Laboratory culture of the freshwater benthic gastropod *Bellamya aeruginosa* (Reeve) and its utility as a test species for sediment toxicity

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Abstract

This study aimed to develop original laboratory culture and sediment toxicity testing protocols for the freshwater gastropod *Bellamya aeruginosa* (Reeve), a new potential species for sediment toxicity testing. *B. aeruginosa* was successfully cultured with an effective culture system under proposed laboratory conditions. Optimal *ad libitum* feeding levels for larvae, juveniles, and adults were 2.0, 6.0, and 16.0 mg fish food/(snail·day), respectively. Mean survival rates of juveniles were higher than 90%. The snails could be sexed at 9 weeks of age, and their generation time is approximately 4 months. Reproduction continued all year around; the mean fecundity was 0.55 newborn/(female·day). The utility of this species for bioassays was evaluated in both 10-day and 28-day case studies with artificial sediments. The 10-day LC_{50} of Cu for larvae was 480 μg/g dry weight (dw), and the lowest observed effects concentration of Cu for survival and growth of larvae was 195 μg/g dw. Survival and growth are reliable indicators of acute toxicity. Larvae accumulated more Cu than adults. *B. aeruginosa* exhibited a higher sensitivity to Cu exposure than standard test species (*Hyalella azteca* and *Chironomus tentans*). The 28-day test of sediment toxicity with adults showed that fecundity was a robust endpoint indicator of reproductive toxicity, and the biochemical endpoints of superoxide dismutase, catalase, and glutathione could be used as sensitive biomarkers for Cu-induced oxidative damage. *B. aeruginosa* can be therefore recommended as a candidate for the standardization of the freshwater sediment toxicity test protocol.

Key words: gastropod; cultures; life history; lowest observed effect concentration; bioaccumulation; oxidative stress

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Introduction

Sediments are core components of aquatic ecosystems, providing food and habitats to benthic organisms, but they are also sinks for multiple contaminants resulting from industrial discharges, urban storm-water runoff, atmospheric deposition and discharges from municipal facilities; thus they constitute a potential threat to both aquatic ecosystems and human health (Adams et al., 1992). Numerous studies have attempted to examine the ecotoxicological effects of sediment-associated contaminants on aquatic ecosystems by sediment toxicity tests (Martinez et al., 2004; Mcgee et al., 2004; Ringenary et al., 2007; Greenstein et al., 2008). Sediment toxicity tests have evolved as powerful tools that provide direct, quantifiable evidence of biological consequences of sediment contamination that can only be inferred from chemical or benthic community analyses (O'Connor and Paul, 2000; USEPA, 2000; Anderson et al., 2004). Sediment toxicity testing depends on suitable test species. To qualify for the assessment of

sediment toxicity, a species should generally meet several requirements, such as ecological importance, wide geographic distribution, year-round availability, convenient culture in the laboratory, and sensitivity to contaminants (ASTM, 2002). Current standard tests for evaluating toxicity of freshwater sediments involve a restricted number of species, such as the amphipod *Hyalella azteca*, the chironomid *Chironomus tentans*, and the oligochaete Lumbriculus variegates (USEPA, 2000; ASTM, 2003). However, in many cases, data based on toxicity testing with a very few species could not sufficiently identify the potential ecotoxicity of a wide range of contaminated sediments due to the differences of biologic traits among species. The establishment of more freshwater benthic macro-invertebrates with important ecological relevance as test species is necessary to assess the ecological risk of contaminated sediments. The gastropods are important members of aquatic ecosystems, and relatively sensitive to contaminants (Oehlmann et al., 2000; Oetken et al., 2005). Although the freshwater gastropod *Lymnea stagnalis* is widely used for evaluating the adverse effects of toxic chemicals or contaminants, it was suitable for water

toxicity testing rather than for sediment toxicity testing due to its pulmonate breathing (Gomot, 1998; Leung et al., 2007). Actually, standardized sediment toxicity tests with specific freshwater gastropods have not been well defined and, therefore, generally have not been used in a regulatory context. Only recently, two prosobranch snails (*Valvata piscinalis* and *Potamopyrgus antipodarum*) have begun to be suggested as potential candidate test species for freshwater sediment toxicity assessment (Ducrot et al., 2006; Duft et al., 2007). Nevertheless, their small size (both approximately 4 mm long when full grown) may make it inconvenient to perform bioassays with multiendpoints; moreover, they are seldom found in Asia. In China, no native benthic macro-invertebrate species has previously been used or validated in freshwater sediment toxicity testing, and consequently an immediate is needed for seeking sediment toxicity testing species.

Recently, some attention has been given to the potential role of the freshwater mudsnail *Bellamya aeruginosa* (Reeve) (Mollusca: Gastropoda, Prosobranchia, Valvatidae) in monitoring environmental pollution. Xiong and You (2002) investigated oxidative stress responses of *B. aeruginosa* to contaminated river sediment in the field, and Zhang et al. (2007) used this species to detect bioaccumulation of microcystins resulting from eutrophication. *B. aeruginosa* is commonly found in various freshwater habitats of lakes, reservoirs, rivers, stream, ditches, and ponds throughout China. As a sediment-dwelling organism, it mainly inhabits soft mud with abundant organic matter (Liu et al., 1993). This species is a dominant community member of freshwater aquatic ecosystems (with density > 400 individuals per square meter in preferred habitats) (Chen et al., 1975; Chen, 1987), and it is therefore an ecologically important representative of benthic macro-invertebrates. Particularly, as it is a deposit feeder, ingesting sediment particles and organic detritus, algae and bacteria in the sediment or other substrate (Liu et al., 1993), it might be highly exposed to sediment-associated contaminants. In addition, *B. aeruginosa* plays an important role in aquatic food chain because it is a primary food item of the snail carp (*Mylopharyngodon piceus*), and also great importance for human consumption. Therefore, potential contaminants may be transferred from this species to higher trophic levels through the food chain. For these reasons, this freshwater gastropod might be considered as a relevant novel species for the assessment of freshwater sediment toxicity. However, the life cycle of *B. aeruginosa* under laboratory conditions has remained unknown, the procedures for assessing sediment toxicity with this species have not been reported.

