Ecotoxicology and Environmental Safety 110 (2014) 208–215

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Developmental toxicity, acute toxicity and mutagenicity testing in freshwater snails *Biomphalaria glabrata* (Mollusca: Gastropoda) exposed to chromium and water samples

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ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form
28 August 2014
Accepted 3 September 2014
Available online 26 September 2014

Keywords:
Biomphalaria glabrata
Dominant lethal test
Developmental toxicity
Potassium dichromate
Toxicity

ABSTRACT

A protocol combining acute toxicity, developmental toxicity and mutagenicity analysis in freshwater snail *Biomphalaria glabrata* for application in ecotoxicological studies is described. For acute toxicity testing, LC_{50} and EC_{50} values were determined; dominant lethal mutations induction was the endpoint for mutagenicity analysis. Reference toxicant potassium dichromate ($K_2Cr_2O_7$) was used to characterize *B. glabrata* sensitivity for toxicity and cyclophosphamide to mutagenicity testing purposes. Compared to other relevant freshwater species, *B. glabrata* showed high sensitivity: the lowest EC_{50} value was obtained with embryos at veliger stage (5.76 mg/L). To assess the model applicability for environmental studies, influent and effluent water samples from a wastewater treatment plant were evaluated. Gastropod sensitivity was assessed in comparison to the standardized bioassay with *Daphnia similis* exposed to the same water samples. Sampling sites identified as toxic to daphnids were also detected by snails, showing a qualitatively similar sensitivity suggesting that *B. glabrata* is a suitable test species for freshwater monitoring. Holding procedures and protocols implemented for toxicity and developmental bioassays showed to be in compliance with international standards for intra-laboratory precision. Thereby, we are proposing this system for application in ecotoxicological studies.

1. Introduction

Acute and chronic toxicity tests have been mostly used in ecotoxicological protocols to evaluate the potential effects of environmental contaminants to natural populations (Cooney, 1995). However, with the need to assess the potential sublethal hazards to ecosystems of pollutants at low concentrations, environmental monitoring programs have encouraged the analysis of effects on gametes, fertilization, reproduction and embryo-larval development (Llanos-Rivera et al., 2009). The effects on reproduction of many pollutants are unknown. Furthermore, among the myriad of chemicals reaching the environment, some compounds classes can directly affect the reproductive potential through the induction of mutations in germ cells (Evenden and Depledge, 1997).

Among aquatic organisms used in ecotoxicological studies, invertebrates have been employed due to their importance in trophic chains and greater sensitivity response to chemical pollutants (Achiorno et al., 2010). Although mollusks are the second largest group in kingdom Animalia, they have not been considered in environmental risk assessment so far, mainly due to the lack of standardized protocols. In this sense, gastropods, the most abundant mollusks, have been successfully used as pollution indicators by different compound classes, such as, metals, pesticides an important group of emerging contaminants, called endocrine disruptors (Salice and Miller, 2003; Oliveira-Filho et al., 2005; Matthiessen, 2008; Ansaldo et al., 2009; Gagnaire et al., 2009; Giusti et al., 2013; Zounkova et al., 2014).

Biomphalaria glabrata freshwater snails have been studied in many aspects because of their role as intermediate hosts of the trematode *Schistosoma mansoni*. Assays with *B. glabrata* (Say, 1818)

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http://dx.doi.org/10.1016/j.ecoenv.2014.09.005
0147-6513/

have demonstrated that this species is a good model for laboratory and monitoring environmental studies (Verrengia Guerrero et al., 1997; Abd Allah et al., 1999; Cochón et al., 2007; Kristoff et al., 2010). *Biomphalaria* shows wide geographical distribution, low dispersion and is easily collected. Snails need little space and exposure systems require relatively small quantities of test samples for analysis hence contributing to reduced costs of aquatic in vivo evaluations. Snails can reproduce throughout the year under controlled conditions and have a short life span: an egg-to-egg monitoring can be done in two months in *B. glabrata*. Being a simultaneous hermaphrodite, different reactions in both sexes do not occur (Nakano et al., 2003). *B glabrata* embryos constitute a good system for studying developmental toxicity. Almost daily, about 20–50 eggs are placed one by one in a single layer forming a transparent plurioval egg capsule allowing the visualization of the embryos throughout development, which takes around seven to nine days at 25 °C. The normal embryonic development has been described in detail (Camey and Verdonk, 1970) and showed well-defined and easily recognizable stages, allowing the detection of morphogenetic effects. This species has been successfully used in our laboratory for more than 30 years to evaluate effects of chemical and physical agents, analyzing aspects such as mortality index, alterations in embryonic development, chromosome aberrations and DNA damage (Kawano et al., 1979; Kawano and Simões, 1986; Okazaki et al., 1996) More recently, studies have been done on the use of the species as a bioindicator for ecotoxicological studies (Nakano et al., 2003; Tallarico et al., 2004; Estevam et al., 2006; Grazeffe et al., 2008). We demonstrated that *B. glabrata* can absorb and activate chemical mutagens from the aquatic environment. The dominant lethal test was established in snails with mitomycin C and cyclophosphamide. This system was efficient, specific and sensitive in the evaluation of germ cell mutations, an important endpoint to assess the effects on reproductive potential of populations (Nakano et al., 2003), including a mutagenicity test to the model.

