Pol. J. Environ. Stud. Vol. 26, No. 6 (2017), 2869-2872
DOI: *10.15244/pjoes/67331* ONLINE PUBLICATION DATE: 2017-10-10

Short Communication

Bioconcentration of Lead and x-ray Microanalysis with SEM in the Freshwater Rotifer *Lecane quadridentata* (Rotifera: Monogononta)

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Received: 6 October 2016
Accepted: 23 November 2016

Abstract

We studied the accumulation of lead as $Pb(NO_3)_2$ in the freshwater rotifer *Lecane quadridentata* using Leadmium Green and x-ray microanalysis by energy dispersion. The results indicate that lead is bioaccumulated in great amounts in the stomach after the rotifer is exposed for 24 h to 0.5 mg L^{-1} of $Pb(NO_3)_2$. When the exposure concentration of $Pb(NO_3)_2$ is increased to 1.0 mg L^{-1}, lead was detected mostly in the cuticle after 24 h. Analysis of x-ray microanalysis by energy dispersion suggests a loss of calcium and rubidium, and an increase of aluminum, niobium, and silicon. A discussion comparing bioaccumulation between the rotifers *Brachionus calyciflorus* and *Lecane quadridentata* is included.

Keywords: aquatic toxicology, environmental toxicology, zooplankton, metals

Introduction

The accumulation of metals depends on: a) the biological species and the chemical element considered, b) the exposure regimen applied, c) cation homeostasis mechanisms, d) life-cycle influences on metal accumulation, and e) an appropriate experimental design in different spatial and temporal scales [1]. Lead is present in environmental matrices and originates from anthropogenic sources, production of batteries, cables, pigments, and chemical additives, mining industries, refineries, and waste disposal [2]. Lead can be removed from the atmosphere and transferred to environmental surfaces and compartments by wet or dry deposition, and when deposited in water, lead partitions rapidly between the sediment and aqueous phases [2]. The distribution of lead within animals is closely associated with calcium metabolism. It is interesting that the tetravalent organic form of lead is generally more toxic than the divalent, inorganic form, and its distribution in organisms may not specifically follow calcium metabolism [3].

Lead enters cells through membrane transporters of divalent cations, potassium channels, cholinergic

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receptors, and mono and polyvalent channels of calcium; the affinity of lead for calcium channels is determined by four residues of glutamate, and enters the cell through an electrostatic interaction between the carboxyl groups of the lateral chains of glutamate [4].

Lead is known to alter biochemical processes and is accumulated in tissues in the freshwater rotifer *Brachionus calyciflorus*, lead produced decalcification after 24-h exposure at 1.0 mg L^{-1} [5]. In a five-day chronic toxicity test using *Lecane quadridentata* there was a 44% reduction in real concentrations of $Pb(NO_3)_2$ (determined by atomic absorption) from day 0 to day 5 as a consequence of rotifer uptake [6]. In contrast, the Pb concentration in EPA medium decreased 52.6% in 24h according to atomic absorption results in the rotifer *B. calyciflorus* when compared to EPA + Pb/24 h versus EPA + Pb/24h residual Pb after rotifer accumulation [5]. This difference suggests either important differences in the detoxification activities or bioconcentration in both species of rotifers.

In rotifers, metal detection in specific places of the rotifer anatomy is scarce; recently, in two species of rotifers trivalent and hexavalent chromium were found after having accumulated in the cuticle of *Lecane quadridentata* and *Brachionus calyciflorus* [7]. Therefore, the present study aims to analyze the accumulation of lead in the cuticle of the freshwater rotifer *Lecane quadridentata* using Leadmium Green to follow the entry of lead after early contact with the rotifer, and then x-ray microanalysis by energy dispersion (through a scanning electron microscope) to study the elemental composition of the rotifers exposed to lead. Finally, we would discuss the differences in lead accumulation and their effects between two freshwater rotifer species: *B. calyciflorus* and *L. quadridentata*.

Experimental

The freshwater rotifer *Lecane quadridentata* were cultured in a bioclimatic chamber with a 16/8 dark/light period at 25ºC [8]. Rotifers were kept in Petri dishes with EPA medium [9] and fed the green alga *Nannochloris oculata* (strain LB2194 of the University of Texas Collection) grown in Bold's basal medium [10].