The objectives of this study were therefore to reveal the life history of *B. aeruginosa* under laboratory conditions, to examine its utility as a test species for assessing sediment toxicity, and to establish protocols for laboratory culture of this species and sediment toxicity assessment.

1 Materials and methods

1.1 Biology of *B. aeruginosa*

B. aeruginosa is a branchiate gastropod. Adults are moderate-sized (with shell lengths of 20–30 mm, referring to the distance from the peristome to its opposite extremity) and are gonochorists: males are readily distinguished from females by the forniciform, stubby right tentacle, which is the copulatory organ (Fig. 1), whereas females have a pair of uniform tentacles (Liu et al., 1993). In natural habitats, the threshold water temperature for breeding is 16–18°C, and the optimal water temperature is 24–26°C. The reproduction is ovoviviparous; when shell lengths reach 15–16 mm (0.81–0.94 g in body weight), females begin to release their first newborn; the reproductive cycle is about 6 months. Adult females are gravid all year round, and can release their newborn continuously in the breeding season; the average number of newborn produced by a female in a year is approximately 50 individuals. The appearance of the newborns is not entirely similar to that of adults. Whereas shell length exceeds shell width in the adults, newborns have their shell length smaller than shell width (Chen and Song, 1975).

1.2 Collection and acclimatization

Original adult snails were collected in April 2006 from the clean sediments of artificial ponds full of soft mud in the Botanical Garden of Wuhan, Hubei, China, and checked for appropriate taxonomy (Liu et al., 1993) and absence of parasites. They were then transported to the laboratory in plastic pails containing site water. On reaching the laboratory, they were placed in a white tray filled with clean water; after they protruded their tentacles, males and females were separated. A maximum of 90 adults consisting of 60 females and 30 males were introduced into each culture chamber in an overlying water recirculation culture system (Fig. 2), which was modified from our previous culture systems for small fish (Ma et al., 2007). The snails were gradually acclimatized to culture conditions (Table 1), which were based on natural habitat conditions. The artificial sediment was used for acclimatization and culture since naturally clean sediment
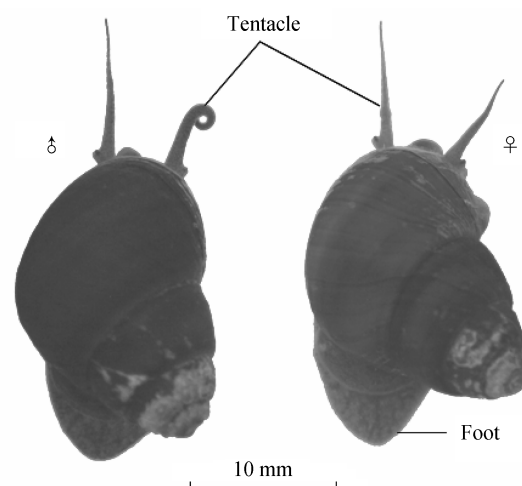

Fig. 1 Sexual dimorphism of adult *Bellamya aeruginosa* (The male is recognized by the forniciform, stubby right tentacle, i.e., copulatory organ).
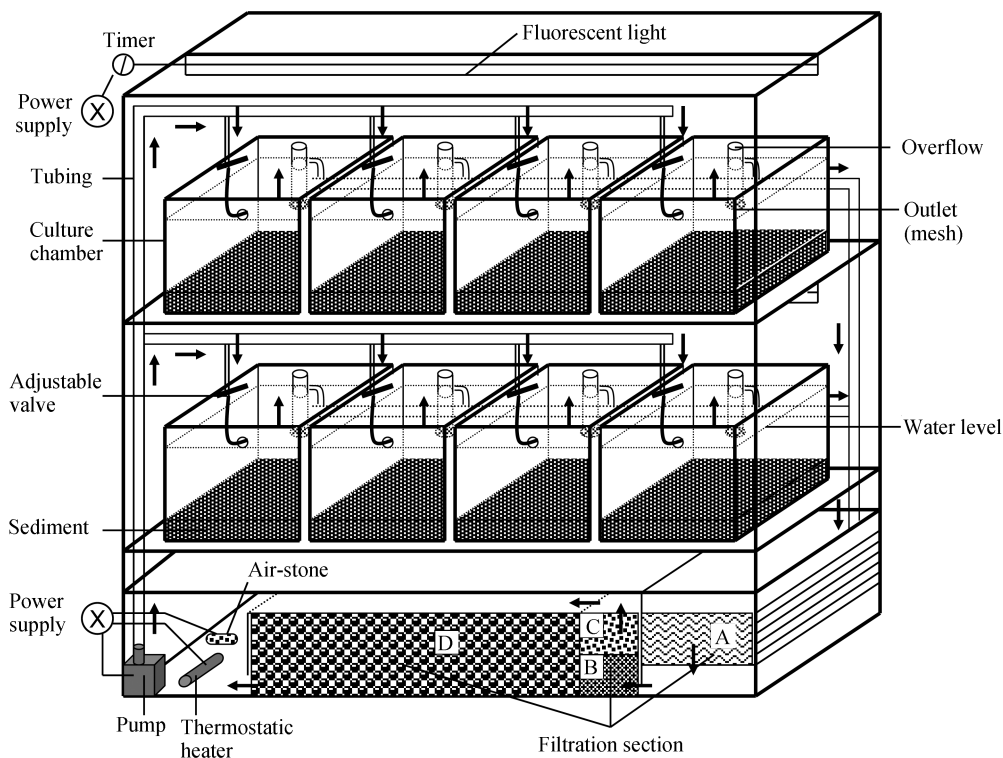
Fig. 2 Schematic of automated overlying water recirculation culture system for *Bellamya aeruginosa*. (A) filter cotton; (B) activated charcoal; (C) ceramic loops; (D) bio-chemical spherules.

