Biomarkers, January–February 2005; 10(1): 41–57

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DNA damage (comet assay) and 8-oxodGuo (HPLC-EC) in relation to oxidative stress in the freshwater bivalve *Unio tumidus*

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Abstract
The relationships between DNA damage and oxidative stress in the digestive gland, gills and haemocytes of the freshwater bivalve *Unio tumidus* were investigated. Two markers of genotoxicity were measured: DNA breaks by means of the comet assay, and oxidative DNA lesions by means of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) measured using high-performance liquid chromatography (HPLC) coupled to electrochemical detection. Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) tissue levels. Effects were studied after exposure of bivalves for 6 days to benzo[*a*]pyrene (B[*a*]P) (50 and 100 $\mu g\,l^{-1}$) and ferric iron (20 and 40 $mg\,l^{-1}$), applied alone or in combination. Lipid peroxidation in the digestive gland and gills resulted from exposure to Fe^{3+} or B[*a*]P whatever the concentrations tested. DNA oxidatively formed lesions were induced in the two tissues at a higher level after B[*a*]P exposure than after Fe^{3+} treatment. No significant dose–response relationship was found with the two compounds and no synergistic effect was observed between Fe^{3+} and B[*a*]P. The gills appeared less sensitive than the digestive gland to DNA lesions expressed as 8-oxodGuo and comet results. Good correlations were noted between 8-oxodGuo and comet. MDA and DNA damage did not correlate as well, although it was stronger in the digestive gland than in the gills. Production of mucus by the gills likely served to prevent lesions by reducing the bioavailability of the chemicals tested, which could explain that dose–effect relationships and synergistic effects were not observed.

Keywords: Unio tumidus, *freshwater bivalve, 8-oxo-7,8-dihydro-2′-deoxyguanosine, comet assay, DNA damage, malondialdehyde*

(Received 10 September 2004; accepted 17 December 2004)

Introduction

The single-cell gel electrophoresis (comet) assay is a microelectrophoretic technique of isolated nuclei in agarose gel first described by Singh et al. (1988) and now extensively used to study genotoxicity. DNA damage measured with this technique applied at $pH \geq 13$ expresses not only direct single- and double-strand breaks, but also alkali-labile sites and secondary damage consecutive to repair enzyme activity (Speit & Hartmann 1995, Horvathova et al. 1998, Menke et al. 2001, Wozniak & Blasiak

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ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.1080/13547500500038783

2003). Under oxidative stress conditions, implication of DNA repair, in the formation of single-strand breaks should represent a minor contribution to the overall strand breaks. Alkali-labile lesions comprised oxidatively generated DNA damage as apurinic and apyrimidinic sites (sites AP) and thymine glycols (Horvathova et al. 1998). DNA breaks assessed by means of the comet assay were often studied in parallel to other parameters in aquatic organisms, in field and laboratory studies. The comet assay has generally been performed to investigate genotoxicity resulting from pollutants, within multimarker approaches in association with the ethoxyresorufin-*o*-deethylase (EROD) activity study (Devaux et al. 1998, Larno et al. 2001, Flammarion et al. 2002), measurements of the expression of cytochrome P450 1A, histopathologic observations (Akcha et al. 2003, Roy et al. 2003), or fluorescent biliary metabolites (Roy et al. 2003, Brown & Steinert 2004).

The 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) was considered as a relevant marker of DNA oxidative damage (Collins et al. 1996). DNA molecules are important targets for radical attacks (Carr & Frei 1999) and up to now the authors have been mainly interested in the modifications of guanine and thymine (Cadet et al. 1999, Evans & Cooke 2004). 8-OxodGuo measurement was the most used parameter to assess DNA oxidative damage in biomonitoring of animal and human populations. The measurement of oxidatively formed DNA damage is of interest because of the biological significance of the lesion and its role in degenerative diseases (Ames et al. 1993, Collins et al. 1996, Griffiths et al. 2002, Cadet et al. 2003). The high-performance liquid chromatography (HPLC) technique coupled to electrochemical detection was shown as an appropriate technique for the measurement of 8-oxodGuo, provided spurious oxidation during analysis is prevented. The introduction of desferoxamine into reagents and buffers, and the chaotropic extraction of DNA by means of sodium iodide improved the quantification of 8-oxodGuo in tissues, by reducing artefactual oxidation during sample preparations (Helbock et al. 1998, Pouget et al. 2000, Cadet et al. 2004).

The present work sought to study the responses of two markers of genotoxicity in the freshwater bivalve *Unio tumidus*: DNA breaks were measured by means of the comet assay and 8-oxodGuo levels in order to assess DNA damage after oxidative stress. The objective was to compare DNA lesions (comet assay), membrane and genomic oxidative damage produced by ferric ions and by a typical genotoxic pollutant — benzo[*a*]pyrene (B[*a*]P). The possible effects of a 6-day exposure of mussels to ferric iron and B[*a*]P were studied in the gills, digestive gland and haemocytes. Lipid peroxidation was measured by evaluating membrane oxidative damage by means of malondialdehyde (MDA).

Iron could trigger *in vivo* the production of oxygen reactive species (ROS) responsible for oxidative stress and lipid peroxidation *via* a mechanism involving the Haber–Weiss reaction catalysed by iron complexed to organic ligands (Castilho et al. 1999).

In vertebrates, B[*a*]P is known for its genotoxicity due to covalent binding of its active metabolites to DNA. In invertebrates, the major activation pathway produces quinones, responsible for oxidative damage through the production of reactive species — anion semiquinone radicals and ROS — *via* the redox quinone cycle (Michel et al. 1995, Mitchelmore et al. 1998).

