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Martinez-haro, Monica; Acevedo, Pelayo; Pais-costa, Antonia Juliana; Taggart, Mark A.; Martins, Irene; Ribeiro, Rui; Marques, Joao Carlos

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Assessing estuarine quality: A cost-effective in situ assay with amphipods

Monica Martinez-Haro[ab*], Pelayo Acevedo[b], Antónia Juliana Pais-Costa[a], Mark A. Taggart[c], Irene Martins[a], Rui Ribeiro[d], João Carlos Marques[a]

[a] MARE – Marine and Environmental Sciences Centre, Department of Life Sciences, University of Coimbra, Portugal

[b] Instituto de Investigación en Recursos Cinegéticos (IREC-CSIC-UCLM-JCCM), Ronda de Toledo s/n, Ciudad Real, Spain

[c] Environmental Research Institute, University of the Highlands and Islands, Castle Street, Thurso, Caithness, Scotland, KW14 7JD, UK

[d] Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, Calçada Martim de Freitas, 3000-456 Coimbra, Portugal

*Corresponding author e-mail address: monica.martinezharo@gmail.com; Phone: + 351239 836386; Fax: + 351239 823603

ABSTRACT

In situ assays based on feeding depression can be powerful ecotoxicological tools that can link physiological organism-level responses to population and/or community-level effects. Amphipods are traditional target species for toxicity tests due to their high sensitivity to contaminants, availability in the field and ease of handling. However, cost-effective in situ assays based on feeding depression are not yet available for amphipods that inhabit estuarine ecosystems. The aim of this work was to assess a short-term in situ assay based on postexposure feeding rates on easily quantifiable food items with an estuarine amphipod.

Experiments were carried out under laboratory conditions using juvenile *Echinogammarus marinus* as the target individual. When 60 *Artemia franciscana* nauplii (as prey) were provided per individual for a period of 30 minutes in dark conditions, feeding rates could be easily quantified. As an endpoint, postexposure feeding inhibition in *E. marinus* was more sensitive to cadmium contamination than mortality. Assay calibration under field conditions demonstrated the relevance of sediment particle size in explaining individual feeding rates in uncontaminated water bodies. An evaluation of the 48-h in situ bioassay based on postexposure feeding rates indicated that it is able to discriminate between unpolluted and polluted estuarine sites. Using the harmonized protocol described here, the in situ postexposure feeding assay with *E. marinus* was found to be a potentially useful, cost-effective tool for assessing estuarine sediment and water quality.

Capsule: Cost-effective in situ assay with amphipods for estuarine quality assessment

Keywords: Cadmium; *Echinogammarus marinus*; Environmental monitoring; Postexposure feeding; Sublethal endpoint.

1. Introduction

Estuaries are almost uniquely amongst aquatic habitats, because of the high degree of variability that prevails in terms of their spatial and temporal physicochemical characteristics (Elliott and Quintino, 2007). Despite these natural stressors, estuaries are also often resilient habitats, which are among the most productive marine ecosystems in the world (Elliott and Quintino, 2007; Underwood and Kromkamp, 1999). In fact, estuaries provide a wide variety of valuable ecosystems services (Barbier et al., 2011). Unfortunately, estuaries are also commonly exposed to high degrees of anthropogenic stress, e.g., due to pollution, exploitation and habitat destruction (Elliott and Quintino, 2007), leading to biodiversity loss that can impair certain critical estuarine ecosystem services (Worm et al., 2006). Within this context, the EU's Water Framework Directive demands effective water quality assessment for all European water bodies. This is considered especially critical for estuarine environments which are already naturally stressed areas (Dauvin and Ruellet, 2009; Elliott and Quintino, 2007).

In recent years, in situ assays have emerged as effective tools when measuring the effects of stressors in aquatic ecosystems (Crane et al., 2007). In situ assays based on feeding depression have been established as non-destructive bioassays that can link physiological organism-level responses to effects at the population and/or community level (Baird et al., 2007; Coulaud et al., 2015; McWilliam and Baird, 2002). In terms of sublethal responses, feeding inhibition can be used as a sensitive indicator of exposure to pollutants – detecting the effects of stress at lower pollutant levels than when using mortality rates alone (Martinez-Haro et al., 2014). This may then facilitate early-warning – indicating that toxic conditions are occurring before population dynamics

become affected (Baird et al., 2007; Maltby et al., 2002; McWillian and Baird, 2002). Even though the vast majority of in situ assays are based on mortality or a chronic response such as differences in size, fertility, sex ratio, etc., following long-term exposure (i.e., 10-d or 28-d; e.g., Castro et al., 2006; Sanz-Lázaro and Marín, 2009), short-term (i.e., 24-h, 48-h or 96-h) assays based on nonlethal endpoints (such as feeding inhibition) have received more attention recently (e.g., Martinez-Haro et al., 2014; McWilliam and Baird, 2002; Moreira et al., 2006a; Rosen and Miller, 2011).

Water-only and sediment toxicity tests using amphipods were first developed in the 1970s - 1980s (e.g., Bellan-Santini and Reish, 1976; Lindén, 1976; Swartz et al., 1979) and since then, have been widely deployed in estuarine and marine habitats (ASTM, 2008; Costa et al., 1998; Rosen et al., 2012; Scarlett et al., 2007; USEPA, 1994; Van Geest et al., 2014). Amphipods are abundant and ecologically important components of aquatic communities and changes in amphipod populations may also affect the structure and functioning of the wider ecosystem by affecting food availability at higher trophic levels (Baird et al., 2007; Coulaud et al., 2015; Kunz et al., 2010; Maltby et al., 2002). Amphipods also have many desirable characteristics that make them useful in ecotoxicology and environmental biomonitoring, i.e., i) their wide geographic distribution, ii) they are easy to handle and to culture under laboratory conditions, and iii) they are highly sensitive to contaminants and adequate species for early-warning monitoring – often being the first macroinvertebrates to disappear from contaminated sites (Dauvin, 1998; Swartz et al., 1982). The most common methodology to study feeding activity of freshwater or marine amphipods uses the loss of mass of preweighed discs (supporting plants or algae) after being offered to an individual for a given time (e.g., Felten et al., 2008; Maltby et al., 2002; Nyman et al., 2013; Pastorinho et al., 2011; Schmidlin et al., 2015). Methodologies based on the loss

of mass require laborious laboratory effort. Discs used in feeding quantification experiments must be dried and weighed accurately and repeatedly, which is time-consuming. However, few studies consider the use of alternative food items (Agostinho et al., 2012; Taylor et al., 1993) or alternative quantification procedures (Coulaud et al., 2015; Gerhardt et al., 1994; Nyman et al., 2013). Thus, here, we aimed to develop and evaluate a short-term, cost-effective, in situ toxicity assay based on the postexposure feeding rates of an estuarine amphipod (*Echinogammarus marinus*) on easily quantifiable food items (nauplii of the brine shrimp *Artemia franciscana*).

