Bull Environ Contam Toxicol (2009) 82:564–568
DOI 10.1007/s00128-009-9657-0

Use of Behavioral Responses of Rainbow Trout *Oncorhynchus mykiss* in Identifying Sublethal Exposure to Hexavalent Chromium

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Received: 29 April 2008 / Accepted: 12 January 2009 / Published online: 4 February 2009

Abstract Laboratory tests were conducted in a flow-through apparatus on 1-year-old rainbow trout *Oncorhynchus mykiss* to evaluate the sensitivity of a number of their behavioral responses to hexavalent chromium (Cr^{6+}). Test fish were exposed to Cr^{6+} concentrations corresponding to 0.001–1 parts of the rainbow trout 96-h LC50 (0.029–28.5 mg Cr/L, respectively) in short-term (15 min) tests. Sensitivity parameter responses could be arranged into the following sequence: latent period of detection response = locomotor activity > gill ventilation frequency > coughing rate. All the rainbow trout responses were sensitive behavioral indicators of sublethal exposure. Behavioral responses meet the criteria as rapid tools for bioassay testing and could be easily standardized using Cr^{6+} as a reference toxicant.

Keywords Fish · Toxicity · Behavioral responses · Hexavalent chromium

The presence of four heavy metals (copper, zinc, nickel, and hexavalent chromium) has been identified as indicators of poor water quality (SCORECARD 2005). Hexavalent chromium (Cr^{6+}) is the most biologically active Cr chemical species and dominant in aquatic ecosystems (Eisler 1986). Chromium is usually produced from anthropogenic sources (Irwin et al. 1997), and is recommended for use as a reference toxicant in standard toxicity tests (EPA US 2002) due to its ability to provide reproducible test results (Dorn et al. 1986).

The acute toxicity of chromium to aquatic life has been extensively investigated and reviewed (EPA US 1985; Eisler 1986; Irwin et al. 1997). Studies evaluating chromium sublethal effects in fish are still insufficient.

Behavioral responses represent an integrated response of fish species to toxicant stress (Scherer 1992; Kane et al. 2005). Changes in spontaneous locomotor activity and respiratory responses are sensitive behavioral indicators of sublethal exposure in fish (Atchison et al. 1987; Scherer 1992). Only a few studies are devoted to fish behavior effects due to Cr^{6+} exposure (see Ellgaard et al. 1978; Van der Putte et al. 1982; Anestis and Neufeld 1986; Gendusa and Beitinger 1992; Svecevičius 2007).

At present a number of fish behavioral procedures have been standardized (see ASTM 2008a, b).

Previous studies have established that rainbow trout were the most sensitive species to the acute toxic effect of Cr^{6+} among four other European freshwater fishes tested and are able to detect and avoid low sublethal concentrations of this heavy metal (Svecevičius 2006, 2007).

The objectives of the present study were to (1) investigate spontaneous locomotor activity and respiratory responses of yearling rainbow trout in response to Cr^{6+} solutions under the same controlled experimental conditions; (2) perform comparative analysis of the sensitivity of the responses studied based on the results of avoidance and acute lethality tests; and (3) evaluate the suitability of Cr^{6+} as a reference toxicant in fish behavioral toxicity tests.

Materials and Methods

Rainbow trout adults (1-year-old) were obtained from Žeimena Hatchery (Švenčionys District, Lithuania). The test fish were acclimated to laboratory conditions for

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1 week prior to testing. The fish were kept in flow-through 1,000-L holding tanks supplied with aerated deep-well water (minimum flow rate 1 L per 1 g of their wet body mass per day), under natural illumination and were fed commercial trout feed daily in the morning; the total amount was no less than 1% of their wet body mass per day. The day before and during the tests the fish were not fed. The average total length of test fish was 100 ± 10 mm and the total weight was 10 ± 2 g (mean ± SEM).

Reagent grade potassium dichromate ($K_2Cr_2O_7$) («REAKHIM» Company, Russia) was used as the toxicant. Stock solution was prepared by dissolving a necessary amount of potassium dichromate in distilled water, the final concentration being recalculated according to the amount of heavy metal ion.

