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Multi-level assessment of chronic toxicity of estuarine sediments with the amphipod *Gammarus locusta*: I. Biochemical endpoints

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Abstract

We report on biomarker responses conducted as part of a multi-level assessment of the chronic toxicity of estuarine sediments to the amphipod *Gammarus locusta*. A companion article accounts for organism and population-level effects. Five moderately contaminated sediments from two Portuguese estuaries, Sado and Tagus, were assessed. Three of them were muddy and two were sandy sediments. The objective was to assess sediments that were not acutely toxic. Three of the sediments met this criterion, the other two were diluted (50% and 75%) with clean sediment until acute toxicity was absent. Following 28-d exposures, the amphipods were analyzed for whole-body metal bioaccumulation, metallothionein induction (MT), DNA strand breakage (SB) and lipid peroxidation (LP). Two of the muddy sediments did not cause chronic toxicity. These findings were consistent with responses at organism and population levels that showed higher growth rates and improvement of reproductive traits for amphipods exposed to these two sediments. Two other sediments, one muddy and one sandy, exhibited pronounced chronic toxicity, affecting SB, MT induction (in muddy sediment), survival and reproduction. Potential toxicants involved in these effects were identified. The last sandy sediment exhibited some loss of DNA integrity, however growth was also enhanced. Present results, together with the organism/population-level data, and also benthic communities information, were analysed under a weight-of-evidence approach. By providing evidence of exposure (or lack of it) to contaminants in sediments, the biomarkers here applied assisted in distinguishing toxicants' impacts in test organisms from the confounding influence of other geochemical features of the sediments.

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1. Introduction

The recognition of the complexity of sediment biogeochemistry and of technical difficulties in the evaluation of the ecological impact of sediment contamination, has led to increasing support for the application of multiple lines of evidence (LOE) in sediment quality assessments, integrated in a weight-of-evidence (WOE) approach (Chapman, McDonald, & Lawrence, 2002; Wenning & Ingersoll, 2002). Sediment toxicity tests are recognized as an essential tool of the WOE approach, although with their inherent strengths and weaknesses. Among the limitations are the recurrent difficulties in discriminating the contaminant-induced impacts in test organisms from those responses attributable to other non-contaminant factors (Wenning & Ingersoll, 2002). In particular, sediment's geochemical properties are known not only to regulate contaminants' bioavailability, but also to be able to directly influence responses of test organisms in various ways (Gunnarsson, Granberg, Nilsson, Rosenberg, & Hellman, 1999; USEPA-USACE, 2001; Wenning & Ingersoll, 2002). Most of the response criteria used in chronic toxicity tests (such as those based on growth and reproduction endpoints) lack specificity, and consequently can be affected both by contaminants and other sediment features (e.g. grain size, amount and quality of organic matter). In this context, additional LOE within the toxicity test are required to determine exposure of test organisms to sediment's contaminants.

Molecular biomarkers provide evidence of exposure to toxicants, and their detection in natural populations provide information about the contaminants bioavailability (Shugart, 2000; Hyne & Maher, 2003). A number of biomarkers have been developed and applied sucessfully

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to various invertebrate species (Langston & Bebianno, 1998; Livingstone, 2001; Hyne & Maher, 2003), but they have been rarely integrated in conventional sediment toxicity tests and/or linked to population-level effects. However, the application of biomarkers in chronic sediment tests can be advantageous for providing evidence of the cause-effect relationship between exposure to sediments' contaminants and ultimate organism and population responses.

Technical difficulties can be one of the reasons for the scarcity of biomarkers in conventional sediment toxicity tests: test organisms must have a short life-cycle in order to assess growth and reproductive effects in a short period and, simultaneously, they must provide large enough biomass for biomarker analysis. In order to fulfil these requirements we selected the amphipod *Gammarus locusta* (L.), a widely distributed species in coastal Atlantic Europe, which groups a number of advantages for application in ecotoxicological studies (Costa & Costa, 2000). Recently, specific methodologies were developed for application of known biomarkers in ecotoxicological studies with this amphipod, namely metallothionein (MT) induction, lipid peroxidation (LP) (Correia, Lima, Costa, & Livingstone, 2002) and DNA strand breakage (SB) (Costa, Neuparth, Costa, Theodorakis, & Shugart, 2002).

Given the critical role that the DNA molecule plays in the life and reproduction of each organism, a number of studies focused on biomarkers of DNA damage to detect genotoxicity in aquatic organisms (Shugart, 1999, 2000). Compared to other techniques used to assess DNA damage, detection of DNA strand breakage by agarose gel electrophoresis has the advantage of determining insult to DNA integrity both qualitatively (single strand-breaks versus double strand-breaks) and quantitatively (number of strand breaks). In addition it can also be applied to DNA extracted from whole organisms, thus not requiring manipulation of the amphipods to collect specific tissues (Costa et al., 2002). Malondialdehyde (MDA), a breakdown product of lipid endoperoxides, is an expression of lipid peroxidation and has been used with success in

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aquatic invertebrates as a general indicator of toxicant stress derived from various types of contamination (Livingstone, 2001). Metallothioneins (MT) are an widely used biomarker of exposure to metallic contaminants (e.g. Cd, Cu, Zn and Hg) which has been applied in numerous aquatic invertebrates, particularly mollusks (Langston & Bebianno, 1998; Livingstone, 2001) and more recently in crustaceans (Moksnes, Lindahl, & Haux, 1995; Barka, Pavillon, & Amiard, 2001; Correia et al., 2002).