2. Materials and methods

2.1. Study species

Echinogammarus marinus was used as it is a widely distributed euryhaline benthic amphipod with an ecologically relevant role in European estuarine food webs (Marques, 1989). It is abundant in intertidal soft-sediment and rocky estuarine habitats covered with the macroalgae *Fucus* spp. (Marques, 1989), making it an appropriate target species for the assessment of environmental pollution in both water and sediment compartments. In Portugal, the Mondego estuary (Western Coast of Portugal: 40º8´10”N, 8º50´51”W) represents the most southerly distribution limit for this species, which extends northwards to Norway and Iceland (Lincoln, 1979). *Echinogammarus marinus* is omnivorous, feeding on a wide range of plant material (including a number of algae species), as well as prey such as hard-bodied isopods and soft-bodied oligochaetes (Alexander et al., 2013a). This species is also an important prey item for birds and fish (McLusky, 1989). Additionally, due to its sensitivity to stress, this

127 species has recently been proposed as potentially useful for environmental monitoring
128 programmes in Northern Atlantic estuaries (Leite et al., 2014; Pastorinho et al., 2011).
129
130 *2.2. Assay organisms*
131
132 All test organisms were collected at low tide from *Fucus vesiculosus* fronds
133 found in the intertidal area of the Mondego estuary where this species maintains a
134 stable and abundant population (Leite et al., 2014; Marques and Nogueira, 1991) and is
135 not affected by microsporidian parasites (I. Martins, personal communication).
136 Organisms were transported to the laboratory in thermally insulated boxes. *Fucus*
137 *vesiculosus* and *Ulva sp.* were also collected during sampling to feed to animals during
138 culture. Clean seawater (~35 g/L salinity) was collected at the mouth of the estuary and
139 transported to the laboratory, diluted with distilled water to 31 g/L salinity (hereafter:
140 culture water), and stored at 4 ºC in darkness for use during subsequent laboratory
141 culture work.
142 Once in the laboratory, organisms were sorted and maintained at 20 ± 1 ºC
143 under a 12-h:12-h light:dark photoperiod in plastic containers filled with culture water
144 which was continuously aerated. Organisms were fed *ad libitum* with *Ulva sp.* and *F.*
145 *vesiculosus*. Prior to toxicity tests using cadmium (Cd) organisms were progressively
146 acclimatized (ASTM, 2002) during a three day period from culture water to
147 reconstituted seawater (RSW) at 33 g/L salinity (Guillard, 1983; RSW consists of 26.4
148 g NaCl, 0.84 g KCl, 1.67 g $CaCl_2.2H_2O$, 4.6 g $MgCl_2.H_2O$, 5.58 g $MgSO_4.7H_2O$, 0.17 g
149 $NaHCO_3$ and 0.03 g H_3BO_3 per litre of deionized water). Prior to all laboratory
150 experiments, tests and in situ assays, organisms were placed in new culture water for a
151 24-h fasting period, to standardize nutritional storage levels across individuals.

The size of the individuals sampled was estimated using the cephalic length following the equation previously determined by Marques and Nogueira (1991): total body length = − 1.211995 + 10.668590 × cephalic length. Except in one experiment, which was carried out to check the effect of animal size, only juveniles with a mean (± SD) total body length of 6.7 ± 1.0 mm (n = 50) were used in experiments (see below).

2.3. Feeding quantification

To quantify the feeding rate of *E. marinus* under laboratory conditions, defrosted nauplii (less than 24-h old) of the brine shrimp *A. franciscana* were used as food (further details in Supplementary material). This food item was selected because this has been successfully used previously for feeding quantification with other invertebrate species (e.g., Agostinho et al., 2012; Martinez-Haro et al., 2014).

Feeding quantification experiments were designed to consider factors that may potentially influence *E. marinus* feeding rates in order to identify an assay that would not result in the consumption of all food items. If all food were consumed a truncated data distribution would be generated which would then pose a problem in terms of data analysis with parametric statistics. Likewise, we used a short recovery period to minimize organism physiological recovery following contaminant exposure (Pais-Costa et al., 2015). The factors assessed were: size class of *E. marinus*, feeding period and number of food items provided.

An initial experiment was carried out to evaluate differences in feeding rates among three size classes of *E. marinus*: small (mean ± SE, 6.5 ± 0.6 mm, n = 12) which corresponds to juveniles, medium (9.4 ± 0.6 mm, n = 12) and large size classes (13.6 ± 0.8 mm, n = 12); the last two classes corresponding to adult animals (Marques and

Nogueira, 1991). For organisms of different size classes we conducted a feeding quantification experiment with three different amounts of food (50, 100 and 150 nauplii per individual). This was performed during a 30-min feeding period. A second experiment evaluated three different feeding periods (30 min, 60 min, and 120 min) for a fixed number of food items (100 nauplii). We optimised food quantity by carrying out a third experiment involving further food item variation (50, 60 and 100 nauplii) over a 30-min feeding period. Finally, we assessed the potential effect of different water volumes added to the glass vials where the feeding of *E. marinus* took place. Four volumes (3, 5, 7 and 10 ml) were tested with a fixed quantity of food and feeding period (60 nauplii and 30 min, respectively). A harmonized protocol for feeding quantification was thus derived and used for the in situ assays.

