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Motionless of snail *Hydrobia ulvae* (Pennant) as response to sediment toxicity and its consequences for the post-exposure feeding

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Abstract

Post-exposure feeding and motionless may be useful endpoints for assessing toxicity. The present study aimed to examine (i) the immobility of the estuarine snail *Hydrobia ulvae* resulting from short-term exposure to copper-spiked sediment, and subsequently, (ii) the potential application of post-exposure feeding (indirectly measured as pellets excreted) as an ecotoxicological response associated with motionlessness. Mobility and post-exposure feeding were influenced by copper contamination. Motionless was noticeable above the concentration of 200 µg Cu g^{-1}, where 40% of the organisms were inactive. Practically all the organisms remained active at the two lowest concentrations: 40 (control) and 60 µg Cu g^{-1}. For 400 and 800 µg Cu g^{-1} the motionless was generally higher than 50%. Mortality higher than 25% was observed at 200, 400 and 800 µg Cu g^{-1}, with values of 27, 40 and 60%, respectively. The feeding inhibition values showed an increasing inhibition from 20% at the lowest concentration (60 µg Cu g^{-1}), up to 59% at 400 µg Cu g^{-1}; at concentration of 800 µg Cu g^{-1}, although the physical activity inhibition had reached 67%, the post-exposure feeding was 1.5 higher in relation to the control. Probably, the starvation period due to motionless during exposure seems to increase the post-exposure feeding and egestion when food was provided; alternatively, increased pellet production could also indicate a detoxification process.

Keywords: copper contamination; physical activity; post-exposure feeding; spiked sediment

INTRODUCTION

Hydrobia ulvae (Pennant) (Gastropoda) is an estuarine benthic mudsnail found in intertidal ecosystems considered an important energy source for many organisms (Lillebø *et al.*, 1999; Pascual & Drake, 2008). Unfortunately, environmental changes and contamination, frequent in estuarine and coastal environments (Redondo-Gómez *et al.*, 2009; Fathallah *et al.*, 2012), threaten seriously *H. ulvae* populations (Lillebø *et al.*, 1999). Because mudsnails live in direct contact with sediment (Haubois *et al.*, 2005), they have been used to provide reliable information about the effects of sediment toxicity (Shipp & Grant, 2006; Hampel *et al.*, 2009; Mauffret *et al.*, 2010; Araújo *et al.*, 2012; Campana *et al.*, 2013). In those studies, focused on different endpoints, such as survival, growth, feeding/egestion rate, total number of veligers or larvae produced, bioaccumulation, avoidance, and other types of behaviour, the organism showed a good discrimination capacity among sediment with different toxicity levels. For *H. ulvae*, physical activity (mobility) and post-exposure feeding are likely suitable measurements for assessing contaminant exposure (Hampel *et al.*, 2009; Krell *et al.*, 2011; Araújo *et al.*, 2012). Mobility is a sub-lethal response that is considered highly relevant based on the assumption that motionless can lead to death (McWilliam & Baird, 2002). In particular, physical activity has been used as the endpoint in studies with a variety of different species (such as amphipods, cladocerans, snails, and tadpoles) and