Our recent research efforts have been directed to the integration of these biomarkers in sediment toxicity tests, by assessing multiple biological effects at several levels of biological organization - from molecular to organism and population levels. This type of approach has been previously tested in laboratory chronic toxicity tests with copper-spiked sediments (Correia, Costa, Neuparth, Diniz, & Costa, 2001; Correia, 2002) and we are now aiming to investigate its usefulness in chronic tests with field-contaminated sediments. Therefore, the goal of the current study was to conduct sediment tests with the amphipod *G. locusta*, integrating biomarker alterations (namely MT, SB and LP) with effects on growth, reproductive performance and recruitment. Here we report biomarker responses, while organism and population-level endpoints were analysed in a parallel paper (Costa et al., submitted this issue). These results were integrated with sediment chemistry and benthic community data in a WOE framework, and discussed in view of the potential of biomarker responses to link sediment's contamination with higher-level endpoints (organism/ population-level).

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2. Material and Methods

2.1. Sediment collection and processing

Control and test sediments were collected from Sado and Tagus estuaries. Figure 1 indicates the locations of the sediments to be analysed and Table 2 summarizes the relevant features of the collection sites. Only one sediment was collected in Tagus estuary, all others were collected in the lower Sado estuary. Test sediments from Sado estuary were collected in several points along the north margin, all located in the euhaline section of estuary. Control sediment was collected in the opposite south margin. The salinity at the collection sites is very close for all sediments and average 32‰, with the exception of sediment P that is about 27‰ (Mucha, 1997). At each site, intertidal surface sediments were sampled using a scoop. In the laboratory, sediments were sieved though a 1500 µm screen to remove macrofauna, and stored at 4ºC for a maximum of 72 h before the initiation of the chronic sediment bioassays. Before the beginning of the assays all test sediments were homogenized for 15 min. with the assistance of a mechanic mixer, after which samples of each sediment were collected for geochemical analysis.

2.2. Sediment geochemical analysis

Sediments were analysed for organic matter content (expressed as percentage of total volatile solids - TVS), bulk concentration of heavy metals (Cd, Cu, and Zn), polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs). The TVS were determined as the percentage weight loss after ignition of dry sediment at 550 °C for 4 hours (Correia & Costa, 2000).

Heavy metal analyses were performed on aliquots of the solid fraction that were freeze-dried, homogenized by grinding, and digested with a mixture of acids according to the method

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described by Rantala and Loring (1977). The digested material was then analysed by air-acetylene flame to determine the concentrations Zn and a pyrolytic graphite furnace equipped with a L'vov platform to establish the concentration of Cu and Cd. The total PAHs were quantified using 16 individual congeners (acenaphthylene, acenaphtene, fluorine, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthrene, benzo(g,h,i)perylene, benzo(k)fluoranthene indeno(1,2,3-cd)pyrene, dibenzo(a,h)anthracene and benzo(a)pyrene). PAHs were extracted from frozen sediment samples using soxhlet extraction, after lyophilization and sift. The sediment extracts were further clean up and fractionated by silica column chromatography, concentrated and quantified by capillary gas chromatography / mass spectrometry / electron capture detection (GC-MS/GC-ECD). PCBs were extracted from frozen sediment samples by first lyophilizing to remove water. Subsequently, the samples were Soxhlet extracted for 16h in hexane. The sediment extracts were cleaned up using Florisil and HCl. Separation of PCBs compounds from non-polar interferences was accomplished using a gas chromatograph equipped with a fused silica capillary column. The injections of each sample aliquots were made with an autosampler. Chlorobiphenyl congeners (CBs) were identified and quantified on the basis of a synthetic mixture of 19 individual congeners (CB 18, 26, 31, 44, 49, 52, 101, 105, 118, 128, 138, 149, 151, 153, 170, 180, 183, 187, 194).

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).

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2.3. Amphipods

The amphipods used in the chronic sediment bioassays were juveniles belonging to the 2-4 mm length class (retained between 1000 and 475µm 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 ºC) with unlimited food (macroalgae *Ulva sp*.).

2.4. Chronic sediment tests

Two independent chronic toxicity tests were performed in this study, which will be hereafter referred to as chronic bioassay 1 and chronic bioassay 2. Sediments P and T were assayed in chronic test 1, and sediments S1, S2 and D were assayed in chronic test 2.

Previous studies on the acute toxicity of sediments P and T (Costa, Correia, & Costa, 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 until acute toxicity was absent. Sediment D was not acutely toxic and thus did not need dilution. Sediments S1 and S2 were assayed in the chronic test at concentrations of 25 % and 50% (v/v), respectively.

The assays were conducted at 20°C with 0.45 µm-filtered seawater at 33±1 ‰ salinity, under a 12-h photoperiod. In both chronic tests five replicates per treatment were employed. The sediments were placed in the respective aquaria (10-L) the day before the start of the assay. The sediment layer had about 1 cm. Seawater was added gently, to minimize sediment resuspention, and aeration was provided with plastic tips placed at least 1 cm above the sediment surface. Before addition of the amphipods, the sediment-overlying water system was allowed to equilibrate overnight.

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The assays started the following day with the allocation of exactly 70 juveniles to each 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 shortage. With the exception of the food supply, that was fresh or frozen *Ulva sp*. in chronic test 1 and 2 respectively, the procedure was the same in both tests. Test chambers were inspected daily for aeration and feeding requirements and to remove dead animals.

At the end of the 28-d exposure period, the contents of each chamber were gently sieved through 1000 and 250 µm mesh sieves to collect surviving adults and their offspring, respectively. Four to five pools of 4 to 6 males from each test sediment (pool wet wt. ~ 0.05 g) were frozen and stored at –80 °C for later quantification of whole body metal bioaccumulation, MT and LP. Fifteen adults (males and non-gravid females) of each tested sediment were sampled for immediate DNA extraction and subsequent analysis of SB.