The concentrations of iron and B[*a*]P tested were representative of polluted aquatic environments. Concentrations were chosen on the basis of quality levels

scored according to the water quality evaluation system (SEQ water) established by the French authorities (Agences de l'Eau 2000a). The concentration of 50 $\mu g\,l^{-1}$ B[*a*]P is the threshold differentiating medium and low grades of water quality (classes 2 and 3, respectively); 100$\mu g\,l^{-1}$ corresponded to a low grade of water quality (class 3).

No quality criteria have been established for iron in surface waters, but a threshold of 50 $mg\,l^{-1}$ has been defined for use in irrigation and cattle watering (Agences de l'Eau 2000b). We chose to study concentrations of 20 and 40 $mg\,l^{-1}$ that are below this threshold.

Materials and methods

Chemicals

Commercial enzymes 2′-deoxyguanosine (dGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine, benzo[*a*]pyrene (B[*a*]P), *N*-laurylsarcosinate, phenylmethyl-sulfonyl fluoride, tetradecyltrimethylammonium bromide and Triton X100 were purchased from Sigma (St-Quentin Fallavier, France). Alkaline phosphatase was purchased from Interchim (Montluçon, France) and the Oxytek DNA Isolation kit reference Zeptometrix 0805001 from Gentaur Molecular Products (Brussels, Belgium). All other reagents were of analytical, chromatographic or molecular biology grade.

Animals

Bivalves, *Unio tumidus* (8–9 cm), were sampled in a control site (La Maxe, France), continuously surveyed for its low level of chemical pollution. After collection from this site, mussels were immediately kept in reconstituted water at controlled temperature (19°C) for 3 days of acclimatization.

Exposure

Groups of five bivalves were exposed to benzo[*a*]pyrene (B[*a*]P1 = 50 $\mu g\,l^{-1}$; B[*a*]P2 = 100 $\mu g\,l^{-1}$) and/or Fe^{3+} (Fe1 = 20 $mg\,l^{-1}$ Fe^{3+}; Fe2 = 40 $mg\,l^{-1}$ Fe^{3+}), for 6 days in polyvinyl chloride (PVC) tanks, in the dark, in reconstituted water at 19°C. Media were renewed every day. The solvent carrier for B[*a*]P was dimethylsulfoxide (DMSO) used at a final concentration of 0.005%. Five animals were exposed to DMSO alone at the same concentration. *U. tumidus* were exposed to Fe^{3+} (Fe1 = 20 $mg\,l^{-1}$ Fe^{3+}; Fe2 = 40 $mg\,l^{-1}$ Fe^{3+}) with or without DMSO. Mussels were fed with algae (7×10^7 cells l^{-1} per animal day^{-1}), 1 h before the renewal of the test medium.

After exposure, haemocytes were harvested from 500 µl haemolymph withdrawn from the pallial cavity by means of a syringe introduced into the anterior muscle. The digestive glands and the gills were dissected out and stored in liquid nitrogen (−196°C) before biochemical analyses. Samples of tissues for DNA damage analyses were kept in phosphate buffered saline (PBS, 0.01 M, Ca^{2+} and Mg^{2+} free, pH 7.4) containing 10% DMSO as cryoprotector and stored at −196°C in liquid nitrogen.

Determination of malondialdehyde levels

Malondialdehyde (MDA) levels were measured on the total tissue homogenates of the digestive gland and gills as a lipid peroxidation index. Sample preparations and MDA determination were performed as described (Cossu et al. 1997, Doyotte et al. 1997, Charissou et al. 2004). In brief, thawed tissues were homogenized with a manual Potter homogenizer in a 50 mM phosphate buffer (pH 7.6) with 1 mM phenylmethylsulfonyl fluoric acid (PMSF) added as protease inhibitor and 1 mM L-serine/borate mixture as inhibitor of the γ-glutamyl transpeptidase. All steps were carried out at 4°C. The homogenization of the digestive gland (0.5 g fresh weight) or the gills (1 g fresh weight) was performed in 2 ml phosphate buffer. The total homogenate was deproteinized for 1 h with 50% ethanol and centrifuged for 30 min at 30 000*g*. A total of 20 µl supernatant was injected onto a reversed-phase LiChrospher 100 RP 18 column (Merck, Strasbourg France).

HPLC elution was performed at 25°C using 25% ethanol buffer (KH_2PO_4 30 mM, Na_2HPO_4 30 mM, pH 6.5), containing 0.5 mM tetradecyl trimethylammonium bromide as the ion-pair reagent. The HPLC system consisted of a Merck model L-6000A, a UV L-4000A detector (Merck-Hitachi, Strasbourg, France) and a chromointegrator D-2500A (Merck). MDA measurements were duplicated for each tissue and each animal. The statistical differences between the control and exposed bivalves were assessed by means of the Student's *t*-test. Total protein content of each fraction was determined according to Bradford (1976) using bovine serum albumin as standard.

Determination of 8-oxodGuo

Measurements of 2′-deoxyguanosine (dGuo) and 8-oxodGuo were performed by means of an HPLC method adapted from Shigenaga et al. (1994) using ultraviolet light detection (290 nm) for dGuo and electrochemical detection for 8-oxodGuo as described (Doyotte 1998, Charissou et al. 2004). DNA isolation was carried out according to the method recommended by Helbock et al. (1998) and Pouget et al. (2000).

