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Bioindication of mercury, arsenic and uranium in the apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae): Bioconcentration and depuration in tissues and symbiotic corpuscles

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HIGHLIGHTS

- The apple snail *Pomacea canaliculata* is a sensitive bioindicator of Hg, As, and U.
- Tissues and symbiotic corpuscles accumulate differentially Hg, As, and U.
- Kidney and digestive gland show different abilities of accumulation and depuration.
- Symbionts detoxify elements by intracellular accumulation and excretion in faeces.
- Tissue and symbionts keep an exposure memory of three toxic elements after depuration.

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ABSTRACT

Pomacea canaliculata is a mollusk potentially useful as a biomonitor species of freshwater quality. This work explores the ability of snail tissues and symbiotic corpuscles to bioconcentrate and depurate mercury, arsenic, and uranium. Adult snails cultured in metal-free reconstituted water were exposed for eight weeks (bioaccumulation phase) to water with Hg ($2\ \mu gL^{-1}$), As ($10\ \mu gL^{-1}$), and U ($30\ \mu gL^{-1}$) and then returned to the reconstituted water for other additional eight weeks (depuration phase). Elemental concentrations in digestive gland, kidney, symbiotic corpuscles and particulate excreta were determined by neutron activation analysis. The glandular symbiotic occupancy was measured by morphometric analysis. After exposure, the kidney showed the highest concentration of Hg, while the digestive gland accumulated mainly As and U. The subcellular distribution in symbiotic corpuscles was ~71%, ~48%, and ~11% for U, Hg, and As, respectively. Tissue depuration between weeks 8 and 16 was variable amongst elements. At week 16, the tissue depuration of U was the highest (digestive gland = 92%; kidney = 80%), while it was lower for Hg (digestive gland = 51%; kidney = 53%). At week 16, arsenic showed a differential pattern of tissue depuration (digestive gland = 23%; kidney = 88%). The symbiotic detoxification of the three elements in excreta was fast between weeks 8 and 10 and it was slower after on. At the end of the depuration, each element distributed differentially in digestive gland and symbiotic corpuscles. Our findings show that symbiotic corpuscles, digestive gland and kidney *P. canaliculata* are sensitive places for biomonitoring of Hg, As and U.

1. Introduction

Metal pollution is a current environmental issue that is originated from natural and anthropic sources (WHO, 2005, 2011, 2012). Abnormalities in metal ion concentrations in living organisms may have different toxic effects and lead towards eco-physiological

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hazards in aquatic environments and also causing many sanitary problems to humans.

Monitoring of metals in the aquatic ecosystems can be evaluated by a combination of chemical and/or biological methods. Amongst them, aquatic bioindicators are widely used as integrators of chemical contamination both with *in situ* studies and transplanted organisms (Belivermiş et al., 2016; Marigómez et al., 2013). Bioindicators have the advantage of detecting the presence of metal pollutants that are quickly diluted in large water volumes and give information on the bioavailability of the compounds to which these elements are associated.

Mollusks have been broadly used to predict environmental risk (Gupta and Singh, 2011) since they are at low trophic levels and they are the entrance doorway of these elements in the trophic web (Burger and Gochfeld, 2001). The apple snail *Pomacea canaliculata* is an amphibious freshwater animal that belongs to Ampullariidae family (Gray 1824), which include more than one hundred and fifty species grouped in nine genera (Hayes et al., 2009). This species is highly extended in tropical and subtropical, lentic and lotic environments of South America (Hayes et al., 2015; Martello et al., 2006; Martín et al., 2001; Martín and De Francesco, 2006; Martınez and Rojas, 2004). Furthermore, it has been anthropogenically distributed to Europe, Asia and Hawaii (Hayes et al., 2008; López-Soriano et al., 2009).

Specific cells of the digestive gland of this snail host two types of pigmented corpuscles named C and K corpuscles (Andrews, 1965; Castro-Vazquez et al., 2002). Evidence indicates that these corpuscles are morphs of a prokaryotic organism (Hayes et al., 2015; Vega et al., 2005, 2006) that reproduces within the epithelial cells of the digestive gland (Koch et al., 2006). Free or packed symbiotic corpuscles within continuous mucus string (Andrews, 1965) are also observed in excreta indicating that they were eliminated from the host (Koch et al., 2006).

Apple snails fulfill the requirements of a species that could be used for environmental monitoring studies (Elder and Collins, 1991) and several field and laboratory studies indicate that these animals are able to accumulate different elements (Hayes et al., 2015; Vega et al., 2012a). Accumulations of Cu, Cd, Pb, and Zn in head-foot and viscera of the ampullariids genera *Pila*, *Lanistes*, and *Pomacea* have been reported (Hayes et al., 2015). Hoang et al. (2008) showed the copper uptake and depuration from water in juveniles of *P. paludosa* (Hoang et al., 2008). *P. scalaris* restricted to the gold mining area is able to increase their Hg concentrations in soft tissues (Callil and Junk, 2001), which bears pigmented symbiotic corpuscles similar to those found in *P. canaliculata* (Castro-Vazquez et al., 2002). *P. canaliculata* receiving effluent from a lead/zinc mine in southern China accumulated high concentrations of these elements (Deng et al., 2008), but its tissue concentrations did not correlate with those found in sediments or macrophytes. Also, the accumulation of Hg, As, and U by *P. canaliculata* from a source of drinking water has been reported (Vega et al., 2012a).

A laboratory study showed the distribution of potentially toxic elements within tissues, eggs and symbiotic corpuscles of *P. canaliculata* (Vega et al., 2012a). Digestive gland and kidney are the main sites of accumulation of different metals. Within the digestive gland, a large fraction of the elements is retained in the symbiotic corpuscles (11.7%–79.7%), which may act as intracellular sites of detoxification. The kidney has been proposed as a place of detoxification of mercury (Hayes et al., 2015).

