

**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 was meant 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 in 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 alongside 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 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. Biochemical responses confirmed that the amphipods exposed to these sediments were distressed with toxicant insult. 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 in short-term exposures (usually 10 days maximum), considering only survival as endpoint, or in some cases including behavior criteria, such as sediment avoidance or failure to rebury (e.g. Traunspurger & Drews, 1996; USEPA, 1999; Chapman & Wang, 2001). 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, Hickey, Morrisey, Nipper, Roper, Williamson et al., 1999; Marsden & Wong, 2001). In Europe, *Corophium volutator* and *Gammarus locusta* are some of 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 (Redmond, Scott, Swartz, & Jones, 1994; Weston, 1996; Emery, Moore, Gray, Duke, Gibson, Wright et al., 1997; USEPA-USACE, 2001), 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; Matthiessen, Bifield, Jarret, Kirby, Law, McMinn et al., 1998; Grant & Briggs, 2002) 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.

Nevertheless, chronic bioassays are an exceptionally valuable environmental assessment tool, since they consider various relevant environmental circumstances that are not addressed in acute sediment tests: 1) most of the wild organisms are most likely chronically exposed to environmental contamination, 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 bioassays 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 bioassays, 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 bioassays 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, 1997), 4) analysing the impact of non-contaminant variables (Costa, Correia, & Costa, 1996; Costa, 1997), establishing the experimental conditions to conduct chronic tests (Neuparth, 1999; 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 with biochemical markers. Here we focus on organism/population-level effects, while biomarker responses were reported in a companion paper – part I (Neuparth et al., submitted this issue).

## 2. Materials and Methods

### 2.1. Sediments

Control and test sediments were collected from Sado and Tagus estuaries. Relevant features of the collection sites are presented in Table 1. Sediments were analysed for organic matter content (expressed as percentage of total volatile solids - TVS), bulk concentration of heavy metals (Cd, Cu and Zn), polychlorinated biphenyls – PCBs, and polynuclear aromatic hydrocarbons (PAHs). The description of sediments sampling and processing, and geochemical analysis, is presented in part I of this study (Neuparth et al., submitted this issue). Concentrations of contaminants in the sediments were compared with the following sediment quality guidelines: a) Effects Range Low (ERL), that is indicative of contaminant concentration below which adverse effects are rarely observed and b)

Effects Range Median (ERM), that points out the concentration above which biological effects frequently occurs (Long, MacDonald, Smith, & Calder, 1995).

## 2.2. *Amphipods*

The amphipods used in the chronic sediment bioassays were juveniles belonging to the 2-4 mm length class (retained between 1000 and 475 $\mu$ m sieves), obtained from a laboratory culturing system, maintained as described in Costa (1997). Twenty-four hours before the beginning of the experiments a stock of juveniles was isolated from the main culture and kept at the assay temperature (20°C) with unlimited food (macroalgae *Ulva sp.*).

## 2.3. *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.

Previous studies on the acute toxicity of sediments P and T (Costa et al., 1998; Neuparth, 1999) did not reveal acute toxicity. Therefore, only sediments D, S1 and S2 were subjected to screening tests of acute toxicity as described in (Costa et al., 1998). Sediments showing acute toxicity were diluted with control sediment as much as required to remove lethal toxicity. Sediment D was not acutely toxic and thus did not need dilution. Sediments S1 and S2 were tested in the chronic bioassay at the concentrations of 25% and 50% (v/v), respectively.

The experimental conditions of the chronic sediment tests is described below. With the exception of the food supply that was fresh or frozen *Ulva sp* in chronic test 1 and 2 respectively, the procedure used in both chronic tests was the same. The assays were conducted at 20°C with 0.45  $\mu$ m-filtered seawater at 33 $\pm$ 1 ‰ salinity and a 12-h

photoperiod. In both chronic tests there were five replicates per treatment. The sediments were placed in the respective aquaria (10-L) the day before starting the assay, with a sediment layer of about 1 cm. Seawater was added gently, to minimize sediment resuspension, and the aeration was provided with plastic pipette tips placed at least 1 cm above the sediment surface. The sediment-overlying water system was allowed to equilibrate overnight, before the addition of the amphipods.