with constant physicochemical properties is not always available for a relatively long culture period or frequent tests with this moderate-sized snail. The artificial sediment was prepared from unpolluted terrestrial soil (Pasteris et al., 2003), collected from an uncultivated plot at Dehang Nature Reserve, Jishou, China. The analysis of this soil showed low background concentrations of heavy metals (with 53.41, 31.34, 19.74, 44.98, 0.38, 18.91, and 187.76

μg/g of Cr, Ni, Cu, Zn, Cd, Pb, and Mn, respectively), a total organic carbon (TOC) content of 4.5% and pH 7.78. This soil has been proved to be a suitable substrate in a pre-culture experiment. The soil was prepared as the culture substrate by a procedure modified from Pasteris et al. (2003). Briefly, after the top 1–2 cm layer of the soil was scraped and discarded with a wooden spade, the soil was collected to a depth of 20 cm. The collected soil was dried at 80°C in an oven, then finely ground, and finally sieved through a 150-μm nylon mesh to remove coarse particles. This processed soil was mixed (1:1, *V/V*) with deionized water. The resulting slurry was allowed to settle for 72 hr at 4°C and then overlying water was decanted. During culture, 50% of the total volume of the substrate in each culture chamber was renewed monthly with fresh artificial sediment. The research by Ducrot et al. (2006) showed that best survival and growth of the gastropod *V. piscinalis* were obtained with aquarium fish food, and thus during the acclimatization and culture, *B. aeruginosa* was fed with ground aquarium fish food (Sanyuan®, Beijing, China). Quality control analysis showed that levels of adverse heavy metals (Pb 0.12 μg/g, Cd 0.02 μg/g, Cr 0.27 μg/g) were far below the corresponding standard limit of the Hygienical Standard for Feeds of China (GB 13078-2001).

1.3 Stock cultures

Newborn snails were collected manually from adult stock chambers, and transferred into prepared culture chambers. Initially, a maximum of 500 newborn were added to each culture chamber. The cultures were checked

Table 1 Culture conditions for *Bellamya aeruginosa*

Parameter	Conditions
Substrate (sediment)	Artificial sediment
Temperature	(24 ± 1)°C
Light	Wide-spectrum fluorescent lights
Illuminance	1000 lux
Photoperiod	12L: 12D
Culture chamber	52-L glass aquarium, 65 cm × 30 cm × 27 cm
Ratio of sediment to water	1:4, i.e., 7 L sediment of 6.5 cm in depth and 28 L water
Overlying water	Charcoal-filtered, dechlorinated tap-water[a]
Overlying water quality checks	Temperature and dissolved oxygen (DO ⩾ 4.0 mg/L) daily; hardness, alkalinity, conductivity, pH, and total ammonia weekly
Renewal of overlying water	Recirculation, filtration, aeration, and routine supplement for water loss
Cleaning	Once a month
Feeding	Ground Sanyuan® aquarium fish food

[a] Water-quality parameters (with SE): pH (7.99 ± 0.15), conductivity (267 ± 4) μS/cm, total ammonia (0.41 ± 0.05) mg/L, alkalinity (2.4 ± 0.3) mmol/L, and hardness (78.4 ± 3.3) mg/L.

at four-week intervals by sieving the sediment. After eight weeks, females and males were separated from the cultures, and released into separated chambers at a density of 90 snails per chamber. In the preliminary culture, the food ration and frequency of feeding for snails depended on the feeding activity of the snails and the amount of residual food. Generally, once the food on the surface of sediment was exhausted, the snails would burrow into the sediment for organic detritus, and excessive remaining food would blacken the sediment surface as it decomposed.

1.4 Determination of *ad libitum* feeding levels

The *ad libitum* feeding levels of *B. aeruginosa* were determined at three stages of life: larvae (7-day-old), juveniles (28-day-old), and adults (4-month-old) with tests lasting 28 days. The tests were conducted in a test system in which test chambers (16.5 cm × 16.5 cm × 15 cm) containing 4 cm of clean artificial sediment were held in a temperature-controlled water bath; continuous renewal of overlying water (4–5 volume renewals/day) was implemented with a multichannel peristaltic pump (Jieheng, Chongqing, China). Each chamber was covered with a nylon lid with a hole of 5 cm in diameter in its center for feeding. Other conditions were the same as those of culture. Larvae were offered 1.0, 2.0, or 4.0 mg food/(snail·day); juveniles 4.0, 6.0, 8.0, or 10 mg food/(snail·day); and adults 8, 12, 16, or 20 mg food/(snail·day). At each feeding level, five replicates were arranged with 50 larvae, 30 juveniles or 10 female plus 5 male adults each. The shell length growth rate of larval and juvenile snails and the fecundity (newborn/(female·day)) of adult females were measured to evaluate optimal feeding level.

