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PRIMARY RESEARCH PAPER

The valve movement response of three freshwater mussels *Corbicula fluminea* Müller 1774, *Hyriopsis cumingii* Lea 1852, and *Anodonta woodiana* Lea 1834 exposed to copper

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Abstract Biological early warning system (BEWS) is an innovative system for real-time water quality monitoring based on different behavioral responses of aquatic organisms. Mussels easily meet the requirement for optimal organisms in BEWS. However, little emphasis has been placed on freshwater mussels. In this study, the Hall element sensor system has been used to investigate exposure–response relationships of valve movement in freshwater *Corbicula fluminea*, *Hyriopsis cumingii*, and *Anodonta woodiana* exposed to a copper (Cu^{2+}) gradient of 0, 0.01, 0.1, 1, and 10 mg l^{-1}. The measured behavioral parameters were compared to determine if a response could be elucidated, including the amplitude of valve opening (AVO) and frequency of valve adduction (FVA). The results suggest that the mean AVO was significantly decreased in *C. fluminea* exposed to 0.1 and 1 mg l^{-1}, and decreased for *H. cumingii* and *A. woodiana* after exposure to 10 mg l^{-1}. The mean FVA was significantly decreased in *H. cumingii* exposed to 1 and 10 mg l^{-1}, while *A. woodiana* revealed lower frequencies only at 10 mg l^{-1}. The three species showed inherent rhythms of valve movements and dose-dependent responses upon copper exposure, and *H. cumingii* showed the most obvious profile of a copper dose-valve movement response.

Keywords Valve movement · Hall element sensor system · Copper · Freshwater mussels

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Introduction

The development of industry and agriculture promotes the rapid increase of aquatic metal pollution. Consequently, the threat posed from waterborne metals to human health is significant, but pales beside the resulting disruption of the natural environment (Zhou et al., 2008; Dailianis, 2010). Copper is a widespread, persistent metal with high toxicity (Naimo, 1995;

Zhou et al., 2008). Worldwide copper concentrations have been reported at 0.04–294 μg l^{-1} in natural water (with extreme values up to 20 mg l^{-1} in highly contaminated water), and as high as 750 μg l^{-1} in the drinking water in some areas of Canada (summarized by Tran et al., 2004). In China, the National Water Quality Standard for Fisheries (Standard Code: GB11607-89) has emphasized that waterborne copper concentration should be less than 0.01 mg l^{-1} in waters that support fish populations. However, concentrations of copper have been frequently reported above the permissible limit (Li et al., 2007; Yang et al., 2008a). Excessive copper sulfate is often added to aquaculture ponds to eradicate algae, and this application has made copper the main source of contamination in fishery environments (Li et al., 2007; Yang et al., 2008a). Thus, the impact of a waterborne metal such as copper on aquatic organisms and human health is a primary concern, and there is an urgent need for effective monitoring systems.

Biomonitoring is a multidisciplinary applied science based on either short-term laboratory tests or the monitoring of biological responses of organisms in the field to pollutants, including heavy metals (Gerhardt et al., 2006; Newton & Cope, 2007). Compared to traditional chemical methods of water monitoring, biological monitoring is a more direct and realistic assessment of the potential toxicity and the degree of biohazard present. Furthermore, biological monitoring provides a more straightforward interpretation of the dynamics of pollutants that influence organisms and aquatic ecosystems. This allows a more effective investigation and confirmation of ecotoxicological mechanisms and effects for traditional, emerging and unknown pollutants (Markert et al., 2003). A large number of aquatic organisms have been used as bioindicators for both active and especially passive monitoring, including algae, macrophytes, zooplankton, insects, bivalves, gastropods, fish, amphibians, mammals, and others (Gerhardt et al., 2006; Zhou et al., 2008). However, it is very difficult to rapidly monitor effects of pollutants and the health of an aquatic ecosystem because traditional physical and chemical measurements are based on lethal sampling of organisms for chemical analysis of tissues, utilize complicated pretreatment processes, and require expensive analytical instruments. Recently, the need for convenient, quick, reliable approaches to assess pollutant toxicities has become even more essential (Ferro et al., 2012; Bae & Park, 2014). Unlike passive monitoring with aquatic organisms, which mainly focus on past adverse consequences of pollution at the population level, and accumulation of toxicants in specimen/organ/tissue at the individual level (Salánke et al., 2003), the real-time analysis of active monitoring (i.e., biosensors) offers the advantage of rapidly detecting the presence of pollutants before they cause any damage (Ferro et al., 2012). This is done by studying the response of populations, behavioral patterns of specimens, special functions of organs, and cellular/subcellular events (Salánke et al., 2003). A typical example of this is the biological early warning system (BEWS) that was developed based on the response behaviors of organisms, and continuously detects a wide range of pollutants for effective water quality monitoring and management (Bae & Park, 2014).

In recent years, many aquatic organisms, including bacteria, algae, daphnia, mussels, and fish, have been used for biosensor applications of BEWS in both short and long-term environmental assessments (reviews: Gerhardt et al., 2006; Bae & Park, 2014). This approach was first proposed by Cairns et al., (1970) to provide a visual image and measurable data integrating an automated detection system and living organisms. Behavioral changes or reactions are continuously tracked by the automated detection system and can be used to assess the rhythmical valve movement in bivalves (Englund & Heino, 1994; Ortmann & Grieshaber, 2003), avoidance behavior of swimming fish (Kim et al., 2011), bioluminescence in microbes (Ivnitski et al., 1999) and potentially other behavioral or physiological responses to different habitat conditions.