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contaminants (e.g., cadmium, fluoride ion, phenol, zinc, and acid mine drainage effluent) (Brent & Herricks, 1998; Macedo-Sousa *et al.*, 2007; Alonso & Camargo, 2012; Denoël *et al.*, 2013). Hampel *et al.* (2009) and Araújo *et al.* (2012) observed a reduction in the mobility of *H. ulvae* with consequent retraction into its shell in order to avoid contact with LAS (linear alkylbenzene sulphonate) and naturally contaminated sediment samples, respectively. Campana *et al.* (2013) showed that exposure to metal-spiked sediments altered the behaviour of snails (e.g., burying, floating, crawling and sinking). Post-exposure feeding has also been considered a sensitive endpoint for assessing environmental contamination (Maltby *et al.*, 2002; McWilliam & Baird, 2002). Basically, post-exposure feeding assays consist of a period of exposure to the contaminant, followed by an incubation period in clean medium, where the rate of feeding is measured (McWilliam & Baird, 2002). It is assumed that the impact on the organism during exposure will persist after the exposure has ended, and this will be reflected in post-exposure feeding. Exposure to different contaminants (e.g., carbendazim, chlorpyrifos, copper, dimethoate, naturally contaminated sediment, profenofos, pulp mill effluent, and zinc) demonstrated to affect the post-exposure feeding of the prawn *Macrobrachium rosenbergii* (Satapornvanit *et al.*, 2009), the midge *Chironomus riparius* (Soares *et al.*, 2005), the amphipod *Echinogammarus meridionalis* (Agostinho *et al.*, 2012), the crab *Carcinus maenas* (Moreira *et al.*, 2006), the polychaete *Hediste* (*Nereis*) *diversicolor* (Moreira *et al.*, 2005), and the cladoceran *Daphnia magna* (McWilliam & Baird, 2002; Rosa *et al.*, 2010). Recently, Krell *et al.* (2011) also showed that contamination affect the post-exposure feeding of the snail *H. ulvae*.

Based on the hypothesis that the motionless, or even merely reduced physical activity, could have important implications on feeding, and that periods of starvation can also change feeding pattern of the organisms (McMahon & Rigler, 1965; Calow, 1975), the objectives of the present study are to examine (i) physical activity of the snail *H. ulvae* resulting from exposure to different copper-spiked estuarine sediment, and (ii) the potential application of post-exposure feeding as an ecotoxicological response associated with motionlessness. Spiked sediment was used because it guarantees organisms are exposed to sediment with similar geochemistry characteristics and therefore misleading in interpretation of the toxicity response by using different sediment samples are prevented (Hutchins *et al.*, 2009). Copper was chosen as pollutant model because it is highly toxic to *H. ulvae* (Campana, 2006).

MATERIAL AND METHODS

Sediment: sampling, treatment and characterisation

Sediment sample (top 0.5 cm) with no toxic effect (Araújo *et al.*, 2012) collected in a clear zone at the Guadalquivir estuary (SW Spain) was used at the spiking matrix. Sediment was mixed, placed in plastic flasks and maintained at 4 ºC until processing (4 days after sampling). In order to eliminate the autochthonous biota, in the laboratory, sediment was placed in a plastic tray, forming a fine layer of sediment, on which liquid nitrogen (-196 ºC) was poured (Araújo *et al.*, 2009). After evaporation of the liquid nitrogen and thawing, the sediment was spiked. Sediment was not sieved. Physical-chemical characterization of the sediment and background metal concentration values are provided in Table 1. Values of pH and redox potential (Eh) were measured (pH Meter 330 WTW) directly in sediment; organic matter (OM, in %) was determined after calcination (at 450 ºC for 48 h); grain size (silt–clay: <0.063 mm) was determined by wet sieving; and for the determination of total organic carbon (TOC, in %) the technique described by El-Rayis (1985) was used. Sediment subsamples (grain size < 0.063 mm) were freeze-dried and digested in a microwave oven (CEM Corporation; Microwave Accelerated Reaction System 5) in order to analyze concentrations (in $\mu g\ g^{-1}$ dry weight) of most important metals usually found in the sediment of the Bay of Cádiz (Araújo *et al.*, 2010), following the procedure described by Loring & Rantala (1992). Concentrations of Al, Co, Cr, Cu, Fe, Li, Mn, Ni, and Zn were determined by inductively-coupled plasma-optical emission spectrometry (ICP–OES, Perkin Elmer Optima 2000 DV); As, Cd, Pb, and Sn concentrations were determined by inductive-coupled plasma mass spectrometry (ICP-MS, Thermo, Serie X7). Reference material analysis (Community Bureau of Reference 277 and Marine Estuarine Standard Sediment-1, National Research Council of Canada) showed the accuracy of the method was higher than 83%.