1.5 Characterization of life history

The survival, growth and reproduction of *B. aeruginosa* were monitored in culture chambers. Under the conditions specified in Table 1, the survival and growth trial were initiated with newborn of homogenous size (shell length (3.33 ± 0.37) mm). These organisms came from the same birth cohort and had been reared for 7 days (freshly-born snails were not used because they are relatively weak). The snails of each life stage (larvae, juveniles, or adults) were offered fish food at the previously determined optimal feeding levels with five replicates containing 90 snails each. The survival and shell length were measured every 4 weeks; sediments were gently sieved through a 300-μm mesh, survivors were counted, shell lengths were measured with a caliper, and then the survivors of each chamber were released into new chamber prepared one week before transfer organisms. The trial lasted 20 weeks, which was beyond the sexual maturation of the snails. At the end of this trial, 40 females and 20 males were randomly selected from each replicate and transferred to new chambers for another 28-day reproduction trial. When the reproduction assays ended, the sediment was sieved, and live and dead juvenile snails were counted together for the estimation of fecundity. The females were dissected and embryos were counted.

1.6 Sediment toxicity tests

1.6.1 Sediment spiking

Sediment spiking was performed following the procedures of Pasteris et al. (2003) with minor modifications. The 10 g/L Cu stock solution was prepared by dissolving analytical grade $CuSO_4 \cdot 5H_2O$ (CAS NO 7758-98-7, Sinopharm, Shanghai, China) in deionized water (18 MΩ·cm). A series of spiking solutions were then prepared from dilutions of the stock solution. Spiking was initiated by mixing the required weight of wet artificial sediment calculated from dry/wet weight ratio with the required Cu solution in a glass jar at a volume ratio of 1:1. The content of each jar was manually stirred with a wooden spoon for at least 6 hr to achieve a uniform distribution of spiked toxicants throughout the sediment, and then kept at 20°C for 14 days to ensure chemical equilibrium between the sediment and water (Simpson et al., 2004). The target nominal Cu concentrations used were 0 (control), 20, 60, 180, 540, and 1080 μg/g dry sediment, which covered the range of environmentally relevant Cu concentrations found in contaminated aquatic environments in China (Ren et al., 2007). At the beginning and end of the following tests, total particulate Cu concentrations in the sediment were determined by inductively coupled plasma-atomic emission spectroscopy (ICP-OES, iCAP6300 Radial, ThermoFisher Scientific, USA) after hydrochloric acid-nitric acid-perchloric acid-hydrofluoric acid digestion. Two procedural blanks were also prepared with each batch of digestion set using the same analytical procedure and reagents. Certified sediment reference material (GBW 07408 (GSS-8), NRCG, China) was analyzed through the same procedure to validate the methodology, and the measured values were found within the certified range (recoveries were within 6% of the certified values). Concentrations of the metals in the sediments were expressed as μg/g on a dry weight basis.

1.6.2 Sediment toxicity test for 10 days with larvae

The acute toxicity of the Cu-spiked sediment to larvae was evaluated over a period of 10 days according to the test conditions described in Table 2 for all target nominal concentrations. At the start of the test, the sediment was added to each of 5 replicate test chambers per treatment to obtain a 2-cm depth; overlying water was then added gently to minimize sediment disturbance. The sediment was allowed to settle for 24 hr for the reestablishment of redox gradients. Meanwhile, the shell lengths of homogenous 7-day-old larvae were measured, and 20 active individuals were gently released into each test chamber. The snails were fed every day. On day 10, the contents of each chamber were sieved, the surviving snails were recovered and counted, and their shell lengths were measured. After depuration in clean water for 24 hr, the snails were rinsed with 10 mmol/L EDTA solution (Sinopharm, Shanghai, China) for 5 min to get rid of the Cu ions that present on their shells (Cœurdassier et al., 2005), and then transferred to a deionized water bath, and finally dried on filter paper. The snails from each replicate of the treatments were

Table 2   Conditions for sediment toxicity testing with *Bellamya aeruginosa*

Parameter	Conditions
Temperature	(24 ± 1)°C, maintained by the temperature-controlled incubator
Light	Wide-spectrum fluorescent lights
Illuminance	1000 lux
Photoperiod	12L: 12D
Test chamber	4-L glass jar (16.5 cm × 16.5 cm × 15 cm), covered with a nylon lid with a 5-cm hole in diameter in its center for feeding
Sediment depth	10-Day larvae test: 2 cm 28-Day adults test: 4 cm
Overlying water	Charcoal-filtered, dechlorinated tap-water
Overlying water quality checks	10-Day larvae test: temperature and DO daily. Hardness, alkalinity, conductivity, pH, and total ammonia at test initiation and termination. 28-Day adults test: temperature and DO daily. Hardness, alkalinity, conductivity, pH, and total ammonia weekly.
Renewal of overlying water	Continuous renewal, 4–5 volume renewals/day, implemented by an automated water-delivery system adjusted by a multichannel peristaltic pump (Jieheng, Chongqing, China).
Number of replicates/ treatment	5
Feeding	Ground Sanyuan® aquarium fish food, China

pooled for determining whole-body Cu burdens with ICP-OES after acid digestion.

