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Responses of thioredoxin 1 and thioredoxin-related protein 14 mRNAs to cadmium and copper stresses in *Venerupis philippinarum*

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ABSTRACT

Thioredoxin (abbreviated as Trx) is an important ubiquitous disulfide reductase, which can protect organisms against various oxidative stresses. In the present study, thioredoxin 1 (named as VpTrx1) and thioredoxin-related protein (named as VpTrp14) were identified from *Venerupis philippinarum*, respectively. Similar to most Trx1s, VpTrx1 possessed all conserved features critical for the fundamental structure and function of Trx1s, such as the conserved catalytic residues (C-G-P-C), but lacked the other cysteine residues, while VpTrp14 contained the conserved motif (C-P-D-C). Quantitative Real-time PCR assay showed that VpTrx1 and VpTrp14 transcripts were distributed in a wide array of tissues most abundantly expressed in the hepatopancreas. The expression of VpTrp14 mRNA in the hepatopancreas was significantly up-regulated after exposure to 10 and 40 μg/L Cd, while the VpTrx1 expression level was kept relatively constant. Both the expression levels of VpTrx1 and VpTrp14 in the hepatopancreas were induced after exposure to Cu, and increased to the peak value at 96 h under the 40 μg/L Cu exposure. These results showed that VpTrp14 transcripts responded to metal stress more acutely than VpTrx1, and both Trxs responded to Cu stress more sensitively than Cd. Together, it was suggested that VpTrx1 and VpTrp14 perhaps played important roles in the antioxidant responses against metal stress in *V. philippinarum*.

1. Introduction

Manila clam, *Venerupis philippinarum* is widely distributed along the Bohai Sea, a semi-enclosed continental shelf sea in China located between 37°07′N to 41°N and 117°35′E to 121°10′E (Jiang et al., 2000; Liang et al., 2004), and has been listed as a sentinel organism in "Mussel Watch Program" launched in China. Cadmium (Cd) and copper (Cu) are two severe heavy metal contaminants along the Bohai coast (Mao et al., 2009; Peng et al., 2009). Both Cd and Cu have been demonstrated to be toxic to marine bivalves and can strongly affect their physiological status by causing oxidative stress (Isani et al., 2003; Dovzhenko et al., 2005). It has been well acknowledged that the antioxidant enzymes such as SOD, Se-GPx and catalase worked cooperatively against oxidative stress, and their activities have been detected in many mollusks (Isani et al., 2003; Abele and Puntarulo, 2004; Franco et al., 2006). Recently, Trxs (thioredoxins) in marine invertebrates have received attention because of their critical roles in regulation of cellular redox homeostasis and fighting against the oxidative stress (Watson et al., 2004).

Thioredoxins (Trxs) are members of an evolutionarily conserved family of redox-active proteins containing a conserved active site dithiol motif C-G-P-C (Holmgren, 1985), which exists in several forms with the cytosolic (Trx1) and mitochondrial (Trx2) forms being the most prevalent. Trx1 is a 12 kDa protein and in general it has 105 amino acids in length, whereas Trx2 mRNA encodes a larger protein than Trx1 but lacks other cysteine residues existing in Trx1 (Hirota et al., 2002). Trxs have been found to possess a variety of biological functions, such as scavenging reactive oxygen species (ROS) (Das and Das, 2000), regeneration of oxidative-damage proteins (Fernando et al., 1992), regulation of gene expression, and controlling apoptosis as regulation of NF-κB transcription factor (Saitoh et al., 1998). Molecular studies have demonstrated that both Trx1 and Trx2 protect organisms against oxidative stress, and Trx1 is inducible in response to oxidative stress (Chen et al., 2002; Jikimoto et al., 2002). Recently, proteins containing the Trx-like active sites have also been identified in various species and classified as new members of the Trx superfamily (Matsuo et al., 2002;

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Nakamura, 2005; Carvalho et al., 2006). Among them, thioredoxin-related protein of 14 kDa (Trp14), a cytosolic protein with conserved motif of C-P-D-C, has been found that acts as a disulfide reductase like Trx1 (Jeong et al., 2004). Trp14 has the redox potential similar to that of Trx1, and it receives the electrons from Trx reductase 1 as does Trx1.

To our knowledge, only a few Trx genes have been identified from the marine invertebrates so far (Aispuro-Hernandez et al., 2008; Mu et al., 2009; Ren et al., 2010). Although Trp14 have been identified in a wide range of organisms from bacteria to mammals (Jiang et al., 2007; Jeong et al., 2009), there were scanty reports about Trp14s and their functions (Jeong et al., 2004; Jiang et al., 2007). As far as we know, no information is available concerning how the Trx1 or Trp14 genes respond to metal stress in marine invertebrates. In the present study, the full-length cDNAs of Trx1 and Trp14 were identified from *V. philippinarum*, and the expression profiles of VpTrx1 and VpTrp14 transcript responded to Cu and Cd exposure were also investigated.

2. Materials and methods

2.1. Animal culture and metal exposure

Adult clams *V. philippinarum* (shell-length: 3.0–4.0 cm) were collected from a local culturing farm and acclimatized in aerated seawater (33 psu) at 25 °C for 10 days before commencement of the experiment. During the acclimatization period, the clams were fed with *Chlorella vulgaris*, and the water was totally exchanged daily. After the acclimatization, the clams were divided into 5 groups and cultured in 20 L aquarium tanks, each containing 30 individuals. Four groups of clams were exposed to Cd (as $CdCl_2$, 10 and 40 μg/L) and Cu (as $CuCl_2$, 10 and 40 μg/L) respectively. The untreated group was employed as the control group. The hepatopancreas of six clams from each tank were randomly sampled for gene expression analysis after 24, 48 and 96 h of exposure, respectively. Total mRNA was immediately extracted using Trizol reagent according to the manufacture's protocol (Invitrogen, USA).

2.2. Cloning the full-length cDNA of VpTrx1 and VpTrp14

BLAST analysis of all expressed sequence tag (EST) sequences from a self-made cDNA library of *V. philippinarum* revealed that two ESTs were highly similar to the previously identified Trx1 and Trp14, respectively. The 5′ and 3′ ends of VpTrx1 and VpTrp14 were obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's recommendations. The PCR products were gel-purified and subcloned into pMD18-T simple vector (Takara). After being transformed into the competent cells of *Escherichia coli* Top10F′, three positive clones were sequenced on an ABI3730 Automated Sequencer (Applied Biosystems, USA).

