Full length article

Molecular cloning, characterization, and the response of Cu/Zn superoxide dismutase and catalase to PBDE-47 and -209 from the freshwater bivalve *Anodonta woodiana*

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ABSTRACT

Polybrominated diphenyl ethers-47 (PBDE-47) and -209 are significant components of total PBDEs in water and can catalyze the production of reactive oxygen species (ROS) in the organisms. Anti-oxidant enzymes play an important role in scavenging the high level of ROS. In the current study, two full-length cDNAs of Cu/Zn superoxide dismutase (CuZnSODs) and catalase (CAT) were isolated from freshwater bivalve *Anodonta woodiana* by rapid amplification of cDNA ends approach and respectively named as AwSOD and AwCAT. The nucleotide sequence of AwSOD cDNA had an open reading frame (ORF) of 465 bp encoding a polypeptide of 155 amino acids in which signature 1 GKHGFHVHEFGDNT and signature 2 GNAGARSACGVI of SODs were observed. Deduced amino acid sequence of AwSOD showed a significant similarity with that of CuZnSODs. AwCAT had an ORF 1536 bp encoding a polypeptide of 512 amino acids which contains a conserved catalytic site motif, and a proximal heme-ligand signature motif of CATs. The time-course expressions of AwSOD and AwCAT in hepatopancreas were measured by quantitative real-time PCR. Expressions of AwSOD and AwCAT showed a significant up-regulation in groups at a low concentration treatment of PBDE-47, a biphasic pattern in groups with a high concentration treatment. Administration of PBDE-209 could result in an up-regulation of AwSOD and AwCAT expressions with time- and dose-dependent matter. These results indicate that up-regulations of AwSOD and AwCAT expression of hepatopancreas of freshwater bivalve *A. woodiana* contribute to eliminate oxidative stress derived from PBDE-47 and -209 treated.

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1. Introduction

Polybrominated diphenyl ethers (PBDEs) are currently important components in flame retardants and other industrial processes, and recognized as ubiquitous environmental contaminants [1,2]. They are widely appeared in commercial products used in daily life, such as electronic equipment, plastics, textiles and building materials [3,4]. Concurrent with their increased application, PBDEs have become a common freshwater organic pollutant of

increasing environmental concern due to their persistence and high bio-accumulative capacity [5]. Generally, these compounds are not naturally degraded by microbes in environment, easily bio-accumulate in fatty tissues and bio-magnify throughout food chains [6]. But, excess PBDEs deposited in organism could catalyze the production of reactive oxygen species (ROS) [7]. Several lines of evidence pointed that the mitochondria is the main site of PBDEs acted on [8,9]. PBDEs have been shown to inhibit mitochondrial respiration. Mitochondria are a major contributor of cellular ROS; ROS produced in the mitochondria can also target the electron transport chain (e.g. complex I), resulting in a cycle generating more ROS, followed by ATP depletion and ultimately cell death [10]. In addition, higher ROS concentrations could bring about the risk of DNA damage, lipid peroxidation and protein carbonylation.

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Therefore, oxidative stress is acknowledged as the most important mechanisms for pollutant-induced toxicity in organisms [11,12]. Among of these PBDEs, PBDE-47 and -209 have received great attention due to they are significant components of total PBDEs in air, water and sediment [7–9,13].

In recent years, increased release of PBDEs has caused a seriously effect on freshwater aquatic organism in many countries [14,15]. Bivalve molluscs are ubiquitous, sedentary filter feeders, which can bio-accumulate pollutants to a high degree [16,17]. The Chinese pond mussel (the Eastern Asiatic freshwater clam or swan-mussel), bivalve *Anodonta woodiana* functions as a main criteria required for a bio-indicator organism [18,19]. Earlier studies have revealed the ability of *Sinanodonta woodiana* to accumulate trace elements and pesticides [20,21], as well as its potential to detect genotoxicity [22]. In addition, these mussels have important ecosystem functions such as particle filtration and processing, nutrient release, and sediment mixing [23]. However, it has indicated that freshwater mussels have been considered as the most critically endangered group in the worldwide [15,16,24]. In North America alone, about 36 species (10%) are already presumed extinct, and three of the eight unionoid species of the North and Central European region are currently listed in the IUCN Red List of Threatened Species (IUCN 2011) [24]. Considering here, their decline of population can profoundly affect aquatic ecosystem [23]. Better understanding of defense ability of freshwater bivalve is a prerequisite for developing and carrying out measures for these animals' conservation.

Strong scavenging ability of excessive ROS is an essential approach for organism survival. This event is performed by anti-oxidant enzymes system including superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase, glutathione S-transferase and metallothionein. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide and divalent oxygen, then CAT catalyze the reduction of hydrogen peroxide [25,26]. SOD and CAT have been considered as biomarkers of exposure for environmental monitoring programs. An induction of SOD activity was observed in *Mytilus galloprovincialis* after urban pollution [27,28]. Increasing activity of CAT was demonstrated in response to exposure to organochlorines in *Perna viridis* and *Ruditapes philippinarum* [29,30].

Considering of bivalve *A. woodiana* as a perfect sentinel species to study the quality of freshwater environment, it is great interest to explore effect of PBDEs on the *A. woodiana*. Thus, in the current study, complete sequences of SOD and CAT were cloned and respectively named AwSOD and AwCAT, and their temporal expressions derived from PBDE-47 and PBDE-209 exposure were determined by quantitative real-time PCR.

2. Materials and methods

2.1. Ethics statement

All handling methods of mussel were conducted in accordance with the guidelines on the care and use of animals for scientific purposes set up by the Institutional Animal Care and Use Committee of Nanyang Medicine College, Nanyang, China.

2.2. Materials

Approximately 1-year-old of *A. woodiana* (shell length, 6.5 ± 0.5 cm) were obtained from the Baihe River of Nanyang, Henan Province, China. Prior to experiment, animals were maintained in a recirculation system containing filtered freshwater at 24 °C for 2 weeks in laboratory. PBDEs-47 and -209 were obtained from AccuStandard (New Haven, CT) and stock solutions were

prepared by dissolution in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO). The 48 h acute toxicity of selected PBDEs-47 and -209 to *A. Woodiana* was carried out in rectangular plastic boxes (40 cm × 25 cm; 10 cm height) containing 15 L artificial pond water and the concentration of DMSO in the water was not excess 0.1%. Clams *A. Woodiana* were randomly grouped into PBDEs treated groups in which animals were respectively administrated with 5 test concentrations of PPBDE-47 and -209, and control group treated with similar volume DMSO. Concentrations range for viability studies were as follows: 6.25 µg/L, 12.5 µg/L, 25 µg/L, 50 µg/L, 100 µg/L for PPBDE-47 as well as 10 µg/L, 20 µg/L, 40 µg/L, 80 µg/L, 160 µg/L for PPBDE-209. Each group has a triplicates per 10 clams and those were not fed during the study. Survival was assessed at 48 h and median lethal concentration (LC50) value calculated using the log-probit method.

According to above mentioned way of animal treatment, 330 clams were selected to determine the expressions of AwSOD and AwCAT. Various of tissues including foot, gill, hepatopancreas, adductor muscle, heart and mantle were collected at 0, 6, 12, 24 and 48 h, then immediately frozen in liquid nitrogen and stored at –80 °C until total RNA extraction.

2.3. Total RNA isolation and reverse transcription

Total RNA was extracted using TRIzol (Invitrogen Life Technologies, USA) according to the manufacturer's protocol. Quality of RNA was monitored by 1.2% agarose gel electrophoresis. First-strand cDNA was synthesized using M-MLV First-Strand cDNA synthesis Kit (Takara, China) according to the manufacturer's instructions and used as the template for PCR reaction.