All sample preparation steps were carried out at 4°C in a nitrogen atmosphere. Extraction of tissue DNA was conducted using the Oxytek DNA isolation kit, according to the recommendations of the manufacturer (Zeptometrix Corporation, Buffalo, NY, USA). This kit provided a chaotropic extraction procedure of DNA, with desferoxamine in all buffers and sodium iodide as the chaotropic agent. DNA concentration was quantified spectrophotometrically at 260 nm and its purity was assessed by ensuring that A260/A280 >1.75. Quantities of DNA extracted averaged 135 ± 17 µg in all samples.

DNA was then digested by nuclease P1 (final concentration of 65 $\mu g\,ml^{-1}$, incubation for 10 min at 65°C). pH was then adjusted to 8.5 with TRIS-HCl (1 M) and nucleotides hydrolysed in nucleosides by alkaline phosphatase (0.2 $U\,\mu l^{-1}$, incubation for 1 h at 37°C). A volume of 114 µl chloroform was then added and the mixture was centrifuged at 12 000*g* for 5 min. A total of 50 µl supernatant were injected onto a reversed-phase C18 Supelco Supelcosil LC-18 DB column (Merck). The HPLC System was equipped with a ultraviolet light detector L-4000A (Merck-Hitachi), in line with a ESA 5100 Coulochem II detector (Eurosep Instruments, Cergy-Pontoise, France). The electrochemical detector was equipped with a

conditioning cell (adjusted to 110 mV) and a 5010 analytical cell (electrodes E1 and E2 adjusted to 125 and 350 mV, respectively, with a sensitivity of 50 nA). The levels of 8-oxodGuo were expressed as the number of lesions per 10^5 dGuo. The statistical differences between the control and exposed bivalves were assessed by means of the Student's *t*-test.

Alkaline single-cell gel electrophoresis (comet) assay

DNA damage was quantified in haemocytes and cells of the digestive gland and gills by means of the comet assay as described by Singh et al. (1988) with minor modifications. Assays were performed under non-actinic light at 4°C. The cellular suspensions from solid tissues (gills and digestive gland) were obtained by mechanical dissociation of 100 mg tissue in 10 ml PBS (0.01 M, Ca^{2+} and Mg^{2+} free, pH 7.4) with 10% DMSO added as a scavenger of $^{\bullet}OH$ radical as recommended by Tice et al. (2000). Differential centrifugations were used to isolate cells from other debris. For haemolymph, a single centrifugation allowed concentration of samples. Each tissue was analysed independently. Cell viability was determined using the trypan blue stain exclusion method immediately after treatment.

Slides were prepared in triplicate for each sample. They were pre-coated the day before with standard agarose 0.8% in PBS. A total of 300 µl cell suspension was added to 300 µl 1% low-melting point (LMP) agarose. A total of 75 µl was transferred onto slides, covered with a coverslip and left on ice 10 min to allow agarose to solidify. The coverslip was gently removed and the third layer (75 µl 0.5% LMP agarose) was spread, covered and left on ice for 10 min. After the removal of the coverslips, the slides were immersed in freshly prepared cold lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, 1% *N*-laurylsarcosinate, 1% Triton X100 and 10% DMSO) for 2 h (1 h only for haemocytes), in order to isolate the nuclei and eliminate the cytoplasm and membranes. Thereafter, slides were placed in an electrophoresis tank, covered with electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA) to allow DNA unwinding for 21 min, and then electrophoresed (300 mA, 21 min, 20 V).

Slides were washed twice for 10 min in neutralizing buffer (0.4 M Tris, pH 7.5) before dehydration in a batch of absolute ethanol for 10 min. Nuclear DNA was stained with 25 µl ethidium bromide (20 µg ml^{-1}) and observed under a ultraviolet light using an epifluorescence microscope (BX60 Olympus, Rungis, France) connected to an image analysis system (Komet 3:1 Kinetic Imaging Ltd). Twenty-five to 50 cells were analysed per slide, in random fashion, on the central part of the gel. Slides were coded and scored blind. No indication of apoptosis was observed with the comet assay and with the trypan blue stain exclusion method.

For each electrophoresis, an aliquot of a suspension of the cell type analysed, previously exposed to hydrogen peroxide (250 µM, 1 h at 4°C), was included as a positive control of cell sensitivity and as an internal standard of electrophoresis conditions.

The percentage of DNA in the tail was chosen to express DNA damage because of the small interexperimental and interelectophoresis variation registered using this parameter (De Boeck et al. 2000). Averaged tail DNA and standard deviation were calculated. Arbitrary classes of DNA damage in cell populations were defined according to the percentage of DNA in the tail: 0–20% (class 0), 20–40% (class 1), 40–60% (class 2), 60–80% (class 3) and >80% (class 4). As comet data were not

normally distributed, non-parametric statistical tests were used. The Kruskal–Wallis and the median tests, applied on raw data, were used to compare the different modalities. Cell distributions based on the degree of damage were analysed by means of the χ^2-test. The differences in cells of treated bivalves as compared with controls and solvent controls were considered as significant when $p \leq 0.01$.

Results

During the 6-day experiment, no sign of toxicity was registered in bivalves exposed to the chemicals. Filtering capacities were maintained. Yet, production of mucus was noted in bivalves exposed to chemicals, but not in controls.

Lipid peroxidation

Basal MDA levels averaged 3.7 ± 0.5 ng mg^{-1} protein in the digestive gland and 4.0 ± 0.4 ng mg^{-1} protein in the gills. Lipid peroxidation increased significantly in both tissues for each treatment (Figure 1).

