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Benzo{*a*}pyrene-induced DNA damage in *Mytilus galloprovincialis*: measurement of bulky DNA adducts and DNA oxidative damage in terms of 8-oxo-7,8-dihydro-2′-deoxyguanosine formation

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Bulky DNA adducts and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) were measured in gill DNA of benzo[*a*]pyrene (B[*a*]P)-exposed mussels (50 mg kg^{-1} dw day^{-1}), respectively by the ^{32}P-post-labelling technique and high performance liquid chromatography coupled to electrochemical detection assay. A time-course study was performed for both biomarkers and their potential use for marine biomonitoring discussed for the sentinel species studied. In gills, B[*a*]P-related DNA adducts were positively correlated with B[*a*]P concentration in whole mussel, and were produced in a time-dependent manner relative to exposure. Comparison of adduct levels recorded in this paper in gills (0.149 ± 0.079 (standard deviation) to 0.480 ± 0.139 adduct per 10^8 normal nucleotides) with previous measures carried out in the digestive gland of the same animals (0.010 ± 0.005 to 0.251 ± 0.062 adduct per 10^8 normal nucleotides) (Akcha *et al*. in press) showed higher levels in the former tissue ($p < 0.001$). As gills appeared more sensitive to B[*a*]P effects than the digestive gland, applying the post-labelling technique in gill DNA may allow an earlier detection of pollutant genotoxic effects. 8-OxodGuo formation was assessed as a measure of oxidative DNA damage. No increase in the level of 8-oxodGuo by B[*a*]P exposure was recorded in gill DNA. A methodological study clearly demonstrated the effect of DNA extraction procedure on 8-oxodGuo measurement. No difference in 8-oxodGuo levels was observed between the chloroform/isoamyl alcohol and the phenol/chloroform DNA extraction protocols for digestive gland (34.8 ± 9.3 and 25.6 ± 4.8 8-oxodGuo per 10^5 dGuo) and gill (6.6 ± 0.8 and 7.3 ± 2.4 8-oxodGuo per 10^5 dGuo) tissues ($p > 0.05$), whereas by the chaotropic method lower 8-oxodGuo levels ($0.02 p < 0.05$) were measured for both tissue (8.3 ± 2.0 and 4.8 ± 1.1 8-oxodGuo per 10^5 dGuo respectively). Contributory factors to the lack of observed increase in gill 8-oxodGuo level by B[*a*]P exposure could be due to the selected way of exposure (via the feed supply) for which gills were not the target tissue of exposure and artifactual DNA oxidation during sample processing that could have masked the possible B[*a*]P oxidative DNA damage.

Keywords: *Mytilus galloprovincialis*, benzo[*a*]pyrene, DNA adducts, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

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Abbreviations: 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; B[*a*]P, benzo[*a*]pyrene; CAT, catalase; dGuo, 2′-deoxyguanosine; dNps, 3′-monophosphate deoxyribonucleosides; dw, dry weight; ECD, electrochemical detection; EIMS, electron impact mass spectrometry; GC, gas chromatography; HPLC, high performance liquid chromatography; MFO, mixed-function oxygenase; NAD(P),H, β-nicotinamide adenine dinucleotide phosphate reduced form; PAH, polycyclic aromatic hydrocarbon; RAL, relative adduct labelling; ROS, reactive oxygen species; SD, standard deviation; TLC, thin layer chromatography.

Introduction

Marine pollution by petroleum and its derivatives is of great environmental concern as annual inputs are estimated to be in the range of million tons per year for the global ocean, with around 28 % contaminating highly productive coastal waters (UNEP 1990). Polycyclic aromatic hydrocarbons (PAHs) are widely studied crude oil components known for their bioconcentration in aquatic organisms, especially bivalves (Botello *et al.* 1997). In exposed animals, several deleterious effects such as DNA damage (Bolognesi *et al.* 1996), tumour development (Gardner *et al.* 1992) and decrease in reproduction success (Lowe and Pipe 1986), have already been identified as a pollution-mediated toxicity of this predominant class of pollutants. The development of the biomarker concept (Shugart *et al.* 1992)—molecular, biochemical histological or physiological alterations that can be used in organisms for the assessment of either pollutant exposure within their environment or pollution-induced effects (Michel *et al.* 1998)—has led to an important increase in the number of studies carried out in environmental toxicology. Because of its bio-ecological particularities, *Mytilus* sp. is an organism of choice for marine environment biomonitoring, and many pollution biomarkers have already been proposed for this sentinel species.

