Bull. Environ. Contam. Toxicol. (1992) 48:237–242

Environmental Contamination and Toxicology

External Biomarkers to Assess Chromium Toxicity in Adult *Lepomis macrochirus*

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Chromium is widely used in the production of stainless steel, bricks, pigments, dyes, and in the tanning, textile, and chemical industries (Bodek et al. 1988), commonly found in the aquatic environment (Moore and Ramamoorthy 1984), and known to elicit acute and chronic toxicity to aquatic life (U.S. EPA 1984). In water, chromium tends to speciate into Cr^{+3} (trivalent) and Cr^{+6} (hexavalent). Speciation of chromium is primarily dependent upon water chemistry, e.g., oxygenation, pH, organic content and amount of particulate matter (Moore and Ramamoorthy, 1984; Schmidt 1984) and the bioavailability of each chromium form is different mainly because of the low solubility of Cr^{+3} (Gendusa et al. 1991).

The objective of this research was to evaluate the efficacy of eight potential biomarkers to indicate stress of chromium exposure in adult bluegill (*Lepomis macrochirus*).

MATERIALS AND METHODS

This objective was assessed in a series of 96 - hr exposures, in which adult (14.5 to 18 cm total length) bluegill (*Lepomis macrochirus*) were observed for sublethal effects of chromium toxicity. Fish obtained from Texoma Fish Hatchery, Denton, Texas, were held in the laboratory for approximately 60 days prior to toxicity tests. Although bluegills were fed during holding, fish were not fed during the 96 - hr exposures.

City of Denton tap water, dechlorinated to levels of 2 μg chlorine/L or less by activated charcoal filtration and vigorous aeration, was used for holding and all toxicity trials. Temperature, pH, alkalinity, hardness, and dissolved oxygen were measured initially and at the end of the 96 - hr tests.

Trials occurred in 40 L aquaria, each containing three bluegills. Each concentration was replicated three times, i.e., a total of nine bluegills were exposed to each chromium concentration. Fish were held at 23 to 26° C and a photoperiod of LD 16:8. Oil - free pumps maintained dissolved oxygen above 72 percent of saturation.

All Cr^{+6} and Cr^{+3} stock solutions were made in Mili - Q water from potassium dichromate ($K_2Cr_2O_7$) and chromic chloride ($CrCl_3 \cdot 6H_2O$), respectively, and were added to dilution water in varying concentrations as 1000 mg/L standard and manually stirred with a glass rod.

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Since Cr^{+3} solutions had low pHs, acid tolerance tests were conducted. Results indicated that Cr^{+3} required buffering with $NaHCO_3$/NaOH to minimize the effects of low pH from compromising the toxicity of Cr^{+3}. Buffering produced an obvious precipitate which was allowed to settle for 24 hours prior to the introduction of test fish. Fish were added either 1 hr (Cr^{+6}) or 24 hr (Cr^{+3}) after preparation of the test solutions.

Water samples (2 ml) for chromium determinations were withdrawn prior to the addition of test fish and again at the end of each trial, acidified with concentrated nitric acid to a pH of about 1.5 and stored under refrigeration for later analysis by atomic absorption with graphite furnace. The detection limit for total chromium in water was 1 μg/L.

Eight potential biomarkers, including changes in appearance, physiology and behavior, identified from initial range - finding tests, were retained in definitive tests. Test fish were observed within one hr of introduction and at each 12 hrs throughout the 96 - hr trials. In addition, fish were observed for mortality at 4 - hr intervals.

RESULTS AND DISCUSSION

All 45 bluegills survived the static 96 - hr pH trials nominal pH levels of 5, 6 and 8 associated with our test chromium exposure solutions.

Acute toxic effects were not observed in bluegills during 96 - hr tests with buffered Cr^{+3}. All control and test fish survived, and no sublethal effects were noted.

Although nominal Cr^{+3} concentrations ranged from 0 to 200 mg/L, measured concentrations ranged from <0.001 to 5.03 mg/L. These trivalent chromium recoveries were similar to those reported by Gendusa et al. (1991). Based on these results, Cr^{+3} was considered inappropriate for use in sublethal toxicity testing with bluegills and Cr^{+3} experiments were terminated.

