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RESEARCH ARTICLE

Response of the freshwater diatom *Halamphora veneta* (Kützing) Levkov to copper and mercury and its potential for bioassessment of heavy metal toxicity in aquatic habitats

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Abstract This study investigates the effects of copper and mercury on growth rate, chlorophyll a content, superoxide dismutase (SOD) activity, SOD mRNA gene expression, and frustule morphology of the benthic freshwater diatom *Halamphora veneta* (Kützing) Levkov and the potential utility of each for toxicity assessment in aquatic habitats. Results showed the following: (1) Compared to mercury, exposure to copper resulted in greater growth inhibition of *H. veneta* even at low concentrations and after short durations of exposure; (2) high accumulation of chlorophyll a in *H. veneta* is a stress response to the presence of heavy metals; (3) SOD activity and SOD gene expression varied in *H. veneta* according to the concentration, exposure time, and type of heavy metal; and (4) exposure to mercury resulted in deformity in the shape and an increase in size of the frustule of *H. veneta.* Growth rate, chlorophyll a content, SOD activity and gene expression, and frustule morphology of *H. veneta* are all potential candidates for the toxicological assessment of copper and mercury in aquatic habitats.

Keywords Chlorophyll a content · Frustule morphology · Growth rate · Superoxide dismutase activity · SOD gene expression

Weijie Mu and Kun Jia contributed equally to this work.

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Introduction

Heavy metals of pollutants of global importance are derived from numerous sources including industrial processes, mining, transport, agricultural, and urban activities (Ouyang et al. 2017). The toxicity of heavy metals to aquatic animals is well documented (Mzimela et al. 2003; Zhang et al. 2005), and in recent years, increasing attention is being paid to the development of protocols for environmental risk assessment of water environments (Fang et al. 2016; Marks et al. 2017; Zhang et al. 2017).

Copper (Cu) is a necessary micronutrient, which plays important functional roles in the biochemistry of aquatic organisms, but becomes toxic at high concentrations (Ahsan et al. 2007; Castruita et al. 2011). Mercury is considered one of the most dangerous metals in the environment (Goyer et al. 1995), mainly because it can accumulate in biological food chains and ultimately affected human health (Campbell et al. 2005; MacDougal et al. 1996; Raimundo et al. 2014). Copper and mercury contamination in the aquatic environment is mostly derived from industrial processes and anthropogenic activities (Ciji and Bijoy Nandan 2014; Nriagu and Pacyna 1988). The development of rapid and efficient biological monitoring technology to evaluate the effects of heavy metal contamination is an urgent scientific problem needed to be solved (Dai et al. 2014; Torres et al. 2008). It is showed that the nontarget organisms especially microalgae were frequently useful in providing information on the physicochemical characteristics of the aquatic environment (Celekli et al. 2017).

Microalgae exhibit various physiological and biochemical responses to changes in water quality (Leung et al. 2017). These include inhibition of growth and damage to photosynthetic pigments including chlorophyll a. It has long been known that such changes can be used as indicators of environmental stress (Allen et al. 1983). In addition, the heavy

metal pollutants can induce oxidative stress by generating reactive oxygen species (ROS) in microalgae. For example, Choudhary et al. (2007) reported that heavy metal stress is closely associated with the induction of oxidative stress biomarkers in *Spirulina*. Pollutants in the environment can lead to the inducement or inhibition of the antioxidant enzyme activity in aquatic organisms; therefore, such enzymes can be used as biomarkers of the toxicity of these pollutants (Lionetto et al. 2003; Monserrat et al. 2007). Superoxide dismutase (SOD) is an enzyme which can be induced by exogenous pollutants such as heavy metals and pesticide and has been used as a biomarker to detect oxidative stress in plants (Ibrahim et al. 2014; Piotrowska-Niczyporuk et al. 2012). Furthermore, morphological changes in algae can also be used as indicators of environmental pollution (Adshead-Simonsen et al. 1981; Cattaneo et al. 2004).

Diatoms are abundant in terrestrial, freshwater, and marine habitats and are a predominant group of microalgae (Torres et al. 2008). They play important roles in the food chain as primary producers, thereby sustaining food webs (Armbrust et al. 2004). Due to their cosmopolitan distribution, short life cycles, and rapid response to environmental perturbations, they are commonly employed in ecotoxicological and environmental studies for evaluating the effects of chemicals and other stressors in the marine environment (Falasco et al. 2009; Guo et al. 2013; Pandey et al. 2014).

Regarding the diatoms' toxic responses to a variety of heavy metals, there are several reports available from physiological endpoints such as growth, photosynthetic efficiency, and antioxidant enzyme activity. For example, cadmium exposure can lead to inhibition of cell growth in *Phaeodactylum tricornutum* (Torres et al. 2000). Increasing heavy metal concentrations can result in a decrease in chlorophyll and induce cellular superoxide dismutase in *Odontella mobiliensis*, *Nitzschia palea*, and *Chaetoceros calcitrans* (Anu et al. 2016; Branco et al. 2010; Manimaran et al. 2012). At the molecular level, it has been reported that exposure to heavy metals including copper and nickel can induce the stress-associated biomarker HSP70/90 in *Ditylum brightwellii* (Guo et al. 2013). Diatoms subjected to heavy metal stress also exhibit morphological changes, including changes in valve outline, striae pattern, costae and septae modification of the raphe, and raphe canal pattern (Gautam et al. 2017). These morphological changes can be excellent specific indicators of metal contamination (Cattaneo et al. 2004). Benthic diatoms in particular have been used in a number of monitoring studies and for a variety of different stressors (Bellinger et al. 2006; Szczepocka and Szulc 2009). However, information regarding the interacting effect of copper or mercury on benthic diatoms is still limited.

Halamphora veneta is a widely distributed freshwater benthic diatom that can be easily cultured in the laboratory (Dedić et al. 2015; Novais et al. 2014). In this study, *H. veneta* was used as a test organism to elucidate the toxicity response of copper and mercury exposure under different treatment concentrations. An integrative measurement of growth, photosynthetic efficiency, enzyme activity, molecular responses, and morphological change was employed to reveal the biochemical defense mechanisms used by this diatom to cope with heavy metal stress. We also attempted to evaluate the best potential biomarkers in diatoms for risk assessment in aquatic ecosystems.