1.6.3 Sediment toxicity test for 28 days with adults

The effects of chronic toxicity of Cu-spiked sediment on adult snails were evaluated over a period of 28 days according to the test conditions described in Table 2 for the following nominal concentrations: 0 (control), 20, 60, 180, and 540 μg/g dry sediment. The selected adults (120 days old, mean shell length: ♂ (17.07 ± 0.74) mm; ♀ (17.20 ± 0.89) mm) that had just reached puberty were used for the test. On day 0, the shell lengths of the snails were measured and 10 females and 5 males (based on the measured population sex ratio in the stock culture) were added to each test chamber. The snails were fed every day. Newborn visible in the test chamber were removed daily and were counted and recorded. On day 28, the contents of each test chamber were sieved and the surviving adults and their offspring were counted and recorded. The surviving adults were measured for shell length. The shells of adults were cracked with forceps, and females were inspected for gravidity (dead females were deemed to be non-gravid). Then the hepatopancreas including gonad, and muscular foot were carefully separated from the visceral hump. The hepatopancreas was cut into two parts: one part was immediately frozen in liquid nitrogen and stored at –80°C until further analysis, and another part of the hepatopancreas, gonad, and muscular foot were dried to constant weight at 60°C. The Cu contents of these tissues were determined with ICP-OES after acid digestion. Three pivotal biomarkers including superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) in the hepatopancreas, which are involved in the primary defense against oxidative damage resulting from heavy metal stress in aquatic organisms (Livingstone, 2001), were determined

using bioassay kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) to reveal oxidative stress responses of *B. aeruginosa* to chronic exposure to Cu-contaminated sediments.

1.7 Statistical analyses

Data were analyzed with SPSS 13.0 and results are presented as mean standard deviation (SD). Concentrations of Cu in the sediment and snail tissues are expressed as μg/g on the basis of dry weight. The endpoint values in response to Cu concentrations were analyzed by one-way analysis of variance (ANOVA) technique followed by post-hoc test after ensuring that the data conformed to homogeneity of variance and normality. Chi-square tests were performed to determine statistically significant differences in survival between treatments and the control. Statistical significance was declared when P value was less than 0.05. The lowest observed effects concentrations (LOECs) were determined by statistically significant differences relative to controls. The median lethal concentration (LC_{50}) was calculated for the 10-day assessment of acute toxicity with the trimmed Spearman-Karber method (Hamilton et al., 1977).

2 Results

2.1 General observations under laboratory culture conditions

The snails generally remained on the surface of the sediment when food supply was sufficient. Once the food on the sediment surface became insufficient or consumed, snails would burrow into the sediment. When dissolved oxygen in the overlying water was below 4.0 mg/L, the snails crawled up the walls of the culture chambers. Under the present culture conditions, reproductive activity was maintained all year around. The newborns were generally found on the shell of an adult snail or on the walls of the culture chambers; and 2–3 days later, they inhabited the surface of the sediment. The ratio of female to male from the cultured population was around 2:1.

2.2 *ad libitum* feeding levels

Individual performances in the 28-day feeding test are shown in Table 3. The optimal growth rates of larval and juvenile snails were achieved when they were fed 2.0 and 6.0 mg fish food/(snail·day), respectively. The optimal feeding level for reproduction was 16.0 mg fish food/(snail·day).

2.3 Life history

Survival and growth patterns of *B. aeruginosa* are presented in Fig. 3. The survival rate of larvae was as high as 91.84%; five weeks later, it remained above 80% until the end of the test, indicating the favorable culture conditions for the life cycle of *B. aeruginosa*. Under 9 weeks of age, snails grew at approximately 190 μm/day (shell length) in a rapid, linear pattern. Growth (30 μm/day) slowed down obviously after the onset of reproduction. No significant difference in size existed between sexes;

Table 3 Growth and fecundity (mean ± SD) of *Bellamya aeruginosa* at different life stages subject to various feeding levels during a 28-d test

Life stage	Feeding level (mg fish food/ (snail·day))	Growth rate of shell length (μm/day)	Fecundity (newborns/ (female·day))
Larvae (7-day-old)	1.0	151 ± 26 [a]	–
	2.0	195 ± 29 [b]	–
	4.0	191 ± 35 [b]	–
Juveniles (28-day-old)	4.0	127 ± 18 [a]	–
	6.0	165 ± 13 [b]	–
	8.0	155 ± 26 [b]	–
	10.0	168 ± 15 [b]	–
Adults (5-month-old)	8.0	–	0.28 ± 0.08 [a]
	12.0	–	0.46 ± 0.12 [b]
	16.0	–	0.64 ± 0.13 [c]
	20.0	–	0.68 ± 0.16 [c]

[a, b, c] Mean values bearing different superscripts within a life stage differed significantly ($P < 0.05$, ANOVA post-hoc Tukey's honest significance difference test).
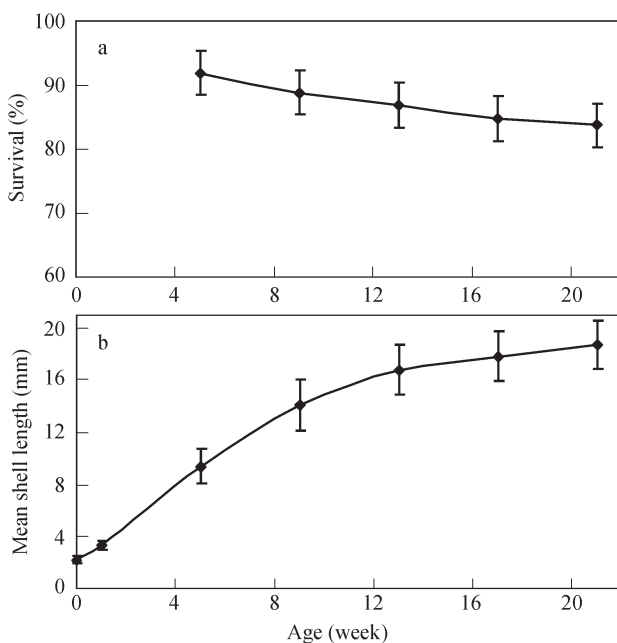
Fig. 3 Survival (a) and growth (b) of *Bellamya aeruginosa* during a culture period of 20 weeks. Error bars represent standard errors of means.

the shell lengths of 21-week-old females and males were (17.76 ± 1.46) mm and (17.59 ± 1.68) mm, respectively. The snails could be sexed at 9 weeks of age (shell length (14.12 ± 1.97) mm. Female snails released their first newborn at approximately 18 weeks of age; thus they were estimated to reach maturity at around 16–17 weeks of age (i.e., 112–119 days old). The fecundity of freshly matured adult females was (0.55 ± 0.13) newborn/(female·day), and the average number of embryos carried by a gravid female was 19.33 ± 2.54.

