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

2

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27 **ABSTRACT**

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

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

48

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

50 *Keywords:* Cadmium; *Echinogammarus marinus*; Environmental monitoring;

51 Postexposure feeding; Sublethal endpoint.

52 **1. Introduction**

53

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

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

77 become affected (Baird et al., 2007; Maltby et al., 2002; McWilliam and Baird, 2002).
78 Even though the vast majority of in situ assays are based on mortality or a chronic
79 response such as differences in size, fertility, sex ratio, etc., following long-term
80 exposure (i.e., 10-d or 28-d; e.g., Castro et al., 2006; Sanz-Lázaro and Marín, 2009),
81 short-term (i.e., 24-h, 48-h or 96-h) assays based on nonlethal endpoints (such as
82 feeding inhibition) have received more attention recently (e.g., Martinez-Haro et al.,
83 2014; McWilliam and Baird, 2002; Moreira et al., 2006a; Rosen and Miller, 2011).

84 Water-only and sediment toxicity tests using amphipods were first developed in
85 the 1970s - 1980s (e.g., Bellan-Santini and Reish, 1976; Lindén, 1976; Swartz et al.,
86 1979) and since then, have been widely deployed in estuarine and marine habitats
87 (ASTM, 2008; Costa et al., 1998; Rosen et al., 2012; Scarlett et al., 2007; USEPA,
88 1994; Van Geest et al., 2014). Amphipods are abundant and ecologically important
89 components of aquatic communities and changes in amphipod populations may also
90 affect the structure and functioning of the wider ecosystem by affecting food
91 availability at higher trophic levels (Baird et al., 2007; Coulaud et al., 2015; Kunz et al.,
92 2010; Maltby et al., 2002). Amphipods also have many desirable characteristics that
93 make them useful in ecotoxicology and environmental biomonitoring, i.e., i) their wide
94 geographic distribution, ii) they are easy to handle and to culture under laboratory
95 conditions, and iii) they are highly sensitive to contaminants and adequate species for
96 early-warning monitoring – often being the first macroinvertebrates to disappear from
97 contaminated sites (Dauvin, 1998; Swartz et al., 1982). The most common
98 methodology to study feeding activity of freshwater or marine amphipods uses the loss
99 of mass of preweighed discs (supporting plants or algae) after being offered to an
100 individual for a given time (e.g., Felten et al., 2008; Maltby et al., 2002; Nyman et al.,
101 2013; Pastorinho et al., 2011; Schmidlin et al., 2015). Methodologies based on the loss

102 of mass require laborious laboratory effort. Discs used in feeding quantification
103 experiments must be dried and weighed accurately and repeatedly, which is time-
104 consuming. However, few studies consider the use of alternative food items (Agostinho
105 et al., 2012; Taylor et al., 1993) or alternative quantification procedures (Coulaud et al.,
106 2015; Gerhardt et al., 1994; Nyman et al., 2013). Thus, here, we aimed to develop and
107 evaluate a short-term, cost-effective, in situ toxicity assay based on the postexposure
108 feeding rates of an estuarine amphipod (*Echinogammarus marinus*) on easily
109 quantifiable food items (nauplii of the brine shrimp *Artemia franciscana*).

110

111 **2. Materials and methods**

112

113 *2.1. Study species*

114

115 *Echinogammarus marinus* was used as it is a widely distributed euryhaline
116 benthic amphipod with an ecologically relevant role in European estuarine food webs
117 (Marques, 1989). It is abundant in intertidal soft-sediment and rocky estuarine habitats
118 covered with the macroalgae *Fucus* spp. (Marques, 1989), making it an appropriate
119 target species for the assessment of environmental pollution in both water and sediment
120 compartments. In Portugal, the Mondego estuary (Western Coast of Portugal:
121 40°8'10"N, 8°50'51"W) represents the most southerly distribution limit for this species,
122 which extends northwards to Norway and Iceland (Lincoln, 1979). *Echinogammarus*
123 *marinus* is omnivorous, feeding on a wide range of plant material (including a number
124 of algae species), as well as prey such as hard-bodied isopods and soft-bodied
125 oligochaetes (Alexander et al., 2013a). This species is also an important prey item for
126 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 °C in darkness for use during subsequent laboratory
141 culture work.

