Addressing the recovery of feeding rates in post-exposure feeding bioassays: *Cyathura carinata* as a case study

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Abstract

Post-exposure bioassays are used in environmental assessment as a cost-effective tool, but the effects of organism's recovery after exposure to pollutant has not yet been addressed in detail. The recoveries of post-exposure feeding rates after being exposed to two sublethal concentrations of cadmium during two different exposure periods (48 h and 96 h) were evaluated under laboratory conditions using the estuarine isopod *Cyathura carinata*. Results showed that feeding depression was a stable endpoint up to 24 h after cadmium exposure, which is useful for ecotoxicological bioassays.

Keywords: aquatic invertebrates; Cd; feeding inhibition; sublethal endpoint.

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1. Introduction

The feeding inhibition of aquatic animals has been shown to be a rapid, sensitive and ecologically relevant nonlethal toxicity test endpoint that can be used to assess toxic stress in ecototoxicology (Allen et al., 1995; Maltby et al., 2002). Since directly measuring feeding rates (FR) during an assay can be extremely difficult, post-exposure feeding inhibition has received a great deal of attention during laboratory and field assays in recent years (e.g., McWilliam and Baird, 2002; Moreira et al., 2005). Post-exposure feeding inhibition is based on the fact that the FR of an organism that is exposed to sublethal concentrations of contaminants may remain inhibited after transfer from a contaminated to an uncontaminated medium. However, few studies address the recovery of feeding inhibition in bioassays based on post-exposure feeding depression (McWilliam and Baird, 2002; Taylor et al., 1998).

The objective of this research was therefore to determine the time period during which the FR of a test species remains inhibited after the exposure period, i.e., without contact with the pollutant (post-exposure time), in order to establish a safe window in which the FR endpoint signals the contact with the toxicant. This led us to evaluate the stability of the post-exposure FR response up to 24 hours after two different contaminant exposure periods (48 h and 96 h) for two sublethal concentrations of cadmium (Cd, as a reference toxicant) in laboratory conditions. The experiments were performed using the isopod *Cyathura carinata* for which the inhibition of median feeding rate after 48-h post exposure to Cd concentration has been described to be 7-fold lower than the corresponding 48-h LC50 (Martinez-Haro et al., 2014). *Cyathura carinata* is a widely distributed and abundant species. It is an ecologically important element of soft bottom benthic communities, playing a major role in estuarine trophic webs (Marques et al., 1994). As an omnivorous species, it feeds on macroalgae, small

invertebrates and detritus (Wägele, 1980). These features make to *C. carinata* a good test species for large-scale monitoring of the ecological status of estuaries.

2. Material and Methods

2.1. Sample Collection

Animal sampling was carried out at low tide in the Mondego estuary (Western Coast of Portugal: 40°08′ N, 8°50′ W), where this species is abundant and well established (Marques et al., 1994). The animals were collected by sieving the first 3 cm of the sediment with local water through a 1-mm mesh. They were then placed in plastic containers with field water and transported to the laboratory in insulated boxes. See Martinez-Haro et al. (2014) for complete details of the sample collection.

2.2. Culture conditions

Once in the laboratory, the animals were sorted by size. Mean total body length (± SD), which was estimated using cephalic measurements following the equation: total body length = 0.745 + 9.01 x cephalic length (see Marques et al., 1994), was 5.9 ± 0.6 mm, n = 20. Since sexual maturation has been reported to occur from 8.13 mm (Marques et al., 1994), the organisms used in the present work were undifferentiated animals correspond to juvenile phase. The animals were maintained at 20 ºC during a 12 h:12 h light:dark photoperiod, in plastic containers filled with a 2-cm layer of clean fine aquarium sand (<0.5 mm) and a 5- to 8-cm height of field water that was continuously aerated (for details, see Martinez-Haro et al., 2014). They were fed daily *ad libitum* with the defrosted nauplii (less than 24-h old) of brine shrimp *Artemia franciscana* Kellogg. The organisms were progressively acclimatized from field to reconstituted seawater (hereafter RSW; ~33 g/l salinity; J. McLachlan's modification of the artificial seawater