In this work, we selected Hg, As, and U for bioindication studies by their frequencies of occurrence, toxicities, and potential for human exposure and the environment (ATSDR, 2017; https://www.atsdr.cdc.gov/spl/index.html) and these elements represent the three empirical categories of metals proposed by Nieboer and Richardson (1980). The atmospheric Hg (class B) deposition is the main source of Hg contamination in aquatic systems (Slemr and Langer, 1992; Wang et al., 2004). In addition, small-scale silver and gold mining have increased mercury emissions in several countries of Latin-American (Aula et al., 1995). In Argentine, the effects of As (intermediate class) on environmental and human health are associated with the entering of As in sources of drinking water and groundwater by leaching from geological formations and volcanic activity (Bardach et al., 2015; Ng et al., 2003). Uranium (class A) is often associated with oxygen (uranyl ion UO_2^{2+}) in natural water sources (Sandino and Bruno, 1992) and its presence in the aquatic system could be a result of the leaching from the natural mineral deposit (WHO, 2012).

Since *P. canaliculata* has been proposed as a bioindicator of water quality (Vega et al., 2012a), it is necessary not only to know the capacity of the animal to bioconcentrate a toxic element, but also it is important to elucidate the ability to retain it within tissues for a long period after the exposure has ended. The bioconcentration and depuration processes of mercury (class B), arsenic (intermediate class) and uranium (class A) were investigated. The role of the intracellular symbiotic corpuscles as sites of bioconcentration and detoxification was also assessed. Since the symbiotic corpuscles occupy an important part of the glandular acinus and they are continuously eliminated in feces, the glandular symbiotic occupancy and the elemental concentration in the excreta were also measured along the experimental period.

2. Materials and methods

2.1. Animals and culturing conditions

Animals from a cultured strain of *P. canaliculata* were used. The strain's original stock was collected at the Rosedal Lake (Palermo, Buenos Aires, Argentina). Snails were grown from hatching until 4 months old in aquaria containing metal-free reconstituted water (Vega et al., 2012a). Control and experimental groups (see section 2.3) were formed by four adult snails in aquaria containing 6 L of reconstituted water. The temperature was regulated between 23 and 26 °C. Artificial lighting was provided 14 h per day. Aquarium water was changed thrice a week. Animals were fed *ad-libitum* with fresh lettuce which was provided after the water change. These culturing conditions were approved by the Institutional Animal Care and Use Committee of the Facultad de Ciencias Médicas of the Universidad Nacional de Cuyo (Approval Protocol N° 55/2015).

2.2. Materials used

HNO_3 (Merck, 100452.2500) and HCl (Merck, 100317.2500) were used for cleansing of laboratory ware. NaCl (Merck 1.06404.1000.1026) and NaN_3 (Tetrahedron) were used to isolate C and K corpuscles. American Society for Testing and Materials (ASTM) type I water, $CaCO_3$ (Biopack 978007), KCl (Biopack, 163106), $MgCl_2$ $6H_2O$ (Biopack 9620.08), Na_2SO_4 (Mallinckrodt, 8024), $NaHCO_3$ (Tetrahedron), $CaCl_2$ (Sigma-Aldrich, 101551865) were used for culturing snails. $HgCl_2$ (Sigma-Aldrich, M1136), Na_3AsO_4 $7H_20$ (Sigma-Aldrich, S9663), UO_2CH_2COOH (Ted Pella Inc. 19481) were used for exposure experiments, at concentrations recommended by the US Environmental Protection Agency Maximum Limit Concentration (EPA' MLC) for drinking water. Suprasil 312 quartz (with low content of metals and lanthanides) was used for the ampoules.

2.3. Experiments

2.3.1. Elemental distribution in tissues and symbiotic corpuscles

An experiment was run to know the elemental preference by tissues and symbiotic corpuscles since Hg, As and U have different chemical properties (Nieboer and Richardson, 1980). Hg (class B) has a high affinity for reduced sulfur groups of aquatic ecosystems (Ravichandran, 2004; Sharma et al., 1993) (i.e. Maltez et al., 2009; Quig, 1998; Shen et al., 2005). As (intermediate class) has affinity for sulfur and oxygen groups present in natural minerals (Bostick et al., 2003; Gao and Mucci, 2001; Saada et al., 2003) and in cellular metallothioneins (Albores et al., 1992; Diniz et al., 2007; Merrifield et al., 2004); U (class A) has a high affinity for phosphates groups of sources of environmental water and of animal's tissues and organs (Hursh and Spoor, 1973; Kurttio et al., 2005).

Snails were cultured from hatching until 4 months old in metal-free reconstituted water (section 2.1) and then four groups of four snails were exposed to (a) reconstituted water, (b) 2 μg/L of Hg, (c) 10 μg/L of As and (d) 30 μg/L of U) during eight weeks (Vega et al., 2012a). After exposure, animals were sacrificed and samples of digestive gland, symbiotic corpuscles and kidney were collected and stored at −80 °C for further determination by neutron activation analysis (section 2.6). The fractions of each element accumulated in both symbiotic corpuscles (inventories) after exposures were calculated as (((ECS ×100)/ECD) x SGO)/100, where ECS is the elemental concentration in each symbiotic morphotype (milligram per kilogram of dry mass), ECD is the elemental concentration in digestive gland (milligram per kilogram of dry mass), and SGO is the symbiotic glandular occupancy by each symbiotic corpuscle (section 2.7).