2.4. Cloning of AwSOD and AwCAT cDNA

AwSOD cDNA fragment was amplified using two degenerate primers AwSOD1 and AwSOD2, and AwCAT using AwCAT1 and AwCAT2 (Table 1), which were respectively designed according to conserved domains of Cu/Zn SOD and CAT of other species including bivalve, gastropod, insect, crustacean and vertebrate. PCR products were subcloned into the pMDT-19 (Takara, China), sequenced from both directions (Invitrogen Life Technologies, China) and identified SOD and CAT partial cDNA sequences. Highly

Table 1
Sequences of PCR primers.

Primer	Sequence (5'–3')
AwSOD1	TGANTGAGANAGGGGAGANAGGT
AwSOD2	CAATAGNAGGCNACNAGAGG
AwCAT1	AGAGAGNCCGTGTTNCTGCCA
AwCAT2	CAAACNCCTTCNATCTCNTTCTGC
5'Race Innerprimer	CATGGCTACATGCTGACAGCCTA
5'Race Outerprimer	CGCGGATCCACAGCCTACTGATGATCAGTCCGATG
AwSOD-5-1	AAGCGGTGCGGGTCAAAG
AwSOD-5-2	TGCAGTTCCTGGGGCCA
AwCAT-5-1	CGTTGAAGTATGTGCGACTGG
AwCAT-5-2	CGCATCGTCTCCAGAACATC
3'Race Outerprimer	TACCGTCTGTTCCACTAGTGATTT
3'Race Innerprimer	CGCGGATCCTCCACTAGTGATTTCACTATAGG
AwSOD-3-1	GGAGAAGTTGTCTTCATCAGC
AwSOD-3-2	TGGTTGACGCTCTTCTCCA
AwCAT-3-1	TGCCAGGCGTTCCTCAAACG
AwCAT-3-2	AGCATAGTTTCTCCCTTAC
AwSOD-F	GCCCACTTCAACCTAGCAAG
AwSOD60-R	CCTGCCAAGGTCATCTTCATCC
AwCAT-F	CCAACTACTTCCCCAACAGTTCC
AwCAT-R	AGCCTCTGTCTGCTTCAGGGT
β-F	CATCCCTGCTCTCCAATATG
β-R	CTGGAAGGTAGAGAGAGAAGCCAAG

GACTACAGCAGCGTCATGATAGCTACGGTCATTCCACTCTTTTAAATAACCGATAACGC	60
AAGTTAGACCTGCATAAATCACC ATG TCCATTAAGGCTGTTTGGCTACTGAGGGGTGACA	120
M S I K A V C V L R G D	12
GTGAAGTTAAAGGAAGTGTCAAGTTTTACAAGAGGGAAGTGGTGCAGTGAACATAACTG	180
S E V K G T V K F L Q E G S G A V N I T	32
GTGAGATAACTGGCCTGGCTGCAGGAAAGCATGGATTCCATGTCCATGAATTTGGAGATA	240
G E I T G L A A G K H G F <u>H</u> V <u>H</u> E F G D	52
ACACTAATGGCTGTACTAGTGTGGGGCCCACTTCAACCCTAGCAAAGCAGGAACATGCAG	300
<u>N</u> <u>T</u> N G C T S A G A <u>H</u> F N P S K Q E <u>H</u> A	72
GACCCGAAGATGCCTCCAGACATGCTGGTGACCTGGGTAATGTCGTAGCTGGAGATGATG	360
G P E D A S R <u>H</u> A G <u>D</u> L G N V V A G D D	92
GAGTGGCCACGTCAACATCAAGGACAGCGTGATCTCACTTACAGGACCAAATTCATCA	420
G V A H V N I K D S V I S L T G P N S I	112
TTGGCAGAACCATGGTGATTCATGCGGATGAAGATGACCTTGGCAGGGGTGGACATGAGT	480
I G R T M V I <u>H</u> A D E D D L G R G G H E	132
TGAGCAAGACAAGTGGCAATGCTGGTGCACGTTCCGCATGTGGTGTATTGGAATTCGA	540
L S K T T <u>G</u> N A G A R S A C G V I G I S	152
AATTGGATT GA GTGAATCATTGCTTGTGATACTCTTTATAATGTTCTGTTGTAATTTG	600
K L D *	155
CAGAAATTATGTTCTGATTTATCCTGTTGCTTACAAAATAGTTTTTAGAATATTTTTTTT	660
TTACATTTTATTGAGGAAGAGAAGAACTAAGAATGATTTTTGTTGTCTTTTGTATGATTTTCA	720
TTTATTCAAGGCTAACATTTTCAGTATTCTTTCGAAAACAAATGCCTAGTGTAACCCAG	780
AGTTTGTATAGGAAGCAAGCTGTGAACAGTGAATATACTTGAGTCCTTAGTATGTGTTA	840
CGGCCAAGATGTAGTGGCAGATTAAGCAATAACAAATTGTAATGTAAATATAAAC	900
AGTTTGGTAATTTTGTCTATTTAAGGAGTTATGAAAAAAAAAAAAAAAAA	949

Fig. 1. Nucleotide and the deduced amino acid sequences of AwSOD. The start and stop codons are indicated in bold. Putative polyadenylation signal "AATAAA" is showed with wavy line. Two predicted CuZnSOD family signatures are marked with underline. The amino acids required for copper (His-46, -48, -64, and -120) and zinc (His-64, -72 and -81 and Asp-84) binding are showed with shadow. Two cysteines (Cys-57 and Cys-146) formed a disulphide bond are marked with boxe.

stringent primers (Table 1) designed from the partial cDNA sequences were used to characterize the 5' and 3' regions of the AwSOD and AwCAT cDNAs by rapid amplification of cDNA ends (RACE) approaches (Takara, China) according to the manufacturer's protocol. 5' Raceouter primer and AwSOD-5-1 (Table 1) were used for the first-round PCR of AwSOD 5' RACE, 5' Raceouter primer and AwCAT-5-1 for AwCAT. 3' Raceouter primer and AwSOD-3-1 (Table 1) were used for the first-round PCR of AwSOD 3' RACE, 3' Raceouter primer and AwCAT-3-1 for AwCAT. Subsequently, the 5' first-round PCR product of AwSOD was used as the template to perform the 5' nested PCR using 5' Raceinner primer included in the kit and AwSOD-5-2, 3' first-round PCR products for 3' nested PCR using 3' Raceinner primer and AwSOD-3-2 (Table 1). Like mentioned above, Raceinner primers (5'iner, 3'iner) and gene specific primers AwCAT-5-2, AwCAT-3-2 were used for AwCAT nest PCR. The 5' RACE and 3' RACE nest PCR products were cloned and five clones were sequenced using the method described above.

2.5. Sequence and phylogenetic analysis

AwSOD and AwCAT sequences were analyzed and compared using the BLAST program with a GenBank database search ([www](http://www.ncbi.nlm.nih.gov/blast)).

[ncbi.nlm.nih.gov/blast](http://www.ncbi.nlm.nih.gov/blast)). Multiple sequence alignments of AwSOD and AwCAT gene were performed using the DANMEN analysis program. The signal peptide was predicted by signal program (<http://www.cbs.dtu.dk/services/SignalP>). The protein domain was predicted with the Simple Modular Architecture Research Tool (<http://smart.embl-heidelberg.de/>). Secondary structure and 3-D structure of prediction of AwSOD and AwCAT were performed by Swiss-model (<http://swissmodel.expasy.org/>). Phylogenetic trees constructed from the alignment were generated by the Neighbor-joining method using MEGA5.0 software. Reliability of trees obtained was assessed by bootstrapping, using 1000 bootstrap replications.