2.5. Biological responses

2.5.1. Quantification of whole-body heavy metals and MT levels. Pools of whole animals (pool wet wt. about 0.05 g) were homogenized at 4 ºC in 4 ml of 0.02 M Tris-HCl buffer (pH 8.6) and sub-samples taken for determination of heavy metals and MT. Whole-body metal analyses were carried out on dried, HNO_3-digested sub-samples using flame atomic absorption spectrophotometry. Analysis of dogfish muscle (DORM-1) and liver reference (DOLT-1) material (National Research Council of Canada, Canada) was carried out, using the same treatment, in order to validate the metal analyses. The values measured for Cu, Zn and Cd, were within the certified range and the concentrations were expressed as $\mu g.g^{-1}$ dry wt. of whole body homogenate. MT determination was performed by differential pulse polarography

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(DPP), essentially as described in Bebianno and Langston (1989). An aliquot of the sub-sample homogenate (2 ml) was centrifuged at 30 000 x g for 1 hour at 4 ºC. The cytosol was heat-treated at 80 ºC for 10 minutes to precipitate the high molecular weight proteins, and subsequently centrifuged at 30 000 x g for 1 hour at 4 ºC. Aliquots (150 - 250 µl) of the heat-treated cytosol were taken for quantification of heat-stable MT using DPP with a static mercury drop electrode. A Metrohm 693 VA Processor and the 694 VA Stand was used for that purpose. The Brdicka supporting electrolyte containing 1 M NH_4Cl, 1M NH_4 OH and 2 mM $[Co(NH_3)_6]Cl_3$ was prepared weekly and stored at 4 ºC (Palecek & Pechan, 1971). In the absence of a purified amphipod MT, quantification was by reference to standard additions of rabbit liver MT-1 (Sigma, Portugal). The values obtained were expressed as mg rabbit-MT equivalents g^{-1} dry wt. of whole body homogenate.

2.5.2. Lipid peroxidation. Malondialdehyde (MDA) was determined by the thiobarbituric acid method of Ohkawa et al. (1979) with minor modifications. Pools of whole animal (0.05 - 0.08 g wet wt) were homogenized at 4 ºC in 1:4 wet wt./buffer volume ratio in 50 mM NaH_2PO_2/Na_2HPO_4 pH 7.4 containing 15 % glycerol (w/v), and centrifuged at 9000 x g for 15 minutes at 4 ºC. Sub-samples (62.5 µl) of tissue homogenate were treated with 25 µl of 8.1 % dodecyl sulphate sodium, 187 µl of 20 % tricloroacetic acid (pH 3.5) and 187 µl of thiobarbituric acid. The mixture was made up to 0.5 ml with distilled water and then heated for 60 minutes in boiling water. After cooling, 125 µl of distilled water and 625 µl of a mixture of n-butanol and pyridine (15:1, v/v) were added. The mixture was shaken vigorously before centrifugation at 4000xg for 10 minutes. The organic layer was then recovered and its absorbance measured at 532 nm. MDA concentrations were derived from a standard curve and the values expressed in terms of MDA ηmol equivalents per g wet wt. tissue.

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2.5.3. DNA Strand breakage analysis. DNA was isolated individually from whole amphipods, immediately after the 28-day exposure. An outline of the DNA extraction procedure and DNA strand breakage analysis is presented below, while detailed descriptions are provided in Costa et al. (2002). Briefly, the DNA isolation involved extractions with PCI (phenol:chloroform:isoamyl alcohol, 25:24:1, v/v/v) and subsequently chloroform, before and after digestions with ribonuclease A and proteinase K. Strand breakage analysis comprised electrophoresis of the DNA extracts under alkaline (pH 12) and neutral (pH 8) conditions, thus allowing for determination of total (single and double) and double-stranded breaks in the DNA, respectively. Migration of the DNA within the gel matrix is size dependent, and detection is easily accomplished after staining with ethidium bromide.

Photographs of ethidium-bromide stained gels were analysed with the software QWin Lite V2.3 (Leica Microsystems) in order to obtain densitometric profiles of the migration of each DNA sample. Finally the average molecular length (L_n) was computed from these data. The average molecular length is inversely proportional to the number of DNA strand breaks according to the formula:

$$\text{Number of strand breaks per } 10^5 \text{ nucleotides} = 1/L_n \times 100 \qquad [1]$$

In order to normalize results among gels, it was required to determine the relative number of total (RNTSB) and double strand breaks (RNDSB). This was accomplished by calculating the difference in the number of strand breaks between every treatment sample and the respective control sediment mean within each gel:

$$\text{samples from total-strand break gel: } RNTSB = 1/L_n\,(s_{ij}) - 1/L_n\,(Cm_j) \qquad [2]$$

$$\text{samples from double-strand break gel: } RNDSB = 1/L_n\,(s_{ij}) - 1/L_n\,(Cm_j), \qquad [3]$$

where s_{ij} is the sample *i* from gel *j* and Cm is the respective control mean from gel *j*

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Accordingly the relative number of single strand breaks (RNSSB) was determined as follows:

$$\text{RNSSB per } 10^5 \text{ nucleotides} = \text{RNTSB}_i - (2 \times \text{RNDSB}_i), \quad [4]$$

where *i* is the sample number.

2.6. Statistical analyses

A one–way analysis of variance (ANOVA) was carried out for each studied variable (tissue level of heavy metals, MT, LP and SB variables - L_n TSB, L_n DSB, RNTSB, RNDSB and RNSSB) to determine if differences in responses between exposed and control amphipods could be attributed to exposure to contaminated sediments. Significant differences were established at $P<0.1$. The Fisher's Least Significant Difference test (LSD) was used for multiple comparisons between pairs of means.