Spiking of the sediment

After collection and processing as described, sediment was spiked (Simpson *et al.*, 2004) with copper as copper sulphate ($CuSO_4 \cdot 5H_2O$, Merck, 99% purity). Glassware used for spiking

Table 1 - Physical-chemical characterization of the sediment sample (total metal concentration are given in $\mu g\ g^{-1}$).

Parameters	Values
pH	7.06
Eh (mV)	-27
OM (%)	5
TOC (%)	1
Grain size(% >0.63 μm)	91
Al	83125
As	17
Cd	0.4
Co	15
Cr	87
Cu	40
Fe	37500
Li	41
Mn	750
Ni	35
Pb	35
Sn	3
Zn	106

was cleaned with nitric acid at 10% and rinsed several times with Milli-Q ultrapure water. Copper solutions were prepared in artificial seawater (ASTM, 1975) with pH of 8.2, salinity 35 and dissolved oxygen (OD) concentration lower than 0.3 mg L^{-1}. In order to reach this value of OD, the solutions were purged with nitrogen gas for 5 h in order to reduce the oxygen concentration and, consequently, bacteria activity. Seawater solution and sediment, in a 1:1 ratio (25 g sediment and 25 mL sea-water), were mixed in plastic 50 mL centrifuge tubes. Immediately, the tube was purged with nitrogen gas for 30 s before closing. Then, the tube contents were homogenized using a Rotabit orbital stirrer (Selecta) for 48 h, at 150 rpm. Spiked sediment was stored at 20 ºC, in darkness, for 15 days. On the 7th day all the tubes were purged with nitrogen gas again for 60 s. After 15 days the tubes were centrifuged at 2700 *g* for 5 min before the tests, in order to eliminate interstitial water. Nominal values for final copper concentrations in the batches of sediment assayed were: 60, 120, 200, 400 and 800 µg g^{-1} plus non-spiked reference sediment (40 µg g^{-1}), hereafter named control. Exposure concentrations chosen were based on a previous study developed by Campana (2006).

H. ulvae individuals

Adult individuals (>3 mm length) of the snail *H. ulvae* were collected by hand picking and in a shallow coastal lagoon considered unpolluted from the Bay of Cadiz (SW Spain), where snails' populations has been monitored for long years (Drake & Arias, 1995; Pascual & Drake, 2008). The culture was maintained for three weeks in aquaria containing 2 L of filtered seawater (0.7 µm pore size filter), collected from the Bay of Cádiz, which was renewed weekly. Temperature (20 ± 2 ºC), salinity (33 ± 2), and photoperiod (16/8 h light/dark) were monitored. Organisms were fed with aliquots *ad libitum* of lyophilized *Ulva* sp. Mortality during the acclimatization period was less than 10%. Twenty four hours before the assays, organisms were starved in order to stimulate them to forage (i.e. to increase their potential mobility). During this starvation period, water was renewed three times in order to prevent coprophagia (feeding on produced pellets).

Effects on physical activity

Spiked and control sediment (0.5 g) were spread evenly in plastic Petri dishes (23.8 cm^2) by triplicate. A volume of 8mL of artificial seawater was added to dishes, vigorously mixed with the sediment and then allowed to settle and equilibrate during 24 h. A shallow layer of water was formed above the sediment, but not sufficient to permit floating activity. On the inner sides of the dishes, sheets of sandpaper were attached with non-toxic silicone, in order to ensure that snails could not climb out, ensuring thus constant contact with sediment. Five organisms were randomly distributed in dishes containing a copper treated sediment sample each. All dishes were non-hermetically closed and incubated under the same conditions of culture. After 24, 48, 72 and 96 h the immobility of each organism was recorded. To assess snail immobility, each replicate was individually submitted to stereomicroscopy with continuous light and during a ten minute period, and each organism was checked for movement. An organism that presented crawling activity or moving into or out of its shell was considered active. All measurements were always carried out at 10:00 am in order to avoid behavioural changes due to differences in the observation hour (potential circadian cycles of activity/inactivity).