Bivalve mussels possess unique characteristics (e.g., sedentary lifestyle, a gradient of tolerance to both chemical contamination and physical alterations, being sufficiently sturdy to survive laboratory and field studies, straightforward interpretation of their reaction to toxicants) of their stress response that make them very suitable for sensing environmental perturbations (Tanabe & Subramanian, 2006; Grabarkiewicz & Davis, 2008, Bae & Park, 2014). These mussels easily meet the requirement for optimal monitoring organism in BEWS (Kramer et al., 1989) and rhythm of valve movement (e.g., closure reaction) was strongly affected by waterborne stressors (Basti et al., 2009). In recent decades, the use of animal-attached remote-sensing technology has increased. In particular, Hall element sensors, which measure mussel valve movements, provide insight into environmental changes by means of the relationship between valve gape and environmental fluctuations. Consequently, mussel-based BEWS have played an increasing role in the surveillance of environmental quality (Kramer et al., 1989; Gerhardt et al., 2006; Robson et al., 2009). So far, there have been many marine shellfish species, including blue mussel (*Mytilus edulis* Linnaeus 1758), northern astarte (*Astarte borealis* Schumacher 1817), Akoya pearl oyster (*Pinctada fucata* Gould 1850), short necked clam (*Ruditapes philippinarum* Adams and Reeve 1850), Pacific blue mussel (*Mytilus trossulus* Gould 1850), great scallop (*Pecten maximus* Linnaeus 1758), common cockle (*Cerastoderma edule* Linnaeus 1758), among others, which have been adopted as surrogate animals and applied in BEWS for continuous and automatic biological monitoring of marine environments (Wilson et al., 2005; Nagai et al., 2006; Basti et al., 2009; Robson et al., 2009). Actually, due to how relatively easy and inexpensive it is to monitor, valve movement has promise as a biological response to contaminants (Newton & Cope, 2007). However, relatively little emphasis has been placed on freshwater mussels, which can be used for monitoring freshwater environments. Nevertheless, a few studies evaluating valve movement of freshwater mussels have been published, and gaping reactions to natural variation (e.g., natural rhythms of filtering activity) (Englund & Heino, 1994; Ortmann & Grieshaber, 2003; Wilson et al., 2005; Liao et al., 2009), as well as changes caused by pollutants (e.g., the response to toxicants) (Kramer et al., 1989; Tran et al., 2004; Liao et al., 2009; Moroishi et al., 2009) have been examined. Therefore, it is essential that tests on a wide range of species are conducted to assess the toxicity of aquatic contaminants, and to establish effective regulatory systems for the development of BEWS. Freshwater mussels would thus provide additional suitable species for assessing toxicity thresholds and responses, and allow monitoring of freshwater environments.

In this study, we investigate valve responses to copper (Cu^{2+}) toxicity using three freshwater mussel species common in China: the Asian clam *Corbicula fluminea* Müller 1774, and the Unionid mussels, triangle-sail mussel *Hyriopsis cumingii* Lea, 1852 and swan mussel *Anodonta* (*Sinanodonta*) *woodiana* Lea 1834. These three species have long been found in the Chinese diet (Yang et al., 2008b; Liu & Yang, 2012) and used for pearl cultivation (Sakai et al., 1997; Yang et al., 2008b). Moreover, *C. fluminea* and *A. woodiana* have largely expanded their range of distribution throughout the world, especially in Europe in recent decades (Hubenov et al., 2013; Guarneri et al., 2014). It is noteworthy that these species have been involved as sentinel organisms for monitoring the status and temporal changes of environmental contaminants in several countries, due to their wide distribution, sessile lifestyle and high tolerance to chemical contaminants (Baudrimont et al., 1999; Yokoyama & Park, 2002; Chen & Xie, 2005; Liu et al., 2010). Thus, these freshwater mussels have the potential to become behavior-based BEWS mussels for assessing toxicity thresholds and responses, and allow rapid monitoring of pollution. In the present study, the Hall element sensor system was used to compare the different behaviors of *C. fluminea*, *H. cumingii*, and *A. woodiana* exposed to copper sulfate. Since we hypothesized that copper exposure would disrupt the valve activities of these three species, and that the disruption would be species- and dose-specific, our efforts were aimed at qualitatively and quantitatively characterizing the spontaneous natural activity of the mussels, conducting an assessment of behavioral changes in valve closing/opening reactions to copper exposure, and assessing the feasibility of developing a BEWS using these freshwater bivalves.

Materials and methods

Chemicals

Copper sulfate ($CuSO_4 \cdot 5H_2O$, analytical reagent grade quality) was used as the test chemical (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The stock solution of $CuSO_4$ (700 mg l^{-1}) was prepared by dissolving the chemical in dechlorinated tap water. The measured background concentrations of copper (Cu^{2+}), sodium (Na^{+}), magnesium (Mg^{2+}), potassium (K^{+}), calcium (Ca^{2+}) in this dechlorinated water was 0.001, 26.7, 6.38, 3.0, 25.33 mg l^{-1}, as determined with an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500ce, Agilent Technologies, USA). Test concentrations were made by the addition of adequate volumes of the stock solution to the water.

Mussels

Freshwater clams, *C. fluminea* ($N = 18$, body weight 6.7 ± 0.7 g, shell length 26.2 ± 0.9 mm, shell height 24.5 ± 1.1 mm, shell width 16.8 ± 1.0 mm, mean ± SD) were collected from Taihu Lake at Wuxi, Jiangsu Province, China in May 2013 using a trawl net. All the clams were estimated to be adults based on shell lengths (Majdi et al., 2014). Triangle-sail mussels, *H. cumingii* ($N = 19$, 5 month-old, body weight 3.1 ± 0.4 g, shell length 34.6 ± 1.7 mm, shell height 33.0 ± 2.8 mm, shell width 6.8 ± 0.6 mm) and swan mussels, *A. woodiana* ($N = 16$, 5-month-old, body weight 2.4 ± 0.5 g, shell length 30.8 ± 1.6 mm, shell height 20.1 ± 1.1 mm, shell width 9.3 ± 0.8 mm) were collected from mussel ponds in Wuyi City, Zhejiang Province from July to October 2013 and from Nanquan Aquatic Base of our laboratory in Wuxi City, Jiangsu Province of China, respectively. These mussels were transported to the laboratory and held in plastic tanks ($50 \times 35 \times 30$ cm) containing continuously aerated dechlorinated tap water under a natural photoperiod for at least 7 days. There was no substrate at the bottom of the tank. Mussels were fed with about 1 g l^{-1} of *Chlorella* sp. (Chlorella Industry Co., Ltd., Tokyo, Japan) three times per week and the water in the tank was renewed once a week. During the experiment, water temperature was maintained between 18 and 24°C, pH ranged between 7.3 and 8.2 and dissolved oxygen (DO) was kept between 7.3 and 9.6 mg l^{-1}. The survival of *C. fluminea* was over 95%, and no mortality was observed in either *H. cumingii* or *A. woodiana* during the acclimation period.