In the present study, two compounds were chosen to the establishment of *B. glabrata* as a model for ecotoxicological tests. First of all, in the evaluation of the organism for acute toxicity testing, a reference toxicant, which is a standard chemical, with well-established chemical characteristics, stability, solubility and toxicity was used. These compounds are known as reference substances, and used as positive control, using a series of multiple concentrations, detecting effects outside the normal range (Rand et al., 1995). Several organics and inorganics compounds, are indicated to be used as reference substances in sensitivity tests. According to Environment Canada (1990), the most suitable reference toxicants for a wide variety of tests are two organics (sodium pentachlorophenate and phenol) and two inorganics (hexavalent chromium and zinc). In this sense, potassium dichromate was used to establish the control chart to *B. glabrata* (Tallarico et al., in press) and to evaluate the suitability of candidate organisms for toxicity testing. Cyclophosphamide was selected as the positive control for the induction of mutations. This compound is a nitrogen mustard used as antineoplasic in the treatment of cancer (Korolkovas and Burckhalter, 1988; Calabresi and Chabner, 1990) and already used in other studies with aquatic snails (Singh and Agarwal, 1981; Nabih and El Hamid, 1984). In the dominant lethal test, cyclophosphamide was used as the reference mutagen to show that the snails could metabolically activate indirect mutagens (Nakano et al., 2003).

In the present study, we developed a protocol combining techniques for acute toxicity assessment, developmental toxicity and mutagenicity for *B. glabrata*. Further, influents and effluents water samples from a Wastewater Treatment Plant (WWTP) were evaluated to assess the applicability of the model for testing purposes.

The relevance of the data obtained in this work is associated to the site selected for the study. The WWTP (Suzano municipality) is located near to the source of the Tietê River. A large amount of complex effluents is released in the Suzano WWTP, the influent is represented by domestic sewage, industrial effluents, and waste from septic tanks and landfill leachate. In addition, the treated effluents are released in the river. With great historic and economic importance for the country, this river crosses the São Paulo state and metropolitan area. Although contaminated, the river still participates in the development of the region and its waters are used in supplying and production of electricity, which warrants studies aimed at its preservation, with reduction of organic and toxic loads.

2. Materials and methods

Two experiment series were performed: 1—to characterize the sensitivity of *B. glabrata* for toxicity testing purposes, adult snails and embryos were exposed to the reference toxicant, chromium; 2—to assess the applicability of the model for ecotoxicological studies, water samples were assessed for toxicity, developmental effects and mutagenicity.

2.1. Test substances and surface water samples

Potassium dichromate [7778-50-9] ($K_2Cr_2O_7$, Merck, PA 99.5 percent) was used as a reference toxicant to standardize the sensitivity assay, as recommended by US EPA (1993). Potassium dichromate is a reddish-orange compound containing the three elements potassium (26.58 percent), chromium (35.35 percent), and oxygen (38.07 percent). Each 2.829 g of potassium dichromate contains one gram of the element chromium. Cyclophosphamide [6055-19-2] ($C_7H_{15}Cl_2N_2O_2P \cdot H_2O$, Sigma Aldrich), previously established as an inducer of dominant lethal mutations in *B. glabrata* (Nakano et al., 2003), was the reference mutagen.

The sampling site for the environmental study was located in Suzano, metropolitan region of São Paulo, and comprises the Suzano Wastewater Treatment Plant (WWTP) (Fig. 1). The water treatment in the WWTP is based on activated sludge (secondary level) with an average flow of 800 L/s. The system was projected for sewage, although it receives several hard industrial effluents.

Surface water samples were taken in: I—August 2006, II—February 2007, III—August 2007 and IV—March 2008; these samplings were chosen to include both high and low pluviometric gradient Four sites were selected: (S0) Ponte Nova Dam (Salesópolis City, near to the river source)–negative control, (S1) 200 m upstream of the plant, (S2) WWTP influent (collected at the medium grids), (S3) treated wastewater (effluent) and (S4) 200 m from the discharge of the effluent in the Tietê River (Fig. 1). Sampling, physical chemical analysis and transportation were carried out by SABESP (Basic Sanitation Company of the State of Sao Paulo). Composed samples (24 h) of 20 L were prepared by SABESP for influent and effluent samplings. The company performs a continuous monitoring of the physical–chemical analysis (pH, water and air temperature, conductivity, oxygen dissolved, biochemical and chemical oxygen demand, nitrogen, total solids and total organic carbon) and the results were previously described by Hamada et al. (2011).