Promising results for PAH–DNA adduct formation in human and vertebrates motivated the investigations on this type of DNA damage as a possible biomarker for coastal water biomonitoring. As a PAH-model in chemical carcinogenesis studies, benzo[*a*]pyrene (B[*a*]P) metabolic activation to DNA adducts is well documented in vertebrates. Different pathways of DNA adduct formation have been identified (Sims *et al.* 1974, Cavalieri *et al.* 1990, Flesher *et al.* 1990, Stansbury *et al.* 1994), particularly the diol-epoxide one (Grover 1986). In the latter pathway, following oxidation by cytochrome P450-dependent monooxygenases and hydration by epoxide hydrolase, B[*a*]P is biotransformed into B[*a*]P *trans*-dihydrodiols. The latter metabolites can be subjected to cytochrome P450-oxidation, leading to highly reactive B[*a*]P diol-epoxides that can covalently bind to DNA to produce bulky aromatic DNA adducts. The major stable adduct formed by this pathway results from *trans* addition of (+)-anti-B[*a*]P-*trans*-7,8-dihydrodiol-9,10 epoxide to the exocyclic N^2 amino group of guanine. PAH-DNA adducts have been extensively studied by the highly sensitive ^{32}P-post-labelling technique (Randerath *et al.* 1989). The formation of persistent B[*a*]P–DNA adducts in target tissues has been shown to be closely related to cancer development in humans and rodents (Lee *et al.* 1998). As DNA adducts can be responsible for base pair substitutions and frameshift mutations (De Vries *et al.* 1997), they are thought to be involved in the initiation of the multi-stage cancer process by inducting mutations in the p53 tumour-suppressor gene (Quan and States 1996) and in the *ras* proto-oncogene (Chaturvedi and Lakshman 1996). As the formation of DNA adducts in an organism involves toxicokinetic parameters such as absorption, tissue distribution, metabolism, detoxification and

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DNA repair, they may be a powerful molecular dosimeter for genotoxic compound exposure and cancer risk assessment.

Among the types of DNA damage induced by pollutant exposure, oxygen radical injury would appear to be highly relevant for marine environmental biomonitoring. As a matter of fact, several pollutants present in the water column and the sediment have been identified as pro-oxidants in marine organisms (Livingstone *et al.* 1990). Oxidative DNA damage results from the production of reactive oxygen species (ROS) that can react directly or indirectly with DNA, causing base modifications, apurinic/apyrimidinic site formation, DNA strand breaks and protein–DNA crosslinks. ROS are endogenously produced by cellular metabolism as well as by exogenous sources such as chemical carcinogen exposure (Kim and Lee 1997). ROS include singlet oxygen (O_2), the superoxide anion radical ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical ($^{\cdot}OH$). Base modifications have been widely studied, particularly the prevalent 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodGuo) that is the main oxidation product of the reaction of singlet oxygen and hydroxyl radical with DNA (Cadet *et al.* 1997a). It has been determined that ROS play a role in carcinogenesis, mutagenesis and aging processes (Klaunig *et al.* 1998). Like bulky adducts, they can affect the expression of several genes either by modification of the DNA methylation pattern or by mutations in proto-oncogenes and tumour suppressor genes (Cerda and Weitzman 1997). Different analytical methods have been developed to measure oxidized DNA base levels. In this respect, the accurate high performance liquid chromatography coupled to electrochemical detection (HPLC/ECD) has received numerous applications for the measurement of 8-oxodGuo (Floyd *et al.* 1986).

In the present paper, bulky DNA adducts and 8-oxodGuo were measured in gill tissue from benzo[*a*]pyrene-contaminated mussels using the ^{32}P-post-labelling technique and the HPLC/ECD assay respectively. A time-course study was performed for both biomarkers, and their potential use in coastal biomonitoring was considered for the sentinel species studied. Mussels were exposed according to the method previously used for a DNA adduct study (Akcha *et al.* 1999). Enzymatic biomarker measurements relative to DNA adduct levels in the digestive gland and B[*a*]P bioaccumulation for mussels studied in this experiment have been reported elsewhere (Akcha *et al.,* in press). Results reported in the present paper in gills were so enlightened by these previous findings. 8-OxodGuo levels were measured in mussel gills, and a methodological study was carried out to determine the effect of DNA extraction procedure on the measured gill and digestive gland 8-oxodGuo levels. In fact, artifactual DNA oxidation can lead to an overestimation of oxidized base levels, complicating interstudy comparisons and the detection of ROS effects. As phenol has been pointed out as a causative agent of artifactual oxidation, a phenol- and a non-phenol-based protocol were tested as well as the promising chaotropic method (Helbock *et al.* 1998). The comparison of 8-oxodGuo levels measured by each protocol enabled us to improve the proposed HPLC/ECD assay for 8-oxodGuo measurement in mussels.

