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Detection of DNA strand breakage in a marine amphipod by agarose gel electrophoresis: exposure to X-rays and copper

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This article describes the leading steps to develop an assay of DNA damage for the marine amphipod *Gammarus locusta,* using agarose gel electrophoresis (AGE). To test the sensitivity and feasibility of the AGE technique, X-ray assays were performed with naked DNA and with live amphipods. These positive controls demonstrated the effectiveness of the AGE technique to not only discriminate distinct levels of DNA strand breakage in a dose-dependent manner, but also to identify and quantify the type of strand breakage induced. It was also shown that it is possible to detect DNA damage using whole-body DNA extracts from amphipods. To explore the potential of this technique for use in ecotoxicological studies with amphipods, a 96-h waterborne-copper toxicity test was performed. Copper-induced DNA strand breakage was first observed after 24 h of exposure, and was recorded again at 96 h, at a copper concentration of 20 $\mu g l^{-1}$. The absence of strand breakage after 48 h of exposure is discussed in the light of the underlying mechanisms of copper toxicity and DNA repair. These studies demonstrated the feasibility of including DNA damage as a biomarker in ecotoxicological studies with amphipods. Information gained from the use of this biomarker would help with the interpretation of chronic toxicity tests and would contribute to our understanding of the impact of genotoxic insult in marine invertebrates, particularly crustaceans.

Keywords: DNA strand breakage, agarose gel electrophoresis, amphipod, *Gammarus locusta*, X-rays, copper

Introduction

The amphipod crustacean *Gammarus locusta* (L). has been proposed for acute sediment toxicity testing in Europe (Costa *et al.* 1998, Costa and Costa 2000) due to a number of characteristics, including availability all along Atlantic Europe, ecological relevance, sensitivity to toxicants and amenability for culturing and testing. In addition, a chronic sediment toxicity test using this species is currently under development (Correia *et al.* 2001, Neuparth *et al.* 2002).

Amphipod sediment toxicity tests have relied on acute or chronic exposures in which the endpoints used are mostly survival, behaviour, growth and

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reproduction. The main difficulties associated with these tests derive from two aspects: (1) the lack of specificity of the response criteria used, despite of their ecological relevance; and (2) the fact that amphipods can be affected by sediment's natural geochemical features, particularly during chronic exposures. We have experienced these difficulties in our endeavour for an accurate understanding of chronic sediment toxicity tests with the amphipod *G. locusta*.

To help bridge the gap between sediment contamination and population responses, and also to provide more insight into the mechanisms of sediment toxicity, biomarkers have been used in ecotoxicological studies with *G. locusta* (Correia *et al*. 2001, 2002a). A biomarker of DNA damage is particularly desirable for this purpose, due to the vital importance of DNA to an organism's life and its progeny and the subsistence of the species.

Maintenance of DNA integrity is of paramount importance for all living organisms, and this is reflected in the very efficient and intricate mechanisms that exist for the protection of the genetic material and the consequent low mutational rate (Shugart 1999, Steinert 1999). Molecular changes in DNA and/or damage to its integrity may potentiate a cascade of detrimental effects from the cellular to the organism level and ultimately to the population level (Forbes 1999, Shugart 1999, Moustacchi 2000). If DNA damage occurs in germ cells of an individual, subsequent generations may be directly affected (Depledge 1998, Shugart 1999).

During the last decade many chemicals of anthropogenic origin have been found to interact with DNA (Depledge 1998). Because of the ubiquity of DNA-reactive or genotoxic agents in the environment, biomarker assays to detect genotoxicity have been increasingly developed and applied to a number of aquatic animals (Hose 1994, Black *et al*. 1996, Depledge 1998, Forbes 1999, Shugart 1999, De Maagd and Tonkes 2000, Dixon and Wilson 2000), especially to fish (Theodorakis *et al*. 1994, 2000, Everaarts 1995, Devaux *et al*. 1998, Choi *et al*. 2000, Lacorn *et al*. 2001).

Little is known about the mechanisms of genotoxicity in marine invertebrates: how they are affected by genotoxicants, which physiological processes are disrupted and how these organisms cope with genotoxic insult at cellular, physiological and population level. As a result of their studies on DNA strand breakage in mussels, Black *et al*. (1996) and Ching *et al*. (2001) recognized that research on the aetiology of DNA damage and repair processes is required.

Among aquatic invertebrates, DNA damage has been mostly investigated in bivalves (Black *et al*. 1996, Bolognesi *et al*. 1999, Steinert 1999, Jha *et al*. 2000, Ching *et al*. 2001, Coughlan *et al*. 2001), but also rarely in crustaceans. In particular, genotoxicity research in amphipods is lacking, even though these animals have been widely used in ecotoxicological studies (ASTM, 1992, Hill *et al*. 1994, DeWitt *et al*. 1999), and play an important role in the structure, function and biodiversity of freshwater and marine ecosystems worldwide (Thomas 1993, Conlan 1994).