Attachment of sensor and measurement of background conditions

Measurement of valve movement was performed according to the methods described by Nagai et al., (2006), Basti et al., (2009) and Moroishi et al., (2009). The set-up for the measurement is shown in Fig. 1A. A valve movement-measuring device, DC-104R (Tokyo Sokki Kenkyujo Co., attenuator amplifier, Japan), was supplied with four sensors (Fig. 1A) to score external magnetic field changes in the output voltage (V_h) in microvolts (μV) from a Hall element sensor ($10 \times 10 \times 4$ mm) and a small ferrite magnet (diameter 6 mm) (Fig. 1B), which corresponded to the gap of valves between opening and closing postures (Fig. 1C). The change of V_h was recorded at a speed of six times per second.

The Hall element sensor was first glued to one half of a mussel's shell using a polyethylene resin and the magnet glued to the other shell. The sensors were then sealed in an underwater paste used for drying reef bond for at least 3 h. For each experiment, 3–4 individual mussels were placed in a 1 L plastic test chamber ($130 \times 400 \times 100$ mm) filled with aerated dechlorinated tap water (17–24°C; DO: 7.3–9.6; pH: 7.3–8.2 mg l^{-1}) and circulated using a peristaltic pump FPC100-1515 (Cheap Sun Japan CO., Ltd., Japan) with a flow rate of 150 ml min^{-1}. Measurement of mussel valve movement was usually undertaken under dark conditions in previous studies in the literature (e.g., Nagai et al., 2006; Moroishi et al., 2009). Moreover, it was demonstrated that the filtering activity of freshwater mussels was highest at night (Englund & Heino, 1994). Therefore, exposure tests of the present study were carried out in darkness. Prior to exposure, mussels with attached sensor were acclimated to the dark conditions (<1 Lux, monitored by a TR-74Ui Illuminance UV Recorder, T&D CORP.; Japan) for more than 12 h in a dark room and food was withheld.

Exposure to copper and measurement of movement

The copper gradient (0, 0.01, 0.1, 1, and 10 mg Cu^{2+} l^{-1}, nominal concentration) used in the current study was based on those of Moroishi et al., (2009) (0, 0.1, 1, or 10 mg l^{-1}) and the aforementioned permissible limit listed in the Chinese GB11607-89 standard (0.01 mg l^{-1}). *C. fluminea*, *H. cumingii*, or *A. woodiana* were exposed to the respective copper concentration (prepared from $CuSO_4{\cdot}5H_2O$) in the continuous flow-through recirculation system. Numbers of mussels attributed to each group were *C. fluminea*: 3 (0 mg l^{-1}), 3 (0.01 mg l^{-1}), 4 (0.1 mg l^{-1}), 4 (1 mg l^{-1}), 4 (10 mg l^{-1}); *H. cumingii*: 4 (0 mg l^{-1}), 4 (0.01 mg l^{-1}), 4 (0.1 mg l^{-1}), 3 (1 mg l^{-1}), 4 (10 mg l^{-1}); *A. woodiana*: 3 (0 mg l^{-1}), 3 (0.01 mg l^{-1}), 3 (0.1 mg l^{-1}), 4 (1 mg l^{-1}), 3 (10 mg l^{-1}).