2.7. Weight-of-evidence (WOE) approach

Based in Chapman et al. (2002) procedure, a WOE framework was applied to our data in order to assemble and interpret the information derived from the multiple lines of evidence (LOE) produced in this investigation. This approach entailed the setting up of an ordinal ranking system to categorize our LOE, followed by construction of the WOE interpretation matrix.

Table 1 summarizes the ordinal ranking system adopted. The various LOE considered comprise concentrations of sediment's contaminants, acute toxicity data, chronic toxicity, and information on benthic community structure obtained from other study (Mucha & Costa, 1999). The LOE for chronic toxicity comprised bioaccumulation of metallic contaminants and biomarker responses (MT, LP and SB) here reported, and organism/population responses reported in Costa et al (submitted this issue). Three categories of responses compared to

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control treatment were considered: negative, neutral and positive. Within negative and positive categories two levels of intensity were considered: moderate and high.

The various LOE differ in the type and relevance of information provided for ecotoxicological assessment of the tested sediments. Therefore, the global evaluation of the quality of each tested sediment required a best professional judgment (BPJ) approach (Chapman et al., 2002). This approach enabled a qualitative analysis of the WOE matrix, attending to the particular information (and its relevance) that can be obtained from each LOE.

3. Results

3.1. Sediment geochemistry

Results of geochemical analyses of sediments are presented on Table 2. 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 sediments 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. Bioaccumulation

Only heavy metals were measured in amphipod tissues. The results for whole-body heavy metal accumulation are displayed in Table 2. The accumulation of Cu was significantly higher in amphipods exposed to sediments P, S2 and D, with 7, 21 and 66 % higher levels,

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respectively, compared with the values of control organisms ($p< 0.1$, $p< 0.05$ and $p< 0.01$ respectively).

Zn detected in organisms exposed to sediment P was also higher in about 26% compared to control ($p< 0.1$). No significant bioaccumulation of Cd was observed in amphipods in any of the contaminated sediments, except sediment S2. On the contrary, for sediments P and D significantly lower body-burdens of Cd were detected ($p< 0.01$).

3.3. MT induction and LP

In chronic test 1 no induction of metallothionein (MT) was observed in animals exposed to contaminated sediments (sediments P and T) compared to control levels (1.3 mg MT g^{-1} dry wt). In contrast, lipid peroxidation (LP) in sediments P and T was about 30% and 40% higher than control (13.5 ηmol MDA g^{-1} wet wt.; $p< 0.05$ and $p< 0.01$ respectively). In chronic test 2, significantly higher MT induction was detected only in amphipods exposed to sediment D compared to control levels of 1.4 mg MT g^{-1} dry wt ($p< 0.1$). Effects on LP were observed only in animals from sediment S1, where values were 36 % higher than control (24.4 ηmol MDA g^{-1} wet wt., $p< 0.01$) (Table 3).

3.4. DNA integrity

The differences in the relative number of total and single strand breaks (RNTSB and RNSSB) of amphipods exposed to contaminated and control sediments are shown in Table 3. In general, for all the contaminated sediments tested, no effects were observed on double strand breakage parameters (L_n DSB and RNDSB).

In chronic test 1, no effects on DNA integrity were detected in exposed amphipods (sediment P and T) compared to control organisms. The RNTSB and RNSSB of amphipods from sediments P and T did not differ from control values ($p> 0.1$). In chronic test 2, the

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results showed significant differences between amphipods exposed to each of the contaminated sediments (S1, S2 and D) compared to control, when the L_n of TSB was examined ($p< 0.05$, $p< 0.01$ and $p< 0.01$ respectively). Loss of DNA integrity was also observed in the relative number of strand breaks (total and single) determined in organisms exposed to sediments S2 and D (RNTSB - $p< 0.01$ and $p< 0.1$, respectively and RNSSB - $p< 0.01$ and $p< 0.05$, respectively). In sediments S2 and D, amphipods had on average twice as many single strand breaks per 10^5 nucleotides than control. In amphipods from sediment S1, RNTSB and RNSSB values were also higher than control, but they were not significantly different ($p> 0.1$).

3.5 Weight-of-evidence (WOE) approach

Table 4 provides a global WOE interpretation of the results here reported (sediment chemistry, metal bioaccumulation, biomarker responses)], together with organism/population endpoints (Costa et al., submitted this issue), and also benthic community information from other study (Mucha & Costa, 1999). Overall, sediments S1, S2 and D, were considered to present a sizeable negative impact and ecological risk. Sediments' S1 and S2 impact is thought to be related in part with PAHs toxicity, while copper contamination appears to have a role in sediment's D toxicity. The two remaining sediments – T and P – did not show negative impact and appear to have a beneficial effect in the test species. However, sediment P have a detrimental ecological impact *in situ*, as indicated by benthic communities' information, that could not be detected through toxicity testing. The reasons for these global sediment quality assessments are specified in the Discussion under a BPJ perspective.

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4. Discussion

4.1 Biomarker responses

Considering the biomarker responses on the whole, sediments evaluated in chronic test 1 (sediments P and T) did not exhibit contaminant-induced stress in the amphipod *G. locusta*. By comparison with control organisms, no adverse effects were detected on MT induction and DNA integrity. Whereas in sediment T only Zn concentrations raise some concern, in sediment P several contaminants (Cu, Zn and PAHs) exceeded the ERL, and therefore biomarker responses (over chronic exposures) might be anticipated. The fact that bioaccumulation of Cu and Zn was only little higher than control levels (approximately one fold higher than control), and apparently insufficient to induce MT, indicates low bioavailability of metallic contaminants in sediments P. The lower levels observed for Cd body-burdens, compared to control in this sediment (and also in sediment D), most likely resulted from interactions with the essential metals Cu and/or Zn that were bioaccumulated by *G. locusta*. These interactions can take place at different stages of absorption, distribution in the organism, and excretion of the above-mentioned metals (Brzoska & Moniuszko-Jakoniuk, 2001). Some studies showed that increased Zn supply may reduce Cd absorption and accumulation and prevent or reduce the adverse action of Cd (Brzoska & Moniuszko-Jakoniuk, 2001).

