Assessment of physiological effects of sublethal cadmium on *Cyprinus carpio*

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Abstract: The effects of sublethal and short-term exposure to Cd were assessed with the purpose of studying the energy metabolism parameters in young *Cyprinus carpio* under laboratory conditions. The fish were exposed to Cd solutions (0.20 mg Cd. L^{-1}) over a two-week term. The assayed concentration was environmentally realistic since it was frequently found in most highly polluted peri-urban water bodies in Argentina. No mortality was recorded among the animals during the experiments. The relationships between assimilation efficiency (U), oxygen consumption as a measure of specific metabolic rate (SMR) and ammonium excretion (AE) were determined. After exposure to Cd, a significant correlation was found between SMR and AE. It was concluded that under the assayed conditions the metal caused alterations in energy-related homeostasis of fish. Most of the responses may be interpreted as indicative of adaptations to compensate for increased energy requirements due to the physiological impairments caused by Cd.

Keywords: *Cyprinus carpio*; cadmium; assimilation efficiency; oxygen consumption; SMR; specific metabolic rate; ammonium excretion; surface response.

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1 Introduction

Fish are good indicators of water pollution. Several of their biochemical responses are used as biomarkers of stress caused by contaminants. A prolonged stress situation leads to an increase in blood glucose and cortisol concentrations (Hanke et al., 1983; Mommsen et al., 1999). Selye (1956) proposed the concept of *stress* to describe the integrated effect of adverse environmental influence on an organism's physiological conditions when exposed to stressors. The physiological response follows a pattern given the name of *General Adaptation Syndrome*, which is characterised by three phases:

- *Alarm*: When the physiological systems adjust to compensate for the effects and reestablish a balance; in fish this phase is characterised, among other symptoms, by loss of appetite, alterations in equilibrium and behavioural changes.
- *Resistance*: associated to a compensatory increase in the metabolic rate.
- *Exhaustion*: when the homeostatic system works at higher rates than normal, which can lead to the death of the animals.

The effects of sublethal concentrations of heavy metals also induce in fish a variety of adverse changes in critical organ morphology, physiological alterations (affecting reproduction, growth, development, swimming capacity, breathing, circulation), biochemical modifications (blood chemistry, enzyme activity), and endocrine and behavioural alterations, among others (Newman and Clements, 2008; Rani et al., 2014).

Cadmium (Cd) is toxicologically and ecotoxicologically a very important heavy metal. Anthropic activity removes it from its natural insoluble deposits and distributes it across different environmental compartments, with the aquatic medium being the most significant end deposit site for soluble forms of this element (Nriagu and Pacyna, 1988). The environmental dynamics of the metal affect the aquatic biota by chronic exposure causing consequences both at individual and population level (WHO, 1992). Impacts therefore may vary, from effects at suborganismal level up to alterations in the ecosystem (Goering et al., 1995; Wright and Welbourn, 1994).

Acute toxicity of Cd on aquatic organisms is highly variable, even among species that are phylogenetically closely related, and is connected to their speciation in the aquatic medium, and most particularly to concentration. In addition, freshwater fish can take up waterborne Cd *via* the gills and gut epithelia, both of which routes are non-selective. After uptake, the metal can be distributed and accumulated in gills, liver, kidney and gastrointestinal tract.

There is a great deal of bibliography about the effect of metals on different physiological parameters in fish (Almeida et al., 2001; Beyers et al., 1999; De Smet and Blust, 2001; Eissa, 2009). The energy unbalance caused by stress occurs jointly with alterations in swimming behaviour, ammonium excretion, and feeding behaviour (Ferrari et al., 2011). In general, these responses are not toxin-specific, although several authors have studied the integration of some of such responses (Handy and Depledge, 1999; Wood, 2011).

The purpose of this study was to evaluate the stress suffered by *C. carpio* exposed to a sublethal concentration of Cd under laboratory conditions using some parameters determining the animal's energy balance; the surface response methodology has been used to predict the integrated effect of these parameters.