2.3. Sequence analysis of VpTrx1 and VpTrp14

The cDNA and amino acid sequences of VpTrx1 and VpTrp14 were analyzed using the BLAST algorithm at NCBI web site (http://www.ncbi.nlm.nih.gov/blast) and the simple modular architecture research tool (SMART) program (Letunic et al., 2009). Multiple alignments of VpTrx1 and VpTrp14 were performed with the ClustalW program (http://www.ebi.ac.uk/clustalw/). A neighbor-joining (NJ) phylogenetic tree was constructed with Mega4.1 software (Kumar, et al., 2008) using the Poisson correction model based on the alignment of amino acids (Saitou and Nei, 1987). Bootstrap analysis was used with 1000 replicates to test the relative support for the branches produced by the NJ analysis (Felsenstein, 1985).

2.4. Tissue-specific expression of VpTrx1 and VpTrp14 mRNA

Hemocytes, foot, gill, hepatopancreas, mantle and adductor muscle were taken aseptically from four clams and subjected to total RNA extraction. One microgram of total RNA was used for cDNA synthesis according to the manufacture's protocol (Promega, USA). qRT-PCR was carried out in an ABI 7500 Real-time Detection System by using the SYBR ExScript qRT-PCR Kit (Takara) as described previously (Li et al., 2010). The expression level of VpTrx1 and VpTrp14 was analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) with β-actin mRNA as the control (Li et al., 2010). The primers used to amplify the β-actin gene were P1 (5′-CTCCCTTGAGAAGAGCTACGA-3′) and P2 (5′-GATACCAGCAGATTC CATACCC-3′). Two sets of gene-specific primers, P3 (GTAACGGCT

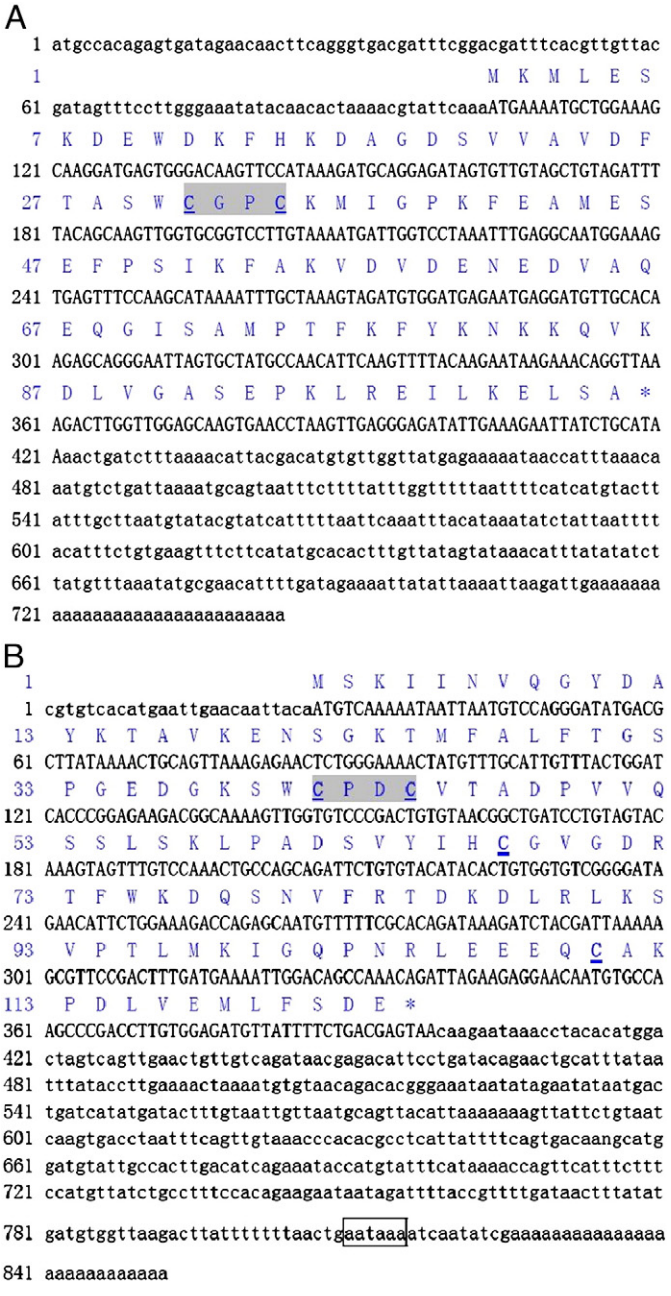

Fig. 1. Nucleotide and deduced amino acid sequences of VpTrx1 (A) and VpTrp14 (B). The asterisk (*) indicates the stop codon. Polyadenylation signal is included in a box. The active cysteine residues are in bold and underlined. The catalytic center is shaded in dark. The other two cysteine residues in VpTrp14, Cys^{67} and Cys^{110}, are double underlined.

GATCCTGTAGT) and P4 (TCTAATCTGTTTGGCTGTCCA) for VpTrx1, P5 (AAGTTCCATAAAGATGCAGGAG) and P6 (TGGCATAGCACTAATTCCCT) for VpTrp14, were designed to amplify products of 189 bp and 185 bp, respectively. All data are given in terms of relative mRNA abundance, expressed as means plus or minus standard errors of the means (S.E.).

2.5. Temporal expression profile of VpTrx1 and VpTrp14 mRNA in the hepatopancreas after Cd or Cu exposure

The tissue of hepatopancreas was selected to analyze temporal expression profile of VpTrx1 and VpTrp14 challenged by cadmium and copper, respectively. The RNA extraction, cDNA synthesis, reaction component, thermal profile, and the data analysis were conducted as previously described (Li et al., 2010).

2.6. Statistical analysis

SPSS 16.0 software (SPSS Inc., USA) was used for statistical analysis. All data were given in terms of relative mRNA expression as means ± S.E. One-way analysis of variance (one-way ANOVA) was performed on all data and $P<0.05$ was considered statistically significant.