111 with L. Provasoli's metal mix PI; described in Guillard, 1983) and maintained in RSW
112 at least 48 h before experiments (ASTM, 2002). Finally, the organisms were kept under
113 fasting conditions (in new cultures with burnt sand and fresh RSW) the last 24 h before
114 the experiments in order to standardize their nutritional storage levels.
115
116 *2.3. Test concentrations*
117 In accordance with our previously published paper, we chose two concentrations of Cd,
118 5 and 0.5 mg/l to test two Cd concentrations in which a significant decrease in the FR
119 could be detected with lower mortality (Martinez-Haro et al., 2014). Stock solutions of
120 500 and 50 mg/l Cd were prepared using CdCl2 (Acros Organic, Geel, Belgium) and
121 nanopure water (conductivity <5 µS/cm; Seralpur PRO 90 CN, Seral, Ransbach-
122 Baumbach, Germany). The test concentrations were obtained by diluting stock solutions
123 with RSW.
124
125 *2.4. Experimental design*
126 Two different exposure periods, 48 h and 96 h, were tested in order to simulate the most
127 common exposure times considered in short-term assays (see e.g., Moreira et al., 2005;
128 Satapornvanit et al., 2009). For this purpose, five different experimental groups were
129 designed: one control group without exposure to pollutant, plus two more for each of
130 the Cd concentrations, one for each exposure period. A total of 440 animals were used
131 for the experiment, 88 in each experimental group. The groups were placed in
132 polyethylene containers filled with 600 ml of test medium and 300 g of sand, and no
133 food was provided to animals during the experiments.
134 Feeding tests were carried out at five different times in each experimental group:
135 a) in the case of the animals in the control group the feeding tests were performed

immediately before starting the experiment (0 h - the result from this test represented the baseline for all experimental groups), and at 48 h, 72 h, 96 h and 120 h after the start of the experiment; b) in the case of the animals that had been exposed to Cd, the tests were carried out immediately after the exposure period (EP), and at EP+3 h, EP+6 h, EP+12 h and EP+24 h (post-exposure times). The experimental design included to randomly subtracting 14 animals (each replicate one animal) of each experimental group at each time to carry out the FRs. In Table 1 the number of replicates analysed per experimental group and time were summarized. Test media and sand were daily renewed. Dead animals were counted and removed from the containers on a daily basis along the experiment. At the end of each exposure periods (48 h or 96 h), and prior to the feeding assays, the animals were washed with RSW and placed in new containers filled only with RSW.

Table 1. Number of replicates analysed per experimental group and time.

Experimental group	**Time from the start of the experiment**				
Control	**0 h**	**48 h**	**72 h**	**96 h**	**120 h**
	n=14	n=14	n=14	n=14	n=14
	Post-exposure time				
	EP	**EP+3 h**	**EP+6 h**	**EP+12 h**	**EP+24 h**
0.5 mg/l – 48 h EP	n=14	n=14	n=14	n=14	n=14
0.5 mg/l – 96 h EP	n=14	n=13	n=14	n=14	n=14
5.0 mg/l – 48 h EP	n=14	n=14	n=14	n=14	n=14
5.0 mg/l – 96 h EP	n=14	n=14	n=14	n=14	n=13

Post-exposure FRs were then performed by following the methodology previously described for this species (Martinez-Haro et al., 2014). Briefly, animals were individually allowed to feed on 100 defrosted nauplii for 30 min at 20 ºC and in darkness. At the end of the feeding period, the remaining nauplii were counted using a