Here we report for the first time, to our knowledge, an investigation of DNA strand breakage in an amphipod crustacean. This paper describes the leading steps in developing a biomarker of DNA damage for the marine amphipod *G. locusta* using agarose gel electrophoresis (AGE). Unlike other techniques commonly used to assess DNA damage (e.g. comet assay), gel electrophoresis does not require isolation of a particular target tissue of the assay organism, since DNA extracts

from the whole organism may be used. This is critical when using amphipods due to their small size (often less than 10 mm) and the inherent difficulties to isolating tissues. Furthermore, by employing this technique each individual can be analysed several times and DNA extracts may be archived for posterior analysis such as DNA fingerprinting (e.g. Theodorakis and Shugart 1998).

However, in AGE DNA extracts from selected tissues have usually been used (Theodorakis *et al.* 1994, Black *et al.* 1996), and it is not known whether this technique can detect DNA damage using DNA extracts from whole amphipods. Therefore, to test the sensitivity and feasibility of this technique, a positive control was required. For this purpose treatment with X-rays – a known inducer of DNA strand breakage (Snyder 1994, Theodorakis and Shugart 1998) – was selected. In order to explore the potential of this technique for use in ecotoxicological studies with amphipods, a copper toxicity test was performed.

Various heavy metals have been shown to elicit DNA damage, although there is no general consensus as to the genotoxic potential of copper (Bolognesi *et al.* 1999), there is evidence that, directly or indirectly, copper generates genotoxicity (Nojima 1992, Bolognesi *et al.* 1999, Yusof and Ercal 1999). Copper was selected for this study since it is one of the major metallic contaminants recorded in sediments from the Sado estuary, a large estuarine system located on the central west coast of Portugal. This estuary has been a reference location for ecotoxicological studies with *G. locusta*, particularly sediment toxicity assessments (Costa *et al.* 1998; our unpublished data). Industrial activities in the outer estuary and historical contamination from mine-fields upstream resulted in high heavy metal concentrations in the sediments. Quintino *et al.* (1995) reported copper concentrations in the sediment ranging from 136–220 $\mu g\, g^{-1}$. Another reason to select copper for this study is that a large and diverse set of data on the toxicity of this metal to *G. locusta* already exists, including acute and chronic toxicity (Correia and Costa 2000, Correia *et al.* 2001), bioaccumulation, metallothionein induction, lipid peroxidation and histochemical detection (Correia *et al.* 2002a, Correia *et al.* 2002b).

Material and Methods

Test organisms

G. locusta were collected from a clean location on the south margin of Sado and estuary, were cultured in a laboratory at 15°C in plastic aquariums with 0.45 µm filtered seawater of 33–34/10‰ salinity and a sediment layer of about 1 cm. The culturing system is semi-static, with 100% water replacement twice a week. Water was sieved through a battery of screens of decreasing mesh size (1500 µm, 1000 µm, 475 µm and 250 µm) to sort the animals into four size classes (adults, subadults, juveniles and newborns), which were distributed to separate aquariums. Food consisted of the macroalgae *Ulva lactuca* and/or *Ulva rigida*. Forty-eight hours before the beginning of the waterborne copper assay, a stock of subadults was isolated from the culturing system and moved to an aquarium at the assay temperature (20°C) with an unlimited food supply.

X-ray assays

The assays performed and respective test designs are summarized in table 1. X-ray exposures used DNA from three sources: (1) coliphage T4 DNA (used as a reference DNA standard); (2) DNA extracts from individual amphipods; and (3) live amphipods. The amphipods used for DNA extraction and X-ray exposure were subadults from our laboratory culture (< 10 mm total length, retained between the 1000 and 1500 µm sieves). The procedure for DNA extraction is described further below. X-ray exposures for all assays were conducted in an ENRAF Nonius FR 591 apparatus set at 4.5 kW. For each exposure DNA extracts or individual amphipods were put in a 1.5 ml plastic microtube and placed in the X-ray apparatus, assuring that the samples would be permanently exposed to the X-ray beam

Table 1. Summary of the experimental design of the tests performed.

	X-ray		
Test design	Naked DNA	Amphipods	Waterborne copper
Exposure conditions	4.5 kW	4.5 kW	20°C, 33–34‰ 0, 5, 10, 20 µg l^{-1} Cu
Sampling times/exposure period	0, 10, 20, 30 min	0, 30 min	24, 48, 96 h
Number of replicates per sampling time	Coliphage T4 DNA: 1 DNA extracts of *G. locusta*: 3	6 amphipods per treatment	12 amphipods per copper concentration and sampling time

Waterborne copper assay

The protocol for the waterborne copper test was modified from the general procedure outlined in Costa *et al.* (1998). The temperature was 20°C and the salinity 33–34/10‰. Only subadult amphipods (males < 10 mm total length, retained between the 1000 and 1500 µm sieves) from the laboratory culture were used. One amphipod was placed in each beaker containing 100 ml of control or contaminant solution, which had nominal concentrations of 5, 10 or 20 µg l^{-1} of $CuCl_2$. These sublethal copper doses were selected according to preliminary tests. Survival was high and did not differ from controls in any of the copper treatments. The amphipods were not fed during the experiment. Twelve animals were sampled from each treatment after 24, 48 and 96 h.