The high content of organic matter of sediment P (8.7% TVS) possibly caused low bioavailability of both metals and PAHs to *G. locusta*, reducing or eliminating the potential negative impacts that would be anticipated from contaminant concentrations alone. It has been extensively shown that either metallic or organic contaminants may become unavailable to biota in sediments with high organic content, due to the strong sorption affinity of contaminants to sediment's organic carbon matrix (Gunnarsson et al., 1999; Lawrence &

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Mason, 2001). Previous results from laboratory studies with *G. locusta* where the sediment-Cu LC50s increased considerably and directly with sediment's organic carbon content (Correia & Costa, 2000), also support this premise. Low contaminant bioavailability as a result of binding to organic carbon matrix may also explain the absence of adverse effects recorded at organism/population level in sediment P. Actually amphipods exposed to this sediment exhibited high individual performance, in particular a significant improvement of individual growth and pregnancy ratio compared to control animals. Similar stimulating effects on growth were also observed in sediment T, although not as significant as in sediment P (Costa et al, submitted this issue).

As opposed to these results, the higher levels of lipid peroxidation detected in animals exposed to sediments P and T, would indicate occurrence of toxicant-induced stress. Although these sediments differ in contamination levels, they have in common the high level of organic content (8.7% and 11.5% TVS, respectively), a feature that was the most likely cause for the growth stimulation observed in amphipods exposed to both sediments. As there is no other evidence showing contaminant-induced insult from all remaining parameters, composite data suggests that LP resulted from other causes than oxidative stress derived from exposure to oxyradical-generating compounds (e.g. Cu or Zn and/or PAHs). Effectively, these LP results are congruent with known evidence that endogenous variables (e.g. nutritional status, age, sex, growth and reproduction) may themselves influence the peroxidation status of organisms (Viarengo, Canesi, Pertica, & Livingstone, 1991), including *G. locusta* (Correia, Costa, Luis, & Livingstone, 2003), therefore contributing to confound contaminant-induced effects. Earlier studies with *G. locusta* revealed that increases in LP could be attributed to the improvement of the physiological condition of animals and not directly to damage derived from exposure to copper (Correia, 2002).

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Results from chronic test 2 globally showed that sediments S2 and D generated chronic toxicity to the amphipod *G. locusta*. According to DNA damage data, sediments S2 and D can produce DNA strand breakage, mainly single strand breaks. The loss of DNA integrity was observed by the significantly higher L_n TSB, RNTSB and RNSSB in animals exposed to both sediments, but it was much more pronounced in sediment S2. The detection of these adverse effects in amphipods from sediments S2 and D provide evidence of exposure and bioavailability of genotoxicants in both sediments. Although the complex chemical nature of sediments may make unclear which were the compound(s) responsible for the DNA damage observed, the approach here followed may help identifying probable candidates. Compound analyses of the various parameters provide significant weight-of-evidence that copper contributed to the detrimental effects observed in amphipods exposed to this sediment D: 1) high concentration of Cu, namely exceeding ERM, 2) significant Cu bioaccumulation and 3) MT induction. Although copper is an essential metal, it may become toxic if intracellular concentrations exceed the organisms' requirements and its detoxification capability (Viarengo, 1989; Schenk, Davis, & Griffin, 1999; Livingstone, 2001). The available scientific evidence referred to in the literature, indicates the potential of copper and other heavy metals as genotoxicants (Bolognesi, Landini, Roggieri, Fabbri, & Viarengo, 1999; Jha, Cheung, Foulkes, Hill, & Depledge, 2000). Moreover in previous studies with *G. locusta*, Cu has been shown to induce MT (Correia et al., 2002) and DNA single strand breaks (Costa et al., 2002).

Effects of MT induction were only observed in amphipods exposed to sediment D. A positive correlation was also detected between MT concentration and whole-body levels of Cu ($r = 0.5035$, $p < 0.05$), indicating that induction of MT was closely associated with Cu in sediment D. The simultaneous presence of SB and MT in animals exposed to sediment D, and the absence of MT induction in amphipods from sediment S2 suggests that the dynamics of toxicity differed in the latter. Although not as high as in sediment D, there was still some Cu

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bioaccumulation in sediment S2, and also levels of Cd little higher compared to control. In this respect it is noteworthy that precisely in amphipods from sediment S2, which apparently lacked MT "protection", DNA damage was especially severe. Although other possible interpretations for these observations cannot be discounted, according the available data the most plausible is that effects on DNA integrity were caused mainly by other genotoxicants, which are not detoxified by MT. The high levels of PAHs (over ERL) in sediment S2 are particular relevant in a sandy sediment where the bioavailable fraction of organic contaminants is potentially higher compared to muddy sediments.

Chronic toxicity of sediments S2 and D was also recorded at the organism/population levels. As described in Costa et al (submitted this issue), an extensive impaired condition was observed in amphipods exposed to these sediments that was expressed by lower survival, an unbalanced sex ratio, low proportion of gravid females, and the lowest number of offspring, by comparison with control amphipods. Hence, the biochemical endpoints are on the whole in agreement with changes seen at higher-levels of biological organization (specially evident on SB), providing more conclusive evidence on the prevalence of contaminant-induced stress in these sediments.