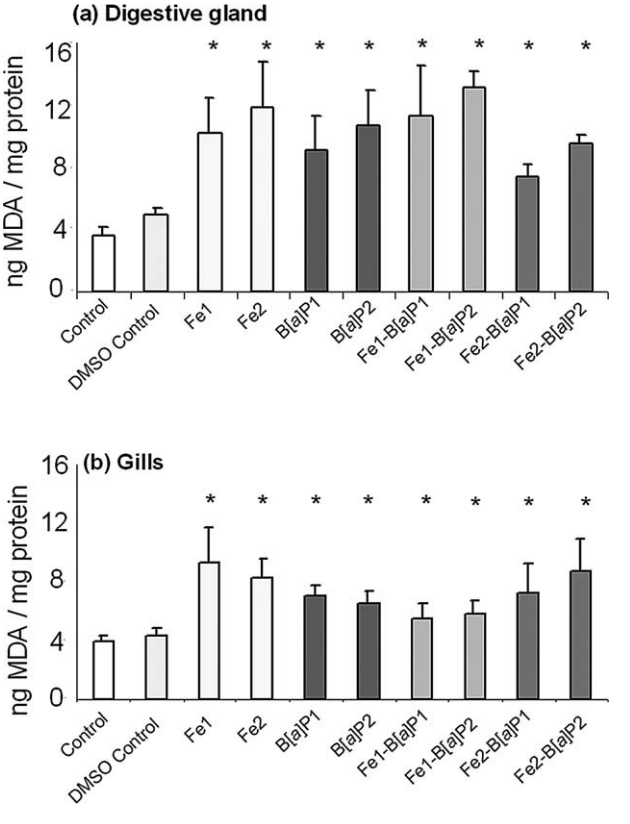

Figure 1. Malondialdehyde (MDA) as lipid peroxidation index in the digestive gland (a) and the gills (b) of *Unio tumidus* exposed to benzo[*a*]pyrene (B[*a*]P1 = 50 µg l^{-1}; B[*a*]P2 = 100 µg l^{-1}) and/or ferric iron (Fe1 = 20 mg l^{-1} Fe3+; Fe2 = 40 mg l^{-1} Fe3+) during 6 days (five animals per treatment); *significant difference with respective control ($p \leq 0.05$); DMSO, dimethylsulfoxide.

In the digestive gland, MDA concentrations were found in the range 10–12 ng mg^{-1} protein after exposure to Fe^{3+} and 9–11 ng mg^{-1} protein after exposure to B[*a*]P. These MDA levels corresponded to a 3-fold increase compared with controls in case of exposure to Fe^{3+} and a 2-fold increase in case of B[*a*]P exposure. Although the intensity of the effects seemed to increase with the chemical concentrations, the difference was not significant between the two concentrations of iron or B[*a*]P tested. No increased effect was found when both compounds were mixed compared with the effects of the chemicals alone (Figure 1).

In the gills, lipid peroxidation was not so high as in the digestive gland, whatever the chemical tested. No synergistic effect resulted from the mixture of the two chemicals.

Oxidatively generated DNA damage

The basal levels of 8-oxodGuo averaged 2.6 ± 0.5 lesions per 10^5 dGuo in the digestive gland and 3.1 ± 0.8 lesions per 10^5 dGuo in the gills. In the digestive gland, the level of oxidatively generated DNA damage increased significantly in bivalves exposed to B[*a*]P (50 and 100 $\mu g\,l^{-1}$) and to iron (40 $mg\,l^{-1}$) (Figure 2a).

In the gills, no effect of iron was observed, while the level of oxidatively generated DNA damage increased significantly after exposure to B[*a*]P (50 and 100 $\mu g\,l^{-1}$) (Figure 2b). The 8-oxodGuo levels were not enhanced with B[*a*]P concentration.

The co-exposure to B[*a*]P and Fe^{3+} did not significantly increase the level of oxidatively generated DNA damage as compared with the effects of the single compounds. In contrast, 8-oxodGuo levels decreased after a co-exposure to 100 $\mu g\,l^{-1}$ B[*a*]P and 20 $mg\,l^{-1}$ Fe^{3+} in the gills and after a co-exposure to 100 $\mu g\,l^{-1}$ B[*a*]P and 40 $mg\,l^{-1}$ Fe^{3+} in the digestive gland.

DNA breaks

The basal percentage of tail DNA in controls averaged $3.6 \pm 3\%$ in haemocytes, $4.4 \pm 1.2\%$ in digestive gland cells and $2.2 \pm 0.9\%$ in gill cells.

In all cell types of control mussels, the majority of cells presented little or no damage (class 0), while 3–4% were ranked in class 1 (percentage of tail DNA between 20–40%).

The percentage of haemocytes with DNA damage increased significantly in mussels exposed to iron and/or B[*a*]P compared with controls (Figure 3a). The intensity of DNA damage and the number of DNA damaged cells were high after B[*a*]P treatment with 5–6% of cells in class 2 and 1–2% in class 3 (60–80% DNA in the tail). A co-exposure to iron and B[*a*]P did not increase the effects observed with each chemical applied alone.

A similar pattern of response was registered in the digestive gland (Figure 3b).

In the gills, DNA damage in treated mussels was lower than in haemolymph and in the digestive gland. The number of DNA damaged cells was significantly increased after iron and/or B[*a*]P exposure, except in iron-treated mussels in the presence of DMSO (Figure 3c).

