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Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta:* II. Organism and population-level endpoints

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Abstract

This study aimed to test the performance of the amphipod *Gammarus locusta* (L.) in chronic sediment toxicity tests. It constitutes part of a multi-level assessment of chronic toxicity of estuarine sediments, integrating organism and population-level endpoints with biochemical markers responses. Here we account for organism and population-level effects, while biomarker responses were reported in a companion article. Five moderately contaminated sediments from Sado and Tagus estuaries were tested, comprising 3 muddy and 2 sandy sediments. These sediments either did not show acute toxicity or were diluted with control sediment as much as required to remove acute toxicity. Subsequent chronic tests consisted of 28-day exposures with survival, individual growth and reproductive traits as endpoints. Two of the muddy sediments induced higher growth rates in the amphipods, and improved reproductive traits. This was understood to be a consequence of the amount of organic matter in the sediment, which was nutritionally beneficial to the amphipods, while concurrently decreasing contaminant bioavailability. Biomarker responses did not reveal toxicant-induced stress in amphipods exposed to these sediments. One of the sandy sediments was acutely toxic at

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50% dilution, but in contrast stimulated amphipod growth when diluted 75%. This was presumed to be an indication of a hormetic response. Finally the two remaining contaminated sediments showed pronounced chronic toxicity, affecting survival and reproduction. The sex ratio of survivors was highly biased towards females, and offspring production was severely impaired. The particulars of the responses of this amphipod were examined, as well as strengths versus limitations of the sediment test. This study illustrates the utility of this chronic test for toxicity assessment of contaminated estuarine sediments, with potential application all along Atlantic Europe.

Keywords: Chronic toxicity; Estuarine sediment; Amphipod; Europe; Population; Growth; Reproduction; Hormesis; Sado; Tagus

1. Introduction

Toxicity tests have been increasingly applied for assessment of freshwater, marine and estuarine sediment quality (Traunspurger & Drews, 1996). Numerous whole sediment tests have been developed, comprising various invertebrate taxa. Yet, most of them consist of short-term exposures (usually 10 days maximum), considering only survival as the endpoint, or in some cases including behavior criteria, such as sediment avoidance or failure to rebury (e.g., Chapman & Wang, 2001; Traunspurger & Drews, 1996; USEPA, 1999). On the contrary, "methods that measure sub-lethal effects have not been available or have not been routinely used to evaluate sediment toxicity in marine and estuarine sediments" (USEPA-USACE, 2001).

Amphipod crustaceans are among the most widely used invertebrate taxa in acute marine and estuarine whole sediment toxicity tests (Chapman & Wang, 2001). In North America, standardized protocols have been developed for five amphipod species (ASTM, 1992; Environment Canada, 1992) and in New Zealand, several protocols are under development (DeWitt et al., 1999; Marsden & Wong, 2001). In Europe, *Corophium volutator* and *Gammarus locusta* are among the few amphipod species for which there are published protocols for marine and estuarine whole sediment tests (Bat & Raffaelli, 1998; Costa, Correia, & Costa, 1998, respectively). Compared to acute tests using amphipods, very few chronic tests have been developed, standardized and widely applied. An exception is the North American amphipod *Leptocheirus plumulosus*, and to a certain extent *Ampelisca abdita*, for which chronic toxicity protocols have been developed (Emery et al., 1997; Redmond, Scott, Swartz, & Jones, 1994; USEPA-USACE, 2001; Weston, 1996), and which have been used in various studies of chronic sediment toxicity. To the best of our knowledge, no such chronic test with European amphipod species has been fully developed and/or protocol has been described (cf. Nendza, 2002). For the amphipod *C. volutator* a standardized protocol for acute marine sediment toxicity has been created under the auspices of the Oslo and Paris Commissions (PARCOM, 1993) and given its wide use and amount of toxicological information, can be considered an European "benchmark" species (in the sense of Chapman, 1995). Yet, most of the published

studies with *C. volutator* still concern only acute sediment toxicity (Ciarelli, Vonck, & van Straalen, 1997; Grant & Briggs, 2002; Matthiessen et al., 1998) and very few considered chronic exposures or other endpoints than survival and burrowing behavior (e.g., Ciarelli, Vonck, van Straalen, & Stronkhorst, 1998; Conradi & Depledge, 1999). Therefore, despite the growing use of sediment toxicity tests as a fundamental component of ecotoxicological assessments, and the enormous scientific effort devoted to them in the last decade, the new generation (Moore & Dillon, 1993) of chronic marine and estuarine sediment tests is yet to be developed, at least in Europe.

Chronic toxicity tests are an exceptionally valuable environmental assessment tool, since they consider various relevant environmental circumstances that are not addressed in acute sediment tests: (1) chronic exposure to contaminants is much more common in natural environments than acute exposure, (2) moderately contaminated sediments are more common than highly contaminated ones and (3) biological effects of contamination other than survival may be of greater ecological relevance for understanding the impact of contaminants on organisms and ecosystems (Emery et al., 1997; Ingersoll, Brunson, Dwyer, Hardesty, & Kemble, 1998; USEPA-USACE, 2001).