2.2. Test organisms

Adults and egg masses were obtained from a population of pigmented wild-type strain of *B. glabrata* originally from Barreiro de Baixo (Minas Gerais, Brazil) and a non-pigmented albino strain from Amaralina (Bahia, Brazil), reared under laboratory conditions for more than 30 years. The snails are maintained, with a maximum of 50 individuals, in plastic aquaria ($50 \times 23 \times 17$ cm)

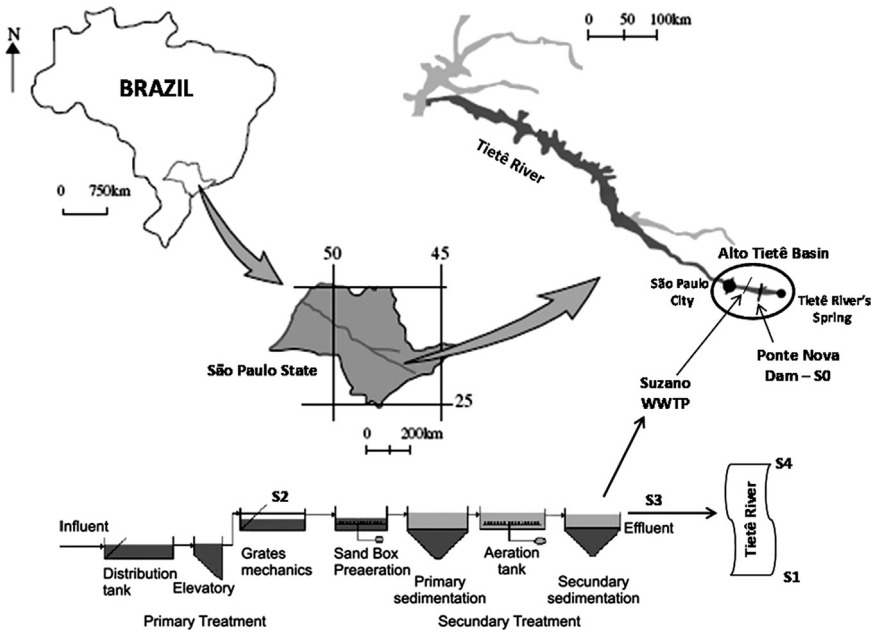

Fig. 1. Map showing the Tietê River in São Paulo State (Brazil), Suzano Wastewater Treatment Plant (WWTP), (S0) Ponte Nova Dam (Salesópolis City) and flowchart of the fluid portion of the WWTP with the sites sampled: (S1) 200 m upstream of the plant, (S2) WWTP influent, (S3) effluent and (S4) 200 m from the discharge of the effluent in the Tietê River (Adapted from Cutolo et al. (2006), Rocha et al. (2009).

with 20 L of filtered, dechlorinated and aerated water. The feeding is fresh lettuce ad libitum and a balanced diet. The room temperature is at $25 \pm 2^{\circ}$ C with a 12 h light period. The culture rotations are realized weekly, removing the egg masses deposited in the aquaria.

2.3. Toxicity testing

Acute toxicity assays were performed with adult snails and embryos. They were exposed to chromium and environmental water samples during 24 h to determine LC_{50} (concentration lethal to 50 percent of the exposed organisms) to adults and EC_{50} values to embryos (median effective concentration in terms of lethality and malformations).

Regarding the assays with environmental effluents, the criteria established for classifying the acute toxicity based on LC_{50} and EC_{50} values were adapted from Arkhipchuk et al. (2006), classifying the intensity of toxic effects based on lethality and embryonic malformations as endpoints as following: (1) Non-toxic: 100 percent of effluent, (2) Slightly toxic: 99–50 percent, (3) Toxic: 49–20 percent and (4) Highly toxic: < 20 percent.

2.3.1. Adult snails toxicity assay

Adult snails at least two months old and with a minimal shell diameter of 10 mm and were not fed 24 h before test start. The mortality before assay start should not exceed 10 percent. Toxicity assays were conducted at 25 ± 2 °C with a 12 h light period for 24 h with ten snails of 10–13 mm shell diameter in 180 mL glasses. Preliminary bioassays with 50, 100 and 200 mg/L were done to determine the range of toxicant concentrations to be used and to define further experimental conditions (data not shown). In the definitive assay, animals were exposed to chromium at selected concentrations of 25, 50, 75, 100, 150 and 200 mg/L prepared by dilution of a stock solution with filtered and dechlorinated tap water. For effluents and influents, concentrations of 10, 25, 50 and 75 percent were selected based on preliminary experiments with 50 percent dilution and with the raw samples. A negative control group was maintained in filtered and dechlorinated tap water under the same experimental conditions.