2 Materials and methods

2.1 *Test organism*

Young *Cyprinus carpio* (Linnaeus, 1758) were used. Body weight and the total length of the fish were: 6.04 ± 0.27 g and 7.78 ± 0.12 cm (N, 20); 10 specimens were used in each test (Controls and Cd exposed).

Seven days before the assays, fish were kept in individual glass containers with continuously aerated and dechlorinated tap water (TW; hardness 70–80 mg $CaCO_3.L^{-1}$) and fed *ad libitum* with commercial fish food composed of (g/100g): carbohydrates 30.0; proteins 42.7; fat 10.5; ashes 10.5; moisture 6.3; caloric value, 14.316 $Joules.g^{-1}$. During the acclimatisation and exposure periods fish were maintained under constant environmental conditions (12D : 12N photoperiod; 21 ± 2ºC) and continuous aeration.

2.2 *Physicochemical parameters*

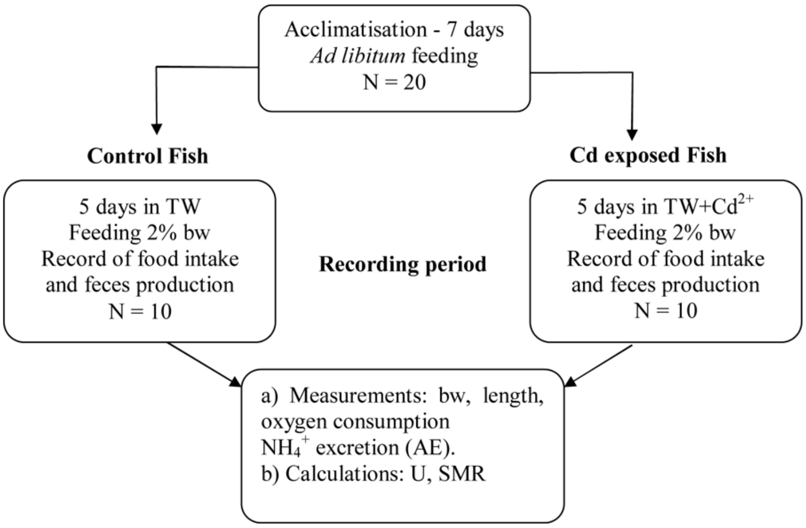
During the assays, the following parameters were daily recorded in all containers: temperature, pH, conductivity ($\mu S.cm^{-1}$), dissolved oxygen (DO, $mg.L^{-1}$), hardness (mg $CaCO_3.L^{-1}$). Cadmium concentration (0.20 $mg.L^{-1}$) was measured by atomic absorption spectrophotometry with air/acetylene flame in an Instrumentation Laboratory, Model 457 equipment, at 228 nm (APHA, 1998).

Assayed Cd^{+2} concentration was determined as sublethal at our laboratory (de la Torre et al., 2000; Eissa et al., 2006; Ossana et al., 2009).

2.3 *Experimental design*

The experimental protocol is schematically shown in Figure 1.

Figure 1 Experimental protocol



2.4 Physiological parameters

During the control and Cd exposure periods, the following parameters were determined daily for each treatment.

- *Food intake* (*I*): Food was offered for 2 h; excess food was then removed by siphoning, filtered and dried at 60ºC up to constant weight. Food intake was estimated as the difference of food weight offered minus the weight of remaining food.

  In order to ensure standard experimental conditions, the last feeding was 36 h before the end of the period.

- *Faeces production* (*F*): Collection of faeces was carried out by siphoning prior to food offer, starting 24 h after the first food offer. Faeces were filtered and dried at 60ºC to constant weight. I and F were expressed as J/ g dw/day.

- *Assimilation efficiency* (*or utilisation*) (*U)*: was calculated as $U = (I - F/I) \times 100$ (Alcaraz and Espina, 1997) and expressed as %.