Energy-dispersive x-ray microanalysis by scanning electron microscopy is used to determine the elemental concentration of aluminum, calcium, carbon, gold, lead, niobium, oxygen, rubidium, and silicon, and all rotifers intoxicated and not intoxicated were used for determining lead by x-rays with SME and Leadmium Green AM dye, according to the protocol of Alvarado-Flores et al. [5]. For x-ray determination we intoxicated 100 rotifers with 1.0 mg L^{-1} of $Pb(NO_3)_2$, and another group of 100 rotifers were used as a non-toxic control (the experiments took place in a 24-well polystyrene plate with a final volume of 2 mL in standard culture conditions). Then we collected the control group and the intoxicated group of rotifers and placed them in an Eppendorf tube in a final volume of 1 mL of EPA medium and added 100 µl of 3% formaldehyde. Then the samples were coated with gold using a DESK II camera, and photographed with JEOL

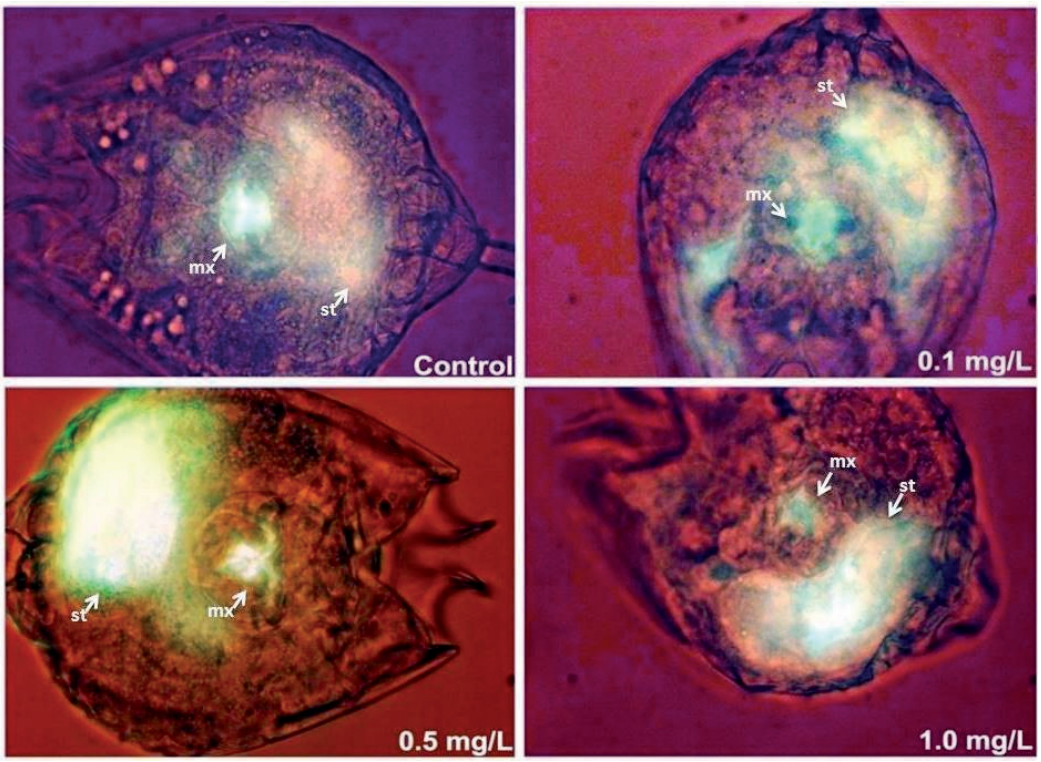

Fig. 1. Analysis of in vivo intracellular lead uptake by Leadmium Green AM dye in the rotifer *Lecane quadridentata*. Exposure time 24 hours, st = stomach, and mx = mastax.

5000 LV SEM. The corresponding x-ray spectra were at 20 kV with an acquisition time of 200 s, working distant 10 mm. The range of x-ray penetration is 0-1,000 nm. For quantitative analysis we used the commercial software INCA suite 3.04 (Oxford 2.6 statistical instrument).

The analysis of *In vivo* intracellular lead uptake using Leadmium Green AM was made as follows: another group of 30 rotifers was used as a control without toxic. Additionally we intoxicated rotifers (n = 30 for each concentration) with 0.1, 0.5, and 1.0 mg L^{-1} of $Pb(NO_3)_2$ for 24 hours (experiments took place in a 24-well polystyrene plate with a final volume of 2 mL with EPa medium). Finally, all rotifers of each group were analyzed according to the protocol of Alvarado-Flores et al. [5]. Fluorescence was determined with an excitation spectrum of 450-490 nm and an emission barrier of 515 nm. The photographs were taken with the camera Cool SNAP PRO coupled to an Axioscop 40 Zeiss microscope.

Results and Discussion

The Leadmium Green study allowed us to localize $Pb(NO_3)_2$ in *L. quadridentata* after 24 h of exposure with different concentrations (0.1, 0.5, and 1.0 mg L^{-1}). The accumulation of $Pb(NO_3)_2$ occurs mainly in the stomach and other parts of the digestive system and in the vitellarium (Fig. 1). In the stomach the greatest accumulation of lead occurred at an exposure concentration of 0.5 mg L^{-1} of $Pb(NO_3)_2$. The x-ray microanalysis showed detection of lead in *L. quadridentata* after 24 h of exposure at 1.0 mg L^{-1} $Pb(NO_3)_2$ (Table 1). Also, we observed a decrease in calcium and an increase in silicon and aluminum in intoxicated rotifers when compared with control animals (Table 1). The only two elements were we found statistical significant differences in were gold and lead. Gold is used as a part of sample preparation for SEM. Both controls and intoxicated animals were prepared in the same way. Therefore, we really have no way of explaining the differences in gold content between both groups. The most important part of the manuscript is related to the significant differences between lead in the control group and the group of rotifers exposed to lead. All we can say is that the values of the elements (especially carbon and oxygen) are consistent with the values and number of replicates reported in previous works (Hernández-Ruiz et al., 2016; Alvarado-Flores et al., 2012). The presence of gold, niobium, and rubidium are related to the process of preparation of the animals for SEM, and we do not have a clear explanation of why some concentrations are higher in the control than intoxicated animals or the other way around regarding these three elements.