Materials and methods

Chemicals

^{32}P-Post-labelling reagents. RNAse A, α-amylase, rat spleen phosphodiesterase, micrococcal nuclease, potato apyrase and SEVAG (chloroform: isoamyl alcohol, 24: 1) were purchased from Sigma-

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Aldrich Chemical. RNAse T1, Nuclease P1, proteinase K and T4 polynucleotide kinase were bought from Roche Molecular Biochemicals. [γ^{32}] ATP (specific activity of > 5000 Ci mmol^{-1}) and Hyperfilm MP were purchased from Amersham Pharmacia Biotech. ^{3}H-*anti*-(±)-*trans*-7,8-dihydroxy-*anti*-9, 10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (^{3}H-BPDE) was from NCI, Chemical Carcinogen Standard Repository, Bethesda, MD. Polyethylenimine cellulose thin layer chromatography (TLC) plates were from Macherey-Nagel. Kodak XAR-5 films were purchased from Eastman Kodak.

HPLC/ECD reagents: RNAse, A, proteinase K, protease, 2′-deoxycytidine (dCyd), 2′-deoxyguanosine (dGuo), 2′-deoxyadenosine (dAdo), thymidine (dThd), 8-hydroxy-2′-deoxyguanosine (8-oxodGuo), alkaline phosphatase, sodium iodide, SEVAG, chloroform, isopropanol alcohol and deferoxamine mesylate were purchased from Sigma-Aldrich Chemical. Nuclease P1 and RNase T1 were from Roche Molecular Biochemicals and HPLC grade methanol from Riedel-de-Haën AG.

All other reagents were of analytical grade. Marine Liquifry was obtained from Interpet.

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Animal treatment

Mytilus galloprovincialis individuals (7.0 ± 0.5 cm length) were rope-growing mussels originating from Thau Bay (Etablissement Archimbaud, Bouzigues, France). After explantation from their natural environment, they were kept in 35 % seawater-filled glass aquariums. Water was directly pumped from the Bay of Arcachon (Atlantic Ocean, France) and oxygenated by a continuous air-bubbler system. Water (1.5 l per individual) was renewed daily, and each sampled animal was systematically replaced to maintain a constant load in the aquarium. The temperature during the experiment was 16–17°C. Each individual was fed daily with 28 mg equivalent dw of Marine Liquifry, a commercial nutritive solution for marine invertebrates. A peristaltic pump was used 8 h a day to bring feed into the aquariums.

Animals were contaminated with benzo[*a*]pyrene via their feed supply at a daily individual and theoretical dose of 50 mg kg^{-1} dw mussel. This dose corresponds to the total PAH concentration recorded in whole mussels from the highest contaminated sites of the French Biomonitoring Networks RNO (RNO 1995). As food was given as a solution, this dose also corresponds to a calculated B[*a*]P water concentration of 33.4 ppb.

Mussels were exposed for 28 days after an acclimatization period of 4 days. Sampling was performed in both control and assay aquariums at day 0, 3, 6, 10, 17, 24 and 28. Three pools of 10 individual gills were collected each time for bulky DNA adduct analysis and 8-oxodGuo measurement. Samples were stored at –80°C prior to analysis.

DNA extraction

DNA was isolated in the same way for bulky DNA adduct and 8-oxodGuo analysis. The protocol used was a slightly modified version of that of Venier and Canova (1996). Each sample was homogenized in 1 mM EDTA/0.5 % SDS (10 ml per g of tissue). A 1 ml aliquot was briefly centrifuged at 500 *g* for a 30 s at 4°C and 500 µl supernatant were recovered. Samples were digested with a total amount of 600 µg RNAse A, 1000 U RNAse T1 and 10 µg α-amylase for 45 min at 37°C. Protein digestion was carried out by incubation at 37°C for 2 h with 200 µg proteinase K. RNA and protein digestions were performed in two stages by adding half of the enzyme amount at mid-incubation course. Following adjustment of the SDS concentration to 1 %, samples were heated for 10 min at 60°C with 125 µl of 5 M sodium perchlorate. DNA was extracted once with 700 µl of SEVAG. After 30 min of mild shaking at room temperature, samples were centrifuged at 14 000 rpm for 15 min at 4°C. The aqueous phase was recovered and precipitated overnight in 2 vol. of glacial absolute ethanol. DNA pellets were recovered after centrifugation at 14 000 rpm for 20 min at 4°C and precipitated in ethanol (1 vol.) once more after the addition of 50 µl of 2.5 M sodium acetate. Each DNA pellet was dried with a speedvac (Savant Bioblock Scientific) and resuspended in 400 µl sterile MilliQ water. DNA titration was performed by UV spectrophotometry at 260, 280 and 230 nm (260/280 ratio was 1.8 ± 0.5 and 260/230 ratio around 2). Samples were stored at below 20°C prior to analysis.