2.3.2. Bioconcentration and depuration in digestive gland and kidney

Animals were cultured from hatching until 4 months old in free-metal reconstituted water (section 2.1). Three experimental groups were used to explore changes in elemental concentration in tissues of snails that were exposed for eight weeks to EPA' MLC in drinking water for Hg, As or U and then transferred to aquariums with free-metal reconstituted water for an additional period of eight weeks. Five aquaria (N = 4 snails) by element were exposed for 8 weeks and then tissue samples collected at weeks 8 (exposure phase) and at weeks 9, 10, 12 and 16 (depuration phase). Additionally, six aquaria containing non-exposed snails (control) was used to assess the basal elemental concentration between day 0 and week 16. Samples were stored at −80 °C for further processing (section 2.6).

2.3.3. Bioconcentration and depuration in symbiotic corpuscles

An experiment was run to know the role of the intracellular symbiotic corpuscles as sites of bioconcentration and elemental depuration since the digestive gland showed a differential elemental depuration in the precedent experiment. The culture and experimental conditions were identical to the precedent experiment (see section 2.3.2). Animals were sacrificed and samples of digestive glands were collected to (a) measure the elemental concentration in symbiotic corpuscles and (b) quantify the glandular symbiotic occupancy (Table S1). Elemental concentration and glandular symbiotic occupancy were studied at day 0 and week 8 (bioconcentration phase) and at weeks 8, 9, 10, 12 and 16 (depuration phase), respectively. Also, we used the mean relative abundance of pigmented corpuscles in the digestive gland to estimate the fraction of elements accumulated in the symbiotic corpuscles (Table S2-S4).

2.3.4. Elemental concentration in particulate excreta

Sediment samples containing fecal droppings were collected from the aquaria at day 0 and weeks 8, 9, 10, 12 and 16 (as described in section 2.3.2). These samples were assessed microscopically and then processed for neutron activation analysis.

2.4. Sample collection and processing for neutron activation analysis

Animals were anesthetized by immersion in an ice bath for 10 min. Once the shell was removed, whole kidney and samples of the digestive gland (~100 mg) were dissected with glass knives. The remainder digestive gland (~800 mg) was used to isolate the C and K symbiotic corpuscles (section 2.5.). Samples of excreta were collected from aquarium's bottom using a Pasteur's pipette. Then, these samples were decanted at 4 °C in a 50 ml tube; the supernatant was discarded while the sediment was stored at −80 °C. All samples were weighted, freeze-dried in plastic tubes and weighed again. Finally, they were sealed in quartz ampoules and stored until neutron activation analysis (see section 2.6). The mean water contents of the digestive gland and kidney were of 0.80 and 0.90, respectively.

2.5. Isolation of C and K corpuscles from digestive gland

Both corpuscles were differentially isolated from the digestive gland according to the fractionation method described previously (Vega et al., 2005). The purity of fractions was assessed microscopically. The C fractions contained less than 5% of K corpuscles while the K fractions contained less than 1% of C corpuscles. These fractions were stored at −80 °C. All fractions were weighted, freeze-dried in plastic tubes and weighed again. They were sealed in quartz ampoules and stored until neutron activation analysis (see section 2.6). The mean water contents of the C and K fractions were of 0.95 and 0.99, respectively.

2.6. Neutron activation analysis

All laboratory ware was first washed in HCl during a week (17.5%, v/v) and then in HNO_3 (32.5%, v/v) for another week. Finally, the remaining acid was eliminated with three passages in abundant American Society for Testing and Materials (ASTM) type I water and then oven-dried at 60 °C and stored until use.

Neutron activation analysis was performed to quantify mercury, arsenic, and uranium in apple snail's samples and excreta. The samples in the quartz ampoules were irradiated for 20 h in a 1 MW MTR pool type reactor (thermal neutron flux ~$1.5 \times 10^{13} n.s^{-1}cm^{-2}$) with neutron flux monitors. Gamma activity was measured by high-resolution gamma-ray spectrometry with a high-purity coaxial Ge detector. Spectra were obtained after 7 and 20 days of decay. The elemental concentrations were calculated with the absolute parametric method using current tables (Kolthoff and Elvin, 1986).

In order to check the accuracy of the method, four certified reference materials (NRCC-DORM2, NRCC-TORT, IAEA-MA-A-2 and IAEA-140/TM) were analyzed together with the samples for the determination of Hg and As (Table S5). For uranium, NIST-2704 and IAEA-SL1 were analyzed. The limits of detection and quantification (LOD and LOQ) in the neutron activation analysis are dependent on the matrix. In all cases, LOQs were lower than the elemental concentration by a factor of 10. The percent coefficient of variation ranged from 15% to 20%, due mostly to the fact that the samples were analyzed sealed in quartz vials to avoid losses of the element of interest. Since the distribution of mass inside the ampoules may not be homogeneous (i.e. because of evaporation of the element

and subsequent deposition in the ampoule walls), there is an additional geometrical uncertainty in the gamma-ray counting processes. Element concentrations from digestive gland, kidney, symbiotic corpuscles and aquarium's excreta were expressed as milligram per kilogram of dry mass.

2.7. Light microscopy and morphometric analysis

Light microscopy preparations were obtained from 3 individuals of each experimental group by cutting 1–2 mm thick slices of the digestive gland with a razor blade from the gland's surface, close to the kidney's boundary (Koch et al., 2006). The samples were fixed in 4% paraformaldehyde for 24 h. Then, they were kept in 70% ethanol, subsequently dehydrated in increasing concentrations of alcohol, embedded in Histoplast® and sectioned (7 μm). Ten separate sections per animal were studied in snails under control, exposure and depuration conditions. Digital micrographs at 100× magnification were obtained with a color video camera on a microscope Nikon ALPHAPHOT-2 YS2. Both intracellular corpuscular types, C and K, were identified by its natural pigmentation (Castro-Vazquez et al., 2002; Koch et al., 2006) and then were contoured using Image Pro-Plus 6.0 (Media Cybernetics, Silver Spring, MA, USA). Glandular symbiotic occupancy was calculated dividing the sum of the total area occupied by each type of symbiotic corpuscle by the acinar area and then multiplying by 100 (Table S1).