The assays started the following day with the allocation of exactly 70 juveniles to each replicate test chamber. The water was renewed every 10 days (80% of the volume). The organisms were fed with macroalgae *Ulva sp.* on a *ad libitum* basis, assuring that food was never in short supply. Test chambers were inspected daily for aeration and feeding requirements and to remove dead animals.

At the end of the assays the overlying water of each replicate was sieved through 1000  $\mu\text{m}$  and 250  $\mu\text{m}$  screens, to collect surviving adults and their offspring, respectively. Sediments were washed at least 5 times to assure that all organisms were removed. The recruiters 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 each of 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 part I (Neuparth et al., submitted this issue). All pregnant females were preserved in 70 % ethanol for posterior analysis as described below.



#### *2.4. 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 reproductive 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 was 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 -  $\frac{4}{3}\pi r_{\max}r^2$  (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's 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.

## 2.5. 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 Table 1. All contaminants were below ERM, except copper in sediment D and phenanthrene in sediment S1. As a general trend, heavy 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. PAHs concentrations were as a rule high in sediments S1, S2 and P. Most of individual PAHs, high or low molecular weight PAHs, and total PAHs, were higher than ERL in these three sediments. PCBs concentrations were low in all tested sediments and in none exceeded ERL.

### 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 a master table (Table 2).

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 of them. No differences were found when considering male and female survival separately. In chronic test 2, total survival was close to 60% for N.C. and S1 sediments, and significantly lower for sediments S2 and D ( $p < 0.05$  and  $p < 0.01$ , respectively), resulting primarily from lower male survival (Table 2).

Surviving females exceeded males in all sediments as evidenced by the sex ratios lower than 1. Sediment S1 was the exception, presenting an even proportion of males and females in average. Sex ratios did not differ in chronic test 1, but in chronic test 2 sediment D had a significant 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 of test. 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 ( $p < 0.5$ ), that was 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 2). This is congruent with differences observed in female growth. In 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 with 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 this sediment had significantly lower percentage of gravid females. Offspring production was low and very irregular in the second test. Several replicates from all sediments did not have any neonates, which precluded the estimation 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 the test 1, control gravid females were still smaller than in sediments T and P, but the differences were not so marked as with comparing 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 females length than control and sediment S1 (Table 2).

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 2). 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 was higher in females from the second test. Pooled data within each test shows 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, that 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. In 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 2).

## **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 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 is 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 on 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's detritic materials lead to higher growth in amphipods, this would also suggest that they were well exposed to contaminants bound to

organic matter. Bioaccumulation data and biomarker responses discussed further ahead may help solving this paradox.

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 (Sibley, Benott, & Ankley, 1997; Ingersoll et al., 1998; Green, Moore, & Farrar, 1999).

Under a context of significant mortality, the potential consequences of various uncontrolled experimental covariants cannot be accounted for. For instance it may be questioned whether growth rates and overall condition of survivors can be positively affected as a result of 1) lower crowding-associated stress or 2) higher incidence of cannibal diet. Regarding crowding, earlier studies have shown that, over a 28 day period, growth rates of *G. locusta* can be inversely affected by organism density (Costa et al., 1996). Cannibalism is a well-known incident in *Gammarus* spp. (Dick, 1995), including *G. locusta* (Christie & Kraufvelin, 2003, our personal observations). *Gammarus* cannibalism is nutritionally advantageous for cannibals, improving overall their fitness (Dick, 1995). Incidence of cannibalism is higher upon more vulnerable victims such as conspecifics in poor health condition. Thus, it is not known if, in the current sediments, cannibalism upon ill and sensitive conspecifics may have promoted growth and fitness of surviving amphipods. In view of the potential presence of these confounding factors in a situation of significant mortality, no conclusive interpretation of the impact of contaminant stress in growth can be reached for sediments S2 and D.