Twenty replicates were used for each experimental group (for each assessed factor, a group was the organisms within the same age class), except for the size class experiment (in which 12 replicates were used). Each replicate consisted of one organism housed in a 30-ml glass beaker filled with 5 ml of RSW, except for the water volume experiment (in which 3, 5, 7 and 10 ml were used). Amphipods were allowed to feed for a given period of time (30 min, except for the feeding period experiment in which 30, 60 and 120 min were used) at 20 ºC in darkness. Dark conditions were chosen because feeding activity increases at night in this species (e.g., Alexander et al., 2013b). At the end of the feeding period, nauplii remaining were counted under a stereomicroscope and feeding rates estimated as the number of nauplii ingested per amphipod in a given time (nauplii/amphipod/30 min, except for the feeding period experiment in which 30 min, 60 min, or 120 min were also considered).

Differences in feeding rates among size classes were analysed using General Linear Models, in which individual feeding rate (ingested nauplii/amphipod/30 min)

was the response variable, and size class and number of food items provided were fixed factors. Similarly, differences in feeding rates among the three feeding periods and the set number of food items provided were analysed, using individual feeding rate as the response variable, and feeding period and number of food items as factors. In cases where factors had more than two levels, post-hoc differences were evaluated using the Tukey Honestly Significant Difference test.

2.4. Lethality and postexposure feeding tests

Cadmium was the reference toxicant (USEPA, 1994) used to determine the 48-h median lethal concentration (48-h LC_{50}) for the lethality assay and the 48-h median effective concentration (48-h EC_{50}) for the postexposure feeding assay. A stock solution containing 250 mg/L Cd was prepared using $CdCl_2$ (Acros Organic, Geel, Belgium) and nanopure water (conductivity < 5 µS/cm; Seralpur PRO 90 CN, Seral, Ransbach-Baumbach, Germany). Test solutions were obtained by serial dilution of the stock solution with control RSW. Preliminary feeding tests were carried out to establish a suitable Cd concentration range. The following six and eight Cd concentrations were used for the lethal and sublethal tests, respectively: 0, 0.125, 0.25, 0.5, 1, and 2 mg Cd/L, and 0, 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg Cd/L.

For both lethal and sublethal tests, three replicates were set up per test concentration, each consisting of five individuals in a 250 ml plastic vial filled with 200 ml of the test solution and small pieces of silicon aquarium tube to provide shelter. Both tests were conducted at 20 ± 1 ºC under a 12-h:12-h light:dark photoperiod without food. Mortality was checked daily. In the sublethal test, postexposure feeding was quantified after 48-h of exposure, following the procedure developed in the

previous section. Before and after exposure, salinity (Wissenschaftlich Technische Werkstätten Cond315i/SET conductivity meter, WTW, Weilheim, Germany), dissolved oxygen (WTW OXI 92 oxygen meter) and pH (WTW 537 pH meter) were recorded twice in each test solution.

Cadmium concentrations were analysed by atomic absorption spectroscopy (AAnalyst800, PerkinElmer). The minimum detection limit was 0.2 µg Cd/L. The Cd concentration in RSW (control) was below the limit of detection. To observe whether Cd concentrations were maintained during the experiment, concentrations from test solutions were measured at the beginning and end of each experiment. Because real and nominal concentrations of the test solutions differed by less than 10%, the latter were used for toxicity endpoint estimates. Median effective lethal (48-h LC_{50} value) and postexposure feeding (48-h EC_{50} value) Cd concentrations, and their respective 95% confidence intervals (CI), were derived using the Trimmed Spearman–Kärber method (Hamilton et al., 1977).

2.5. In situ design

2.5.1. Environmental sites

Six environmental sites were selected for in situ assays at a reference and a polluted estuarine site, both located in the Northwest of the Iberian Peninsula, along the Portuguese coast (see **Table 1**): Minho river estuary (Caminha, Portugal) and Lima river estuary (Viana do Castelo, Portugal). The Minho river estuary is an undisturbed estuary subject to low human pressure (Ferreira et al., 2003) that is a NATURA 2000 site. Previous studies have described very low levels of metals and organic

252 contaminants at the mouth of this estuary (Gravato et al., 2010; Guimarães et al., 2012).
253 Four environmental sites (R1-R4; coded from the mouth to upstream) were selected in
254 the Minho river estuary. These enabled us to determine baseline postexposure feeding
255 rates over an environmental gradient, i.e., of salinity, temperature and sediment
256 characteristics such as particle size distribution and organic matter content (see Table
257 1). We studied the relationship between environmental parameters and feeding rate
258 within the reference estuary in order to determine which environmental factors
259 (confounding factors) could potentially influence feeding rate for the study species.
260 In contrast, the Lima estuary receives greater levels of anthropogenic pollution.
261 These relate to shipyards, commercial seaport operations, commercial fishing, marina
262 activities, and those due to dredging of the navigational channel. Additionally, the
263 estuary receives discharges related to agricultural runoff, a paper mill effluent and
264 urban and industrial sewage (Costa-Dias et al., 2010). As such, the Lima estuary has
265 been the focus for numerous biological studies (e.g., Gravato et al., 2010; Guimarães et
266 al., 2012). Two environmental sites (P1-P2) were selected in the Lima river estuary in
267 order to assess the sensitivity of the in situ assay in a polluted scenario.
268
269 *2.5.2. In situ assay procedure*
270
271 Chambers for the in situ assay were adapted from those used previously for
272 another invertebrate species, the isopod *Cyathura carinata* (Martinez-Haro et al.,
273 2014), with minor modifications (see details in Supplementary material).
274
275

Table 1. Geographical coordinates, water-column salinity and temperature (ºC) (at low and high tide), total suspended solids, particulate organic matter and sediment organic matter content (mean ± SD), and particle size distribution at the reference (R1 – R4) and polluted (P1 – P2) environmental sites in the estuaries of the Minho and Lima rivers, respectively, where the 48-h in situ assays with *Echinogammaurs marinus* were deployed.