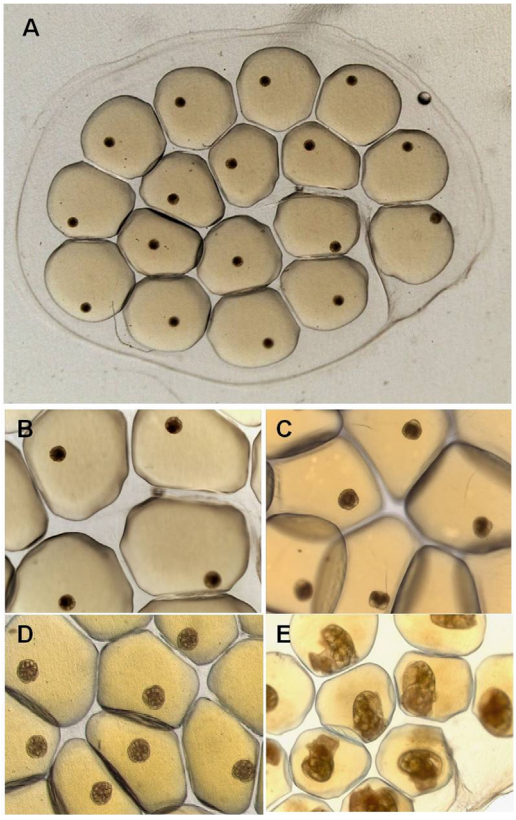

Fig. 2. (A) Spawning of *Biomphalaria glabrata*, embryonic developmental stages—(B) Blastulae, (C) Gastrulae, (D) Trochophore and (E) Veliger stages.

After 24 h of exposure, snails were washed and observed daily (24, 48, 72, 96 and 168 h). Mortality and abnormal responses, as retraction or extending of body and release of mucus and hemolymph, were recorded at each observed time. Usually, the average

duration of acute toxicity tests is 96 h. To check for further effects, we extended the analysis for 7 days after the end of the exposure. Death was ascertained by the absence of heart beatings for 2 min. A pale color of the shell as consequence of hemolimph loss was also observed after death. All experiments were repeated three times. Toxicity was assessed by recording the number of viable organisms at different concentrations of chromium and tested samples. All results are expressed as LC_{50} (lethal concentration) values, the concentration that affected 50 percent of the organisms tested in different concentrations of chromium and water samples.

2.3.2. Developmental toxicity assay

Transparent plastic sheets served as substrate for oviposition. Each snail lays one egg-mass with a variable number of eggs (15–50/animal/day) (Fig. 2A). Non damaged egg masses were collected and maintained in Petri dishes with filtered and dechlorinated tap water. Egg masses were observed with a stereomicroscope and classified according to the developmental stage. Egg masses with embryos at blastulae (0–15 h after the first egg cleavage), gastrulae (24–39 h), trocophore (48–87 h) and veliger (96–111 h) stages, according to Camey and Verdonk (1970) (Fig. 2B to E), were exposed to chromium and to water samples during 24 h. The entire *B. glabrata* embryonic development occurs in 8 days. Egg masses assays were conducted at 25 ± 2 °C with a 12 h light period.

Based on LC_{50} values for adult snails, embryos were exposed to chromium concentrations of 1.30, 3.13, 6.25, 12.50, 25 and 50 mg/L. Regarding the tests with water samples, preliminary assays with raw samples of the WWTP and Tietê River water samples were done with embryos; the influent sample (S2) was diluted to 25, 35, 40, 50, 60, 75 and 100 percent. A control group was maintained in filtered and dechlorinated tap water at the same experimental conditions. Approximately five egg masses (with a minimum of the 100 embryos in total) of each stage were exposed to the solutions. At the end of exposure, egg masses were washed with dechlorinated tap water and observed daily for sublethal (malformation effects) and lethal effects during eight days.

Effects on embryonic development were classified according the following criteria:

- Dead: embryos that died before hatching;
- Malformed: embryos with multiple affected structures, presenting abnormal developmental stage, usually classified as teratomorphic or hydropic;
- Normal: embryos without anatomic anomalies, which showed regular development.

The rate of affected embryos was used to estimate the median effective concentration (EC_{50}) values. Assays were repeated three times with approximately 100 embryos for each concentration. The sum of dead and malformed embryos was used to estimate the EC_{50} values.

2.4. Mutagenicity testing

2.4.1. Dominant lethal test

The protocol for the dominant lethal test was standardized with the reference mutagens cyclophosphamide, mitomycin C (Nakano et al., 2003) and ionizing radiation (Tallarico et al., 2004). Briefly, wild-type snails are exposed to the test agents or samples and crossed with non-exposed albino snails at different times after exposure. The frequencies of malformations in the heterozygous progeny of non-exposed albino snails indicate the induction of lethal dominant mutations.

Table 1
Effect of potassium dichromate ($K_2Cr_2O_7$) in *Biomphalaria glabrata* adults exposed for 24 h.

Concentration (mg/L)	Mortality			Total (%)	Mean ± SD	LC_{50} (mg/L) (CI)
	Exp 1	Exp 2	Exp3			
200	10	10	10	30 (100)	10 ± 0.00	
150	10	9	10	29 (96.67)	9.67 ± 0.58	
100	4	3	3	10 (33.33)	3.33 ± 0.58	97.07
75	3	3	1	7(23.33)	2.33 ± 1.15	(80.07–117.74)
50	2	1	1	4 (13.33)	1.33 ± 0.58	
25	0	2	0	2 (6.67)	0.67 ± 1.15	
0	0	0	0	0 (0.00)	0.00 ± 0.00	

LC_{50}=50% lethal concentration; CI=confidence intervals; SD=standard deviation.

All snails used in the experiments were selected by previous analysis of background frequencies of embryonic malformations described by Nakano et al. (2003).