142 Once in the laboratory, organisms were sorted and maintained at 20 ± 1 °C
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 CaCl₂.2H₂O, 4.6 g MgCl₂.H₂O, 5.58 g MgSO₄.7H₂O, 0.17 g
149 NaHCO₃ and 0.03 g H₃BO₃ 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.

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

157

158 *2.3. Feeding quantification*

159

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

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

173 An initial experiment was carried out to evaluate differences in feeding rates
174 among three size classes of *E. marinus*: small (mean \pm SE, 6.5 ± 0.6 mm, $n = 12$) which
175 corresponds to juveniles, medium (9.4 ± 0.6 mm, $n = 12$) and large size classes ($13.6 \pm$
176 0.8 mm, $n = 12$); the last two classes corresponding to adult animals (Marques and

177 Nogueira, 1991). For organisms of different size classes we conducted a feeding
178 quantification experiment with three different amounts of food (50, 100 and 150 nauplii
179 per individual). This was performed during a 30-min feeding period. A second
180 experiment evaluated three different feeding periods (30 min, 60 min, and 120 min) for
181 a fixed number of food items (100 nauplii). We optimised food quantity by carrying out
182 a third experiment involving further food item variation (50, 60 and 100 nauplii) over a
183 30-min feeding period. Finally, we assessed the potential effect of different water
184 volumes added to the glass vials where the feeding of *E. marinus* took place. Four
185 volumes (3, 5, 7 and 10 ml) were tested with a fixed quantity of food and feeding
186 period (60 nauplii and 30 min, respectively). A harmonized protocol for feeding
187 quantification was thus derived and used for the in situ assays.

188 Twenty replicates were used for each experimental group (for each assessed
189 factor, a group was the organisms within the same age class), except for the size class
190 experiment (in which 12 replicates were used). Each replicate consisted of one
191 organism housed in a 30-ml glass beaker filled with 5 ml of RSW, except for the water
192 volume experiment (in which 3, 5, 7 and 10 ml were used). Amphipods were allowed to
193 feed for a given period of time (30 min, except for the feeding period experiment in
194 which 30, 60 and 120 min were used) at 20 °C in darkness. Dark conditions were
195 chosen because feeding activity increases at night in this species (e.g., Alexander et al.,
196 2013b). At the end of the feeding period, nauplii remaining were counted under a
197 stereomicroscope and feeding rates estimated as the number of nauplii ingested per
198 amphipod in a given time (nauplii/amphipod/30 min, except for the feeding period
199 experiment in which 30 min, 60 min, or 120 min were also considered).

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

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

208

209 *2.4. Lethality and postexposure feeding tests*

210

211 Cadmium was the reference toxicant (USEPA, 1994) used to determine the 48-h
212 median lethal concentration (48-h LC₅₀) for the lethality assay and the 48-h median
213 effective concentration (48-h EC₅₀) for the postexposure feeding assay. A stock
214 solution containing 250 mg/L Cd was prepared using CdCl₂ (Acros Organic, Geel,
215 Belgium) and nanopure water (conductivity < 5 µS/cm; Seralpur PRO 90 CN, Seral,
216 Ransbach-Baumbach, Germany). Test solutions were obtained by serial dilution of the
217 stock solution with control RSW. Preliminary feeding tests were carried out to establish
218 a suitable Cd concentration range. The following six and eight Cd concentrations were
219 used for the lethal and sublethal tests, respectively: 0, 0.125, 0.25, 0.5, 1, and 2 mg
220 Cd/L, and 0, 0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg Cd/L.

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

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

231 Cadmium concentrations were analysed by atomic absorption spectroscopy
232 (AAAnalyst800, PerkinElmer). The minimum detection limit was 0.2 µg Cd/L. The Cd
233 concentration in RSW (control) was below the limit of detection. To observe whether
234 Cd concentrations were maintained during the experiment, concentrations from test
235 solutions were measured at the beginning and end of each experiment. Because real and
236 nominal concentrations of the test solutions differed by less than 10%, the latter were
237 used for toxicity endpoint estimates. Median effective lethal (48-h LC₅₀ value) and
238 postexposure feeding (48-h EC₅₀ value) Cd concentrations, and their respective 95%
239 confidence intervals (CI), were derived using the Trimmed Spearman–Kärber method
240 (Hamilton et al., 1977).