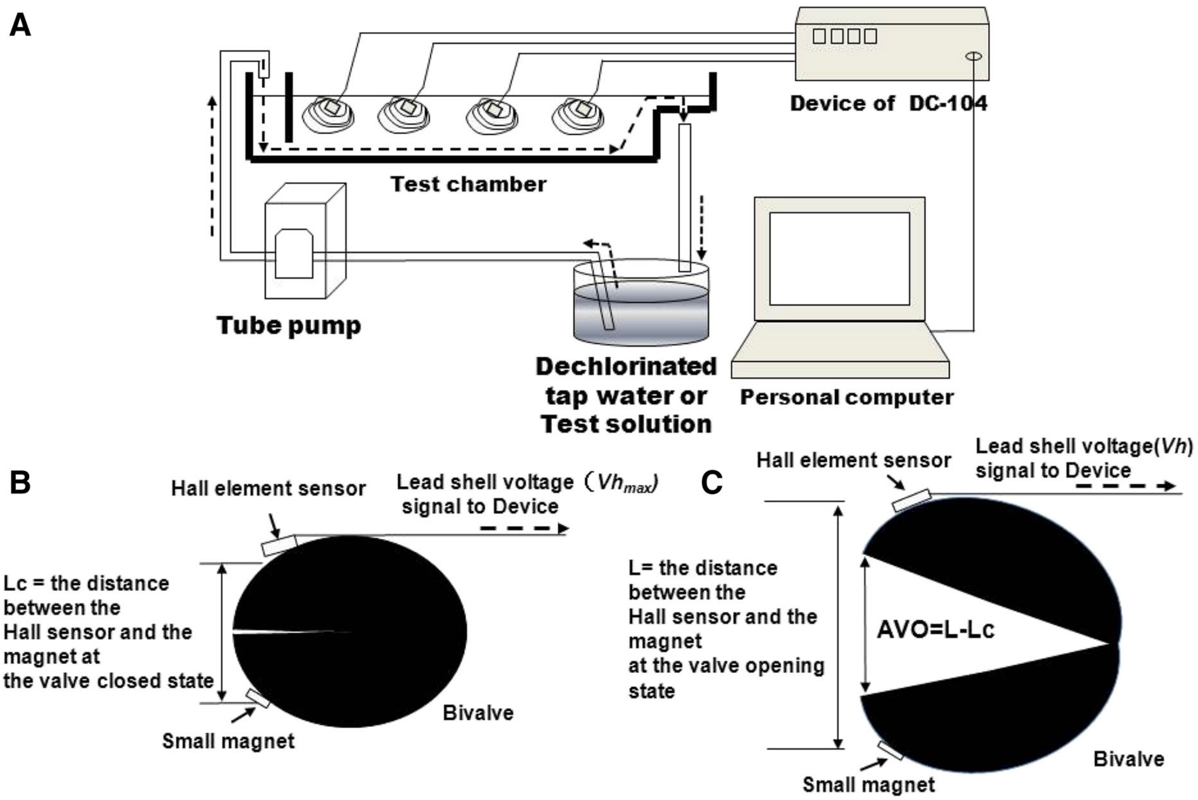


Fig. 1 Experimental set-up of the mussels' valve movement monitor

Recording of the valve movements was initiated at 9:00 a.m. every day in the same dark room. After checking closure of shells, V_h was recorded for each mussel using a computer for an exposure period of 10,800 s (i.e., 3 h). When the measurement was finished, excess water was blotted off the shell of the mussel and the Hall element sensor and magnet were removed.

Conversion of the sensor output voltage into the gap distance between the valves

First, with a ruler we separated the Hall element sensor and small ferrite magnet by a series of distances (L) (i.e., 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 18, 20, 24, 30 mm). Therefore, we obtained corresponding V_h data from V_{hmax} of 3,820 μV to V_{hmin} of 0 μV. Next, in accordance with Nagai et al., (2006), we created an X (i.e., L^{-2} values) by Y (i.e., V_h values) correlation graph and performed goodness of fit analysis. Consequently, the linear equation for conversion of V_h to L was obtained as follows (Figs. 1, SI Fig. 1):

$$L_C = \sqrt{\frac{101665}{V_{hmax} - 68.273}} \tag{1}$$

$$L = \sqrt{\frac{101665}{V_h - 68.273}} \quad (R^2 = 0.9798) \tag{2}$$

$$\mathrm{AVO} = L - L_C \tag{3}$$

There is a considerably strong correlation between V_h and L ($R^2 = 0.9798$) (Fig. 2). This correlation demonstrates the feasibility for accurate calculation of L using V_h. We first transformed the recorded sensor V_h to absolute value and picked out the absolute V_{hmax} from the recorded data of individuals. V_{hmax} was considered as the absolute lead shell voltage recorded by the device of the gap in closed state and the formula (1) was used to calculate the distance (L_C) between the Hall sensor and the magnet at the valve closed state of

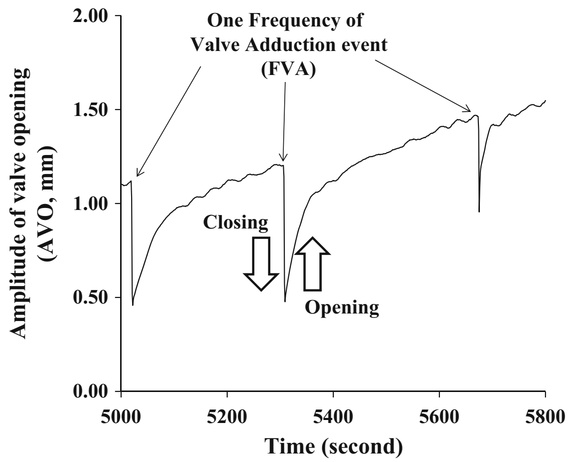


Fig. 2 A sketch map showing mussel FVA event from opening to closing

each mussel. Afterwards, the formula (2) converted the recorded data V_h to the distance (L) between the Hall sensor and the magnet at the valve opening state of each mussel. The real-time amplitude of valve opening (AVO) is the value of $L - L_C$ to produce a Y axis wave pattern (Fig. 2).

Count of valve adduction frequency

In the present study, a rapid decreasing followed by opening and then once more rapid closure curve in the Y axis was referred to as one valve adduction cycle (Fig. 2) and frequency of valve adduction (FVA) was counted over the 3 h experimental period.

Data analysis

The mean AVO (mm) and the mean FVA (number h^{-1}) were used as population-level parameters for a comparative statistical analysis between the control and exposure groups (each mussel was exposed to one concentration only). Dunnett's test was used to compare the differences between control and treatment groups. A P value <0.05 was considered statistically significant. All statistical analysis was performed using SPSS Statistics v.16.0 (IBM Corp., Armonk, NY, USA) and StatView software V. 5.0 (SAS Institute Inc., Cary, NC, USA). Considering our small sample size for the FVA tests, we also conducted a post-hoc power analysis to demonstrate how large of a sample size would be required to produce statistically significant results (at $P = 0.05$, two-sided tests) with a power of 0.8 (i.e., large effect). The power analysis was performed using R (*pwr* package).

Results

Amplitude of valve opening (AVO) as a reaction to copper exposure

The mean AVO (i.e., mean value of AVO during each period of 600 s) was used to evaluate valve movement reaction after exposure to different copper regimes. Under the control conditions, the mean AVO was in the range of 0.09–1.88 mm (Fig. 3A) for *C. fluminea*, 0.46–0.86 mm for *H. cumingii* (Fig. 3B), and 0.03–1.02 mm for *A. woodiana* (Fig. 4C), respectively. In *H. cumingii*, valves tended to alternate between partial closures and extended period opening postures (Fig. 3B, also see SI Fig. 2). In *C. fluminea* (Fig. 3A, also see SI Fig. 3) and *A. woodiana* (Fig. 3C, also see SI Fig. 4), valves showed a closure tendency for nearly half of the recording period, followed by a period of extended opening.