2.6. Quantification of AwSOD and AwCAT expression by real-time PCR

To determine the levels of AwSOD and AwCAT transcript derived from hepatopancreas and other tissues, real-time quantitative PCR was performed following the manufacture instruction of SYBR Premix Ex TaqTM (TaKaRa, China). AwSOD and AwCAT primers as well as β -actin primers (Table 1) were designed based on isolated sequences of *A. woodiana*. PCR was performed using an ABI 7500

<i>Anodonta woodiana</i>	MSIKAVCVLRGDSEV—KGTVKFLQEGSGA—VNITGEITGLAAGK GHVHEFGDNT <u>NGC</u>	57
<i>Aplysia californica</i>	—MVKAVCVLAAGSSTSITGTITFTQEGPADSTIVTGEVKGLAPGK GHVHEFGDNT <u>NGC</u>	59
<i>Danio rerio</i>	MVNKAVCVLKGTEV—TGTVYFNQEGEKKPVKVTGEITGLTPGK GHVHEFGDNT <u>NGC</u>	58
<i>Xenopus laevis</i>	—MVKAVCVLAGSGDV—KGVVHFEQDE—GAVSVEGKIEGLTDGL GHVHEFGDNT <u>NGC</u>	57
<i>Mus musculus</i>	MAMKAVCVLKGDPV—QGTIHFEQKASGEPVVLSGQITGLTEG GHVHEFGDNT <u>QGC</u>	58
<i>Homo sapiens</i>	MATKAVCVLKGDPV—QGIINFEQKESNGPVKVGSIKGLTEGL GHVHEFGDNT <u>AGC</u>	58
<i>Anodonta woodiana</i>	TSAGAHFNPSKQEHAGPEDASRHAGDLGNVVGDDGVAHVNIKDSVISLTGPN SIIGRTM	117
<i>Aplysia californica</i>	MSAGGHFNPLGATHGGPDDAVRHAGDLGNIIAGDDGVAKVEIKDPQVPLIGENSIVGRSL	119
<i>Danio rerio</i>	ISAGPHFNPHDKTHGGPTDSVRHVGLGNVTADASGAKIEIEDAMTL SGQHSIIIGRTM	118
<i>Xenopus laevis</i>	MSAGSHFNPNKNGHAGPDTDRHVGLGNVTAE G—GVAQFKITDSLISLKGPNSIIGRTA	117
<i>Mus musculus</i>	TSAGPHFNPSKKGPPADEERHVGLGNVTAGKDGVANV SIIEHRVISLSGEHSIIIGRTM	118
<i>Homo sapiens</i>	TSAGPHFNPLSRKHGGPKDEERHVGLGNVTADKDGVDV SIIESVISLSGDHCIIGRTL	118
<i>Anodonta woodiana</i>	VIHADEDDLGRGGHEL SKTGNAGARSACGVI ISKLD	155
<i>Aplysia californica</i>	VVHEKEDDLGKGGNEESL KTGNAGPRVACGVI GITK—	155
<i>Danio rerio</i>	VIHEKEDDLGKGGNEESL KTGNAGGRLACGVI GITQ—	151
<i>Xenopus laevis</i>	VVHEKADDLGKGGN ESLKTGNAGGRLACGVI GYSF—	151
<i>Mus musculus</i>	VVHEKQDDLKGGNEES TKTGNAGSRLACGVI GIAQ—	151
<i>Homo sapiens</i>	VVHEKADDLGKGGNEES TKTGNAGSRLACGVI GIAQ—	151

Fig. 2. The nucleotide acid sequence and the deduced amino acid sequence of AwCAT. The start codon (ATG) and stop codon (TAA) are indicated in bold. Putative polyadenylation signal "AATAAA" is showed with wavy line. The highly conserved catalytic site motif (FDRERIPERVVHAKGAGA) and proximal heme-ligand signature motif (RLFSYSDTH) are underlined. The potential N-glycosylation residues are marked with shadow.

Real-Time Detection System (Applied Biosystems, USA) according to instruction. Based on standard curve constructed, expression levels of AwSOD and AwCAT were calculated by $2^{-\Delta\Delta C_t}$, and the data obtained were subjected to the statistical analysis followed by an unpaired sample *t*-test. Significant difference was accepted at $P < 0.05$.

3. Results

3.1. Characterization of AwSOD and AwCAT

A 949 bp nucleotide sequence representing the cDNA sequence of AwSOD was obtained by RACE, and deposited in GenBank under accession number KU363382. The full-length cDNA consisted of a 5' untranslated region (UTR) of 83 bp, a 3' UTR of 401 bp and an open reading frame (ORF) of 465 bp encoding a polypeptide of 155 amino acids with an estimated molecular mass of 15.78 kDa and theoretical isoelectric point of 6.22 (Fig. 1). The termination signal (AATAAA) was detected at the positions 891–896 in the 3' UTR. Multiple alignment of the deduced amino acid sequence (Fig. 1) with other closely related cytoplasmic Cu/Zn SOD sequences showed that three cysteines (Cys7, Cys 57 and Cys 146) were present in the mature AwSOD. Cys 57 and Cys 146 are conserved in all Cu/Zn SODs and it is believed that those form an intramolecular disulfide bond. Conserved amino acids required for binding of copper (His-46, -48, -64, and -120) and zinc (His-64, -72 and -81 and Asp-84) were also observed in AwSOD (Fig. 2). Two highly conserved sequences of Cu/Zn SOD family were found in the deduced amino acid sequence of AwSOD, signature 1 was GKHGFHVHEFGDNT and signature 2 was GNAGARSACGVI. BLAST analysis showed that the deduced amino acid sequence of AwSOD has high identity with the Cu/Zn SODs, 97.42% identity to *Cristaria plicata*, 61.39% to *Aplysia californica*, 64.10% to *Danio rerio* and 62.18% with *Homo sapiens*.

A 1784 bp cDNA sequence of AwCAT was obtained by RACE, and deposited in GenBank under accession number KU363383. The full-length cDNA consisted of a 5' untranslated region (UTR) of 14 bp, a

3' UTR of 234 bp and an open reading frame (ORF) of 1536 bp encoding a polypeptide of 512 amino acids with an estimated molecular mass of 58.05 kDa and theoretical isoelectric point of 7.23 (Fig. 3). The termination signal (AATAAA) was detected at the positions 1743–1748 in the 3' UTR. CAT highly conserved sequences were also identified in AwCAT by multiple alignments. His72, Asn145 and Tyr355 in CATs were predicated to be the catalytic amino acids which were conserved in AwCAT (Fig. 3). Two CAT family signature sequences were found in the deduced amino acid sequence of AwCAT, a highly conserved catalytic site motif (61FDRERIPERVVHAKGAGA77) and a proximal heme-ligand signature motif (351RLFSYSDTH358) (Fig. 4). In addition, NFS (residues 436–438) was predicted to be the glycosylation sites. Amino acid sequence alignment revealed that AwCAT contained putative residues involved in NADPH-binding (N145, H191, F195, S198, R200, Y212, K234, W300, Q302, Y355) and heme binding (R69, H72, R109, N145, F150, R351, Y355, R362) (Fig. 4). BLAST analysis showed that the deduced amino acid sequence of AwCAT has high identity with the CATs, 91.99% identity to *C. plicata*, 73.88% to *A. californica*, 66.09% to *Bombyx mori*, 65.21% to *D. rerio*, 66.98% to *Rattus norvegicus* and 66.98% to *H. sapiens*.

Predicted secondary structure of AwSOD showed a high similarity with that of Cu/Zn SOD of mollusc *Alvinella pompejana*, had 9 β -sheets and 2 α -helixes and a disulphide bond formed by Cys 57 and Cys 146 (Fig. 5A). AwCAT showed a high similarity with CAT of domestic cow *Bos taurus*, has 12 β -sheets and 23 α -helixes (Fig. 5B). The 3-D structure alignments of them (Fig. 6) respectively showed a high-degree similarity with that of Cu/Zn SODs and that of CATs.