Development of chronic bioassays involves much greater scientific and technological effort than acute bioassays, which may explain why they are not used so often. Selection of test organisms is one of the critical topics. Few test organisms have appropriate characteristics, and not all species that are adequate for acute toxicity tests can be used in chronic testing. For chronic tests, preference is given to organisms with a relatively short life cycle, enabling assessment of biological effects at endpoints such as growth and reproductive features within a few weeks period (e.g., Emery et al., 1997; Ingersoll et al., 1998). Also the ecology of the test species, particularly demographic and reproductive features, must be given careful consideration. A step-wise procedure should be followed to determine experimental conditions of toxicity tests, sensitivity to confounding variables, and response criteria, after exposure to controlled amounts of a reference sediment toxicant in the laboratory (e.g., DeWitt, Redmond, Sewall, & Swartz, 1992). Finally the test must be thoroughly evaluated using trial toxicity tests with field-collected sediments in order to determine the test's sensitivity, to analyse the biological responses, and identify test features requiring modification and/or improvement.

This study constitutes the culminating step of a series of integrated studies aiming to develop a chronic sediment test with the amphipod *G. locusta*. This strategy entailed: (1) update of the available information concerning the distribution and ecology of wild populations of *G. locusta* (Costa & Costa, 2000), (2) analysis of its life history at our reference location (Sado estuary) (Costa & Costa, 1999), (3) development of a culturing system (Costa, Correia, & Costa, 1996; Costa, 1997), (4) analysing the impact of non-contaminant variables (Costa et al., 1996), establishing the experimental conditions to conduct chronic tests (Neuparth, Costa, & Costa, 2002) and investigating the impact of chronic exposure to a reference toxicant using laboratory-spiked sediments (Correia, Costa, Neuparth, Diniz, & Costa, 2001, our unpublished data). In addition, recently several biomarkers of exposure and stress at the molecular, biochemical and cellular levels have been developed for this

amphipod (Correia, Lima, Costa, & Livingstone, 2002; Costa, Neuparth, Costa, Theodorakis, & Shugart, 2002). By integrating biomarkers in sediment tests we seek to differentiate toxicant-induced impacts from other effects derived from sediments' geochemical features.

Here, we provide a first assessment of the performance of *G. locusta* under chronic exposure to moderately contaminated estuarine sediments, this way enabling optimisation of the developing chronic sediment test. The current study is a component (part II) of a larger investigation that consisted on the assessment of chronic sediment toxicity with *G. locusta*, integrating organism – and population-level endpoints (survival, growth and reproductive traits) with biochemical markers. Here, we focus on organism/population-level effects, while biomarker responses were reported in a companion paper – part I (Neuparth, Correia, Costa, Lima, & Costa, 2004).

2. Materials and methods

2.1. Sediments

Control and test sediments were collected from Sado and Tagus estuaries. Relevant features of the collection sites, description of sediments sampling and processing, and geochemical analysis, is presented in part I of this study (Neuparth et al., 2004).

2.2. Chronic sediment tests

Two independent chronic toxicity tests were performed in this study, which will be hereafter referred to as chronic test 1 and chronic test 2. Sediments P and T were tested in chronic test 1, and sediments S1, S2 and D were tested in chronic test 2. A general description of the chronic tests experimental conditions is presented in part I (Neuparth et al., 2004).

At the end of the assays the overlying water of each replicate was sieved through 1000 and 250 μm screens, to collect surviving adults and their offspring, respectively. Sediments were washed at least five times to assure that all organisms were removed. The neonates were transferred to plastic flasks and preserved in 70% ethanol with Bengal rose for latter counting.

A binocular microscope coupled with an imaging workstation (Leica) was used to photograph all adult survivors and to store the respective pictures in a database before further processing. *G. locusta* has prominent sexual dimorphism (Lincoln, 1979) and sex could be easily distinguished. An evenly distributed number of males per replicate was then used for biomarker analysis, as described in Neuparth et al. (2004). All pregnant females were preserved in 70% ethanol for posterior analysis as described below.

2.3. Sample processing and determination of individual and population endpoints

Organism and population endpoints were determined separately for each sex (when applicable) and comprised survival, sex ratio, individual growth and repro-

ductive traits. The latter included percentage of gravid females, offspring production, fecundity, embryo size and embryo development stage. All of these endpoints were measured separately in each replicate, at the end of the experiment. Number of male and female survivors was converted to percentage. Sex ratio in each replicate was determined as the number of surviving males divided by the number of surviving females.