2.4 Assessment of sediment toxicity for 10 days with larvae

The acute toxicity of Cu-spiked sediments to the larvae of *B. aeruginosa* is shown in Table 4. No mortalities occurred in the control over the 10-day test, indicating that the test conditions were appropriate for sediment toxicity testing. Cu concentrations below 195 μg/g dw had no effect

on survival. Cu concentrations of 570 and 872 μg/g dw led to sharply decreased survival rates; only few snails survived at 872 μg/g dw. The calculated 10-day LC_{50} of Cu for larvae was 480 μg/g ($CI_{95\%}$: [416; 536]). Because of the high mortality, data of growth and whole-body Cu burden are not presented for the 872 μg/g dw treatment. The lowest observed effect concentration (LOEC) of Cu for both survival and growth of larvae was 195 μg/g dw since significant decrease of survival and growth occurred at this level and higher. The average whole-body Cu burden of the snails subjected to each Cu-spiked treatment was significantly higher than that of the control, and increased in a concentration-dependent manner; a sharp increase occurred at 570 μg/g dw.

2.5 Assessment of sediment toxicity for 28 days with adults

The data of survival, growth, tissue Cu burden, and biochemical endpoints are presented as combined values from both sexes due to the relatively small number of male samples in each replicate (Table 5 and Fig. 4). Compared to the controls, only Cu concentration of 570 μg/g significantly decreased the survival rate and growth rate; otherwise the Cu concentrations had no effect on the
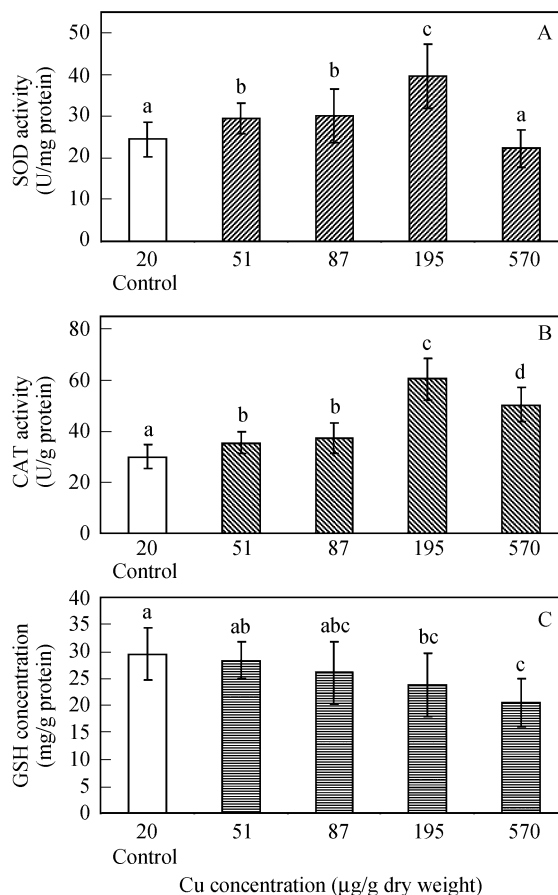

Fig. 4 SOD (A) and CAT activity (B), and the GSH concentration (C) in the hepatopancreas of adult *Bellamya aeruginosa* following 28-day chronic exposure to Cu-spiked sediment. Error bars represent standard errors of means. Treatments with different letters differed significantly ($P < 0.05$, ANOVA post-hoc Tukey's honest significance difference multiple comparison test).

Table 4 Mean survival rate, growth rate of shell length, and whole-body Cu burden of the larvae of *Bellamya aeruginosa* following 10-day exposure to Cu-spiked sediment

Endpoint	20 μg Cu/g dw (control)	51 μg Cu/g dw	87 μg Cu/g dw	195 μg Cu/g dw	570 μg Cu/g dw	872 μg Cu/g dw
Mean survival rate (%)	100	100	100	88.33* (2.89)	46.67* (7.64)	5.00* (8.66)
Growth rate of shell length(μm/day)	220.71 (14.75)	217.48 (19.70)	234.97 (17.32)	127.63* (19.25)	123.83* (19.86)	–
Whole-body Cu burden (μg/g dw)	26.92 (0.52)	38.89* (1.19)	60.52* (1.48)	88.48* (2.36)	185.09* (6.51)	–

* Values significantly different from the corresponding control ($P < 0.05$, Chi-square test for survival; ANOVA post-hoc Dunnett's test for growth and whole-body Cu burden). The value in parenthesis means SD.