155 stereomicroscope, and FR were estimated as the mean number of nauplii ingested per
156 individual in 30 min.
157 Cadmium concentrations were analysed by means of atomic absorption
158 spectroscopy (AAnalyst800, PerkinElmer) in the test solutions and in a reserved batch
159 of sand that had been used before. The results were 0.4 ± 0.002 mg/l and 5 ± 0.143
160 mg/l, for Cd concentrations of 0.5 and 5 mg/l, respectively. Since the actual and
161 nominal Cd concentrations differed by more than 10% for 0.5 mg/l, the actual Cd
162 concentration was subsequently used. Blanks, and a certified soil reference material
163 (CRM, GBW07406), were also processed in each batch of digestions (to provide quality
164 control data). The Cd concentrations in the control and in the clean sand were below the
165 limit of detection (LOD 0.003 mg/l and 0.004 μg/g, respectively). The percentage of Cd
166 recovery for soil CRM was 98%.
167
168 *2.5. Data analysis*
169 The stability of the FR over time in the control group was tested using one-way
170 ANOVA, with the individual FR as the response variable and time (5 categories; 0 h, 48
171 h, 72 h, 96 h, and 120 h) as the factor. For each exposure period (48 h or 96 h), the
172 effect of Cd concentrations (0 mg/l, 0.4 mg/l or 5 mg/l) on FR was tested using one-way
173 ANOVA, and a post-hoc Tukey's test to check for differences among Cd
174 concentrations. Differences in the FR between the control and the groups exposed to Cd
175 for each post-exposure time interval (EP, EP+3 h, EP+6 h, EP+12 h and EP+24 h) were
176 checked using a one-way ANOVA. Differences in the FR between post-exposure times
177 were tested using a one-way ANOVA for each exposure period with a Dunnett's post-
178 hoc test to check differences between EP and each one of the remaining post-exposure

time intervals. Residuals of the models were checked for normality and homoscedasticity, throught Kolmogorov-Smirnov and Levene tests, respectively.

The tests were performed using IBM SPSS Statistics 20.0, with a level of statistical significance of 0.05.

3. Results

The mortality in the control group was 2.6% of animals at the end of the test (after 120 h). For the animals exposed to Cd, a higher metal concentration and longer exposure resulted in higher mortality rates (2.3% and 3.4% for individuals exposed to 0.4 mg/l Cd after 48 h and 96 h of exposure, respectively; and 9.1% and 17.0% for individuals exposed to 5 mg/l Cd after 48 h and 96 h of exposure, respectively).

The mean FR in the control group (mean ± SE: 38 ± 1 nauplii/ind/30 min) did not differ over time ($F_{4,64}$ = 1.307, p = 0.277; Fig. 1), although a slight decrease was observed at 96 h from the start of the experiment, which may be a sign of the effect of starvation. The FR for this experimental group achieved a mean coefficient of variation of 23% (14% - 34%, min-max). The FR of animals exposed to 5 mg Cd/l for both exposure periods were significantly lower than FR detected in controls ($p < 0.001$; Figs. 2a, 2b). The same pattern was not observed in animals exposed to 0.4 mg Cd/l for any of the exposure periods ($p > 0.05$; Figs. 2a, 2b).

