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Bioaccumulation of Chromium in Red Swamp Crayfish (*Procambarus clarkii*)

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Abstract

Crayfish were exposed to a range of potassium dichromate concentrations (0.15, 0.30, 3.0 and 30 $mg\,l^{-1}$) for periods up to 7 weeks. Chromium bioaccumulation in all tissues over the 7 week exposure period was not consistently time- and dose-dependent. The order of distribution of chromium into the various tissues was dependent upon the exposure concentration of the metal. Chromium clearance studies conducted 1 and 3 weeks following exposure demonstrated a concentration reduction in most tissues only at the highest exposure concentration of chromium (30 $mg\,l^{-1}$). Histological studies demonstrated damage to both the gills and hepatopancreas at the lowest exposure concentration. The results suggest that the red swamp crayfish, *Procambarus clarkii*, is a useful biomarker for chromium exposure. © 1997 Elsevier Science B.V.

Keywords: Bioaccumulation; Chromium; Red Swamp Crayfish (*Procambarus clarkii*)

1. Introduction

Chromium is an essential trace element in its trivalent oxidation state but is a toxic and carcinogenic metal in its hexavalent form [1,2]. Chromium's low abundance in

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natural ecosystems has precluded its prominence as a biological hazard until recent industrial activities, such as dying, tanning, metallurgy and metal plating, introduced the metal into localized environments [3]. Chromium from such sites has been found in concentrations in excess of 50 $g\,kg^{-1}$ in soils and as much as 14 $g\,l^{-1}$ in groundwater [4,5]. Hexavalent chromium (chromate) has been found to cause lesions in DNA of mammalian cells, DNA–protein complexes and DNA–protein crosslinks [6,7]. This genotoxicity is most likely the result of the formation of hydroxyl radicals following the intracellular reduction of chromium VI [Cr(VI)] to chromium III [Cr(III)] [8]. The biochemistry of Cr(III) has also been implicated in some of the metal's toxic effects [9].

Much of the environmental concern involving chromium is based upon its biological fate after its release into the environment. The mobile chromate anion can be adsorbed onto soils or leached into groundwater and eventually is taken up by plants or animals [10]. Some recent work has focused on the effect of environmental toxins on crayfish species [11–14], and it has been proposed that the freshwater crayfish (*Procambarus clarkii*) could be used as a biomarker for heavy metal contamination [15]. The crayfish is also currently being commercially farmed for human consumption. It is in these interests that the characterization of chromium accumulation and clearance in this species has been undertaken and presented in this report.

2. Materials and methods

2.1. Reagents and supplies

Certified atomic absorption chromium standards (EM Science, Gibbstown, NJ) were used in specimen analyses, with an independent control certified standard (Aldrich Chemical Co., Milwaukee, WI) used for validation of the standard curve. Crystalline potassium dichromate (Fisher Scientific, Fair Lawn, NJ) was used to prepare the chromium spiking solution for the crayfish exposure. Specimens for routine metal analyses were digested in instrumental grade nitric acid (EM Science, Tracepur Plus). Diphenylcarbazide, used in analysis of chromium in crayfish exposure water, was obtained from Aldrich Chemical Co. Reagents used in the preparation of the matrix modifier for chromium analysis (cuprous nitrate and magnesium nitrate) were obtained from Perkin–Elmer.

Reagents for the hemolymph anticoagulant solution were obtained from the following sources: sodium chloride (S-671) and citric acid (A940) were from Fisher Scientific (Pittsburgh, PA); glucose (G5250), trisodium citrate dihydrate and EDTA (E5134) were from Sigma Chemical Co. (St. Louis, MO).

Buffered Formalin (1:10) was obtained from Curtin Matheson Scientific (Houston, TX). Laboratory Grade (Type II) water was obtained using a Life Scientific, Inc. (St. Louis, MO) water purification system.

2.2. Test crayfish

Red swamp crayfish (*Procambarus clarkii*) were purchased from a local vendor and separated as to size and gender. Female crayfish with carapaces measuring 20 to 48 mm

(10 to 35 g) in length were used in this study. Twenty to 30 crayfish were maintained together (by treatment group) in plastic aquaria containing 4 l of constantly aerated tap water. All crayfish were housed under controlled conditions of temperature (22 °C) and light (12 h light/dark) [16]. Aquaria water was changed 4 times/week, with spiking and mixing of the exposure water occurring before the animals were returned. Crayfish were fed commercial crayfish food (People's Moss Gin Co., Milwaukee, WI) three times a week, at least an hour before the water was changed. All crayfish were housed under the above conditions for two weeks before being included in the chromium-exposure study. Molting animals were eliminated during exposure and clearance.

