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Cnesterodon decemmaculatus Juveniles as Test Organisms in Toxicity Assessment: Cadmium Case

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Abstract The poeciliid *Cnesterodon decemmaculatus* is one of the native species of southern South America recently recommended for use as a test species in biomonitoring. Therefore, it is important to characterize its responses to stress conditions caused by pollution. The aim of this work was to determine the toxicity of the reference toxicant cadmium (Cd) and to evaluate the lethality response of juveniles of *C. decemmaculatus* exposed to an environmental sample with a high degree of pollution (Luján River, Buenos Aires, Argentina). The LC_{50} values at 24 and 96 h were 6.00 and 2.27 mg Cd/L, respectively. The uptake of Cd was significantly greater in the first 24 h in relation to the total time of exposure in the bioassay. The toxicity of the water was in agreement with the level of contamination. A Cd contaminant pulse exerted an important additive effect on the toxicity of the environmental sample. The results provide information regarding the sensitivity of a native species to be used as a test organism in environmental monitoring.

Keywords *Cnesterodon decemmaculatus* · Cadmium toxicity · Bioconcentration · Water toxicity assessment

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The assessment of water quality requires a great quantity and variety of analytical data, often integrated into a water quality index (WQI) (Torres et al. 2009), as well as the evaluation of ecotoxicity on the aquatic biota. Because of the trophic position of fish as top consumers in the food chain of aquatic ecosystems, they are widely recognized indicators of pollution and commonly used as test organisms (Ferrari et al. 2005). The micro-pollutants present in the aquatic environment are likely to accumulate in fish and thus represent a potential risk not only to fish themselves, but also to piscivorous birds and mammals, including humans (Bervoets et al. 2009).

Acute and short-term chronic toxicity tests have been used as tools in the evaluation and monitoring of environmental toxicity. Currently, there is a growing trend to use native organisms in toxicity assessment. We have previously carried out diverse studies of the Reconquista River by means of physicochemical assessment of the water quality and chronic and acute toxicity bioassays, using native species as test organisms under field and laboratory conditions (Ferrari et al. 2005). Some native fish species, such as *Cnesterodon decemmaculatus,* have been recommended for use in bioassays (IRAM 2008). This guideline emphasizes the importance of using reference toxicants and developing control charts. The control chart demonstrates the sensitivity of the cohort used, the stability of the biological response, and the repeatability of the results obtained. This chart is generated from the results of successive tests with a reference toxicant, and the mean effective concentration of the reference toxicant, i.e., the lethal concentration for 50 % of the test organisms (LC_{50}), is then obtained.

Cadmium (Cd) is one of the most toxic metals to aquatic biota (Mebane 2006), whether dissolved in water or deposited in sediment, and constitutes a contamination

source for the various aquatic food chains (Barhoumi et al. 2009). It is one of the substances recommended as a reference toxicant (USEPA 2001). Taking into consideration that *C. decemmaculatus* is a native species that is commonly most used in regional ecotoxicological assessments, and that it is essential to increase the knowledge of its response to different toxic chemicals, the aims of this work were: (a) to establish the degree of sensitivity of *C. decemmaculatus* to Cd in standardized conditions for use as a positive control (reference toxicant) in environmental monitoring tests, through the calculation of LC_{50} values at 24 and 96 h of exposure, (b) to determine the bioconcentration of Cd in sublethal concentrations, and (c) to evaluate the toxicity of an environmental sample with and without the addition of a contaminant pulse of Cd.

Materials and Methods

The study was approached from three perspectives: lethality, Cd uptake and response to an environmental sample. All bioassays were conducted with juveniles of *C. decemmaculatus* obtained from our laboratory culture, according to Somma et al. (2011) following the protocol of (IRAM 2008), with minor modifications. The test conditions for conducting the lethality bioassay are indicated in Table 1. Mortality was checked every 24 h. Fishes exhibiting neither heartbeat nor responsive behavior to gentle prodding were considered dead, and were removed from containers. The results were expressed in terms of accumulated mortality. All the glassware material used in the assays was prewashed with 5 % nitric acid for 24 h and then thoroughly rinsed with double distilled water several times. All the solutions were prepared using analytical grade reagents and double distilled water.