Discussion

This study confirmed that exposure to iron and B[*a*]P produced an oxidative stress in bivalves. B[*a*]P induced membrane oxidative damage as well as DNA damage. On the

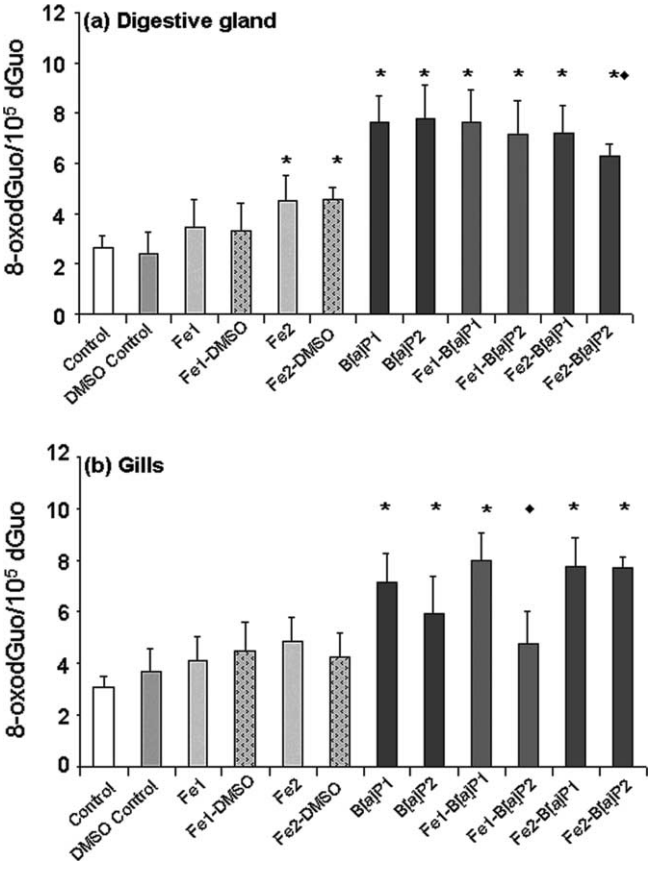

Figure 2. 8-OxodGuo levels in the digestive gland (a) and the gills (b) of *Unio tumidus* exposed to benzo[*a*]pyrene (B[*a*]P1 = 50 $\mu g\,l^{-1}$; B[*a*]P2 = 100 $\mu g\,l^{-1}$) and/or ferric iron (Fe1 = 20 $mg\,l^{-1}$ Fe3+; Fe2 = 40 $mg\,l^{-1}$ Fe3+) during 6 days (five animals per treatment); *significant difference with respective control ($p \leq 0.05$); ♦, significant decrease compared with B[*a*]P alone, applied at the same concentration ($p \leq 0.05$); 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DMSO, dimethylsulfoxide.

other hand, Fe^{3+}-induced oxidative stress did alter membranes, but oxidatively formed DNA lesions were low. Whereas lipid peroxidation was high in the two tissues, the gills appeared less sensitive to DNA oxidative damage than the digestive gland.

DNA breaks assessed with the comet assay were observed in haemocytes of the chemical-exposed mussels to the same extent as in cells of the digestive gland.

In general, good correlations were found between tail DNA (comet assay) and 8-oxodGuo in the digestive gland ($p = 0.00001$) and in the gills ($p = 0.0004$) (Figure 4a). Correlations between lipid peroxidation and DNA damage — 8-oxodGuo or tail DNA — in the digestive gland and in the gills were poor, although better in the digestive gland than in the gills, and with tail DNA rather than 8-oxodGuo (Figure 4b, c). Poor correlations between DNA and membrane oxidative damage had also been obtained in *Unio* from field studies by Charissou et al. (2004). These authors also found a higher degree of correlation in the digestive gland than in the gills, and a correlation coefficient ($r^2 = 0.38$) between 8-oxodGuo and MDA in the digestive

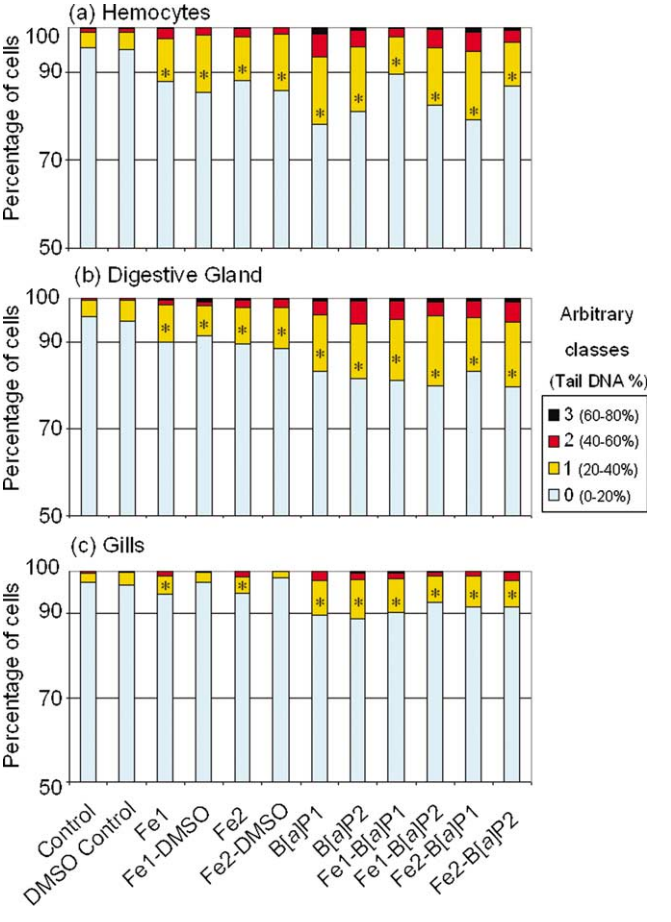

Figure 3. Distributions of DNA damage (percentage of tail DNA) in the three cell populations: (a) haemocytes, (b) digestive gland and (c) gills of *Unio tumidus* exposed to benzo[*a*]pyrene (B[*a*]P1 = 50 $\mu g\,l^{-1}$; B[*a*]P2 = 100 $\mu g\,l^{-1}$) and/or ferric iron (Fe1 = 20 $mg\,l^{-1}$ Fe^{3+}; Fe2 = 40 $mg\,l^{-1}$ Fe^{3+}) during 6 days; *significant difference with respective control ($p \leq 0.01$); DMSO, dimethylsulfoxide.