Since amphipods used at the beginning of the assay were all juveniles from a rigorously limited and known size class (2–4 mm), individual growth was determined as the individual length at the end of the assay. For that purpose, the metasomatic length (ML) was used, which is defined as the distance between the anterior end of the rostrum and the posterior end of the last metasomatic segment (e.g., DeWitt et al., 1992). Length was determined from the individual photographs with the help of the software Leica QWin (Version 2.3) which enables measuring the curve along the dorsal side of the amphipods (the image analysis system was previously calibrated with a 10.0 mm standard). For statistical comparisons length data from each sex were pooled within each treatment (sediment).

Gravid females were carefully manipulated under the stereomicroscope to extract embryos from the brood pouch without damaging them. Fecundity, embryo size (diameter and volume) and embryo development stages were determined. Four embryo development stages (I–IV) were considered according to Fish (1975). Fecundity or brood size is the number of embryos present in the brood pouch. For brood size and embryo size, evaluation of the last stage of embryonic development (IV) – newly hatched young – was not considered, since they can freely leave and enter the brood-pouch. Similar to amphipod length, embryo diameter was measured with the assistance of Leica QWin (Version 2.3). A sample of 10 embryos from each female's brood was measured and the respective average was considered the embryo size of that brood. Because embryos are not completely spherical, the longest and shortest diameter were measured, and the average diameter determined. The volume of the embryos was determined as a prolate spheroid $-4/3\pi r_{\max} \cdot r^2$ (Steele & Steele, 1991), where $r_{\max}$ and r are the rays corresponding to the longest and shortest diameter, respectively. Offspring production was quantified as the number of newborns produced per gravid female in chronic test 1, whereas in chronic test 2, given the low number of neonates obtained, the total number of offspring per treatment was used.

In *Gammarus* spp. the mean number of embryos per brood (mean fecundity) is inversely proportional to mean embryo size and directly proportional to female's body size (Sutcliffe, 1993). However, as in 28-day chronic bioassays only a limited range of female body sizes is under analysis, reliable brood size/female length and embryo size/female length relationships cannot be determined. Consequently, in the current study we refrain from comparing the slopes of these curves, which is only suitable when the whole range of female body sizes is covered. (e.g., Neuparth et al., 2002). Instead, mean gravid female length, mean fecundity and mean embryo size (diameter and volume) were calculated independently for each treatment and the two former allocated side by side in the same graphic for better visualization. These reproductive traits were analysed collectively for each sediment (treatment), to eliminate bias in the data derived from the low representation of gravid females in some

replicates. On these same grounds, data from stage I and II embryos were pooled, and stage III embryos were left out of the analysis. This was meant to minimize variation due to egg loss and egg mortality within a brood as maintained by Skadsheim (1984). Embryo development stages were analysed by determining the relative percentage of each stage of pooled data from each treatment.

2.4. Statistics

The effects of sediments on organism and population-level endpoints were analysed by one-way ANOVA separately for each dependent variable. Post-hoc comparisons were carried out using Fisher's least significant difference (LSD) test. Significant differences were considered at $p < 0.1$.

3. Results

3.1. Sediment geochemistry

A summary of results of sediment geochemical analyses of the sediments is presented in part I (Neuparth et al., 2004, Table 1). As a general trend, trace metal concentrations were higher in sediment D, P and T. Copper concentration exceeded ERM or ERL in sediment D and P, respectively, and zinc levels exceeded ERL either in D, P or T sediments. PAH concentrations were as a rule high in sediments S1, S2 and P.

3.2. Biological responses

A total of 1123 amphipods survived from both chronic tests (493 from test 1, plus 630 from test 2), of which 494 were males and 627 females. Length was determined for every survivor and reproductive traits determined for all gravid females. All results concerning biological responses (survival, growth and reproductive traits) recorded in both chronic tests, together with the respective statistics, are compiled in Table 1.

3.2.1. Survival / sex-ratio

Mean total survival in chronic test 1 did not differ between sediments and was very close to 50% in all cases. No differences were found when considering male and female survival separately. In chronic test 2, total survival was close to 60% for Control and S1 sediments, and significantly lower for sediments S2 and D ($p < 0.05$, $p < 0.01$, respectively), resulting primarily from lower male survival (Table 1). Control survival was within the expected range for 28-d test periods with *G. locusta* (Correia et al., 2001; Neuparth et al., 2002). Cannibalism most likely accounts for a significant portion of control mortality in laboratory tests with this species. Cannibal behaviour is well-known in *Gammarus* spp. (Dick, 1995), including *G. locusta* (Christie & Kraufvelin, 2003, our personal observations).