	Minho				Lima	
	R1	R2	R3	R4	P1	P2
Coordinates						
Latitude	41º 52´ 02.55´´N	41º 52´ 01.71´´N	41º 51´ 59.92´´N	41º 52´ 16.42´´N	41º 41´ 17.65´´N	41º 40´ 59.05´´N
Longitude	8º 51´ 35.16´´W	8º 51´ 20.61´´W	8º 51´ 01.08´´W	8º 50´ 40.10´´W	8º 48´ 53.81´´W	8º 49´ 40.65´´W
Water-column						
Salinity (g/L)	22.6 – 33.9	26.2 – 33.8	20.5 – 32.1	23.0 – 33.4	29.4 – 32.5	22.3 – 34.4
Temperature (ºC)	18.4 – 14.5	17.9 – 14.4	18.4 – 15.8	19.5 – 14.7	18.5 – 16.5	17.0 – 15.6
Total suspended solids (mg/L)	62.1 ± 70.8	20.5 ± 10.6	44.4 ± 40.7	29.3 ± 15.9	19.7 ± 10.7	40.7 ± 37.1
Particulate organic matter (mg/L)	8.8 ± 6.8	3.3 ± 0.9	6.4 ± 5.3	3.9 ± 0.1	6.6 ± 0.6	5.5 ± 3.9
Sediment						
Organic matter content (%)	1.3 ± 0.08	1.2 ± 0.12	2.1 ± 0.25	5.0 ± 0.07	2.0 ± 0.07	1.0 ± 0.25
Particle size distribution (%)						
< 63 µm	5	20	13	45	8	6
63 – 125 µm	3	3	8	30	4	3
125 – 250 µm	3	9	19	13	19	29
250 – 500 µm	55	53	50	8	47	44
500 – 1000 µm	24	16	9	2	12	11
1000 – 2000 µm	4	1	0	1	6	5
> 2000 µm	6	0	0	0	2	1

Field deployments were carried out simultaneously in both estuaries. Amphipods were transported to environmental sites in plastic containers (completely filled with seawater from the collection site) in thermally insulated boxes. For each environmental site, a set of three chambers was deployed at low tide. Each chamber was pushed into the sediment to a depth of approximately 14 cm. Up to 1 cm of lateral window protruded from the sediment, guaranteeing the exchange of both overlying water and sediment pore water (through the 200-µm meshed windows). Ten organisms were released into each chamber, and a small piece of local *Fucus sp*. was added to provide shelter and simulate natural habitat (Marques and Nogueira, 1991). The top of the chamber was immediately covered with the cap to prevent escape of organisms and ingress of indigenous fauna.

After a 48-h exposure period, chambers were retrieved at low tide (more details given in in Supplementary material), individually placed inside a labelled small plastic bag and transported to the laboratory in a thermally insulated box. Water was also collected at each environmental site and transported to the laboratory for further use. All field sites were less than a 4-h drive from the laboratory. Once in the laboratory, organisms were immediately retrieved from the sediment and cleaned with water collected from each respective environmental site. Finally, postexposure feeding rates (nauplii/amphipod/30 min) were quantified individually according to the developed procedures. Feeding assays were all performed within 5 h of chamber retrieval from the field.

2.5.3. Environmental gradients

304 To characterize the environmental conditions at each environmental site, salinity
305 and temperature were measured in the water column at low and high tides (YSI
306 Professional Plus handheld multiparameter probe). A water sample was also collected
307 and filtered (Whatman GF/C glass fibre filters) to quantify the particulate organic
308 matter (% volatile solids, mg/L) and total suspended solids (mg/L). Both were
309 measured according to standard procedures described in Strickland and Parsons (1972)
310 and APHA (1995). Finally, from each environmental site, a composite sediment sample
311 (2–5 cm upper layer) was collected for organic matter content and particle size analysis
312 (see below). Sediment samples from each environmental site were first homogenized,
313 visible indigenous animals and large debris were removed with tweezers, and then the
314 sample was divided into four subsamples. Three were used for organic matter content
315 analysis and one for particle size distribution analysis following the procedure
316 described in Martinez-Haro et al. (2014). Organic matter content was determined as the
317 percentage of initial dry weight (after heating at 105 ºC for 24 h) remaining after
318 igniting the sample in a muffle furnace (Thermo Fisher Scientific, Thermolyne F6000,
319 Essex, United Kingdom) at 550 ºC for 4 h (Buchanan and Kain, 1971).
320
321 *2.6. Expected feeding rates*
322
323 To calibrate an environmental model able to predict expected feeding rates
324 without pollutant inhibition for the studied species, a General Linear Model was created
325 using the data from the reference estuary. First, co-linearity among predictors was
326 checked using variance inflation factors (VIF) to avoid bias in the model
327 parameterization. Only predictors with a VIF of < 3 were considered for modelling
328 (e.g., Zuur et al., 2010). Postexposure feeding rate was the response variable (after

arcsine transformation, since proportion of total food given was used) and environmental parameters were the predictors (**Table 1**). A forward-backward stepwise procedure was used to select the most parsimonious model (hereafter final model) using the Akaike Information Criteria (Akaike, 1974). Assessing normality, homocedasticity, independence and linearity with the standardized residuals we validated the final model. The final model was used to predict feeding rates at all environmental sites (both at reference and polluted estuary sites), which in turn was used to assess the sensitivity of the in situ assay.

Following a procedure described previously, the ratio between predicted values (from modelling) and observed values was calculated for each environmental site (see Martinez-Haro et al., 2014). In addition, the model was used to predict expected feeding rates considering the mean environmental characteristics prevailing at the polluted estuary. This feeding rate was then multiplied by the above-mentioned ratio to obtain adjusted feeding rates for each site; among site variability due to differences in environmental characteristics (other than that related to contamination) were thus removed. A detailed description of this mathematical rationale can be found in Supplementary material.

The comparison between adjusted postexposure feeding rates among the four reference environmental sites was made with a one-way nested ANOVA. Then, the adjusted postexposure feeding rate of the reference estuary (coded as a whole) was compared with the two polluted estuary environmental sites using a one-way ANOVA, with replicates nested within site. Least Significant Difference tests for the marginal means were used to check for differences among sites. Except when otherwise stated, tests were performed using IBM SPSS Statistics 20.0 using a level of statistical significance of $p \leq 0.05$.

3. Results

3.1. Feeding quantification

Lower feeding rates for *E. marinus* were observed in the small size class when compared to the other two size classes ($F_{2,96}$ = 8.726, p < 0.001; **Fig. 1**). There were also statistically significant differences in feeding rates in relation to the number of food items provided ($F_{2,96}$ = 8.109, p = 0.001), i.e., rate was lower when 150 nauplii were provided than when 50 or 100 were given. The interaction between size class and food number provided was not significant ($F_{4,96}$ = 0.778, p = 0.542). Regarding the percentage of food items ingested, a clear reduction was observed as an increased number of food items was provided ($F_{2,96}$ = 99.829, p < 0.001).