Concerning sediment S1, no MT induction was detected and the loss of DNA integrity was not as high and clear than in amphipods exposed to S2 and D (significant effects were only observed in Ln TSB). Similar to what was observed in the first chronic toxicity test, effects on LP were also detected in amphipods exposed to this sediment. For the reasons previously mentioned this response is again interpreted as related with the higher average length observed in S1 amphipods. Despite the high level of contamination and acute toxicity of this sediment, the 75% dilution with control sediment was effective in almost eliminating chronic toxicity. However, this dilution may be just near the toxicity threshold, as residual traces of toxicity and some evidence of stimulating effects on growth suggest occurrence of

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hormesis. Hormesis is a biological response to sub-inhibitory doses of stressor characterized by stimulatory effects on biological indicators of insulted organisms, particularly growth, compared to non-stressed control organisms (Stebbing, 1982, 1997). The event of hormesis has been document in vertebrate and invertebrates in response to a variety of stressors (Calabrese, Baldwin, & Holland, 1999). As showed in a previous study, where evidence was found of copper-induced hormesis in *G. locusta*, stimulatory effects of low doses of toxicant can be coupled with induction of LP (Correia, 2002) and MT (Correia et al., 2001). The toxicity of sediment S1 was patent even at 50% dilution. By further diluting this sediment with control sediment, contaminant concentrations were reduced possibly to a hormetic level, with stimulatory effects on growth and LP, but not on MT, plausibly because hormesis inducer was not a metallic contaminant.

4.2 Weight-of-Evidence (WOE) approach

In summary, by way of a WOE interpretation of the multiple biological effects examined, the following conclusions can be drawn from this investigation:

1) Sediments enriched in organic matter may have a significant positive impact in *G. locusta*'s growth in chronic bioassays (e.g. sediments T and P). These potential effects should be considered in advance when designing either acute or chronic bioassays with benthic organisms, where multiple-level assessments may play an useful role.
2) On the whole, sediments T and P did not reveal contaminant-induced stress to the amphipod *G. locusta*. Despite sediment P showed some chemical contamination, the high organic content of this sediment may have had some influence in reducing the bioavailability of toxicants and consequently eliminating symptoms of toxicity. On the other hand, the same factor probably promoted growth and fecundity of *G. locusta*. All previous (Costa et al., 1998; Neuparth, 1999) and current tests with sediment P failed

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to show toxicity. However, these findings do not match with benthic macrofauna studies that recorded a disturbed community in the sediment collection site (Mucha & Costa, 1999). The development of extreme anoxic conditions under high load of organic matter, has been proposed as explanation for this impoverished benthic community (Mucha and Costa, 1999).

3) As opposed to sediment P, results from the second chronic test show that a high content of organic matter in the sediment is not necessarily a shield from contaminant insult. That is the case for sediment D, which despite being the sediment with highest organic content tested, was also one of chronically toxic sediments. Although other possibilities cannot be discounted, there is some WOE that the origin of the sediment D toxicity is associated with copper, namely considering the sediment's copper above ERM, bioaccumulation of copper and induction of MT.

4) The origin of the sediments S1 and S2 toxicity is apparently related with the nearby industrial effluent at the sampling site, as it is evidenced by reduction of sediment toxicity as a function of the distance from the effluent – S1 more toxic than S2. The high levels of PAHs detected in these sediments probably played a role in the serious detrimental effects recorded. The current findings of severe toxicity match with data showing a highly disturbed benthic community at these sediments' collection site (Mucha & Costa, 1999).

5) A presumed hormetic response occurred after exposure of the amphipods to serial dilutions of sediment S1. Only recently hormesis started to receive more widespread recognition (Calabrese & Baldwin, 1999) but its relevance for ecological risk assessments should be given proper appreciation promptly [Calabrese, 1999 #310; (Chapman, 2002). The application of multiple-level assessments and WOE approaches in chronic bioassays is likely to improve the ability to detect and understand better this

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relevant phenomenon. Particularly considering that hormesis has been documented for a multitude of stressors, toxicants being one among many others.

6) Sediments' dilutions contribute to establish more clearly dose-responses and enable ranking of sediment toxicity, which in this study was S1>S2>D. Sediment S1 was the most toxic since a 75% dilution was required to eliminate acute toxicity and reduce chronic toxicity to the putative hormetic response. Sediment S2 was the second most toxic sediment since it still exhibited chronic toxicity after a 50% dilution, and sediment D was the least toxic of the three sediments given that it only showed chronic toxicity, but not acute toxicity when tested in full.

This study illustrated how integration of biochemical markers in chronic sediment tests within a WOE framework, can help backing-up interpretation of organism and population-level responses. By providing evidence of exposure (or lack of it) to contaminants in sediments under examination, the biomarkers here applied assisted in the identification of the grounds for organism-level responses. Namely they contributed to distinguish responses induced by sediment contaminants from those responses derived from other factors, such as for example the amount of organic matter in the sediment.

Further research is encouraged in what concerns with the use of a multi-level assessment of chronic sediment toxicity, as for example the inclusion of the biomarkers tested here, and eventually other potential biochemical endpoints, in long-term tests where responses are determined on a time-course basis. This will enable a better understanding of pathways of contaminant metabolism, detoxication and toxic action, allowing to follow more closely the whole toxicological process up to the organism/population levels.

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Table 1. Ordinal ranking scheme applied to sediment quality data in order to build the weight-of-evidence tabular interpretation matrix provided in Table 4.