Table 1
Chronic effects of field sediments on survival, growth and reproductive traits of *G. locusta*, and respective statistics[a,b,c,d]

Endpoints	Chronic test 1			Chronic test 2			
	Control sediment	Sediment T	Sediment P	Control sediment	Sediment S1	Sediment S2	Sediment D
Survival							
Total	50.0 ± 10.3	49.7 ± 4.8	49.3 ± 4.3	60.0 ± 6.6	59.4 ± 2.5	**▲▲35.4 ± 16.5	***▲▲ 32.3 ± 11.7
Males	22.3 ± 6.4	19.1 ± 4.6	21.8 ± 7.6	27.7 ± 3.4	30.6 ± 4.0	*▲▲15.0 ± 12.3	**▲▲▲ 12.0 ± 5.4
Females	27.7 ± 7.4	30.6 ± 4.1	27.5 ± 7.2	32.3 ± 7.4	28.8 ± 2.9	20.4 ± 11.5	20.3 ± 11.4
Sex-ratio	0.85 ± 0.37	0.64 ± 0.20	0.90 ± 0.59	0.89 ± 0.16	1.0 ± 0.23	▲▲0.75 ± 0.22	**▲▲▲ 0.63 ± 0.11
Growth							
Males ♂	11.00 ± 1.61	*11.44 ± 1.71	***11.71 ± 1.24	10.02 ± 1.59	10.36 ± 1.48	10.12 ± 1.31	▲▲9.67 ± 1.47
	n = 78	*n* = 67	*n* = 61	*n* = 97	*n* = 107	*n* = 42	*n* = 42
Females♀	9.3 ± 1.49	***▲▲9.91 ± 1.26	***10.41 ± 1.16	▲8.77 ± 1.27	*9.10 ± 1.17	▲ 8.68 ± 1.43	▲▲8.56 ± 1.41
	n = 97	*n* = 107	*n* = 81	*n* = 113	*n* = 101	*n* = 57	*n* = 71
Reproductive traits							
Gravid females(%)	47.3 ± 12.0	***▲ 71.0 ± 11.1	***83.4 ± 2.5	27.6 ± 20	42.6 ± 14.4	▲▲11.9 ± 13.3	▲▲ 17.0 ± 13.4
Offspring production (per gravid female)[b]	4.6 ± 1.8	1.8 ± 0.5	3.0 ± 2.5	49	27	1	10
Gravid females length (mm)	10.04 ± 1.07	*10.43 ± 0.82	**10.55 ± 0.84	9.96 ± 0.71	10.01 ± 0.63	10.38 ± 0.52	10.10 ± 0.60
	n = 34	*n* = 51	*n* = 41	*n* = 18	*n* = 35	*n* = 11	*n* = 12
Fecundity (number of stage I and II embryos per gravid female)	21.4 ± 12.7	23.5 ± 12.0	23.4 ± 11.3	17.7 ± 6.4	16.0 ± 5.8	17.0 ± 8.8	18.2 ± 6.9
	n = 34	*n* = 51	*n* = 41	*n* = 18	*n* = 35	*n* = 11	*n* = 12
Embryo diameter (I and II) (mm)	0.510 ± 0.027	0.499 ± 0.031	0.504 ± 0.035	0.518 ± 0.024	0.516 ± 0.037	0.522 ± 0.042	0.525 ± 0.016
Embryo volume (I and II) ($mm^3 \times 10^{-3}$)	63.4 ± 10.6	59.4 ± 11.0	61.1 ± 12.9	66.2 ± 9.1	66.7 ± 14.2	69.0 ± 16.5	69.7 ± 6.3
Embryo development stage (relative proportion (%))							
I	43.6	32.4	43.9	48.3	53.7	50.0	69.2
II	18.2	36.5	18.2	13.8	31.7	28.6	23.1
III	18.2	18.9	12.1	24.1	9.8	21.4	7.7
IV	20.0	12.2	25.8	13.8	4.9	0.0	0.0

[a] Data reported as mean ± standard deviation; *n* = number of individuals analysed.
[b] For chronic test 2 the total number of offspring per treatment is reported.
[c] * – Indicates significant differences from control :* = $p < 0.1$, ** = $p < 0.05$, *** = $p < 0.01$.
[d] ▲– Indicates significant differences from Sediment P (for chronic test 1) and significant differences from sediment S1 (for chronic test 2): ▲ = $p < 0.1$,▲▲ = $p < 0.05$, ▲▲▲ = $p < 0.01$.

Surviving females exceeded males in all sediments as evidenced by the sex ratios lower than one. Sediment S1 was the exception, presenting an even proportion of males and females in average. Sex ratios did not differ on chronic test 1, but on chronic test 2 sediment D had a significantly lower sex ratio than control ($p < 0.05$) and both sediments S2 and D differed significantly from sediment S1 ($p < 0.05$ and $p < 0.01$, respectively), hence showing an abnormally low proportion of surviving males.

3.2.2. Individual growth

Average length of males and females was significantly higher in sediments T and P than in the control sediment. Sediment P had the largest amphipods, particularly the females, with an average length of 10.4 mm that exceeded the highest male average size of test 2, which was recorded in sediment S1. The average length of sediment S1 females was significantly higher compared to control and sediments S2 and D ($p < 0.1$, $p < 0.1$ and $p < 0.5$, respectively). Sediment S1 males had also significantly higher length than sediment D ($p < 0.5$), the sediment in which the amphipods of both sexes had the lowest average lengths.