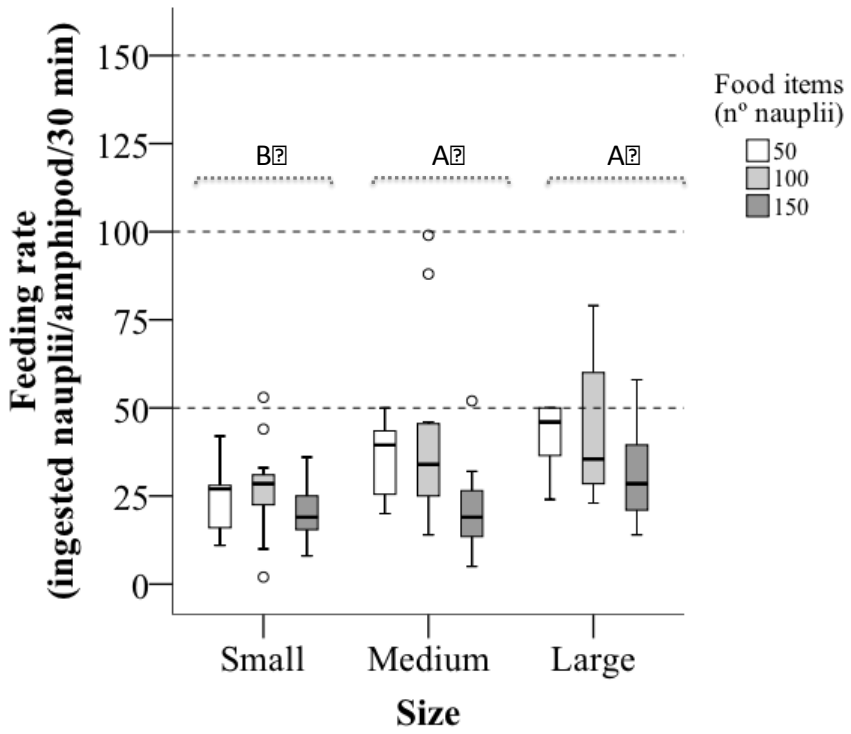

Fig. 1. Box plots showing feeding rate (ingested nauplii/amphipod/30 min) for organisms of three size classes (small, medium and large) of *Echinogammarus marinus*, when a different quantity of food was given (using a 30-min feeding period). Dashed lines represent the maximum number of food items available for each experimental group (i.e., the maximum possible feeding rate). Size classes that share the same capital letter did not significantly differ (Tukey HSD test, $p > 0.05$), when the same number of food items was supplied to amphipods.

No statistical differences were detected among the feeding periods tested ($F_{2,50} = 2.232$, $p = 0.118$; **Fig. 2**). Statistically different feeding rates were however detected when amphipods were allowed to eat 50, 60 or 100 nauplii ($F_{2,57} = 4.651$, $p = 0.013$). Finally, no significant differences were found among the test volumes ($F_{3,76} = 0.477$, $p = 0.699$).

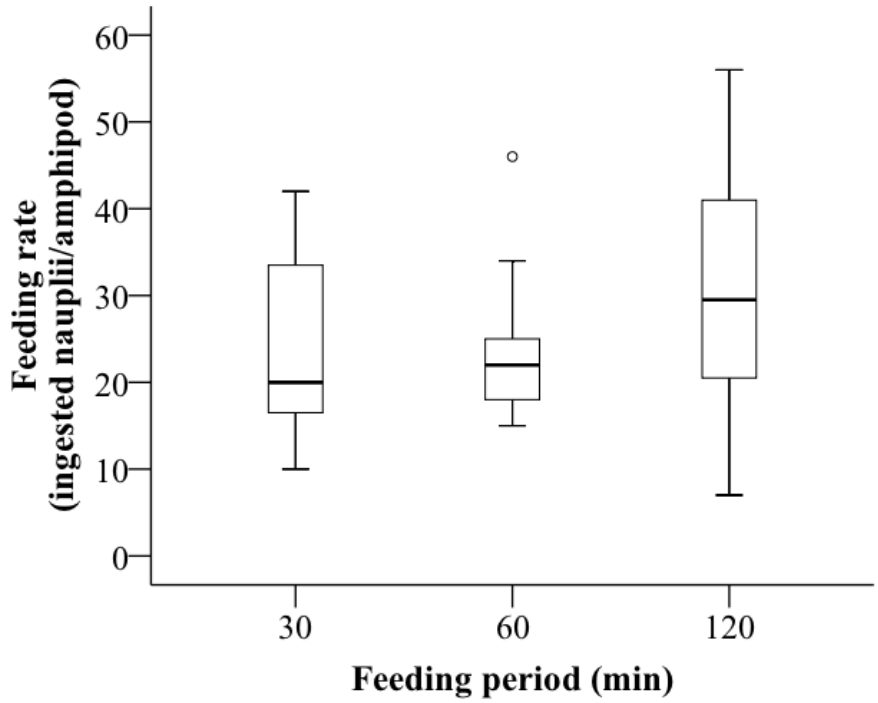

Fig. 2. Box plots showing the feeding rate (ingested nauplii/amphipod/X min) for juveniles *Echinogammarus marinus* when 100 nauplii per individual were provided for three different feeding periods. Means sharing the same capital letter did not differ significantly (Tukey HSD test, $p > 0.05$).

3.2.Cadmium lethality and postexposure feeding tests

Control survival for all toxicity tests was 100%. The mean 48-h LC_{50} value (with 95% CL values) for Cd was 0.93 (0.57 – 1.5) mg/L, and the mean 48-h Cd EC_{50} was 0.66 (0.34 – 1.23) mg/L; the 48-h EC_{50} for postexposure feeding was 1.4-fold lower than the corresponding 48-h LC_{50}.

Dissolved oxygen in each test chamber was above 60% saturation at all times for both the lethal and postexposure feeding tests (ASTM, 2002). Mean (± SD) values for pH and salinity were 7.6 ± 0.03 and 33.5 ± 0.22 g/L in the lethal test and 7.6 ± 0.02 and 33.4 ± 0.24 g/L for the postexposure feeding test.