3.2. Phylogenetic analysis

To examine the relationships between AwSOD and various Cu/Zn SODs as well as AwCAT and CATs, phylogenetic trees were generated by the MEGA 5.0 neighbor joining method using different corresponding family members selected from vertebrate and invertebrate species respectively including of *H. sapiens*, *Mus musculus*, frog *Xenopus laevis*, zebra fish *D. rerio*, shrimp *Litopenaeus*

GAAAACCATAGCAATGGCTAACAGAGACAAGGCATCAAAACAGCTGAATGACTTCAAGAAAGCTCAAAACCAACCGATGTGCTGACTA	90
M A N R D K A S N Q L N D F K K A Q T K P D V L T	25
CAGGTAGTGGGGCCCCAGTGGGTACCAAGACAGCCACAATGACTGTCGGTCCAAGGGTCCAGTCCGTGATGCAGGACTTTGTCTTCACGG	180
T G S G A P V G T K T A T M T V G P R G P V L M Q D F V F T	55
ATGAAATGGCTCATTTTGACAGAGAAAGATCCCTGAGAGAGTTGTCCATGCAAAAGGAGCTGGTGGCTTTGGTTACTTTGAGTGCACAC	270
D E M A H F <u>F D R E R I P E R V V H A K G A G A F G Y F E C T</u>	85
ACGACATAACGTCACTGCAAGCCAAGCCTTTTGAATATACTGGCAAGAAGACACCTTTGGCTGCGCGTTTTTCAACAGTTGGTGGAG	360
H D I T S Y C K A K P F E Y T G K K T P L A A R F S T V G G	115
AGAGTGGCTCAGCTGACACGGCTAGAGATCCTCGAGGATTGTCTGCAAAATTTTACTGAAGATGGTAACGGGATTGGCTGGGAATA	450
E S G S A D T T A R D P R G F A V K F Y T E D G N W D L A G <u>N</u>	145
ACACTCCCATTCTTCCATCCGAGACCAATGTTTCCCAAATTCATCCATACCCAAAAGAGGAATCCTCAGACTCATTAAAGGACC	540
N T P I F F I R D P M L F P N F I H T Q K R N P Q T H L K D	175
CTGACATGTTTGGGACTTCATCACCTTCGTCCAGAAACCAACCCAGGTGTCTTCTCTTCCGACCGTGGTACCCAGATGGT	630
P D M F W D F I T L R P E T T H Q V S F L F S D R G T P D G	205
ATCGTCACATGGACGGCTATGGCAGTCACACATACAAAATGGTGAACAAGGACGGCAAGCCAGTCTACTGCAAGTTCCACTGGAAGACTG	720
Y R H M D G Y G S H T Y K M V N K D G K P V Y C K F H W K T	235
ACCAGGTATCAAGAAGTACCAGCAAAAGCAGCAGAAATGGCTGGCAGTGACCTGATTATCCATAAAAGACCTGTACAATGCCA	810
D Q G I K N L P A N K A A E M A G S D P D Y S I K D L Y N A	265
TCGCTGAGGGCACTACCATCTGGTCACTGTATATCCAGGTCACTACCTTTGAGGAGGCAGAGAGTTCCGATTATATCCATTGACC	900
I A E G N Y P S W S L Y I Q V M T F E E A E R F R F N P F D	295
TTACCAAGATTGGCCACAAGGGGAGTATCCACTGATACCTGTGGGAAGATGGTGCTCAACAGAAATCCAAGAATTACTTTGCTGAAG	990
L T K I W P Q G E Y P L I P V G K M V L N R N P K N Y F A E	325
TGGAGCAGATTGCTTTTCCACAGCTCAGATGATCCCTGGCAGTACAGCCAAAGTCCAGATAAAATGCTGCAGGACGCTGTTTCTTACT	1080
V E Q I A F S P A H M I P G I E P S P D K M L Q G <u>R L F S Y</u>	355
CGGACACTCATCGTCACTGGGAAGTAACCTTCAGATTCTGTCAATTGCCATACAACGGGAATGTCAAGAATTACAGAGGG	1170
<u>S D T H</u> R H R L G S N Y L Q I P V N C P Y N G N V K N Y Q R	385
ATGGCCCAATGTGTTAATGATAACCAAGCTGGAGCACCAAACTACTTCCCAACAGTTCAGTGGCCCTCAAGATGATACCAACACA	1260
D G P Q C V N D N Q A G A P N Y F P N S S S G P Q D D T K H	415
TGGAGACACCACACAGTGAGTGGAGATGTGGCTCGCTACAATACTGCTGATGAGGACAACCTTTCCCAAGTCAGACCTACTGGGAAA	1350
M E H T T T V S G D V A R Y N T A D E D N F S Q V S T Y W E	445
AGGTTTGAACCTGAAGCAAGACAGAGGCTGTGTGAGGACATTGCCGGCCATGCAAAAGGATGCCAGGAGTTCATCCAGAAGAGAATTA	1440
K V L N P E A R Q R L C E D I A G H A K D A Q E F I Q K R I	475
TCAATCAGTGGACCCAGGTTGACCCAGAATGTGGACAGACAATCCAAAACTCTGCTTAAATACAAAGCTGAGGAGCAGAAAAGAAGGT	1530
I N Q W T Q V D P E C G Q T I Q K L L L K Y K A E E Q K R R	505
CTGGAGTTACTGCCAACCTGTAGACGCTCATAGTGGGATTCTTGACGGCTTTGCTACTGAAAATGGATTATATGTGAATAACAGATC	1620
S G V T A N L *	512
CTGATCCCATGTTGTATGGCATAGGACTGAGACGACTACATTAATAGGGTCTAATATAACCTGTAACCTTCATATCCAAGTGCTAGGTTGT	1710
GATGATTCTCTGTTACTATTCAATGTATCCGAATAAACCATTTTATACAATTTTGTGAAAAGAAAAA	

Fig. 3. Multiple alignment of AwSOD with other CuZnSODs. Two signature motifs sequences of CuZnSOD are marked as red box. The amino acids required for copper (His-46, -48, -64, and -120) and zinc (His-64, -72 and -81 and Asp-84) binding are shaded. Two cysteines (Cys-57 and Cys-146) formed a disulphide bond are showed with double lines.

vannamei, insect *Drosophila virilis*, gastropod *Biomphalaria glabrata*, clam *Crassostrea gigas* and so on (Fig. 7). The most relationship of evolution of AwSOD was that of freshwater bivalve *C. plicata*, next was insect and marine bivalve, last was vertebrate, gastropods and crustacean (Fig. 7A). In addition, phylogenetic analysis showed the most relationship of evolution of AwCAT is that of freshwater bivalve, then was gastropods, and marine bivalve, last was vertebrate, insect and crustacean (Fig. 7B). In evidenced sequences of mollusk, the most relationships of evolution with AwSOD and AwCAT were respectively those of fresh water clams. The phylogenetic analysis of AwSOD and AwCATs respectively reflects early divergence and independent evolution from other Cu/Zn SODs and CATs.

3.3. Tissue distribution of AwSOD and AwCAT mRNA

Results of real time PCR showed that expressions of AwSOD and AwCAT were widely distributed in foot, gill, hepatopancreas, adductor muscle and mantle of *A. woodiana* (Fig. 8). In selected tissues, the highest expression of AwSOD mRNA was observed in the hepatopancreas, intermediate ones in the foot, adductor

muscle, and gill, lower ones in heart and mantle (Fig. 8). The mRNA level of AwCAT showed a high expression in the hepatopancreas and gill, a moderate level in adductor muscle and mantle, a lower level in heart.

3.4. Acute toxicity

48 h acute toxicity tests in *A. woodiana* indicated that the LC50 of PPBDE-47 is 33.36 $\mu\text{g/L}$ (Table 2). The highest concentration of PPBDE-209 tested (160 $\mu\text{g/L}$) had no impact on *A. woodiana* survival after 48 h (Table 2), so LC50 values were not determined.