- Oxygen consumption and ammonium excreted (AE) were determined for each fish upon completion of their exposure. For this purpose, the fish were transferred to an aerated plastic flask containing 300 ml TW. The initial DO (iO) and the final DO (fO) were measured from samples taken before and after the container was sealed for 45 min to prevent the access of air; an additional sample was taken for the determination of ammonium concentration. DO was determined using the Winkler method and ammonium excreted (AE) using a Merck kit (Spectroquant 1.14752) for a range of 0.03–3.00 mg $NH_4.L^{-1}$. Oxygen consumption was calculated as the difference between iO and fO; the specific metabolic rate (SMR) was calculated from the results of the above-mentioned individual measurements as mg $O_2^{-1}g^{-1}$ dw.h^{-1}.

Results of oxygen consumption and ammonium excretion were expressed as J.g(dw)$^{-1}$.day^{-1} (Elliot and Davison, 1975).

2.5 Statistical analysis

The significance of differences between groups was tested by *t Test*; the level of significance was set at $p < 0.05$.

In describing the relationships between the three variables considered (U, AE and SMR), the following polynomial quadratic model was used:

$$Y = \beta 0 + \beta 1 x_1 + \beta 2 x_2 + \beta 3 x_1^2 + \beta 4\, x_2^2 + \beta 5\, x_1 x_2 + \varepsilon,$$

where *Y* is the response variable, $\beta 0$ to $\beta 5$ are the partial polynomial coefficients for estimating the relationship between U regressors (*y*-axis), SMR (*x*-axis) and AE (*z*-axis) for the control group and the group exposed to the metal; ε: error of the model.

The resulting polynomial was analysed using linear regression (by forming pairs with the three variables) and the Durbin-Watson correlation (Montgomery and Peck, 1982). Statistical analyses were conducted using the Statistica program. Results are shown graphically using three-dimensional plots of the measured responses.

3 Results

3.1 Physicochemical results of the assay media used (Table 1)

Except for cadmium content, no significant differences in the physicochemical parameters of the media were found between the control and treated groups. In addition, no mortality of treated fish was recorded. OD levels were within the species' tolerance limits, causing no additional stress to animals. The Cd concentrations remained constant; in the control aquarium metal concentration was below the analytical detection limit (<20 µg $Cd.L^{-1}$).

Table 1 Physicochemical parameters of the media in control and Cd aquaria

Parameter	*Controls*	*Cd exposed*
DO ($mg.L^{-1}$)	4.06 ± 0.23 (12)	4.00 ± 0.47 (12)
pH	8.50 ± 0.05 (12)	8.62 ± 0.01 (12)
Hardness (mg $CaCO_3.L^{-1}$)	75 ± 2 (5)	75 ± 2 (5)
Cd^{2+} ($mg.L^{-1}$)	ND (11)	0.22 ± 0.03 (11)*

Data are given as mean ± SEM; in brackets number of measurements. ND: not detected.
*Significant differences between groups ($p < 0.05$).

3.2 Physiological parameters (Table 2)

Based on the experimental data, calculations were made for assimilation efficiency (U), ammonium excreted (AE) and SMR.

Table 2 Physiological parameters in control and Cd-exposed fishes

	Controls ($N = 10$)	*Cd exposed* ($N = 10$)	*% Change*
Assimilation efficiency (U) (%)	87.23 ± 3.07	83.06 ± 3.28	–4.8
Ammonium excreted (AE) ($J.day^{-1}.g^{-1}$ dw)	166.30 ± 20.10	217.02 ± 39.70*	+30.5
Specific metabolic rate (SMR) ($J.day^{-1}.g^{-1}$ dw)	168.12 ± 10.36	206.60 ± 7.99*	+22.9

Data are given as mean ± SEM. N, number of fish. *Indicates significant differences between groups ($p < 0.05$).

Table 3 shows the results of applying the polynomial used, regression and the values corresponding to the Durbin-Watson correlation. Regression for the pairs of variables was found to be significant for the SMR-AE pair ($p < 0.05$) among fish exposed to Cd.