2.3. Water monitoring

Aquarium water was tested 3 to 4 times a week, before feeding and after water changes, to monitor the following parameters: Dissolved oxygen concentration (as mg O_2 l^{-1}), pH, water hardness (as mg Ca^{2+} l^{-1}), and total chromium concentration (as mg Cr l^{-1}). Oxygen concentration was measured using a membrane-type dissolved-O_2 meter (Model 55, YSI Inc., Yellow Springs, OH); pH was measured with an Orion (Boston, MA) model 420A pH meter. Both instruments were calibrated daily according to the manufacturer's recommendations. Water hardness was measured using a LaMotte, Inc.

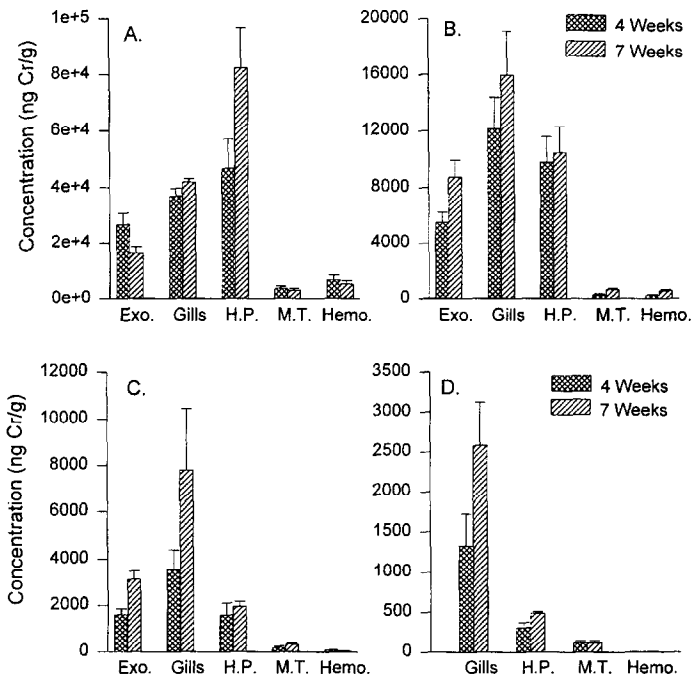

Fig. 1. Comparison of crayfish tissue concentrations of chromium at 4 and 7 weeks: (A) 30 mg l^{-1} exposure; (B) 3.0 mg l^{-1} exposure; (C) 0.3 mg l^{-1} exposure; (D) 0.15 mg l^{-1} exposure. (Abbreviations: Exo. = exoskeleton, H.P. = hepatopancreas, M.T. = muscle tissue, Hemo. = hemolymph).

(Chestertown, MD) model AB-H water hardness test kit, following the manufacturer's protocol. Because of the large number of samples involved, the routine testing of chromium concentration in aquarium water was accomplished spectrophotometrically using the more rapid diphenylcarbazide technique [17], rather than by atomic absorption.

2.4. Chromium exposure study

A chromium stock solution was prepared by dissolving 3.6 g $K_2Cr_2O_7$ in 1.0 l deionized water (12.2 mM, 1.3 $g l^{-1}$ Cr). Crayfish were exposed to four different concentrations of chromium (0.15 $mg l^{-1}$, 0.30 $mg l^{-1}$, 3.0 $mg l^{-1}$ and 30 $mg l^{-1}$) in a total of three experiments, detailed below, each with a respective control group which received no chromium exposure. The results of these three experiments were then combined. All of the chromium solutions were prepared by dilution of the stock solution

