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Article

Bioconcentration of Cd and Pb by the River Crab *Trichodactylus fluviatilis* (Crustacea: Decapoda)

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Foi avaliada a bioconcentração de cádmio e chumbo pelo caranguejo fluvial *Trichodactylus fluviatilis*. Trinta animais foram expostos a 200 μg L^{-1} de cádmio e chumbo por 7, 14 e 21 dias. Após dissecação e digestão nitro-perclórica, os metais foram determinados nas brânquias, hepatopâncreas e músculo por espectrometria de emissão óptica com plasma acoplado indutivamente. O chumbo foi detectado apenas nas brânquias, sem diferenças significativas entre diferentes períodos de exposição. O cádmio foi encontrado em todos os tecidos após exposição. Diferenças significativas entre as concentrações de cádmio em animais expostos por diferentes períodos sugerem um processo de acumulação, com concentração estabilizada após 14 dias. Usando um procedimento de extração em fase sólida com *Saccharomyces cerevisae* e com o objetivo de avaliar a transferência e estocagem de cádmio nos tecidos, foi realizado o fracionamento de cádmio na forma livre (ou lábil) e cádmio-proteína (possivelmente metalotioneína) (Cd-P) em animais expostos a 200 μg L^{-1} de cádmio por 21 dias. A determinação de cádmio livre e Cd-P nos tecidos mostrou que nas brânquias o metal foi encontrado principalmente na forma livre, enquanto que no hepatopâncreas o metal foi encontrado principalmente ligado à proteína. Pode-se inferir que, absorvido através das brânquias, o cádmio foi transferido e estocado no hepatopâncreas dos animais.

The bioconcentration of cadmium and lead by the freshwater crab *Trichodactylus fluviatilis* was evaluated. Thirty animals were exposed to 200 μg L^{-1} of cadmium and lead for 7, 14 and 21 days. Both metals were determined in gills, hepatopancreas and muscle after dissection and digestion by inductively coupled plasma optical emission spectrometry. Lead was detected only in gills, but without significant difference among different exposure periods. Cadmium was found in all tissues after exposure. Significant differences among cadmium concentrations in animals exposed for different periods suggest an accumulation process, with concentration stabilized after 14 days. Fractionation of free (or labile) cadmium and cadmium protein (possibly metallothionein) (Cd-P) in gills and hepatopancreas were carried out to assess the cadmium transference and storage in the tissues using a solid phase extraction procedure with *Saccharomyces cerevisae*. Fractionation of free (or labile) cadmium and cadmium protein (possibly metallothionein) (Cd-P) in animals exposed to 200 μg L^{-1} for 21 days in gills and hepatopancreas were carried to assess the cadmium transference and storage in the tissues using a solid phase extraction procedure with *Saccharomyces cerevisae*. In gills, cadmium was found mainly in the free form, while in hepatopancreas the metal was found mainly bound to the protein (Cd-P). It may be inferred that, absorbed through gills, cadmium was transferred and stored in the hepatopancreas.

Keywords: bioconcentration, biomonitor, metallothionein, cadmium, lead

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Introduction

Freshwater environments nearby industries and cities frequently have high levels of contamination by metals. Mining, house waste disposal, effluents from industry and agriculture are the major responsible for these contaminations.[1-4] Cadmium and Pb show no functional activity in organism metabolism and their toxic effects on biota are dependent on several factors, such as the chemical speciation in the aquatic environment.[3,5]

Cadmium is considered one of the most potentially toxic metals in the environment. The anthropogenic sources of environmental contamination of this metal are batteries, synthetic pigments, residues of galvanoplastic factories and fertilizers.[5,6] In aquatic environments, Cd has higher motility and it is found as Cd^{2+} (hydrated ion II) or as an ionic complex with other organic or inorganic substances.[6]

Lead is relatively abundant in the earth crust and it has a tendency to accumulate in sediments. As it has low solubility, the metal can be accessible to the food chain for a long time.[6] Lead is found in aquatic environment as Pb(II) in the free form (hydrated ion II) or containing organic or inorganic ligands. Normally, the free form is comparatively more toxic. The main natural sources of lead are volcanic emissions and weathering of rocks, but they are considered insignificant when compared to anthropogenic ones, like mining and metallurgy activities, chemical industries, electric battery factories, paints and pigments.[6] Organic Pb forms are released in the environment through direct sources (production, transportation and storage of gasoline with Pb and consequent traffic emissions) and chemical/biological methylation of inorganic lead in anaerobic sediments.[6] Metals from natural sources and contaminated effluents can be taken up and subsequently accumulated in aquatic animal tissues according to their physiologic characteristics (species, metabolism, feeding habits, size, sex).[3-4,7-12] Besides, characteristics from the aquatic environment (solubility, concentration and/or different metals interaction, pH, salinity, dissolved oxygen) can also interfere in the accumulation process.[3,4,6,13] Bioindicator organisms can provide real evidence of bioavailability and effects of contaminants in the environment.[14] In aquatic environments, bioindicators can take up and accumulate substances in concentrations several times higher than those found in water.[3,11] Some studies show Cd accumulation in aquatic animals in concentrations from 100 to 1000 times higher than those in the water. The related bioconcentration factors range from 113 to 18,000 for invertebrates and from 3 to 2,213 for fish.[6]