The results obtained in the acute toxicity assays with environmental samples were used to determine the concentration range for the dominant lethal test. Snails were exposed to raw samples in all assays, except first sampling (I) of S2 assessment because the storage time had exceeded the limit recommended by ISO-10 706 (2000). The second sampling (II) of S2 was diluted to 25 and 50 percent, and the third sampling (III) was diluted to 1 and 5 percent because this sample shown to be more toxic to snails than second (II) in acute toxicity assay. A negative control group was kept in filtered and dechlorinated tap water; cyclophosphamide was used as in the positive control group.

Three groups of 10 adult wild-type snails were exposed to the samples for 7 days. Animals were fed daily with fresh lettuce during the exposure period. Twenty-four hours after the end of the exposure, each wild-type snail was put together with a non-exposed albino snail for 24 h in individual aquaria (pairing) with perforated covers to allow breathing. Animals are crossed with albino snails at different intervals to evaluate the effects in the germ cells at the different stages of spermatogenesis (10, 17, 24, 31, 38, 45, 52 and 59 days), according to Tallarico et al. (2004). After this period, animals were isolated and egg capsules were collected from albino snails. To evaluate the effects on crossing rates, numbers of effective crossings, that is, albino snails that produced heterozygous embryos were compared among the different groups.

Egg capsules were collected daily. Plastic sheets were placed on the water surface to propitiate oviposition and egg capsules were then transferred to cell culture plates and maintained in climatic chambers at 25 °C until the end of the analysis. After a crossing between homozygous wild-type snails and albino snails, both groups of animals produce embryos; for this study, only the offspring of the albino snails was analyzed. Among the offspring of the albino snails, the following types of embryos are produced: (i) heterozygous wild-type embryos produced by cross-fertilization and (ii) albino embryos produced by self-fertilization or by cross-fertilization with other albino snails before the onset of the experiment. Phenotypically wild-type embryos can be identified among the offspring of albino snails by the presence of pigmentation in the eyes, visible from the fourth day on of embryonic development. Thus, we analyzed the wild-type embryos, originated from sperm of the exposed wild-type snails; albino embryos were discarded.

Embryonic malformations analyses were conducted following a procedure previously reported (Nakano et al., 2003). Embryos were observed for eight days from the beginning of the development until nearly hatching using a stereomicroscope (Leica). The

Table 2
Effects of potassium dichromate ($K_2Cr_2O_7$) in *Biomphalaria glabrata* embryonic stages exposed for 24 h. The results are expressed as the sum of the three assays.

Embryonic stages	Concentration (mg/L)	Number of embryos	Dead embryos (%)	Malformed embryos (%)	Total of affected embryos (%)	Mean ± SD	EC_{50} (mg/L) (CI)
Blastulae	50.00	325	325 (100.0)	0 (0.00)	325 (100.0)	108.33 ± 2.52	20.37
	25.00	325	118 (36.31)	127 (39.09)	245 (75.38)	81.67 ± 14.43	(19.17–21.63)
	12.50	349	7 (2.01)	7 (2.01)	14 (4.02)	4.67 ± 4.16	
	6.25	320	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	
	3.13	326	2 (0.61)	0 (0.00)	2 (0.61)	0.67 ± 1.15	
	1.30	320	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	
	0.00	313	1 (0.32)	0 (0.00)	1 (0.32)	0.33 ± 0.58	
Gastrulae	50.00	329	329 (100.0)	0 (0.00)	329 (100.0)	106.33 ± 7.09	17.59
	25.00	308	103 (33.44)	157 (50.97)	260 (84.41)	86.67 ± 16.04	(16.55–18.71)
	12.50	322	5 (1.55)	44 (13.66)	49 (15.21)	16.33 ± 11.24	
	6.25	329	1 (0.30)	0 (0.00)	1 (0.30)	0.33 ± 0.58	
	3.13	316	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	
	1.30	336	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	
	0.00	307	2 (0.66)	0 (0.00)	2 (0.66)	0.67 ± 1.15	
Trochophore	50.00	339	339 (100.0)	0 (0.00)	339 (100.0)	113.00 ± 12.77	11.04
	25.00	312	233 (74.78)	54 (17.30)	287 (81.51)	95.67 ± 11.15	(10.26–11.88)
	12.50	311	69 (22.19)	151 (48.55)	220 (70.74)	73.33 ± 31.50	
	6.25	333	9 (2.70)	25 (7.51)	34 (10.21)	11.33 ± 16.29	
	3.13	334	0 (0.00)	3 (0.90)	3 (0.90)	1.00 ± 1.73	
	1.30	312	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	
	0.00	305	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	
Veliger	50.00	308	308 (100.0)	0 (0.00)	308 (100.0)	102.67 ± 1.53	5.76
	25.00	309	309 (100.0)	0 (0.00)	309 (100.0)	103.00 ± 3.61	(5.14–6.06)
	12.50	316	208 (65.82)	77 (24.37)	285 (90.19)	95.00 ± 2.65	
	6.25	322	24 (7.45)	198 (61.49)	222 (68.94)	74.00 ± 24.58	
	3.13	321	21 (6.54)	10 (3.12)	31 (9.66)	10.33 ± 11.68	
	1.30	314	2 (0.64)	0 (0.00)	2 (0.64)	0.67 ± 1.15	
	0.00	301	0 (0.00)	0 (0.00)	0 (0.00)	0.00 ± 0.00	

EC_{50} = 50% lethal concentration; CI = confidence intervals; SD = standard deviation.

