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The toxicity of cadmium to three aquatic organisms (*Photobacterium phosphoreum*, *Daphnia magna* and *Carassius auratus*) under different pH levels

R.-J. Qu, X.-H. Wang, M.-B. Feng, Y. Li, H.-X. Liu, L.-S. Wang, Z.-Y. Wang *

State Key Laboratory of Pollution Control and Resources Reuse, School of Environment, Xianlin Campus, Nanjing University, Nanjing 210023, Jiangsu, PR China

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ABSTRACT

This study investigated the effect of pH on cadmium toxicity to three aquatic organisms: *Photobacterium phosphoreum*, *Daphnia magna* and *Carassius auratus*. The acute toxicity of Cd^{2+} to *P. phosphoreum* and *D. magna* at five pH values (5.0, 6.0, 7.0, 8.0, and 9.0) was assessed by calculating EC_{50} values. We determined that Cd^{2+} was least toxic under acidic conditions, and *D. magna* was more sensitive to the toxicity of Cd than *P. phosphoreum*. To evaluate Cd^{2+}-induced hepatic oxidative stress in *C. auratus* at three pH levels (5.0, 7.25, 9.0), the activity of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase), the level of glutathione and the malondialdehyde content in the liver were measured. Oxidative damage was observed after 7 d Cd exposure at pH 9.0. An important finding of the current research was that Cd^{2+} was generally more toxic to the three test organisms in alkaline environments than in acidic environments.

1. Introduction

The global increase in freshwater contamination by numerous natural and industrial chemical compounds is a major environmental problem in the world (Schwarzenbach et al., 2006). Heavy metals are important contaminants of aquatic environments worldwide. Among these metals, cadmium has received considerable attention in recent years because its concentration in water body has been markedly increased by human activities such as sewage treatment, production of pulp and paper, and processing of metals (Hare, 1992). As a nonessential element, cadmium may endanger the growth and development of aquatic life. For example, cadmium may inhibit the bioluminescence of bacteria (Ishaque et al., 2006), cause limited activity even death in daphnias (Canizares-Villanueva et al., 2000), and induce oxidative stress in fish (Livingstone, 2001). The toxicity of cadmium in contaminated ecosystems depends not only on the concentration of this metal but also on the water chemistry. An important environmental stressor that affects most chemical and biological processes in water is pH. The pH of aquatic systems can be decreased or increased by a variety of anthropogenic sources, including agriculture, urbanization, industry, and mining (USEPA, http://www.epa.gov/caddis/ssr_ph4s.html). Fluctuations in pH may lead to changes in cadmium speciation, thereby influencing its bioavailability and toxicity to exposed organisms. Thus, it is of significance to study cadmium toxicity to different aquatic species as a function of pH.

In aquatic toxicological studies, bacteria, daphnids and fish are the most frequently used test species (Farre and Barcelo, 2003). These organisms represent different trophic levels in the aquatic food chain and are capable of reflecting the water quality. A bioluminescence inhibition assay using the marine bacterium *Photobacterium phosphoreum* is often chosen as the first toxicity screening method in a test battery because it is fast and cost effective (Davoren et al., 2005; Pandard et al., 2006; Girotti et al., 2008). In the assay, light production is directly proportional to the metabolic activity of the bacterial population, and inhibition of enzymatic activity causes a corresponding decrease in luminescence intensity. Recently, this simple and sensitive biotest has been widely used to investigate the toxicity of various inorganic and organic compounds in water samples (Ren and Frymier, 2005; Trang et al., 2005; Gueune et al., 2009; Katritzky et al., 2010).

Acute toxicity testing using freshwater daphnids, particularly *Daphnia magna*, is a popular bioassay used internationally for toxicity screening of chemicals and for monitoring of effluents and contaminated waters (Persoone et al., 2009). *D. magna* has been recommended as a standard test organism by many international organizations (e.g., ISO and OECD). The use of *D. magna* has many

* Corresponding author. Fax: +86 25 89680358.
E-mail address: wangzun315cn@163.com (Z.-Y. Wang).

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advantages, such as a high sensitivity and a short reproductive cycle. Since its initial application in 1928, *D. magna* has been used routinely in toxicological studies (Biesinger and Christensen, 1972; Hermens et al., 1984; De Schamphelaere et al., 2004).