3. Results

3.1. CDNA cloning and sequence analysis of VpTrx1 and VpTrp14

Two nucleotide sequences of 743 bp and 852 bp representing the complete cDNA sequence of VpTrx1 (Fig. 1A) and VpTrp14 (Fig. 1B) were obtained by overlapping EST and the amplified fragments. The

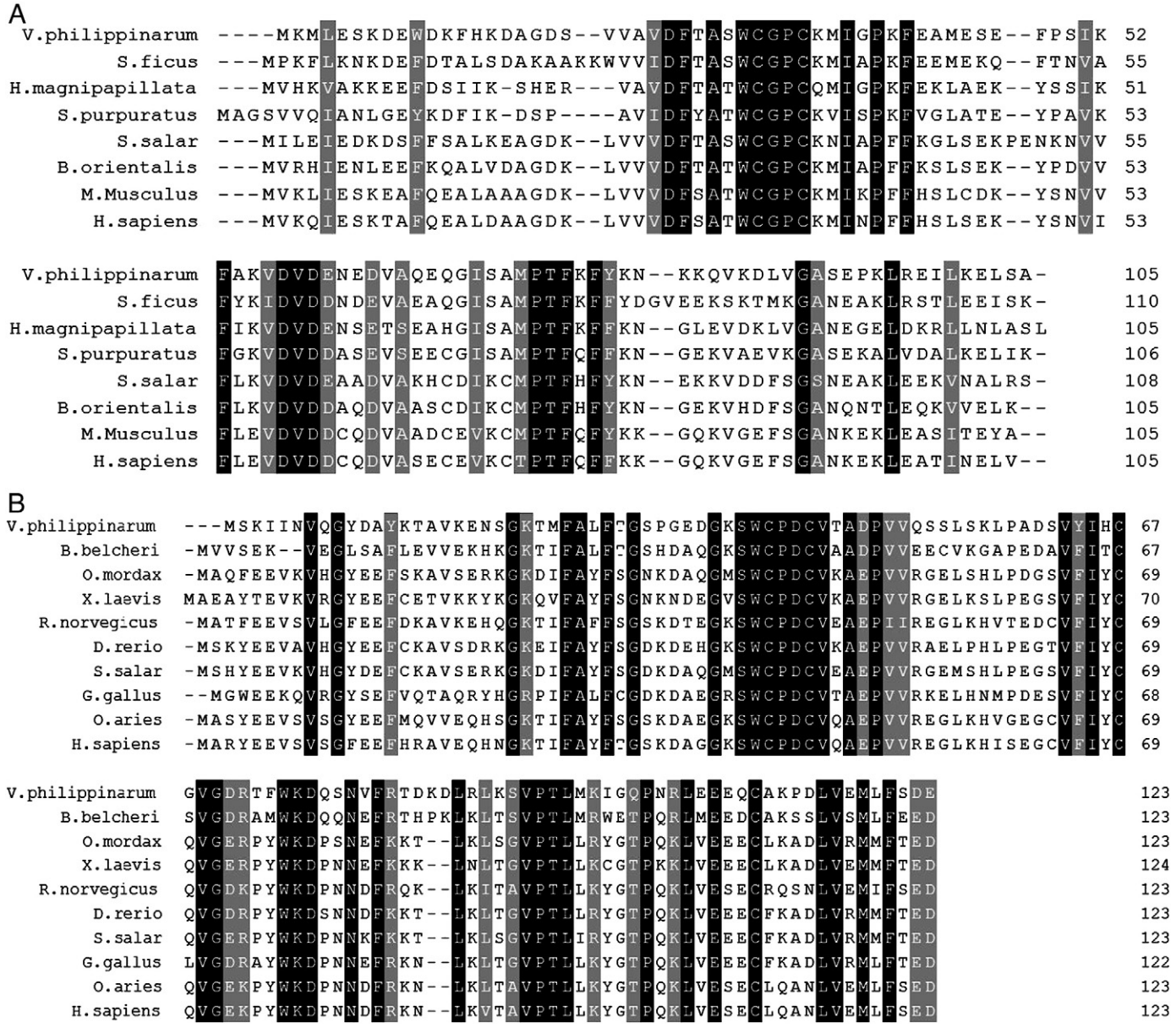

Fig. 2. Multiple alignment of VpTrx1 (A) and VpTrp14 (B) protein sequences with corresponding counterparts deposited in GenBank. The black shadow region indicates positions where all sequences share the same amino acid residue. Gaps are indicated by dashes to improve the alignment. The species and the GenBank accession no. are CAG25528.1 (*Suberites ficus*), XP_002157684.1 (*Hydra magnipapillata*), XP_787070.1 (*Strongylocentrotus purpuratus*), ACI69512.1 (*Salmo salar*), ACJ12082.1 (*Bombina orientalis*), AAH94415 (*Mus musculus*), AAF86466 (*Homo sapiens*), ABK63289.1 (*Branchiostoma belcheri*), ACO09547.1 (*Osmerus mordax*), NP_001134971.1 (*Salmo salar*), NP_001003456.1 (*Danio rerio*), NP_001089800.1 (*Xenopus laevis*), XP_415925.1 (*Gallus gallus*), NP_001099275.1 (*Rattus norvegicus*), NP_001156529.1 (*Ovis aries*) and NP_116120.1 (*Homo sapiens*) from GenBank.

sequences of VpTrx1 and VpTrp14 were deposited in GenBank under accession no. GQ384404 and HQ174261, respectively. The deduced amino acid sequences of VpTrx1 and VpTrp14 were shown below the corresponding nucleotide sequence in Fig. 1.

The complete sequence of VpTrx1 cDNA encoded a polypeptide of 105 amino acids with the predicted molecular weight of 11.85 kDa and the theoretical isoelectric point of 5.04. SMART program analysis revealed that VpTrx1 contained a typical Trx1 domain ranging from Glu^{5} to Leu^{103}. The full-length cDNA of VpTrp14 encoding a polypeptide of 123 amino acids with the predicted molecular weight of 13.68 kDa and the theoretical isoelectric point of 5.17. SMART program analysis revealed that VpTrp14 contained a Trx-like domain ranging from Asn^{6} to Asp^{122}.