Many studies have shown that Cd and Pb can accumulate in freshwater decapods.[3,4,10,11] Nevertheless, the bioconcentration of these metals depends on the biological characteristics of each species and it can vary according to physiological patterns of accumulation, metabolism, size, sex, and individual variability factors of the organisms, like growing, build up or loss of gametes and energy reserves.[3,13-15]

Invertebrates have many processes of cellular detoxification which can decrease potentially toxic metal concentrations in circulation, like intracellular mechanisms involving high-affinity binding with low molecular weight proteins, known as metallothioneins.[12,13] Metallothioneins are found in almost any vertebrate including many species of fish and aquatic invertebrates, mainly mollusks and crustaceans.[16] In animals, they are more abundant in parenchymatic tissues (liver, kidney, pancreas and intestine), but their occurrence and biosynthesis have also been shown in other tissues and cellular types.[17]

The binding of potentially toxic metals with metallothioneins represents a sequestration function which leaves the metals unable to interact with other proteins, like enzymes, conferring protection against metal toxicity at the cellular level.[18] Tissues which are directly involved in metal uptake, storage and excretion have a high capacity of synthesizing metallothioneins.[13,16] In aquatic animals, these proteins have been identified in hepatopancreas and gills of mollusks and crustaceans.[16,18-22]

The objective of this study was to investigate Cd and Pb bioconcentration processes using the freshwater crab *T. fluviatilis* as bioindicator species. The effect of sex, time of exposure to metals and their distribution in different tissues of the animal was evaluated. Additionally, the metal storage process in *T. fluviatilis* was evaluated by determination of the concentration of cadmium inorganic forms (possibly Cd(II) or "free-Cd") and organic forms (possibly Cd-metallotioneins, Cd-P) in gills and hepatopancreas.

Experimental

Field work

Thirty-one *Richodactylus fluviatilis* specimens (Table 1) were caught in Ribeirão Claro river (S 22º 41'35,3" W 47º32'26,1") - Corumbataí watershed - São Paulo, Brazil, using traps containing cat food, placed on the river bank, near vegetation.

Table 1. Biometric data of collected specimens of *T. fluviatilis*, with Cl and Cw corresponding to length and width of cephalotoracic carapace, respectively

	n	Average mass (g)	Cl (cm)[a]	Average Cl (cm)	Cw (cm)[a]	Average Cw (cm)
Males	11	11.0	2.2 - 2.9	2.5	2.4 - 3.3	2.9
Females	20	7.11	2.1- 3.0	2.4	2.4 - 3.4	2.7

[a]minimum value - maximum value.

Bioassays

Bioassay 1: Total Cd and Pb in water and tissues

All glassware and plastics used during exposure, sample preparation and determination of Cd and Pb concentrations were previously decontaminated with a 10% v/v nitric acid (HNO_3) solution for 8 h, followed by rinsing with distilled and deionised water. All solutions were made with purified water (18 MΩ cm).

Stock solutions of 1000 mg L^{-1} Cd and Pb (High-Purity Standard, Charleston, SC, USA) were used in both bioassays. The digestion process was carried out using HNO_3 and $HClO_4$ *pro analisi* (p.a.) grade (Merck, Darmstadt, Germany). All solutions were made with purified water (18 MΩ cm).

Ribeirão Claro river water was used for the exposures, once previous analysis has shown Cd and Pb concentrations (dissolved fraction) below the limits of detection (4 and 30 ng mL^{-1} for Cd and Pb, respectively). All crabs were individually placed in plastic bottles containing 900 mL of water from the river, with controlled temperature (25 °C). The exposure to both metals was made by adding 180 µL of each stock solution to the water, resulting in exposure solutions of 200 µg L^{-1} of Cd and Pb.