To establish the sensitivity of the organisms to Cd, four bioassays were performed. Juveniles were exposed to the following nominal concentrations of Cd: 0 (control), 0.5, 1.0, 2.0, 4.0, 6.0 and 8.0 mg Cd/L. This range of concentrations was selected based on a preliminary bioassay. The different Cd concentrations were obtained from a stock solution containing 1,000 mg Cd/L (prepared from $CdCl_2{\cdot}2.5H_2O$, J. T. Baker, Phillipsburg, NJ, USA, in double distilled water). The effective concentration of Cd in the solutions assayed was measured by atomic absorption spectroscopy (Jarrell-Ash, Waltham, MA, USA) in aliquots of the exposure solutions. The detection limit was 0.05 mg/L. This concentration of Cd in solution was defined as analytical Cd (Cd_{an}). The 24 and 96 h Cd–LC_{50} values and their intervals of acceptable variation were calculated as the mean of four acute toxicity assays (chart control). The upper and lower limits of such intervals were obtained by either adding or subtracting, respectively, two standard deviations, from the average, according to IRAM (2008).

Table 1 Test conditions for conducting 96 h lethality bioassays with juveniles of *C. decemmaculatus*

Parameter	Condition
Exposure type	Static
Dilution medium	Moderate hard water (MHW), Weber (1993). $MgSO_4{\cdot}7H_2O$, $NaHCO_3$, KCl and $CaSO_4$ salts in distilled water. Final concentrations of Mg, Ca, Na and K were 0.50, 0.35, 1.14 and 0.054 mM respectively. Prior to use, aerated and the hardness content, pH and DO measured
Dilution medium renewal	At 48 h
Acclimation period	48 h
Exposure time	96 h
Temperature	23 ± 1°C
Photoperiod conditions	16 h light/8 h darkness
Container material	Polypropylene
Loading of organisms	1 g organism/L dilution medium
Interval age of the organisms	15–21 days
Interval size of the organisms	7–14 mm long
Feeding regime	Unfed during exposure time
Number of replicates	Minimum of three per exposure
Number of individuals per replicate	10
Endpoint	Lethality (LC_{50}): 24 and 96 h
Acceptability criteria	Negative control mortality below 10 %

The LC_{50} for each assay was calculated with the PROBIT method, using the EPA Probit Analysis Program Version 1.5 (Norberg-King 1993).

To determine Cd uptake, fish were exposed to 0.5 and 1.0 mg/L (sublethal concentrations under the experimental conditions). The bioconcentration was calculated as Cd content in whole fish on the basis of dry weight (μgCd/gDW). The uptake was measured in surviving individuals pooled at 24 and 96 h of exposure. Each pool of organisms was rinsed with distilled water and dried at 60°C to determine dry weight. Then, the pools were transferred to borosilicate tubes and digested with concentrated nitric acid (about 2–3 mL per pool) at 100°C. Finally, the samples were diluted with concentrated nitric acid to 5 mL, and Cd content was determined with a Shimadzu model 6701 atomic absorption spectrometer (Kyoto, Japan) equipped with a GFA 6000 graphite furnace. Recovery of the procedures was checked using a certified reference material (Antartic krill, MURST-ISS-A2, supplied by Istituto Superiore di Sanità, Rome, Italy). Blanks were run with each batch of samples. The mean recovery percentage for Cd (five replicates) was 95.4 % ± 2.3 %. Coefficients of variation ranged from 2.4 % to 4.3 %. The detection limit was 0.0001 mg Cd/L.

The toxicity of an environmental sample was evaluated by a bioassay, following the same protocol as for the reference toxicant. A sample of surface water (20–30 cm depth) was taken from downstream of a discharge of sewage water in the Luján River (34°32′56″S, 59°06′57″W), stored in a polyethylene container and transported to the laboratory at 4°C within 5 h of sampling. The river water was refrigerated until used. The exposure phase of the bioassay began on the day that the river water sample was collected. Just prior to use in the bioassay, the water was warmed to a test temperature and aerated. The effect of 1 mg Cd/L (as $CdCl_2{\cdot}2.5H_2O$) as a contamination pulse was evaluated according to de la Torre et al. (1997). Therefore, the following treatments were tested:

(a) Moderately hard water (control; MHW),
(b) Luján River water sample without dilution (100 %),
(c) Luján River water sample diluted (50 %) with MHW,
(d) Luján River water sample diluted (50 %) with MHW + 1 mg/L Cd.