241

242 *2.5. In situ design*

243

244 *2.5.1. Environmental sites*

245

246 Six environmental sites were selected for in situ assays at a reference and a
247 polluted estuarine site, both located in the Northwest of the Iberian Peninsula, along the
248 Portuguese coast (see **Table 1**): Minho river estuary (Caminha, Portugal) and Lima
249 river estuary (Viana do Castelo, Portugal). The Minho river estuary is an undisturbed
250 estuary subject to low human pressure (Ferreira et al., 2003) that is a NATURA 2000
251 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

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

	Minho				Lima	
	R1	R2	R3	R4	P1	P2
Coordinates						
Latitude	41° 52' 02.55''N	41° 52' 01.71''N	41° 51' 59.92''N	41° 52' 16.42''N	41° 41' 17.65''N	41° 40' 59.05''N
Longitude	8° 51' 35.16''W	8° 51' 20.61''W	8° 51' 01.08''W	8° 50' 40.10''W	8° 48' 53.81''W	8° 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 (°C)	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

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

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

301

302 2.5.3. *Environmental gradients*

303

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 °C for 24 h) remaining after
318 igniting the sample in a muffle furnace (Thermo Fisher Scientific, Thermolyne F6000,
319 Essex, United Kingdom) at 550 °C 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

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

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

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

354

355 3. Results

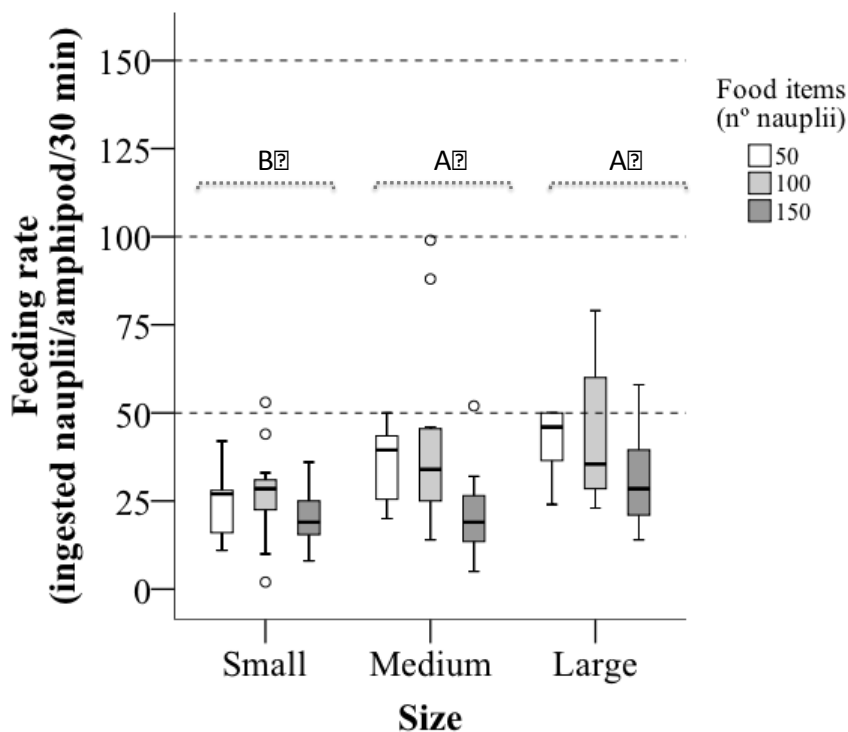
356

357 3.1. Feeding quantification

358