2.8. Statistical analysis

Comparisons amongst elemental concentrations in digestive gland, kidney, and symbiotic corpuscles were made with Generalized Linear Mixed Models (GLMMs) and the Least Significant Difference (LSD) of Fisher. Individual snails were used as a random factor. Data analyses were performed using STATGRAPHICS Centurion XVI (version 16.0.07). The significance level was fixed at $p < .05$.

Comparisons of elemental concentrations (tissue or symbiotic) amongst different endpoints (weeks 8, 9, 10, 12 and 16) of the depuration phase were made by One-way ANOVA and then multiple comparisons tests (Tukey and Bonferroni). Comparisons of elemental concentration between non-exposed and exposed snails at week 8 were made by the Mann-Whitney unpaired test. Data analyses were performed using GraphPad Prism 6®. The significance level was fixed at $p < .05$.

3. Results

3.1. Elemental distribution in tissues and symbiotic corpuscles

As expected, concentrations of Hg, As, and U in digestive gland, C and K corpuscles and kidney were significantly higher in snails exposed to the EPA's MCL of these elements than in non-exposed snails (Mann-Whitney test, Table S5).

Fig. 1 shows the elemental concentration in tissues and symbiotic corpuscles obtained from snails that were exposed for 8 weeks to EPA's MLC for either Hg, As or U. Kidney and C corpuscles showed higher Hg concentrations than digestive gland and K corpuscles (GLMMs; LSD of Fischer). K symbiotic corpuscles showed higher As and U concentrations than C symbiotic corpuscles and kidney. No statically significant differences in the variance were found when GLMMs was run using the individual as the random factor ($p = .73$ for Hg; $p = .25$ for As; $p = .08$ for U). The distribution of the elemental inventories of C and K corpuscles in the digestive gland was calculated (Table 1). The accumulations in pigmented corpuscles were approximately of 48% for mercury, 11% for arsenic and 71% for uranium.

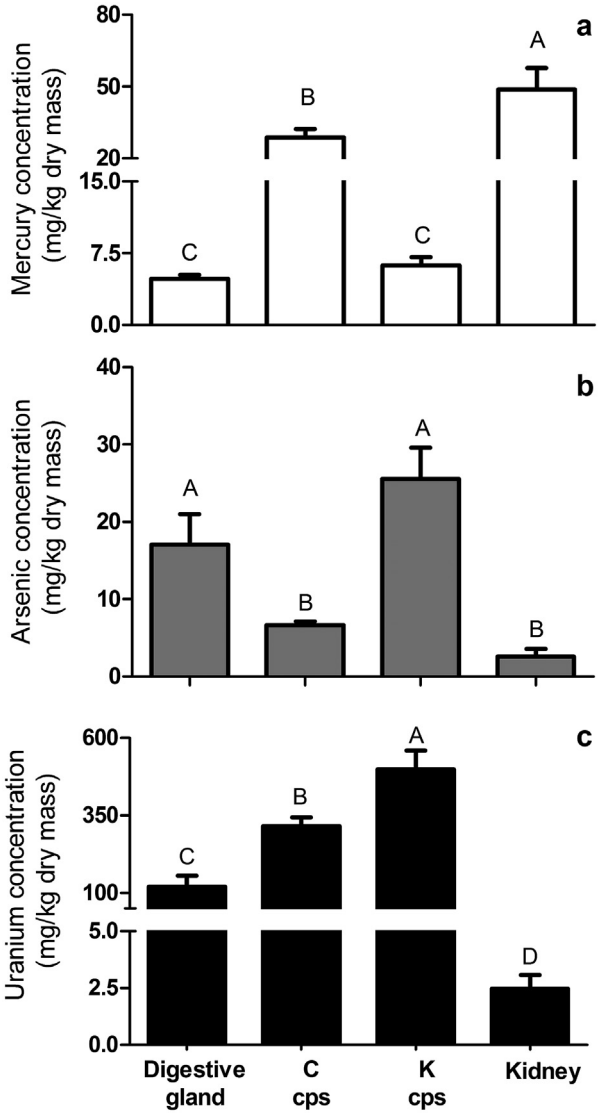

Fig. 1. Elemental concentration (mg/kg dry mass) in digestive gland, C and K corpuscles, and kidney in snails exposed for eight weeks to mercury (a), arsenic (b) or uranium (c). Mean ± SEM was computed for each group. N = 4. Different letters indicate statistically significant differences (GLM; Least Significant Difference of Fisher).

3.2. Bioconcentration and depuration in digestive gland and kidney

Results of the three exposures and elemental depuration experiments in tissues of apple snails are shown in Fig. 2.

An analysis of variance (One-way ANOVA) for each element in digestive gland and kidney from exposed snails at different endpoints (weeks 8, 9, 10, 12 and 16) of the depuration phase was made. For mercury, digestive gland showed an increase at week 9 followed by a significant decrease between weeks 12 and 16 (Fig. 2a). Furthermore, the kidney had a significant fall at week 16 (Fig. 2b).

For arsenic, digestive gland did not show significant temporal changes in the elemental concentration along the depuration phase (Fig. 2c; One-way ANOVA, $p > .05$). On the other hand, kidney showed a decrease at weeks 12 and 16 (Fig. 2d).