The physicochemical profile of the sample was determined by evaluating the following parameters: pH, temperature, chlorides (Cl^-) and ammonium concentrations ($N–NH_4^+$), biochemical oxygen demand (BOD_5), and dissolved oxygen (DO). The physicochemical analyses were carried out in duplicate, following standard methods APHA (2005). Water quality was also characterized by means of the application of a WQI for organic pollution. This index is determined considering temperature, DO, BOD_5 and $N–NH_4^+$ and Cl^- concentrations. A WQI value of ten is equivalent to an original pure state, while 0 corresponds to a highly polluted state, such as an untreated sewage effluent (Berón 1984).

Results and Discussion

The values for DO, pH and hardness in MHW at time zero ranged between: 8.2–9.1 mg O_2/L, 7.6–7.8 and 94.3–101.7 mg $CaCO_3$/L, respectively.

The measured concentrations of Cd [mean + SD (n = 5)] in the acute toxicity test exposure chambers with nominal concentrations of 0.5, 1, 2, 4, 6, and 8 mg Cd/L were 0.5 ± 0.1 (n = 4), 0.8 ± 0.0 (n = 10), 2.0 ± 0.1 (n = 10), 3.8 ± 0.2 (n = 9), 5.7 ± 0.6 (n = 5) and 7.4 + 0.5 (n = 7) mg Cd^{2+}/L, respectively. The Cd values measured in the solutions were 10 % lower than the nominal values, except for the 1.0 solutions mg/L, which were 20 % lower than the nominal values. Therefore, the nominal concentrations of Cd in solution were used to calculate the LC_{50} values.

Table 2 shows the values of LC_{50} (mg Cd^{2+}/L) obtained for the different times of exposure and bioassays. The results showed that the LC_{50} values obtained for the 24 h exposure were more variable between the assays, and that the confidence intervals were greater than those obtained at the final time of exposure (96 h). These results allowed for the establishment of the first control chart for Cd in juveniles of *C. decemmaculatus*. The mean LC_{50} values at 24 and 96 h exposure were 6.00 (3.10–8.90) and 2.27 (1.83–2.71) mg Cd/L, respectively (Fig. 1). These can be used as reference toxicant values for this species.

The toxic effects of Cd show great variability depending on the organisms, the dilution medium and assay conditions. This accounts for the variable EC_{50} and LC_{50} values, even within the same species and development stage (Mebane 2006). In general terms, toxicity is lower in marine organisms, while in freshwater environments it is lower in invertebrates than in vertebrates, even at early development stages (Achiorno et al. 2010). For some fish species, such as *Danio rerio, Poecilia reticulata, Gambusia affinis* and *Oncorhynchus mykiss*, the sensitivity to Cd is between 0.45 and 10.4 mg/L (USEPA 2013). Since the results obtained in this study showed values of sensitivity to Cd comparable to those reported for other teleosts, Cd may be considered as moderately toxic to *C. decemmaculatus* under our test conditions.

The concentrations at which Cd uptake was determined corresponded to values of lethal concentrations lower than the LC_1 and LC_{10} for 24 and 96 h, respectively. For both concentrations, the greater incorporation of Cd occurred in the first 24 h of exposure (Fig. 2). The body burdens of Cd

Table 2 Lethal concentrations of Cd (LC_{50} mg Cd^{2+}/L) in moderately hard water (MHW, 23°C, 16 h light/8 h dark) obtained for *C. decemmaculatus* juveniles at two exposure times in four bioassays

Exposure time (h)	Bioassay	LC_{50} (mgCd^{2+}/L)	Confidence limits	Slope	Intercept	χ^2	χ^2 (critical value)
24	I	5.21	4.54–6.04	4.82	1.54	0.44	7.82
	II	4.41	3.57–5.18	5.02	1.77	4.01	7.82
	III	6.80	5.68–8.83	5.60	0.34	0.05	3.84
	IV	7.59	6.03–11.27	3.36	2.04	4.15	9.49
96	I	2.16	1.47–2.91	3.05	3.98	1.62	5.59
	II	2.07	1.49–2.63	2.70	4.15	3.45	9.49
	III	2.58	1.47–3.43	3.64	3.50	3.43	7.82
	IV	2.28	1.63–2.82	4.02	3.56	0.55	3.84