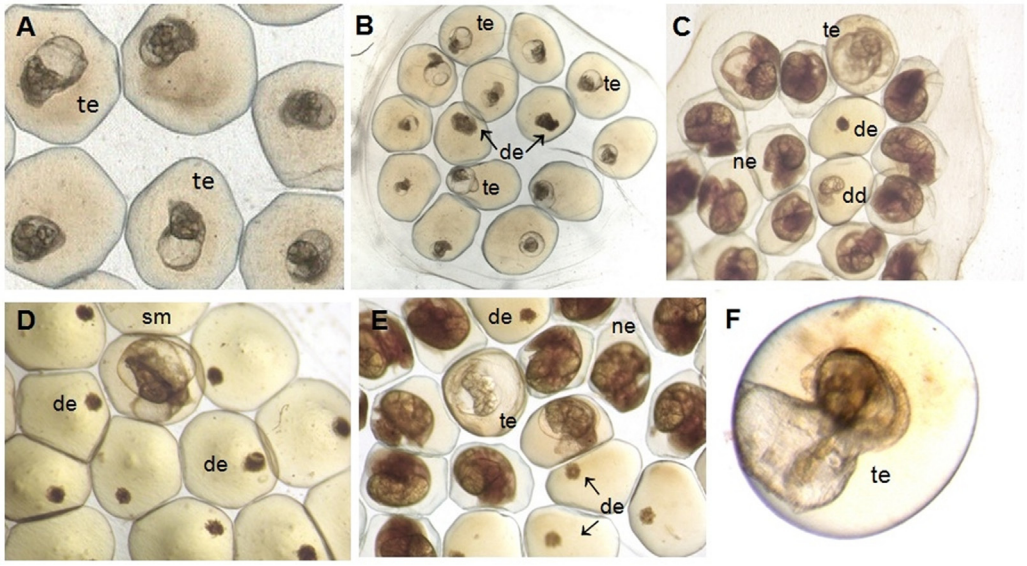

Fig. 3. *Biomphalaria glabrata* embryos in static assay (24 h exposure) observed daily during 8 days. (A) and (B) Embryos exposed to chromium ($K_2Cr_2O_7$); (C) to (F) Embryos exposed to WWTP influent (S2). Normal embryo (ne), dead embryos (de), developmental delay (dd), shell malformation (sm) and abnormal development, denominated teratomorphic or hydropic embryos (te).

number of teratomorphic or hydropic wild-type embryos – in which multiple structures are affected – was scored among the offspring of albino snails. All analyses were carried out in coded scoring.

2.5. Statistical analysis

Median lethal concentration (LC_{50}) and median effective concentration (EC_{50}) for chromium, as well as the samples tested in *B. glabrata* adults and embryos, including the 95 percent confidence intervals were estimated using Trimmed Spearman–Karber method (Hamilton et al., 1977).

3. Results

Results of the experiments with *B. glabrata* adults and embryos exposed to chromium are shown in Tables 1 and 2. Fig. 3A and B

Table 3
Adults and embryos of *Biomphalaria glabrata* exposed to influent samples of the municipal wastewater system (S2).

Samplings	*Biomphalaria glabrata*–24 h				
	Adults LC_{50} (%) (CI)	Blastulae EC_{50} (%) (CI)	Gastrulae EC_{50} (%) (CI)	Trochophore EC_{50} (%) (CI)	Veliger EC_{50} (%) (CI)
I	> 100 (–)	43.04 (41.92–44.19)	41.56 (41.20–41.91)	57.16 (55.78–58.58)	60.06 (59.36–60.76)
II	41.25 (32.61–52.18)	NT	NT	NT	NT
III	84.16 (75.34–94.02)	48.24 (47.17–49.34)	43.71 (42.39–45.06)	55.43 (54.16–56.73)	62.64 (61.55–63.75)
IV	NT	NT	NT	NT	NT

Samplings I–08/2006; II–02/2007; III–08/2007; IV–03/2008; CI–confidence intervals; LC_{50}–50% lethal concentration; EC_{50}–50% effective concentration; NT–non toxic.

show the teratomorphic embryos after exposure to salt. For adult snails, the 24 h LC_{50} of chromium was 97.07 mg/L (80.07–117.74 mg/L) (Table 1). Snails exposed to the highest concentration presented behavioral changes, such as the attempting to escape from the exposure solution and mucus release. Moreover, with 50 mg/L, unlike observed in the other groups, the surviving animals did not resume normal reproductive activity, as can be seen by the lack of egg masses.