3.2. Homology and phylogenetic analysis

Blast analysis of VpTrx1 protein revealed that it shared significant homology with Trx1s from hydra *Hydra magnipapillata* (XP_002157684.1, 55% identity), sea urchin *Strongylocentrotus purpuratus* (XP_787070.1, 55% identity), sponge *Suberites ficus* (CAG25528.1, 54% identity), amphioxus *Branchiostoma belcheri* (AAK72483.1, 52% identity) and salmon *Salmo salar* (ACI69512.1, 50% identity), while VpTrp14 displayed high similarity with Trp14s from *B. belcheri* (ABK63289.1, 56% identity), *Osmerus mordax* (ACO09547.1, 50% identity), *S. salar* (NP_001134971.1, 49% identity), *Danio rerio* (NP_001003456.1, 49% identity), *Xenopus laevis* (NP_001089800.1, 49% identity), *Gallus gallus* (XP_415925.1, 50% identity), *Rattus norvegicus* (NP_001099275.1, 47% identity) and *Homo sapiens* (NP_116120.1, 47% identity). Multiple alignments revealed that the C-G-P-C and C-P-D-C motif were highly conserved in all analyzed Trx1s (Fig. 2A) and Trp14s (Fig. 2B), respectively.

According to the phylogenetic tree (Fig. 3A), the Trx1 members from animals were mainly clustered into two groups. VpTrx1 was at first clustered with Trx1 from the invertebrate *S. ficus*, and then formed a sister group with Trx1 from cephalochordata and further grouped with those from vertebrates. The Trp14s from animals were also mainly clustered into two groups. VpTrp14 formed a sister group with Trp14 from cephalochordata and further grouped with the vertebrate Trp14s (Fig. 3B). The relationships displayed in the phylogenic trees were in good agreement with traditional taxonomy.

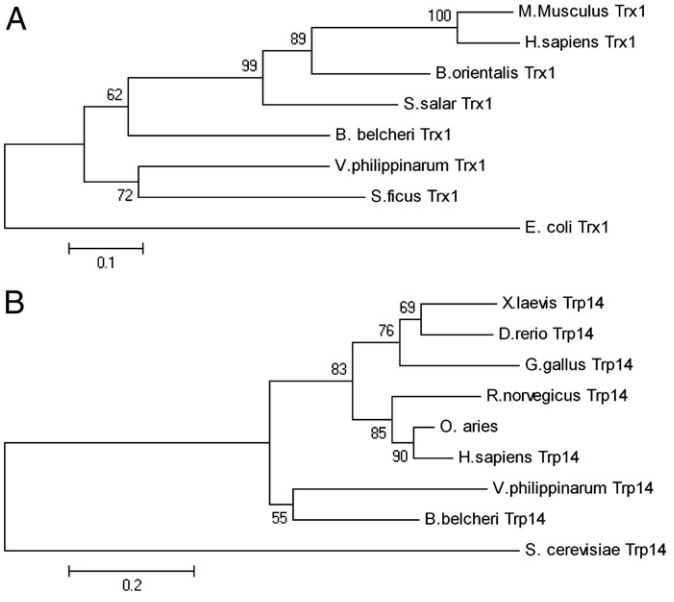

Fig. 3. Neighbor-joining (NJ) tree based on the amino acid sequences of Trx1 (A) and Trp14 (B) from different organisms. The numbers at the forks indicate the bootstrap. The species and the GenBank accession no. are as follows: CAG25528.1 (*Suberites ficus*), AAK72483.1 (*Branchiostoma belcheri*), ACI69512.1 (*Salmo salar*), ACJ12082.1 (*Bombina orientalis*), AAH94415 (*Mus musculus*), AAF86466 (*Homo sapiens*), YP 003046833.1 (*Escherichia coli*), ABK63289.1 (*Branchiostoma belcheri*), ACO09547.1 (*Osmerus mordax*), NP_001134971.1 (*Salmo salar*), NP_001003456.1 (*Danio rerio*), NP_001089800.1 (*Xenopus laevis*), XP_415925.1 (*Gallus gallus*), NP_001099275.1 (*Rattus norvegicus*), NP_001156529.1 (*Ovis aries*), NP_116120.1 (*Homo sapiens*) and NP_013468 (*Saccharomyces cerevisiae*).

3.3. Tissue-specific expression of VpTrx1 and VpTrp14

The tissue distribution of VpTrx1 and VpTrp14 mRNA was investigated by Quantitative Real-time PCR with β-actin as internal control. For VpTrx1, VpTrp14 and β-actin genes, there was only one peak at the corresponding melting temperature in the dissociation curve analysis, indicating that the PCR product was specifically amplified (data not shown). The mRNA transcripts of VpTrx1 and VpTrp14 were detected in all the examined tissues, including hepatopancreas, hemocytes, muscle, mantle and gills. The VpTrx1 transcript was dominantly detected in the tissue of hepatopancreas, and moderately expressed in gills, hemocytes and mantle (Fig. 4A). A similar tissue-specific expression pattern was also observed for VpTrp14 transcript with the highest expression level in the hepatopancreas, and moderate levels in mantle, hemocytes and gills (Fig. 4B).

3.4. Temporal expression profile of VpTrx1 and VpTrp14 mRNA in the hepatopancreas after Cd-exposure

For 10 μg/L Cd-treated group, the expression level of VpTrx1 transcript in the hepatopancreas increased after exposure for 24 h, then decreased at 48 and 96 h. When the concentration increased to 40 μg/L, the expression of VpTrx1 mRNA displayed a time-dependent pattern with a 2.2-fold increase after exposure for 96 h (Fig. 5A). However, no significant difference in the VpTrx1 expression level was observed between all treated groups and the control group ($P>0.05$).

As illustrated in Fig. 5B, in 10 μg/L Cd-exposed group, VpTrp14 mRNA expression level increased significantly at 24 and 48 h compared with that of the control group ($P<0.05$), and then decreased at 96 h. 40 μg/L Cd induced VpTrp14 mRNA expression significantly from 24 h to 96 h in a time-dependent manner ($P<0.05$). The peak expression level of VpTrp14 was detected with 3.6-fold increase at 96 h in 40 μg/L Cd-treated group. However, there was no significant difference between the two treated groups at the same exposure time.