3.2.3. Reproductive traits

The proportion of gravid females in chronic test 1 was distinctly higher than in the second test (Table 1). This is congruent with differences observed in female growth. On average, more than 80% of the females exposed to sediment P were gravid. The second highest percent of gravid females was recorded in sediment T, followed by control sediment from test 1, in a sequence similar to that observed with female growth. Sediments T and P differed significantly from control ($p < 0.01$) and between each other ($p < 0.05$). The sediment from test 2 with largest females, S1 – was also the sediment showing the highest proportion of gravid females, differing significantly from sediments S2 and D, but not from control 2.

Offspring production results appear not to be related to pregnancy ratio and female growth. The highest average number of offspring per gravid female in chronic test 1 was recorded in the control sediment, despite the fact that this sediment had a significantly lower percentage of gravid females. Offspring production was low and very irregular in second test. Several replicates from all sediments did not have any neonates, which precluded the estimation of the mean number of offspring produced per gravid female per replicate. Hence, the results presented were the total number of offspring recorded in each sediment. Control 2 had the highest number of neonates, despite having a lower mean pregnancy ratio than sediment S1, and sediments S2 and D a very low number of offspring.

Differences in female growth between the two chronic tests were not so pronounced when only gravid females were compared. In test 1, control gravid females were still smaller than in sediments T and P, but the differences were not so marked compared to all females ($p < 0.1$ and $p < 0.05$, respectively). In the second test dissimilarities were even less pronounced, and no significant differences were found in average length of gravid females. Immature and/or non-gravid females contributed to the comparatively low average females' length in sediments S2 and D. Both of

these sediments had higher values for gravid female length than control or sediment S1 (Table 1).

There were no significant differences in fecundity – average number of pooled embryos I and II per gravid female – in any of the chronic tests. This reproductive trait displayed considerable variation within treatment, particularly in test 1. In this test, sediments T and P had practically the same average fecundity – 23.5 and 23.4 embryos per female, respectively. Fecundity was lower in the second test. The highest value recorded in sediment D – 18.2 embryos per gravid female, still lower than the lowest value recorded in test 1 – 21.4 embryo per gravid female in control sediment (Table 1). Given the variability of the average fecundity values in both tests, there was no direct correlation with the respective length of gravid females.

As opposed to fecundity, embryo diameter and volume were higher in females from the second test. Pooled data within each test show an inverse relationship between fecundity and egg size, which is consistent with the regular reproductive patterns in *Gammarus* amphipods (Sutcliffe, 1993). There were no significant differences found in egg size, either diameter or volume, in any of the chronic tests. Embryo diameter and volume showed the same variation pattern. Test 1 control had the highest values for embryo size, while sediment D was the highest in the second chronic test.

The relative proportion of stage I embryos was always higher and above 40% for all sediments except sediment T, which showed a higher proportion of stage II embryos. Sediment T was the only sediment that had a percentage of stage I embryos lower than 40% and had a lower percentage of stage IV embryos compared with the remaining sediments from chronic test 1. On average, the percentage of stage IV embryos was lower in the second test, where no embryos at this development stage were scored in females from sediments S2 and D (Table 1).

4. Discussion

4.1. Chronic sediment toxicity: individual and population responses

Sediments assayed in chronic test 1 did not show toxicity, in the sense that toxicity is regularly understood: an impaired condition (fitness) detected by comparison with the experimental control (negative control sediment), as assessed by the various endpoints. On the contrary, amphipods exposed to sediments T and P had an overall and distinctly improved condition compared with the control. Improved condition was detected by the higher average length – especially in total females and gravid females – and higher pregnancy ratio. It has been observed before with *G. locusta* (and as well in other amphipods/invertebrates), that growth responses have a direct reflection in reproductive performance, since female maturation and brood size are a function of growth (Correia et al., 2001; Neuparth et al., 2002). It does appear to be the case in this experiment, since no reproduction-specific effects were detected in the brood size and embryo size.

The high content in organic matter is the most likely reason for the growth promotion in sediments T and P. Gammarid amphipods are known to have a diverse diet which includes deposit-feeding, macroalgae grazing, intraguild-predation, cannibalism, etc. (Brun & Dumay, 1974; Macneil, Dick, & Elwood, 1999; Costa & Costa, 2000; Christie & Kraufvelin, 2003). In the test aquaria *G. locusta* can be seen roaming the sediment surface, grasping sediment particles and picking up pieces of detritic material. Previous studies with *G. locusta* suggested that growth might be promoted in organically rich sediments (Costa, 1997). Therefore, it can be concluded that the positive impacts of sediments T and P on growth reflect a richer and more efficient diet provided by those sediments.