For uranium, digestive gland showed a pronounced decrease in the elemental concentration at weeks 12 and 16 (Fig. 2e) but this

Table 1
Mean element concentrations (mg/kg dry mass) in digestive gland and pigmented corpuscles from apple snails exposed for eight weeks to either (a) 2 μg/L of Hg, (b) 10 μg/L of As or (c) 30 μg/L of U.

Metals	Digestive gland	C corpuscles	K corpuscles	Pigmented corpuscles (C + K)
Hg	5.1 (100)	28.68 (36.2)	6.19 (12.0)	48.2%
As	17.1 (100)	6.64 (2.1)	25.6 (9.3)	11.4%
U	120 (100)	316 (18.8)	499 (52.2)	71.0%

The percent distribution of each element amongst the digestive gland (100%) and C and K corpuscles is given in parentheses. We used the mean symbiotic glandular occupancy by K and C corpuscles (Table S1) to estimate the fraction of each element accumulated in the pigmented corpuscles.

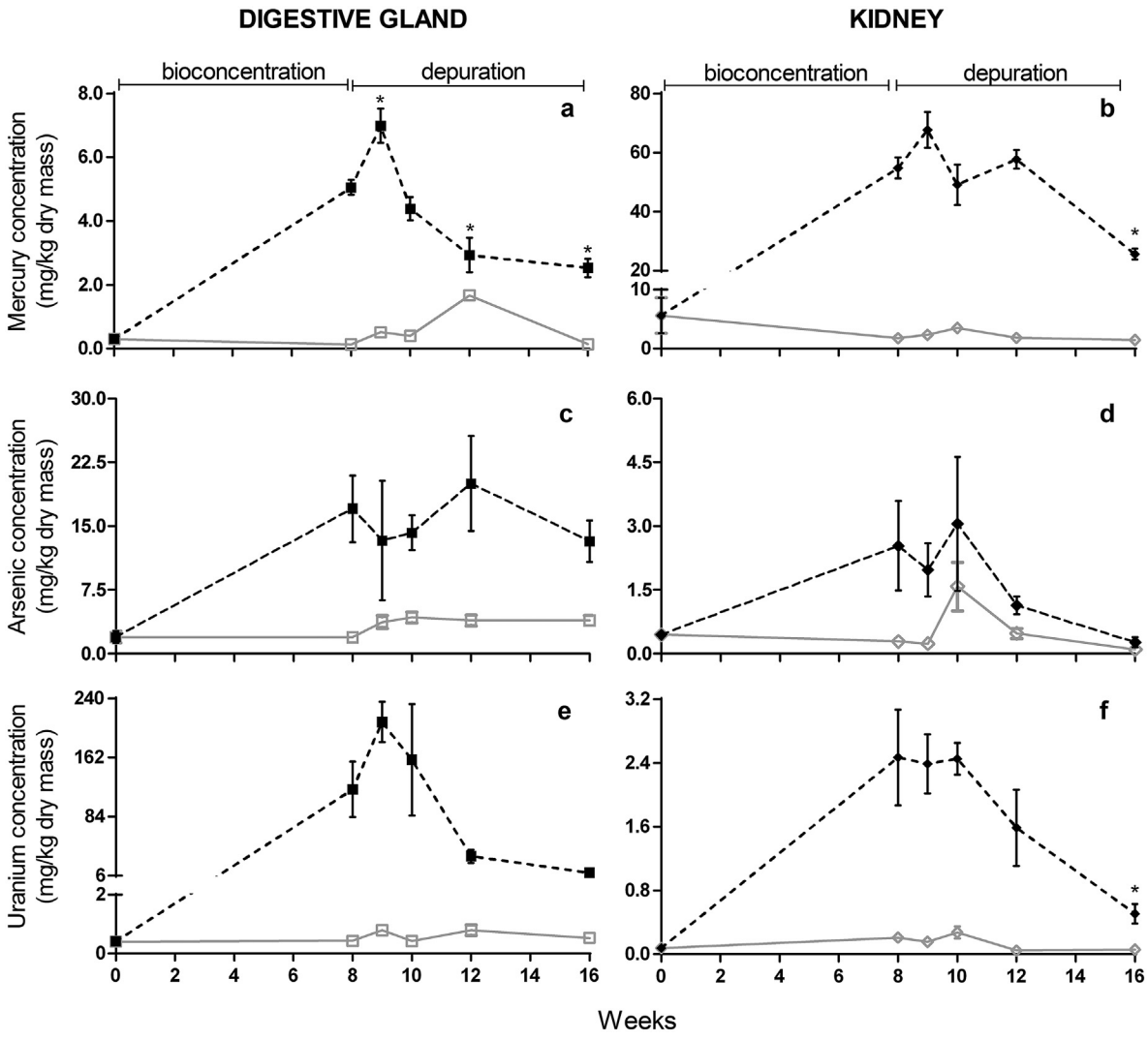

Fig. 2. Elemental concentrations (mg/kg dry mass) in digestive gland and kidney of non-exposed (grey) and exposed (black) snails for eight weeks to mercury (a–b), arsenic (c–d) and uranium (e–f) and then maintained for eight additional weeks in free-metal reconstituted water. Mean ± SEM was computed for each group. N = 4. Asterisks indicate statistically significant differences (ANOVA; Tukey and Bonferroni post-tests) among groups of exposed snails (blacks) along depuration phase (weeks 8, 9, 10, 12 and 16).

was not significant. At week 16, kidney significantly diminished its elemental concentration (Fig. 2f).

Also, we used the mean concentrations in digestive gland and kidney at the beginning (week 8) and at the end (week 16) of the depuration phase to estimate the fraction of elements retained in both tissues. At the end of depuration phase, the retention of mercury, arsenic, and uranium in digestive gland was of approximately 49%, 77%, and 8%, respectively, while the percent of elements retained in the kidney was of approximately 47%, 12%, and 20%, respectively.