In contrast to Cr^{+3}, measured values of Cr^{+6} agreed well with nominal values; recovery efficiencies from test waters ranged from 96 to 113 percent of nominal. Aqueous exposures of Cr^{+6} ranged from 0 to 250 mg/L (nominal) and from less than 0.001 to 281.3 mg/L (measured) and were constant during the 96 - hr exposures. Three replicates of three fish each were exposed to six concentrations: control, 30, 60, 120, 180, and 250 mg/L.

Adverse changes were observed in all eight biomarkers and these were highly positively correlated to Cr^{+6} concentration (Spearman rank correlation, p ranged from 0.0001 to 0.0009, see Figure 1, panels A through H).

Dark coloration, an indicator of general stress, was the most commonly observed biomarker in this study (Panel A). Fully 63.4 % of the test bluegills were darkly colored. Similarly, in the controls, dark coloration was the most frequently observed (median = 33 %) of all eight biomarkers. Exposure to Cr^{+6} increased the percentages of darkly colored bluegills. Ninety - six hr medians ranged from 56 to 89 % among the five chromium exposed groups. Changes in color appear to be too sensitive for use as a toxicity biomarker. A sudden change in lighting or mild vibrations (such as light tapping on the test aquaria) produced rapid color changes in test fish. Color changes at higher concentrations of chromium appeared to last longer (at times irreversible) than the transient color changes noted at lower concentrations or during the initial exposure time periods.