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 for this event 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, submitted this issue) 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, 2002; 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 content of those sediments. Still, this explanation does not clarify how the amphipods fed on sediment's decaying materials and greatly benefit in their condition, without suffering from contaminant toxicity. Several hypothesis can be considered, one of them is that the nutritional quality of the diet and amphipod condition was so overwhelmingly enhanced that it exceeded by large the physiological and energetic needs for coping with pollutant 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 individual/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 were 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 for the toxicity observed.

Alongside to what was noted on chronic bioassay 1, the stimulation of growth in sediment S1 was also accompanied by induction of lipid peroxidation. Yet, in this case growth stimulation is presumed to be a hormetic response, instead of a result of sediment's 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.

Globally, biomarker data contributed to ascribe whether organism/population responses were contaminant-induced - sediments S1, S2 and D - or influenced by sediment features -sediments T and P.

#### 4.3. Appraisal of the Chronic Sediment Test with the amphipod *Gammarus locusta*

Overall this study (part I and II) and previous research of the team (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 practical 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, and 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 (USEPA, 1999; Nendza, 2002). 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 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 (Neuparth, 1999; 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 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 (Costa, 1997; 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 for its role in coastal ecosystems as a consumer and as a prey (Costa & Costa, 2000). In recent studies this species is indicated among the most prominent mesograzers in various locations (Karez, Engelbert, & Sommer, 2000; Lotze & Worm, 2000; Christie & Kraufvelin, 2003). Christie and Kraufvelin (2003) draw attention to the potential of this species to develop enormous population densities 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 salinities 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 rising 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 5-7‰.

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's 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 is 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 and 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, Griscom, Lee, Choi, Koch, Luoma et al., 2000) - an efficient and ecological relevant route of exposure. Furthermore the 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 sediment bioassays is illustrated by the freshwater epibenthic amphipod *Hyaella 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's 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 in growth. This constitutes evidence 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 sensitivities to contaminants, and also that species sensitivity may be selective depending on the toxicant (Luoma, 1996; Weston, 1996; McPherson & Chapman, 2000). 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 (USEPA, 1999; Chapman, Ho, Munns Jr., Solomon, & Weinstein, 2002).

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 exists. There is some information concerning the toxicity of heavy metals in the water, namely tin and lead acute toxicity (Zencirci, 1980) and about heavy 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 ( $\gamma$ -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, 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.

4.3.4. *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's 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's 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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Table 1. Sediments' collection sites, sediment features, and chemical contaminants of each sediment tested in chronic toxicity tests.<sup>a, b, c</sup>