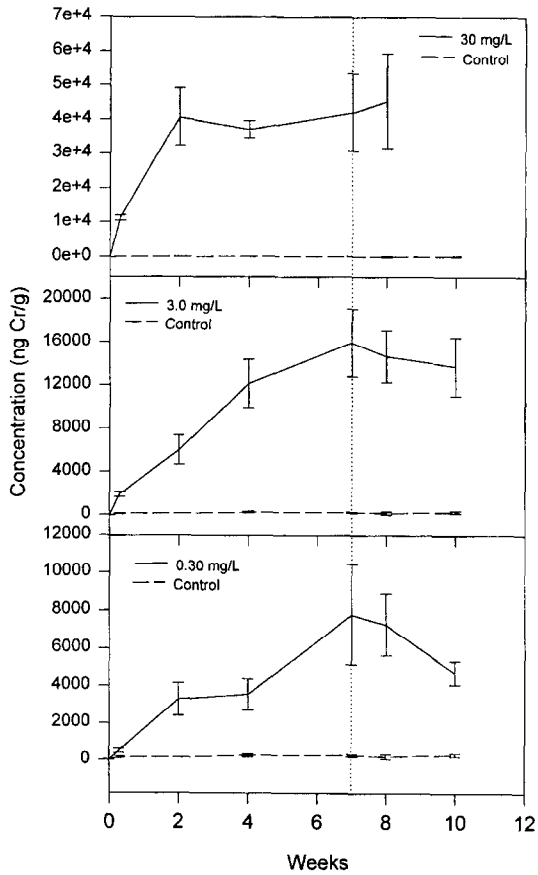

Fig. 2. Chromium accumulation versus time in crayfish gills. Partition at seven weeks represents the onset of the clearance experiment.

in tap water, and the controls were maintained in tap water. Exposure time was between 0 and 7 weeks.

At the end of each exposure period, the following protocol was followed. Crayfish were weighed and hemolymph was collected using a 1 cc syringe coated with hemolymph anticoagulant (0.14 M sodium chloride, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, and 10 mM EDTA adjusted to pH of 4.6). The volume of hemolymph was recorded (approximately 1.0 ml) and was then added to a plastic tube containing 0.33 ml of the anticoagulant solution. Crayfish were then sacrificed and the following tissues were collected and weighed for total chromium analysis and/or histopathology evaluation: hepatopancreas, gills, abdominal muscle and exoskeleton (carapace).

The preliminary experiment involved a single exposure group of 0.15 $mg\,l^{-1}$ and control. Sampling ($n = 5$) from this experiment occurred at time intervals of 4 and 7 weeks. Tissues at both time points were fixed for later histopathological studies. The collection of exoskeleton samples was not included in this experiment.

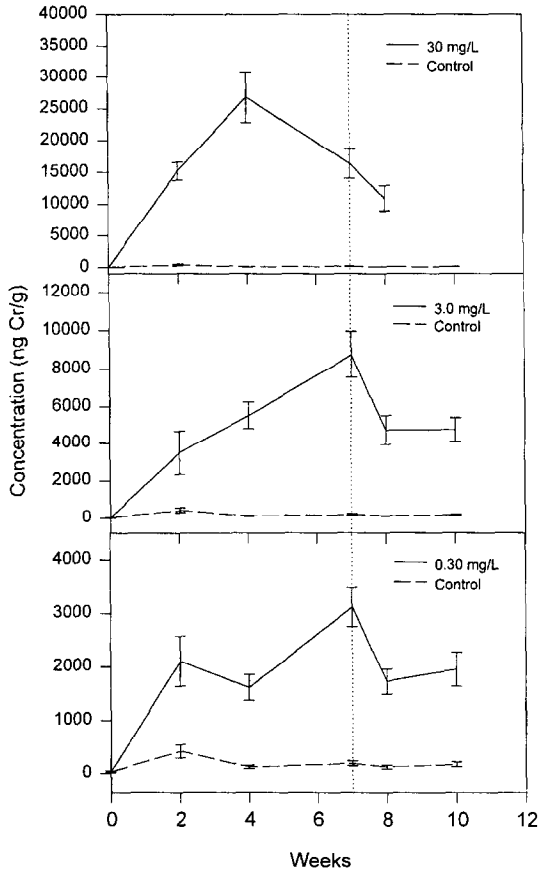

Fig. 3. Chromium accumulation versus time in crayfish exoskeleton (carapace). Partition at seven weeks represents the onset of the clearance experiment.

Two follow-up experiments increased the exposure concentrations to the higher levels listed above. Sampling ($n = 5$) from the first of these experiments occurred at $t = 0$, 2 days, 2 weeks, 4 weeks, and 7 weeks. After the final exposure time, crayfish were placed in clean tap water for three weeks in a clearance study; crayfish were evaluated at 1 and 3 weeks during this period. In a repeat experiment, samples were taken at 0, 4, and 7 weeks, giving the combined results a sampling of $n = 10$ for these respective time points.