3.5. Temporal expression of AwSOD and AwCAT in hepatopancreas exposure to PBDE-47 and PBDE-209

Temporal expressions of AwSOD and AwCAT mRNA in hepatopancreas were significantly affected by PBDE-47 challenge using RT-qPCR with β -actin as internal control (Fig. 9). In all the PBDE-47 treated groups (6.25, 12.5, 25, 50 and 100 $\mu\text{g/L}$), levels of AwSOD mRNA increased more than 2.47 folds ($P < 0.05$) at 6 h compared with that of control group, 2.8 folds at 12 h (Fig. 9A). At 24 h and

<i>Anodonta woodiana</i>	–MANRDKASNQLNDFKK–AQTk–PDVLTGSGAPVGTATMTVGPRGPVLMQDFVFTD	56
<i>Drosophila melanogaster</i>	–MAGRDAASNQLIDYKN–SQTVSPGAITTGNGAPIGKIDASQTVGPRGPILLQDVNFLD	57
<i>Danio rerio</i>	MADDREKSTDQMKLWKEGRGSQR–PDVLTGAGVPIGDKLNAMTAGPRGPILLQDVVFTD	59
<i>Rattus norvegicus</i>	MADSRDPASDQMKQWKEQRAQK–PDVLTGGGNPIGDKLNIMTAGPRGPILLQDVVFTD	59
<i>Homo sapiens</i>	MADSRDPASDQMQHWKEQRAAQK–ADVLTGAGNPVGDKLVITVGPRGPILLQNVVFTD	59
<i>Anodonta woodiana</i>	EMAHFDREIRIPERVVHAKGAGAFGYFECTHDTISYCKAKPFYTGKKTPLAARFSTVGG	116
<i>Drosophila melanogaster</i>	EMSHFDREIRIPERVVHAKGAGAFGYFEVTHDITQYCAAKIFDKVKKRTPLAVRFSTVGG	117
<i>Danio rerio</i>	EMAHFDREIRIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHIGKTTPIAVRFSTVAGE	119
<i>Rattus norvegicus</i>	EMAHFDREIRIPERVVHAKGAGAFGYFEVTHDITRYSKAKVFEHIGKRTPIAVRFSTVAGE	119
<i>Homo sapiens</i>	EMAHFDREIRIPERVVHAKGAGAFGYFEVTHDITKYSKAKVFEHIGKRTPIAVRFSTVAGE	119
<i>Anodonta woodiana</i>	SGSADTARDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDPMLFPNF IHTQKRNPQTHLKD	176
<i>Drosophila melanogaster</i>	SGSADTARDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDPILFPPSFIHTQKRNPQTHLKD	177
<i>Danio rerio</i>	AGSSDTRDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDTLLFPPSFIHSQKRNPQTHLKD	179
<i>Rattus norvegicus</i>	SGSADTARDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDAMLFPSPFIHSQKRNPQTHLKD	179
<i>Homo sapiens</i>	SGSADTARDPRGFAVKFYTEDGNWDLVGNNTPIFFIRDPILFPPSFIHSQKRNPQTHLKD	179
<i>Anodonta woodiana</i>	DMFWDFITLRPETTHQVSFLFSDRGTPDGYRHMNGYSGHTYKMNKDGKPVYCKFHWKTD	236
<i>Drosophila melanogaster</i>	DMFWDFITLRPEAHQVCILFSDRGTPDGYCHMNGYSGHTFKLINAKGEPYIAKFHFKTD	237
<i>Danio rerio</i>	DMVWDFWLSRPESLHQVSFLFSDRGIPDGYRHMNGYSGHTFKLVNAQCGQPVYCKFHYKT	239
<i>Rattus norvegicus</i>	DMVWDFWLSRPESLHQVTFILFSDRGIPDGYRHMNGYSGHTFKLVNANGEAVYCKFHYKT	239
<i>Homo sapiens</i>	DMVWDFWLSRPESLHQVSFLFSDRGIPDGYRHMNGYSGHTFKLVNANGEAVYCKFHYKT	239
<i>Anodonta woodiana</i>	QGIKNLPANKAAEMAGSDPDYSIKDLYNAIAEGNYPWSLYIQVMTFEEAERFRNPFDL	296
<i>Drosophila melanogaster</i>	QGIKNLDVKTADQLASTDPDYSIRDLYNRKTKFPSTWYIYQVMTFEQAQKFKYNPFDL	297
<i>Danio rerio</i>	QGIKNIPVEEADRLAATDPDYSIRDLYNAIANGNFPSTWYIYQVMTFEQAENKWNPFDL	299
<i>Rattus norvegicus</i>	QGIKNLPVEEAGRLAQEDPDYGLRDLFNAIASGNYPSTWYIYQVMTFKEAETFPNPFDL	299
<i>Homo sapiens</i>	QGIKNLSVEDAARLSQEDPDYGLRDLFNAIATGKDPSTWYIYQVMTFNQAEETFPNPFDL	299
<i>Anodonta woodiana</i>	TKIWPQGEYPLIPVGKMLNRNPNKYFAEVEQIAFSPAHPMIPGIEPSDKMLQGRFLFSYS	356
<i>Drosophila melanogaster</i>	TKVWSQKEYPLIPVGKMLDRNPNKYFAEVEQIAFSPAHLVPGVEPSDKMLHGRFLFSYS	357
<i>Danio rerio</i>	TKVWSHKEFPLIPVGRFVLRNPNVNYFAEVEQLAFDPSNMPPGIEPSDKMLQGRFLFSYP	359
<i>Rattus norvegicus</i>	TKVWPHKDYPLIPVGKLVLRNPNVNYFAEVEQMAFDPSNMPPGIEPSDKMLQGRFLFAYP	359
<i>Homo sapiens</i>	TRVWPHKDYPLIPVGKLVLRNPNVNYFAEVEQIAFDPSNMPPGIEASPDKMLQGRFLFAYP	359
<i>Anodonta woodiana</i>	DTHRHRLGSNYLQIPVNCYPYNGVKNYQRDGPQCVNDNQAGAPNYFPNSSSGPQDDTKHM	416
<i>Drosophila melanogaster</i>	DTHRHRLGPNYLQIPVNCYPYKVIENFQRDGMNVTDNQDAPNYFPNSFNGPQECPRAR	417
<i>Danio rerio</i>	DTHRHRLGANYLQIPVNCYPYRTRVANYQRDGMCMHDNQGGAPNYFPNSFAPDVPQRF	419
<i>Rattus norvegicus</i>	DTHRHRLGPNYLQIPVNCYPYRVARVANYQRDGMCMHDNQGGAPNYFPNSFAPEQQGSAL	419
<i>Homo sapiens</i>	DTHRHRLGPNYLHIPPVNCYPYRVARVANYQRDGMCMQDNQGGAPNYFPNSFGAPEQQPSAL	419
<i>Anodonta woodiana</i>	EHTTT–VSGDVARYNTADEDNFS–QVSTYWEKVLNPEARQLCEDIAGHAKDAQEFIQK	473
<i>Drosophila melanogaster</i>	ALSSCCPVTGDVRYSSGDTEDNFGQVTDFFVHVLDKCAKKRLVQNIAGHLSNASQFLQE	477
<i>Danio rerio</i>	ESKCK–VSPDVARYNSADDDNVT–QVRTFTTQVLNEAERERLCQNMAGHLKGAQLFIQK	477
<i>Rattus norvegicus</i>	EHHSQ–CSADVCRFNSANEDNVT–QVRTFTYTKVLNEEERKRLCENIANHLKDAQLFIQR	477
<i>Homo sapiens</i>	EHSIQ–YSGEVRRFNTANDDNVT–QVRAFVNVNLNEEQRKRLCENIAGHLKDAQIFIQK	477
<i>Anodonta woodiana</i>	RIINQWTQVDECGQTIQKLLKYKAEQKRRSGVTANL–	512
<i>Drosophila melanogaster</i>	RAVKNFTQVHADFGRLMTEELNLAKSKF–	505
<i>Danio rerio</i>	RMVQNLMVHSDYGNRVQALLDKHNAEGKNTVHVYSRGGASAVAAASKM–	527
<i>Rattus norvegicus</i>	KAVKNFTDVHPDYGARVQALLDQYNSQPKNAIHTYVQAG–SHIAAKGKANL	528
<i>Homo sapiens</i>	KAVKNFTEVHPDYGSHIQALLDKYNAEKPKNAIHTFVRSG–SHLVAREKANL	528