Despite high inherent variability in valve movement behavior between mussel species, there was a decreasing trend in the distance between valve movements detected, especially in the high exposure groups. Exposure of *C. fluminea* to different copper regimes generally resulted in a decreasing trend (mean AVO: close to 0.1 mm) for the mean AVO in the latter half of the recording period at 0.01 mg l^{-1}, and valve nearly closing (mean AVO usually <0.1 mm) after 30 min at 0.1 mg l^{-1} ($P = 0.002$) (Fig. 3A, also see SI Fig. 3). Although there were several partial opening postures, almost all of the mussels displayed asymptotic behavior of nearly closing (mean AVO < 0.1 mm) at 1 mg l^{-1} ($P < 0.0001$) (Fig. 3A). Moreover, an unexpected valve opening behavior was found in *C. fluminea* when exposed to 10 mg l^{-1} (Fig. 3A, also see SI Fig. 3). A generally wider mean AVO (near 2.0 mm) was observed at this level than those of other exposure groups (Fig. 3A).

Following exposure of *H. cumingii* to copper concentrations between 0.01 mg l^{-1} to 10 mg l^{-1}, the mean AVO in the group of 0.01 were generally congruent with that of the control group (Fig. 3B). A slight decline in mean AVO was found in the group of 1 mg l^{-1} (Fig. 3B) although an unexpected high peak of AVO occurred at the time of 380–390 s in an

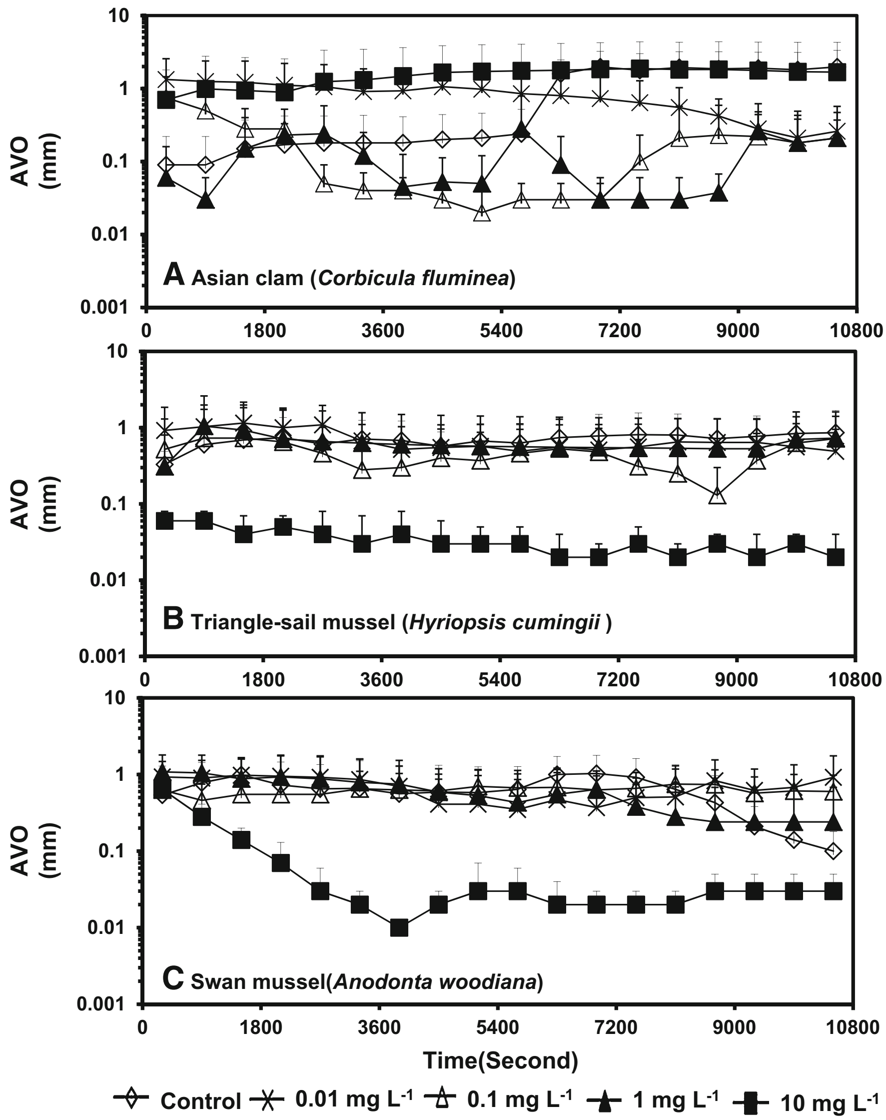


Fig. 3 Distance between the valves under control and increasing copper concentrations over 3 h. Data are shown as mean ± SD during each 600 s. A log scale was used on the *Y* axis. **A** The result of Asian clam *Corbicula fluminea*, (*open triangle*) 0.1 mg l^{-1} (Dunnett *t*-tests, $P = 0.002$) and (*filled triangle*) 1 mg l^{-1} (Dunnett *t*-tests, $P < 0.0001$) show significant differences compared to the control group; **B** the result of triangle-sail mussel *Hyriopsis cumingii* (*filled square*) 10 mg l^{-1} show significant differences compared to the control group (Dunnett *t*-tests, $P < 0.0001$); **C** the result of swan mussel *Anodonta woodiana*, (*filled square*) 10 mg l^{-1} show significant differences compared to the control group (Dunnett *t*-tests, $P < 0.0001$)