		●●	●	⊖	○	○○
	CHEMISTRY (Metals - PAHs - PCBs)	One or more contaminants exceed ERM	One or more contaminants exceed ERL	All contaminants are below ERL	----------	----------
	ACUTE TOXICITY TESTS	Mortality higher than 20%	20% ≤ Mortality ≥ 10%	Mortality lower than 10%	----------	----------
CHRONIC TOXICITY TESTS	**Bioaccumulation** (Cu – Zn - Cd)	Contaminant uptake higher than control, at least for one of the metals ($p < 0.01$)	Contaminant uptake higher than control, at least for one of the metals ($0.01 \leq p < 0.1$)	No significant increase of contaminants uptake compared to control ($p \geq 0.1$)	Contaminant uptake lower than control, at least for one of the metals ($0.01 \leq p < 0.1$)	Contaminant uptake lower than control, at least for one of the metals ($p < 0.01$)
	Biomarkers (MT - LP - SB)	Significant induction of biomarker responses compared to control levels ($p < 0.01$)	Significant induction of biomarker responses compared to control levels ($0.01 \leq p < 0.1$)	No significant induction of biomarker responses compared to control levels ($p \geq 0.1$)	Significant reduction of biomarker responses compared to control levels ($0.01 \leq p < 0.1$)	Significant reduction of biomarker responses compared to control levels ($p < 0.01$)
	Survival	Significant reduction of survival compared to control ($p < 0.01$)	Significant reduction of survival compared to control ($0.01 \leq p < 0.1$)	No significant difference on survival compared to control ($p \geq 0.1$)	Significant increase of survival compared to control ($0.01 \leq p < 0.1$)	Significant increase of survival compared to control ($p < 0.01$)
	Growth	Significant reduction of growth compared to control ($p < 0.01$)	Significant reduction of growth compared to control ($0.01 \leq p < 0.1$)	No significant difference on growth compared to control ($p \geq 0.1$)	Significant increase of growth compared to control ($0.01 \leq p < 0.1$)	Significant increase of growth compared to control ($p < 0.01$)
	Reproductive traits	Substantial impairment in reproductive traits (compared to control)	Moderate impairment in reproductive traits (compared to control)	No symptoms of impairment or improvement in reproductive traits (compared to control)	Moderate improvement in reproductive traits (compared to control)	Substantial improvement in reproductive traits (compared to control)
	BENTHIC COMMUNITY STRUCTURE	Diversity and/or abundance substantially lower than control	Diversity and/or abundance lower than control	Diversity and/or abundance not different from control	----------	----------
	OVERALL ASSESSMENT	**(Severe adverse effects)**	**(Moderate adverse effects)**	**(Neutral effects)**	**(Moderate beneficial effects)**	**(High beneficial effects)**

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Table 2. Sediments' collection sites, sediment features, and chemical contaminants of each sediment tested in chronic toxicity tests.[a, b, c]

	Control Sediment	Chronic Test 1		Chronic Test 2		
		Sediment T	Sediment P	Sediment S1	Sediment S2	Sediment D
Sediments' collection sites	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.
Sediment Type	sand	mud	mud	sand	sand	mud
TVS (%)	0.7	11.5	8.7	0.4	0.4	12.7
Metals (µg.g^{-1} dry wt)						
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*
PAHs and PCBs (ηg.g-1 dry wt)						
naphthalene	17	19	41	4	65	5
acenaphthylene	3	3	13	BDL	21	5
acenaphtene	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
∑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 [b]	81	93	970	1390	1596	335
benzo(g,h,i)perylene [b]	BDL	BDL	492	BDL	BDL	BDL
benzo(k)fluoranthene [b]	BDL	BDL	BDL	BDL	BDL	BDL
indeno(1,2,3-cd)pyrene [b]	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
∑High mol wt PAHs	340	389	4900*	9779*	6879*	1574
∑Total PAHs	434	495	5312*	11627*	7247*	1742
Total PCBs	0.83	0.83	2.51	0.68	0.89	1.78

[a] BDL = Bellow detection limit
[b] ERL and ERM guidelines not available
[c] Values above ERL = *; Values above ERM = **

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Table 3. Compilation of biomarker responses of *Gammarus locusta* to tested estuarine sediments.[a,b]

	Chronic Test 1			Chronic Test 2			
	Sediments						
	C	T	P	C	S1	S2	D
Bioaccumulation ($\mu g.g^{-1}$ dry wt)							
Cu	59.1±5.0	53.4±8.1	63.5±8.5*	42.4±3.9	44±3.6	51.3±6.7**	70.0±11.4***
Zn	42.2±5.5	48.1±11.4	53.4±9.3*	60.2±9.7	52.4±6.5	53.9±5.7	78.9±0.2
Cd	0.21±0.06	0.18±0.05	0.08±0.02***	0.61±0.2	0.56±0.15	0.89±0.29*	0.25±0.11***
Metallothionein (mg.g-1dry wt)							
	1.4±0.1	1.2±0.3	1.2±0.3	1.4±0.1	1.6±0.1	1.5±0.4	1.8±0.2*
Strand Breakage							
L_n TSB	27.9±5.6	26.6±8.5	28.5±7.4	27.1±8.1	21.8±7.3**	16.3±5.4***	19.8±4.9***
L_n DSB	38.3±6.3	40.3±8.7	36.3±6.0	48.0±10.8	45.3±12.1	43.9±12.9	48.1±11.5
RNTSB	0.12±0.67	0.55±0.37	0.15±0.96	0.65±1.8	1.41±1.64	3.34±2.12***	1.70±1.44*
RNSSB	-0.009±0.92	0. 59±2.17	-0.27±1.23	0.54±1.50	1.081±1.56	2.70±2.34***	1.79±1.78**
Lipid peroxidation (ηmol MDA g^{-1} wet wt.)							
	13.5±0.8	19.3± 2.9***	17.9±2.2**	24.4±2.9	33.1±2.7***	27.7±5.6	24.9±4.1

[a] L_n TSB and L_n DSB - average molecular length of total and double strand breakage, respectively; RNTSB and RNSSB – relative number of total and single strand breakage, respectively.
[b] Asterisks indicate significant effects compared with control: * = p< 0.1; ** = p< 0.05; *** = p< 0.01.