3.3. Bioconcentration and depuration in symbiotic corpuscles and particulate excreta

Results of bioconcentration and depuration experiments in symbiotic corpuscles from apple snails are shown in Fig. 3.

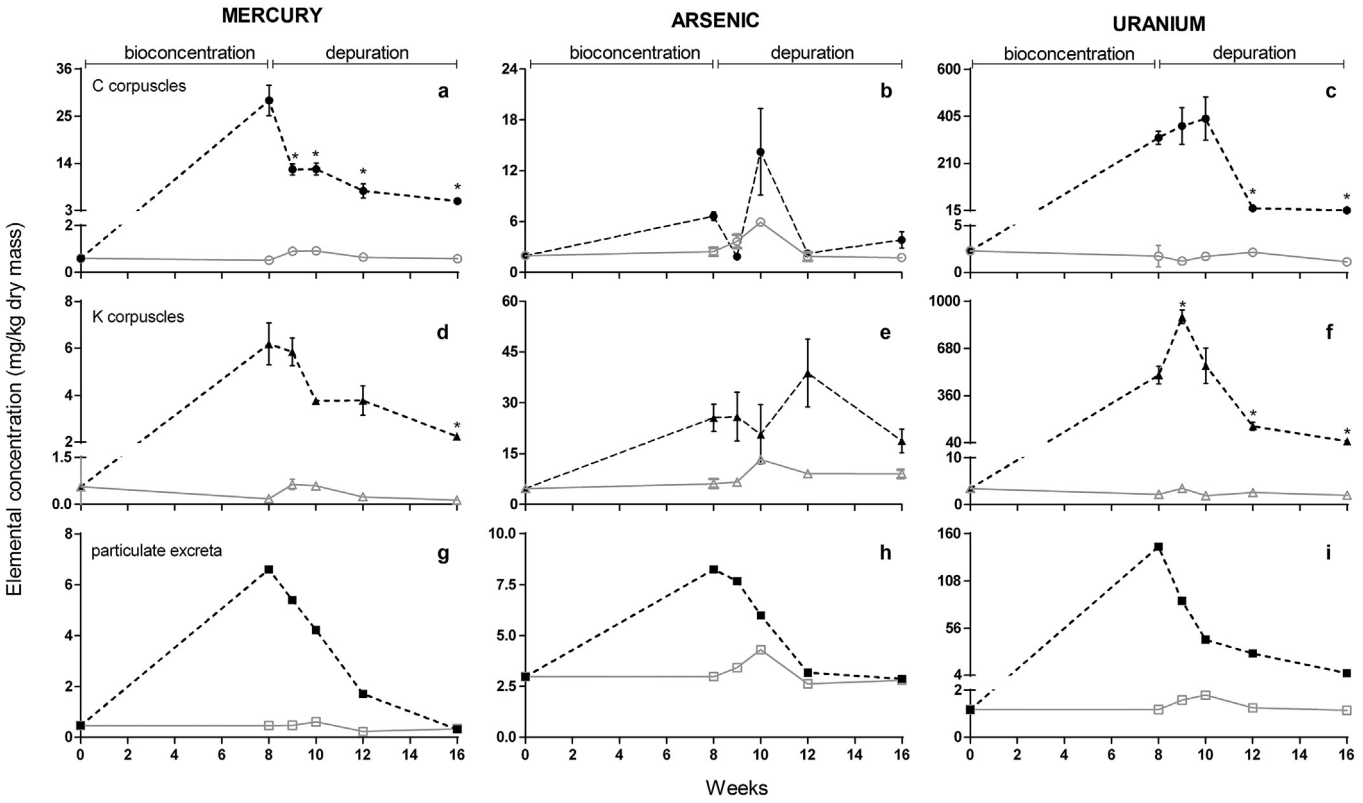

Fig. 3. Elemental concentration (mg/kg dry mass) in C and K symbiotic corpuscles isolated from the digestive gland and in particulate excreta of non-exposed (grey) and exposed (black) snails for eight weeks to mercury (a, d and g), arsenic (b, e and h) and uranium (c, f and i), and then maintained for eight additional weeks in metal-free reconstituted water. Mean ± SEM was computed for each group. N = 4. Asterisks indicate statistically significant differences (ANOVA; Tukey and Bonferroni post-tests) among groups of exposed snails (black) among depuration phase (weeks 8, 9, 10, 12 and 16).

An analysis of variance (One-way ANOVA) of each metal in C and K symbiotic corpuscles from exposed snails along the depuration phase was made. For mercury, the elemental concentration in C corpuscles decreased at week 9 and this trend was maintained until the end of the depuration period (Fig. 3a). Mercury concentration in K corpuscles decreased on week 16 (Fig. 3d). For arsenic, neither type of symbiotic corpuscles (C and K) showed significant changes during the depuration phase (Fig. 3b and e). The uranium concentration in C corpuscles remained constant up to week 10, but then a significant decrease was observed in weeks12 and 16 (Fig. 3c). Concentration in K corpuscles was increased at week 9 and then it has a significant decrease in weeks 12–16 (Fig. 3f).

The relative abundance of symbiotic corpuscles in the digestive gland was fluctuant (Table S1), thus an estimation of elemental inventories of these intracellular corpuscles at the beginning and the end of depuration phase was made. At week 8, the percent of Hg, As and U that accumulated in both symbiotic corpuscles was 48%, 11%, and 71%, respectively (Tables 2–4). At the end of depuration (week 16), the percent of corpuscular retention of mercury, arsenic, and uranium was approximately about 25%, 18%, and 54%, respectively.