Exposure periods were 7 (for 4 males and 6 females), 14 (5 males and 5 females) and 21 (1 male and 7 females) days. For control, 1 male and 1 female were allowed to stand at the same conditions without metals in water for a 7-day-period, since background concentrations for the species is known from previous studies. In these studies Cd and Pb concentrations for *T. fluviatilis* caught in river water without laboratory exposures (16 crabs) were below 0.5 and 8 mg kg^{-1}, respectively. In addition, river water concentrations of dissolved Cd and Pb were below 4 and 30 ng mL^{-1}, respectively. Analysis of control individuals and river water used for the exposures confirmed these previous results: animals exposed to low concentrations of Cd and Pb (≤ 4 and 30 ng mL^{-1}) shows tissues concentrations below 0.5 and 8 mg kg^{-1}.To avoid accumulation of residues during the exposure period, the water was changed every 72 h. Each crab was fed with small pieces of fish in freshly changed water. In order to prevent metal uptake from food, Cd^{2+} and Pb^{2+} stock solutions were added only after certifying that there were no fish remains in the water.

Cadmium and Pb concentrations in exposure solutions were monitored by periodic sampling from 0 to 144 h of total exposure. The initial exposure solution (0 to 72 h of total exposure) was called solution 1 and the exposure solution after first water exchange (72 to 144 h of exposure) was called solution 2. Additionally, the pH of the exposure solutions was measured each 12 h, for 3 days.

After exposure, animals were euthanized by chilling at −10 °C and classified by sex, mass, carapace length and width. Using stainless material, gill, hepatopancreas and muscle tissues were removed from each individual, weighed and stored in 2 mL Eppendorf tubes at −10 °C until analysis.

Wet tissues samples (0.08-0.24 g of gills, 0.01-0.19 g of hepatopancreas and 0.03-0.18 g of muscle) were transferred to digestion tubes. The tissues were predigested by adding 2.5 mL of concentrated HNO_3 (Merck) in each tube and allowed the material to stand overnight.

The digestion process was completed in a digestion block by heating the tubes at 100 °C (for 1 h) and 160 °C (for about 2 h), until a solution without suspended fragments was obtained. After cooling, 0.2 mL of concentrated perchloric acid (*p.a.* grade, Merck) was added in each tube, followed by heating at 160 °C for 15 min, 190 °C for 30 min and 210 °C for about 2.5 h. The addition of perchloric acid was particularly necessary to ensure effective digestion of hepatopancreas samples. In addition, it gives a residual acid concentration which is similar to all samples. After cooling, the obtained extracts were quantitatively transferred to volumetric flasks and diluted to 20 mL with ultrapure water, produced in a Milli-Q system (Millipore, Billerica, MA, USA).

A GBC model Integra XL ICP OES spectrometer (Melbourne, Australia) with a V-Groove nebulizer (VeeSpray, Glass Expansion, Melbourne, Australia) installed in a cyclonic spray chamber (Glass Expansion) was used for determination of cadmium and lead concentrations in water samples and biological digests/extracts. The spectrometer was operated under the following conditions: forward power = 1200 W; plasma gas flow rate = 10 L min^{-1}; auxiliary gas flow rate = 0.5 L min^{-1}; nebulizer gas flow rate = 0.6 L min^{-1}; sample introduction flow rate = 3.2 mL min^{-1}; observation height (radial viewing) = 10 mm; sample introduction flow rate = 2.8 mL min^{-1}. Cadmium and Pb measurements were performed at 226.502 and 220.353 nm, respectively. External calibration curves were used for quantification of both metals. Replicates (n = 2) were made to all samples. Standard reference materials (SRM) of Fish Homogenate (SRM MA-A-2/TM, International Atomic Energy Agency, Vienna, Austria) and Copepod Homogenate (SRM MA-A-1/TM, International Atomic Energy Agency) were used to check the accuracy of the analytical procedure.

Bioassay 2: Metallothioneins (Cd-binding biomolecules) and metal speciation

For this work stage other 12 *T. fluviatilis* specimens were caught and exposed to 200 µg L^{-1} Cd solutions for 21 days, using the same strategy described previously. After the exposure phase, the animals were euthanized, classified by sex, mass, carapace length and width. Gills and hepatopancreas were removed from each individual, weighed and stored in 2 mL Eppendorf tubes at −10 °C until analysis. This bioassay

aimed to evaluate the presence of Cd-binding biomolecules (possibly metallothioneins) in gills and hepatopancreas.