Deep-well water was used as the dilution water. Average hardness of the water was approximately 284 mg/L as $CaCO_3$, alkalinity was 244 mg/L as HCO_3^-, pH was from 7.9 to 8.1, temperature was maintained at 10.5–11.5°C, and oxygen concentration was maintained at a range of 8–10 mg/L.

The tests were performed in a flow-through test apparatus consisting of 6 isolated test boxes connected into one battery (Svecevičius 2005). Each test box was of 0.5-L volume and was supplied with incoming and out coming dilution water (flow rate 25 mL/min). Circulating water flow was established in each test box by changing the amount of air supply in order to induce a slight rheotaxis in the test fish for their better orientation in one direction.

The following behavioral responses of test fish were investigated:

– Latent period of detection response (in second)
– Spontaneous locomotor activity (in grades)
– Two types of fish behavioral-respiratory responses were also registered: gill ventilation frequency and coughing rate in counts per minute, respectively.

The test was conducted as follows: fish were placed into test boxes (one individual into each) and acclimated for 24-h period. All the data were obtained by direct observation, during a 1-min period for each parameter (locomotor activity, ventilation frequency, and coughing rate) during an initial control period and then during an exposure period within 15 min of exposure after the latent period of detection response was measured (Svecevičius 2005).

The relative sensitivity of the behavioral responses was evaluated by comparing their threshold values. Threshold-effect-concentration (TEC) was estimated by defining the geometric mean between the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) (Van Leeuwen and Hermens 1995).

The amount of oxygen in the water as well as temperature and pH were measured routinely with a hand held multimeter (WTW Multi 340i/SET, Germany). Water samples taken from the test boxes were measured for total chromium with an atomic absorption spectrophotometer (SHIMADZU AA-6800, Japan) with the flame or graphite furnace techniques using proprietary software. Each sample was analyzed three times. Mean measured concentrations were within 5%–10% of target.

Overall, 12 test fish were examined at each Cr^{6+} concentration. Each of the 12 fish was tested only once. Data were analyzed using one-way ANOVA followed by Tukey's HSD post-hoc test at $p < 0.05$ through STATISTICA (Version 6.0) and GraphPad Instat™ (Version 2.04) softwares.

Results and Discussion

The data obtained showed that with an increase in Cr^{6+} concentration, the latent period of detection response rapidly decreased, while the intensity of other responses studied increased (Table 1).

Table 1 Behavioral responses of rainbow trout to the effect of hexavalent chromium (mean ± SEM, N = 12)

Hexavalent chromium concentration (parts of 96-h LC50)	Latent period of detection response (s)	Locomotor activity (grades)	Gill ventilation frequency (counts/min)	Coughing rate (counts/min)
0 (Control)	–	0.16 ± 0.04	80 ± 1.7	1.2 ± 0.3
0.001	Immeasurable	0.15 ± 0.04	86 ± 1.7	1.1 ± 0.3
0.005	75 ± 11.8	0.30 ± 0.03*	85 ± 1.6	1.2 ± 0.3
0.01	70 ± 10.1	0.35 ± 0.05*	88 ± 1.2*	1.8 ± 0.2
0.05	40 ± 7.5	0.53 ± 0.05*	93 ± 1.0*	2.2 ± 0.3
0.1	32 ± 6.1	0.60 ± 0.04*	95 ± 0.7*	2.3 ± 0.2*
0.5	20 ± 3.7	0.75 ± 0.04*	98 ± 1.2*	2.5 ± 0.3*
1	17 ± 3.1	0.77 ± 0.05*	99 ± 1.0*	2.6 ± 0.1*

* Denote values significantly different from control ($p < 0.05$)

Locomotor activity increased most rapidly, followed by coughing rate and gill ventilation frequency. At a Cr^{6+} concentration equal to the 96-h LC50, locomotor activity increased five fold, and coughing rate increased 2.2 times, while gill ventilation frequency increased only 1.3-fold as compared to control. The intensity of the locomotor activity was significant at a Cr^{6+} concentration of 0.05, gill ventilation frequency at 0.01, and coughing rate at 0.1 parts of 96-h LC50, respectively. Consequently, locomotor activity was found to be the most sensitive response to the effect of Cr^{6+} among the responses, although latent period of detection response (a specific indicator) was of the same level of sensitivity allowing determining Cr^{6+} at a concentration of 0.14 mg Cr/L.