Effects on post-exposure feeding

After the last measurement of mobility, organisms were placed individually in one well of a microplate (12-wells suspension culture-plates, 17.8/16 MM and 127.8/85/19 MM CELLSTAR, Greiner Bio-One) containing 5 mL of clean artificial sea-water, without sediment, and 2 mg of lyophilized *Ulva* sp. provided as food. Organisms were maintained in these wells for 3 h and, during this period, the presence or absence of movement in individuals determined as non-active in the previous test was verified. It was expected that food supply would stimulate any non-active organism to feed and display movement; therefore any organism that remained inactive during that period was considered dead. No mortality was registered in control treatment. After recording incidence of mortality (after 3 h), snails were kept in the same wells containing 5 mL artificial seawater, without sediment and with food, for post-exposure feeding test for 24 h. In this phase, feeding rate was measured indirectly by number of produced faecal pellets; this is considered a good indirect measure of snail feeding (Shipp & Grant, 2006). After 24 h feeding, snails were transferred to wells with clean water and without food and sediment where the pellets were counted for 3 h. In this time period, 80% of total pellets are produced (Pascual & Drake, 2008). Every 30 min all the pellets produced were removed in order to prevent coprophagia and erosion of the pellets.

Statistical analysis

Active individuals (%) were calculated from the total number (15) of snails exposed for treatment. Mobility was based on organism total number in order to reduce the importance of each organism in the analysis, as 5 organisms were introduced by replicate. Most important information about physical activity response was to verify that in increasing concentrations there were more inactive individuals and so more individuals in starvation. However, faecal pellet production was determined on an individual basis. For calculating the percentage reduction in feeding, the number of pellets produced by organisms exposed to control sediment was taken as reference (100%). Statistical difference in pellet production was checked using Anova and Tukey's post-hoc test ($p<0.05$).

RESULTS

There was no mortality in the control treatment after 96 h exposure. Physical activity of snails recorded at short-

term (throughout the 96 h) of exposure to the copper-spiked sediment decreased with increasing concentration (Fig. 1). This effect was pronounced above the concentration of 200 µg Cu g^{-1}, where 40% of the organisms were inactive. Practically all the organisms remained active at the two lowest concentrations: 40 (control) and 60 µg Cu g^{-1}. For 400 and 800 µg Cu g^{-1} the motionless at 24, 48, 72 and 96 h were generally higher than 50%. Results clearly showed that mobility was inhibited throughout the assay as a function of the input of copper and that the motionless recorded at the end of the assay was similar to that at the beginning.

Because mortality is difficult to measure in snails (dead individuals can be confused with inactive individuals), it was recorded after stimulation for 3 h with food, as previously described. Individuals that remained inactive were considered dead. Mobility was recovered after this stimulation in two organisms classified as inactive at a concentration of 60 µg Cu g^{-1}; in one organism at 400 µg Cu g^{-1}; and in one at 800 µg Cu g^{-1}. Mortality found to be higher than 25% were observed at concentrations of 200, 400 and 800 µg Cu g^{-1}, with values of 27, 40 and 60%, respectively.

The number of faecal pellets counted for each organism and treatment after feeding is plotted in Fig. 2. When exposed to control sediment, 16 pellets per individual were produced. At increasing concentrations (up to 400 µg Cu g^{-1}) the egestion decreased as a function of the input of copper; however, at 800 µg Cu g^{-1}, production of pellets was high, around 24 pellets per individual. Mean numbers of pellets (±SD) produced by individuals for each concentration (control, 60, 120, 200, 400, and 800 µg Cu g^{-1}) were 16 (±6), 12.5 (±9), 11.7 (±5), 8.7 (±5), 6.4 (±2), and 24.2 (±13) respectively, with statistically difference ($p<0.05$; $F_{5,42}=3.593$) between 800 µg Cu g^{-1} and the concentrations 200 and 400µg Cu g^{-1}. Based on the egestion shown by organisms exposed to control sediment, the feeding inhibition values for each concentration showed an increasing