^{32}P-postlabelling assay

DNA samples were precipitated as indicated above and resuspended in 2 mM Tris–HCl, pH 7.5, to reach a final concentration of 2 µg DNA µl^{-1}. DNA (5 µg) was then hydrolysed to 3′-monophosphate deoxyribonucleosides (dNps) by addition of 1.6 U micrococcal nuclease and 0.015 U spleen phosphodiesterase in 100 mM sodium succinate, 50 mM calcium chloride, pH 6 and incubation for 3.5 h at 37°C. Normal dNps were dephosphorylated by addition of 1.9 µg nuclease P1 in 30 mM zinc chloride, 0.25 M sodium succinate, pH 5 and incubation for 30 min at 37°C. Reaction was stopped with 2.5 µl 0.5 M Tris–HCl, pH 9. Adducted dNps were labelled by incubation for 40 min at 37°C with [γ^{32}]ATP (2 µl per sample) and kinase T4 (2.5 U per sample) in 10 mM bicine, pH 9.5, 10 mM magnesium chloride, 10 mM dithiothreitol, 1 mM spermidine. The ^{32}P-ATP excess was then hydrolysed by incubation with 0.05 U potato apyrase for 40 min at 37°C.

Thin layer chromatography was conducted on polyethylenimine plates in the following buffers: D1, 1 M sodium phosphate, pH 6.8; D2, 5.3 M lithium formate, 8.5 M urea, pH 3.5; D3, 1.2 M lithium chloride, 0.5 M Tris–HCl, 8.5 M urea, pH 8; D4, 1.7 M sodium phosphate, pH 6.

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Autoradiography of the TLC plates allowed a qualitative analysis of the bulky B[*a*]P-related DNA adducts of each sample. Quantification of the radioactive spots was carried out by Cerenkov counting of blank and radioactive areas. Results are expressed in relative adduct labelling (RAL), i.e. adducts per 10^8 normal nucleotides. Because the assay is time-consuming and expensive, DNA adduct levels were assessed in control animals only at day 0 and 28.

HPLC/ECD assay

HPLC/ECD assay was conducted according to the protocol of Melamede *et al*. (1996). Each DNA sample was reprecipitated and resuspended in Tris–HCl 10 mM, EDTA 1 mM, pH 8, to reach a final concentration of 1 µg DNA µl^{-1}. For each sample, 50 µg DNA were digested to 2′-deoxyribonucleosides for 8-oxodGuo determination. Following denaturation for 5 min at 90°C, samples were rapidly cooled in ice. Then, 5 µl of sodium acetate 0.5 M, pH 5.1, were added per sample before digestion for 1 h at 37°C with 5 U nuclease P1. After the addition of 20 µl Tris–HCl 0.4 M, pH 8, and 6 U alkaline phosphatase, samples were incubated for 1 h at 37°C. Proteins were precipitated by centrifugation with half a volume of chloroform for 8 min at 12 000 *g*. Supernatants were recovered and dried with a speedvac. Pellets wee suspended in 100 µl MilliQ water and stored in the dark at –20°C prior to injection.

8-OxodGuo determination was carried out by HPLC (Philips PU 4100) coupled to electrochemical detection (Kontron 405). Separation of 8-oxodGuo from 2′-deoxyribonucleosides was performed on a Supelguard LC-18-S pre-column (Supelco, 20×4 mm) and a Satisfaction C8+ column (CIL Cluzeau, 15×4.6 mm). Elution was performed in isocratic mode using a mobile phase composed of methanol 10%, citric acid 10 mM, sodium acetate 25 mM, sodium hydroxide 30 mM and acetic acid 10 mM. The elution flow rate was set at 1 ml min^{-1}. The detection sensitivity of the electrochemical detector was 0.5 nA V^{-1} for an oxidation potential of 650 mV. 8-OxodGuo quantification was done in accordance with a calibration curve previously obtained with known pmole amounts of authentic 8-oxodGuo. For standard expression in number of 8-oxodGuo residues per 10^5 dGuo, deoxyguanosine was also quantified by coupling a UV detector (Philips PU 40218520) at the output of the HPLC column. The UV apparatus was set at a wavelength of 260 nm. A calibration curve was also obtained for this compound in the nmole range. For the conditions described, the retention times of both 8-oxodGuo and dGuo were respectively 10.5 and 7.0 min.