Figure 2 provides a graphic representation of the development of parameters determined by applying the appropriate polynomials to SMR, U and AE in control and Cd-exposed carps.

Table 3 Coefficients for each term in the linear regression model; parameters for the resulting polynomial

	β0	*β1*	*β2*	*β3*	*β4*	*β5*	R^2	*F*	*p*	*D-W*
Control										
U vs. SMR	397.24	–20.84	+6.20	+0.15	–0.02	–0.01	0.042	0.349	0.571	
U vs. AE							0.045	0.381	0.554	
SMR vs. AE							0.062	0.529	0.487	
Cd exposed										
U vs. SMR	295.87	–2.89	–1.77	+0.06	–0.03	+0.01	0.212	2.153	0.180	
U vs. AE							0.029	0.243	0.635	
SMR vs. AE							0.443	6.367	*0.035*	2.48*

R: correlation coefficient; *F*: Fisher statistic; *p*: probability. D-W: Durbin-Watson correlation. *Significant negative correlation.

4 Discussion

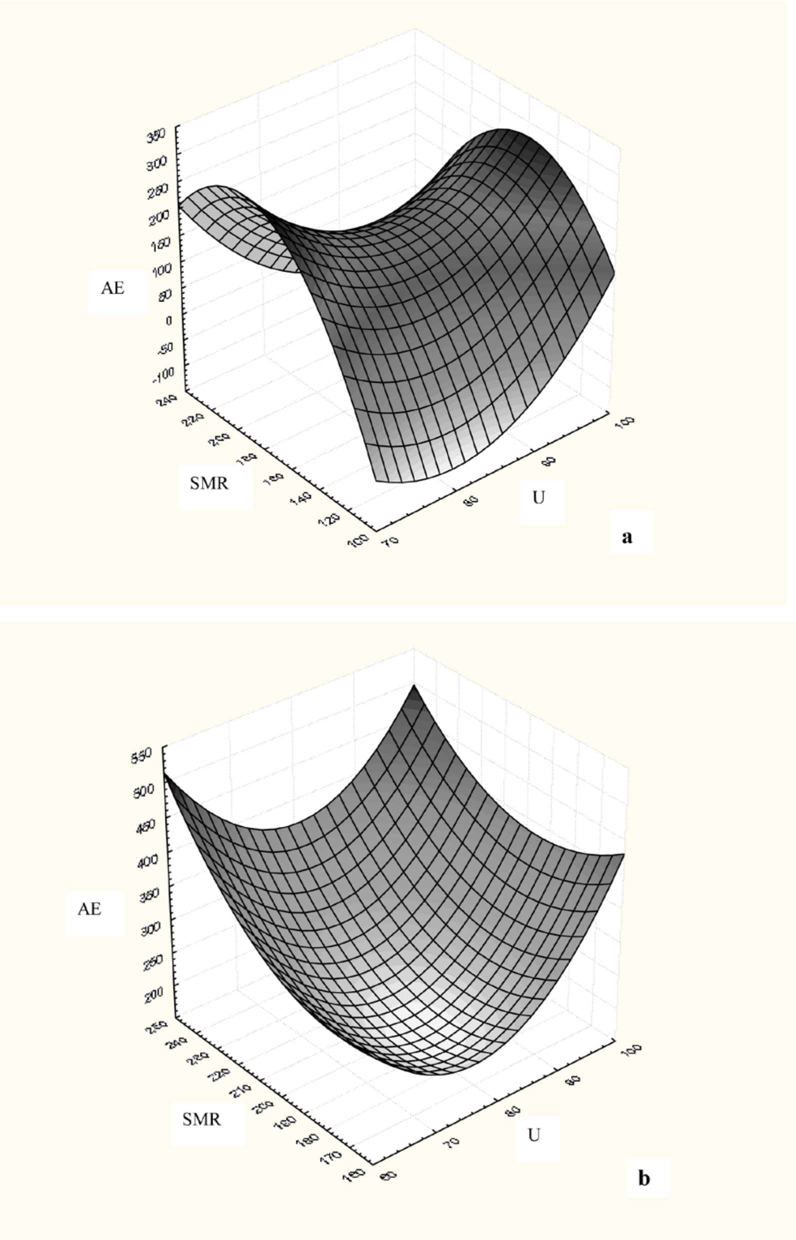
When fish are exposed to chemical stressors, certain physiological mechanisms are activated to resist the environmental stress and maintain homeostasis within its normal range (Wendelaar Bonga, 1997); this parameter may affect an entire set of processes (i.e., digestion, assimilation, respiration and excretion) (Evans et al., 2005), which means an environmental stressor such as (sublethal) Cd interfering in them would cause growth alterations (Hansen et al., 2002). The assayed concentration of Cd (Table 1) was environmentally realistic since it was frequently found in most highly polluted peri-urban water bodies in Argentina. In our case, it was noted that in comparison with the control specimens, Cd-exposed experienced increases in NH_4 excretion and in SMR, though no changes were noted in the effectiveness of assimilation efficiency (U) (Table 2).