Sample preparation

Based on previously described methods,[23-26] the following procedure was used to extract the cytosol from the selected tissues of exposed *T. fluviatilis.* About 0.1 g of hepatopancreas and gills from each specimen was homogenized using 10 mL 0.05 mol L^{-1} TRIS (tris(hydroxymethyl)aminomethane) (pH 7.0 and 4 °C) in 30 mL centrifuge tubes. Then, the mixtures were centrifuged on a 15000 g and 4 °C for 30 min using a JOUAN centrifuge MR 23i with controlled temperature (St. Herblain, France). The supernatants were transferred to 50 mL tubes and the final volume of each sample was made up to 50 mL by adding 40 mL of the same 0.05 mol L^{-1} TRIS solution. The obtained extracts were used for determining total cadmium concentrations (direct determination on ICP OES) and Cd(II) and Cd-binding biomolecules (Cd-P) concentrations (after fractionation).

The procedure used for Cd fractionation on cytosols was the same as proposed by Menegário *et al.*,[23] where the baker yeast *Saccharomyces cerevisae* is used as sorbent material to separate Cd(II) and Cd-P (possibly Cd metallothioneins or Cd bound to other proteins) from cytosols of biological extracts. After contact with the yeast and centrifugation, the inorganic fraction of cadmium (Cd(II) or "free" Cd) is expected to be found in solid phase (retained by the yeast) while the Cd-P is expected to be found in the liquid phase.

Solid phase extraction

Aliquots of 10 mL of the obtained extracts were transferred to 15 mL centrifuge tubes containing 0.0625 g of *Saccharomyces cerevisiae*. The tubes were manually agitated until complete mixture of yeast and solution (about 30 s) and maintained in water bath at 25 °C for 10 min. Subsequently, the tubes were centrifuged at 1900 g for 7 min using a JOUAN B4i centrifuge (St. Herblain, France). After phase separation, the liquid phase was transferred to other flask and the solid phase was mixed with 10 mL 2% v/v HNO_3.

Liquid and solid phase were agitated and directly introduced into the ICP OES, using a V-Groove nebulizer, to determine Cd-P and Cd(II) concentrations, respectively. The efficiency of V-groove nebulizer to introduce slurry of yeast has been previously demonstrated.[23] The yeast slurry basically contains individual cells of the microorganism with diameter size changing from of 5 to 10 μm, while the tolerance to particulates for the nebulizer is typically up to 300 μm. Acidification of solid phase results in a slurry characterized by a matrix effect lower than 20% (as compared with nebulization of HNO_3 solution), while the matrix effect on liquid phase is insignificant.

Statistical analysis

Taking into account that the non-parametric test of Kruskal-Wallis allows multiple comparisons among independent samples, it was used to compare differences between concentrations of Cd and Pb due to sexes and exposure periods. When significant differences between the variables tested were confirmed ($p < 0.05$), Dunn's statistical test was used for multiple comparisons, pair to pair, of the independent samples.

To determine if Cd concentrations found in different tissues were significantly different ($p < 0.05$), Friedman test was used, once it allows comparisons between dependent variables (different tissues from the same animals). The same test was used to compare Cd(II) and Cd-P concentrations in gills and in hepatopancreas (different forms of cadmium in the same tissues) of 21 days exposed animals. Statistical tests were performed using the program BioEstat 5.0, with 95% of confidence.

Results and Discussion

Digestion of reference material was carried out using the procedure described in bioassay 1 item, using 0.5 g of sample. Cadmium and Pb concentrations obtained for standard reference materials by using the digestion procedure described in bioassay 1 are shown in Table 2. For fish sample, concentrations of Cd and Pb were lower than detection limits. For copepod sample, the obtained results were according to certified values (95% level), which indicate that the procedure was suitable for analysis of biological materials.

Table 2. Average concentrations (dry weight) ± confidence interval (n = 4, t = 3.182, 95% level) in standard reference materials

	Certified (mg kg^{-1})		Found (mg kg^{-1})	
	Fish	Copepod	Fish	Copepod
Cd	0.066 ± 0.004	0.75 ± 0.03	< 0.2[a]	0.5 ± 0.3
Pb	0.58 ± 0.07	2.1 ± 0.3	< 2.0[a]	3 ± 1

[a]Limit of detection (3 σ , dry mass) found with the procedure described in bioassay 1 using 0.5 g of sample.

Cadmium and Pb concentrations in exposure solutions

There was no significant pH alteration in the exposure solution during 72 h of incubation. For this period, values between 7.4 and 8.0 were obtained.

Potentially bioavailable (total dissolved) Cd and Pb were determined in exposure solutions. With this purpose, a filtration in 0.45 μm acetate cellulose membrane was made, followed by acidification with HNO_3.