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

367



368

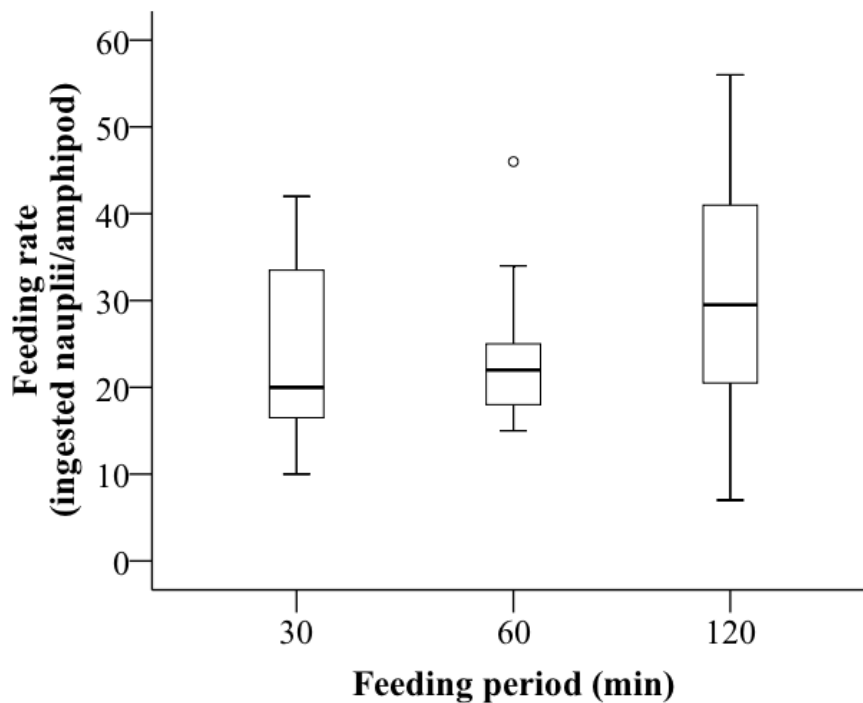
369

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

377

378 No statistical differences were detected among the feeding periods tested ($F_{2,50}$
379 = 2.232, $p = 0.118$; **Fig. 2**). Statistically different feeding rates were however detected
380 when amphipods were allowed to eat 50, 60 or 100 nauplii ($F_{2,57} = 4.651$, $p = 0.013$).
381 Finally, no significant differences were found among the test volumes ($F_{3,76} = 0.477$, p
382 = 0.699).

383



384

385

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

390

391 3.2. Cadmium lethality and postexposure feeding tests

392

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

397 Dissolved oxygen in each test chamber was above 60% saturation at all times
398 for both the lethal and postexposure feeding tests (ASTM, 2002). Mean (\pm SD) values
399 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
400 and 33.4 ± 0.24 g/L for the postexposure feeding test.

401

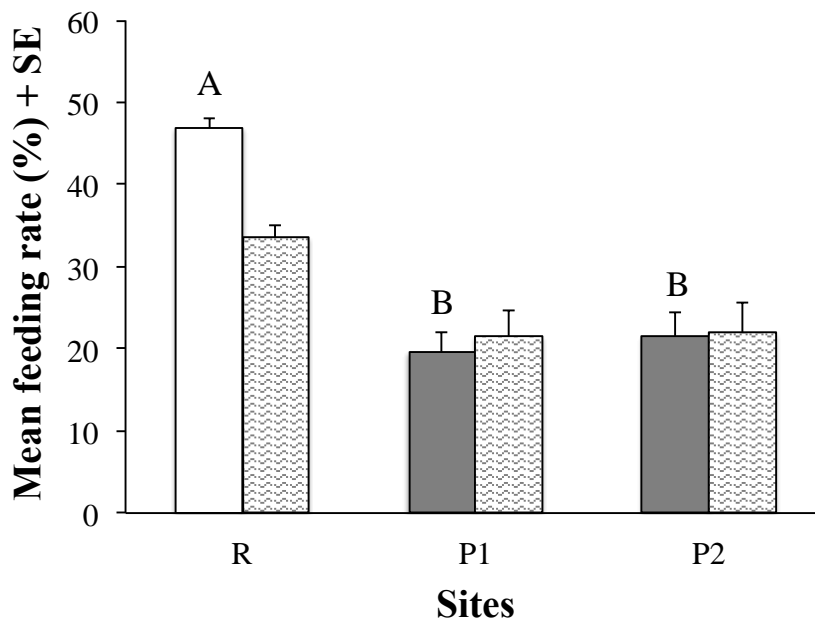
402 3.3. In situ assay

403

404 Table 1 summarizes values for the environmental parameters at each
405 environmental site. At the end of the in situ assay deployment, all chambers were found
406 intact and in their original position. After controlling for multicollinearity, salinity at
407 low tide, salinity at high tide, proportion of sediment present with a size of between
408 500-1000 μ m and 1000-2000 μ m, and organic matter content, were considered for
409 modelling. Statistical modelling showed that feeding rates at the reference estuary were
410 influenced by the proportion of sediment present with a size of between 500-1000 μ m