Methodological development on 8-oxodGuo measurement

Chaotropic DNA extraction method. The method applied was derived from that of Helbock *et al*. (1998). Typically, 100–150 mg of tissue per sample were homogenized in 2 ml buffer A (320 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris–HCl, 0.1 mM deferoxamine mesylate, 1% triton X-100, pH 7.5) and centrifuged at 1500 *g* for 10 min at 4°C. The supernatant was then discarded and the pellet washed with 2 ml buffer A. Following centrifugation for 10 min at 1500 *g*, the pellet was recovered and resuspended in 600 µl 5 mM EDTA-Na_2, 10 mM Tris–HCl, 0.15 mM deferoxamine mesylate (pH 8). After addition of 35 µl 10% SDS, RNA digestion was performed by incubation with 60 µg RNAse A and 10 U RNAse T1 for 15 min at 50°C. Protein digestion was carried out by incubation with 600 µg protease for 1 h at 37°C. The sample was then centrifuged at 5000 *g* for 15 min at 4°C and the supernatant was recovered in a 5 ml sterile tube. Following addition of a 1.2 ml sodium iodide solution (20 mM EDTA-Na_2, 7.6 M NaI, 40 mM Tris–HCl, 0.3 mM deferoxamine mesylate, pH 8) and 2 ml isopropanol, the tube was centrifuged for 15 min at 5000 *g*. The pellet was then recovered and resuspended in 2 ml 40% isopropanol. Following centrifugation, the pellet was washed in 2 ml 70% ethanol and resuspended in 100 µl 0.1 mM deferoxamine mesylate. Samples were stored at –20°C prior to digestion.

SEVAG and phenol/chloroform DNA extraction methods. The SEVAG and phenol/chloroform methods used to study the effect of phenol on 8-oxodGuo level differed only in the nature of the organic solvent used to extract DNA. Mussel samples were homogenized in 10 mM EDTA, 50 mM Tris–HCl, pH 7.5, 0.5% SDS, 2.5 mM deferoxamine mesylate (10 ml g^{-1} tissue). In a subsequent step, 1 ml of homogenate was recovered and centrifuged for 30 s at 500 *g*. DNA extraction was then carried out on 500 µl of homogenate. RNA digestion was performed by incubation for 1 h at 37°C with 600 µg RNAse A and 1000 U RNAse T1. Protein digestion was carried out by incubation for 2 h at 37°C with 325 µg proteinase K. The total amount of enzyme was reached in two stages, with half added at mid-incubation course. Following the addition of 25 µl 10% SDS, the sample was heated to 60°C for 10 min and the proteins were precipitated with 125 µl 5 M sodium perchlorate.

(a) *SEVAG:* DNA was extracted with 800 µl of SEVAG. After a mild agitation for 20 min at room temperature, the sample was centrifuged at 14 000 rpm for 15 min at 4°C and the aqueous phase was recovered.

(b) *Phenol/chloroform:* DNA was extracted by addition of 800 µl phenol/chloroform (1: 1). Following agitation for 5 min at room temperature, the 14 000 rpm supernatant fraction was recovered and the extraction then proceeded for two additional periods. DNA extraction was then achieved by three successive extractions with 800 µl chloroform. DNA was precipitated from the aqueous phase by addition of 50 µl 2.5 M sodium acetate and 2 vol. of glacial absolute ethanol and overnight storage at

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–20°C. Following centrifugation at 14 000 rpm for 20 min at 4°C, DNA pellets were recovered and dried with a speedvac. DNA was then resuspended in 400 µl MilliQ water and the DNA was UV quantified. Samples were stored at –20°C prior to digestion.

Enzymatic digestion. For samples prepared by the SEVAG and the phenol/chloroform methods, 100 µg DNA were digested to deoxyribonucleosides. With the chaotropic method, the 100 µl DNA solution was entirely digested. DNA digestion proceeded as follows. After the addition of 10 U nuclease P1 (1 U μl^{-1} in 300 mM sodium acetate, 1 mM $ZnSO_4$, pH 5.5) and 1 U alkaline phosphatase, samples were incubated for 90 min at 37°C. Proteins were precipitated with 100 µl chloroform. Then, the samples were centrifuged at 10 000 *g* for 5 min and the supernatants recovered for injection.

Statistical analysis

DNA adduct levels recorded in mussel gills were analysed by calculating correlation coefficients with respect to B[*a*]P exposure time and B[*a*]P concentration in whole mussel (Statistica Soft Inc.). Data on 8-oxoGuo measurement were subjected to a two-factor analysis of variances, ANOVA, using both the time period animals were kept transplanted in the aquarium and B[*a*]P exposure as factors (Statistica Soft Inc.). The comparison of the 8-oxodGuo levels measured in each tissue by the different protocols was performed by Student *t*-tests for paired samples. For each protocol, intertissue comparison of 8-oxodGuo levels was performed by a multiple factor ANOVA, taking both tissue nature and pool identification as factors. Each calculated mean was characterized by its standard deviation (SD) value.