gland similar to the one we obtained. Yet, they correlated individual values rather than means of groups as done here. A link could have been expected because products of lipid peroxidation such as alkoxy ($RO^{\bullet}$), peroxy ($ROO^{\bullet}$) and hydroxyl ($^{\bullet}OH$) radicals as well as reactive aldehydes are well known for their role as agents of oxidative stress, protein and DNA damage (Slater 1984, Cerutti 1985).

No synergy was registered between B[*a*]P and Fe^{3+}. In contrast, the effects of the two chemicals on lipid peroxidation and oxidative DNA injuries seemed antagonistic in some circumstances, although the decreased effects were not always significant. Our hypothesis is that the bioavailability of the chemicals, when combined or tested in the presence of DMSO, was reduced due to the production of mucus. The latter has not been evaluated here because it was unexpected, but this should be measured in further studies to support this hypothesis. An additional hypothesis is that faster repair of oxidative damage may have occurred leading to reduced lesions at day 6 after co-exposure to both chemicals.

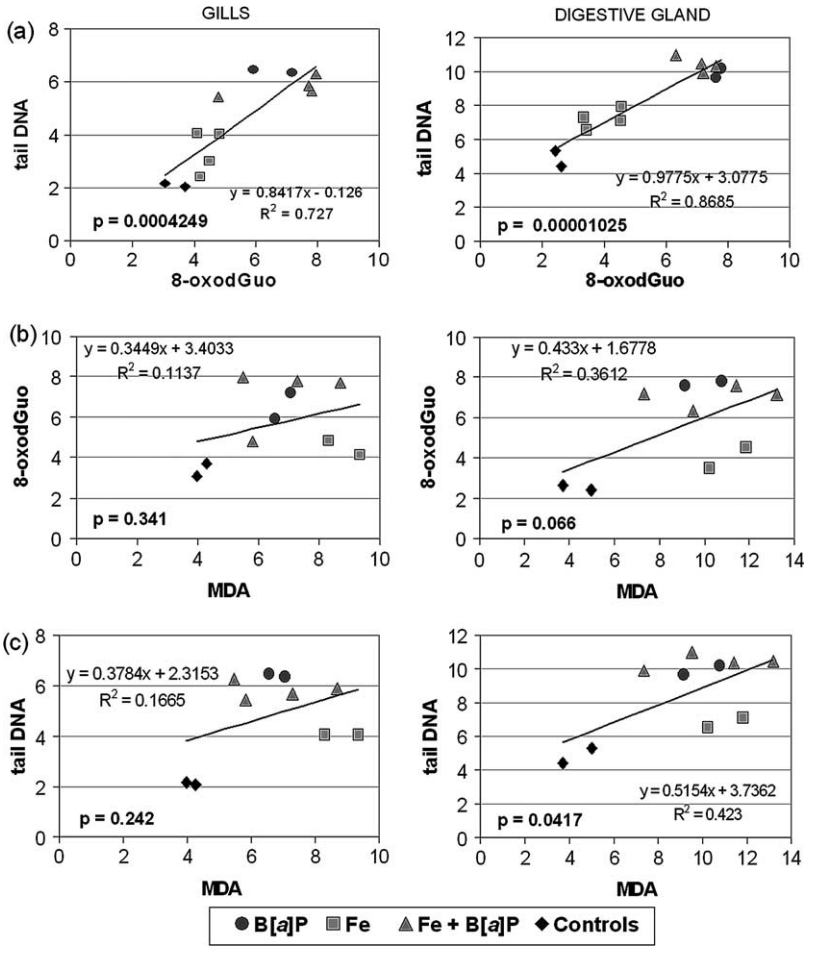

Figure 4. Relationships between the responses of the three parameters: DNA breaks (tail DNA), oxidatively generated DNA damage (8-oxodGuo) and lipid peroxidation (MDA), in the digestive gland and the gills of *Unio tumidus* after 6 days of exposure; benzo[*a*]pyrene (B[*a*]P: ●; 50 and 100 μg/L); ferric iron (Fe: ■; 20 and 40 mg/L Fe^{3+}). Controls (◆); mixtures of both compounds (Fe+B[*a*]P:▲).

In the present study, the measurement of 8-oxodGuo in tissues of bivalves had been optimized with the use of oxygen-free conditions during analysis, which reduced oxidative artefacts as shown by Nakajima et al. (1996) and Doyotte (1998) in our laboratory, and with the use of chaotropic extraction of DNA as recommended by Helbock et al. (1998). The inclusion of desferal in buffers during DNA extraction also limited auto-oxidation. Moreover, the hydrolysis of more than 100μg DNA per sample was performed in all cases to minimize errors as suggested by Nakae et al. (1995) and Helbock et al. (1998). With the improved method using chaotropic DNA extraction, the baseline level of 8-oxodGuo had fallen consistently, especially in the gills as shown from the comparison with levels obtained by Charissou et al. (2004) in the same *Unio* species (Table I).