Materials and methods

Diatom species and culture conditions

H. veneta was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences and cultured by the Aquatic Laboratory, Harbin Normal University, China, as follows: Cells of *H. veneta* were inoculated into 500 mL sterile flasks containing 200 mL modified CSI medium (Watanabe et al. 1988) and incubated at 20 ± 1 °C with 12:12 h light-dark photoperiod using a cool-white fluorescent light (4000 lx/cm^2). The composition of nitrate, phosphate, silicate, trace metals, and vitamins used in the CSI medium is presented in Table 1. All the flasks were shaken three times per day to keep the diatoms under good growth conditions (Foster et al. 2008). Diatoms in the exponential growth phase were harvested for the following tests.

Toxicity treatments

H. veneta cells were exposed to Cu (0, 0.1, 0.2, 0.5, 1, and 2 mg L^{-1}) and Hg (0, 0.1, 0.5, 1, 2, and 5 mg L^{-1}), with each dose series being tested at four time intervals (24, 48, 72, and 96 h). Each treatment was performed in triplicate in 500-mL

Table 1 Chemical composition of CSI medium

Components	Final concentrations in medium
$Ca(NO_3)\cdot 4H_2O$	150 g L^{-1}
KNO_3	100 g L^{-1}
$MgSO_4\cdot 7H_2O$	40 g L^{-1}
β-Na_2 glycerophosphate$\cdot 5H_2O$	25 g L^{-1}
Vitamin B_{12}	0.1 μg L^{-1}
Soil leachate solution	30 ml L^{-1}
Na_2EDTA	0.75 g L^{-1}
$FeCl_3\cdot 6H_2O$	0.097 g L^{-1}
$ZnCl_2\cdot 7H_2O$	0.05 g L^{-1}
$CoCl_2\cdot 6H_2O$	0.002 g L^{-1}
$MnCl_2\cdot 4H_2O$	0.041 g L^{-1}
$Na_2Mo_4O_4\cdot 2H_2O$	0.004 g L^{-1}

flasks. During the acute toxicity experiment, the growth inhibition rates, chlorophyll a content, SOD activity, and gene expression of *H. veneta* were measured at each time interval.

Growth rate test

Samples of 250 mL were taken at each time interval (24, 48, 72, and 96 h). Optical density values were measured using UV-1700 UV/vis spectrophotometer (Unico Instrument, Shanghai, China) at 540 nm. The growth rate was calculated by the logistic equation (Wang et al. 2010):

$$\mu_{i-j} = \left(\ln X_j - \ln X_i\right)/t_j - t_i \ \left(\text{day}^{-1}\right)$$

where μ_{i-j} is the average growth rate from time i to j, X_i is the optical density at time i, and X_j is the optical density at time j.

Determination of chlorophyll a content

Measurements of chlorophyll a (Chl a) content were applied as previously described (Harborne 1998). In brief, triplicates of 8 mL blended cultures were centrifuged at 4000×*g* for 10 min every 24 h. After the supernatant was removed, 5 mL acetone was added to the retentate, and the mixture was ultrasonically disrupted for about 30 min and placed in the dark at 4 °C for 24 h. The methanol-extracted samples were centrifuged at 4000×*g* for 10 min and the supernatants were transferred into tubes. The absorbance value at 646 and 663 nm were measured. The Chl a content was computed by the following equation:

$$\text{Chl a}\left(\text{mg L}^{-1}\right) = 12.21A_{663} - 2.81A_{646}$$

where A_{663} is the absorbance value at 646 nm and A_{646} is the absorbance value at 646 nm.

Assay of total superoxide dismutase content

Total superoxide dismutase (T-SOD) activity (including Mn-SOD and CuZn-SOD) was determined according to Ji (1991), using an enzymatic method by using the SOD assay kit (Nanjing Jiancheng, China). At each time interval and each treatment with heavy metals, 200 mL of cultured diatoms was centrifuged at 6000×*g* for 5 min, and then diatoms were washed in 0.1 mol/L PBS buffer. Assay conditions were 65 μmol phosphate buffer, pH 7.8, 1 μmol hydrochloric hydroxylamine, 0.75 μmol xanthine, and 2.3×10^{-3} IU xanthine dismutase. One hundred microliters of the supernatant was incubated in the system for 40 min at 37 °C, and the reaction was terminated with 2 mL 3.3 g L^{-1} *p*-aminobenzene sulfonic acid and 10 g L^{-1} naphthylamine. An SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation (monitored at 550 nm) by 50%.

The SOD activity was calculated as the following equation:

$$A_{\text{SOD}}\left(\text{U mg prot}^{-1}\right) = (\text{absorbance at 550 nm of the control} - \text{absorbance at 550 nm of the sample})$$
$$/\text{absorbance at 550 nm of the control}/50\% \times \text{total volume of the reaction}$$
$$/\text{the volume of sample}/\text{the protein concentration of the sample}\left(\text{mg prot mL}^{-1}\right)$$

Quantitative analysis of the messenger RNA expression of superoxide dismutase enzymes by real-time PCR

After exposure to heavy metals, cells of the treated and control groups were harvested, and the total RNA was extracted from the diatoms by TranZol Up (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. The total RNA integrity was confirmed using 1% formaldehyde/agarose gel with ethidium bromide (EtBr) staining and UV transilluminator (Bio-Rad, USA). The quality of RNA samples was determined by using Beckman DU800 spectrophotometric measurements of the ratio of absorbance 260 and 280 nm ($A_{260/280}$) in a range from 1.8 to 2.0. The cDNA was synthesized from 1 μg of the total RNA, using a kit of Transcript One-Step gDNA Removal and cDNA Synthesis Supermix (TransGen Biotech, Beijing, China). Real-time PCR assays (25 μL) of each individual sample were run in triplicate wells using UltraSYBR Mixture (with ROX) on an Applied Biosystems 7500 Fast Real-Time PCR System (ABI 7500, USA). The PCR cycling conditions comprised an initial polymerase activation step of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 10 min. Primers of *sod* and *18s* are shown in Table 2. The housekeeping gene *18s* was used as an internal standard. To confirm the primer specificity, melting curve analysis of amplification products was performed at the end of each PCR reaction to ensure that only one PCR product was amplified and detected. The relative expression levels of different genes were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Scanning electron microscopy

To study the morphological changes induced by heavy metals, *H. veneta* was exposed to copper and mercury at concentrations of 1.5 and 4.8 mg L^{-1} (i.e. their own 96 h EC_{50}),

Table 2 Primers used for real-time reverse transcription polymerase chain reaction assessment of superoxide dismutase gene expression in *H. veneta*

Gene	Oligo name	Sequence (5′–3′)	Amplicon size (bp)
18S	RT-F	TTCCACCACGATAAGCACCAC	145
	RT-R	GAGGTAGTAAGCGTGTTCCCA	
SOD	RT-F	TCTTACTTACGACTACGCCTCCCTC	284
	RT-R	CAGAAAAGAGTGTGGTTCAAGTGGC	

respectively. The diatoms were cleaned by acid treatment, then cells were harvested by centrifugation at 2500×*g* for 5 min, and frustules were washed by centrifugation several times with ultrapure water. The samples were sputter-coated with gold/palladium and viewed under a Hitachi S-4800 scanning electron microscope at 20 kV.