3.3. In situ assay

Table 1 summarizes values for the environmental parameters at each environmental site. At the end of the in situ assay deployment, all chambers were found intact and in their original position. After controlling for multicollinearity, salinity at low tide, salinity at high tide, proportion of sediment present with a size of between 500-1000 μm and 1000-2000 μm, and organic matter content, were considered for modelling. Statistical modelling showed that feeding rates at the reference estuary were influenced by the proportion of sediment present with a size of between 500-1000 μm

and 1000-2000 μm. The relationship between feeding rate (*PEF*) and the percentage of the two size fractions of sediment (*X1*: sediment between 500-1000 μm; *X2*: sediment between 1000-2000 μm,) was (± SE): arcsine *PEF* = 45.586 (± 2.012) – 1.059 (± 0.207)·*X1* + 1.871 (± 1.190)·*X2* (Adjusted $R^2 = 0.260$, $p < 0.001$).

After adjusting to the mean value of predictors in the polluted estuary (11.9% of sediment at 500-1000 μm and 5.4% of sediment at 1000-2000 μm), no differences in feeding rate among sites or replicates were detected at the reference estuary ($F_{3,99} = 0.009$, $p > 0.05$; $F_{8,99} = 0.936$, $p > 0.05$, respectively). Differences in adjusted postexposure feeding rates were detected between animals from reference and polluted estuaries ($F_{2,139} = 28.446$, $p < 0.001$; **Fig. 3**), but not among replicates ($F_{14,139} = 0.718$, $p > 0.05$).

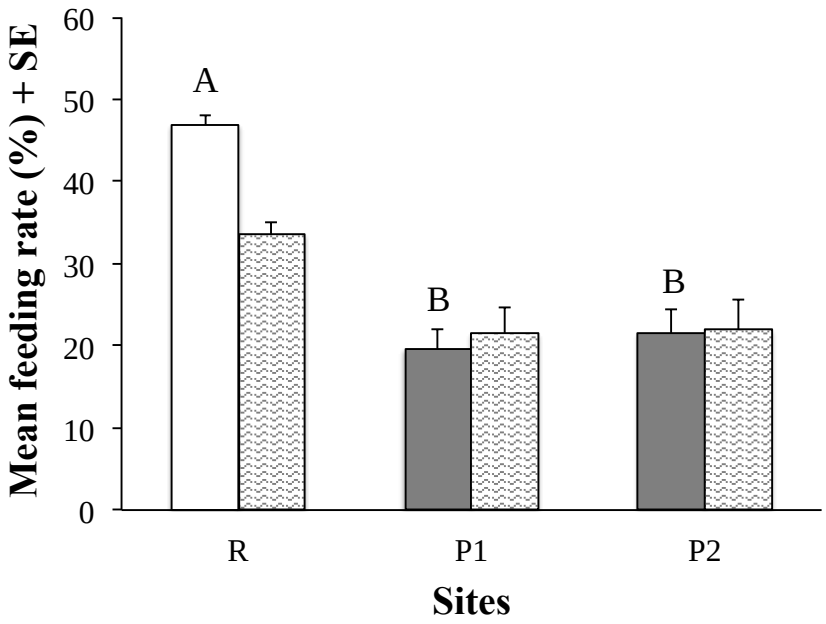

Fig. 3. Adjusted and observed (bars waves) postexposure mean feeding rates (% of food items ingested in 30 min) of *Echinogammarus marinus* after a 48-h exposure period, for the reference estuary of the Minho river (R, white column) and for the two sites at the polluted estuary of the Lima river (P1-P2, grey columns). Error bars indicate

+1 standard error. Means sharing the same capital letter did not differ significantly (Least Significant Differences, $p > 0.05$).

4. Discussion

4.1. Food items

Traditionally, the feeding activity of freshwater or marine amphipods has been quantified through the loss of mass of weighed discs (made from plant or algae) after being offered to an individual for a given exposure time (e.g., Maltby et al., 2002; Nyman et al., 2013; Pastorinho et al., 2011; Schmidlin et al., 2015). Methodologies based on the loss of mass require laborious laboratory effort, as repeated drying and accurate weight measurements are needed. Here, we used *A. franciscana* nauplii that are quick and easy to obtain, manipulate and quantify in laboratory conditions. This is also a cost-effective prey item, which is ideal when quantifying feeding activity in freshwater amphipods (Agostinho et al., 2012; Taylor et al., 1993). We showed that *Artemia* nauplii appear to be a suitable and practicable prey that can be used in assays based on feeding quantification for the estuarine amphipod *E. marinus*.

4.2. Feeding quantification

Three different *E. marinus* size classes (juveniles and two classes of adults) along with four different numbers of food items (50, 60, 100 and 150 nauplii provided) and three feeding periods (30, 60 and 120 min) were tested in order to optimize the laboratory conditions to perform a feeding assay. Regarding organism size, lower

453 feeding rates were detected for the small size class, which is in agreement with
454 Alexander et al. (2013a) for *E. marinus* feeding on live size-matched isopods. The
455 lower feeding rates observed for the smaller organisms may be because adults have a
456 higher ability to catch, handle and process food in relation to the less experienced
457 juveniles. In our study, consumption of all food items was observed in some organisms
458 of the medium and large size class when 50 food items were supplied. Similar results
459 were obtained for medium size fed with 100 items. Overall, a greater decrease was
460 detected in the absolute numbers of food items ingested in 30 min especially when 150
461 nauplii were provided. This reduction on food items ingested could be related to the
462 combined effect of the higher densities (150 nauplii) to which amphipods were exposed
463 and the limited feeding time provided (only 30 min). Regarding, the percentage of food
464 items ingested, this clearly tended to decrease when higher densities of food were
465 provided, which is compatible with the tendency for predatory efficiency to increase as
466 prey density reduces, a response previously described for this species (Alexander et al.,
467 2013a). A reduction in efficiency could be also suppose a lower number of caught prey
468 at very high densities as was observed here in the absolute numbers of food items
469 ingested. When 150 prey items were provided, a large proportion of them were not
470 consumed and therefore higher effort is required to quantify feeding rate. In order to
471 avoid a truncated data distribution in feeding rate and to optimize laboratory effort,
472 small *E. marinus* (i.e, juveniles) are recommended for use in this in situ feeding assay,
473 as are 50-100 prey items (see below). In addition, juveniles are a good target size
474 because i) they are more sensitive to pollutants than adults (e.g., McCahon and Pascoe,
475 1988), ii) they tend to accumulate higher levels of metals than adults (Correia et al.,
476 2004; Pastorinho et al., 2009), and iii) they represent the most abundant size class

almost throughout the year - corresponding to > 50% of the population in our study area (Leite et al., 2014).