χ^2 lower than χ^2 critical value denotes goodness of-fit ($p < 0.05$)

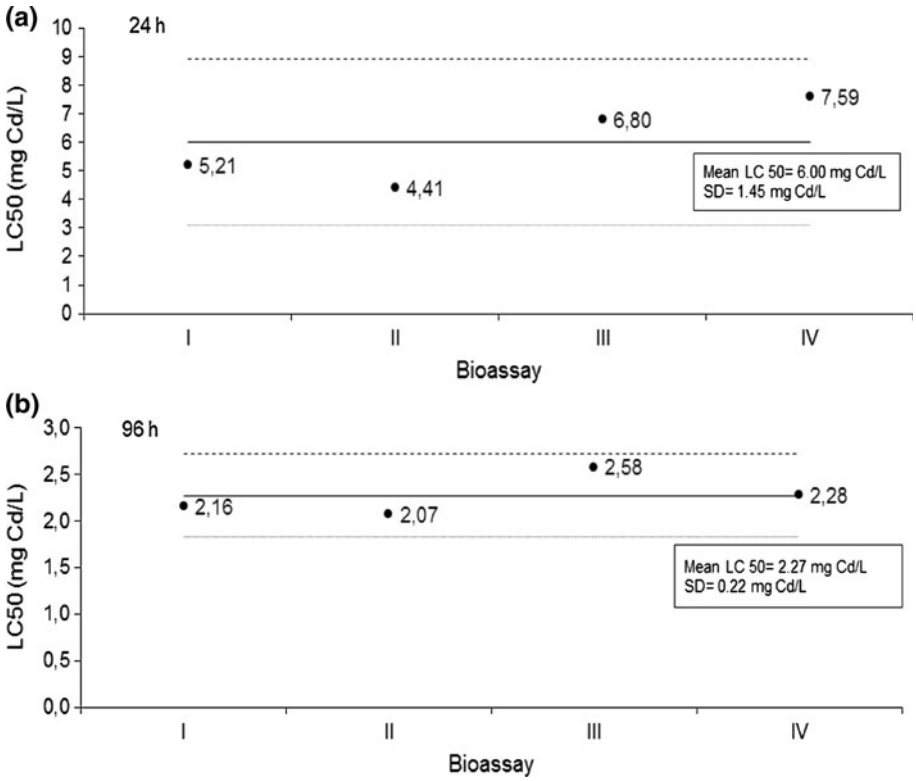

Fig. 1 Obtained cadmium control chart in MHW (23°C, 16 h light/8 h dark), for *C. decemmaculatus* juveniles at 24 h (**a**) and 96 h (**b**) of exposure *solid line* LC50 mean value *dashed line upper* and *dotted line lower* limits of the interval of acceptable variation. The mean and standard deviation (SD) are indicated in the *boxes*

were slightly greater in fish that were exposed to the higher concentration of Cd over both 24 and 96 h of exposure. To produce lethality, a chemical usually must first enter the organism, and then reach the site of action at an internally lethal concentration. This will cause the death of the organism, regardless of the external exposure conditions (Penttinen et al. 2011). In our study, the concentrations at which uptake was measured were not lethal. Therefore, it may be inferred that the measured body burdens of Cd were within the tolerance range. This is the first report of bioaccumulation of Cd in *C. decemmaculatus*, and it can be inferred that *C. decemmaculatus* has the ability to concentrate large amounts of Cd in its tissues.