Fig. 4. Multiple alignment of AwCAT with other CATs. The highly conserved catalytic site motif and proximal heme-ligand signature motif are respectively marked in red box and blue box. The putative NADPH-binding residues (N145, H191, F195, S198, R200, Y212, K234, W300, Q302, Y355) are marked with bold, heme binding (R69, H72, R109, N145, F150, R351, Y355, R362) are showed in under line. Conserved catalytic amino residues (His72, Asn145 and Tyr355) are indicated with shadow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

48 h exposure, significant increase of AwCAT expression was still observed in groups at a low concentrations of PBDE-47 treatment

(6.25, 12.5 and 25 $\mu\text{g/L}$), but a down-regulation of AwSOD was occurred in ones of a high concentrations of PBDE-47 treatment (50

Fig. 5. Predicted secondary structures of AwSOD and AwCAT. A: The secondary structure of AwSOD. B: The secondary structure of AwCAT.

and 100 $\mu\text{g/L}$) (Fig. 9A). Like that of AwSOD, expression of AwCAT showed a significant up-regulation in the low concentration of PBDE-47 treated groups (6.25, 12.5 and 25 $\mu\text{g/L}$), and a biphasic manner in the high concentration of PBDE-47 treated ones (50 and 100 $\mu\text{g/L}$) (Fig. 9B). In all the PBDE-209 treated groups (10, 20, 40, 80 and 160 $\mu\text{g/L}$), expressions of AwSOD and AwCAT were all up-regulated with a time- and dose-dependent pattern during experiment observed (Fig. 9C,D).

4. Discussion

In predicted amino acid sequence, no signal peptide was found in AwSOD indicating that it belongs to a member of the cytoplasmic CuZnSOD family. The multiple alignment of the deduced amino acid sequence of AwSOD with other CuZnSODs revealed that two cysteines (Cys 57 and Cys 146) are consistent with that of CuZnSODs, and are likely to form one disulphide bond that is insistent with that of predicted secondary structure. The amino acids

Fig. 6. Predicted 3D structures of AwSOD and AwCAT. A: The 3D structure of AwSOD. B: The 3D structure of AwCAT.

Fig. 7. Phylogenetic relationship of AwsOD and AwCAT from other organisms. A: The phylogenetic relationship of AwsOD. B: The phylogenetic relationship of AwCAT.

Fig. 8. Real-time PCR analysis of AwsOD and AwCAT transcript from different tissues. n = 3 replicates. A: The tissues distribution of AwsOD. B: The tissues distribution of AwCAT.

required for copper and zinc bindings were conserved in AwsOD and CuZnSODs, suggest that these conserved amino acids are contribute to the structure stability and catalyze function of AwsOD. It has established that these amino acids play a potential role in stabilizing Cu/Zn SODs conformation under harsh environmental conditions due to copper and zinc ions have critical functions in stabilizing the quaternary structure and therefore in the kinetic properties of Cu/Zn SOD [31–34].

As a result of multiple alignments using different CATs, conserved catalytic amino acids, such as His72, Asn145, and Tyr355, were observed in AwCAT and other CATs. In addition, proximal heme ligand signature sequence and of proximal active site signature, the signal sequences of CATs, were also identified in AwCAT and CATs of bivalves. Therefore, the high similarity and two conserved characteristic sites of AwCAT, indicating that AwCAT is a member of the CAT family. Notably, the peroxisome targeting signal in the C-terminus of AwCAT is ANL which is consistent with the canonical signal of CAT of human, mouse and rat, suggest AwCAT likely shares potential characterizations of peroxisomal glycoprotein like most members of CAT family of vertebrate. In mammals, NADPH is considered to be tightly bound to CAT, and to offset substrate (H_2O_2) to enzyme [35,36]. This event is fulfilled through

electron tunneling between binding site of NADPH on the surface of CATs and the heme group within enzyme [36]. 12 amino acid residues in CAT sequence are identified to be responsible for formation of NADPH binding site [37]. Among them, 10 are identified and significantly conserved in the sequence of CATs including N145, H191, F195, S198, R200, Y212, K234, W300, Q302, Y355. These residues are also found in AwCAT. In order to further elucidate the function of AwCAT, the presumed secondary structure and tertiary structure of the molecule are established using the SWISS-MODEL prediction algorithm. The contents of α -helix and β -sheet in AwCAT are very similar to that of vertebrate CAT. Combination conserved residues and spatial structures, we postulate that AwCAT is involved in eliminating H_2O_2 like CAT of human and other vertebrate, but this hypothesis is need be confirmed in the future [35,38].

AwsOD and AwCAT had a constitutive but distinct expression pattern in various tissues. The highest level distribution of AwsOD and AwCAT mRNA in selected tissues was observed in hepatopancreas that is related to the roles of hepatopancreas as the main metabolic tissue and the main defense tissue against oxidative stress [39]. Besides hepatopancreas, expression levels of AwsOD and AwCAT in other tissues are not similar, suggesting that ROS elimination is a complex process. Notably, clams *A. woodiana*

Table 248 h LC50 values for *A. Woodiana* exposed to PBDE-47 and -209.

PBDEs	LC50 ($\mu\text{g/L}$)	95% confidence intervals	
		Lower	Upper
PBDE-47	33.36	23.740	49.876
PBDE-209	/	/	/

are filter-feeding freshwater bivalves, gill is continuously exposed to abundant microorganism in the freshwater environment, thus, strong expression of AwCAT in gill could contribute to elimination of ROS or H_2O_2 .

Our results show LC50 for PBDE-47 is 33.36 $\mu\text{g/L}$, but PBDE-209 was not determined in selected concentrations of experiment, assume that *A. woodiana* has more sensitive response to PBDE-47 than PBDE-209 at a similar exposure concentration. Relative observations of aquatic organism response faced PBDEs were also reported in other studies [40]. The LC50 for 48 h acute toxicity tests in *Daphnia magna* indicated that PBDE-28 is the least toxic in terms of lethality, followed by PBDE-100, PPBDE-47, and then PBDE-99 [40,41]. Meanwhile, 2.5 mg/L of PPBDE-209 tested had no impact on daphnid survival after 48 h [41]. In adults of another crustacean species, the copepod *A. tonsa*, LC50 concentration of PBDEs-28, -47, -99 and -100 for 48 h was 108, 370, 705 and 520 $\mu\text{g/L}$, respectively [40,42]. One possible explain of difference of acute toxicity derived from different PBDEs is that higher lipophilicity of PBDE-47, -99 and -100, result in a weak defense barrier to overcome their injury

[6,40].

In present study, 6 h post exposure of different concentrations of BDE47 could result in a significant up-regulation of AwSOD and AwCAT expressions in contrast with that of control group, suggest administration BDE47 can initiate antioxidant system of *A. woodiana* to cope with ROS injury. In healthy organism, total ROS level stay a relatively low level and ROS will be rapidly eliminated by an array of anti-oxidant enzymes to maintain a balance between ROS level and anti-oxidant enzyme activity [39,43]. Orchestra of anti-oxidant enzymes is a key hallmark of organism homeostasis. Moderate oxidative stress in a shorter time may function as an important player to induce different anti-oxidant enzyme expressions to deal with different ROS [25,26,43].