Contamination from sediment T, and mostly from sediment P, apparently did not negatively affect the amphipods, as far as we could determine from our results. Sediment geochemical properties, particularly organic matter, are known to interfere inversely with contaminant bioavailability, by providing a matrix for contaminant binding (Correia & Costa, 2000; Lawrence & Mason, 2001). This would explain the absence of toxic effects. On the other hand, if feeding on sediment detritic materials led to higher growth in amphipods, this would also suggest that they were well exposed to contaminants bound to organic matter.

In chronic test 2, sediments S2 and D were significantly toxic. Toxicity was expressed by the lower survival, particularly males, and a biased sex ratio, more evident in sediment D. These severe toxic effects were not apparent in growth of surviving animals. However, caution must be taken interpreting growth data from chronic tests when there were significant reductions in survival (Green, Moore, & Farrar, 1999; Ingersoll et al., 1998; Sibley, Benott, & Ankley, 1997). One main concern is that growth may have been affected by reduction of organism density on the course of the test. Earlier studies with *G. locusta* have shown that, over a 28 day period, growth rates can be inversely affected by organism density (Costa et al., 1996). Another concern, particularly relevant in tests with gammarids, is cannibalism. Surviving amphipods from sediments with high mortality that eventually preyed upon conspecifics would be in nutritional advantage (see Dick, 1995) compared to amphipods from sediments with low mortality. In view of the potential presence of these confounding factors, growth results from sediments S2 and D must be examined with some reserve. Concurrently, effects on reproduction of amphipods exposed to these sediments must be interpreted with some caution. There are symptoms of detrimental effects in several reproduction endpoints, which are consistent with the severe toxicity detected on survival, namely the low pregnancy ratios, the absence of stage IV embryos and the very low number of offspring produced in these sediments.

Amphipods condition in sediment S1 seems to be slightly superior to control, as evidenced by no differences in survival, the sex-ratio of 1:1, higher average length of males and females and higher pregnancy ratio. As opposed to chronic test 1, here healthier condition cannot be attributed to sediments organic matter since sediment S1 had lower organic content than control. A possible explanation is the phenomenon called hormesis, which can be identified as a growth enhancement induced by exposure to low doses of contaminant (Stebbing, 1997). This type of biological response has been already detected in *G. locusta* exposed to sediments spiked with

copper (Correia et al., 2001) and has been increasingly reported in ecotoxicological studies, as for example sediment toxicity assessments with amphipods (Green et al., 1999) and other invertebrates (Martinez-Madrid, Rodriguez, Perez-Iglesias, & Navarro, 1999).

4.2. Integration with biomarker data

Molecular and biochemical data (Neuparth et al., 2004) confirm the absence of contaminant-induced stress in sediments T and P from chronic test 1. No significant bioaccumulation of metals was recorded, metallothioneins were not induced, and no DNA damage was detected above control background. The exception was lipid peroxidation that was significantly higher in sediments T and P. However, it was observed in other studies with *G. locusta* (Correia, Costa, Luis, & Livingstone, 2003) that lipid peroxidation levels are closely and directly related with growth and age. Differences in the nutritional status of animals (see above) may also affect this parameter, and therefore LP induction cannot be ascribed to toxic effects in this context.

Hence, pooled data from different levels of biological organization did not detect toxic effects in sediment T, and particularly in sediment P, that presented concentrations above ERL for various contaminants. The growth stimulation is attributed to the comparatively high content of organic matter of those sediments. Still, it does not clarify how the amphipods fed on sediment decaying materials and greatly benefit in their condition, without suffering from contaminant toxicity. One possible explanation is that the physiological benefits derived from the sediment organic matter diet exceeded largely the energetic and physiological needs to cope with contaminant stress and detoxification. Under this assumption, the healthy condition of the animals would enable them to cope with contaminant stress so effectively, that no toxicity traces were detected, at least as far we could determine.

Corroborating findings at the organism/population level, biomarkers also detected contaminant-induced stress in sediments S2 and D. DNA damage was detected on both sediments although much more pronounced in sediment S2. The type of DNA damage identified was high levels of single strand breaks, indicating the presence of genotoxicants in those sediments. Yet, no metallothionein induction was recorded in sediment S1. Sediment D was the only sediment where MT induction was observed, thereby indicating that metals may have contributed to the toxicity observed.

Stimulation of growth in sediment S1 was also accompanied by induction of lipid peroxidation, like it was observed in sediments from chronic test 1. Yet, in this case growth stimulation is presumed to be a hormetic response, instead of a result of sediment organic matter. The high levels of lipid peroxidation are congruent with hormesis. Both metallothioneins and LP have been clearly linked with hormetic responses in previous studies with *G. locusta* (Correia, 2002). There is also a slight amount of DNA damage detected in S1 that may constitute a residual trace of toxicant insult still present in low amounts in this 75% diluted sediment.