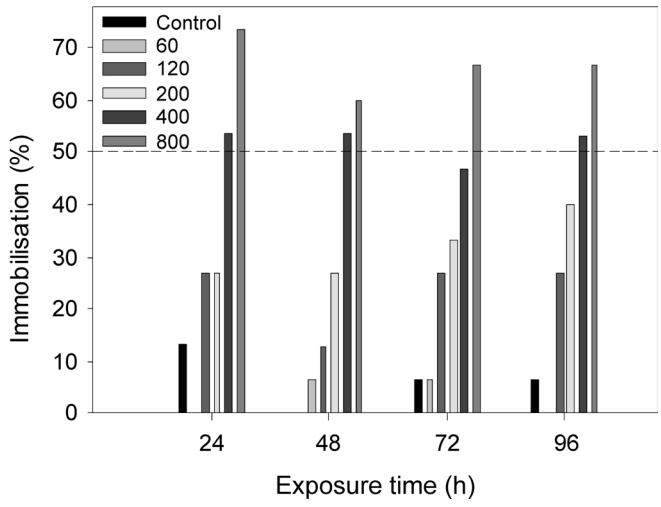

Fig. 1 - Percentage of immobile individuals of snail *Hydrobia ulvae* exposed to copper-spiked sediment during 96 h. The dashed line indicates 50% effect. Nominal copper concentrations (top left) are expressed in µg g^{-1}. Data where bar is missed indicate 0% of immobilisation.

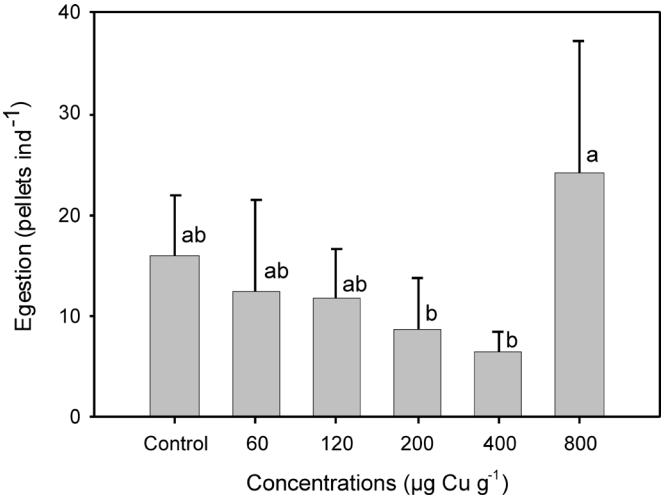

Fig. 2 - Mean egestion/excretion (nº. of pellets per organism) of snail *Hydrobia ulvae* after exposure to copper-spiked sediment. Different letters indicate statistically ($p<0.05$) different means.

inhibition from 20% at the lowest concentration, up to 59 % at 400 µg Cu g^{-1}; but this response decreased to a negative value (-53%) when organisms had previously been exposed at 800 µg Cu g^{-1}.

DISCUSSION

Physical activity is an endpoint able to discriminate between different inputs of copper in sediment. The sensitivity of motionless as an endpoint has also been reported in other studies with *H. ulvae* (Hampel *et al.*, 2009; Araújo *et al.*, 2012; Campana *et al.*, 2013). Observed effects of copper at short-term exposure on snails' physical activity in this study are in accordance with results described by Campana (2006), who observed that, at concentrations of 0, 50 and 100 µg Cu g^{-1} in spiked sediment, the activity of *H. ulvae* was similar to that when exposed to the control sediment, with organisms presenting three main activities: floating, burrowing and crawling; in contrast, at 200 and 400 µg Cu g^{-1} those activities were reduced and mortality began to occur. Post-exposure feeding, determined by pellets production, also demonstrated to be a response capable of discriminating among different sediment copper toxicity levels, after previous short-term exposure to copper. According Krell *et al.* (2011) post-exposure feeding was found to be three times more sensitive than lethality, but less sensitive than growth, when *H. ulvae* was exposed to copper in water.