Comparisons by Akcha et al. (2000b) of different protocols of DNA extraction in *M. galloprovincialis* showed the dramatic lowering of 8-oxodGuo levels consecutive to

Table I. 8-OxodGuo levels expressed as 8-oxodGuo/10^5 dGuo in tissues of aquatic invertebrates and vertebrates from reference sites measured by means of high-performance liquid chromatography coupled to electrochemical detection (HPLC-EC) and using different methods for DNA extraction and desferoxamine (DF) as radical scavenger.

		Tissues			
Organisms and species	Method for DNA extraction	Liver or digestive gland	Gills	Other tissue	References
Bivalves					
Mytilus galloprovincialis	chaotropic (NaI) DF^{as}	7.9 ± 2	4.8 ± 1.1		Akcha et al. (2000a)
Mytilus galloprovincialis	chaotropic (NaI) DF^{as}	8.3 ± 2	4.8 ± 1.1		
	phenol/CH_3Cl DF^{ext}	25.6 ± 4.8[1] 34.3 ± 9.5[2]	7.3 ± 2.4[1] 13.9 ± 3.7[2]		Akcha et al. (2000b)
	SEVAG DF^{ext}	34.8 ± 9.3[1]	6.6 ± 0.8[1]		
Mytella guyanensis	chaotropic (NaI) DF^{as}	1.48 ± 0.4			Torres et al. (2002)
Perna perna	chaotropic (NaI) DF^{as}	3.08 ± 0.9		mantle 5.1 ± 2.0	Almeida et al. (2003a)
Perna perna	chaotropic (NaI) DF^{as}	1.28 ± 0.5	0.58 ± 0.5	mantle 1.36 ± 0.4	Almeida et al. (2003b)
Unio tumidus	phenol/CH_3Cl N_2	3.2 ± 0.4	16.8 ± 0.9		Charissou et al. (2004)
Unio tumidus	chaotropic (NaI) DF^{ext} N_2	2.6 ± 0.5	3.1 ± 0.8		*this study*
Fish					
Sparus aurata	phenol/CH_3Cl DF^{ext}	5.0 ± 0.6 7.2 ± 1.6[3]	5.9 ± 1.5[3]	blood 1.2 ± 0.2[3]	Rodriguez-Ariza et al. (1999)
Ictalurus punctatus *Ameiurus nebulosus*	Pronase E Na acetate BHT	2–4			Ploch et al. (1999)

[1,2]Mussels from two different sets of samples.
[3]Controls injected with corn oil in laboratory experiments.
DF^{as}, desferoxamine in all solutions; DF^{ext}, desferoxamine in extraction buffers; N_2, sample preparation in nitrogen atmosphere; DF, desferoxamine; SEVAG, chloroform/isoamyl alcohol; BHT, 2,6-di-*tert*-butyl-para cresol.

the use of chaotropic DNA extraction and desferal in buffers. Yet, it is clear that improvements in protocols can still be attained by using the optimized method of DNA extraction recently published by Ravanat et al. (2002).

The steady-state levels of 8-oxodGuo in the digestive gland and gills found in *Unio* in the present study were in the lowest range of values as opposed to those measured in other bivalves using HPLC-EC and the same improved methods for DNA extraction (Table I). In all studies conducted with optimized methods including a

chaotropic-NaI extraction and use of desferoxamine in all reagents, the range of estimates of 8-oxodGuo in tissues of aquatic invertebrates and vertebrates was 5-fold higher than values measured by HPLC-EC in the DNA of human cells and recommended by ESCODD (Collins et al. 2004). The artefactual oxidation of DNA due to methodological conditions cannot explain on its own the higher levels of 8-oxodGuo in these organisms. Indeed, the steady-state level of 8-oxodGuo was 2.3 ± 0.06 residues/10^6 dGuo in control rat liver DNA (Matos et al. 2001) and it was more than 5-fold higher in the digestive gland DNA of the bivalve *Perna* when measured using the same technique of analysis (Almeida et al. 2003a,b). Therefore, higher levels in aquatic species than in mammals cannot be excluded. Depleted antioxidant capacities of aquatic species compared with mammals may explain higher oxidant status (Nakae et al. 1995). In addition, bivalves are known as filtering species that bioaccumulate pollutants liable to raise the baseline level of 8-oxodGuo.

The present 8-oxodGuo results in the digestive gland were in accordance with the findings of a study carried out on *U. tumidus* by Charissou et al. (2004). The duration of 6 days of exposure was chosen with regards to this field study. Indeed, the 8-oxodGuo levels in the digestive gland were greater after 7 days than after a 21-day field exposure of the bivalves in a large pond with a water of medium grade quality. Charissou et al. (2004) noted that DNA of the freshwater *Unio tumidus* was sensitive to oxidatively formed lesions and that appearance of 8-oxodGuo lesions was time-dependent, with an earlier occurrence in the digestive gland than in the gills.

The present 8-oxodGuo results can also be compared with those of Canova et al. (1998) who studied B[*a*]P contamination levels in water close to the concentrations tested here. The authors exposed *Mytilus galloprovincialis* to B[*a*]P (in the range 0–0.5–5–50–100–500–1000 $\mu g\, l^{-1}$ for 0, 48 and 72 h) and registered an increase of the oxidized base but no dose–response relationship in 8-oxodGuo induction was observed. Comparisons with results of Akcha et al. (2000a,b) were more difficult, because the authors used high B[*a*]P concentrations in food (0–500–100 $mg\, kg^{-1}$).

The comet assay protocol we used incorporated an internal calibration in every electrophoresis by including a control constituted of a suspension of the cells studied (gills, digestive gland or haemocytes) treated with H_2O_2 (250 μM; 1 h). No external calibration was made in order to quantify DNA breaks. An external control could be constituted by a suspension of cells submitted to gamma irradiation, where 1 Gy exposure is alleged to generate 1000 DNA strand breaks per molecule of DNA in mammalian cells (Gulston et al. 2002). Such controls are especially useful to compare comet assay results and 8-oxodGuo measurements in assessing oxidatively formed DNA damage (Collins et al. 1997).