411 and 1000-2000 μm . The relationship between feeding rate (*PEF*) and the percentage of
412 the two size fractions of sediment (*X1*: sediment between 500-1000 μm ; *X2*: sediment
413 between 1000-2000 μm ,) was (\pm SE): $\arcsin PEF = 45.586 (\pm 2.012) - 1.059 (\pm$
414 $0.207) \cdot X1 + 1.871 (\pm 1.190) \cdot X2$ (Adjusted $R^2 = 0.260$, $p < 0.001$).

415 After adjusting to the mean value of predictors in the polluted estuary (11.9% of
416 sediment at 500-1000 μm and 5.4% of sediment at 1000-2000 μm), no differences in
417 feeding rate among sites or replicates were detected at the reference estuary ($F_{3,99} =$
418 0.009 , $p > 0.05$; $F_{8,99} = 0.936$, $p > 0.05$, respectively). Differences in adjusted
419 postexposure feeding rates were detected between animals from reference and polluted
420 estuaries ($F_{2,139} = 28.446$, $p < 0.001$; **Fig. 3**), but not among replicates ($F_{14,139} = 0.718$, p
421 > 0.05).



422

423

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

428 +1 standard error. Means sharing the same capital letter did not differ significantly
429 (Least Significant Differences, $p > 0.05$).

430

431 **4. Discussion**

432

433 *4.1. Food items*

434

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

446

447 *4.2. Feeding quantification*

448

449 Three different *E. marinus* size classes (juveniles and two classes of adults)
450 along with four different numbers of food items (50, 60, 100 and 150 nauplii provided)
451 and three feeding periods (30, 60 and 120 min) were tested in order to optimize the
452 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

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

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

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

496

497 *4.3. Cadmium lethality and postexposure feeding tests*

498

499 Results obtained from our toxicity tests indicated that postexposure feeding
500 activity was a more sensitive endpoint than mortality for *E. marinus*. The 48-h EC₅₀ for
501 Cd for postexposure feeding was approximately 1.4 times lower than the 48-h LC₅₀

502 (0.66 mg/L vs 0.93 mg/L, respectively). For comparative purposes, the relationship
503 between mortality and feeding inhibition, over the same Cd exposure period, was also
504 reported by Felten et al. (2008) using the freshwater amphipod *G. pulex*. Therein, the
505 reported 168-h LC₅₀ was 21.6 µg Cd/L, and a feeding rate reduction of 36% was
506 observed after 168 h in amphipods exposed to 15 µg Cd/L (i.e., 1.4 times lower than
507 the 168-h LC₅₀). The 48-h EC₅₀ value reported in our study is higher than the 96-h EC₅₀
508 value reported for this species previously when using a 24-h postexposure feeding
509 period and weighed discs of *F. vesiculosus* (0.21 - 0.73 µg/L; Pastorinho et al., 2011).
510 Whilst the 96-h EC₅₀ value would normally be expected to be lower than the 48-h EC₅₀
511 and the LC₅₀ (e.g., Martinez-Haro et al., 2014), differences in methodological
512 procedures could explain these discrepancies. In one case, amphipods were exposed for
513 96-h and then fed for 24 h with weighed discs of *F. vesiculosus* (Pastorinho et al.,
514 2011). In our study, amphipods were exposed for 48-h and then fed for just 30 min with
515 *Artemia* nauplii. Another explanation could be related to the different Cd content in the
516 *Fucus* and *Artemia* (i.e., 0.28 vs 0.025 µg Cd/g, respectively; Pastorinho et al., 2009).
517 Perhaps, a higher Cd content in *Fucus* may increase sensitivity in animals previously
518 exposed. However, no data on Cd content in the discs of *F. vesiculosus* offered to *E.*
519 *marinus* were reported by Pastorinho et al. (2011). Likewise, the *Artemia* nauplii used
520 here were not analysed (these were hatched from brine shrimp eggs using Ocean
521 NutritionTM, assumed to have an insignificant metal content).