Concentrations of analytes in the initial exposure solution (0 to 72 h of total exposure), solution 1, and in the exposure solution after first water exchange (72 to 144 h of exposure), solution 2, are shown in Figures 1 and 2, respectively.

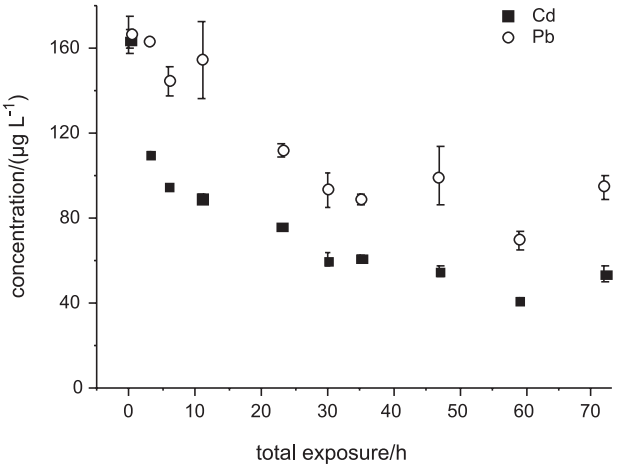

Figure 1. Cadmium and Pb concentrations (mean ± SD) in water samples taken from initial exposure solution (solution 1).

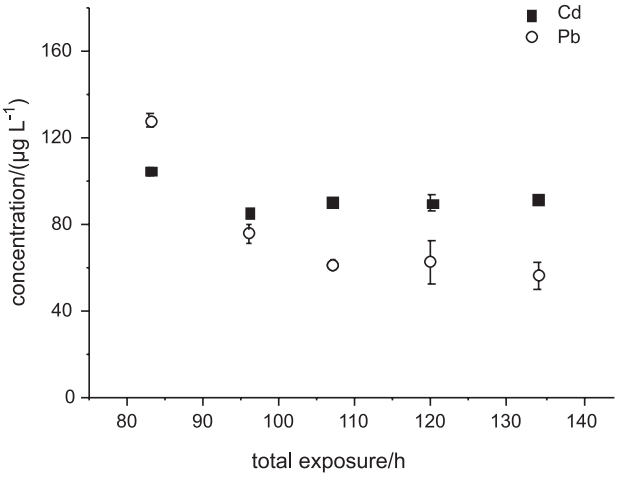

Figure 2. Cadmium and lead concentrations (mean ± SD) in water samples taken from first changed exposure solution (solution 2).

It is observed a trend towards decreasing concentrations of Cd and Pb in exposure solutions during the incubation period, mainly in the first 20-25 h. These results indicate metal uptake by the animals with different rates during the incubation period (72 h). Nevertheless, it should be taken into account that this difference can be consequence of possible interactions of Cd and Pb with the carapaces of animals, with particulate material and/or with other compounds present in the river water used for the exposures.

Lead and Cd concentrations in tissues

Three animals died between 19-21 days. After exposure, Pb was detected in gills of 26 individuals. The average metal concentrations found in gills for different periods of exposure are shown in Figure 3. Lead was also detected in hepatopancreas of only 3 animals (average concentration of 8.42 ± 6.85 mg kg^{-1}) and muscle of 4 animals (average concentration of 11.33 ± 1.29 mg kg^{-1}). The mean concentration of the metal in gills was 15.61 mg kg^{-1}. From this value and considering moisture of 75%,[27] a mean concentration of 62.45 mg kg^{-1} in dry weight can be estimated for gills. The results obtained for Pb suggest that the transference of the element from gills to other tissues did not occur during the exposure period. There is little information in the literature about interactions of Pb within crabs. For comparison, Turoczy *et al.*,[28] related significant differences in lead concentrations in tissues of some species of marine crabs (*Paralithodes camtschatica, Portunus pelagicus, Tachypleus tridentatus* and *Callinectes spp.*).

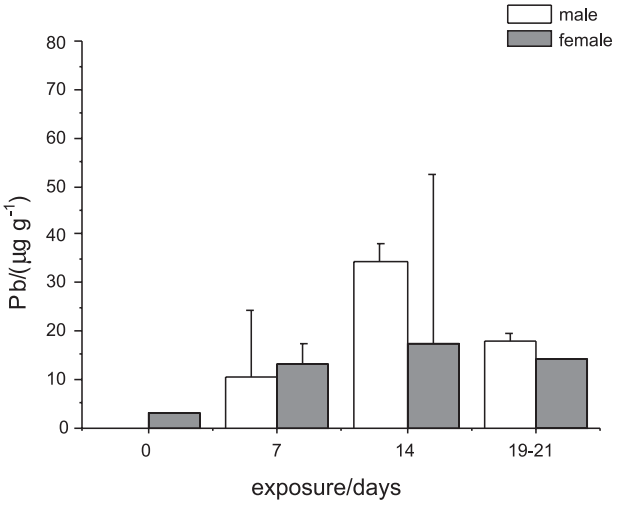

Figure 3. Lead concentrations (mean ± SD) in gills of *T. fluviatilis* males and females according to the period of exposure.