Statistical analysis

All experiments were performed in triplicate and results were presented as mean value ± standard deviation (SD). The experimental data was analyzed by SPSS version 18.0. The EC_{50} values with 95% confidence intervals were calculated by probit analysis. One-way ANOVA with Tukey's test was used to determine whether the outcomes were significantly different from the control treatments without heavy metals ($p < 0.05$).

Results

Influence of heavy metal on *H. veneta* growth rates

Exposure of *H. veneta* to copper and mercury induced changes in growth rate during the assay. Figure 1a shows the effects of copper on the growth rates of *H. veneta*. A clear dose-response trend was observed during the 96-h exposure. Compared with controls, growth rates were significantly lower in all tested treatment concentrations at 24 and 48 h of exposure ($p < 0.05$). In 0.1, 0.2, 0.5, 1, and 2 mg L^{-1} copper treatments, growth rates decreased by 28.2, 41.6, 43.8, 45.0, and 70.1% at 24 h and by 47.9, 58.0, 46.6, 47.1, and 64.8% at 48 h compared with the control group, respectively. However, after 48 h exposure, the growth rate of *H. veneta* was significantly lower in the treatments with low concentrations relative to the controls (0.1, 0.2 mg L^{-1}), and there was no significant difference between the other concentrations at 48 and 96 h ($p < 0.05$). Figure 1b illustrates the effects of mercury on the growth rates of *H. veneta*. In 2 and 5 mg L^{-1} mercury treatments, growth of *H. veneta* was lower compared with the controls at 24 h exposure ($p < 0.05$). There was no significant difference between the controls and the treatments with mercury at 48 h exposure ($p < 0.05$). After 72 h exposure, mercury significantly inhibited the growth of *H. veneta* in all treatment concentrations ($p < 0.05$).

Effects of heavy metal on chlorophyll a content

The effects of copper and mercury on the chlorophyll a content differed in *H. veneta*. As shown in Fig. 2a, the Chl a content of *H. veneta* was significantly increased at higher concentrations of copper (0.2, 0.5, and 1 mg L^{-1}), at 24 and 96 h exposure, compared to the control group. The Chl a content was 3.6, 3.12, and 2.65 mg L^{-1} in 0.2, 0.5, and 1 mg L^{-1} copper treatments, respectively, which were higher by 68.2, 45.8, and 19.2%, respectively, compared with that of the controls at 24 h exposure. In addition, Chl a content was 3.93 mg L^{-1} in 0.2 mg L^{-1} copper, significantly increased by 47.2% compared with that of the controls at 96 h exposure (Fig. 2a, $p < 0.05$). Meanwhile, the Chl a content of *H. veneta* was significantly lower at higher concentrations of copper (e.g., 1 mg L^{-1}) at 48 and 72 h. Figure 2b indicates that the Chl a content of *H. veneta* was obviously affected by mercury at higher concentration during 96 h exposure relative to the controls. But the Chl a content in 0.1 mg L^{-1} mercury treatment was significantly higher than that of the controls at 72 and 96 h exposure. The Chl a content in 0.1 mg L^{-1} mercury treatment was significantly higher, i.e., by 33.1 and 69.3%, compared with that of the controls at 72 and 96 h exposure, respectively (Fig. 2b, $p < 0.05$).

Effects of heavy metal on SOD enzyme activities

SOD activities increased gradually with increasing treatment concentrations (Fig. 3). For the 0.5 and 2 mg L^{-1} copper treatments, SOD activity was higher by 92.5 and 57.6%, respectively, compared to the control at 24 h exposure (Fig. 3a, $p < 0.05$). At 48 h, SOD activity was higher in all the tested concentrations, i.e., by 95.4, 110.1, 97, 91, and 105.8% compared with the control at 0.1, 0.2, 0.5, 1, and 2 mg L^{-1} copper, respectively. SOD activity in *H. veneta* did not increase significantly in copper treatments at 72 h ($p < 0.05$) but was significantly higher at 96 h, i.e., by 70.7 and 57.8% in 0.1 and 0.2 mg L^{-1} copper treatments ($p < 0.05$), respectively, relative to the controls (Fig. 3a). As shown in Fig. 3b, mercury caused a significant increase of SOD activity, by 32.7, 36.9, and 38.9% in 0.1, 0.5, and 5 mg L^{-1}, respectively, at 24 h; by 39.8, 79.3, and 51.3% in 0.1, 0.5, and 2 mg L^{-1}, respectively, at 48 h; and by 54.7 and 37.3% in 0.1 mg L^{-1} at 72 and 96 h exposure, respectively ($p < 0.05$, Fig. 3b). By contrast, SOD activity was significantly lower, i.e., by 73.4 and 55.6% in