DNA extraction

DNA was isolated from individuals immediately after exposure to the X-rays or copper (preliminary studies indicated that immediate extraction of DNA directly from live amphipods produces DNA extracts with the best quality, a critical goal for this analysis). Whole amphipods were homogenized in a 1.5 ml microtube containing 500 µl TEN (50 mM Tris, 25 mM ethylene diamine tetra-acetic acid EDTA and 100 mM NaCl). Following addition and mixing with 50 µl of 10% sarcosyl, the resultant solution was extracted with phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, v/v/v) and subsequently with chloroform. The DNA was then precipitated from the aqueous phase with ethanol, and stored at − 20°C for at least 2 h or overnight if necessary to pause the extraction procedure. The samples were then washed in 70% ethanol, vacuum dried and resuspended in TEN. The extracts were then digested with ribonuclease A and proteinase K for 1 and 2 h, respectively, in a 55°C water bath, extracted a second time with PCI and chloroform, followed by precipitation with ethanol and redissolution in TE (10 mM Tris and 1 mM EDTA, pH 8.0). This extraction procedure is designed to minimize DNA shearing and produces partly purified, high molecular weight, DNA extracts (Theodorakis *et al.* 1994). DNA concentration of each sample was quantified spectrophotometrically (Unicam Helios) by reading the absorbance at 260 nm.

AGE

AGE and densitometry analysis has been described in detail in Theodorakis *et al.* (1994), and was applied in this study with some minor adaptations. Briefly this technique comprises the electrophoresis of DNA extracts under alkaline (pH 12) and neutral (pH 8) conditions, thus allowing for determination of total (single and double) and double strand 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. Only total strand break (TSB) quantification was performed for samples from coliphage T4, whereas both TSBs and double strand breaks (DSBs) were quantified for the remaining X-ray assays and for the copper toxicity test. Since it was necessary to run several gels to process all samples, the number of control and treatments were distributed as evenly as possible between the gels.

TSB gel. For the quantification of TSBs 0.8% agarose gels (50 mM NaCl, 4 mM EDTA) were prepared and soaked for 30 min prior to allocation of the DNA samples in the electrophoresis chamber containing alkaline running buffer (30 mM NaOH, 2 mM EDTA, pH 12). Appropriate volumes of DNA stock were diluted in sterilized double-distilled H_2O (ddH_2O) in order to obtain 1000 ng of DNA in each sample. Then 1.5 µl of 0.5 M NaOH was applied to each sample for 15 min prior to placement in the gel. DNA samples were allocated in the gel by leaving one empty well after every sample pair, in order to account for background brightness in the densitometric analysis. Molecular size standards containing coliphage T4 DNA plus Hind III digests of λ DNA and a 100 bp ladder were run in lanes

flanking the samples in each gel. The alkaline buffer was cooled during the run by circulating it through coiled tubing immersed in an ice bath. Electrophoresis was conducted at 75 V for 3 h or for 7 cm. The gels were then neutralized in 100 mM Tris for 30 min, stained for 30 min in an ethidium bromide solution (0.002 mg ml^{-1}), destained for 30 min in ddH_2O and photographed under UV light (figure 1a).

DSB gel. DNA was loaded into 0.3% agarose gels, cast onto a 3.0% agarose gel basement for support, and run in TBE buffer (90 mM tris, 90 mM boric acid, 2 mM EDTA, pH 8). DNA dilutions were performed to obtain 500 ng of DNA per sample. The DNA extracts and molecular size standards were allocated in the gel as described above. The electrophoresis was performed in TBE buffer at 25 V