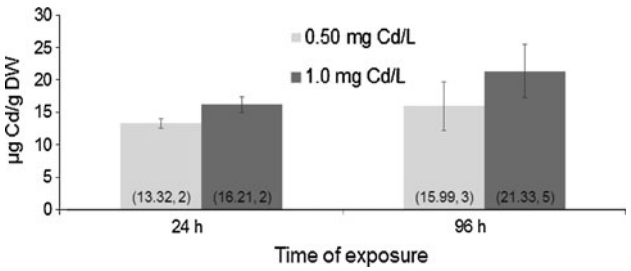

Fig. 2 Cadmium uptake determined for *C. decemmaculatus* juveniles exposed to 0.5 and 1.0 mg Cd/L (lethal concentrations lower than LC_1 and LC_{10} for 24 and 96 h respectively, MHW, 23°C, 16 h light/8 h dark). *Error bars* represent the mean standard deviation. (Mean value, n)

The physico-chemical characterization of the surface water used in the bioassay to evaluate the toxicity of an environmental sample yielded the following: 19.5°C, 142 mg Cl^-/L, 11 mg N–NH_4/L, 5.2 mg O_2/L and 170 mg O_2/L for BOD_5. These data were used for the calculation of the WQI, which was 2.4, a value corresponding to a very high level of pollution. The physico-chemical profile of the water sample from the Luján River shows an important deterioration of quality. The WQI obtained here supports that diagnosis, pointing out that the contamination of the river is a consequence of the effluent poured into the river without any previous treatment.

The cumulative mortality at 96 h in the assay using water from the Lujan River is shown in Fig. 3. At 96 h of exposure, the river water caused mortality close to 50 %, whereas the dilution to 50 % with MHW reduced mortality to 33 %. The addition of a contaminant pulse with a sublethal concentration of Cd in the diluted river water increased mortality by 50 %.

The toxicity of the river water on test organisms was in agreement with the level of contamination according to the WQI obtained, showing a good correspondence between the results of the bioassay and the environmental chemical stress conditions. The contaminant pulse had a very important additive effect. The results of the bioassay with the environmental sample suggest a high sensitivity of the species to the pollution of the environment. It is important to point out that *C. decemmaculatus* is a common species of the Luján River.

The toxicity evaluation by means of acute bioassays is an initial, rapid and inexpensive tool, which provides valuable information for toxicity assessment of environmental samples. These bioassays acquire increased ecological relevance in environmental risk evaluations if they are carried out with test organisms belonging to the native fauna. However, it must be kept in mind that there is an uncertainty factor when laboratory results are extrapolated to field conditions, because of the simultaneous influence of a number of environmental and biological factors (bioavailability, toxicokinetics, sensitivity of organisms, etc.).

The toxic effect of a Cd polluting pulse was determined by adding a known concentration as a reference toxicant in previous studies (Weber 1993; Demichelis et al. 2001; Ferrari et al. 1997, 2005; García et al. 1998, 2010). Cadmium has also been spiked into sediments as a reference toxicant in toxicity tests that evaluated sediment quality (Ciarelli et al. 1997; Giusto et al. 2008; Giusto and Ferrari 2008). Although these tests do not take into account the "real world" contamination, they are important tools in developing chemical-specific and site-specific criteria. It is known that water quality characteristics may influence the availability of a metal to aquatic organisms, depending on whether the constituent is present in solution or as an adsorbed species within the mixture (Mastrángelo et al. 2011). However, in most cases, the toxicity of the water samples from polluted sites increases after the addition of Cd (de la Torre et al. 1997; Ferrari et al. 2005).

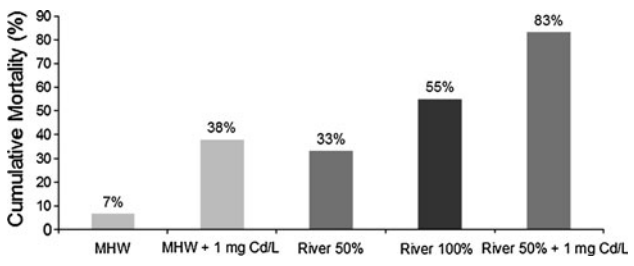

Fig. 3 Cumulative mortality of *C. decemmaculatus* juveniles at 96 h exposure in the environmental sample bioassay (23°C, 16 h light/8 h dark)

This study provides basic information about the sensitivity of a native species to a reference toxicant for use as a positive control in the biomonitoring of regional water bodies. It must be emphasized that our methodology is an appropriate tool that is applicable to aquatic toxicity assessments as part of a battery of tests in biomonitoring programs (Ferrari et al. 2005).

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