Results

Detection of B{a}P-related DNA adducts in gill tissue

DNA adduct levels measured in gills of the exposed mussels are reported in table 1. To allow the comparison of the DNA adduct levels recorded in both gills and digestive gland of mussels from the same experiment, adduct levels recorded in the digestive gland are also provided in this table (Akcha *et al.* in press). B[*a*]P concentrations in whole mussel are also cited to give an idea of the internal tissue dose reached during exposure (Akcha *et al.* in press). A positive correlation was found in the present study between DNA adduct level in gill tissue and time of B[*a*]P exposure ($R = 0{,}747$, $p < 0.001$) (figure 1). As in the digestive gland, B[*a*]P-related DNA adducts were produced in a time-dependent manner relative to exposure, and adduct level was positively correlated with B[*a*]P concentration in whole mussel tissues ($R = 0.714$, $p < 0.001$) (figure 2).

Table 1. B[*a*]P-related DNA adduct levels in gills and digestive gland of B[*a*]P-contaminated mussels.

	Gills		Digestive gland[a]		B[*a*]P Concentration[b]
B[*a*]P exposure (days)	Mean RAL ($n = 3$)	SD	Mean RAL ($n = 3$)	SD	mg B[*a*]P kg^{-1} dw ($n = 1$)
0	0.149	0.079	0.010	0.005	0.005
3	0.186	0.041	0.032	0.015	48.363
6	0.254	0.011	0.044	0.015	107.724
10	0.227	0.069	0.088	0.030	90.47
17	0.302	0.040	0.060	0.062	337.344
24	0.405	0.236	0.269	0.082	274.231
28	0.480	0.139	0.251	0.062	581.057

[a] Akcha *et al.* (2000).
[b] B[*a*]P concentration recorded in whole mussel.

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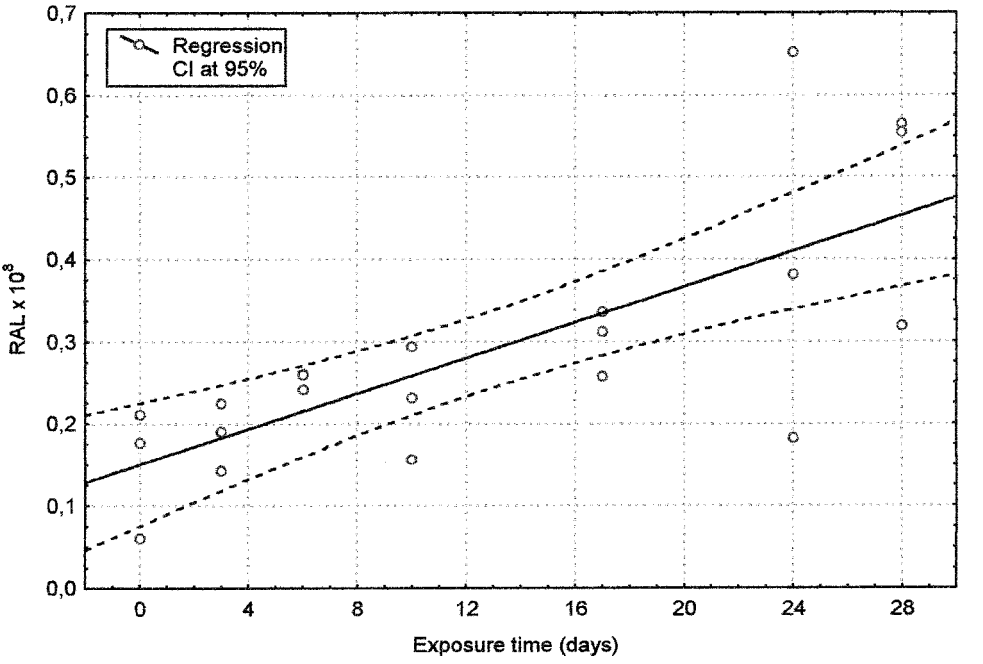

Figure 1. Time-dependent formation of B[*a*]P-related DNA adducts in mussel gills. RAL $\times 10^8$ = 0.150 + 0.011 exposure time (days), $r = 0.747$, $p < 0.001$.