The main way of entry of $Pb(NO_3)_2$ in rotifers is through the digestive system, and absorption and adsorption takes place in the stomach. It is probably the interchange of calcium for silicon caused by $Pb(NO_3)_2$ exposure to a mechanism of detoxification in rotifers, as suggested by our x-ray diffraction results. The accumulation of lead in the cuticle of *L. quadridentata* happens after 24 h exposure; instead, in *B. calyciflorus* there is no lead accumulation after 24-h exposure at 1.0 mg L^{-1} [5]. In both species there is decalcification and an increase in silicon in the cuticle (compared to our results with those of [5]). These similarities in the effects of lead exposure in both species suggest that $Pb(NO_3)_2$ alters the biochemical mechanisms of transport and distribution of both calcium and silicon in the cuticle of the rotifers. Recent studies of chromium III and VI exposure in the same two species also resulted in decalcification; however, the levels of silicon behave in different ways in both species: *L. quadridentata* showed an increase in silicon after exposure to chromium III, but silicon was not detected after exposure to chromium VI. In *B. calyciflorus,* exposure to both chromium III and VI resulted in no detection of silicon [7].

All these results can be explained at the biochemical level with a scenario in which lead enters the cells and is distributed mainly through the transport of divalent cations (MTCD), potassium channels, cholinergic

Table 1. X-ray diffraction elemental analysis of composition of *Lecane quadridentata* organisms control and intoxicated with $Pb(NO_3)_2$. The values are the mean ± one standard deviation. CV% is the coefficient of variation.

Element	% Composition *L. quadridentata* Control (n=8)	CV%	% Composition *L. quadridentata* Intoxicated Pb(NO3)2 (n=12)	CV%
Aluminium	0.94 ± 0.80	85.11	1.79	0
Calcium	0.52 ± 0.09	17.31	0.47 ± 0.38	80.85
Carbon	65.07 ± 1.47	2.26	65.60 ± 5.44	8.29
Gold	0	0.00	25.32	0
Lead	0	0.00	2.28 ± 1.73	75.88
Niobium	17.18 ± 1.73	10.07	20.29 ± 17.40	85.76
Oxygen	29.23 ± 6.72	22.99	30.18 ± 8.84	29.29
Rubidium	1.86 ± 0.19	10.22	0.47 ± 0.08	17.02
Silicon	0.64 ± 0.21	32.81	0.78 ± 0.38	48.72

receptors, and channels of calcium mono and polyvalents: Na^{+2}, K^{+2}, Ca^{+2}, Si^{+2}, Li^{+2}, Cs^{+2}, and Ba^{+2} [4]. Once inside, the cells lead can be fixed to the cuticle or be detoxified by several mechanisms in which the differences between rotifer species explain why decalcification occurs in both species, but silicon behaves differently. Perhaps the fact that *L. quadridentata* has the thickest cuticle and could be benthic and *B. calyciflorus* has a less thick cuticle and is completely planktonic might explain the differences in observed silicon levels.

The sensitivity to $Pb(NO_3)_2$ between both genera and species also differ. For example, in *B. calyciflorus* and *B. plicatilis* the LC50 value is 4.0 mg L^{-1}, while in *B. patulus* it is 6.15. In contrast, in *L. quadridentata* the LC50 it is 3.7 mg L^{-1}, in *L. hamata* it is 0.68, and in *L. luna* it is 0.14 mg L^{-1} [11]. It seems by looking at the small set of data that the *Brachionus* species are more tolerant to lead than species of *Lecane*. This fact might be related to a scenario in which species of *Brachionus* detoxify metals better than species of *Lecane*. In general, the range of acute toxicity in rotifers for $Pb(NO_3)_2$ goes from 0.035 to 56.2 mg L^{-1} [12].

Conclusions

In conclusion, exposure to lead decalcifies the cuticle of freshwater rotifers with a related increase in silicon. Based on a small set of data, we can affirm that species of the genus *Brachionus* are more tolerant to lead in terms of acute toxicity, perhaps because its detoxification mechanisms are more efficient than species of the genus *Lecane*.

Acknowledgements

The authors wish to thank Javier Ventura-Juárez, Marcelo Silva-Briano, and Aracely Adabache for their support.

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