Results of post-exposure feeding obtained in the highest concentration tested were unusual, and they indicate that information obtained by using this endpoint must be treated with caution. In sediment with the highest copper concentration, the number of pellets produced was 1.5 times greater than in the control sediment. This result could be associated with the longer 24 h post-exposure feeding period employed. Since other authors have recommended a very short post-exposure feeding period (sometimes <1 h) in order to minimize any possible physiological recovery by the organism from the

effects of toxicants suffered during exposure (Krell *et al.*, 2011), in our study a shorter post-exposure feeding period of 3 h rather than 24 h was initially used, and the number of pellets produced by organisms exposed to the most contaminated sediment was still 1.3 times greater than pellets produced in control sediment (data not shown). This indicates that shorter post-exposure feeding period had little influence on the results.

In studies in which post-exposure feeding is used to assess previous exposure, no food is provided, in order to avoid interference in the results. When post-exposure feeding is used to assess previous exposure to sediment, detritus, microalgae and bacteria associated with the sediment could be used as a food source during the exposure. The higher post-exposure feeding observed at the highest concentration could likely be linked to the response of a major reduction in physical activity. Individuals exposed to most contaminated sediment did not feed during the test due to motionless; in contrast, individuals exposed to lower concentrations were more active and were able to feed, albeit suffering the effects of contamination, what was recorded in subsequent feeding conditions. Once the exposure was ended and food was provided, possibly those organisms exposed to the most contaminated sediment increased their feeding to compensate for the period in starvation. Calow (1975) showed that, when food was available after varying periods of starvation, the ingestion rate for two freshwater gastropods, as well as the absorption efficiency, increased. It was demonstrated that *Daphnia magna* submitted to a starvation period, when they were exposed to non-limiting food concentration after starvation, ingested food more rapidly than the animals that had been fed (McMahon & Rigler, 1965). These authors indicated that inhibitory or toxic foods should also decrease food intake in a similar way to imposed starvation. On the other hand, the higher egestion obtained at the highest concentrations could also be a mechanism of detoxification caused by the ingestion of toxic material with consequent egestion of this material and mucus associated. An increased external mucus production has been described for *H. ulvae* when exposed to different contaminants like LAS, copper, cadmium, zinc (Hampel *et al.*, 2009; Campana *et al.*, 2013) and the role of the mucus in preventing the assimilation of aluminum has also been evidenced (Balance *et al.*, 2002). Therefore, given that gastropods produce mucus within their guts (Davies & Hawkins, 1998), and that after the starvation the activity and secretion of digestive juices could increase (Calow, 1975), the increase of toxicity might have generated a high level of mucus being secreted to generate a barrier and limit direct exposure (Brooks & White, 1995; Desouky, 2006). At the end of the acute exposure, the mucus, granules and toxic material would then be excreted by fecal pellets (Brooks & White, 1995; Desouky, 2006). Therefore, we could hypothesize that snails exposed to highly contaminated sediment could excrete higher quantity of pellets. To test this hypothesis it would be necessary to examine the composition of the pellets regarding constitutive material and presence of copper. This would be studied in further researches.

Post-exposure feeding of *H. ulvae* demonstrated to be a sensitive and reliable endpoint for toxicity assessment. However, it needs to be analysed with caution in whole-sediment toxicity tests because interference can exist due to food availability present in sediments. In this sense, two factors should be taken into account: (i) snails are able to alter the ingestion (Haubois *et al.*, 2005) and (ii) particularly *H. ulvae* can adopt different feeding strategies as a function of the sediment contamination levels (Granberg & Forbes, 2006). In addition, if motionless is observed during exposure to avoid continuous exposure to contaminants, probably the organisms will be in starvation and the measurements of the post-exposure feeding could be misinterpreted by a further increasing in ingestion.

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