3.5. Temporal expression profile of VpTrx1 and VpTrp14 mRNA in the hepatopancreas after Cu-exposure

In 10 μg/L Cu-treated group, the expression level of VpTrx1 slightly increased to *approx.* 3-fold of the control, however, no significant difference was observed between the treated and the control groups. When the concentration increased to 40 μg/L, the expression of VpTrx1 increased to 5.2-fold at 24 h after exposure, then decreased to 3.8-fold at 48 h, and reached maximum of 13.0-fold at 96 h after exposure. However, only the expression level of VpTrx1 at 96 h was significantly higher than that of the control ($P<0.05$) (Fig. 6A).

Significant increase of VpTrp14 expression was found at 24 h (8.4-fold higher than the blank, $P<0.05$) after 10 μg/L Cu exposures. After that, the expression of VpTrp14 transcript decreased to 4.0-fold and 3.8-fold of the control at 48 and 96 h, respectively. For 40 μg/L Cu-exposed group, the expression level of VpTrp14 was significantly induced from 24 to 96 h after exposure ($P<0.05$). The maximum expression level of VpTrp14 occurred at 96 h in 40 μg/L Cu-treated groups, with a value of 18.8-fold (Fig. 6B).

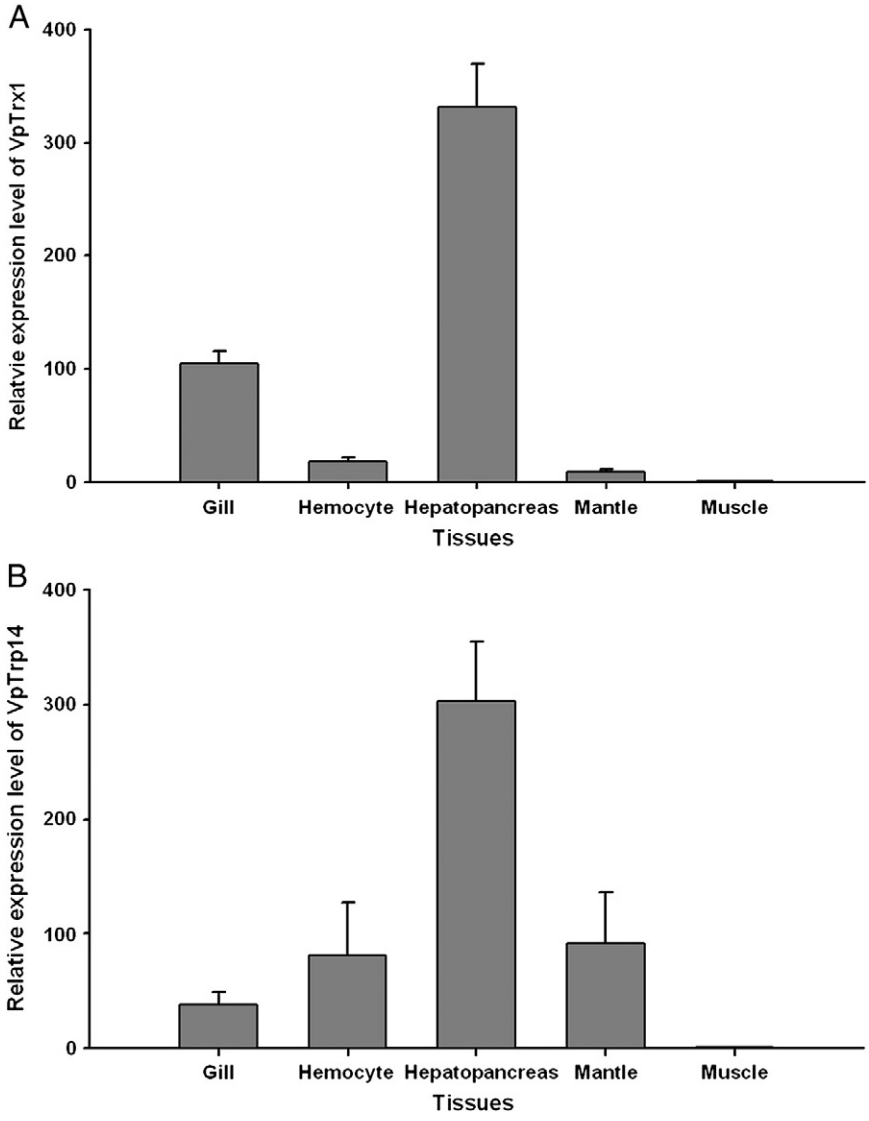

Fig. 4. VpTrx1 (A) and VpTrp14 (B) mRNA expression level in different tissues of adult clams detected by Real-time PCR. VpTrx1 (A) and VpTrp14 (B) transcript levels in hemocytes, gills, mantles, and hepatopancreas were normalized to that of adductor muscles, respectively. Each bar represented mean ± S.E. (n = 4). Significance across control was indicated with an asterisk at $P<0.05$.

4. Discussion

The thioredoxin system, mainly consisting of thioredoxin, thioredoxin reductase and NADPH, is a major antioxidant system to maintaining the intracellular redox state (Arner and Holmgren, 2000). Trx can serve as electron donors for enzymes such as thioredoxin peroxidases (peroxiredoxins, Prx) to counteract oxidative stress and can also scavenge ROS. The oxidized Trx is reduced by thioredoxin reductase to the reduced dithiol state (Tonissen and Di Trapani, 2009). Knowledge about Trx system in mollusks is still limited, and only a few reports have demonstrated that Trx system plays an important role in disk abalone *Haliotis discus discus* against physical stress, metal and peroxide exposure (De Zoysa et al., 2008; De Zoysa and Lee, 2009). Recently, thioredoxin reductase activity has been used as an antioxidant parameter in toxicological study of the mussel *Mytilus edulis* (Tedesco et al., 2010; Trevisan et al., 2011). However, little information about the role of Trx in maintaining redox homeostasis is available in bivalves. With this study, the existence of Trx1 and Trp14 in bivalves is first confirmed. In addition, Trx1 and Trp14 have the potential to be used in toxicological studies in marine invertebrates.