Fish are an indispensable component of integrated toxicity testing of aquatic environments. The prominence of fish in environmental risk assessment is demonstrated by several toxicity tests in the OECD guidelines. In particular, the fish acute toxicity test is a mandatory component of the base set of data requirements for ecotoxicity testing (Lammer et al., 2009). However, in routine acute tests with mortality as the endpoint, it is difficult to evaluate the physiological changes that occur in experimental fish. Thus, in the present study, we evaluated contaminant-induced oxidative stress in fish, which may ultimately lead to cell death. Several oxidation-related biomarkers of the liver, including the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), the level of nonenzymatic antioxidant glutathione (GSH), and the concentration of malondialdehyde (MDA), were measured in the goldfish *Carassius auratus*. This freshwater fish was chosen as the test animal due to its extensive distribution in China and the widespread use in ecotoxicological researches (Sun et al., 2006; Zhu et al., 2008; Zhao et al., 2011).

To the best of our knowledge, previous toxicity testing concerning the influence of pH on cadmium toxicity was either performed within a narrow pH range or was limited to only a single species. These shortcomings prompted us to conduct the current study. By investigating the toxicity of cadmium to three aquatic organisms (*P. phosphoreum*, *D. magna* and *C. auratus*) across a relatively wide pH range (5.0–9.0), we were able to characterize the contribution of pH to cadmium toxicity in different organisms. The results may provide useful information for evaluating the toxicological effects of Cd in various environments with different pH.

2. Materials and methods

2.1. Chemicals and instruments

Chemicals: Cadmium sulfate, hydrochloric acid and sodium hydroxide, bought from Sinopharm Chemical Reagent Co., Ltd., are of analytical grade. 3-(*N*-morpholino) propanesulfonic acid (MOPS) with a purity of 99% was supplied by Aladdin® Reagent. The kits for the analysis of oxidative stress biomarkers were purchased from Nanjing Jiancheng Bioengineering Institute.

Instruments: METTLER-TOLEDO S20 SevenEasy pH Meter (METTLER-TOLEDO, China), Tecan Infinite 200® PRO multimode microplate reader (Tecan, Switzerland), PRX-250B Intelligent Artificial Climate Chamber (Safe , China), Eppendorf 5417R centrifuge (Eppendorf, Germany), IKA T10 homogenizer (IKA, Germany), TU-1800 UV–vis spectrophotometer (Persee, China), and Atomic absorption spectrophotometry (SOLLAR M6, Thermo, USA).

2.2. Test species and corresponding treatments

Freeze-dried powder of *P. phosphoreum* (T3 mutation) was obtained from the Institute of Soil Science, Chinese Academy of Sciences (Nanjing, China). After injection of 0.5 mL of cold sterilized 2.0% NaCl solution into a vial containing 0.5 g of freeze-dried powder, the solution was mixed thoroughly by shaking. After 2 min, *P. phosphoreum* was revived, and 10 μL of the bacterial liquid was diluted with 2 mL of 3.0% NaCl solution to serve as the working fluid for subsequent tests.

The *D. magna* strain was supplied by the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Beijing, China). Tap water that had been adsorbed by activated carbon and aerated for more than 48 h was used as culture water. Daphnids were kept in the culture water (pH 7.25 ± 0.25) in a 14 h: 10 h light: dark cycle at 20 °C, and were fed daily with green algae, *Scenedesmus obliquus*. Juvenile fleas that had undergone three generations of parthenogenesis (6–24 h old) were used in the experiment.

C. auratus (weight: 30.15 ± 4.35 g; length: 13.8 ± 0.9 cm) were purchased from a local aquatic breeding center. Before the experiments, the goldfish were acclimatized in tanks containing 150 L dechlorinated and aerated water at 20 ± 1 °C for 10 days. The water used for acclimation and subsequent experiments had a pH of 7.25 ± 0.25, conductivity of 340.6 ± 16.4 μs/cm, total hardness of 135.5 ± 9.3 mg $CaCO_3$/L, and alkalinity of 40.7 ± 5.2 mg $CaCO_3$/L. The following ion levels were measured: Na^+, 11.2 ± 0.2 mg/L; K^+, 2.34 ± 0.07 mg/L; Mg^{2+}, 7.74 ± 0.02 mg/mL; Ca^{2+}, 41.07 ± 0.82 mg/L and Cl^-, 28.3 ± 1.2 mg/L. The aquaria were aerated with air stones attached to an air compressor to saturate with oxygen (6.76 ± 0.84 mg O_2/L). The fish were fed twice a day with commercial pellets. The experiments were initiated when the total mortality fell to below 1%.