Our results showed that the number of food items ingested was similar for the three feeding periods used, i.e., a greater number of prey were not ingested if the feeding period was longer. This may be due to constraints related to the digestive process (Penry and Jumars, 1990). Given this, a 30-min feeding period (the shortest period) was used for subsequent tests and we recommend this period for future implementation of the in situ assay. Short feeding periods are also recommended in order to avoid bias due to the potential effect of an organisms' postexposure recovery (Pais-Costa et al., 2015).

Finally, an experiment was conducted to fine-tune the number of prey provided to juveniles (since initial tests suggested this should be between 50 and 100 prey items). Results indicated that 60 nauplii would be an ideal food quantity (for a feeding period of 30 min), because: i) there was a lower probability of obtaining a truncated data distribution (than when 50 nauplii were supplied), and ii) fewer prey remained after feeding (i.e., less effort was required to determine feeding rate), with respect to the use of 100 nauplii. Summarizing, results indicated that 60 nauplii per individual was adequate, as was a short feeding period of 30 minutes (in dark conditions) to quantify feeding rates for this juvenile amphipod.

4.3.Cadmium lethality and postexposure feeding tests

Results obtained from our toxicity tests indicated that postexposure feeding activity was a more sensitive endpoint than mortality for *E. marinus*. The 48-h EC_{50} for Cd for postexposure feeding was approximately 1.4 times lower than the 48-h LC_{50}

(0.66 mg/L vs 0.93 mg/L, respectively). For comparative purposes, the relationship between mortality and feeding inhibition, over the same Cd exposure period, was also reported by Felten et al. (2008) using the freshwater amphipod *G. pulex*. Therein, the reported 168-h LC_{50} was 21.6 μg Cd/L, and a feeding rate reduction of 36% was observed after 168 h in amphipods exposed to 15 μg Cd/L (i.e., 1.4 times lower than the 168-h LC_{50}). The 48-h EC_{50} value reported in our study is higher than the 96-h EC_{50} value reported for this species previously when using a 24-h postexposure feeding period and weighed discs of *F. vesiculosus* (0.21 - 0.73 μg/L; Pastorinho et al., 2011). Whilst the 96-h EC_{50} value would normally be expected to be lower than the 48-h EC_{50} and the LC_{50} (e.g., Martinez-Haro et al., 2014), differences in methodological procedures could explain these discrepancies. In one case, amphipods were exposed for 96-h and then fed for 24 h with weighed discs of *F. vesiculosus* (Pastorinho et al., 2011). In our study, amphipods were exposed for 48-h and then fed for just 30 min with *Artemia* nauplii. Another explanation could be related to the different Cd content in the *Fucus* and *Artemia* (i.e., 0.28 *vs* 0.025 μg Cd/g, respectively; Pastorinho et al., 2009). Perhaps, a higher Cd content in *Fucus* may increase sensitivity in animals previously exposed. However, no data on Cd content in the discs of *F. vesiculosus* offered to *E. marinus* were reported by Pastorinho et al. (2011). Likewise, the *Artemia* nauplii used here were not analysed (these were hatched from brine shrimp eggs using Ocean Nutrition™, assumed to have an insignificant metal content).

To the best of our knowledge, this is the first study to consider the lethal toxicity of a reference toxicant to *E. marinus.* Previously, Pastorinho et al. (2009) evaluated Cd uptake and bioaccumulation in *E. marinus* at different life stages (including juveniles) after 96 h of Cd exposure (at 1 mg/L). These authors also evaluated the concentration causing a 50% feeding decay after 96 h of exposure to different metals (Zn, Cd, Cu and

Ni), and their mixtures, in *E. marinus* from populations along a latitudinal gradient. However, neither study reported data regarding mortality rates. Our data highlighted that *E. marinus* is a medium sensitivity amphipod when compared with other species commonly used for ecotoxicological surveys (Table 2). Since the bioavailability of Cd has been shown to decrease with increasing salinity for a number of crustacean species (e.g., Wright, 1995), a direct comparison between LC_{50} values for freshwater and brackish water amphipods should however be considered with caution.

535 Table 2. Acute toxicity, 48-h and 96-h LC50 (mg Cd/L), of Cd reported in literature for amphipods used in bioassays.

Species	Habitat	Age	48-h LC50	96-h LC50	Reference
Bathyporeia pilosa	BW	J	1.46	0.35	Strode and Balode 2013
Echinogammarus marinus	BW	J	0.93		This study
Eohaustorius estuaries	BW	J		41.9	Meador 1993
Gammarus tigrinus	BW	J	0.068	0.019	Strode and Balode 2013
Leptocheirus plumulosus	BW	J		0.3	McGee et al. 1998
Marinogammarus obtusatus	BW	J		3.5	Wright and Frain 1981
Monoporeia affinis	BW	J	12.6	5	Strode and Balode 2013
Pontogammarus robustoides	BW	J	0.25	0.014	Strode and Balode 2013
Corophium orientale	BW	A		1.2-7.23	Lera et al. 2008, Onorati et al. 1999, Bigongiari et al. 2004
Gammarus tigrinus	BW	A	0.269#		Boets et al. 2012
Chelura terebrans	BW/SW	A		0.63	Hong and Reish 1987
Corophium insidiosum	BW/SW	A		1.27	Hong and Reish 1987
Elasmopus bampo	BW/SW	A		0.57	Hong and Reish 1987
Grandidierella japonica	BW/SW	A		1.17	Hong and Reish 1987
Rhepoxynius abronius	BW/SW	A		0.24	Hong and Reish 1987
Gammarus fossarum	FW	J	0.01*	0.05*	Alonso et al. 2010
Gammarus pulex	FW	J	0.022-1*	0.005-0.5*	Alonso et al. 2010, Strode and Balode 2013, McCahon and Pascoe 1988
Hyalella azteca	FW	J	0.0035-0.015	0.007	Strode and Balode 2013; Gust 2006
Crangonyx pseudogracilis	FW	A	34.6	1.7	Martin and Holdich 1986
Dikerogammarus villosus	FW	A	0.0257		Boets et al. 2012
Echinogammarus berilloni	FW	A	0.602	0.089	Boets et al. 2012
Gammarus fossarum	FW	A	0.015-0.1*	0.0062-0.2*	Alonso et al. 2010, Boets et al. 2012, Musko et al. 1990
Gammarus pulex	FW	A	0.25-8*	0.02-1.5*	Alonso et al. 2010, Boets et al. 2012, Felten et al. 2008, Vellinger et al. 2012, Williams et al. 1985
Gammarus roseli	FW	A		0.0086	Boets et al. 2012