All experimental data obtained here are similaral to the results of a previous study conducted with a heavy metal model mixture (Svecevičius 2005).

The relationship between Cr^{6+} concentration and behavioral response intensity was non-linear (Fig. 1). Regression analysis was conducted and two descriptive models were applied: logarithmic fit for locomotor activity and logistic for latent period of detection response, gill ventilation frequency, and coughing rate.

Comparison of the threshold values for the studied responses showed (Table 2) that avoidance response was the most sensitive to the effect of Cr^{6+}. It was almost 30-fold more sensitive than locomotor activity, 105-fold more sensitive than coughing rate, and 1,035-fold more sensitive than gill ventilation frequency.

The great differences in the behavioral results probably can be explained based on their toxicological reactions. Avoidance response and locomotor activity are referred to the same category of behavioral responses as described by Scherer (1992). However, avoidance response can be initiated through chemosensory irritation, since it was established that the fish olfactory system is involved in the formation of an avoidance response to heavy metals (Brown et al. 1982; Svecevičius 1991). Also Cr^{6+} acts as a strong oxidizing agent, easily penetrating biological membranes and causing cellular damage (Irwin et al. 1997), therefore Cr^{6+} can be a strong sensory irritant. Locomotor activity may reflect a more non-specific stress response resulting in changes in blood cortisol and glucose levels. Gill ventilation frequency and coughing rate are intimately associated with respiratory demands and gill irritation or blockage (Scherer et al. 1986).

The toxicity of Cr^{6+} to fish is dependent upon several physico-chemical factors of water. Toxicity increases with decreasing water pH and decreases as hardness increases (EPA US 1985). Water temperature has a limited influence (Smith and Heath 1979).

Hexavalent chromium at sublethal concentrations is known to elevate swimming and respiratory activity in fish.

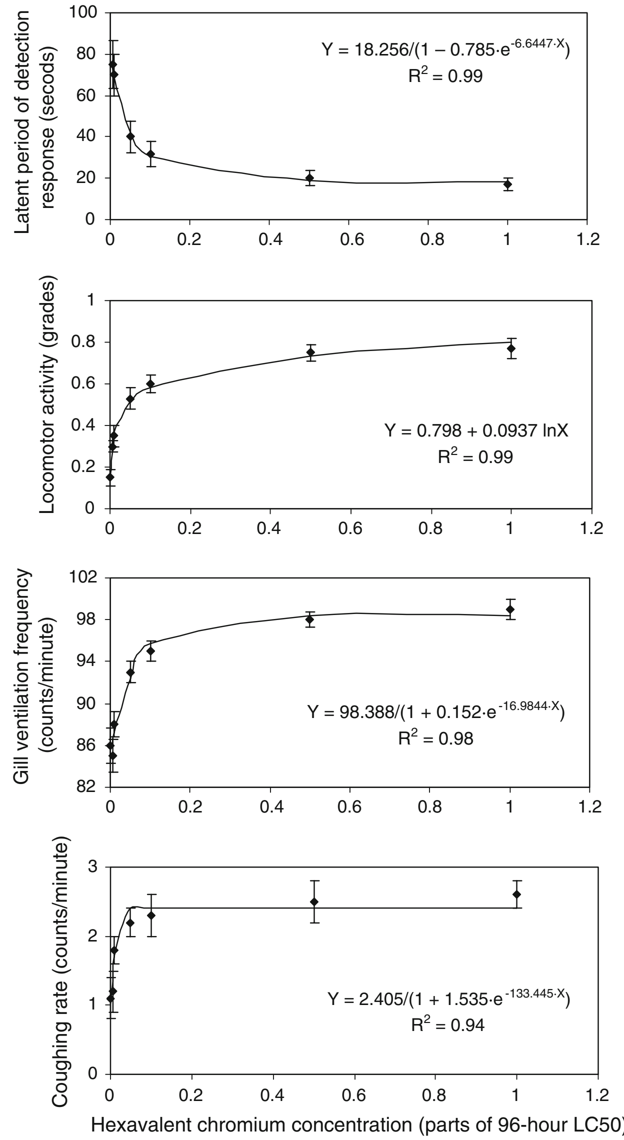

Fig. 1 Relationship between intensity of rainbow trout behavioral responses and Cr^{6+} concentration