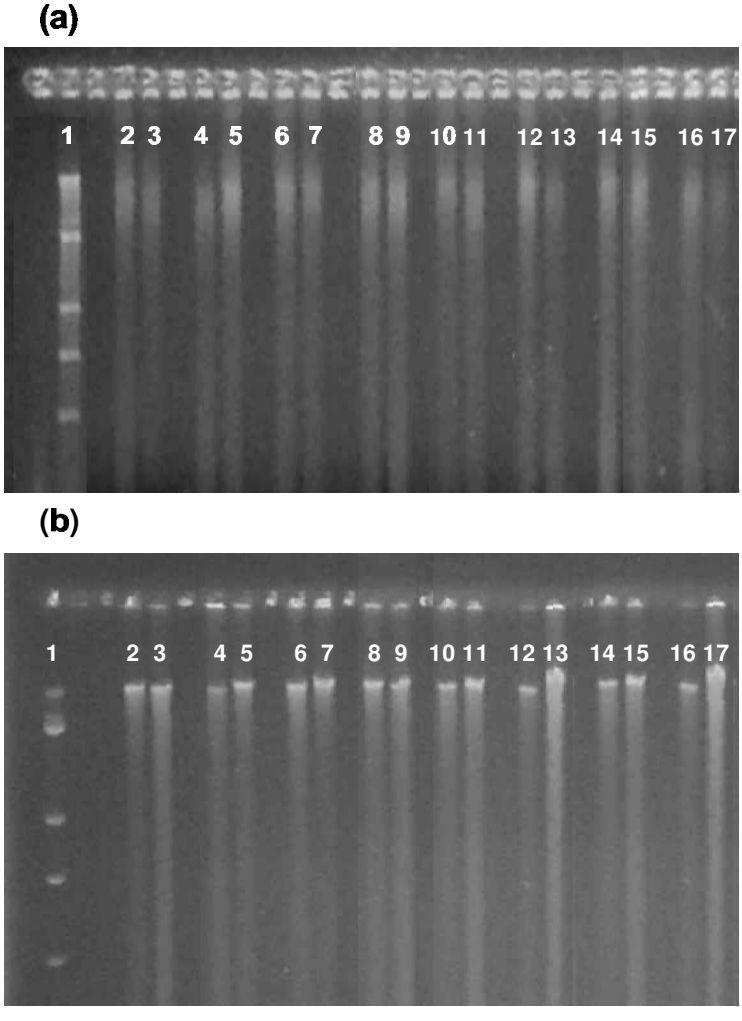

Figure 1. Photographs of agarose gel runs under alkaline (a) and neutral (b) conditions to determine, respectively, total and double DNA strand breakage of *G. locusta* exposed to copper. (a) Lane 1, molecular size standards (coliphage T4 DNA plus Hind III digests of λ DNA); lanes 2–3 and 10–11, controls (unexposed amphipods) for 24 and 48 h, respectively; lanes 4–5, exposure to 5 $\mu g\,l^{-1}$ Cu for 24 h; lanes 6–7, 12 and 15, exposure to 10 $\mu g\,l^{-1}$ Cu for 24, 48 and 96 h, respectively; lanes 8–9, 13–14 and 16–17, exposure to 20 $\mu g\,l^{-1}$ Cu for 24, 48 and 96 h, respectively. (b) Lane 1, molecular size standards (coliphage T4 DNA plus Hind III digests of λ DNA); lanes 2–3, 8–9 and 13, controls (unexposed amphipods) for 24, 48 and 96 h respectively; lane 4, exposure to 5 $\mu g\,l^{-1}$ Cu for 24 h; lanes 5–6 and 14–16, exposure to 10 $\mu g\,l^{-1}$ Cu for 24 and 48 h, respectively; lanes 7, 10–12 and 17, exposure to 20 $\mu g\,l^{-1}$ Cu for 24, 48 and 96 h, respectively.

for 12 h. The staining procedure was the same as for TSB gels, except the neutralization step, which was not necessary for DSB gels (figure 1b).

Densitometry analysis

Photographs of gels stained with ethidium bromide were analysed with the software QWin Lite V2.3 (Leica Microsystems) in order to obtain densitometric profiles of the migration of each DNA sample. The average molecular length (L_n) was computed using the basic program MollenBas specifically created for that purpose (a copy of the program is available from C.W.T. on request). L_n is inversely proportional to the number of DNA strand breaks according to the formula:

$$\text{number of strand breaks per } 10^5 \text{ nucleotides} = 1/L_n \times 100 \quad (1)$$

In order to normalize results among gels, it was required to determine the relative number of TSBs (RNTSB) and DSBs (RNDSB). This was accomplished by calculating the difference in the number of strand breaks between every sample (naked DNA exposed to X-rays or DNA extracted from copper-exposed animals) and the respective control mean (mean values for unexposed DNA samples) within each gel:

$$\text{samples from TSB gel: RNTSB} = 1/L_n(s_{ij}) - 1/L_n(C\text{m}_j) \quad (2)$$

$$\text{samples from DSB gel: RNDSB} = 1/L_n(s_{ij}) - 1/L_n(C\text{m}_j) \quad (3)$$

where s_{ij} is the sample i from gel j, and $C\text{m}_j$ is the respective control mean from gel j.

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.

Statistics

X-ray effects on naked DNA strand breakage were analysed using one-way analysis of variance (ANOVA) separately for each dependent variable (L_n TSB, L_n DSB, RNTSB, RNDSB and RNSSB). Post-hoc comparisons were carried out using the least significant difference (LSD) test. For amphipods exposed to X-rays, Student's t-tests were performed. Data from the copper assay was analysed separately for each dependent variable, using two-way ANOVA, with sampling periods and copper doses as the factors. Post-hoc comparisons were carried out using the LSD test (Statistica®6.0). In all tests the acceptance level of significance was $p < 0.1$.