There were not significant differences in Pb concentrations between males and females (Kruskal-Wallis, p = 0.7934). For natural expositions to the metal, similar results were found for the marine crabs *Callinectes spp.*,[29] and *Tachypleus* tridentatus.[28] On the other hand, differences in Pb concentrations between sexes have been reported for *Paralithodes camtschatica*.[30]

There were no significant differences among Pb concentrations in gills of animals from different exposure periods (Kruskal-Wallis, p = 0.5067). It shows that, at studied conditions, the maximum concentration of Pb in this tissue occurs on the first 7 days of exposure. After this period, there is no more lead accumulation by the animals.

Cadmium was detected in gills and hepatopancreas of all exposed animals. In muscle, metal was detected in 25 animals from the 28 exposed ones. Average Cd concentrations (for males and females) obtained in the three periods of exposure for gills, hepatopancreas and muscle are shown in Figures 4, 5 and 6, respectively.

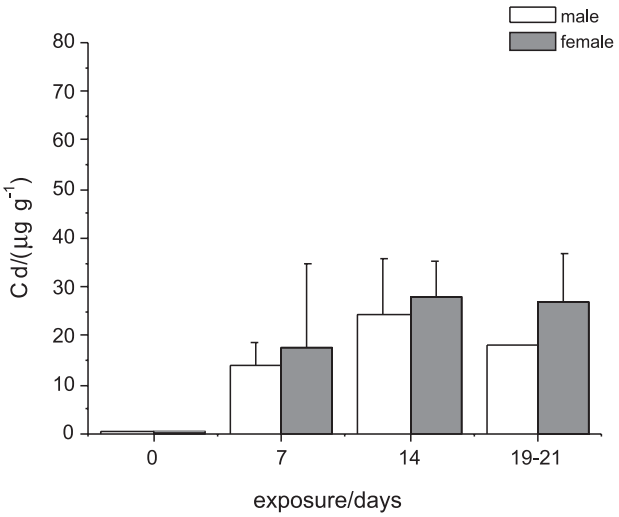

Figure 4. Cadmium concentrations (mean ± SD) in gills of *T. fluviatilis* males and females according to the period of exposure.

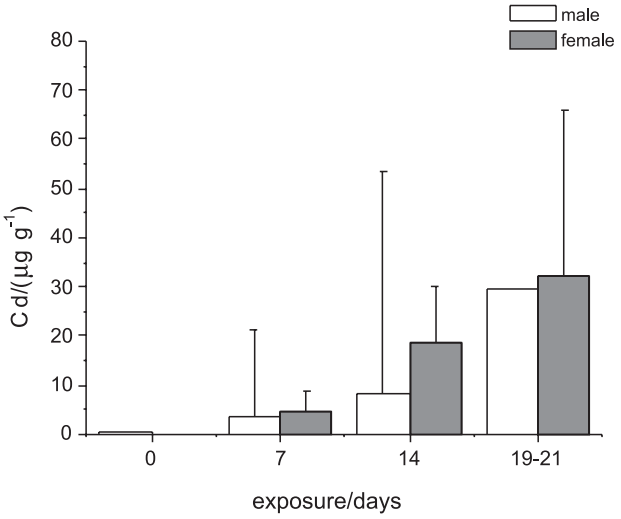

Figure 5. Cadmium concentrations (mean ± SD) in hepatopancreas of *T. fluviatilis* males and females according to the period of exposure.

