, J.L. Sericano, et al., Polychlorinated dibenzo-p-dioxins and dibenzofurans detected in bivalve samples from the NOAA National Status and Trends Program, Mar. Pollut. Bull. 81 (2014) 317–324.
[3] A.D.S. Guerreiro, R.C. Rola, M.T. Rovani, et al., Antifouling biocides: impairment of bivalve immune system by chlorothalonil, Aquat, Toxicology 189 (2017) 194–199.
[4] M. Nunes, P. Marchand, A. Vernisseau, et al., PCDD/Fs and dioxin-like PCBs in sediment and biota from the Mondego estuary (Portugal), Chemosphere 83 (2011) 1345–1352.
[5] J.L. Domingo, A. Bocio, Levels of PCDD/PCDFs and PCBs in edible marine species and human intake: a literature review, Environ. Int. 33 (2007) 397–405.
[6] L.C. Skinner, Distributions of polyhalogenated compounds in Hudson River (New York, USA) fish in relation to human uses along the river, Environ. Pollut. 159 (2011) 2565–2574.
[7] T.C. Della, M. Mariottini, M.L. Vannuccini, et al., Induction of CYP1A and ABC transporters in European sea bass (*Dicentrarchus labrax*) upon 2,3,7,8-TCDD waterborne exposure, Mar. Environ. Res. 99 (2014) 218–222.
[8] G. Charnley, J. Doull, Human exposure to dioxins from food, 1999–2002, Food Chem. Toxicol. 43 (2005) 671–679.
[9] T.C.K. Heiden, C.A. Struble, M.L. Rise, et al., Molecular targets of 2,3,7,8-tetrachlorodibenzo-dioxin (TCDD) within the zebrafish ovary: insights into TCDD-induced endocrine disruption and reproductive toxicity, Reprod. Toxicol. 25 (2008) 47–57.
[10] P. Mocarelli, P.M. Gerthoux, D.G. Patterson, et al., Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality, Environ. Health Perspect. 116 (2008) 70–77.
[11] M. Ikeda, M. Tamura, J. Yamashita, et al., Repeated in utero and lactational 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure affects male gonads in offspring, leading to sex ratio changes in F2 progeny, Toxicol. Appl. Pharmacol. 206 (2005) 351–355.
[12] S.J. Bursian, J. Kern, R.E. Remington, et al., Dietary exposure of mink (*Mustela vison*) to fish from the upper Hudson River, New York, USA: effects on organ mass and pathology, Environ. Toxicol. Chem. 32 (2013) 794–801.
[13] K.M. Xiong, R.E. Peterson, W. Heideman, AHR-Mediated Downregulation of sox9b causes jaw malformation in zebrafish embryos, Mol. Pharmacol. 74 (2008) 1544–1553.
[14] T.C. King Heiden, J. Spitsbergen, W. Heideman, et al., Persistent adverse effects on health and reproduction caused by exposure of zebrafish to 2,3,7,8-tetrachlorodibenzo-p-dioxin during early development and gonad differentiation, Toxicol. Sci. 109 (2009) 75–87.
[15] K. Yoshizawa, A.E. Brix, D.M. Sells, et al., Reproductive lesions in female Harlan Sprague-Dawley rats following two-year oral treatment with dioxin and dioxin-like compounds, Toxicol. Pathol. 37 (2009) 921–937.
[16] S.-K. Kim, J.R. Oh, W.J. Shim, et al., Geographical distribution and accumulation features of organochlorine residues in bivalves from coastal areas of South Kore, Mar. Pollut. Bull. 45 (2002) 268–279.
[17] M.G. Commendatore, M.A. Franco, C.P. Gomes, et al., Butyltins, polyaromatic hydrocarbons, organochlorine pesticides, and polychlorinated biphenyls in sediments and bivalve mollusks in a mid-latitude environment from the Patagonian coastal zone, Environ. Toxicol. Chem. 34 (2016) 2750–2763.

[18] S. Pizzini, C. Sbicego, F. Corami, et al., 3,3'-dichlorobiphenyl (non-Aroclor PCB-11) as a marker of non-legacy PCB contamination in marine species: comparison between Antarctic and Mediterranean bivalves, Chemosphere 175 (2017) 28–35.
[19] N.S. Fracchiolla, C. Annaloro, F. Guidotti, et al., 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) role in hematopoiesis and in hematologic diseases: a critical review, Toxicology 374 (2016) 60–68.
[20] L. Poellinger, Mechanistic aspects - the dioxin (aryl hydrocarbon) receptor, Food Addit. Contam. 17 (2000) 261–266.
[21] P.K. Mandal, Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology, J. Comp. Physiol. B 175 (2005) 221–230.
[22] J. Lindén, S. Lensu, J. Tuomisto, et al., Dioxins, the aryl hydrocarbon receptor and the central regulation of energy balance. A review, Front. Neuroeng. 31 (2010) 452–478.
[23] M. Viluksela, Y. Bager, J.T. Tuomisto, et al., Liver tumor-promoting activity of 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) in TCDD-sensitive and TCDD resistant rat strains, Cancer Res. 60 (2000) 6911–6920.
[24] Y.P. Dragan, D. Schrenk, Animal studies addressing the carcinogenicity of TCDD (or related compounds) with an emphasis on tumour promotion, Food Addit. Contam. 17 (2000) 289–302.
[25] S. Knerr, D. Schrenk, Carcinogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in experimental models, Mol. Nutr. Food Res. 50 (2006) 897–907.
[26] K. Bruner-Tran, T. Ding, R.L. Lister, et al., Paternal developmental TCDD exposure disrupts placental function in a subsequent adult pregnancy in a murine model, Fertil. Steril. 108 (2017) e105.