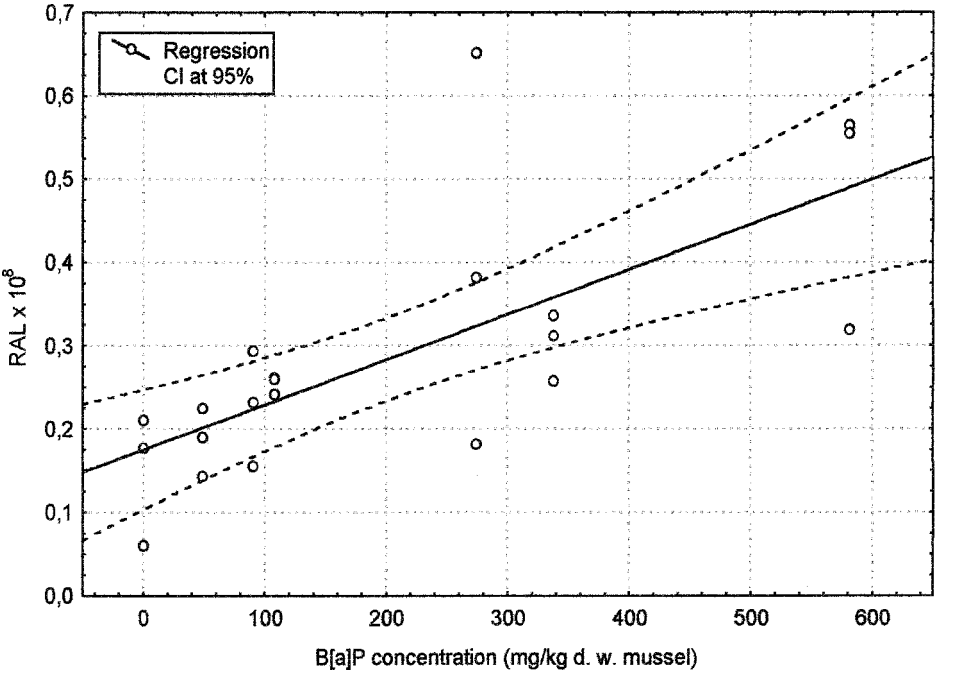

Figure 2. Correlation between DNA adduct level in gill DNA and whole mussel B[*a*]P concentration. RAL $\times 10^8$ = 0.175+0.001 B[*a*]P concentration, $r = 0.714$, $p < 0.001$.

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Measurement of 8-OHdG in gill DNA

Laboratory exposure. 8-OxodGuo levels recorded in both control and B[*a*]P-exposed animals are indicated in table 2. No increased 8-oxodGuo formation was observed during the time animals were kept in the aquariums ($p = 0.827$). In exposed animals, no effect of B[*a*]P exposure on 8-oxodGuo level was observed in gill DNA ($p = 0.051$), despite the high B[*a*]P concentration reached during this experiment in whole mussel tissues. The mean 8-oxodGuo level was $25.83 \pm 6.26/10^5$ dGuo. This absence of effect explains the lack of correlation between 8-oxodGuo level and B[*a*]P concentration ($p > 0.05$).

Methodological development. The results are reported in table 3. No difference in 8-oxodGuo levels was observed between the SEVAG and the phenol/chloroform DNA extraction protocols for digestive gland (34.8 ± 9.3 and 25.6 ± 4.8 8-oxodGuo per 10^5 dGuo) and gill (6.6 ± 0.8 and 7.3 ± 2.4 8-oxodGuo per 10^5 dGuo) tissues ($0.2 < p < 0.3$ and $0.6 < p < 0.7$ respectively). The 8-oxodGuo levels recorded by the chaotropic method were significantly lower in both digestive gland and gill DNA (8.3 ± 2.0 8-oxodGuo per 10^5 dGuo for $0.02 < p < 0.05$, and 4.8 ± 1.1 8-oxodGuo per 10^5 dGuo for $0.02 < p < 0.05$ respectively) than with the phenol/chloroform method.

Intertissue comparisons showed the existence of higher 8-oxodGuo levels in digestive gland than in gill DNA for samples prepared either by the SEVAG- and phenol/chloroform-based protocols ($p < 0.001$) or by the chaotropic method ($p < 0.01$).

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Table 2. Mean 8-oxodGuo level (number of lesions/10^5 dGuo) in gill DNA of both control and B[*a*]P-exposed mussels.

	Control mussels		B[*a*]P-exposed mussels	
Time of experiment (days)	Mean ($n = 3$)	SD	Mean ($n = 3$)	SD
0	25.8	3.3	25.8	3.3
3	23.1	0.8	29.4	9.1
6	21.7	6	24.9	4.5
10	19.3	2.4	24.9	8.6
17	18.9	2.7	27.6	7.2
24	23.3	4.9	25.9	6.0
28	25.2	3.5	22.3	5.1

Table 3. Effect of DNA extraction procedure on 8-oxodGuo (number of lesions/10^5 dGuo) measurement.