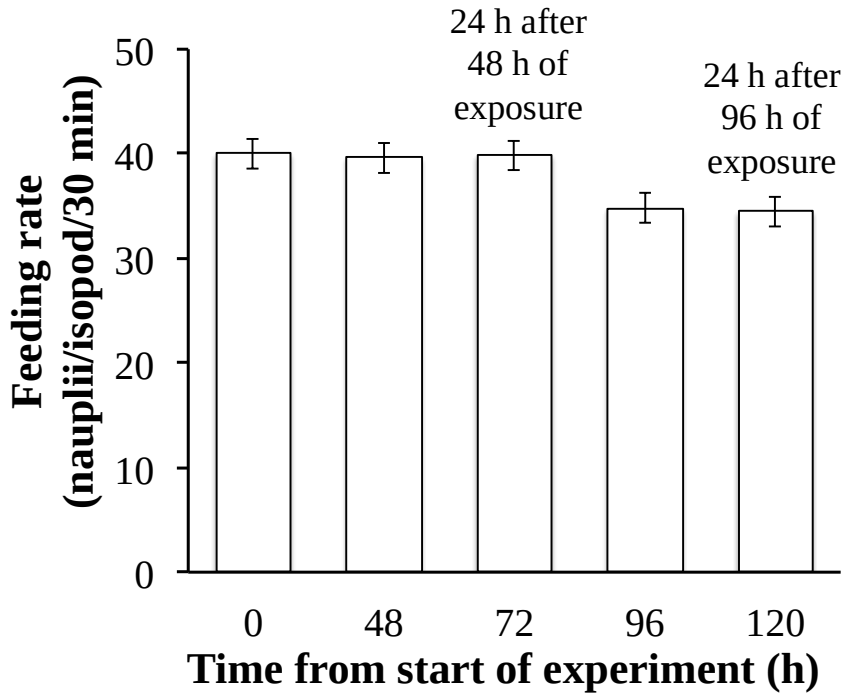

Fig. 1. Mean (± SE) marginal feeding rates (nauplii/individual/30 min) for *Cyathura carinata* in the control group (animals without exposure to cadmium) during the experiment. Correspondences in time with the exposed animals are depicted.

With regard to controls, mean FR were statistically lower in the organism exposed to 5 mg/l for all the post-exposure times tested for both 48 h and 96 h exposure periods ($p < 0.05$). However, no statistical differences were detected for any of the organisms exposed to 0.4 mg/l except for the post-exposure times 12 h and 24 h of the 96 h exposure period ($p < 0.05$). For the groups exposed to Cd, no significant differences between FR at EP and all the remaining post-exposure time intervals were detected at any of the exposure periods (Dunnett's post-hoc test $p < 0.05$; Figs. 2c, 2d). Although not significant, a slight increase on the feeding rate was detected at EP+6 h for 48 h exposure period.