Results

X-ray assays

The variation of L_n of naked DNA, as determined from TSB and DSB gels, with X-ray exposure time is shown in figure 2a. In the TSB gel, the L_n of *G. locusta* DNA decreased gradually and significantly (ANOVA, $p < 0.05$) with the exposure period, reaching as low as 30% of the original L_n after 30 min. Loss of DNA integrity, as a function of X-ray exposure period, was observed to occur exponentially. For the DSB gel, L_n values also appeared to decrease with increasing exposure period, but this effect was not statistically significant ($p > 0.1$). Naked coliphage T4 DNA responded similarly (data not shown).

Relative strand break (single, double and total) analyses indicated a significant effect of X-ray exposure on RNTSB (ANOVA, $p < 0.001$) and RNSSB ($p < 0.05$), but not on RNDSB ($p > 0.1$). After 30 min of X-ray exposure, naked DNA extracts of *G. locusta* had, respectively, 5.35 and 3.90 times more TSBs and single strand breaks (SSBs) per 10^5 nucleotides on average than before exposure (figure 2b). Both RNTSB and RNSSB increased gradually with X-ray exposure period.

The results of exposure of live amphipods to X-rays are shown in table 2. Significant effects of X-rays were observed on L_n from TSB gel (t-test, $p < 0.1$; note $p = 0.0508$), but not on L_n from the DSB gel ($p > 0.1$). The RNTSB was

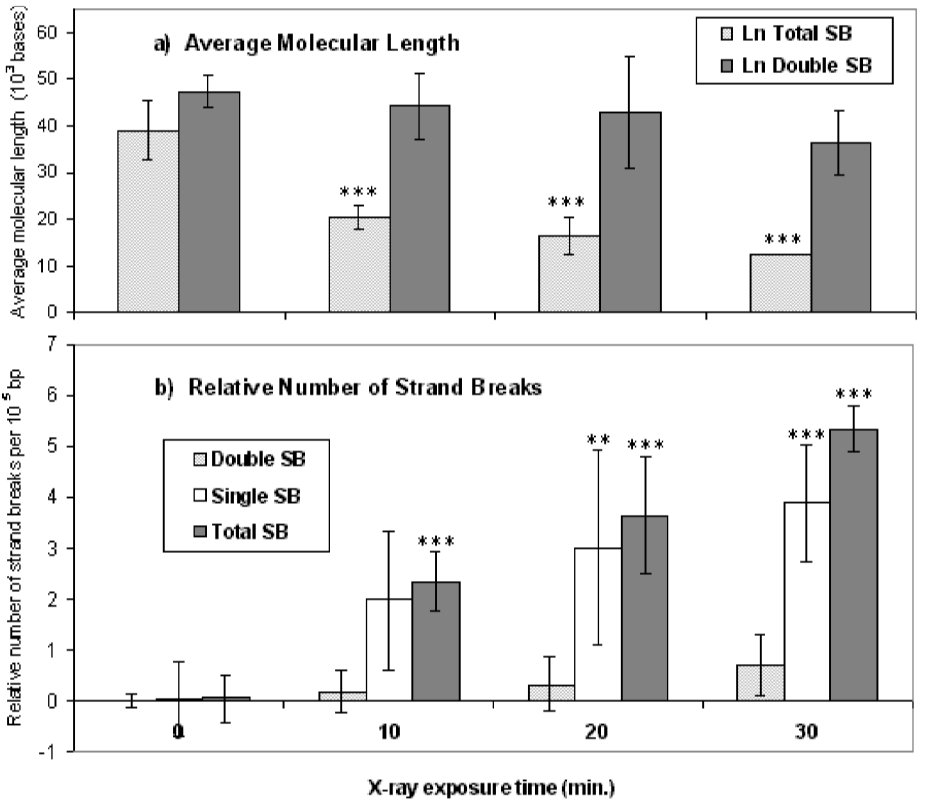

Figure 2. Strand breakage of naked DNA with increasing exposure time to X-rays: (a) average molecular length (L_n) as determined from TSB and DSB gels; (b) relative number of total, double and single strand breaks. The number of samples analysed per exposure period was three. The bars and error bars represent means ± SD. The asterisks indicate significant effects compared with controls: **, $p < 0.05$; ***, $p < 0.01$.

Table 2. DNA strand breakage of *G. locusta* exposed to X-rays for 30 min: average molecular length of the DNA (L_n) as determined from total strand break (TSB) and double strand break (DSB) gels, and the relative number of total, double and single strand breaks (RNTSB, RNDSB, and RNSSB, respectively). Data given are means ± SD.

	L_n				
	TSB gel ($\times 10^3$ bases)	DSB gel ($\times 10^3$ bases)	RNTSB (per 10^5 bp)	RNDSB (per 10^5 bp)	RNSSB (per 10^5 bp)
Control	23.3 ± 5.5	37.1 ± 4.4	0.15 ± 0.9	0.03 ± 0.3	0.10 ± 0.8
X-ray	17.8 ± 2.0*	37.1 ± 5.4	1.41 ± 0.7**	0.05 ± 0.4	1.30 ± 1.2

Total strand breaks = single strand breaks + double strand breaks.
Asterisks indicate significant effects compared with control: *, $p < 0.1$; **, $p < 0.05$

significantly higher in exposed amphipods ($p < 0.05$), but the RNDSB and RNSSB did not differ between control and exposed amphipods ($p > 0.1$).