Sediments' collection sites	Control Sediment	Chronic Test 1		Chronic Test 2		
		Sediment T	Sediment P	Sediment S1	Sediment S2	Sediment D
	clean area of the South margin of Sado estuary	north margin of Tagus estuary near the city of Vila Franca de Xira	north margin of Sado estuary close to a pulp mill effluent	north margin of Sado estuary, near the effluent of a pesticide and fertilizer plant (S1 collected 25 meters from the effluent, and S2 30 meters upstream from S1)		north margin of Sado estuary, in a dockyard nearby an urban effluent of the city of Setúbal.
<b>Sediment Type</b>	sand	mud	mud	sand	sand	mud
<b>TVS (%)</b>	0.7	11.5	8.7	0.4	0.4	12.7
<b>Metals (<math>\mu\text{g}\cdot\text{g}^{-1}</math> dry wt)</b>						
Cd	BDL	0.31	0.32	0.13	0.12	0.6
Cu	0.21	33	85*	14	11	361**
Zn	20	190*	221*	27	62	217*
<b>PAHs and PCBs (<math>\text{ng}\cdot\text{g}^{-1}</math> dry wt)</b>						
naphthalene	17	19	41	4	65	5
acenaphthylene	3	3	13	BDL	21	5
acenaphthene	BDL	BDL	BDL	BDL	BDL	BDL
fluorene	BDL	BDL	55*	104*	18	13
phenanthrene	74	84	303*	1658**	346*	145
anthracene	BDL	BDL	BDL	BDL	BDL	BDL
$\Sigma$ Low mol wt PAHs	94	106	412	1848*	368	168
fluoranthene	90	103	785*	1681*	1411*	355
pyrene	122	139	686*	2186*	1088*	359
benzo(a)anthracene	BDL	BDL	490*	1194*	704*	154
chrysene	47	54	401*	1345*	518*	188
benzo(b)fluoranthrene <sup>b</sup>	81	93	970	1390	1596	335
benzo(g,h,i)perylene <sup>b</sup>	BDL	BDL	492	BDL	BDL	BDL
benzo(k)fluoranthene <sup>b</sup>	BDL	BDL	BDL	BDL	BDL	BDL
indeno(1,2,3-cd)pyrene <sup>b</sup>	BDL	BDL	525	644	657	BDL
dibenzo(a,h)anthracene	BDL	BDL	BDL	BDL	BDL	BDL
benzo(a)pyrene	BDL	BDL	551*	794*	905*	183
$\Sigma$ High mol wt PAHs	340	389	4900*	9779*	6879*	1574
$\Sigma$ Total PAHs	434	495	5312*	11627*	7247*	1742
Total PCBs	0.83	0.83	2.51	0.68	0.89	1.78

<sup>a</sup> BDL = Bellow detection limit<sup>b</sup> ERL and ERM guidelines not available<sup>c</sup> Values above ERL = \*; Values above ERM = \*\*

Table 2. Master table of the chronic effects of field sediments on survival, growth and reproductive traits of *G. locusta*, and respective statistics.<sup>a, b, c, d</sup>

Endpoints	Chronic test 1			Chronic test 2				
	Control Sediment	Sediment T	Sediment P	Control Sediment	Sediment S1	Sediment S2	Sediment D	
<b>Survival</b>								
Total	50.6±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	28.9±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	
<b>Growth</b>								
Males ♂	11.00±1.61 n=78	*11.44±1.71 n=67	***11.71±1.24 n=61	10.02±1.59 n=97	10.36±1.48 n=107	10.12±1.31 n=42	▲▲9.67±1.47 n= 42	
Females ♀	9.3±1.49 n=97	***▲▲9.91±1.26 n=107	***10.41±1.16 n= 81	▲8.77±1.27 n= 113	*9.10±1.17 n=101	▲8.68±1.43 n=57	▲▲8.56±1.41 n=71	
<b>Reproductive traits</b>								
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) <sup>b</sup>	4.6±1.8	1.8±0.5	3.0±2.5	49	27	1	10	
Gravid females length (mm)	10.04± 1.07 n=34	*10.43± 0.82 n=51	**10.55± 0.84 n=41	9.96±0.71 n=18	10.01± 0.63 n=35	10.38± 0.52 n=11	10.10± 0.60 n=12	
Fecundity (number of stage I and II embryos per gravid female)	21.4±12.7 n=34	23.5±12.0 n=51	23.4±11.3 n=41	17.7±6.4 n=18	16.0±5.8 n=35	17.0±8.8 n=11	18.2±6.9 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 <sup>3</sup> x 10 <sup>-3</sup> )	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

<sup>a</sup> Data reported as mean±standard deviation; n = number of individuals analysed.

<sup>b</sup> For chronic test 2 it is reported the total number of offspring per treatment.

<sup>c</sup> \* - Indicates significant differences from control :\*=p<0.1, \*\*=p<0.05, \*\*\*=p<0.01.

<sup>d</sup> ▲ - 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