2.3. Experimental design

2.3.1. P. phosphoreum

The test was carried out according to the National Standard Method of China (Water quality – Determination of the acute toxicity – Luminescent bacteria test. GB/T15441-1995). The metal stock was prepared from $3CdSO_4 \cdot 8H_2O$ by dissolving 6.016 g in 3.0% NaCl solution, resulting in 2.637 g Cd/L. Based on the preliminary test, six gradient concentrations at each pH value (i.e., 1.319, 2.637, 13.185, 26.370, 131.85, and 263.70 mg Cd/L for pH 5.0 and 6.0; 0.264, 1.319, 2.637, 13.185, 26.370, and 131.85 mg Cd/L for pH 7.0; and 0.132, 0.264, 1.319, 2.637, 13.185, and 26.370 mg Cd/L for pH 8.0 and 9.0) were used to determine the EC_{50} values. The concentration series in octuplicate and eight controls were arranged in a 96-well (8 rows × 12 columns) black flat-bottom microplate (GRE, USA.). First, each well in the first column of the microplate was filled with 180 μL of 3.0% NaCl solution to serve as the control group. Second, the same volume of $HgCl_2$ standard solution was added to each of the eight wells in the second column to serve as a reference to verify the reliability of the experimental results. The third column which was injected with 180 μL of the pH-adjusted 3.0% NaCl solution was set as the pH-control group. Next, 180 μL of pH-adjusted metal solutions was added to the wells from the fourth column to the ninth column in order of increasing concentration. Then, 20 μL of bacterial suspension was added into each test well to get a total volume of 200 μL. The metal solutions and controls were adjusted with HCl (0.12 mol/L and 0.012 mol/L) and NaOH (0.05 mol/L and 0.005 mol/L) to obtain final pH values of approximately 5.0, 6.0, 7.0, 8.0, and 9.0. These five pH values were selected because the previous research has shown that within this range, the pH has no effect on light emission by luminescent bacteria (Fulladosa et al., 2004). The non-complexing buffer MOPS was used at a concentration of 2 mM to stabilize the pH.

To accurately determine Cd toxicity to *P. phosphoreum* at different pH levels, it was necessary to adjust the pH without significantly altering Cd concentrations in solution. The pH of the bacterial suspension was approximately 7.58. Repeated trials indicated that the pH of the metal solutions should be adjusted to 4.90, 5.92, 6.95, 8.10, and 9.16 to achieve the desired pH of 5.0, 6.0, 7.0, 8.0, and 9.0, respectively, after the addition of the bacterial liquid. All the final pH values were adjusted to be within ±0.1 of the desired value.

For example, to prepare 263.70 mg Cd/L solution (pH 4.90), the following steps were performed. First, the pH of the 3.0% NaCl solution was adjusted to 4.90 with HCl (0.12 mol/L and 0.012 mol/L). Next, 2.5 mL of the metal stock, 10 mL of 3.0% NaCl solution (pH 4.90), and 2.5 mL of the buffering agent (2 mM) were added to a 50 mL glass beaker. After readjustment to pH 4.90, the mixture was transferred to a 25 mL volumetric flask. The beaker was washed three times with the 3.0% NaCl solution (pH 4.90), which was also transferred to the volumetric flask. Additional 3.0% NaCl solution was added until the liquid level reached the scale line on the volumetric flask. Before use, the solution was poured into the pipetting reservoir, and the pH was remeasured and adjusted to 4.90 if needed. This pH adjustment did not significantly alter the Cd concentration in solution because the amount of HCl or NaOH added was negligible (less than 0.03 mL). After mixing in a 9:1 volume ratio with the bacterial suspension, the pH and the metal concentration were 5.0 and 237.33 mg Cd/L, respectively. Due to dilution by the bacterial liquid, all the concentrations were scaled by a factor of 0.9 for EC_{50} calculation. The exposure solution was used immediately after preparation. The bioluminescence of various treatments and controls was determined using a Tecan Infinite 200® PRO multimode microplate reader after exposure of 15 min at 25 °C. The toxicity of each treatment was expressed as the relative light rate (RLR, %), which is calculated as follows:

$$\mathrm{RLR}(\%) = L/L_0 \times 100\%,$$

where L_0 and L are the average light units of the controls and the treatments, respectively.

By fitting a straight line between the RLR values falling within the 10–90% range and the corresponding concentrations with the linear regression method, the regression equations were obtained and used to calculate EC_{50} values (i.e., the concentration at which RLR is 50%).

2.3.2. D. magna

The acute toxicity of the metal-spiked samples to *D. magna* was determined in accordance with the National Standard Method of China (Water quality—Determination of the acute toxicity of substance to Daphnia (*D. magna* straus) GB/T 13266-1991). Preliminary experiments were performed to investigate the effect of pH on *D. magna*. The experiments revealed that the activity of the organism was not reduced by 24 h exposure to culture medium of pH 5.0, 6.0, 7.0, 8.0 or 9.0,