Waterborne copper assay

L_n values from TSB and DSB gels as a function of copper dose and exposure period are shown in figure 3. The respective RNTSB, RNDSB and RNSSB values are displayed in figure 4. Two-way ANOVA detected significant effects of copper dose on L_n TSB ($p < 0.05$), RNTSB ($p < 0.01$) and RNSSB ($p < 0.1$). No effects were observed on L_n DSB and RNDSB ($p > 0.1$). The highest copper concentration used ($20\,\mu g\,l^{-1}$) produced values significantly different from controls after 48 h of exposure for L_n TSB ($p < 0.05$), at 24 and 96 h ($p < 0.01$) for RNTSB and at 24 ($p < 0.05$) and 96 h ($p < 0.01$) for RNSSB.

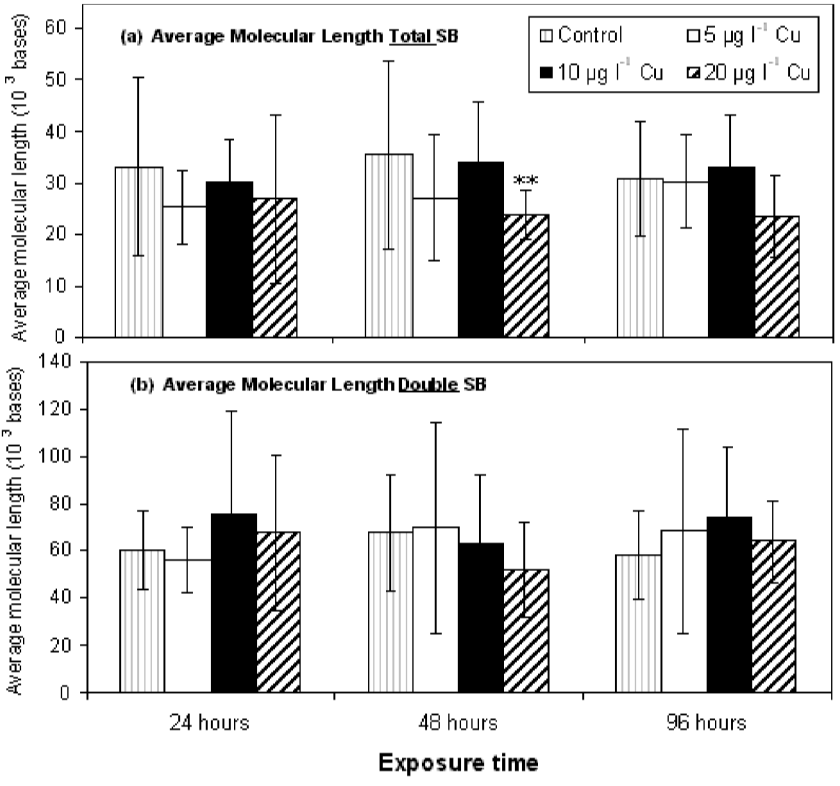

Figure 3. DNA strand breakage of *G. locusta* exposed to copper: average molecular length (L_n) as determined from (a) TSB gel and (b) DSB gel. The number of samples analysed, from left to right, were: (a) 24 h – 10, 8, 11 and 11; 48 h – 9, 10, 9 and 9; 96 h – 11, 9, 11, 11; (b) 24 h – 12, 6, 12 and 12; 48 h – 12, 11, 11 and 12; 96 h – 9, 11, 12 and 12. The bars and error bars represent means ± SD. The asterisks indicate significant effects compared with controls: ** $p < 0.05$