Embryos at the veliger stage were seventeen times more sensitive to chromium than adults. The chromium salt promoted an increase in the occurrence of malformed embryos and lethality in all stages of *B. glabrata*. Embryos exposed after gastrulation showed higher rates of malformations, the veliger stage was the most susceptible to the substance, showing lethality at lower concentrations–9.66 percent with 3.13 mg/L. At veliger stage, embryos were three times more sensitive to chromium than those at blastulae stage. The EC_{50} values were 20.37 mg/L (19.17–21.63 mg/L) for blastulae, 17.59 mg/L (16.55–18.71 mg/L) for gastrulae, 11.04 mg/L (10.26–11.88 mg/L) for trochophore and 5.76 mg/L (5.14–6.06 mg/L) for veliger stages (Table 2).

Table 3 shows the data from the study with water samples. Each sample was tested in embryos and adult snails. Samples from the Ponte Nova Dam (S0), Tietê River upstream (S1) and downstream (S4) did not show acute toxicity to adults and embryos of *B. glabrata*. The WWTP influent (S2) showed acute toxicity to *B. glabrata* adult snails in the samplings II and III; sampling II was toxic to snails (LC_{50}=41.25 percent) and sampling III was slightly toxic (LC_{50}=84.16 percent) and no toxicity was observed in the sampling IV.

Samplings I and III induced similar effects to embryos. Embryos were more sensitive than adult snails to the WWTP influent in these samplings considering the developmental stage. The influent was classified as toxic to embryos at blastulae, 43.04 percent (I) and 48.24 percent (III); and at gastrulae, 41.56 percent (I) and 43.71 percent (III). The influent was slightly toxic for embryos at trochophore, 57.16 percent (I) and 55.43 percent (III); and at veliger stage, 60.06 percent (I) and 62.64 percent (III) (Table 3). The sensitivity was inversely proportional to the developmental stage: the veliger stage was 20 percent less sensitive than blastulae and gastrulae. The influent promoted, in most cases, unspecific abnormal development, with teratomorphic embryos (Fig. 3C, E and F). Some embryos showed developmental delay (Fig. 3C) and shell malformation (Fig. 3D). All embryos exposed to S2 samples died (Fig. 3D and E). In the second sampling (II), the influent (S2) was toxic only to adults (LC_{50} of 41.25 percent).

The environmental water samples did not induce dominant lethal mutations in *B. glabrata* in all samplings. The frequencies of non-viable embryos – total of dead and malformed embryos – in offsprings of non-exposed albino snails crossed with wild-type snails after exposure to environmental samples remained below 5 percent, which is established as the background level for experiments with embryos. In all experiments, dominant lethal mutations were induced by the reference mutagen cyclophosphamide (500 mg/L and 1000 mg/L); the frequencies of malformations were between 6.43 and 52.38 percent.

4. Discussion

Freshwater snails have shown to be suitable for water monitoring. In this sense, we proposed a protocol combining acute toxicity, developmental toxicity and mutagenicity analysis using *B. glabrata* adults and embryos as a test organism. The standardization and validation of this native species for ecotoxicological studies are relevant, mainly for Latin American where policies and regulations regarding the environment are still quite underdeveloped compared to the others countries. Furthermore, water samples from Suzano WWTP were analyzed to verify the potential of snails as bioindicator for environmental analyses.

Hexavalent chromium has been widely investigated for toxicity and used as reference substance in ecotoxicological studies to different aquatic organisms; however, there are few studies on freshwater snails and embryos (Ravera, 1977; Coeurdassier et al., 2005; Factor and Chavez, 2012). Regarding species from different taxonomic groups, *Ceriodaphnia rigaudi* and *Bosmina longirostris* are among the freshwater invertebrates most sensitive to Cr (48-h LC_{50} of 0.002 mg/L and 96-h LC_{50} of 0.05 mL/mL, respectively) (Achiorno et al., 2010). The 48-h LC_{50} for *Daphnia similis* was 0.027 mg/L (Rodgher et al., 2010). Direct comparisons of our results on *B. glabrata* with these data should be made with caution, since we have used 24 h exposure time. Nevertheless, *B. glabrata* embryos at veliger stage were as sensitive as the microcrustaceans *Bryocamptus minutus* and *Attheyella crassa* (96-h LC_{50} values of 3.56 mg/L and 3.82 mg/L, respectively), even considering the lower exposure time of *B. glabrata* assays.

Mechanisms determining chemicals toxicity in snails are still poorly understood, as well as those involved in the induction of developmental effects in these species. In general, embryos and adult *Biomphalaria* snails show different sensitivities depending on the chemical nature of the tested agent (Oliveira-Filho et al., 2005; Rapado et al., 2011). To reach the embryos, molecules should penetrate the egg gelatinous capsule and the egg membrane, which is influenced by characteristics as solubility, polarity, and molecule size (Miyasato et al., 2012). Wastewater frequently contains apolar compounds or substances not water-soluble, making the egg capsule penetration difficult to occur. The absense of toxic effects of WWTP influent on embryos in the sampling II, ranked as toxic for adult snails, could be explained by these factors.

Lack on information is also remarkable regarding the effects of chemicals on different developmental stages of mollusks. Embryos can also show a differential sensitivity depending on the developmental stage (Kawano and Simões, 1986) and the studies are limited to blastulae and juveniles (Salice and Roesijadi, 2002; Khangarot and Das, 2010; Bandow and Weltje, 2012). In this way,

the responses in all developmental stages appear to require more complex explanation and require more investigation.