4.3. Appraisal of the chronic sediment test with the amphipod Gammarus locusta

Overall this study (part I and II) and previous research (summarized in the introduction), provide a comprehensive and integrated approach for addressing sediment chronic toxicity with an European amphipod species. As a corollary of this investigation it is important to discuss the relevance of the findings here reported, both from a pragmatic point of view regarding application in monitoring programs, as well as from a scientific perspective. Costa et al. (1998) enumerated the reasons and interest of the application of *G. locusta* in acute sediment bioassays, considering the criteria advanced by Hill, Matthiessen, & Heimbach (1994) to select test organisms. Here we update those comments adding the chronic toxicity scope and introducing the new data produced in the meantime.

4.3.1. Availability and amenability

The availability of organisms for testing is a prime consideration for selecting a test species. Preference is given to native species from the particular region under examination in order to improve the ecological relevance of the test (Nendza, 2002; USEPA, 1999). Each estuary or coastal ecosystems has its own particularities and in many of them it may not be easy to find infaunal candidate amphipods that cover the basic requirements for a test species, or that constitute the best choice in the particular context of the ecosystem being studied. For example, *Corophium volutator* is very rare in the Portuguese coast, despite its widespread occurrence in northern Europe and Canada. Culturing is possible but not sufficiently competent to produce a continuous and large enough provision of organisms for sediment tests (Nendza, 2002). The currently known distribution range of *G. locusta* covers all Atlantic European coast, from Iceland and Norway to the Strait of Gibraltar, including the British Isles and the Baltic Sea (Costa & Costa, 2000). This species is a common and abundant component of the benthic fauna in many coastal ecosystems of the Atlantic Europe, and therefore is available from the wild in many locations. But the most important is its amenability for culturing and producing large numbers of organisms in laboratory (Neuparth et al., 2002). We have developed a culturing system for this species that has been maintained for several years now in our facilities. When extremely necessary, we were able to keep this culture for up to seven months without external provision of animals from the wild. This culturing potential is certainly one of the most important strengths of this species as a candidate for test organism.

4.3.2. Ecological relevance

A recent review on the ecology of *G. locusta* confirmed its ecological relevance, not only for its distribution and abundance in numerous locations of the European coast, but as well by its role in the coastal ecosystems as a consumer and has a prey (Costa & Costa, 2000). In recent studies this species is indicated among the most prominent mesograzers in various locations (Christie & Kraufvelin, 2003; Karez, Engelbert, & Sommer, 2000; Lotze & Worm, 2000). Christie & Kraufvelin (2003) draw attention to the potential of this species to develop enormous population den-

sities in macroalgae habitats, and to its large contribution for the secondary production in these communities.

4.3.3. Salinity and sediment tolerance

Neuparth et al. (2002) established new temperature and salinity ranges for chronic testing with *G. locusta*, expanding the testing conditions that were previously established for acute sediment tests. Sediments from estuarine sections over 20‰ salinity can be safely tested, providing that the test animals undergo proper acclimation to the test salinity. This chronic sediment test is therefore limited by the salinity tolerance of *G. locusta*, and accordingly other species should be used to test sediments from sections of estuaries with lower salinity. However this test can still cover a considerable area of some estuaries, as for example in the case of Sado estuary, where most of the sediments of environmental concern are located at salinities over 20‰. Plus it should be kept in mind that there is one remarkable exception to this lower salinity limit: the Baltic Sea, where populations of *G. locusta* live permanently at salinities between 5‰ and 7‰.

Regarding sediment tolerance, no impairment in biological functions has been diagnosed so far, and in that context *G. locusta* can be used for testing all sediment types. This characteristic is one of most relevant strengths of this test, considering that many successful sediment bioassay organisms have constraints in the testable types of sediment. Given that sediment organic matter content may have a beneficial impact, compared with low organic content sediments, potential effects must be considered in advance when designing chronic sediment tests. An adequate action is the inclusion of reference sediments with the closest possible features to the sediments under testing (Chapman & Wang, 2001).

4.3.4. Exposure to sediment toxicants and sensitivity

Guidelines and standards (e.g., ASTM, 1992) give preference to the use of infaunal amphipods over epibenthic species, based on the assumption that exposure and sensitivity to sediment contamination are higher in free-burrowing or tube-dwelling amphipods than their epibenthic counterparts. Although this may be a reasonable assumption, it should not be taken as a definitive conclusion, and requires analysis under a wider ecological scope (see Lawrence & Mason (2001) for a thorough discussion on bioavailability of sediment contaminants). Some ecological features account for exposure to sediment contamination, other than living in the sediment, as for example the feeding mode (Lawrence & Mason, 2001). Some tube-dwellers for instance are active suspension-feeders and along the toxicity test end-up being mostly exposed to overlaying water (Warren, Tessier, & Hare, 1998) whereas some epibenthic species are active deposit-feeders, this way being exposed to sediment contamination through dietary uptake (Lee et al., 2000) – an efficient and ecologically relevant route of exposure. Furthermore, contaminant bioavailability may be related with the digestive systems of the benthic organisms (Chapman & Wang, 2001). Evidence of the applicability and interest of using epibenthic amphipods in whole sediment bioassays is illustrated by the freshwater epibenthic amphipod *Hyalella azteca*,

that has been successfully and widely used in acute and chronic sediment toxicity assessments (Environment Canada, 1997; Ingersoll et al., 1998).