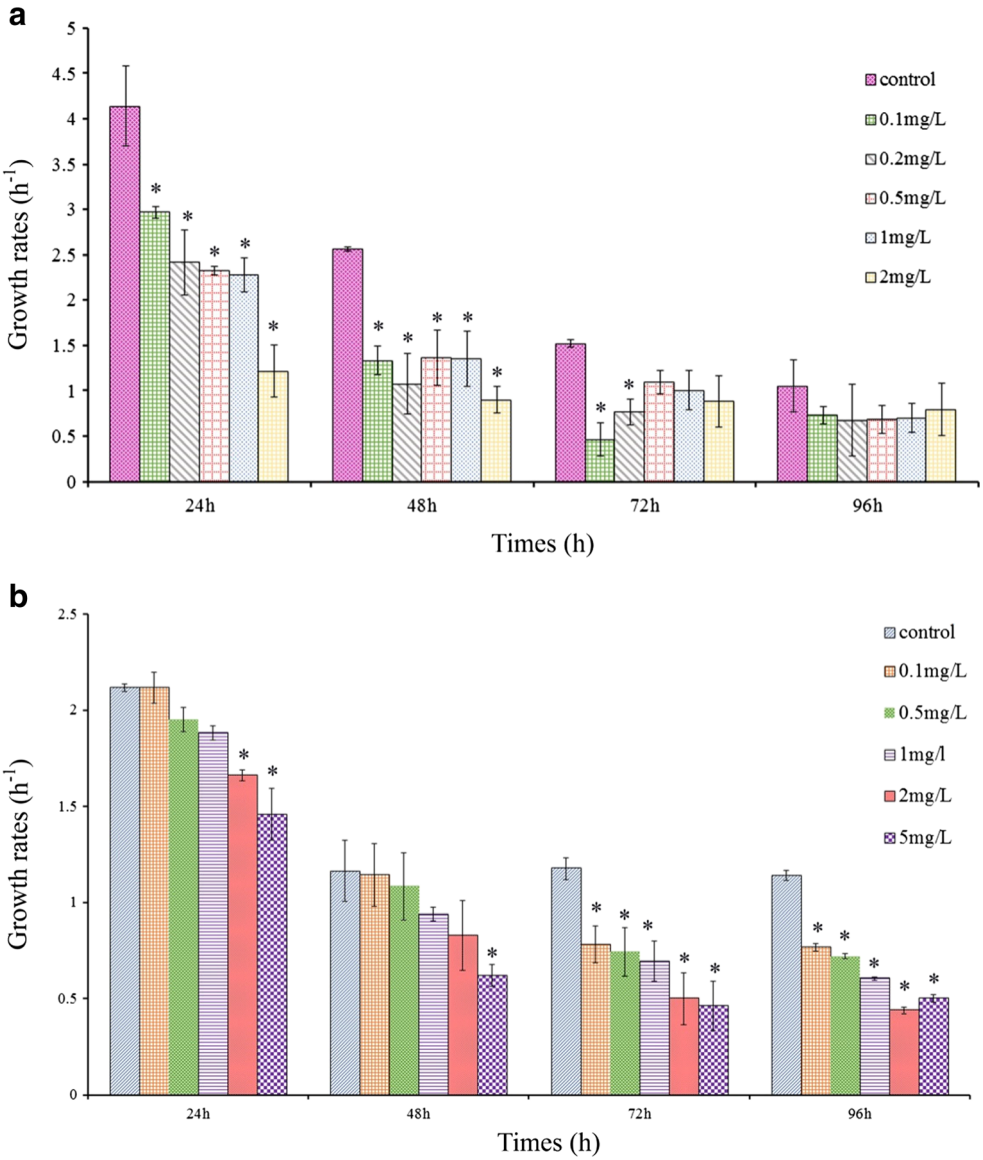

Fig. 1 Growth rates of *Halamphora veneta* after 96 h exposure to different concentrations of copper (**a**) and mercury (**b**)

5 mg L^{-1}, at 72 and 96 h mercury exposure, respectively ($p < 0.05$, Fig. 3b).

Messenger RNA relative expression levels of SOD

The effects of copper on SOD messenger RNA (mRNA) expression were significantly decreased in *H. veneta* during 96 h exposure, whereas SOD expression level increased in lower concentrations of mercury with increasing times of exposure (Fig. 4). SOD mRNA levels decreased rapidly and were statistically lower than controls after 96 h of Cu exposure ($p < 0.01$), except the concentration of 0.5 mg L^{-1} (Fig. 4a). In contrast to copper treatment, the SOD mRNA levels after 96 h of Hg exposure at different time points under different concentrations did not show a trend of change (Fig. 4b). At 24 h from the beginning of the exposure, mRNA accumulation was temporarily higher than controls at 0.5, 2, and 5 mg L^{-1} ($p < 0.01$). Then, SOD mRNA levels significantly increased over the controls after 48 h of Hg exposure at the lower concentration of 0.1 mg L^{-1} ($p < 0.01$). In addition, significantly decreased SOD mRNA levels compared to the controls after 48 h of Hg exposure were observed at 0.5, 1, and 5 mg L^{-1} ($p < 0.01$). At 72 h exposure, mRNA accumulation was temporarily higher than controls at 1 and 2 mg L^{-1}, lower than the controls at 5 mg L^{-1} ($p < 0.01$). At 96 h exposure, the mRNA level was significantly higher than the controls at 2 mg L^{-1} ($p < 0.01$).

Morphological changes after heavy metal treatments

The *H. veneta* cells in control cultures had a semilanceolate, or slightly concave, shape with both ends narrowly rounded or slightly curved inward (Fig. 5). Cells treated with copper were not conspicuously deformed (Fig. 6). However, after mercury