[27] L. Canesi, G. Frenzilli, T. Balbi, et al., Interactive effects of n-TiO_2, and 2,3,7,8-TCDD on the marine bivalve *Mytilus galloprovincialis*, Aquat. Toxicol. 153 (2014) 53–65.
[28] W. Shi, Y. Han, C. Guo, et al., Immunotoxicity of nanoparticle nTiO_2 to a commercial marine bivalve species, *Tegillarca granosa*, Fish Shellfish Immunol. 66 (2017) 300–306.
[29] W. Su, S. Zha, Y. Wang, et al., Benzo[a]pyrene exposure under future ocean acidification scenarios weakens the immune responses of blood clam, *Tegillarca granosa*, Fish, Shellfish Immun 63 (2017) 465–470.
[30] W. Su, J. Rong, S. Zha, et al., Ocean acidification affects the cytoskeleton, lysozymes, and nitric oxide of hemocytes: a possible explanation for the hampered phagocytosis in blood clams, *Tegillarca granosa*, Front. Physiol. 9 (2018) 619.
[31] V. Dethlefsen, Status report on aquatic pollution problems in Europe, Aquat. Toxicol. 11 (1988) 259–286 1988.
[32] C. Kim, I. Lee, D. Jung, et al., Reconnaissance of dioxin-like and estrogen-like toxicities in sediments of Taean, Korea-seven years after the Hebei Spirit oil spill, Chemosphere 168 (2017) 1203–1210.
[33] J.P. Giesy, P.D. Jones, K. Kannan, et al., Effects of chronic dietary exposure to environmentally relevant concentrations to 2,3,7,8-tetrachlorodibenzo-p-dioxin on survival, growth, reproduction and biochemical responses of female rainbow trout (*Oncorhynchus mykiss*), Aquat. Toxicol. 59 (2002) 35–53 2002.
[34] S. Liu, W. Shi, C. Guo, et al., Ocean acidification weakens the immune response of blood clam through hampering the NF-κB and toll-like receptor pathways, Fish Shellfish Immunol. 54 (2016) 322–327.
[35] Z. Zhu, L. Xu, X. Wu, et al., Morphological, structural characteristics and phagocytic and enzymatic activities of haemocytes in blood clam *Tegillarca granosa*, J. Fish. China 35 (2011) 1494–1504.
[36] C. Chi, S.S. Giri, J.W. Jun, et al., Immune response of the bay scallop, *Argopecten irradians*, after exposure to the algicide palmitoleic acid, Fish Shellfish Immunol. 57 (2016) 371–378.
[37] T.H.D. Ong, Y. Na, G.K. Meenashisundaram, et al., Insight into cytotoxicity of Mg nanocomposites using MTT assay technique, Mater. Sci. Eng. C 78 (2017) 647–652.
[38] H. Gong, Y. Zhang, K. Jiang, et al., p73 coordinates with Δ133p53 to promote DNA double-strand break repair, Cell Death, Differentiation 25 (2018) 1063–1079.
[39] J.T. Brosnan, P.E. Mccullough, G.K. Breeden, Smooth crabgrass control with indaziflam at various spring timings, Weed Technol. 25 (2016) 363–366.
[40] X.C. Zhou, L. Cao, High sensitivity microgravimetric biosensor for qualitative and quantitative diagnostic detection of polychlorinated dibenzo-p-dioxins, Royal. Soc. Chem. 126 (2001) 71–78.
[41] H. Katsumata, S. Kaneco, T. Suzuki, et al., Sonochemical degradation of 2,3,7,8-tetrachlorod -ibenzo-p-dioxins in aqueous solution with Fe(III)/UV system, Chemosphere 69 (2007) 1261–1266.
[42] Z.J. Wang, W. Ma, J.M. Yang, et al., Effects of Cu^{2+},on alkaline phosphatase from *Macrobrachium rosenbergii*, Int. J. Biol. Macromol. 117 (2018) 116–123.
[43] E.A. Abdallah, R.N. Said, D.S. Mosallam, et al., Serial serum alkaline phosphatase as an early biomarker for osteopenia of prematurity, Medicine 96 (2016) e4837.
[44] A.H. D'costa, S. S.K, P.K. M.K, et al., The Backwater Clam (*Meretrix casta*) as a bioindicator species for monitoring the pollution of an estuarine environment by genotoxic agents, Mutat. Res. Gen. Tox. En. 825 (2018) 8–14.
[45] M.L. Hannam, S.D. Bamber, J.A. Moody, et al., Immune function in the Arctic Scallop, *Chlamys islandica*, following dispersed oil exposure, Aquat. Toxicol. 92 (2009) 187–194.
[46] S.S. Ghosh, T.W. Gehr, S. Ghosh, Curcumin and chronic kidney disease (CKD): major mode of action through stimulating endogenous intestinal alkaline phosphatase, Molecules 19 (2014) 20139–20156.
[47] J. Bilski, A. Mazur-Bialy, D. Wojcik, et al., The role of intestinal alkaline phosphatase in inflammatory disorders of gastrointestinal tract, Mediat. Inflamm. 6 (2017) 9074601.
[48] E.W.K. Ching, W.H.L. Siu, P.K.S. Lam, DNA adduct formation and DNA strand breaks in green-lipped Mussels (*Perna viridis*) exposed to Benzo[α]pyrene: dose- and time-dependent relationships, Mar. Pollut. Bull. 42 (2001) 603–610.
[49] S. Zha, S. Liu, W. Su, et al., Laboratory simulation reveals significant impacts of ocean acidification on microbial community composition and host-pathogen interactions between the blood clam and *Vibrio harveyi*, Fish Shellfish Immunol. 71 (2017) 393–398.