In this work, the full-length cDNAs of VpTrx1 and VpTrp14 were identified from Manila clam *V. philippinarum*, and they both possessed the conserved features critical for the fundamental structure and function of the Trx proteins. Most notable was the absence of other cysteine residues in VpTrx1 that was different from most typical Trx1s. However, similar phenomena were also observed in Trx1 from *S. ficus* and hypothetical Trx from *H. magnipapillata*. VpTrp14 possessed the conserved motif C-P-D-C and shared high identity with counterparts from amphioxus and human. All these characterization suggested that VpTrx1 and VpTrp14 were new members of the Trx1 and Trp14 family, respectively.

Like other Trx proteins, VpTrx1 and VpTrp14 were widely expressed in all tested tissues. The enrichment of VpTrx1 and VpTrp14 mRNA in the hepatopancreas indicated that hepatopancreas probably was the major site for pollutants uptake and possesses the highest concentrations of detoxifying enzymes in invertebrates (Livingstone, 1991). The expression levels of VpTrx1 and VpTrp14 were also relatively high in other organs, such as gills, hemocytes and mantles which were constantly confronted with environmental stress factors, suggesting their important roles in redox homeostasis in these organs.

In the present study, the expression levels of VpTrx1 and VpTrp14 in the hepatopancreas were up-regulated after exposure to high-level of metals, which indicated that both VpTrx1 and VpTrp14 expressions

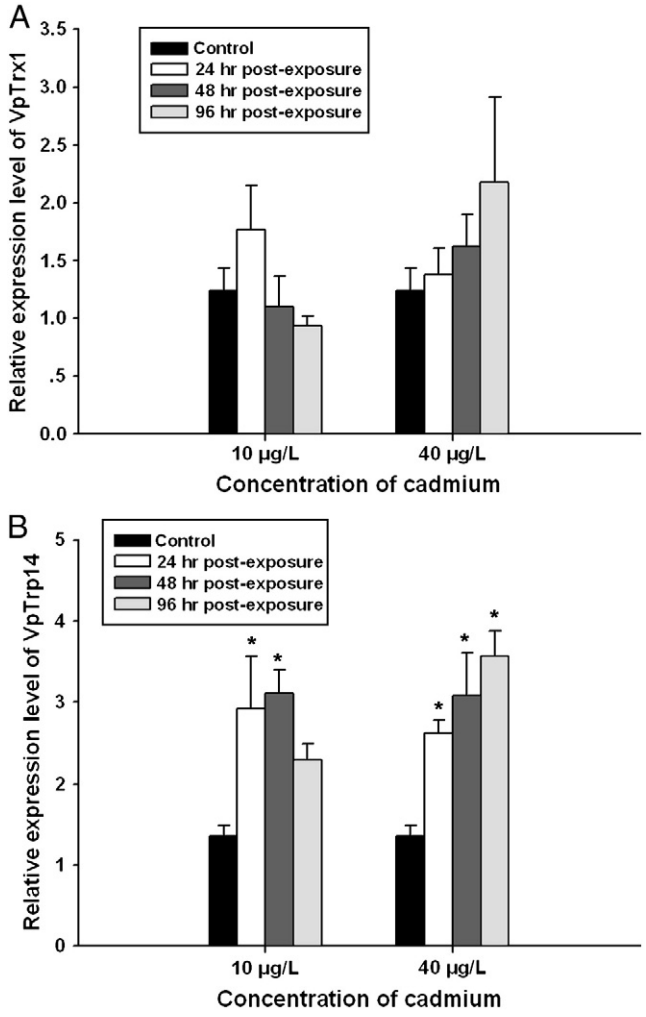

Fig. 5. Temporal expression profile of VpTrx1 (A) and VpTrp14 (B) mRNA in the hepatopancreas after Cd-exposure measured by Quantitative Real-time PCR. Each bar represented mean ± S.E. (n = 4). Significance across control was indicated with an asterisk at $P<0.05$.

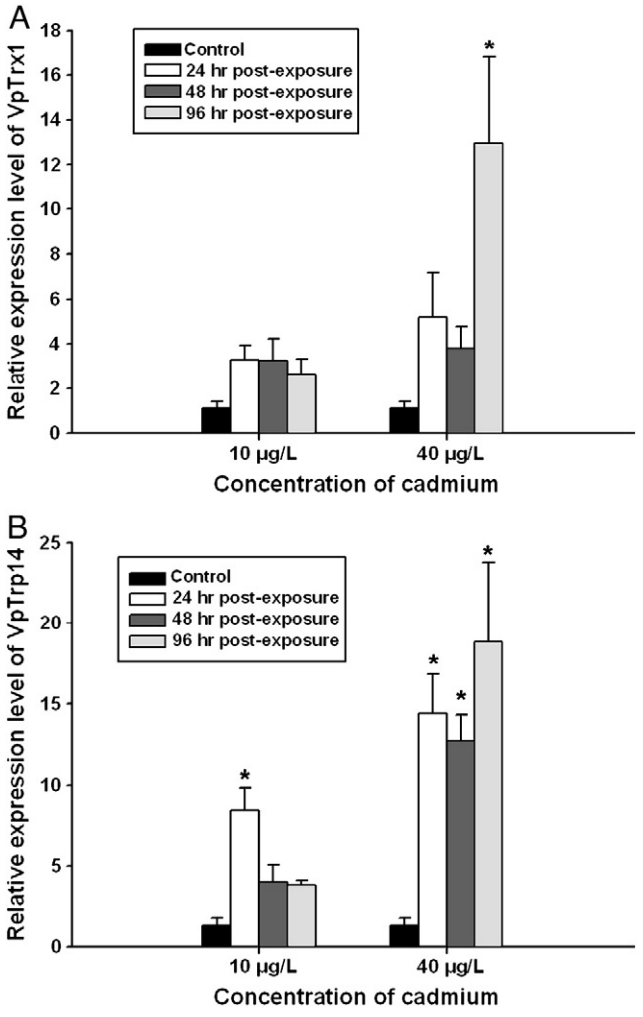

Fig. 6. Temporal expression profile of VpTrx1 (A) and VpTrp14 (B) mRNA in the hepatopancreas after Cu-exposure measured by Quantitative Real-time PCR. Each bar represented mean ± S.E. (n = 4). Significance across control was indicated with an asterisk at $P<0.05$.