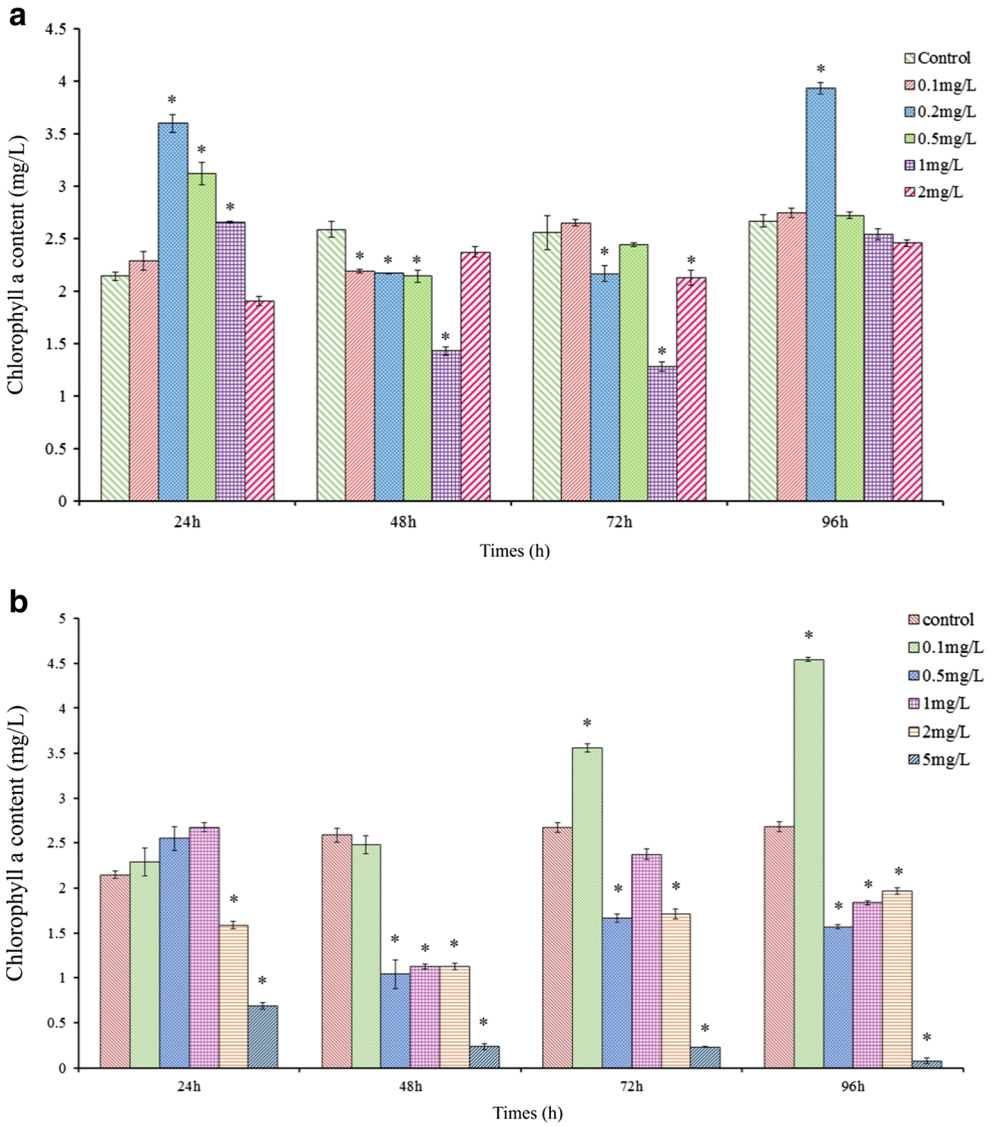

Fig. 2 Chlorophyll a content of *Halamphora veneta* after 96 h exposure to different concentrations of copper (**a**) and mercury (**b**)

treatment, the frustules were enlarged and irregularly deformed with the dorsal margin bulging, the ventral margin concave, and both ends slightly rounded (Fig. 7).

Discussion

Because heavy metals can affect the growth rate of diatoms, it has been suggested that this function can be used as an indicator of heavy metal pollution in water (Kim et al. 2013; Leung et al. 2017). Copper and mercury are common pollutants in many aquatic environments, especially those which are surrounded by densely populated areas (Anu et al. 2016; Mico et al. 2006; Nolde 2007). The toxicity of these two heavy metals to microalgae, such as the marine diatom (*C. calcitrans*) and the green algae (*Tetraselmis chuii* and *Chlorella sorokiniana*), has previously been reported (Anu et al. 2016; Davarpanah and Guilhermino 2015; Gomez-Jacinto et al. 2015). However, there are relatively few data on the acute effects of copper and mercury to freshwater benthic diatoms although Johnson et al. (2007) found that the growth of *Nitzschia closterium* was acclimated in culture medium containing 5 or 25 μg L^{-1} copper. In the present study, the growth rate of *H. veneta* showed differing responses to copper and mercury. It is noteworthy that the concentration of copper in this study was in the range 0.1–2 mg L^{-1}, which was much greater than that used previously for acclimating *N. closterium* (Johnson et al. 2007). One reason for this was that *H. veneta* could not be fully adapted to copper stress in the present work. Furthermore, the sensitivity of *H. veneta* to copper varied with the duration of exposure, the growth rate being significantly lower after 24 and 48 h compared with the control group. This means that copper, at initial treatment time, will give rise to a greater growth inhibition compared with those exposed for longer durations, thereby disrupting the function of the diatoms from the outset. This finding is