No significant difference was found in Cd concentrations between animals from different sexes (Kruskal-Wallis, $p = 0.4442$), as previously observed by Schuwerack *et al.*[11] Also, for most crab species exposed to cadmium in natural aquatic systems (as *Pseudocarcinus gigas*, *Callinectes sp.*, *Tachypleus tridentatus*) had been reported no differences between Cd concentrations related to sex.[3,28] Only Chen *et al.*,[31] reported differences of cadmium concentrations between sexes (cadmium in hepatopancreas of *Thalamita crenata*). The reasons for this difference are still not clear.[3]

There were significant differences between Cd concentrations of exposed animals from different periods (Kruskal-Wallis, $p < 0.05$), suggesting an accumulation process. The results obtained for Dunn's test (Table 3) suggest that in the experimental conditions used, Cd is

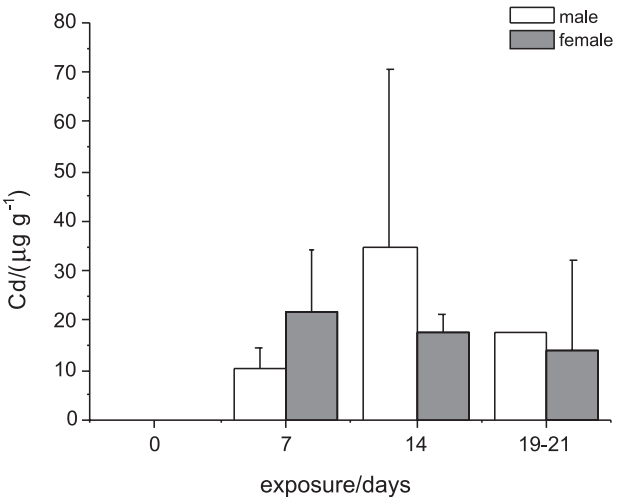

Figure 6. Cadmium concentrations (mean ± SD) in muscle of *T. fluviatilis* males and females according to the period of exposure.

Table 3. Results obtained for Dunn's test for cadmium concentrations found in different exposure periods

Pairs	P
7 days × 14 days	< 0.05
7 days × 21 days	< 0.05
14 days × 21 days	n.s.

n.s.: not significant.

accumulated by the animals until 14 days. After this period the concentration was stabilized.

There were significant differences (Friedman, $p < 0.05$) between Cd concentrations in gills (average of 21.09 mg kg^{-1}) and muscle (average of 2.17 mg kg^{-1}) and between hepatopancreas (average of 10.52 mg kg^{-1}) and muscle. Considering moisture of 75%,[27] we can estimate mean Cd concentrations in dry mass of 84.4, 8.7 and 42.1 mg $kg^{-1}$for gill, muscle and hepatopancreas, respectively.

For most studied species, differences in bioconcentration of metals among tissues and organs are observed and they show to vary according to metals and crustacean species.[3,10,11,14,15,32]

For the freshwater crab *Potamonautes warreni*,[11] exposed to Cd in laboratory and for natural expositions for the marine species *Carcinus maenas*,[9] there were also reported significant differences among metal concentrations in different tissues, following the order: gills > hepatopancreas > muscle.

Similar results to those obtained in this work were reported for *Nephrops norvegicus*,[9] where Cd concentration was higher as well in gill as in hepatopancreas of males and females, even in metal conditions of 1 µg mL^{-1} in

Table 4. Bioconcentration factor (BCF) values for gills, hepatopancreas and muscle according to the exposure period

Exp. time (days)	Cadmium BCF (L kg^{-1})			Lead BCF (L kg^{-1})		
	Gills	Hepatopancreas	Muscle	Gills	Hepatopancreas	Muscle
07	81	22	8	64	-	-
14	123	66	17	88	-	-
19 - 21	122	154	14	78	-	-

environment. For *Pseudocarcinus gigas*,[28] and *Penaeus californiensis,*[31] higher Cd concentrations were found in hepatopancreas, followed by gills and muscle.

Bioconcentration factor (BCF) was calculated according to the equation 1, and the obtained values for tissues, for each one of the exposure periods, are shown in Table 4.

$$BCF = C_b / C_w \quad (1)$$

Where: C_b = average metal concentration in animals, μg kg^{-1}; C_w = metal concentration in water, μg L^{-1}.

For comparison, freshwater amphipods (*Hyalella azteca*) exposed for 5 days to a Cd concentration of 4.4 μg L^{-1} showed BCF of 31.803.[33] Mussels and oysters (bivalves) in natural environments with Pb concentrations of 0.64 μg L^{-1} and Cd concentrations of 0.08 μg L^{-1} have shown bioaccumulation factors (when a chemical substance is absorbed in organism by all routes of exposure as occurs in the natural environment) of 220 e 216 for Pb and Cd, respectively. Crabs in these conditions shown bioaccumulation factors of 145 for Pb and 525 for Cd.[34] Bioconcentration factors obtained in the present study were lower in comparison to those obtained for crabs naturally exposed to Cd and Pb, possibly due to conditions of higher concentrations in exposures.