Table 5 Mean survival rate, growth rate of shell length, gravidity, fecundity (average number of offspring per surviving adult), and tissue Cu burden of adult *Bellamya aeruginosa* following 28-day chronic exposure to Cu-spiked sediment

Endpoint	20 μg Cu/g dw (control)	51 μg Cu/g dw	87 μg Cu/g dw	195 μg Cu/g dw	570 μg Cu/g dw
Mean survival rate (%)	97.72 (3.61)	95.83 (3.69)	97.82 (3.17)	96.89 (4.67)	83.33 (7.22)*
Growth rate of shell length (μm/day)	70.58 (4.83)	71.21 (3.24)	66.65 (6.89)	65.67 (8.13)	54.15 (5.83)*
Gravidity (% gravid females per total number of surviving females)	69.47 (4.79)	68.07 (8.83)	65.07 (7.29)	63.43 (3.98)	41.67 (7.22)*
Fecundity (newborn/(female·day))	2.53 (0.39)	2.63 (0.45)	2.28 (0.26)	1.76 (0.23)*	1.37 (0.30)*
Tissue Cu burden (μg Cu/g dw)					
Hepatopancreas	30.01 (4.98)	33.88 (8.14)	49.94 (15.04)	115.97 (23.45)*	244.95 (52.58)*
Gonad	26.14 (5.85)	30.21 (8.14)	46.04 (15.00)	95.68 (25.67)*	172.73 (32.63)*
Muscular foot	7.75 (2.00)	16.01 (3.45)	20.07 (5.01)	29.68 (5.80)*	43.05 (9.72)*

Values significantly different from the corresponding controls ($P < 0.05$, Chi-square test for survival; ANOVA post-hoc Dunnett's test for other endpoints) The value in the parenthesis means SD.

survival and growth of adults. The gravidity of females was significantly lowered at 570 μg/g dw, whereas the fecundity was significantly decreased by Cu at 195 μg/g dw or higher. The tissue Cu burden was significantly elevated at 195 μg/g dw or higher and ranked in the order of hepatopancreas > gonad > muscular foot. The hepatopancreatic SOD and CAT activity increased initially and then decreased with increasing sediment Cu concentrations (Fig. 4A, B). Both SOD and CAT activity significantly increased as Cu concentrations increased from 51 to 195 μg/g dw, indicating that Cu significantly stimulated SOD and CAT activity. However, the SOD and CAT activities in response to 570 μg/g dw were significantly lower than those at 195 μg/g dw, indicating the presence of inhibition. Compared with the control, GSH concentrations tended to decrease with increasing Cu concentrations, and significant decreases occurred at 195 μg/g dw and beyond (Fig. 4C).

3 Discussion

3.1 Laboratory culture of *B. aeruginosa*

Generally, organisms obtained by either direct field-sampling or purchasing from local suppliers are not recommended as test organisms for sediment toxicity testing because some uncertain characteristics (such as unknown source, unidentified age, health, potential pollution stress) of these organisms may potentially confound the interpretation of bioassay results (USEPA, 2000). Therefore, successful laboratory culture of selected species is prerequisite for developing protocols of sediment toxicity. In the present study, due to its moderate size, relatively long life cycle and low fecundity, a sufficient batch of homogenous individuals of *B. aeruginosa* can rarely be harvested from a population maintained within a confined space. An appropriate culture device is therefore indispensable. The current culture experiment with *B. aeruginosa* showed that the established culture system was a relatively efficient and trouble-free device, in which the optimal conditions of dissolved oxygen and temperature for snails could be stably maintained through the filtration, reaeration, and thermoregulation of the overlying water. Indeed, except for daily cleaning of the filter cotton, timely replenishment of water loss, and routine feeding, no other efforts were demanded, thus minimizing perturbations to sediments and stress on the snails, and enhancing culture performance. In addition, unlike the oviparous gastropods (Ducrot et al., 2006), the reproductive feature of ovoviviparity also facilitated culture since no special care was needed at their early life stage. Therefore, rearing *B. aeruginosa* with this system was neither time nor labor consuming.

Culture experiments also indicated that the quality of the sediments used declined gradually over the culture period due to the decomposition of food residue and dead snails. Quality control of the sediments is necessary to perform long-term culture. A good solution for this issue is to renew sediments periodically (Ducrot et al., 2006), which requires a large amount of sediments. However, the reliable natural sediment sources with consistently acceptable clean sediments are not always available in the presence of extensive aquatic pollution. Artificial sediment based on unpolluted soil may be a practicable alternative because the use of artificial sediments can allow standardization of test organism cultures and eliminate interferences caused by the presence of endogenous organisms in natural sediments (USEPA, 2000; Pasteris et al., 2003). In our laboratory culture, the survival rates as high as 80% and early maturation (4 months), compared with more than 6 months in natural habitats (Chen and Song, 1975), demonstrated clearly that the artificial sediments used were favorable

to the growth and development of *B. aeruginosa*. Under laboratory culture conditions (Table 1), the snails can breed three generations in one year.

The fecundity of the laboratory population was approximately twice that in the field population (0.24 newborn/(female·day)) (Chen and Song, 1975), indicating that the commercial fish food satisfied the nutritional requirements of *B. aeruginosa* for reproduction. The use of eight culture chambers with 90 adults at a female to male ratio of 2:1 could maintain a continuous supply of newborn throughout the year. It should be noted that, in order to realize planned culture, juvenile snails should be sexed in a timely manner and reared in separate culture chambers to avoid unexpected mating. Nevertheless, due to the use of artificial sediments, the obtained values for life-history traits might be different from the values in natural sediments.