G. locusta feeding mode combines mostly macroalgae grazing and deposit feeding (Costa & Costa, 2000). Observation of this species in the test aquaria illustrates its strong attraction to the bottom. *G. locusta* shares its time between feeding suspended in *Ulva* spp. pieces (leaves) and roaming over sediment surface, picking up and feeding on detritic materials and grasping sediment particles for food. The relevance of deposit feeding for *G. locusta* was confirmed in this study by the significant positive impact of sediment organic content on growth. This suggested that dietary uptake is one of the routes of exposure to sediment contamination to consider in chronic bioassays with this amphipod.

Sensitivity to contaminants is another aspect to consider. Several studies indicate great differences in amphipod sensitivies to contaminants, and also that species sensitivity may be selective depending on the toxicant (Luoma, 1996; McPherson & Chapman, 2000; Weston, 1996). Therefore sensitivity depends on the toxicant and on the species being used, and not on the infaunal versus epibenthic habitat. As an example, in water-only cadmium acute toxicity tests comparing *C. volutator* and *G. locusta* sensitivities (Neuparth, 1999), the latter species was almost 10 times more sensitive. Also, in trial sediment toxicity tests, both acute and chronic, *C. volutator* was equally or less sensitive than *G. locusta* (Neuparth, 1999). On the other hand, available information indicates that the tube-builder amphipod *Leptocheirus plumulosus* is more sensitive to cadmium than *G. locusta* (however experimental conditions were not the same – DeWitt et al. (1992)). In this regard, for comprehensive assessments of sediment toxicity, a battery of species with varying sensitivities is recommended (Chapman, Ho, Munns, Solomon, & Weinstein, 2002; USEPA, 1999).

The information available indicates that *G. locusta* is a particularly sensitive species, not only to toxicants but also to environmental disturbance in general (Costa & Costa, 2000). This amphipod has been reported as the least tolerant, both at the toxicological and physiological level, among the European marine and brackish *Gammarus* spp. (Costa & Costa, 2000; Gaston & Spicer, 2001). These amphipods, as a group, constitute a very relevant component of European coastal ecosystems. Given its sensitivity, *G. locusta* can be considered one of the *Gammarus* presenting greater risk from suffering the impact of environmental contamination, and simultaneously the most appropriate sentinel species for preventing and protecting other *Gammarus* spp.

Few data about sensitivity of *G. locusta* to specific toxicants exist. There is some information concerning the toxicity of trace metals in the water, namely tin and lead acute toxicity (Zencirci, 1980) and about trace metal bioaccumulation in the laboratory (Clason & Zauke, 2000) and in the field, namely copper, zinc and iron (Rainbow & Moore, 1986), lead and cadmium (Mesmar, 1987) and zinc, lead and copper (Alliot & Frenet-Piron, 1988). Previous studies that we performed included acute toxicity of copper and cadmium in the water, lindane (γ-hexachlorocyclohexane) and copper-spiked sediments (Costa et al., 1998; Correia & Costa, 2000), and more recently chronic toxicity of copper in the water (Correia et al., 2002) and in the

sediment (Correia et al., 2001; Correia et al., 2002). Finally the current study indicates that this species is chronically sensitive to estuarine sediments with mixed contamination. Nevertheless, clearly much more research is required on the sensitivity of *G. locusta* to specific environmental toxicants.

4.3.5. Potential of expansion and integration of new endpoints

The integrated biological effects approach followed in this study, assisted in disclosing whether effects observed at the organism and population levels were induced by sediment contaminants and, if so, provided some insight to the potential toxicants involved.

The demographic features of *G. locusta* offer testing opportunities that can hardly be met by currently used test species. Its relatively short life cycle, combined with comparatively large size attained during the bioassay (28-day), enables the measurement of biological effects from the molecular to population level in the same organism. This is so because measurement of biochemical endpoints may require a reasonable amount of sample biomass for the analysis, and *G. locusta* is considerably larger than many of the amphipod species currently used in marine and estuarine sediment tests.

The ability to integrate biochemical and individual/population level effects is one of the most innovative and potentially more fruitful strengths of *G. locusta*'s chronic test. A number of new strategies and designs in sediment bioassays may be attempted and novel approaches to address field sediments' toxicity considered. The application of these or similar strategies to this amphipod, and other candidate organisms, may grant a significant contribution to the interpretation and comprehension of the impact of environmental contaminants in marine organisms and ecosystems.

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