Cadmium-binding biomolecules concentrations in tissues

The total Cd, inorganic cadmium (Cd(II)) and organic Cd (possibly Cd-metallothioneins) concentrations obtained from gills and hepatopancreas of exposed *T. fluviatilis* are shown in Table 5. Gills and hepatopancreas were chosen for analysis once they are probably responsible for absorption and storage of Cd, respectively. As only Cd was detected in all tissues and it is expected low interaction between Pb and metallothioneins, the fractionation study focused only the first element. The sum of Cd(II) and Cd-P concentrations for each animal represents values near 100% of total cadmium, determined directly on the extracts, which indicates that all Cd species existing on extracts were determined.

With the exception of animal L, it can be observed that total cadmium (CdT) concentrations in hepatopancreas

Table 5. Total cadmium (CdT), inorganic cadmium (Cd(II)) and organic cadmium (Cd-P) concentrations (wet mass, mg kg^{-1}) in cytosols of hepatopancreas and gills of *T. fluviatilis* exposed to 200 μg L^{-1} of cadmium. The limit of detection (3 σ, wet mass) found with the procedure described in bioassay 2 (using at least 0.09 g of sample) was 4 mg kg^{-1}

Animal	Exp. time (days)	Sex	CdT				Cd(II)				Cd-P			
			Hepatopancreas		Gills		Hepatopancreas		Gills		Hepatopancreas		Gills	
			Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
A	9 [a]	M	18.77	0.56	< 4	-	< 4	-	< 4	0.47	11.28	0.33	< 4	-
B	13 [a]	M	30.44	0.72	< 4	-	4.20	0.83	< 4	2.22	25.24	0.43	< 4	-
C	14 [a]	F	25.50	2.79	6.93	0.01	25.55	4.06	5.40	0.58	9.03	1.04	< 4	-
D	14 [a]	M	14.74	0.26	-	-	< 4	-	-	-	12.14	0.48	-	-
E	14 [a]	M	12.51	0.21	7.59	0.30	< 4	-	7.88	0.22	11.75	0.48	< 4	-
F	15 [a]	M	21.70	1.01	< 4	-	22.30	2.00	< 4	0.79	4.74	0.58	< 4	-
G	20 [a]	M	13.00	0.19	< 4	-	4.60	0.68	< 4	3.28	8.45	1.11	< 4	-
H	21	M	36.33	0.17	8.09	0.62	21.15	0.65	7.15	0.51	12.64	5.70	4.61	1.01
I	21	M	29.42	0.45	6.92	1.13	8.34	0.28	4.03	0.42	20.90	0.76	< 4	-
J	21	M	53.28	0.29	6.59	0.13	20.45	1.39	8.91	1.26	34.48	1.73	< 4	-
K	21	F	15.09	0.63	11.50	0.12	< 4	-	12.07	0.74	11.95	1.27	< 4	-
L	21	F	9.02	0.03	13.82	0.22	< 4	-	12.36	0.55	8.15	0.69	< 4	-

[a]Animals which died before the end of exposure. SD: standard deviation.

are always higher than the ones obtained in gills, indicating an storage function for this tissue, as seen in previous results.

Higher levels of cadmium in hepatopancreas show that the biggest ratio of the metal is stored in the organ.[11,14,16,29] In most crustaceans, hepatopancreas has central function to metabolize, to store and to detoxificate many metals. Elevated concentrations in this organ (when compared to other tissues) probably are associated to its function and to its high amounts of metallothioneins.[14,16,28]

Friedman statistic test results showed no significant differences of Cd(II) and Cd-P concentrations in hepatopancreas ($p = 0.1317$), although it seems to be a trend towards higher values of the organic form (bound to proteins, possibly metallothioneins). On the other hand, in gills, there were found significant differences between the inorganic and organic forms (Friedman, $p = 0.0348$) evidencing that Cd in this tissue was found mainly as Cd(II), the "free" form.

Thus, the obtained results (Cd(II) and Cd-P) and CdT concentrations obtained for both tissues shows that cadmium is possibly absorbed through gills and rapidly transferred to hepatopancreas (at least, during the maximum period of exposure), where it is stored.

Conclusions

After exposure, Pb was found in detectable concentrations only in gills. It was not observed significant increase in Pb concentrations in gills towards periods of exposure (7, 14 and 21 days). Cadmium was found in all tissues after exposures (7-21 days). Considerable increases in cadmium concentration in gills and hepatopancreas indicate that the crab *T. fluviatilis* may be a potential bioindicator species. In addition, results of Cd-P determination in tissues suggest that this metal possibly is absorbed through gills, followed by its transferring and storage in hepatopancreas.

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