At 12 h post exposure, expressions of AwSOD and AwCAT in PBDE-47 treated groups still showed a significant increase compared with that of control group, but a decrease trend compare with that of 6 h, this phenomena is associated with scavenging ability of animals faced persistent production of ROS. Along elongation of exposure time, ROS should gradually be produced and accumulated in the cells in respect with constantly deposition of different concentrations of PBDE-47 in body. On another hand, *A. woodiana* also adjust it's antioxidant ability by enhancing anti-oxidant enzyme expression to eliminate ROS. If generation of ROS are under a weak level, anti-oxidant enzyme expressions should be up-regulated that contribute to endure oxidative stresses [25,26,39,44]. Once the production of ROS is exceed the eliminating ability of cells that cause a shift between oxidants and antioxidants in favor of oxidants, and result in chronic inflammation, apoptosis

Fig. 9. Temporal expressions of AwSOD and AwCAT in hepatopancreas after PBDE-47 and -209 challenge as measured by quantitative real-time PCR. A: Effect of PBDE-47 on AwSOD in hepatopancreas. B: Effect of PBDE-47 on AwCAT in hepatopancreas. C: Effect of PBDE-209 on AwSOD in hepatopancreas. D: Effect of PBDE-209 on AwCAT in hepatopancreas. Bars represented means $\pm$ SE; n = 3/each group/each time point. *P < 0.05, #P < 0.01 vs control group at the same time.

of immune cells, etc [45,46]. In the condition, if animals cannot produce new cells to compensate for death ones, number of living cells should be gradually decreased. Following, expressions of AwSOD and AwCAT are also decline. Thus, treatment of different concentrations of PBDE-47 results in a different degree decrease of AwSOD and AwCAT expression. Considering here, it is not difficult to understand that expressions of AwSOD and AwCAT show a significant down-regulation at 24 and 48 h in high concentration groups compared with that of control group. Notably, the expressions of AwSOD and AwCAT in the high concentration groups of PBDE-47 show a U shape pattern characterized by an up-regulation at early stage and a down-regulation during experiment observed, that is in line with the hormesis phenomenon in toxicology [47–49].

In the PBDE-209 treated groups, up-regulations of AwSOD and AwCAT expression exhibited a time- and dose-dependent pattern. Based above mentioned, suggesting that selected concentrations are not reach to a threshold because administration of this concentration can produce enough ROS to disturb the balance between oxidant and antioxidant. In selected concentrations, LC50 of PBDE-209 was not detected in experiment that is contributes to understand this phenomena.

In conclusion, complete cDNA sequences of AwSOD and AwCAT are cloned from freshwater bivalve *A. woodiana* and their full-length cDNAs contain highly conserved domains and residues important for antioxidant defense. Correlating the tissue specific mRNA expressions and expression inductions of AwSOD and AwCAT derived from PBDE-47 and PBDE-209 suggests that *A. woodiana* is a potential bio-indicator for assessing the impact of freshwater pollutants with the application of gene expression approach. It worth noting that administration of BDE47 is likely to be more detrimental to these non-target organisms than PBDE-209 at a similar concentration. In order to prevent or mitigate extensive negative impacts on aquatic organisms in freshwater and conserve aquatic organism biodiversity, it would thus be of critical importance for government to be aware of the effects of sequential PBDEs exposure and control diffusion of PBDEs.

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References

- [1] H. Fromme, G. Becher, B. Hilger, W. Völkel, Brominated flame retardants-Exposure and risk assessment for the general population, *Int. J. Hyg. Environ. Health* 219 (2016) 1–23.
- [2] S.C. Roberts, P.D. Noyes, E.P. Gallagher, H.M. Stapleton, Species-specific differences and structure-activity relationships in the debromination of PBDE congeners in three fish species, *Environ. Sci. Technol.* 45 (2011) 1999–2005.
- [3] C.T. Chou, Y.C. Hsiao, F.C. Ko, J.O. Cheng, Y.M. Cheng, T.H. Chen, Chronic exposure of 2,2',4,4'-tetrabromodiphenyl ether (PBDE-47) alters locomotion behavior in juvenile zebrafish (*Danio rerio*), *Aquat. Toxicol.* 98 (2010) 388–395.
- [4] M.S. Santos, A. Alves, L.M. Madeira, Chemical and photochemical degradation of polybrominated diphenyl ethers in liquid systems- a review, *Water Res.* 88 (2016) 39–59.
- [5] R. Zhang, J. Guo, F. Wu, Y. Mu, J.P. Giesy, H. Chang, X. Zhao, C. Feng, Toxicity reference values for polybrominated diphenyl ethers: risk assessment for predatory birds and mammals from two Chinese lakes, *Rev. Environ. Contam. Toxicol.* 229 (2014) 111–137.
- [6] R. Cruz, S.C. Cunha, S. Casal, Brominated flame retardants and seafood safety: a review, *Environ. Int.* 77 (2015) 116–131.
- [7] J. Blanco, M. Mulero, J.L. Domingo, D.J. Sanchez, Perinatal exposure to BDE-99 causes decreased protein levels of cyclin D1 via GSK3 β activation and increased ROS production in rat pup livers, *Toxicol. Sci.* 137 (2014) 491–498.
- [8] E. Napoli, C. Hung, S. Wong, C. Giulivi, Toxicity of the flame-retardant BDE-49 on brain mitochondria and neuronal progenitor striatal cells enhanced by a PTEN-deficient background, *Toxicol. Sci.* 132 (2013) 196–210.
- [9] S.C. Huang, G. Giordano, L.G. Costa, Comparative cytotoxicity and intracellular accumulation of five polybrominated diphenyl ether congeners in mouse cerebellar granule neurons, *Toxicol. Sci.* 114 (2010) 124–132.
- [10] M. Ott, V. Gogvadze, S. Orrenius, B. Zhivotovsky, Mitochondria, oxidative stress and cell death, *Apoptosis* 12 (2007) 913–922.
- [11] J. Han, E.J. Won, M.C. Lee, J.S. Seo, S.J. Lee, J.S. Lee, Developmental retardation, reduced fecundity, and modulated expression of the defense in the intertidal copepod *Tigriopus japonicus* exposed to BDE-47 and PFOS, *Aquat. Toxicol.* 165 (2015) 136–143.
- [12] X. Liu, J. Wang, C. Lu, C. Zhu, B. Qian, Z. Li, C. Liu, J. Shao, J. Yan, The role of lysosomes in BDE 47-mediated activation of mitochondrial apoptotic pathway in HepG2 cells, *Chemosphere* 124 (2015) 10–21.
- [13] S. Jin, F. Yang, Y. Hui, Y. Xu, Y. Lu, J. Liu, Cytotoxicity and apoptosis induction on RTG-2 cells of 2,2',4,4'-tetrabromodiphenyl ether (BDE-47) and decabrominated diphenyl ether (BDE-209), *Toxicol. In Vitro* 24 (2010) 1190–1196.
- [14] M. Curcic, K. Durgo, N. Kopjar, M. Ancic, S. Vucinic, B. Antonijevic, Cadmium and decabrominated diphenyl ether mixture: in vitro evaluation of cytotoxic, prooxidative and genotoxic effects, *Environ. Toxicol. Pharmacol.* 38 (2014) 663–671.
- [15] X.K. She, X. Wang, J.B. Zhou, R.S. Zhao, Porous lead(II)-based metal organic nanotubes as an adsorbent for dispersive solid-phase extraction of polybrominated diphenyl ethers from environmental water samples, *J. Chromatogr. A* 1423 (2015) 31–38.
- [16] U.J. Kim, H. Jo, I.S. Lee, G.J. Joo, J.E. Oh, Investigation of bioaccumulation and biotransformation of polybrominated diphenyl ethers, hydroxylated and methoxylated derivatives in varying trophic level freshwater fishes, *Chemosphere* 137 (2015) 108–114.
- [17] P. Soo, P.A. Todd, The behaviour of giant clams (Bivalvia: Cardiidae: Tridacninae), *Mar. Biol.* 161 (12) (2014) 2699–2717.
- [18] S. Kolarević, J. Knežević-Vukčević, M. Paunović, M. Kračun, B. Vasiljević, J. Tomović, B. Vuković-Gačić, Z. Gačić, Monitoring of DNA damage in haemocytes of freshwater mussel *Sinodonta woodiana* sampled from the Velika Morava River in Serbia with the comet assay, *Chemosphere* 93 (2) (2013) 243–251.
- [19] M. Reichard, M. Vrtílek, K. Douda, C. Smith, An invasive species reverses the roles in a host-parasite relationship between bitterling fish and unionid mussels, *Biol. Lett.* 8 (2012) 601–604.
- [20] H. Liu, J. Yang, J. Gan, Trace element accumulation in bivalve mussels *Anodonta woodiana* from Taihu Lake, China, *Arch. Environ. Contam. Toxicol.* 59 (2010) 593–601.
- [21] X. Zhang, Z. Liu, E. Jeppesen, W.D. Taylor, Effects of deposit-feeding tubificid worms and filter-feeding bivalves on benthic-pelagic coupling: implications for the restoration of eutrophic shallow lakes, *Water Res.* 50 (2014) 135–146.
- [22] S. Ciparis, A. Phipps, D.J. Soucek, C.E. Zipper, J.W. Jones, Effects of environmentally relevant mixtures of major ions on a freshwater mussel, *Environ. Pollut.* 207 (2015) 280–287.
- [23] P. Hliwa, B. Zdanowski, G.J. Dietrich, A. Andronowska, J. Król, A. Ciereszko, Temporal changes in gametogenesis of the invasive Chinese pond mussel *Sinodonta woodiana* (Lea, 1834) (Bivalvia: Unionidae) from the Konin lakes system (central Poland), *Folia Biol.* 63 (2015) 175–178.
- [24] M. Parolini, S. Magni, S. Castiglioni, A. Binelli, Amphetamine exposure imbalanced antioxidant activity in the bivalve *Dreissena polymorpha* causing oxidative and genetic damage, *Chemosphere* 144 (2016) 207–213.
- [25] D. Macías-Mayorga, I. Laiz, I. Moreno-Garrido, J. Blasco, Is oxidative stress related to cadmium accumulation in the Mollusc *Crassostrea angulata*? *Aquat. Toxicol.* 161 (2015) 231–241.
- [26] H. Chen, J. Zha, L. Yuan, Z. Wang, Effects of fluoxetine on behavior, antioxidant enzyme systems, and multixenobiotic resistance in the Asian clam *Corbicula fluminea*, *Chemosphere* 119 (2015) 856–862.
- [27] S. Koutsogiannaki, S. Franzellitti, E. Fabbri, M. Kaloyianni, Oxidative stress parameters induced by exposure to either cadmium or 17 β -estradiol on *Mytilus galloprovincialis* hemocytes: The role of signaling molecules, *Aquat. Toxicol.* 146 (2014) 186–195.
- [28] V.L. Maria, M.J. Bebianno, Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper, *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 154 (2011) 56–63.
- [29] S.B. De Luca-Abbott, B.J. Richardson, K.E. McClellan, G.J. Zheng, M. Martin, P.K. Lam, Field validation of antioxidant enzyme biomarkers in mussels (*Perna viridis*) and clams (*Ruditapes philippinarum*) transplanted in Hong Kong coastal waters, *Mar. Pollut. Bull.* 51 (2005) 694–707.
- [30] Q. Wang, Z. Yuan, H. Wu, F. Liu, J. Zhao, Molecular characterization of a manganese superoxide dismutase and copper/zinc superoxide dismutase from the mussel *Mytilus galloprovincialis*, *Fish. Shellfish Immunol.* 34 (2013) 1345–1351.
- [31] Y. Bao, L. Li, Q. Wu, Zhang G. Cloning, characterization, and expression analysis of extracellular copper/zinc superoxide dismutase gene from bay scallop *Argopecten irradians*, *Fish. Shellfish Immunol.* 27 (2009) 17–25.
- [32] H.H. Xu, H. Ma, B.Q. Hu, D.B. Lowrie, X.Y. Fan, Wen CG. Molecular cloning, identification and functional characterization of a novel intracellular Cu-Zn superoxide dismutase from the freshwater mussel *Cristaria plicata*, *Fish. Shellfish Immunol.* 29 (2010) 615–622.
- [33] G. Atli, E.G. Canli, A. Eroglu, M. Canli, Characterization of antioxidant system parameters in four freshwater fish species, *Ecotoxicol. Environ. Saf.* 126 (2015) 30–37.
- [34] H. Park, I.Y. Ahn, J.K. Lee, S.C. Shin, J. Lee, E.J. Choy, Molecular cloning, characterization, and the response of manganese superoxide dismutase from the