2.5. Chromium A.A. analyses

Tissue and hemolymph samples (approximately 0.5 g) were digested in 10 ml of 17% trace metal grade nitric acid using a CEM MDS-2000 Microwave Digestion System. Chromium analyses were performed on a Perkin–Elmer model 5200 ZL atomic absorp-

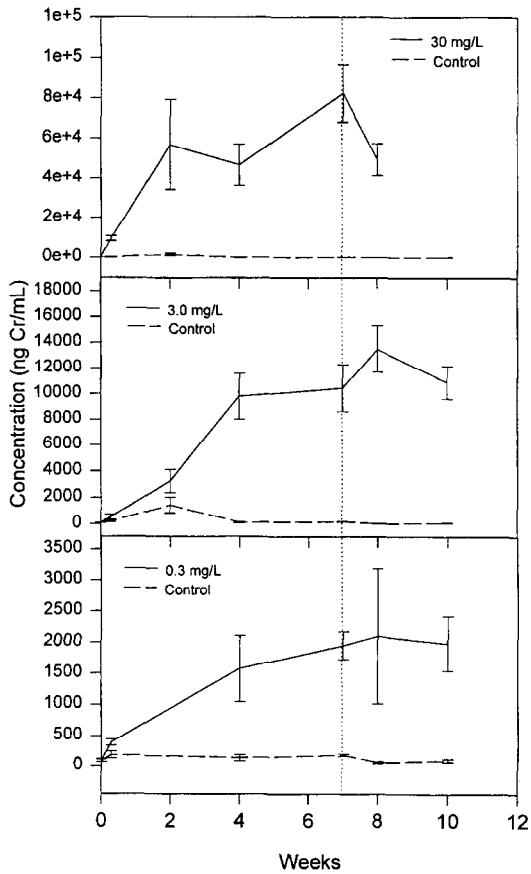

Fig. 4. Chromium accumulation versus time in crayfish hepatopancreas. Partition at seven weeks represents the onset of the clearance experiment.

tion spectrometer equipped with Zeeman background correction and a graphite furnace with autosampler. High purity argon was used. The composition of the matrix modifier solution was a 1:1 mixture of $Cu(NO_3)_2$ (5 $g\,l^{-1}$) and $Mg(NO_3)_2$ (5 $g\,l^{-1}$). The analyses of tissues spiked with chromate showed near 100% recovery. Chromium content of the tissues was calculated in terms of nanograms Cr per gram of tissue (or milliliter of hemolymph), with a sensitivity of ca. 10 $ng\,g^{-1}$.

2.6. Histopathological studies

All tissues were fixed in 10% buffered formalin. Tissues were processed using an MVP I Tissue Processor and embedded in paraffin. Tissue sections of 5 to 7 μm were prepared using a Microm HM 335 E Microtome and affixed to clean glass slides. Mounted sections were stained with hematoxylin and eosin and viewed with a Nikon light microscope.

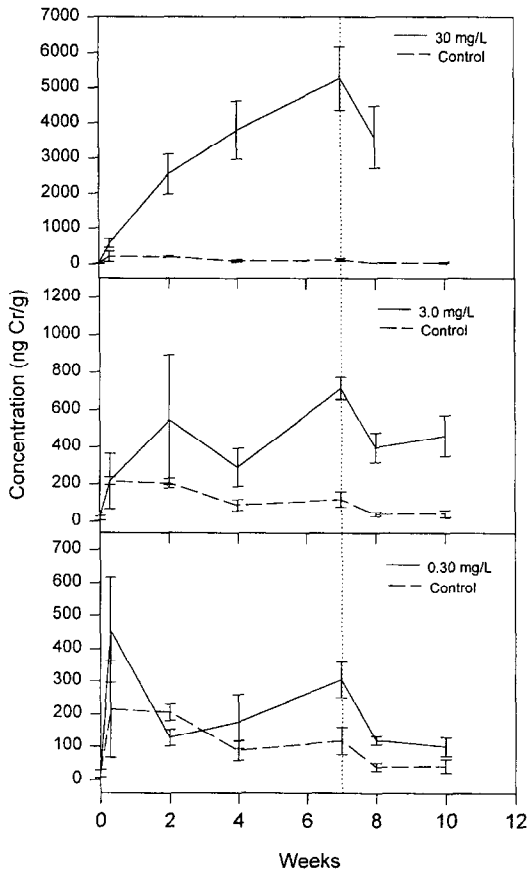

Fig. 5. Accumulation versus time in crayfish abdominal muscle. Partition at seven weeks represents the onset of the clearance experiment.