Frequently, disturbances in the early development frequently lead to arrested development and inviability (Arner et al., 2009). This was observed in our study with embryos at blastulae and gastrulae exposed to WWTP influent. Embryos at gastrulae stages presented the highest rates of malformation that led to death promptly. On the other hand, embryos at veliger stage were the most sensitive to chromium, showing a developmental delay, leading to death with lower concentrations. Chromium was both embryolethal and teratogenic to *B. glabrata* embryos; at this valency state (hexavalent chromium), chromium is highly soluble and can be easily absorbed, passing the cells membrane and crossing the shell surface and the body of embryos and adult snails. The mechanisms by which chromium salts exert teratogenic action may be attributed to the mitosis inhibition of chromium compounds by interacting with DNA, which occurs during reduction of hexavalent chromium to trivalent chromium process, Cr (V) produced from the reduction of Cr (VI) reacted with hydrogen peroxide to generate hydroxyl radical via Fenton-like reactions (Borthiry et al., 2007). These highly reactive radical species released are capable of causing DNA damage (Asmatullah and Shakoori, 1998).

The analysis of available organic compounds performed by SABESP as routine showed that some compounds as tetrachloroethene, ethylbenzene, *m,p*-xylene and xylene have remained in water after the influent treatment, in concentrations not negligible. These compounds are used in different industries, with applications ranging of solvents, dyes, paints, pesticides, pharmaceuticals, detergents, plasticizers, e.g. However, in general, most organic compounds were within the permissible limits of the Brazilian National Environmental Council resolution (CONAMA, 2005). Other parameters were analyzed by SABESP. Regarding temperature, a low variation in water river samples, influent and effluent was observed, with values between 18 °C and 22 °C. These temperatures are suitable for the organic matter biodegradation in the water (between 10 °C to 30 °C). The pH showed similar values, ranging from 7.12 to 7.27 in samples of river water and 7.35 to 8.18 in the effluent. pH control in wastewater is recommended, as well as adjustment, when necessary, because it is an important environmental variable. The great majority of living organisms require a pH within the range of neutrality (6 to 8), and also, an ideal pH for biodegradation is near neutral (Cooney, 1995; Chasin and Pedrozo, 2004).

In organic matter control large variations in BOD values were observed, both upstream as downstream. Moreover, 33 ppm was the average concentration in the final effluent (S3), representing values up to ten times lower as compared with those observed in the influent (S2), with average of 354 ppm. These results show the relevance of the treatment in the removal of organic matter. From a legal standpoint, the WWTP meets the requirement of reducing 80 percent of BOD, resulting in values below the maximum allowable limit of 60 ppm of oxygen to enable the biodegradation of organic matter.

Snail sensitivity was demonstrated in comparison to the standardized bioassay with *Daphnia similis,* a recommended test organism in ecotoxicological studies in Brazil (ABNT, 2004), exposed to the same water samples as described by Hamada et al. (2011). In this study, the acute toxicity was removed after secondary treatment and samples from the final WWTP effluent (S3) did not exhibit effect on *B. glabrata* adults and embryos. Biological treatment effectiveness at the WWTP could also be observed for the *D. similis*: S3 samples were nontoxic for microcrustaceans in all samplings with the exception of the sampling IV, which was slightly toxic (EC_{50} (I)=75 percent). Regarding the performance and sensitivity of the proposed bioassays, there was a good correlation with validated assays: the acute toxicity for *B. glabrata* was qualitatively similar to that obtained with *D. similis*.

The lack of mutagenicity in the dominant lethal test suggests that possible mutagenic contaminants present in the samples were not bioavailable or they were protected by adsorption onto the sediments. This is a pioneer study with mutagenicity testing with an aquatic species; furthermore, there are no data on mutagenicity of environmental samples for this area. The only assessment was made by CETESB (2008) where no mutagenicity was detected in the Ames test in the same period studied, and our results are in agreement with the CETESB data.

In the present study, the dominant lethal test in *B. glabrata* was applied for the first time to the mutagenicity assessment of real samples of a wastewater treatment plant influent, effluent and downstream water. The results showed that studies of environmental samples containing complex mixtures of substances are possible in freshwater snails and demonstrated the relevance of establishing *B. glabrata* as a suitable organism to environmental mutagenicity studies.

5. Conclusion

From the data obtained in this study, we concluded that the protocol combining acute toxicity, developmental toxicity and mutagenicity analysis in *B. glabrata* was effective for the assessment of environmental water samples. The use of a representative species with ecological significance for aquatic environment is a necessary tool for monitoring chemical mixtures and wastewaters. Moreover, the standardization of assay in embryos provides endpoints for the aquatic life risk assessment and protection.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The study was supported by São Paulo Research Foundation (FAPESP grant no. 05/60237-8) and National Counsel of Technological and Scientific Development (CNPq grant no. 141951/2005-2). The authors thank Basic Sanitation Company of the State of São Paulo (SABESP) for sample collection and partnership. The authors are grateful to Cristina Antonia Alba de Albuquerque for the technical assistance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2014.09.005.

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