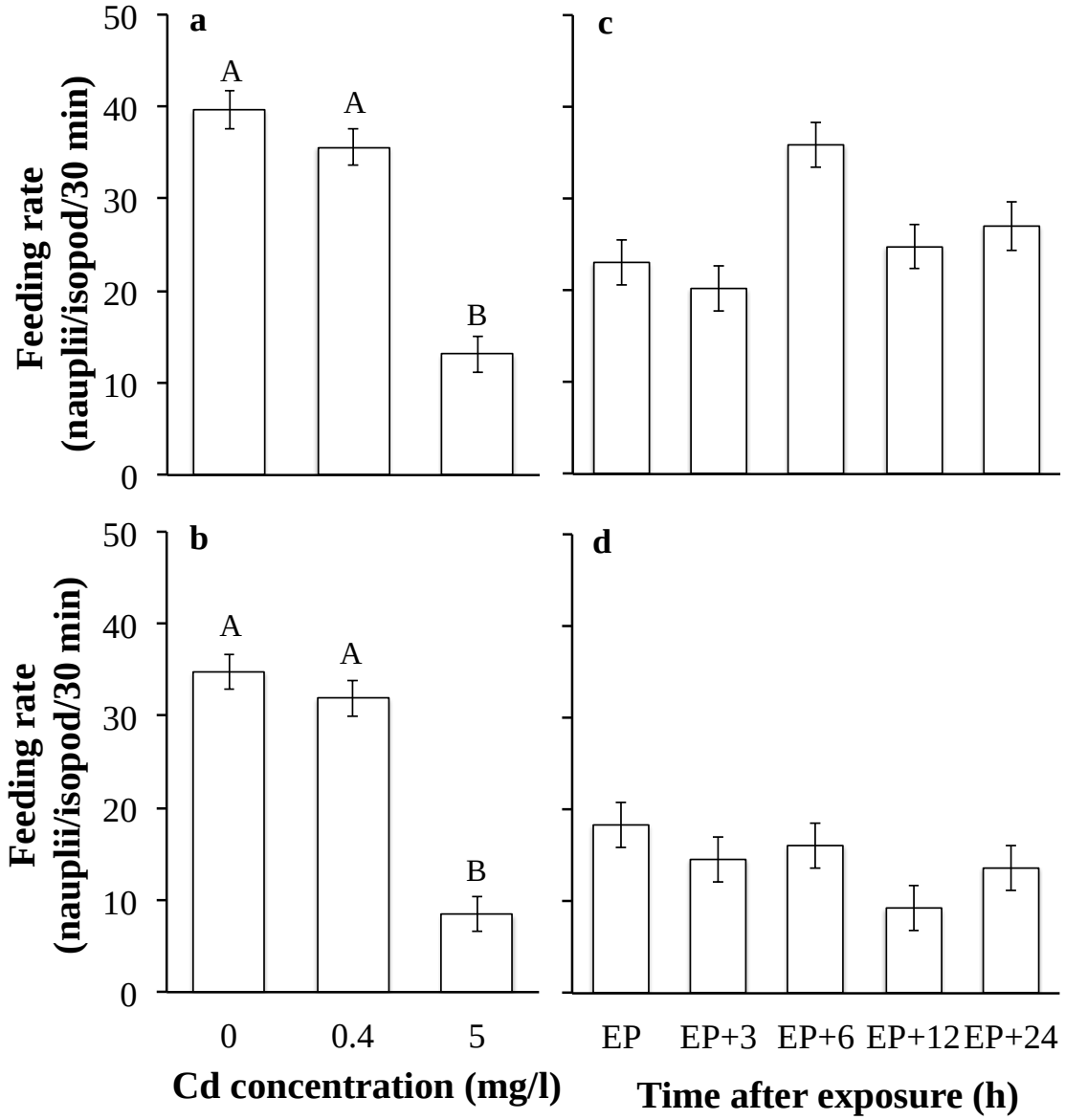

Fig. 2. Mean (± SE) marginal feeding rates (nauplii/individual/30 min) for *Cyathura carinata* after two cadmium (Cd) exposure periods (EP), 48 h and 96 h. Differences among concentrations are shown in a) and b), for exposure periods of 48 h and 96 h, respectively (concentrations with the same capital letter did not differ significantly; Post-hoc Tukey's test, $p > 0.05$). Differences between EP and each post-exposure time are shown in c) and d), for exposure periods of 48 h and 96 h, respectively (Dunnett's post-hoc test, $p > 0.05$).

4. Discussion

The recovery of the organism's response after exposure to pollutants is an important issue that must be controlled in assays based on post-exposure endpoints. As expected, higher concentrations and longer exposure period to Cd resulted in higher mortality rates and a higher feeding inhibition of animals, which coincides with previous studies with other invertebrate and vertebrate species that have been exposed to this metal (Pascoe and Shazili, 1986; Pestana et al., 2007). It should be noted that the exposure to 5 mg Cd/l for 96 h resulted in a mortality of 17% in *C. carinata*, which may be due to the toxic effect of concentration of that concentration plus the starvation period to which the animals were subjected. The absence of a clear recovery as regards the FR at least 24 h after Cd exposure should be considered as a safe window in which post-exposure feeding assays can be carried out without expectations of punctual feeding reduction as a result of the animal's recovery. Only weak and inconsistent fluctuations in the FR were detected for a punctual post-exposure interval (EP+6 h), which is probably owing to certain uncontrolled factors affecting the feeding assays at this time.

The stability in feeding response obtained here is consistent with previous studies focused on metals and some organic pollutants. For instance, feeding depression was detected in *Daphnia* both during the exposure (24 h) and post-exposure (4 h) to sublethal concentrations of contaminants, including Cd and organic pollutants (McWilliam and Baird, 2002; Taylor et al., 1998). Wilding and Maltby (2006) found the FR of *Gammarus pulex* exposed to sublethal zinc concentrations during 6 days did not recover, and even became more depressed, after 6 days of exposure. Similarly, Brent and Herricks (1998) found that *Hyalella azteca* mobility was not recovered for up to 172 h after short (30-240 min) exposure periods to sublethal concentrations of Cd and zinc. Our study corroborates with this previous evidence and supposes the first

assessment of the potential recovery effects up to 24 h after exposure, which is quite relevant as regards applying these new generation ecotoxicological tools.

5. Concluding remarks

The present study has shown that inhibitions in the feeding rates of *C. carinata* after Cd exposure represented a stable response as a bioassay endpoint since no evidence of recovery was detected in 24 h after the exposure period.

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