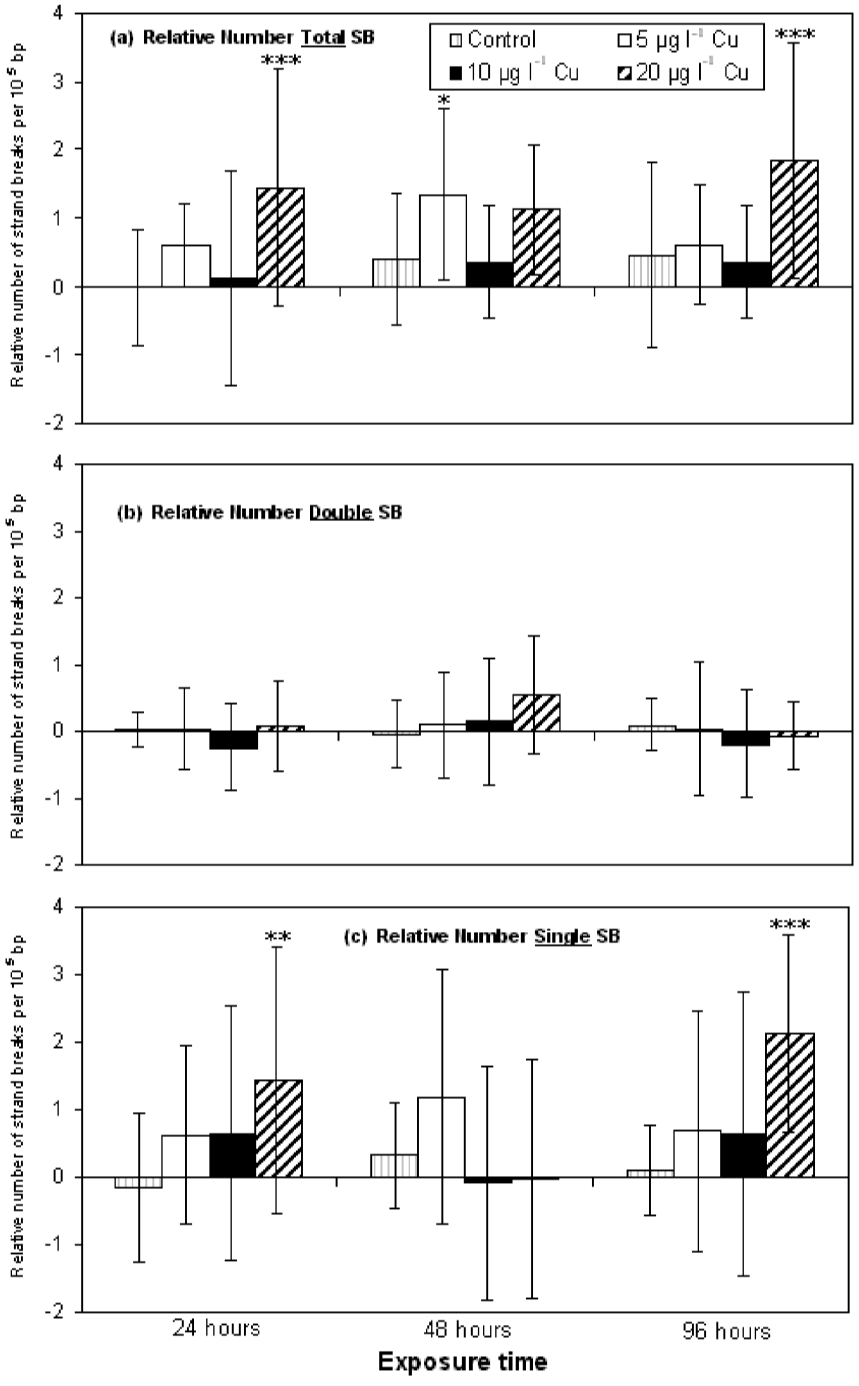

Figure 4. DNA strand breakage of *G. locusta* exposed to copper: relative number of (a) total, (b) double, and (c) single strand breaks as a function of copper dose and exposure period. Number of samples analysed, from left to right, were: (a) 24 h – 10, 8, 11 and 11; 48 h – 9, 10, 9 and 9; 96 h – 11, 9, 11 and 11; (b) 24 h – 12, 6, 12 and 12; 48 h – 12, 11, 11 and 12; 96 h – 9, 11, 12 and 12; (c) 24 h – 10, 6, 11 and 11; 48 h – 9, 9, 10 and 9; 96 h – 9, 8, 11 and 11. The bars and error bars represent means $\pm$ SD. The asterisks indicate significant effects compared with controls: *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$.

Discussion

The X-ray exposure of naked DNA constituted an extreme situation that contributed to the determination of reference values for the measured parameters and tested whether the AGE technique was properly set up. By defining the detection limits, discriminatory ability and range of variation that may be expected, it should be possible to improve the experimental design of DNA strand breakage analysis in ecotoxicological studies with amphipods. The X-ray exposure of amphipods provide an intermediate intercalibration step between the extreme situation of naked DNA and the exposure of amphipods to a genotoxicant in the water.

Results of the X-ray exposure of DNA showed the effectiveness of the AGE technique in discriminating distinct levels of DNA strand breakage in a dose-dependent manner, and identifying and quantifying the various types of DNA strand breakage that were induced. They also demonstrate that the reference genotoxicant used (X-rays) was effective and properly implemented to produce strand breakage.

X-rays also produced DNA strand breakage in live amphipods, showing that it is possible to detect DNA damage by AGE using whole-body DNA extracts from amphipods. Effects on naked DNA were as expected, that is much more intense and clear than with amphipods. While there was a decrease of about 70% in the L_n of amphipod DNA and coliphage T4 DNA (data not shown) exposed to X-rays for the longest period, only a reduction of 24% in DNA integrity, compared with controls, was observed for the same period with amphipods. Concomitantly, the RNTSB and RNSSB values observed with naked DNA were considerably higher compared with those obtained with live amphipods. The lower statistical significance of the effects observed with amphipods compared with naked DNA indicate that a higher number of replicates ($n > 6$) is required in the former situation to improve resolution. This was due to a lower level of DNA strand breakage in living organisms than in naked DNA (as a result of radioprotective or DNA repair activities in living tissue), and an increased variability in DNA strand breakage in live amphipods, presumably arising from differences in inherent susceptibility among individuals, thus reducing the statistical power of the analysis.