responded to the metal stress sensitively. During the exposure period, the VpTrx1 and VpTrp14 transcripts firstly increased at 24 h and then decreased at 96 h in 10 μg/L Cd- or Cu-treated groups. This may be explained by the fact that ROS increased quickly after the metal stress (Viarengo et al., 1999; Gonzalez et al., 2010) and a rapid activation of VpTrx1 and VpTrp14 mRNA transcription was required to ensure the synthesis of Trxs. The ROS induced by low-level metal was easily eliminated by antioxidants including VpTrx1 and VpTrp14, so that both the transcripts decreased at 96 h. As concerned to 40 μg/L Cd- or Cu-stress, the expression level of VpTrx1 and VpTrp14 transcripts increased as the exposure time prolonged. It was suggested that high-level of metal exposure might induce so much ROS formation that the clam had to keep the VpTrx1 and VpTrp14 expression at a steady increasing level. Therefore, both VpTrx1 and VpTrp14 transcripts reached maximum at 96 h after exposure. Previous investigation on prokaryotes reported that the expression of *Bacillus subtilis* Trx was induced by selenium exposure (Garbisu et al., 1996). In unicellular green alga *Chlamydomonas reinhardtii*, the expression of Trx m and Trx h could also be induced by Cd exposure (Lemaire et al., 1999). These studies suggested that Trx and Trx-related proteins might be important to protect organisms from oxidative damage caused by heavy metals.

The differential expression level of VpTrx1 and VpTrp14 responding to metal stress indicated the oxidative stress induced by Cu might be more severe than that induced by Cd in *V. philippinarum*. Moreover, VpTrp14 expression was more sensitive than VpTrx1 expression to the metal stresses. This may be explained by the hypothesis that the disulfide reductase activity of VpTrp14 was higher than that of VpTrx1. It has been demonstrated that the specific activity of Trp14 was about four times than that of Trx1 in human (Jeong et al., 2004). The activity difference between Trx family members depended largely on the identity of the two XX residues, along with the protein context in which the C-X-X-C sequence occurs (Aslund et al., 1997; Mossner et al., 1999; Carvalho et al., 2006).

In conclusion, the full-length cDNAs encoding Trx1 and Trp14 were identified from *V. philippinarum*. VpTrx1 and VpTrp14 were constitutively expressed in all the tested tissues, and both transcripts were up-regulated after high-level metal stress in the hepatopancreas. In addition, VpTrp14 was more sensitive than VpTrx1 to the metal stresses. These results suggested that VpTrx1 and VpTrp14 perhaps played important roles in the antioxidant response against metal stress in *V. philippinarum*.

Acknowledgments

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References

Abele, D., Puntarulo, S., 2004. Formation of reactive species and induction of antioxidant defense systems in polar and temperate marine invertebrates and fish. Comp. Biochem. Physiol. Part A. 138, 405–415.

Aispuro-Hernandez, E., Garcia-Orozco, K.D., Muhlia-Almazan, A., Del-Toro-Sanchez, L., Robles-Sanchez, R.M., Hernandez, J., Gonzalez-Aguilar, G., Yepiz-Plascencia, G., Sotelo-Mundo, R.R., 2008. Shrimp thioredoxin is a potent antioxidant protein. Comp. Biochem. Physiol. Part C. 148, 94–99.

Arner, E.S., Holmgren, A., 2000. Physiological functions of thioredoxin and thioredoxin reductase. Eur. J. Biochem. 267, 6102–6109.

Aslund, F., Berndt, K.D., Holmgren, A., 1997. Redox potentials of glutaredoxins and other thiol-disulfide oxidoreductases of the thioredoxin superfamily determined by direct protein–protein redox equilibria. J. Biol. Chem. 272, 30780–30786.

Carvalho, A.P., Fernandes, P.A., Ramos, M.J., 2006. Similarities and differences in the thioredoxin superfamily. Prog. Biophys. Mol. Biol. 91, 229–248.

Chen, Y., Cai, J., Murphy, T.J., Jones, D.P., 2002. Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. J. Biol. Chem. 277, 33242–33248.

Das, K.C., Das, C.K., 2000. Thioredoxin, a singlet oxygen quencher and hydroxyl radical scavenger: redox independent functions. Biochem. Biophys. Res. Commun. 277, 443–447.

De Zoysa, M., Lee, J., 2009. Expression profiling of antioxidant and selected immune genes in the gill of disk abalone against thermal, low-salinity and hypoxic stresses. Comp. Biochem. Physiol. Part A. 154, S6–S16.

De Zoysa, M., Pushpamali, W.A., Whang, I., Kim, S.J., Lee, J., 2008. Mitochondrial thioredoxin-2 from disk abalone (*Haliotis discus discus*): molecular characterization, tissue expression and DNA protection activity of its recombinant protein. Comp. Biochem. Physiol. Part B 149, 630–639.

Dovzhenko, N.V., Kurilenko, A.V., Bel'cheva, N.N., Chelomin, V.P., 2005. Cadmium induced oxidative stress in the bivalve mollusk *Modiolus modiolus*. Russ. J. Mar. Biol. 31, 309–313.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using bootstrap. Evolution 39, 783–791.

Fernando, M.R., Nanri, H., Yoshitake, S., Nagata-Kuno, K., Minakami, S., 1992. Thioredoxin regenerate proteins inactivated by oxidative stress in endothelial cells. Eur. J. Biochem. 209, 917–922.

Franco, J.L., Trivella, D.B., Trevisan, R., Dinslaken, D.F., Marques, M.R., Bainy, A.C., Dafre, A.L., 2006. Antioxidant status and stress proteins in the gills of the brown mussel *Perna perna* exposed to zinc. Chem. Biol. Interact. 160, 232–240.

Garbisu, C., Ishii, T., Leighton, T., Buchanan, B.B., 1996. Bacterial reduction of selenite to elemental selenium. Chem. Geol. 132, 199–204.

Gonzalez, P.M., Abele, D., Puntarulo, S., 2010. Exposure to excess dissolved iron in vivo affects oxidative status in the bivalve *Mya arenaria*. Comp. Biochem. Physiol. Part C 152, 167–174.

Hirota, K., Nakamura, H., Masutani, H., Yodoi, J., 2002. Thioredoxin superfamily and thioredoxin-inducing agents. Ann. N. Y. Acad. Sci. 957, 189–199.