536 BW = Brackish water; FW = Fresh water; SW = Seawater; A = Adult; J = Juvenile; # Carried out under FW conditions* Obtained from a figure.

4.4. In situ assay

The in situ assay developed and harmonized in this study was able to discriminate between unpolluted and polluted estuaries. It was deployed in a reference estuary along key environmental gradients to factor in feeding response in relation to environmental factors. The assay based on postexposure feeding as an endpoint, appears to be very effective at responding within short time periods (i.e., in just 30 min). Results indicated that the proportion of sediment with a size fraction between 500-1000 μm (coarse sand) and 1000-2000 μm (very coarse sand) were important in terms of modulating feeding rates in *E. marinus*. In this sense, substrates with particle size between 500-1000 μm had a negative influence on post-exposure feeding rate, whereas particle sizes between 1000-2000 μm had a positive influence. After taking into account the strong co-linearity among variables detected, the reason why these environmental variables affected feeding activity for *E. marinus* must be related to intrinsic ecological characteristics. In fact, substrate preferences by two sympatric estuarine amphipods (*E. marinus* and *Eulimnogammarus obtusatus*) were studied by Maren (1975) as a potential factor to influence their spatial distribution. The granulometric properties of sediment are a key factor modulating survival, growth and reproduction for amphipods (e.g., Costa et al., 1998; DeWitt et al., 1988; Emery et al. 1997). Postexposure feeding rates were adjusted according to these factors. Adjusted data showed lower feeding rates in the Lima estuary, confirming its polluted status (e.g., Gravato et al., 2010; Guimarães et al., 2012). Results also demonstrated the suitability of *E. marinus* as a test organism for postexposure in situ assays. Furthermore, the absence of significant differences between replicates of adjusted

postexposure feeding rates after in situ deployment in reference or polluted estuaries suggests a high robustness and precision of this assay.

Several studies have reported on the polluted status of the estuary of the Lima river and the effects that such pollution has had on organisms that inhabit the estuary (e.g., Gravato et al. 2010, Guimarães et al., 2012; Rodrigues et al., 2012). Azevedo et al. (2013) also considered the ecological quality of sites on the Lima estuary (two of which were used in our study: P1 and P2 correspond with the sites L3 and L2, respectively, of their study). These authors described P1 as a heavily disturbed and P2 as a moderately disturbed location. These sites were also evaluated using two community based indices: a macroinvertebrate index based on average macroinvertebrate abundance of species tolerant to disturbance (M-AMBI: Multivariate - AZTI Marine Biotic Index; Muxika et al., 2007) and a demersal index based on demersal fish and crustacean data (EDI: Estuarine Demersal Index; Borja et al., 2004). Based on these indices, Azevedo et al. (2013) classified P1 as having Poor or Moderate Ecological Quality (M-AMBI and EDI, respectively), and P2 as having Good Ecological Quality Status (after M-AMBI assessment; EDI was not performed). Additionally, the toxicity of sediment elutriates was also assessed using ToxScreen, a bioassay based on inhibition (%) of luminescence in a highly sensitive strain of the bacterium *Photobacterium leiognathi* (Ulitzur et al., 2002). Results indicated that both sediments induced a reduction in bacterial luminescence of > 50% which was compatible with a toxic effect (Azevedo et al., 2013). The in situ assay used here was not able to discriminate between P1 and P2, but as in previously described work, it apparently detected higher levels of pollution in P1.

Conclusion

This in situ assay using the amphipod *E. marinus* and based on postexposure feeding, appears to have potential as a tool for environmental estuarine assessment. By using this target species, a new short-term, sublethal, in situ toxicity assay based on 48 h of exposure followed by a 30 min postexposure feeding period is suggested. This adds to other similar assays that are available for estuarine environments, which involve the use of the polychaetes *Hediste diversicolor* and *Neanthes arenaceodentata* (Moreira et al., 2006b; Rosen and Miller, 2011), the isopod *C. carinata* (Martinez-Haro et al., 2014), the gastropod *Hydrobia ulvae* (Krell et al., 2011) and the crab *C. maenas* (Moreira et al., 2006a). These in situ assays are promising tools for environmental monitoring programmes; however, they also have some limitations that require further study. The models developed here and in other similar work have generally been developed under a relatively limited range of environmental conditions - therefore, in order to widen the applicability of these assays, they must be generalized and validated within other differing environmental scenarios and during different seasons. Together these assays encompass a wide range of estuarine trophic levels and therefore ecosystem functions, as such, they have the potential to be included within (or form themselves) a battery of in situ bioassays for use in monitoring programmes (alongside more conventional community based approaches) (Martinez-Haro et al., 2015). Batteries of multi-species assays, which include organisms from different trophic levels, are certainly required, due to their capacity to provide more comprehensive information regarding the impact of environmental pollution (Kokkali and van Delft, 2014; Repetto, 2013). Selection of the most appropriate test species for use in battery bioassays is critical (Narracci et al., 2009), and it is important that the correct battery is also selected for each particular case study (so as to avoid obtaining inappropriate or redundant information). Functional

ecotoxicological tools such as this one are considered of benefit if we are to obtain more accurate and effective ecological quality assessments – something which is ultimately considered crucial within the EU's Water Framework Directive (Reyjol et al., 2014).

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Appendix A. Supplementary material

Supplementary material includes a description of the chambers used for in situ deployments and the Standard Operating Procedure (SOP) for the amphipod *E. marinus* in situ postexposure feeding assay.

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