Table 2 Comparison of rainbow trout behavioral response threshold concentrations with acutely lethal concentration of Cr^{6+}

Behavioral response	Threshold-effect-concentration (TEC) (mg Cr/L)	Part of 96-h LC50
Locomotor activity	0.059	0.002
Gill ventilation frequency	0.2	0.007
Coughing rate	2.1	0.07
Avoidance response	0.002	0.00006

TEC for avoidance response was derived from data of the previous study (Svecevičius 2007). NOEC = 0.0015 and LOEC = 0.003 of mg Cr/L, respectively

Ellgaard et al. (1978) using an observational technique have found hyperactivity in bluegill (*Lepomis macrochirus*) at sublethal Cr^{6+} concentrations of 0.05–24 mg/L (22 ± 1°C, pH = 6.5, hardness = 105 mg/L, alkalinity = 11 mg/L as $CaCO_3$).

Van der Putte et al. (1982) analyzed respiration activity in rainbow trout exposed to Cr^{6+} concentrations ranging from 1 to 50 mg/L at different pH values (6.5 and 7.8, 96-h LC50 20.2 and 65.5 mg Cr/L, respectively, total hardness = 80 mg/L as $CaCO_3$, and alkalinity = 92 mg/L as HCO_3^-). Water temperature was maintained at 13 ± 0.5°C. There was an overall increase in respiration activity with increasing exposure duration and Cr^{6+} concentration. At pH = 6.5, an increase in gill ventilation frequency and coughing rate were observed at much lower concentrations than at pH = 7.8.

Similarly, Gendusa and Beitinger (1992) analyzed a number of external biomarkers in adult bluegill exposed for 96 h to Cr^{6+} concentrations ranging from 30 to 250 mg/L (23–26°C, pH = 7.9–8.1, hardness = 88–108, and alkalinity = 88–120 as mEq/L), and found that rapid opercular rates were observed at a test concentrations greater than 30 mg Cr/L (96-h LC50 value was 99.1 mg Cr/L). Rapid pectoral fin movements were also found to be an informative biomarker.

Overall, the data obtained here are in close agreement with previous studies indicating that locomotor responses of fish are more sensitive indicators of the toxic effect of Cr^{6+} than respiratory behavior. Using data from previous research (see Svecevičius 2005), and the data obtained in this study, it is evident that all the rainbow trout responses studied are sensitive behavioral indicators of low sublethal exposure and meet the criteria as rapid bioassay tools for early warning systems as discussed by Van der Van Scalie et al. (2001). Such responses as locomotor activity and avoidance response allow detection of heavy metals at low concentrations and could be used in determining or revising water-quality guidelines for the protection of aquatic biota (Svecevičius 2005, 2007). Respiratory responses were found to be less sensitive, but also could be successfully used in bioassay testing of treated industrial and municipal effluents before they are discharged into receiving waters.

The development of behavioral methods in fish as important tools in aquatic toxicology has now reached the stage where they have been standardized. A guide covering some general information on methods for qualitative and quantitative assessment of the behavioral responses of fish (locomotory activity, feeding, and social responses) during standard laboratory toxicity tests to measure the sublethal effects of exposure to chemical substances (ASTM 2008a) and a guide covering information on methods to measure and interpret ventilatory behavioral responses of freshwater fish to pollutants (ASTM 2008b) are available.

In every standard test procedure, reference toxicants must be used because reference toxicant tests indicate the reliability of the analytical procedure used for verifying toxicant concentrations, and allow one to control and detect the variability associated with test organisms and their relative health (Dorn et al. 1986). Since Cr^{6+} at the present is one of the most widely used reference toxicants, the data obtained in this study could be useful in developing standard reference toxicant behavioral studies.

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