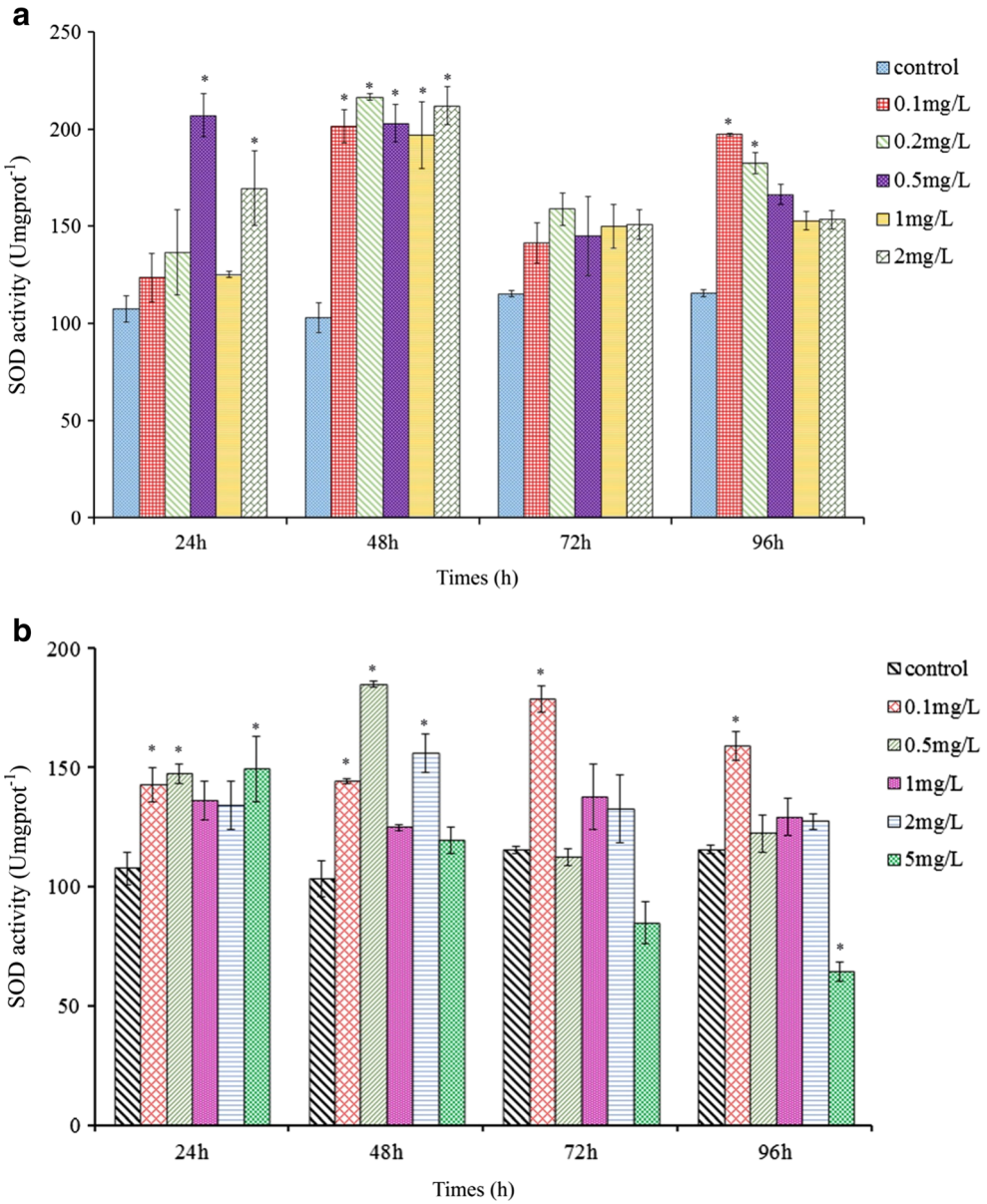

Fig. 3 SOD activity of *Halamphora veneta* after 96 h exposure to different concentrations of copper (**a**) and mercury (**b**)

consistent with of Anu et al. (2016), who reported that the growth rate of *C. calcitrans* was significantly inhibited by copper at concentrations ≥0.18 mg L^{-1} (Anu et al. 2016). However, it is of no significant effect in *H. veneta* at 96 h of copper exposure in this study. It has been established that toxic effects induced by copper depend on many factors, including the species used for the test, initial cell density, light illumination, temperature, media used, and exposure time (Manimaran et al. 2012). Our findings are consistent with the fact that exposure time is one of the most important factors in the impact of heavy metals on diatoms. In addition, our results may indicate that benthic diatoms *H. veneta* may develop some tolerance for copper with the passage of time.

In contrast to copper, mercury inhibits the growth rate of *H. venta* most at concentrations of greater than 2 mg L^{-1} (not the lower concentrations), at 24 h of exposure. At 48 h exposure, there was no significant decrease in the growth rate of *H. venta* (except for 5 mg L^{-1} mercury treated). In addition, very low concentrations of mercury were found to inhibit the growth rate of *H. veneta* at 72 h and 96 h exposure. This is consistent with Horvatić and Peršić (2007) who reported that the growth of the marine diatom *P. tricornutum* was inhibited by mercury at low concentrations of 0.01 mg L^{-1}. In a study of another marine diatom, *Thalassiosira weissflogii*, growth rate gradually decreased from the concentration of 5 to 500 nM under mercury treatments over a 6-day exposure period (Morelli et al. 2009). Furthermore, our results showed that mercury stress has a similar trend at 72 and 96 h on diatom growth rates. Some reports have indicated that diatom growth does not change significantly with time during chronic

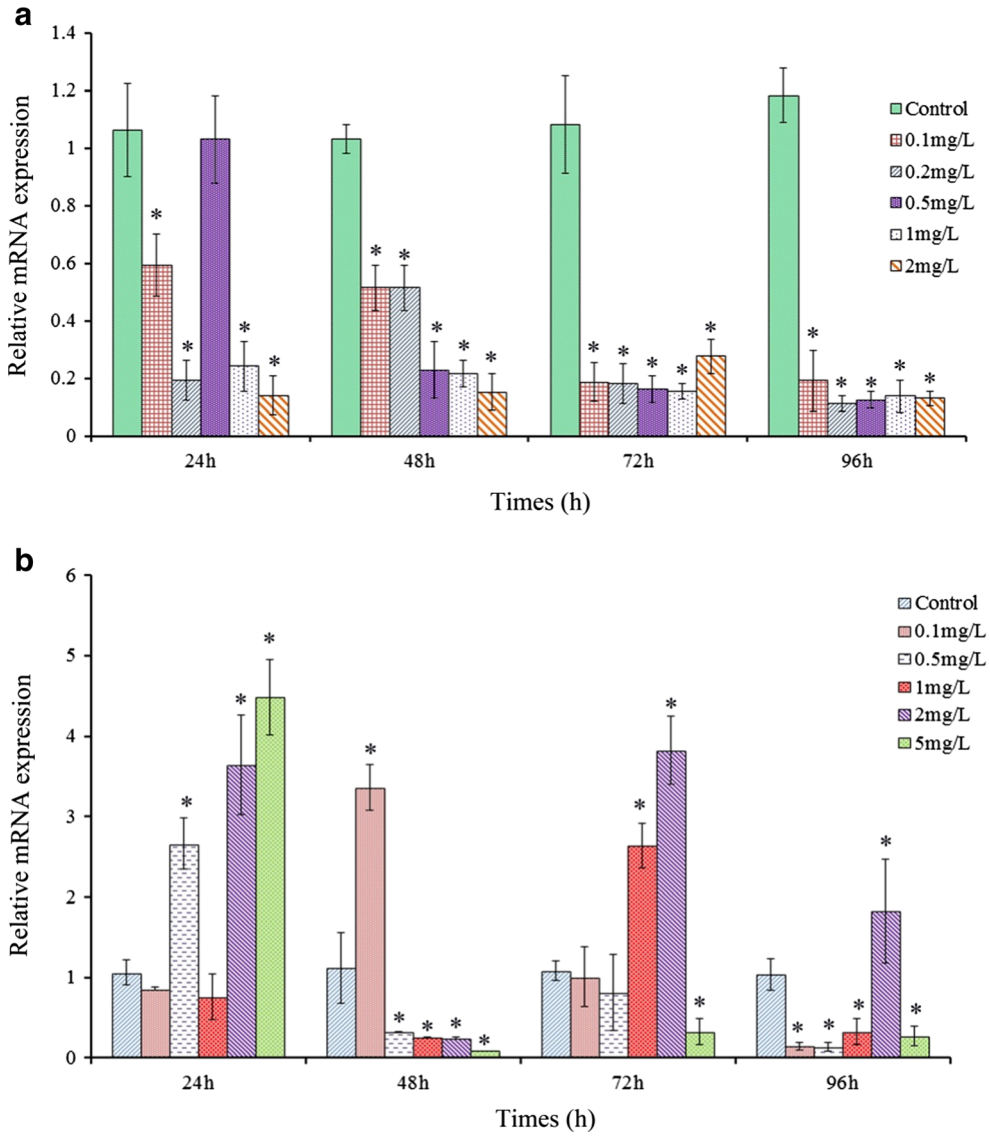

Fig. 4 SOD gene expression of *Halamphora veneta* after 96 h exposure to different concentrations of copper (**a**) and mercury (**b**)