2.7. Statistical analysis

Data were analyzed statistically using a two tailed non-paired t-test for single group analyses. Analysis of variance (ANOVA) test was utilized for multi-group data followed by Scheffe's test for post-hoc comparisons.

2.8. Results

2.8.1. Water parameters

Water parameters (pH, $[O_2]$, [Cr], and hardness) were monitored immediately after the water change, and again before the water was changed two days later. The results of these tests showed no significant differences among the exposure groups, or between the exposure groups and the control aquaria (except in total chromium concentration).

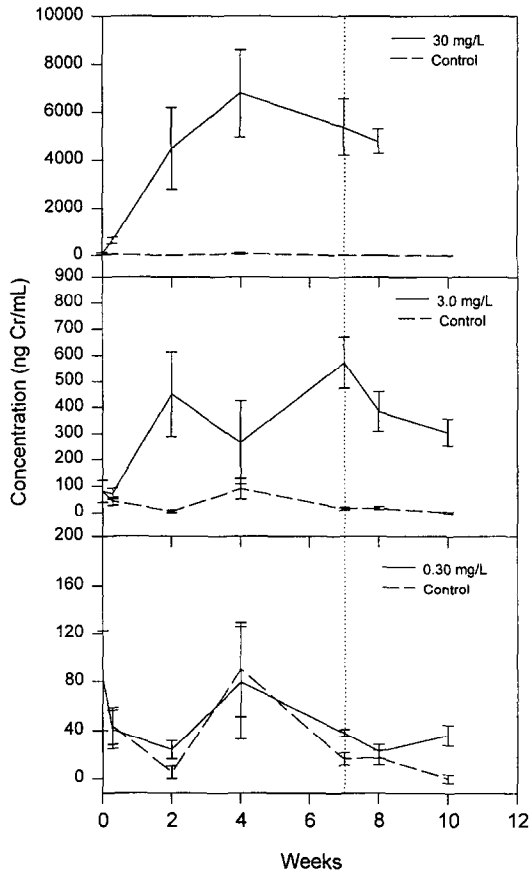

Fig. 6. Chromium accumulation versus time in crayfish hemolymph. Partition at seven weeks represents the onset of the clearance experiment.

Ranges of these parameters over two days in aquaria water were as follows: pH, between 6.5 and 8.5; $[O_2]$, between 5 and 8 $mg\,l^{-1}$; hardness, between 135 and 180 mg $Ca^{2+}\,l^{-1}$; [Cr], within $\pm 10\%$ of intended value. Generally, values for hardness and chromium concentration were observed to rise during the 2 days between water changes. An experiment conducted on identical aquaria, but without animals, demonstrated that this observed rise can be attributed to evaporative effects. These observations also rule out any interference or adsorption by the plastic aquaria.

2.9. *Chromium distribution and accumulation in crayfish tissues*

The results of the exposure experiments have been combined and presented graphically. Accumulation results from the first experiment (0.15 $mg\,l^{-1}$ exposure) are pre-