Control viability was verified by the trypan blue dye exclusion method, which confirmed lack of cytotoxicity and scarcity of very fragmented comet images with DNA percentage in the tail $>80\%$ (class 4) sometimes named as ghost or hedgehog cells (Hartmann et al. 2003).

The haemocytes appeared very sensitive to DNA damage measured by the comet assay. The sensitivity of these cells, added to the ease of sampling and preparation, justifies the wide use of this cell type in environmental genotoxicity studies. In the present work, the higher level of damage in haemocytes and the digestive gland, than in the gills, suggests that absorption of the chemicals studied *via* the digestive tract is predominant compared with filtration through the gills. Indeed, mussels were fed with algae in this experiment, and adsorption of the

chemicals by algae may explain this route of intoxication. The hypotheses that gills are protected by mucus production and dietary adsorption deserve to be more fully investigated.

Basal levels of DNA damage measured in cells of *Unio tumidus* using the comet assay were in the range 2–4% DNA in the tail. They were lower than those observed in marine bivalve, *Mytilus edulis* or *Perna viridis* (Birmelin et al. 1998, Mitchelmore et al. 1998, Shaw et al. 2004, Siu et al. 2004) and equivalent to those observed in freshwater species, such as fish trout, goldfish, zebrafish or amphibians (Belpaeme et al. 1998, Mitchelmore & Chipman 1998a,b, Anitha et al. 2000, Schnurstein & Braunbeck 2001, Roy et al. 2003, Chemeris et al. 2004).

A number of studies measured B[*a*]P-induced DNA damage in bivalves during a short period of time of a few hours. Only a few studies investigating effects in the long term could be compared with the present work. Siu et al. (2004) observed DNA damage in haemocytes after exposure of *Perna viridis* to B[*a*]P up to 30 $\mu g\,l^{-1}$ in the water for 12 days. DNA damage was significant after 3 days of exposure to 0.3 $\mu g\,l^{-1}$; damage peaked after 6 days of exposure to 3 and 30 μg B[*a*]P l^{-1} and lasted until 12 days. On the other hand, some experiments using sediment contaminated with B[*a*]P or other polycyclic aromatic hydrocarbons for a long period showed negative or small DNA damage, probably due to the low bioavailability of hydrocarbons in sediment (Nacci et al. 1996, Coughlan et al. 2002, Hamoutene et al. 2002, Hartl et al. 2004). A B[*a*]P-contaminated diet which consisted in microalgae pre-incubated in 0–0.5–20 $\mu g\,l^{-1}$ B[*a*]P solution, was delivered to *Mytilus edulis*, every day for 14 days; this resulted in DNA damage observed in the digestive gland at days 3, 7 and 14, but no dose–effect–time increase was registered (Large et al. 2002).

In present study, the comet assay was used to assess DNA breaks and 8-oxodGuo measurements to assess oxidatively formed lesions. Although a high degree of correlation was found between the results of the two assays, each biomarker is specific of either DNA breakage or oxidative lesions and cannot be used as an alternative to the other. Indeed, the comet assay performed with the standard alkaline protocol fails to detect base oxidation in DNA, in particular 8-oxodGuo. However, the comet assay becomes quite appropriate to detect DNA oxidative lesions when it is used in conjunction with the formamidopyrimidine glycosylase (Fpg), a specific repair enzyme, which creates single-strand breaks in the DNA at the sites of altered purines (ESCODD 2003). The Fpg based methods seem less prone to the artefact of additional oxidation and appear as a promising alternative technique to measure oxidatively generated purine derivatives (Wozniak & Blasiak 2003, Collins et al. 2004). Additionally, the use of repair enzyme endonuclease III (endo III) could allow the detection of pyrimidine lesions formed by reactive oxygen species and ionizing radiation (Collins et al. 1997, Collins & Horvatova 2001, ESCODD 2003).

Conclusion

Two markers of genotoxicity — DNA breaks assessed using the comet assay and 8-oxodGuo measured by means of HPLC coupled to electrochemical detection — were studied to assess DNA damage in the freshwater bivalve *Unio tumidus* exposed to oxidative stress. Exposure to ferric iron or B[*a*]P resulted in lipid peroxidation in the digestive gland and the gills. B[*a*]P induced clear oxidatively formed DNA damage in

both tissues, while genotoxicity of Fe^{3+} was much lower. The digestive gland and haemolymph were more sensitive to DNA oxidative damage than the gills. No dose–response relationship was observed for either compound, for any of the parameters studied. The measurement of 8-oxodGuo levels confirmed its sensitivity as a marker of oxidatively formed DNA damage and the comet assay its interest as a complement to 8-oxodGuo measurements to assess DNA damage.

Additional studies are necessary to test the hypothesis that mucus production was responsible for reduced bioavailability of chemicals and subsequent decreased toxicity. Further research should also be undertaken to establish that haemolymph is a target tissue for pollutants absorbed *via* the digestive tract and *via* the gills. This would then confirm the usefulness of investigating this tissue in future environmental biomonitoring studies using invertebrates.

Acknowledgements

Work was carried out within the CPER research programme in Environment and within the LITEAU-ERIKA research programme of the Ministry of Ecology and Sustainable Development (MEDD), Paris. The authors thank the Region Lorraine and MEDD for support. They finally thank the anonymous reviewers for thoughtful comments on the first version of the paper.

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