mercury exposure experiments (Horvatić and Peršić (2007); Morelli et al. 2009). This phenomenon might be attributed to a modest allocation of cellular components in diatom which might be integrated into other processes leading to a reduction in damage to cell and enhanced resistance (Calabrese 2015).

Another parameter that has been used in plants and algae as an indicator of environmental pollutants is chlorophyll a content (Lau and Lane 2002). It is noteworthy that, in the present study, the chlorophyll a content of *H. veneta* was higher in the copper and mercury treatments than in the controls. The increase in chlorophyll content following exposure to copper contrasted with previous reports. Dao and Beardall (2016), for example, found that Chl a content in *Scenedesmus acutus* and *Chlorella* sp. declined with increasing heavy metal concentrations at 24 h exposure. Chen et al. (2012) and Branco et al. (2010) both reported that the content of photosynthetic pigments, including Chl a, Chl b, and carotenoids, increased in algae exposed to cadmium and titanium dioxide, respectively. These findings show that the response of chlorophyll content to metal stress varies among different species and with metal concentration. Furthermore, it has also been reported that in algae exposed to toxic compounds, other pigments can convert to Chl a leading to an increase in Chl a content (Fang et al. 1998). Chen et al. (2012) suggested that it might be due to increased ROS which was caused by exogenous substances such as exposure to contaminants. In the present study, we also observed higher Chl a levels in *H. veneta* exposed to copper and mercury compared to the control group, which might be caused by resistance response of diatoms to heavy metals. Knowledge on the tolerance and response of microalgae to heavy metals is important in order to determine the potential utility of microalgae for pollution assessment. Although several previous studies have focused on the biochemical responses of microalgae to heavy metals, the effects

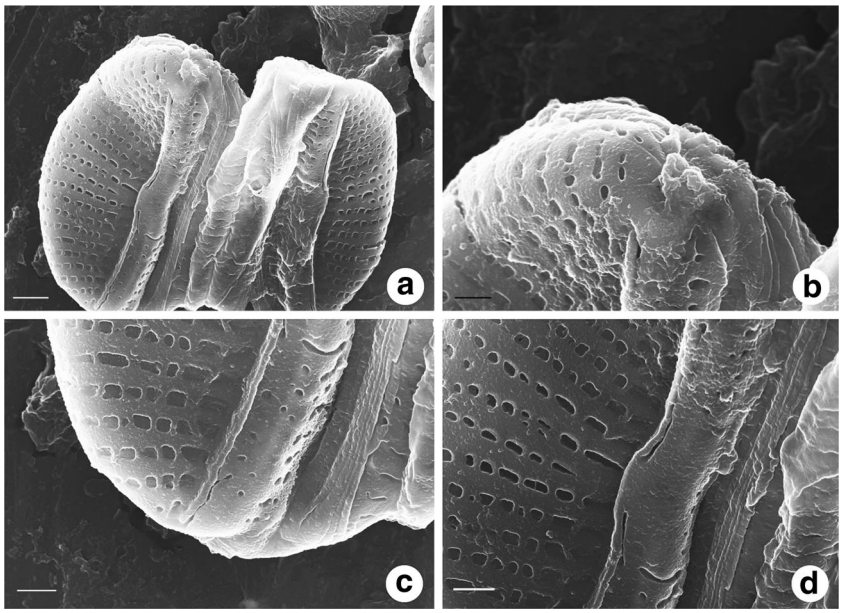

Fig. 5 SEM images of *Halamphora veneta* in the absence of heavy metal treatment: **a** the whole frustule, **b** the anterior end of the frustule, **c** the posterior end of the frustule, and **d** the ventral surface of the frustule. Bars, 2 μm

of copper and mercury on antioxidant enzyme activity coupled with gene expression levels are poorly known (Glanemann et al. 2003). It is established that ROS such as superoxide, hydroxyl radicals, and hydrogen peroxide are produced in microalgae on being exposed to heavy metal contamination (Dao and Beardall 2016; Li et al. 2006). Although ROS play an important role in host defense, overproduction and residuals can cause oxidative damage (Sharma et al. 2012). SOD is an important component in preventing oxidative damage in microalgae and is widely used as an indicator of environmental stress (Blaise and Menard 1998; Peterson and Stauber 1996). In the present study, SOD activity in *H. veneta* was induced by both copper and mercury treatments. This finding is consistent with several previous studies including Li et al. (2006) and Verlecar et al. (2007) for the marine dinoflagellate *Pavlova viridis* and Choudhary et al. (2007) for the cyanobacterium *Spirulina platensis*. Based on the findings of the present study, we concluded that SOD activity in the benthic diatom *H. veneta* is a reliable biomarker of oxidative stress.