Holmgren, A., 1985. Thioredoxin. Annu. Rev. Biochem. 54, 237–271.

Isani, G., Monari, M., Andreani, G., Fabbri, M., Carpenè, E., 2003. Effect of copper exposure on the antioxidant enzymes in bivalve mollusc *Scapharca inaequivalvis*. Vet. Res. Commun. 27, 691–693.

Jeong, W., Yoon, H.W., Lee, S.R., Rhee, S.G., 2004. Identification and characterization of TRP14, a thioredoxin-related protein of 14 kDa. New insights into the specificity of thioredoxin function. J. Biol. Chem. 279, 3142–3150.

Jeong, W., Jung, Y., Kim, H., Park, S.J., Rhee, S.G., 2009. Thioredoxin-related protein 14, a new member of the thioredoxin family with disulfide reductase activity: implication in the redox regulation of TNF-α signaling. Free Radic. Biol. Med. 47, 1294–1303.

Jiang, W.S., Pohlmann, T., Sundermann, J., Feng, S.Z., 2000. A modeling study of SPM transport in the Bohai Sea. J. Marine Syst. 24, 175–200.

Jiang, S., Zhang, S., Vuthiphandchai, V., Nimrat, S., 2007. Human TRP14 gene homologue from amphioxus *Branchiostoma belcheri*: identification, evolution, expression and functional characterization. J. Anat. 210, 555–564.

Jikimoto, T., Nishikubo, Y., Koshiba, M., Kanagawa, S., Morinobu, S., Morinobu, A., Saura, R., Mizuno, K., Kondo, S., Toyokuni, S., Nakamura, H., Yodoi, J., Kumagai, S., 2002. Thioredoxin as a biomarker for oxidative stress in patients with rheumatoid arthritis. Mol. Immunol. 38, 765–772.

Kumar, S., Dudley, J., Nei, M., Tamura, K., 2008. MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief. Bioinform. 9, 299–306.

Lemaire, S., Keryer, E., Stein, M., Schepens, I., Issakidis-Bourguet, E., Gérard-Hirne, C., Miginiac-Maslow, M., Jacquot, J.P., 1999. Heavy-metal regulation of thioredoxin gene expression in *Chlamydomonas reinhardtii*. Plant Physiol. 120, 773–778.

Letunic, I., Doerks, T., Bork, P., 2009. SMART 6: recent updates and new developments. Nucleic Acids Res. 37, D229–D232.

Li, C., Sun, H., Chen, A., Ning, X., Wu, H., Qin, S., Xue, Q., Zhao, J., 2010. Identification and characterization of an intracellular Cu, Zn-superoxide dismutase (icCu/Zn-SOD) gene from clam *Venerupis philippinarum*. Fish Shellfish Immunol. 28, 499–503.

Liang, L.N., He, B., Jiang, G.B., Chen, D.Y., Yao, Z.W., 2004. Evaluation of molluscs as biomonitors to investigate heavy metal contaminations along the Chinese Bohai Sea. Sci. Total Environ. 324, 105–113.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Livingstone, D.R., 1991. Towards a specific index of impact by organic pollution for marine invertebrates. Comp. Biochem. Physiol. Part C 100, 151–155.

Mao, T.Y., Dai, M.X., Peng, S.T., Li, G.L., 2009. Temporal-spatial variation trend analysis of heavy metals (Cu, Zn, Pb, Cd, Hg) in Bohai Bay in 10 years. J. Tianjin Univ. (Sci. Technol.). 9, 817–825 (in Chinese with English abstract).

Matsuo, Y., Hirota, K., Nakamura, H., Yodoi, J., 2002. Redox regulation by thioredoxin and its related molecules. Drug News Perspect. 15, 575–580.

Mossner, E., Huber-Wunderlich, M., Rietsch, A., Beckwith, J., Glockshuber, R., Aslund, F., 1999. Importance of redox potential for the in vivo function of the cytoplasmic disulfide reductant thioredoxin from *Escherichia coli*. J. Biol. Chem. 274, 25254–25259.

Mu, C., Zhao, J., Wang, L., Song, L., Song, X., Zhang, H., Qiu, L., Gai, Y., Cui, Z., 2009. A thioredoxin with antioxidant activity identified from *Eriocheir sinensis*. Fish Shellfish Immunol. 26, 716–723.

Nakamura, H., 2005. Thioredoxin and its related molecules: update 2005. Antioxid. Redox Signal. 7, 823–828.

Peng, S.T., Hu, Y.D., Bai, Z.P., 2009. Pollution assessment and ecological risk evaluation for heavy metals in the sediments of Bohai Bay. J. Waterw. Harbour 1, 57–60 (in Chinese with English abstract).

Ren, Q., Zhang, R.R., Zhao, X.F., Wang, J.X., 2010. A thioredoxin response to the WSSV challenge on the Chinese white shrimp, *Fenneropenaeus chinensis*. Comp. Biochem. Physiol. Part C 151, 92–98.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., Ichijo, H., 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal regulating kinase (ASK) 1. EMBO J. 17, 2596–2606.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Tedesco, S., Doyle, H., Blasco, J., Redmond, G., Sheehan, D., 2010. Exposure of the blue mussel, *Mytilus edulis*, to gold nanoparticle and the pro-oxidant menadione. Comp. Biochem. Physiol. Part C. 151, 167–174.

Tonissen, K.F., Di Trapani, G., 2009. Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. Mol. Nutr. Food Res. 53, 87–103.

Trevisan, R., Mello, D.F., Fisher, A.S., Schuwerack, P.M., Dafre, A.L., Moody, A.J., 2011. Selenium in water enhances antioxidant defenses and protects against copper-induced DNA damage in the blue mussel *Mytilus edulis*. Aquat. Toxicol. 101, 64–71.

Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., Blasco, J., 1999. Role of metallothionein against oxidative stress in mussel *Mytilus galloprovincialis*. Am. J. Physiol. 227, 1612–1619.

Watson, W.H., Yang, X., Choi, Y.E., Jones, D.P., Kehrer, J.P., 2004. Thioredoxin and its role in toxicology. Toxicol. Sci. 78, 3–14.