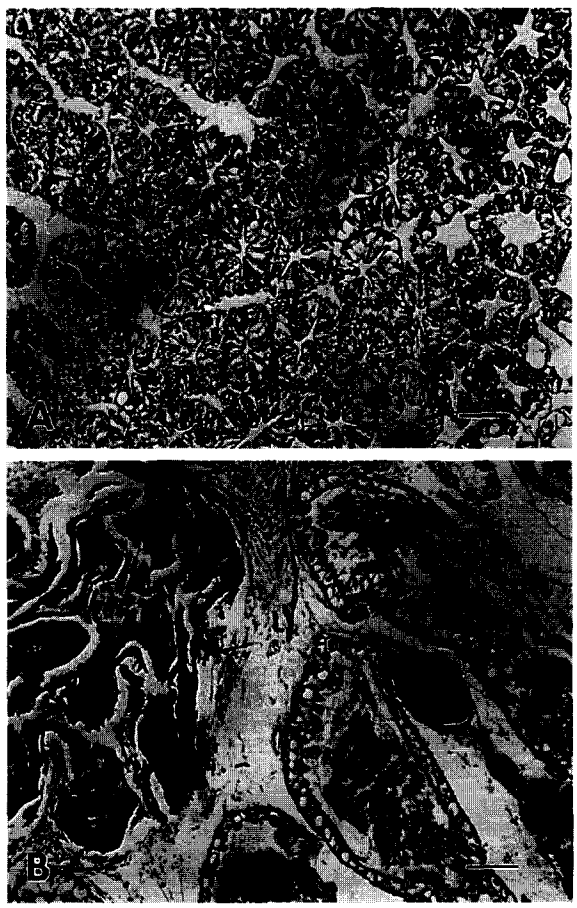

Fig. 7. Haematoxylin and eosin stained section of a crayfish hepatopancreas. (A) Section of control hepatopancreas (B) Section of hepatopancreas from a crayfish exposed to 150 $\mu g\,l^{-1}$ for 7 weeks. Bar = 30 μm.

sented in Fig. 1D; the results of the next two experiments are presented in Figs. 1–6. The onset of the clearance portion of the experiment is delineated on the plots at the 7 week time period. Since there was a greater attrition rate in the highest exposure group, the clearance experiment for this group was ended after one week due to a lack of a sufficient number of crayfish.

Chromium concentrations among the tissues evaluated is seen to be the most concentrated in the exoskeleton and gills, except in the highest (30 $mg\,l^{-1}$, 3.0 $mg\,l^{-1}$) exposure groups. As can be seen from Fig. 1, the distribution of chromium concentration varies among the tissues, and at the highest exposure dosage, the greatest concentration of chromium was found in the hepatopancreas. This trend is observed in both the 4 and 7 week samplings, but appears more pronounced at the 7 week time period. Chromium

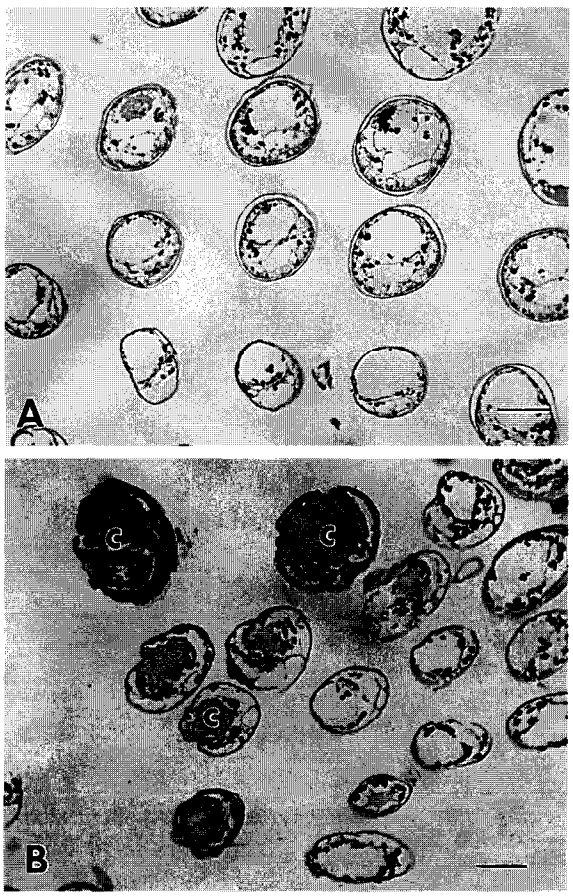

Fig. 8. Haematoxylin and eosin stained section of crayfish gill tissue. (A) Section of control gill (B) Section of gill from a crayfish exposed to 150 $\mu g\,l^{-1}$ for 7 weeks. Note formation of capsules (C) in the gill tubules. Bar = 22 μm.

concentrations in the hemolymph and muscle are consistently low compared to the other tissues sampled. Hemolymph concentrations reflect circulating levels of chromium.

Patterns of chromium accumulation and clearance vary among the crayfish tissues. Accumulation in the gills (Fig. 2), generally occurs in a steady, time-dependent manner. However, at the highest exposure concentration (30 $mg\,l^{-1}$), chromium accumulation appears to cease after approximately 2 weeks, observed as a plateau in the concentration versus time curve at this dosage. Clearance of chromium from the gills is slow in all exposure groups, with insignificant change in concentration over the three week clearance period.