The present study also investigated the expression pattern of the SOD gene in *H. veneta* at different concentrations of copper and mercury. Previous studies have demonstrated changes in expression of the SOD gene in the ciliate *Euplotes vannus* induced by exposure to nitrofurazone (Hong et al. 2015) and in the copepod *Tigriopus japonicus* induced by exposure to copper, zinc, and mercury (Kim et al. 2015). By contrast, SOD gene expression in *T. japonicus* was not affected by exposure to triphenyltin chloride (Yi et al. 2014). Lauritano et al. suggested that SOD gene expression is related both to the pollutant and to the duration of exposure (Lauritano et al. 2012). The present study showed that SOD gene expression was

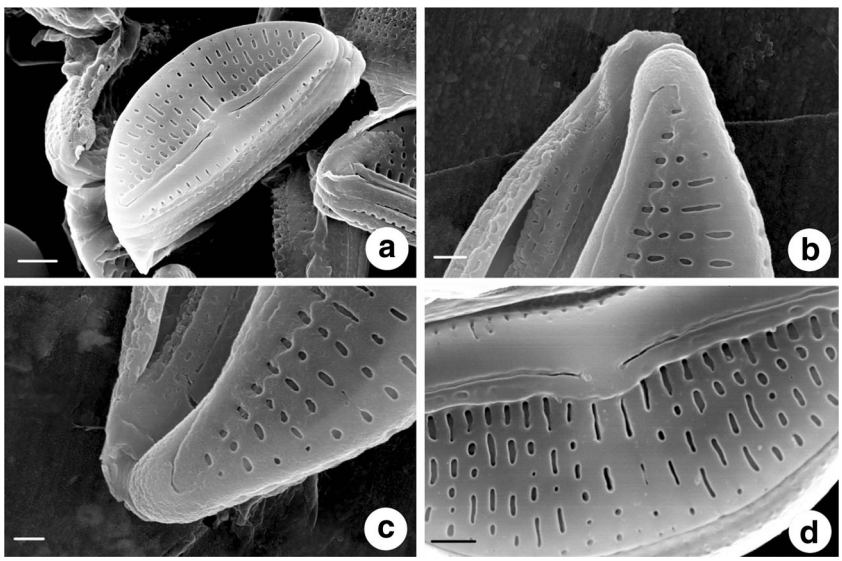

Fig. 6 SEM images of *Halamphora veneta* in copper treatment: **a** the whole frustule (bar, 2 μm), **b** the anterior ends of the frustule (bar, 3 μm), **c** the posterior ends of the frustule (bar, 3 μm), and **d** the ventral surface of the frustule (bar, 3 μm)

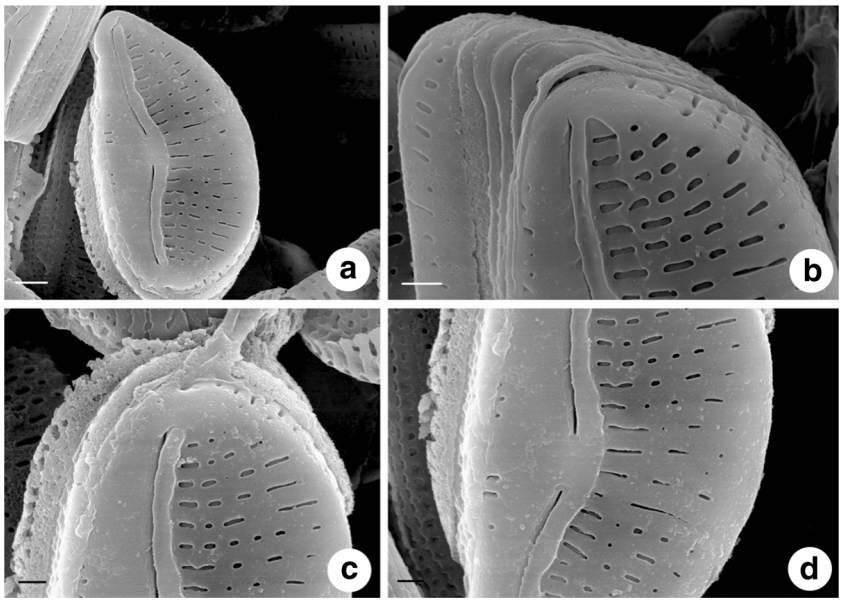

Fig. 7 SEM images of *Halamphora veneta* in mercury treatment: **a** distortion of frustule shape (bar, 3 μm), **b** the rounded anterior end of the frustule (bar, 2 μm), **c** the rounded posterior end of the frustule (bar, 3 μm), and **d** the widened ventral surface of the frustule (bar, 2 μm)

suppressed by exposure to copper at all concentrations except at 0.5 mg L^{-1}. By contrast, SOD gene expression levels were significantly induced at lower concentrations of mercury (0.1, 0.5, 1, and 2 mg L^{-1}) at 96 h. It has been suggested that the differences in gene expression might be explained by the structural differences between the various intracellular targets of these contaminants (Lauritano et al. 2012). In addition, the increased SOD gene expression level in *H. veneta* when exposed to mercury might imply that the cells have developed defense systems consisting of several antioxidant enzymes as protective mechanisms (Kim et al. 2013).

Previous studies have shown that morphological abnormalities in diatom frustules can be used as an effective tool for biomonitoring of heavy metal pollution in water bodies (Falasco et al. 2009; Pandey et al. 2014; Morin et al. 2012). Studies of raphe-bearing diatoms including *Gomphonema parvulum* and *Pinnularia conica* showed deformities at elevated concentrations of heavy metals (Duong et al. 2008; Victoria and Gomez 2010). These findings are consistent with the present study, that is heavy metal stress results in deformation of the frustule in *H. veneta*. Pandey et al. (2014) observed modifications of the raphe in diatoms exposed to Cu. In the present study, the morphological changes in *H. veneta* were more pronounced in cells exposed to mercury than to copper, showing a deformity of shape and increase in size of the frustule. We speculated that mercury could directly interact with the microalgal cells surface, leading to physical effects, such as cell membrane disruption. In a previous study, Branco et al. (2010) reported that a higher concentration than control of cadmium was found loosely bound to the frustule, and concluded that this was evidence of an effective metal-exclusion mechanism that binds the metal to the extracellular structures preventing its entrance into the cell. Morphological changes were far less pronounced in cells exposed to copper. Analysis of the frustule will be required in order to demonstrate the presence of copper and the possible existence of a mechanism for excluding it from the cells of *H. veneta*.

Conclusions

To the best of our knowledge, this is the first study of the effects of copper and mercury on a benthic diatom. Based on our findings, we infer the following conclusions:

1. Copper will lead to a greater growth inhibition, destroying diatom and ecology at the very beginning, and exposure time is one of the important factors.
2. High accumulation of chlorophyll a in the cell is indicative of resistance of the diatom to heavy metals.
3. SOD activity and gene expression are reliable biomarkers of oxidative stress in *H. veneta*.
4. Change in the morphology of the frustule of *H. veneta* is a potential indicator of mercury contamination in water.

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