Results of elemental concentrations in particulate excreta along the exposure and depuration phases are shown in Fig. 3(g–i). In all cases, the sediment was mainly composed of symbiotic corpuscles within the heavily packed fecal droppings as described previously (Castro-Vazquez et al., 2002). Concentrations of Hg, As and U in excreta from snails exposed to the EPA's MCL of these elements were higher than in non-exposed snails. The symbiotic elimination of these elements in excreta was fast at the beginning of the depuration phase (weeks 8–12) but then it decreased gradually (weeks 12–16).

4. Discussion

Although previous studies have evaluated the possibility of using apple snails as sentinels of elemental pollution in freshwater bodies (Adewunmi et al., 1996; Deng et al., 2008; Ezemonye et al., 2006; Frakes et al., 2008; Hoang et al., 2008; Maltez et al., 2009; Vega et al., 2012a) the influences of chemical, biological and temporal factors on the elemental partitioning are not fully understood. The central objective of the current work was to shed light on the role of the kidney, digestive gland and its intracellular symbiotic corpuscles of *P. canaliculata* in the bioconcentration and depuration processes of three elements: mercury (class B), arsenic (intermediate class) and uranium (class A).

4.1. Elemental distribution after metal uptake from water

Kidney and digestive gland of freshwater caenogastropods are involved in a diverse array of physiological functions, including osmoregulation, resorption of ions and organic solutes, uric acid accumulation, excretion, enzymatic digestion, immunological barrier and detoxification of metal pollutants (Andrews, 1981; Fretter and Graham, 1962; Godoy et al., 2013; Little, 1981; Rodríguez, 2017; Taylor and Andrews, 1988; Vega et al., 2007, 2012a).

The present study shows that biological (tissues or symbiotic corpuscles) and chemical (elements) aspects of exposure must be considered when interpreting an organism's internal metal

distribution. The increase in metal concentration detected in kidney of Hg-exposed apple snails can be due to the presence of vacuolated epithelial cells containing dark brown concretions rich in purine and uric acid (Andrews, 1965) and the reabsorption of ions during the formation of diluted urine (Little, 1981; Vega et al., 2012a). Also, the presence of conspicuous hemocytes aggregates in the kidney of *P. canaliculata* (Giraud-Billoud et al., 2013) and their phagocytic function (Cueto et al., 2015) could increase the sequestration of mercury in an immobilized form. Effects on the hemocyte defensive role related to the presence of pollutants have been reported previously (Auffret and Oubella, 1997; Brousseau et al., 2000; Grundy et al., 1996).

Our precedent study (Vega et al., 2012a) showed that the digestive gland of *P. canaliculata* accumulates metals of class A, B and intermediate from drinking water. Here, the metal distribution in digestive gland and in the symbiotic corpuscles differs among the three elements. It is likely that the preferences of accumulation in the digestive gland (U > As > Hg) are associated with EPA's MCL of these elements (U = 30 μg L^{-1}> As = 10 μg L^{-1}> Hg 2 μg L^{-1}), as well as the biochemical and morphological features of the symbiotic corpuscles (Vega et al., 2012b) and of their host cells (Koch et al., 2006). The subcellular distribution in pigmented corpuscles (U ~71% > Hg~48% > As~11%) after exposure confirmed that this symbiotic organism is one of the most important factors of the non-essential elements tolerance and resistance of this snail.

4.2. Accumulation and depuration in kidney and digestive gland

The impact of a metal on an organism may be related to the total metal burden of the organism which is determined by a dynamic balance of uptake, accumulation, deposition and depuration processes.

In the present study, the tissue depuration is variable among the three elements. At week 16, the tissue depuration of U is high (Digestive gland~92% - Kidney~79%), while it is lower for Hg (Digestive gland~50% - Kidney~53%). Arsenic shows a differential pattern of tissue depuration (Digestive gland ~23% - Kidney ~89%). The tissue variability in the arsenic retention in this snail may be associated with the presence of different metal detoxification systems. It has been reported that aquatic organisms sequester metals in intracellular compartments through the binding with metallothioneins-like proteins (MLPs) (Viarengo et al., 1985) and metal-rich cytoplasmic granules (Wallace et al., 2003). Specifically, the expression of MLPs has been shown in the ampullariids *Marisa cornuarietis* and *Pomacea bridgesii* exposed to intermediate and B class metals (Maltez et al., 2009). Also, the absence of changes in the levels of arsenic in the digestive gland during all depuration phase may be indicative of intracellular enzymatic action and redistribution in less toxic organic forms (Francesconi et al., 1998).

4.3. Symbiotic corpuscles as sites of elemental bioconcentration and detoxification

Based on the morphological and molecular evidence it has been hypothesized that pigmented corpuscles found in the digestive gland of Ampullariidae are a prokaryote akin to the Cyanobacteria (Castro-Vazquez et al., 2002; Koch et al., 2006; Vega et al., 2005, 2006). Both symbiotic corpuscles, named C and K corpuscles, live in epithelial cells of the digestive gland of *P. canaliculata* and then are freed into gland ducts and are thought to be involved in the digestion of food (Godoy et al., 2013) and metal detoxification (Vega et al., 2012a).

The accumulation and subsequent detoxification in symbiotic corpuscles have been shown here. This elemental distribution in digestive gland showed that mercury (~36%) was mainly accumulated in C corpuscles while uranium (~52%) and arsenic (~9%) were accumulated in K corpuscles. These findings suggest that the symbiotic corpuscles may have mechanisms of ion metal removal similar to that found in Cyanobacteria (for a review see (Baptista and Vasconcelos, 2006)).