The response surface is based on a group of statistical, mathematical and empirical techniques, which provides the means to create models determining optimum conditions in the presence of complex interactions that contributed to combined overall representation of the changes occurring in the various parameters assessed. In this case, the ammonium excreted (AE) is noted as a critical factor in the responses to exposure in media with sublethal Cd concentrations. The shape of the figure between control and Cd-exposed specimens were significantly different (Figure 2): there was a close relationship between SMR and AE, a slight increase in SMR and U with no changes between AE and U.

In Cd-exposed fish (Figure 2(b)) the SMR/AE relationship was critical. Durbin-Watson correlation testing revealed a negative correlation between these parameters (Table 3). A reduction in SMR implies a reduction in oxygen consumption, which would cause an increase in ammonium excretion due to an increase in the use of anaerobic energy and protein metabolism (Ferrari et al., 2011) suggesting a condition of biochemical stress caused by the metal.

Other prior testing conducted in our laboratory on fish of the same species showed that their exposure to 0.3 mg $Cd^{2+}.L^{-1}$ swimming activity was reduced, which was attributed to diminished oxygen intake capacity (Eissa, 2009). On the other hand, De Boeck et al. (1995, 2006) reported that rainbow trout (*Oncorhynchus mykiss*) exposed to copper exhibited a similar response, with a reduction in swimming activity, an increase in the demand of oxygen and a lower rate of oxygen consumption. One significant difference between carp and rainbow trout lies in that the latter requires high oxygen concentration environments while carp can tolerate low oxygen levels for prolonged periods (De Boeck et al., 2004).

Figure 2 Response surface plot that describes the relationship between ammonia excretion (AE), standard metabolic rate (SMR) and assimilation (U) of *Cyprinus carpio* exposed to Cd: (a) controls and (b) Cadmium-exposed fish (see online version for colours)



Among fish of the same species but greater body weight, exposed for a period of 40 days to sublethal Cd concentrations comparable to those assayed in this study, there was a significant decrease in food intake, faecal production, and food assimilation rates (Moza et al., 1995), while assimilation efficiency, oxygen consumption, oxygen extraction efficiency, SMR and ammonium excretion increased following exposure; the overall balance associated with the Cd-linked stress may be expressed as a highly significant decrease of the scope for growth (De Boeck et al., 1995; Espina et al., 2000; Ferrari et al., 2006, 2011).

Espina et al. (2000) noted comparable early responses of young cadmium-exposed *Ctenopharyngodon idella* to the stress caused by the metal.

Some authors suggest that the effect on physiological parameters induced by pollutants in terms of energy provides a basis to fully understand the effects of stress by exposure to anthropogenic pollutants (Beyers et al., 1999).

The integrated energy parameters found through response surface analysis were useful to evaluate the different physiological responses of fish.

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