- Antarctic bivalve *Laternula elliptica* to PCB exposure, Fish. Shellfish Immunol. 27 (2009) 522–528.
- [35] C.D. Putnam, A.S. Arvai, Y. Bourne, J.A. Tainer, Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism, J. Mol. Biol. 296 (1) (2000) 295–309.
- [36] H.N. Kirkman, M. Rolfo, A.M. Ferraris, G.F. Gaetani, Mechanisms of protection of catalase by NADPH. Kinetics and stoichiometry, J. Biol. Chem. 274 (1999) 13908–13914.
- [37] C. Li, D. Ni, L. Song, J. Zhao, H. Zhang, L. Li, Molecular cloning and characterization of a catalase gene from Zhikong scallop *Chlamys farreri*, Fish. Shellfish Immunol. 24 (2008) 26–34.
- [38] C. Wang, X. Yue, X. Lu, B. Liu, The role of catalase in the immune response to oxidative stress and pathogen challenge in the clam *Meretrix meretrix*, Fish. Shellfish Immunol. 3 (2013) 91–99.
- [39] A. Bigot, P. Vasseur, Rodius F.SOD and CAT cDNA cloning, and expression pattern of detoxification genes in the freshwater bivalve *Unio tumidus* transplanted into the Moselle river, Ecotoxicology 19 (2010) 369–376.
- [40] R. Davies, E. Zou, Polybrominated diphenyl ethers disrupt molting in neonatal *Daphnia magna*, Ecotoxicology 21 (2012) 1371–1380.
- [41] M.G. Evandri, L.G. Costa, P. Bolle, Evaluation of brominated diphenyl ether-99 toxicity with *Raphidocelis subcapitata* and *Daphnia magna*, Environ. Toxicol. Chem. 22 (2003) 2167–2172.
- [42] L. Wollenberger, L. Dinan, M. Breitholtz, Brominated flame retardants: activities in a crustacean development test and in an ecdysteroid screening assay, Environ. Toxicol. Chem. 24 (2005) 400–407.
- [43] L. Tomanek, Proteomic responses to environmentally induced oxidative stress, J. Exp. Biol. 218 (2015) 1867–1879.
- [44] K.Q. de Andrade, F.A. Moura, J.M. Dos Santos, O.R. de Araújo, J.C. de Farias Santos, M.O. Goulart, Oxidative stress and inflammation in hepatic diseases: therapeutic possibilities of N-acetylcysteine, Int. J. Mol. Sci. 16 (2015) 30269–30308.
- [45] S.A. Lozano-Sepulveda, O.L. Bryan-Marrugo, C. Cordova-Fletes, M.C. Gutierrez-Ruiz, A.M. Rivas-Estilla, Oxidative stress modulation in hepatitis C virus infected cells, World J. Hepatol. 7 (2015) 2880–2889.
- [46] N.S. Alamolhodaei, K. Shirani, G. Karimi, Arsenic cardiotoxicity: An overview, Environ. Toxicol. Pharmacol. 40 (2015) 1005–1014.
- [47] R.G. Belz, H.P. Piepho, Statistical modeling of the hormetic dose zone and the toxic potency completes the quantitative description of hormetic dose responses, Environ. Toxicol. Chem. 34 (2015) 1169–1177.
- [48] G. Martínez-Sánchez, G. Pérez-Davison, L. Re, A. Giuliani, Ozone as u-shaped dose responses molecules (hormetins), Dose Response 9 (2010) 32–49.
- [49] E.J. Calabrese, U-shaped dose response in behavioral pharmacology: historical foundations, Crit. Rev. Toxicol. 38 (2008) 591–598.