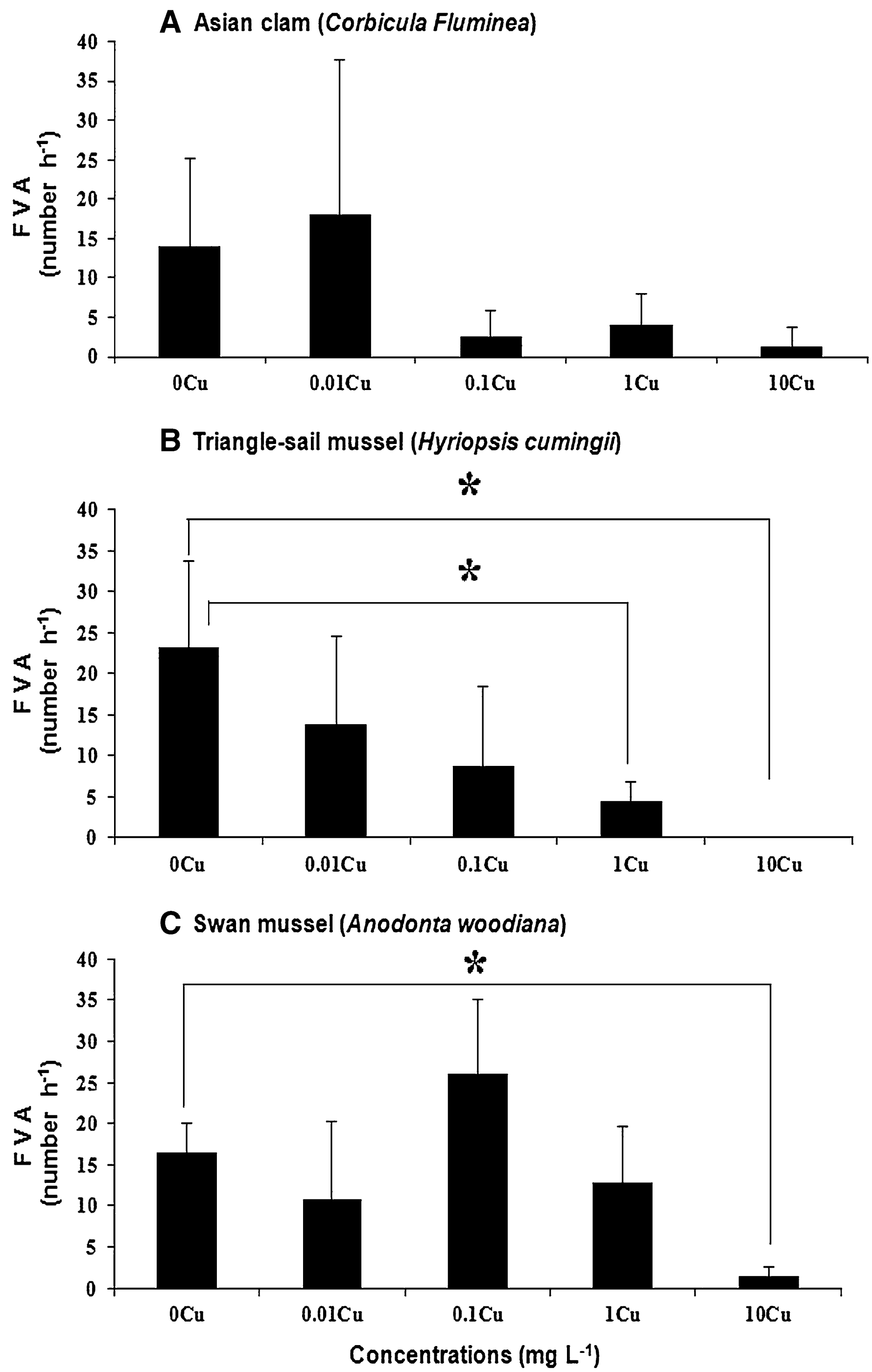
individual (SI Fig. 2). The mean AVO tended to decrease in the 0.1 mg l^{-1} group (generally 30% decrease, but not at a significant level), and was significantly lower (displaying nearly closing behavior, generally a 95% decrease, mean AVO < 0.1 mm) in the 10 mg l^{-1} group in comparison to that of the control ($P < 0.0001$, Fig. 3B, also see SI Fig. 2).

As for the *A. woodiana*, relatively similar valve reactions (mean AVO > 0.1 mm) were found between the control and 0.01, 0.1 and 1 mg l^{-1} exposed groups in comparison to that of control and 10 mg l^{-1} treatment pairs (Fig. 3C, also see SI Fig. 4). A significantly prolonged valve closure (mean AVO < 0.1 mm) was only recorded in these mussels at the 10 mg l^{-1} exposure (generally 84% decrease, $P < 0.0001$), and it lasted for almost the whole 3 h exposure period (Fig. 3C).

Frequency of valve adduction (FVA) reaction to exposure

Except for the 0.01 mg l^{-1} copper-exposed group, exposure of *C. fluminea* to 0.1, 1, or 10 mg l^{-1} of copper seemed to induce a decreasing (83, 72, and 91% decrease, respectively) trend of the mean FVA when compared to those under control conditions,

Fig. 4 Frequency of valve adduction (mean ± SD) under the different copper concentrations. The *asterisk* indicates that the mean values are significantly different from control according to the Dunnett *t*-tests. Triangle-sail mussel *Hyriopsis cumingii*: 1 mg l^{-1} versus control, $P = 0.034$; 10 mg l^{-1} versus control, $P = 0.014$. Swan mussel *Anodonta woodiana*: 10 mg l^{-1} versus control, $P = 0.05$



even though the reaction was not statistically significant with the use of 4 replicates per treatment (Fig. 4A). Post-hoc power analysis revealed that 5 and 6 replicates per treatment would be needed to achieve $P = 0.05$ for a power of 0.8 for 0.1 and 1.0 mg l^{-1} concentrations, respectively. Similarly, *H. cumingii* exhibited a gradually decreasing mean FVA with increasing levels of copper exposure (Fig. 4B). There were 41 and 35% decreases of mean FVA in *H. cumingii*, and *A. woodiana* to copper exposure even at

0.01 mg l^{-1}, although these decreases were not statistically significant. The power analysis detected a need for 10 (for *H. cumingii*) and 21 (for *A. woodiana*) replicates to detect a statistically significant result at this concentration. Significantly decreasing mean FVA was detected both in 1 mg l^{-1} (81% decrease, $P = 0.034$, Fig. 4B) and 10 mg l^{-1} (100% decrease, $P = 0.014$, Fig. 4B) groups of *H. cumingii* when compared to the control, while five replicates would be needed to achieve a statistical significance for the 0.1 mg l^{-1} treatment. Meanwhile, *A. woodiana* revealed a significant decline in mean FVA (92% decrease, $P = 0.05$, Fig. 4C) in the 10 mg l^{-1} copper treatment when compared with those of the control group. In general, *H. cumingii* presented a relatively clearer profile of a copper dose-valve movement response than the two other mussel species.