Dellagnola (Dellagnolla, 2015; Dellagnola et al., 2017) has reported the alcianophilic nature of the cell wall of the C corpuscles and the electrondense layers of the K corpuscles that indicate the presence of glycosaminoglycans. It is likely that these extracellular symbiotic structures were participating in a kind of metal bio-sorption as it occurs in the extracellular polysaccharides (EPS) on the cyanobacteria cell surface (De Philippis et al., 2003; Pereira et al., 2009) which has been related to the stabilization of their extracellular environment (Ahmed et al., 2014). Freire-Nordi et al. (2005) have shown *in vitro* that the EPS of the cyanobacterium *Anabaena spiroides* are able to complex mercury (Freire-Nordi et al., 2005). The presence of amide and deprotonated carboxyl on the EPS of the cyanobacterium *Synechococcus elongatus* strain BDU/75042 have been identified as the main sites of binding of uranyl from water system (Acharya et al., 2009). In addition, polyphosphates are often found in Bacteria, mainly Cyanobacteria (Pettersson et al., 1988), and are involved in the sequestration of U (Acharya et al., 2012).

As in the Cyanobacteria, the symbiotic corpuscles show a low concentration of As and it may be associated with detoxification mechanisms that involve the conversion of As^{+5} to As^{+3} and its subsequent efflux from these prokaryotic cells (Sánchez-Riego et al., 2014; Yin et al., 2011; Zhang et al., 2013) and the enzymatic conversion of As^{+3} in non-toxic volatile methylated species (Yin et al., 2011). Altogether, these putative detoxification mechanisms in intracellular symbiotic corpuscles probably contribute to the higher concentrations of As found in the gland host cells of *P. canaliculata*.

These results are relevant to analyse the role of symbiotic corpuscles in the detoxification process of apple snails since the digestive gland of the host maintains a pool of symbionts, as well as eliminates continuously large quantities of them in faeces (Castro-Vazquez et al., 2002; Koch et al., 2006; Vega et al., 2006). After exposure, the temporal changes of elemental concentrations in both intracellular symbiotic corpuscles were different among the three elements studied; however, the patterns of elimination in excreta were similar.

The early decline (weeks 8–10) of the Hg load of the intracellular C corpuscles is associated with the constant mercurial elimination in particulate excreta which may indicate an increase of the apocrine secretion of C symbiotic corpuscles by host cells. After week 12, the mercurial load of symbiotic corpuscles (Table S2) remains approximately constant but the snails continue eliminating this element in feces. It is likely that kidney's hemocytes were participating in a kind of releasing of this element by diapedesis across the kidney or the digestive tract (Marigomez et al., 1990).

The As load in both pigmented corpuscles and their host cells remained constant, however, this element was continuously detoxified in particulate excreta during the first half of the depuration phase. This unexpected finding may be indicating the As mobilization from putative accumulator tissues to digestive cells where this element is then directed to symbiotic corpuscles (mainly K corpuscles, Table S3) and other intracellular structures (range = 69.6%–88.6%). It is possible that the muscle of *P. canaliculata* delivered arsenic after exposure since this tissue occupies a significant mass of the snail and has some ability to bind different elements (Vega et al., 2012a).

Uranium is quickly eliminated in feces during the first two weeks of the depuration phase but the U load in digestive cells

remained approximately constant. It is likely that U mobilization from putative accumulator tissues to digestive cells is occurring. After week 12, the continuous elimination of U in feces is associated with a decrease in concentration in the host and symbiotic cells.

4.4. Environmental and human health

Apple snails are used as a food source for human and for farmed species (Heuzé and Tran, 2017). *Pomacea canaliculata* and *P. maculata* have been introduced anthropogenically to Asia and Hawaii as a human resource (Hayes et al., 2008; López-Soriano et al., 2009) and became pests for production of rice and taro. Other species of *Pomacea* in America (Diupotex-Chong et al., 2004) and the genus *Pila* in India and Southeast Asia have been used for human consumption. In addition, *P. canaliculata* has been proposed as a substitute food for the diet protein of poultry, pigs, fish, and frogs (Heuzé and Tran, 2017). Since pigmented corpuscles similar to those found in *P. canaliculata* have been found in other species of *Pomacea* and the genera *Marisa*, *Asolene* and *Pila* (Castro-Vazquez et al., 2002; Devi et al., 1981; Meenakshi, 1955) they may have the same bioconcentration capacity of metallic water contaminants. From a toxicological view, the potential use of apple snails as a suitable substitute for protein sources will depend on the identification of pollutants in natural aquatic ecosystems and its accumulation in snails. A warning must be made on the method of processing of the snails because it is probable that an increase of heavy metals by kg of consumed food may occur when the shells are removed and/or the head-foot and viscera of the snails are dried.

5. Conclusion

The freshwater snail *P. canaliculata* is a very sensitive bioindicator that selectively accumulates environmental metal pollutants at high levels in digestive gland, kidney, and symbiotic corpuscles when exposed to safe levels of these elements. In Hg-exposed apple snails, the preferential accumulation in the kidney was 31 times higher than no-exposed snails. In As or U exposed apple snails, the preferential accumulations in digestive gland were 9 and 276 times higher than no-exposed snails, respectively. The accumulation of mercury and uranium in symbiotic corpuscles were of one or two magnitude orders major than no-exposed snails. Tissues and symbiotic corpuscles of this apple snail are able to keep up a record of the exposures after the snails come back to the metal-free environment.

Competing interests

The authors declare that no competing interests exist.

Author contributions

IAV coordinated the experiments. ADCD and IAV designed and conceived the experiments. ADCD performed all the experiments. MAA and SRG executed the neutron activation analysis. MAA and IAV contributed reagents/materials/analysis tools. All authors contributed to the writing and improving the manuscript, and approved the final version.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2017.12.145.

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