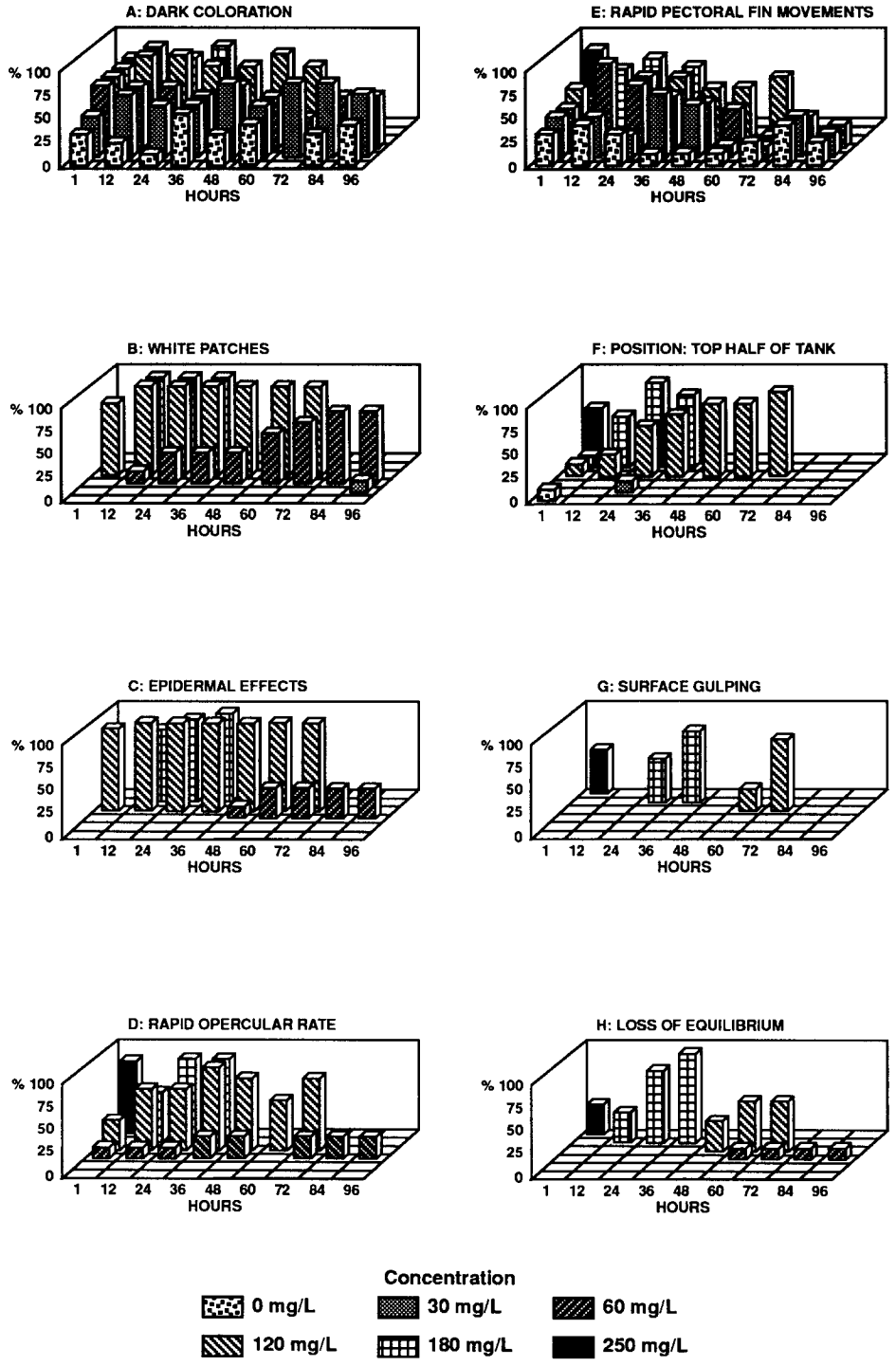

Figure 1, panels A through H. Percentage of bluegill (*Lepomis macrochirus*) exhibiting symptoms of stress from hexavalent chromium exposure.

Although none of the nine control bluegills and only one bluegill at 30 mg /L exhibited white patches during these trials, a majority of the bluegills at 60, 120 and 180 mg Cr^{+6} /L had white epidermal patches. This effect had a strong time component which is clearly seen in fish exposed to 60 mg / L (Panel B). At this concentration a highly significant (r = 0.98) linear relationship existed between time and percentage of bluegills with white patches. At concentrations of 120 and 180 mg /L 100 % of the fish had patches within 12 hrs of exposure. Interestingly, no patching was seen at the highest test concentration, probably because fish died before patching had time to develop.

Not surprising, epidermal effects such as loss of mucous and sloughing of scales (Panel C) closely mirrored the relationship between Cr^{+6} concentration and the appearance of white patches. In fact, the highest correlation measured (r_s = 0.930) occurred between epidermal effects and the appearance of white patches. At the conclusion of the trials 33 % of the fish exposed to 60 mg / L and 100 % of the fish exposed to 120 and 180 mg /L had epidermal effects.

Of the water quality parameters measured, only pH was affected by increasing concentrations of hexavalent chromium. It is likely that acidity corresponding to higher Cr^{+6} contributed to effects on the skin and fins of the test fish. It is unlikely, however, that acidity alone resulted in these observed changes in appearance because pH tolerance tests performed at pHs lower than those in the chromium toxicity trials revealed no observed changes in fish appearance or any other biomarker.

Rapid opercular rates (rates > 1 /sec) which are a sign of stress, appeared in neither controls nor bluegills exposed to 30 mg /L (Panel D). However, a majority (up to 100 %) of the bluegills exposed to higher concentrations of Cr^{+6} had rapid opercular rates. The median number of fish with rapid opercular rates at concentrations of 60, 120, and 180 mg /L were 22, 67 and 72.5 %, respectively. Within one hr, all bluegills at 250 mg /L were operculating rapidly. Also, in the sampling period immediately prior to 100 % death, all fish were exhibiting rapid opercular rates. The frequency of high opercular rate was highly correlated to Cr^{+6} concentration (r_s = 0.844, $p < 0.0001$).

Rapid pectoral fin movements were the second most commonly observed indicator of distress (Panel E). Fully 42.7 % of fish had rapid pectoral fin movements. The frequency of control bluegills exhibiting rapid pectoral fin movement was relatively high (median = 22 %) and constant (arithmetic range: 11 to 44 %). In general, the percentage of bluegills with rapid pectoral movements appeared to decrease over time. Also in contrast to rapid opercular rates, rapid pectoral fin movements were not observed in 100 % of the bluegills just prior to death.