3.2 Assessment of sediment toxicity

The survival rates of the controls in both sediment toxicity tests were 100% and 97.92%, respectively, which are higher than the minimum allowable value required for standardized tests (USEPA, 2000) and indicate that the test conditions were suitable for sediment toxicity tests. The LOEC of the Cu-spiked sediment for survival and growth of the larvae was lower than that in the adults (195 vs. 570 μg/g dw), indicating that the larvae were more sensitive to the Cu-spiked sediment than the adults. This difference in sensitivity to Cu between the larvae and the adults seemed to be associated with their capabilities to metabolize Cu, since the Cu burdens were obviously higher in larvae than that in adults. In any case, tissue Cu burden in adults might not be a useful indicator for chronic exposure to low concentrations. The distribution of Cu in tissues agreed with published observations showing that major microcystins were present in the hepatopancreas of *B. aeruginosa* (Zhang et al., 2007). As far as the 10-day LOEC was concerned, *B. aeruginosa* seemed to be more sensitive to sediment Cu exposure than the two standard test species *H. azteca* and *C. tentans*, with 10-day LOECs for growth and survival of >2010 μg/g (Huggett et al., 1999). The LOEC of toxicity endpoints proved that both survival and growth of *B. aeruginosa* responded sensitively to the adverse effects of Cu in the 10-day assessment of acute toxicity with larvae. In the 28-day assessment of chronic toxicity with adults, fecundity was a reliable endpoint indicating Cu toxicity to reproduction, whereas survival, growth, and gravidity did not appear to be sensitive endpoints.

Antioxidant defense systems have been widely studied in various aquatic organisms (such as mollusks and oligochaetes) exposed experimentally to chemicals or collected from polluted areas, and oxidative stress components such as SOD, CAT, GSH, and MT (metallothionein) have been frequently used as exposure or effect biomarkers of contaminants (Bergayou et al., 2009; Mosleh et al., 2007). It is well known that SOD and CAT are enzymes that detoxify reactive oxygen species in the cells. In the present study, hepatopancreatic SOD and CAT exhibited high sensitivity to Cu stress because Cu exposure at low concentration (51 μg/g) could cause significant increase of their activities. Similar results reported by Xiong and You (2002) showed that SOD and CAT in the whole visceral hump of *B. aeruginosa* were relatively sensitive to *in situ* exposure to polluted river sediments. Therefore, in *B. aeruginosa*, hepatopancreatic SOD and CAT can be used as effective biomarkers for contaminant-induced oxidative damage. Moreover, the distinct hormesis (stimulation at low doses but inhibition at high doses) (Calabrese and Lu, 2006) of SOD activity (Fig. 4A) suggested that SOD can be used as a more suitable biomarker for exposure to low concentrations of Cu than CAT and GSH. In general, GSH reacts readily with heavy metal ions and thus participates in detoxification (Kuroshima, 1995). In our study, the hepatopancreatic GSH content in snails exposed to high concentrations of Cu (570 μg/g dw) significantly decreased, indicating that high concentration of accumulated Cu markedly depleted GSH and thus induced Cu toxicity as evidenced by significantly decreased SOD activity.

Compared to reported small freshwater gastropods, such as *V. piscinalis* (Ducrot et al., 2006) and *P. antipodarum* (Duft et al., 2007), our bioassay with *B. aeruginosa* showed some distinct advantages. Its relatively large size (adults with shell length of more than 17 mm) facilitates experimental handling and provides sufficient tissue biomass for bioassays, and thus enables identification of potential endpoints for sublethal or chronic toxicity. For example, multi-endpoints can be measured simultaneously with the relatively large 50-mg hepatopancreas.

Although our sediment toxicity test used artificial sediments instead of natural sediments, this practice simply provided a general method for evaluating toxicity of sediments spiked with specific chemicals. Actually, the use of artificial sediment may eliminate or control the variation in sediment physico-chemical characteristics (USEPA, 2000). In a similar study about toxicity of Cu-spiked sediments to *Tubifex tubifex*, Pasteris et al. (2003) suggested that when artificial sediment was Cu-spiked and used in bioassays, an adequate and consistent matching between target and measured concentrations and a definite dose-response curve were obtained, which was also confirmed by our bioassay. Nevertheless, the sensitivity of *B. aeruginosa* to Cu in the current study may only be representative of the experimental conditions instead of natural sediments.

The life history traits and the results from the assessment of toxicity of the Cu-spiked sediment proved that *B. aeruginosa* appears to have a great potential as a test species for sediment toxicity tests in China. Based on the life history of *B. aeruginosa*, the assessment of sediment toxicity can be carried out through the developmental stages of larvae (0–28 days old), juveniles (28–112 days old) and adults (over 112 days old). Due to the thin and fragile shell of newborns and their behaviors of inhabiting non-sediment substrates, 7-day-old larvae instead of newly born individuals are recommended for use. However, due to the relatively long life cycle of *B. aeruginosa*, the

assessment of sediment toxicity over a single life cycle or even generations requires an extended experimental period.

Further investigations are warranted to ascertain acute and chronic sensitivity of *B. aeruginosa* to a wide range of contaminants of concern in freshwater ecosystems, and to develop some sensitive bioassay endpoints or biomarkers.

4 Conclusions

Under the designed conditions, *B. aeruginosa* was successfully cultured with ideal culture performances and reached sexual maturity earlier than field populations. The test conditions of our study proved to be suitable for *B. aeruginosa*. Its moderate size facilitated assays with individuals of different life stages. Compared to the routine test species, *B. aeruginosa* seems to be a sensitive species. Our findings suggest *B. aeruginosa* as a suitable species for the assessment of sediment toxicity. Further investigations are still required to understand its acute and chronic sensitivity to a wide range of contaminants, and to identify other effective bioassay endpoints or biomarkers for the assessment of sediment toxicity.

Acknowledgments

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