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Table 4. Weight-of-evidence tabular interpretation matrix of toxicity of sediments from Sado and Tagus estuaries analysed in this study. Matrix and ordinal ranking scheme (see Table 1) based upon Chapman et al. (2002).[a,b]

LINES OF EVIDENCE ↓ SEDIMENTS →	Chronic test1		Chronic test 2		
	Sediment T	Sediment P	Sediment S1	Sediment S2	Sediment D
CHEMISTRY (Metals – PCBs - PAHs)	●- ⊖ - ⊖	● - ⊖ - ●	⊖ - ⊖- ●	⊖ - ⊖- ●	●● - ⊖ - ⊖
ACUTE TOXICITY TESTS	0% dilution ⊖	0% dilution ⊖	0% dilution ●● 50% dilution ●	0% dilution ●● 50% dilution ⊖	0% dilution ⊖
CHRONIC TOXICITY TESTS [a]	(0% dilution)	(0% dilution)	(75% dilution)	(50% dilution)	(0% dilution)
Bioaccumulation (Cu - Zn - Cd)	⊖ - ⊖ - ⊖	● - ● - ○○	⊖ - ⊖ - ⊖	●- ⊖ - ●	●●- ⊖ - ○○
Biomarkers (MT – LP - SB)	⊖ - ●● - ⊖	⊖ - ● - ⊖	⊖ - ●● - ⊖	⊖ - ⊖ - ●●	● - ⊖ - ●●
Individual/Population Endpoints (survival – growth - reproductive traits) (Costa *et al*., submitted this issue)	⊖ - ○○ - ○	⊖ - ○○ - ○○	⊖ - ○ - ○	● - ? - ●	●● - ? - ●
BENTHIC COMMUNITY STRUCTURE (Mucha and Costa, 1999)	NA	●●	●●	●●	NA
OVERALL ASSESSMENT / COMMENTS (based on Best Professional Judgment)	Moderate beneficial effects (high load of organic matter)	- Beneficial effects recorded in chronic toxicity test (high load of organic matter) - Disturbed benthic community (anoxic conditions)	Severe adverse effects (PAHs toxicity)	Severe adverse effects (PAHs toxicity)	Moderate adverse effects (Copper toxicity)

[a] Chemistry data refers to full non-diluted sediments
[b] ? = Inconclusive results - see Costa et al. (submitted this issue) for discussion

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Figures Captions

Figure 1. Sediments' sampling sites in Tagus (above) and Sado (below) estuaries. The sediments from Sado estuary were labelled as C, D, P and S (location for sediments S1 and S2) and the sediment from Tagus estuary as T.

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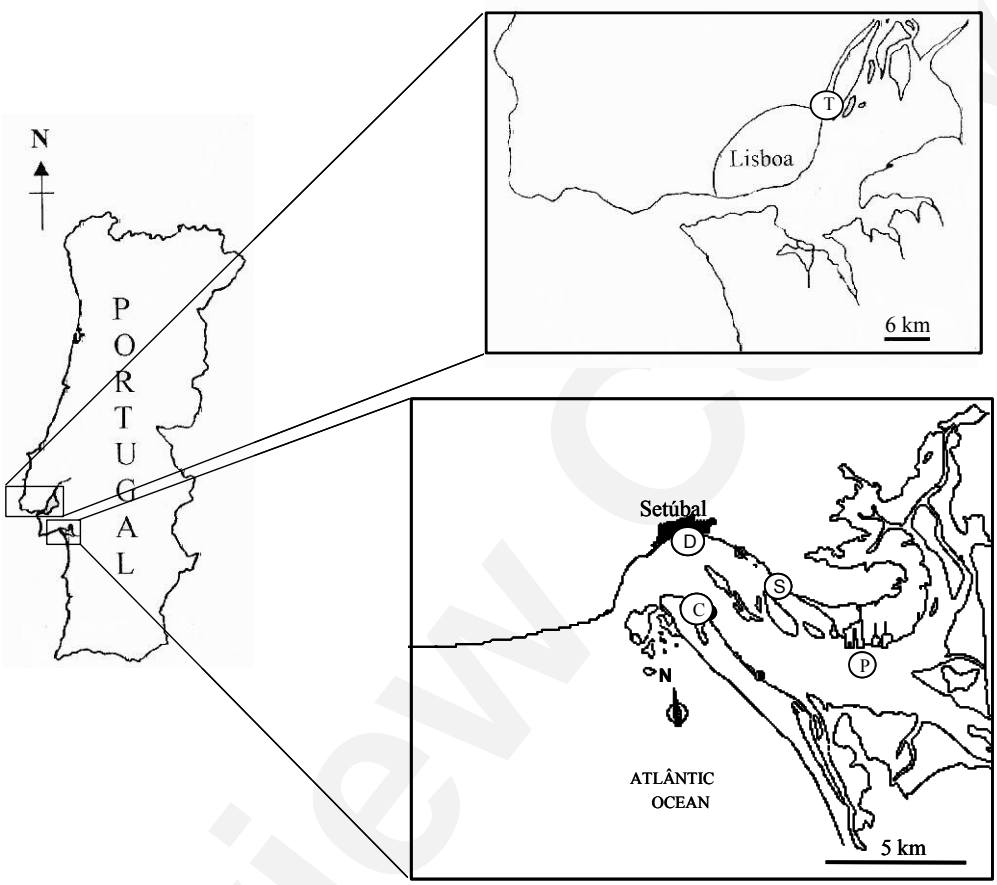

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