Discussion

Selection of the copper exposure gradient

In the present study, the copper exposure gradient for *C. fluminea*, *H. cumingii*, and *A. woodiana* was chosen as 0, 0.01, 0.1, 1, or 10 mg l^{-1}, although to our knowledge, neither LC50 nor EC50 reference data are available, for these three species in the literature. Our choice of the concentration gradient seemed to be verified with the relatively obvious difference observed between control and exposure groups for all three test mussel species. LC_{50} or EC_{50} values exposed to Cu^{2+} were documented by the US EPA (2007) between 0.027 and 0.199 mg l^{-1} in juveniles (including 1–2 days juveniles) of freshwater mussel *Actinonaias* and *Utterbackia imbecillis* Say 1829. Moreover, several recent studies evaluating copper toxicity using mussel-attached sensing technology on *Corbicula* species (Tran et al., 2004; Liao et al., 2005; Moroishi et al., 2009) provided useful reference data for us to select this gradient. Moroishi et al., (2009) conducted a 6 h real-time investigation on *Corbicula japonica* Prime1864 with a copper gradient of 0, 0.1, 1, or 10 mg l^{-1} and effectively documented the resulting behaviors in response to adverse effects, including significant valve closure and reduction in the distance between the valves. However, Tran et al., (2004) estimated that the maximum expected dissolved copper sensitivity within 5 h in *C. fluminea* was 0.004 mg l^{-1}. Additionally, Liao et al., (2005) observed the valve closure behavior of *C. fluminea* over a 24 h period using waterborne copper concentrations of 0.02 and 0.05 mg l^{-1}. These variable responses in sensitivity to copper might be caused by differences in strains/stocks of this species or water quality, similar to the findings of Denic et al., (2015) on freshwater pearl mussel *Margaritifera margaritifera* Linnaeus 1758. The copper gradient of Moroishi et al., (2009) may be a good choice for the present study, as *C. fluminea* was one of the target mussels. However, 0.01 mg l^{-1} is the permissible limit of waterborne copper according to the Chinese GB11607-89 standard promulgated in 1989, which had to be taken into consideration. Therefore, our final copper gradient was selected as 0, 0.01, 0.1, 1, and 10 mg l^{-1} in reference to the concentrations of US EPA (2007), Moroishi et al., (2009) and GB11607-89 standard.

Comparing changes in valve movement response of three freshwater mussel species

It appears that the present study is the first report on the Hall element sensor system to be used in the study of freshwater mussel *H. cumingii* and *A. woodiana*. Our results showed that this approach of automatic continuous recording of valve movements can be applied in the development of BEWS using additional freshwater mussel species. Data collected from AVO and FVA measurements facilitated a qualitative and quantitative illustration of valve closing/opening reactions in *C. fluminea*, *H. cumingii,* and *A. woodiana* when exposed to copper. Amongst the three species, *H. cumingii* might be the best choice for a BEWS bioindicator to copper exposure, as its valve adduction activities were more strongly correlated with the waterborne copper gradient (Fig. 4).

Interspecific variations in valve movement over time have been noted among the three mussel species in both control and copper-exposed groups. In the case of the control mussels, *C. fluminea* and *A. woodiana* alternated their movements between continuous valve closures to repeated opening-closing postures. In contrast, the valve of *H. cumingii* was usually open throughout the study period. Actually, *C. fluminea* may keep its valve closed for even several hours at a time (Ortmann & Grieshaber, 2003; Liao et al., 2009) while no data of *A. woodiana* and *H. cumingii* were available previously.

In the exposure groups, the normal rhythms of valve movement were disturbed and the mussels showed different types of toxicity-related responses when exposed to different copper concentrations. Decreased tendencies of mean AVO in *C. fluminea* and mean FVA in *H. cumingii* and *A. woodiana* to copper exposure were possibly observed at 0.01 mg l^{-1}, the permissible limit of the GB11607-89 Standard, although these differences were not statistically significant. The fact that a measurable response occurred at such a low waterborne copper level suggests that mean AVO and FVA can be potentially used as relatively sensitive parameters to understand copper stress responses in different freshwater mussel species. Another species of freshwater mussel, *Dreissena polymorpha* Pallas 1771, was also found to exhibit the stress-related closure response behavior to the similar concentration of 0.015 mg l^{-1} Cu^{2+} (37.5 μg l^{-1} $CuSO_4$) (Kramer et al., 1989).

After exposure to 0.1 mg l^{-1}, *C. fluminea* would close their valves and the mean AVO would decreased to <0.05 mm within 30 min. Moroishi et al. (2009) reported a similar reaction in *C. japonica*. At the same copper level, *H. cumingii* revealed an obvious decrease in valve adduction activity, but this instead induced a series of rapid closings and openings within several seconds in *A. woodiana*. This decrease, however, was not statistically significant given our relatively limited sample size. This latter phenomenon has also been reported by Tran et al. (2004) in *C. fluminea*.