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

527 Ni), and their mixtures, in *E. marinus* from populations along a latitudinal gradient.
528 However, neither study reported data regarding mortality rates. Our data highlighted
529 that *E. marinus* is a medium sensitivity amphipod when compared with other species
530 commonly used for ecotoxicological surveys (Table 2). Since the bioavailability of Cd
531 has been shown to decrease with increasing salinity for a number of crustacean species
532 (e.g., Wright, 1995), a direct comparison between LC₅₀ values for freshwater and
533 brackish water amphipods should however be considered with caution.
534

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

537 4.4. *In situ* assay

538

539 The *in situ* assay developed and harmonized in this study was able to
540 discriminate between unpolluted and polluted estuaries. It was deployed in a reference
541 estuary along key environmental gradients to factor in feeding response in relation to
542 environmental factors. The assay based on postexposure feeding as an endpoint,
543 appears to be very effective at responding within short time periods (i.e., in just 30
544 min). Results indicated that the proportion of sediment with a size fraction between
545 500-1000 μm (coarse sand) and 1000-2000 μm (very coarse sand) were important in
546 terms of modulating feeding rates in *E. marinus*. In this sense, substrates with particle
547 size between 500-1000 μm had a negative influence on post-exposure feeding rate,
548 whereas particle sizes between 1000-2000 μm had a positive influence. After taking
549 into account the strong co-linearity among variables detected, the reason why these
550 environmental variables affected feeding activity for *E. marinus* must be related to
551 intrinsic ecological characteristics. In fact, substrate preferences by two sympatric
552 estuarine amphipods (*E. marinus* and *Eulimnogammarus obtusatus*) were studied by
553 Maren (1975) as a potential factor to influence their spatial distribution. The
554 granulometric properties of sediment are a key factor modulating survival, growth and
555 reproduction for amphipods (e.g., Costa et al., 1998; DeWitt et al., 1988; Emery et al.
556 1997). Postexposure feeding rates were adjusted according to these factors. Adjusted
557 data showed lower feeding rates in the Lima estuary, confirming its polluted status
558 (e.g., Gravato et al., 2010; Guimarães et al., 2012). Results also demonstrated the
559 suitability of *E. marinus* as a test organism for postexposure *in situ* assays.
560 Furthermore, the absence of significant differences between replicates of adjusted

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

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

584

585 **Conclusion**

586

587 This in situ assay using the amphipod *E. marinus* and based on postexposure feeding,
588 appears to have potential as a tool for environmental estuarine assessment. By using
589 this target species, a new short-term, sublethal, in situ toxicity assay based on 48 h of
590 exposure followed by a 30 min postexposure feeding period is suggested. This adds to
591 other similar assays that are available for estuarine environments, which involve the use
592 of the polychaetes *Hediste diversicolor* and *Neanthes arenaceodentata* (Moreira et al.,
593 2006b; Rosen and Miller, 2011), the isopod *C. carinata* (Martinez-Haro et al., 2014),
594 the gastropod *Hydrobia ulvae* (Krell et al., 2011) and the crab *C. maenas* (Moreira et
595 al., 2006a). These in situ assays are promising tools for environmental monitoring
596 programmes; however, they also have some limitations that require further study. The
597 models developed here and in other similar work have generally been developed under
598 a relatively limited range of environmental conditions - therefore, in order to widen the
599 applicability of these assays, they must be generalized and validated within other
600 differing environmental scenarios and during different seasons. Together these assays
601 encompass a wide range of estuarine trophic levels and therefore ecosystem functions,
602 as such, they have the potential to be included within (or form themselves) a battery of
603 in situ bioassays for use in monitoring programmes (alongside more conventional
604 community based approaches) (Martinez-Haro et al., 2015). Batteries of multi-species
605 assays, which include organisms from different trophic levels, are certainly required,
606 due to their capacity to provide more comprehensive information regarding the impact
607 of environmental pollution (Kokkali and van Delft, 2014; Repetto, 2013). Selection of
608 the most appropriate test species for use in battery bioassays is critical (Narracci et al.,
609 2009), and it is important that the correct battery is also selected for each particular case
610 study (so as to avoid obtaining inappropriate or redundant information). Functional

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

615

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617

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635

636 **Appendix A. Supplementary material**

637

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

641

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