Stressed fish, particularly those in respiratory distress, often move into surface waters. This response was clearly seen in bluegills in these trials. Position in the tank was strongly correlated with concentration (r_s = 0.725, $p < 0.0001$). Whereas bluegills at 0, 30, and 60 mg/L were infrequently observed in the upper half of the aquarium, concentration and time-dependent movements into the upper half of the test aquaria were seen at the three highest concentrations (Panel F). This was particularly evident at 120 mg / L where a strong linear relationship (r = 0.951, p = 0.001) exists between time and percentage of fish in the top of the tank. The time until 50 % of test fish occupied the top half was inversely related to concentration: 1 hr at 250 mg /L, 12 hrs at 180 mg/L and 24 hrs at 120 mg/L. During the sampling period prior to 100 % lethality, between 75 and 100 % of the fish were found in the

upper half of their aquarium.

Of all the biomarkers, surface gulping was the least observed (Panel G). In only 26 out of a total of 486 observations (5.3%) were fish surface gulping. Surface gulping only occurred at Cr^{+6} concentrations causing lethality ($\geq$ 120 mg/L) it was both time and concentration dependent, and appeared at high frequencies during the sampling period immediately before all fish at a particular concentration died. In these trials, surface gulping appeared to be a prelude to death.

Loss of equilibrium is a common endpoint in stress testing (see Beitinger and McCauley 1990). With one exception at 60 mg /L, loss of equilibrium occurred at higher, lethal concentrations (Panel H). Again, both time and concentration-dependent effects were observed. Like surface gulping, more than 50 % of the bluegills were in this state during the sampling period before their death.

Bluegill mortalities were directly related to both Cr^{+6} concentration and exposure time (Table 1). No mortalities occurred among controls and bluegills exposed to 30 mg Cr / L, intermediate numbers of mortalities occurred at 60, 120 and 180 mg/L and all fish died at 250 mg/L. LC50s (96-h) calculated for the three individual trials ranged from 89.5 to 104.7 mg/L. Since the 95 percent confidence intervals about the mean LC50s overlapped among replicates, survival data were pooled. A pooled 96 - hr LC50 of 99.1 mg / L was determined, accompanied by a 95 % confidence interval of 65.6 to 137.6 mg /L.

Table 1. Mortality of bluegills (*Lepomis macrochirus*) exposed to Cr^{+6} in water. Three trials per concentration, three fish per trial. Values listed in rows next to trials 1, 2 and 3 are hours to death for individual bluegills.

	Hexavalent Concentrations (mg / L)					
Trial	0	30	60	120	180	250
1	none	none	88	88 88	42 74	8 8 12
2	none	none	none	80 88	32 64 64	4 8 8
3	none	none	92	92 96	40 56 68	4 4 12
% Dead	0	0	22.2	66.6	88.8	100
X Survival Time (hr)	-	-	90	88.6	55	6.8

LC50 (96 hr) = 99.1 mg/L
95% Confidence interval = 65.6 to 137.6 mg/L

Mortality data indicated that Cr^{+6} at 30 mg/ L is a sublethal concentration, 60 mg /L is beginning to become lethal at 4 days of exposure, concentrations of 120 and 180 mg / L are above the 96 - hr LC50 and, finally, 250 mg / L is acutely lethal, all bluegills died within 12 hrs. Also, our 96 - hr LC50 of 99.1 mg Cr^{+6} /L agrees well with published acute LC50s for bluegills which range from 110.0 (Trama and Benoit, 1960) to 144.5 mg/L (U.S.EPA, 1984).

In contrast to trivalent chromium, clear time and concentration effects were seen in all selected endpoints in bluegills exposed to five hexavlent chromium

concentrations and a control. Changes in coloration and position of fish in aquaria were sensitive indicators of stress in general, but insufficiently specific for use as biomarkers of chromium toxicity. Fin movements, rate of opercular frequency and epidermal changes (e.g., lesions, scale loss, and epidermal sloughing) were sensitive biomarkers, and the intensities of these effects were augmented by increasing Cr^{+6} concentrations and exposure times in these trials. Finally, movement to the surface, surface gulping and loss of equilibrium occurred at high frequencies in bluegills at or near to lethal combinations of concentration and time.

Although we could not establish lethality thresholds for trivalent chromium, a hexavalent chromium 96 - hr LC50 of 99.1 mg/ L with 95 % confidence limits extending from 68.6 to 137.6 mg/L were determined from our exposures.

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Received July 8, 1991; accepted September 30, 1991.