Exposure to the higher concentration of copper (especially 10 mg l^{-1}) tended to result in stronger responses in the three mussel species; *H. cumingii* and *A. woodiana* exhibited prolonged valve closure while *C. fluminea* demonstrated an unusually wide valve opening. When exposed to contaminants, bivalves usually reduce their filtrating-uptake activity to avoid, or respond to toxic effects (Newton & Cope, 2007; Moroishi et al., 2009), reflecting their inherent protective behavioral strategies. Therefore, the valve response of *H. cumingii* and *A. woodiana* appear to be protective behaviors. In contrast, the behavior of the unusually wide valve opening in *C. fluminea* (Fig. 3A, also see SI Fig. 3) might imply that the copper stress of 10 mg l^{-1} was beyond the ability of this behavior to protect the mussel. Sobrino-Figueroa & Cáceres-Martínez (2009) reported a case of damage to the sensory cells located on the mantle edge of juvenile Catarina scallop (*Argopecten ventricosus* Sowerby II 1842) when exposed to Cd, Cr, and a mixture of Cd^{+}, Cr^{+}, and Pb. Serious gill malfunction (Sunila, 1998) and posterior adductor muscle severing (Manley, 1983) were also observed after copper exposure in *M. edulis*. Similarly, this unusual behavior of *C. fluminea* might have not resulted from the death of the mussel individual, but was possibly caused by normal tissue hypofunction interfering with the valve closing response due to the high toxicity of copper. This hypothesis requires further confirmation.

No data for copper LC50 or EC50 values have been available for *C. fluminea*, *H. cumingii*, or *A. woodiana* so far. By means of the copper concentration gradients and FVA data of each species, we attempted to tentatively estimate 3-h EC_{50} values for the three mussel species. Visual inspection of the data in SI Fig. 5 suggested that reduced tendency of FVA occurred at 0.01 mg l^{-1} for *H. cumingii* and *A. woodiana*, and at 0.1 mg l^{-1} for *C. fluminea*. As stated above, worldwide copper concentrations have been reported at 0.00004–0.294 mg l^{-1} in natural waters (summarized by Tran et al., 2004). The highest level (10 mg l^{-1}) of our copper gradient may only exist in highly contaminated water. It may be, therefore, better to estimate the effective concentration (EC) of this study using the plotted data of FVA response against exposure concentrations of 0, 0.01, 0.1, and 1 mg l^{-1} (i.e., excluding 10 mg l^{-1}, see SI Fig. 5). Adopting the similar estimation in microbial communities used by Monavari & Guieysse (1997) for toxicant EC_{100} and EC_{50} from observable effects (endpoints), EC_{50} (i.e., half EC_{100}, EC_{100} is assumed to be the concentration when FVA = 0) values of this study were obtained as 0.7, 0.6, 2.1 mg l^{-1} for *C. fluminea*, *H. cumingii*, and *A. woodiana*, respectively, using the concentration-response curves (see SI Fig. 5). The mussels used in our study were adults or 5-month-old life stages. Our EC_{50} estimates were higher than those reported for juveniles (including 1–2-day-old juveniles) of the freshwater mussels, *Actinonaias* and *U. imbecillis* (0.027–0.199 mg l^{-1}) (US EPA, 2007). We assume that the differences are related to the use of different life stages between the US EPA study and our data. The US EPA mussels were likely very early-stage juveniles, while those used in our study were late-stage juveniles and adults. If 3-h EC_1 is defined as the recommended threshold for safe concentration, the values are calculated as 0.014, 0.012, and 0.042 (i.e., 0.01, 0.01, and 0.04 after rounding down) mg l^{-1} for *C. fluminea*, *H. cumingii*, and *A. woodiana*, respectively. All these data seem to be close to the permissible limit outlined in the GB11607-89 Standard (i.e., 0.01 mg l^{-1}). Therefore, it cannot be concluded that the tolerated limit is an indisputable safe concentration for the three mussel species (especially *C. fluminea*, *H. cumingii*). Alternatively, a simple deterministic approach may provide clues to derive Predicted No Effect Concentrations (PNEC) of copper in our study by dividing a parameter of toxicity (e.g., EC_{50}) by the conservative assessment factor (AF) of 1,000. This number is a conservative, protective AF and applied when only limited data are available (OECD, 2011). As mentioned, the reduced tendencies of FVA were noted at 0.1 mg l^{-1} for *C. fluminea*, and at 0.01 mg l^{-1} for *H. cumingii* and *A. woodiana* (Fig. 4). In the AF case of 1,000, the estimated conservative PNEC are 0.001, 0.001, and 0.002 mg l^{-1} after rounding up for *C. fluminea*, *H. cumingii*, and *A. woodiana*, respectively. These PNEC values seem reasonable and are supported, to some extent, by the result of Tran et al., (2004) in which the sensitivity of waterborne copper within 5 h was found as 0.004 mg l^{-1} in *C. fluminea*.

Conclusions

The Hall element sensor system has been extensively used to investigate the copper exposure–response relationships of valve movement in *C. fluminea*, *H. cumingii*, and *A. woodiana*. We suggest that the concentration gradient of 0, 0.01, 0.1, 1, or 10 mg l^{-1} might be useful not only to assess the protective behavioral strategies or behavior potentially reflecting damaged tissues of the three freshwater mussel species, but also to evaluate the sensitivity of these freshwater mussels to waterborne copper. Interspecific variations have been recorded in temporal dynamics of valve movement among the three mussel species. In particular, all three species showed a drastic alteration in valve responses to 10 mg l^{-1}. *H. cumingii* showed the clearest profile of a copper dose-valve movement response among the three mussel species. Moreover, the alteration of mean AVO and FVA of this study can possibly be used as useful parameters for development of mussel-based BEWS to Cu^{2+} exposure using *C. fluminea*, *H. cumingii*, and *A. woodiana*. The present study provides further evidence that freshwater mussels could be used as ideal environmental monitoring organisms of BEWS. We note, however, that relatively low sample size and high individual variability limit the extent of our conclusions. Further research is required to address the exact EC_{50}/safe concentration for the three mussel species to copper exposure more accurately, and to address their corresponding physiological and histological mechanisms of AVO/FVA reactions using a Before-after-control-impact (BACI) experimental design. In addition, valve movement responses to other stimulating factors such as temperature, pH, DO, and other inorganic and organic pollutants in the laboratory or field are still unknown and require further investigation.

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