This study revealed that exposure to waterborne copper at a concentration of $20\,\mu g\,l^{-1}$ can produce DNA strand breakage in *G. locusta*, namely SSBs. These effects were detected by the significantly higher RNTSB and RNSSB values in animals exposed to the highest copper concentration, but were not evidenced in the parameter L_n. In this study, six gels were required to process all the copper assay samples. Although the AGE settings were rigidly standardized, analytical variations among gels always occur, and may be reflected in L_n quantification. By calculating the relative number of strand breaks (total, double and single), among-gel variability is removed, since exposed and control samples are compared within the same gel. Therefore, when several gels are used, the determination of the relative number of strand breaks constitutes a more sensitive and reliable endpoint than L_n for detecting DNA strand breakage using AGE.

Bolognesi *et al.* (1999) recorded copper to be the second most potent genotoxicant, after mercury, in an investigation of the genotoxic potential of heavy metals in the mussel *Mytilus galloprovincialis*. Nojima (1992) found that various peptide-copper(I) complexes were able to bind to DNA and to produce strand breakage. Also, copper(II)-complexes were found to promote oxidative DNA strand scission

(Ueda *et al*. 1996). It is well known that copper may exert its toxicity by generating reactive oxygen species (ROS), resulting in various types of oxidative damage, including DNA strand breakage and lipid peroxidation (Yusof and Ercal 1999; Livingstone 2001). The pro-oxidant activity of copper has been already reported in toxicity tests with *G. locusta*, in which lipid peroxidation was detected as a result of exposure to waterborne copper (Correia *et al*. 2002a). Therefore the DNA damage observed in the present study may constitute another manifestation of copper's pro-oxidant activity in the species.

Copper effects on DNA integrity were first observed after 24 h of exposure, and were recorded again at 96 h of exposure, but not after 48 h (for which the only effect detected was a lower L_n in the TSB gel). DNA strand breakage is an early biological response which is expected to occur within the time frame of hours to days after exposure to a genotoxicant (Shugart 2000). As opposed to DSBs, SSBs are generally more readily reversible and repaired. It is well established that genetically controlled DNA repair processes are generally common to all organisms from bacteria to humans (Moustacchi 2000) and therefore they must be considered in advance when analysing DNA strand breakage. Persistence of this disruption depends upon factors such as the ability of the organism to repair its damaged DNA and the exposure period and dose of the genotoxicant (Shugart 2000). The dynamics of DNA damage and repair mechanisms has been indicated as responsible for significant time-dependent fluctuations in DNA strand breakage levels in various animals such as mussels (Black *et al*. 1996, Ching *et al*. 2001) and fish (Theodorakis *et al*. 1992, Everaarts 1995, Devaux *et al*. 1998) exposed to distinct genotoxicants. One possible explanation for the absence of strand breakage at 48 h is that DNA repair mechanisms are more strongly induced after 48 h but were again exhausted at 96 h. Another possibility is that amphipods defence mechanisms (i.e. metallothioneins, etc.) may have become effective only after 48 h, alleviating copper toxicity and allowing animals to recover from the recent insult.

According to Yusof and Ercal (1999), copper toxicity is a result of an imbalance between pro-oxidant and antioxidant ratio. By sequestering and inactivating copper, metallothioneins are increasingly recognized to play a protective role against copper-induced cytotoxicity (Yusof and Ercal 1999). These metal-binding proteins are one of the defence mechanisms triggered by *G. locusta* in response to copper exposure (Correia *et al*. 2002a). Elucidation of the interactions between DNA repair systems and cell's defence mechanisms would contribute to improve our understanding of the aetiology of DNA damage induced by environmental genotoxicants.

In summary, results from this study demonstrate that DNA damage in amphipods can be detected using AGE with whole-body DNA extracts. Applying this technique we were able to detect DNA strand breakage in *G. locusta* by exposure to sublethal doses of an environmental contaminant in the water. Therefore there is potential to include biomarkers of DNA damage in ecotoxicological studies with amphipods. This would help interpretation of chronic toxicity tests and contribute to our understanding of the impact of genotoxic insult in marine invertebrates, particularly crustaceans. However, further investigation of the potential of AGE to detect genotoxic insult in amphipods is still required. This should include comparison with other techniques to detect DNA damage, laboratory testing with other environmental genotoxicants and field

samples of contaminated water and sediments, time-course studies with longer exposure periods to highlight DNA damage and repair cycles, and integration with other biomarkers and endpoints to correlate individual effects with effects at the population level.

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