Chromium uptake by the exoskeleton (Fig. 3) shows a similar time-dependent accumulation at low doses to that seen in the gills. The highest exposure group (30 $mg\,l^{-1}$) displays a peak after 4 weeks, with the concentration declining significantly at the 7 week sampling. Clearance of chromium from the exoskeleton appears rapid at first, but appears to level-off (in the lower exposure groups) at a concentration approximately equal to that observed at the 4 week exposure level.

Chromium accumulation in the hepatopancreas (Fig. 4) is suggestive of time and dose-dependence. Concentrations appear to be nearly in direct proportion to the exposure levels, but time-dependence is difficult to characterize due to the high variability among animals. Patterns in clearance are also not particularly distinct because of this variability, but concentrations appear to persist in all but the 30 $mg\,l^{-1}$ exposure group.

Abdominal muscle (Fig. 5) does not demonstrate any significant bioaccumulation of chromium, since muscle concentrations are not significantly different from circulating levels measured in hemolymph (Fig. 6). Hemolymph concentration increases with increasing exposure concentration, which is reflected in the tissue concentration of chromium measured in muscle. There is only a slight tapering of chromium amounts observed in hemolymph in the clearance study. Muscle tissue displays a rapid clearance at 1 week, but then appears to remain stable.

2.10. Histopathology of hepatopancreas and gills

Histological study of the hepatopancreas of crayfish exposed to chromium demonstrates damage to the tubules, even at the lowest concentration tested (0.15 $mg\,l^{-1}$). As seen in Fig. 7, many of the tubules are disorganized and/or degenerating. Control specimens do not demonstrate any such degenerative changes.

Gills collected from chromium-exposed crayfish (0.15 $mg\,l^{-1}$) exhibited a disorganization of the epithelial cells lining the afferent and efferent branchial vessels of the gill filaments (Fig. 8). The presence of capsules is also evident in gills of the chromium exposed animals. Controls did not demonstrate any of these pathological changes.

3. Discussion

This study represents one of very few reports available on the bioaccumulation of chromium in crustaceans. In a report by Hernandez et al. [18], acute exposure (96 h) in

doses of up to 500 $mg\,l^{-1}$ was conducted for determination of accumulation rates in various crayfish tissues, including the antennal glands. The present study focuses instead upon the distribution among soft tissue (excluding the glands) and exoskeleton, and employs much lower chromium exposure concentrations (approximately 10-fold less). The results presented herein are not in disagreement with those reported by Hernandez, showing a time and dose dependency among soft tissues similar to those reported previously. However, the longer term exposure studies presented here have determined that the tissues which are in direct contact with the contaminated medium (i.e. gills and exoskeleton) show that a maximum equilibrium tissue concentration is reached (Figs. 2 and 3). This condition appears as a peak in the exoskeleton at the highest dosage (30 $mg\,l^{-1}$) at 4 weeks. The concentration in gills reaches a plateau in 2 to 4 weeks in both the highest and middle dosages, an observation which is reflected in the hemolymph (Fig. 6). Comparison of different concentration exposures shows that once the gills and exoskeleton reach saturation, the other tissues (especially the hepatopancreas, refer to Fig. 1A) increasingly share a greater burden of chromium contamination. In these tissues, where accumulation appears to occur more slowly, chromium accumulation is time-dependent throughout the exposure.

These results also demonstrate that chromium is cleared rather slowly from most of the tissues examined in this study. These data are characterized by either a flat clearance curve at the end of the exposure period or a quick drop in concentration followed by a flattening-out effect. Histological examination of the tissues sharing the greatest chromium concentrations and with slow clearance (gills and hepatopancreas) demonstrate that chromium exposure is accompanied by considerable morphological changes and tissue damage.

The observation that chromium uptake occurs readily but is only slowly removed implies that some change in the contaminant's chemical state may have occurred. It has been proposed that upon penetrating mammalian cells, chromate is reduced to Cr(III) by intracellular proteins. The trivalent form of chromium traverses cellular membranes poorly and binds to nonspecific proteins [19]. These complexes are slow to exchange, and accumulation of such bound species could result in a chromium depot in those tissues which have, consequently, undergone the most oxidative damage. Such a mechanism could explain the clearance and histopathological results obtained in this study. Further studies which are intended to elucidate the oxidative state of chromium after the metal has entered the tissues are currently underway. Regardless of the mechanism, high accumulation, coupled with slow clearance of chromium, suggests that the red swamp crayfish (*Procambarus clarkii*) is an excellent biomarker for chromium contamination.

Acknowledgements

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