Mussel origin	DNA extraction protocol	Gills	Digestive gland
Rya de la Rosa, Spain (set of samples no. 1)	Phenol/chloroform	7.3 ± 2.4	25.6 ± 4.8
	SEVAG	6.6 ± 0.8	34.8 ± 9.3
Rya de la Rosa, Spain (set of samples no. 2)	Chaotropic	4.8 ± 1.1	8.30 ± 2.03
	Phenol/chloroform	13.9 ± 3.7	34.31 ± 9.50

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Discussion

B{a}P-related DNA adduct formation

The correlation observed in gills and digestive gland between DNA adduct level and B[*a*]P whole mussel content supports the proposition that DNA adducts are a potential biomarker of PAH exposure in mussels. The exposure time-dependent formation of B[*a*]P–DNA adducts provides a similar conclusion. As demonstrated by Canova *et al*. (1998) and Harvey and Parry (1997) in water-column contamination studies, the highest levels of B[*a*]P–DNA adducts were not recorded in the digestive gland but in the gills ($p < 0.001$), which appear to be more sensitive to B[*a*]P exposure. However, in a preliminary toxicokinetic study conducted in the same conditions as in this paper, but using [^{3}H]B[*a*]P, digestive gland displayed the highest B[*a*]P bioconcentration and DNA binding value in mussel (Akcha *et al*. 1999). As animals were exposed to contaminant in their feed, digestive gland was the main target tissue and showed the highest B[*a*]P concentration. Digestive gland is also the main tissue for biotransformation in the mussel (Livingstone and Pipe 1992), being characterized by higher phase I activities due to the presence of both a mixed function oxygenase (MFO) system and a microsomal flavin monooxygenase (FMO) system. Therefore, our observation of a higher DNA adduct level in the gills than in the digestive gland in the study reported in this paper appears to be contrary to these previous findings as higher phase I activities could account for higher DNA adduct formation. However, there are several possible explanations for the differences observed. DNA adduct formation can be regarded as the result of two opposite processes (activation and elimination). Because digestive gland showed higher B[*a*]P uptake and phase I activities than gills, the differences between the two tissues are probably related to the elimination rather than the activation process. In this manner, a decrease in phase II conjugation activities could lead to an unbalanced ratio between phase I and phase II activities, resulting in an increased adduct level. In fact, glutathione-*S*-transferase (GST, EC 2.5.1.18) activity in gills of exposed mussels was found to be significantly depressed by B[*a*]P exposure, whereas the same activity in the digestive gland remained unchanged (Akcha *et al*. in press). Another possible explanation relates to differences in B[*a*]P activation pathways between tissues. It has been shown in vertebrates that B[*a*]P can be metabolized either by the diol-epoxide or the radical cation pathways (Cavalieri *et al*. 1990). The latter activation pathway involved a P450-catalysed one-electron oxidation leading to the production of B[*a*]P radical cations that can react directly with DNA to yield depurinating DNA adduct formation. This kind of adduct, which is not detected by the standard ^{32}P-post-labelling assay, is thought to constitute up to 80% of the total adducts produced in vertebrates (Rogan *et al*. 1993). As B[*a*]P radical cations have been demonstrated to be the precursors of B[*a*]P quinones (Cavalieri *et al*. 1988) and since quinones are the major metabolites of *in vivo* B[*a*]P biotransformation in mussel (Michel *et al*. 1995), the radical cation pathway may also be important in bivalves. Hence, it is possible that the low bulky DNA adduct levels recorded in the digestive gland were mainly due to a predominant radical cation pathway in this tissue. However, the presence of the same adduct pattern in both tissues tends to indicate that differences in the diol-epoxide activation pathway could be quantitative but not qualitative. Nevertheless, this assumption cannot account for the higher DNA binding value observed in the digestive gland. In fact, depurinating adducts are lost during the classical DNA

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extraction procedure and thus do not contribute to the binding index. The [^{3}H]-binding assay used by Akcha *et al.* (1999) is presumably not as specific as the ^{32}P-post-labelling technique and possibly could include adducts not detected by the latter technique.

An observed difference in adduct level between tissues is important for applications in biomonitoring studies. As gills appear to be more sensitive than digestive gland to genotoxic exposure, as indicated by the measurement of bulky DNA adducts, a post-labelling assay in gills may allow an earlier detection of the exposure-related genotoxic effects.

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8-OxodGuo measurement in mussel gills

In aquatic organisms, different processes have been identified as stimulators of pollutant ROS production, including uptake directly or indirectly (following biotransformation processes) of redox cycling xenobiotics, redox reactions with transition metals or other organic free radicals, and induction of cytochrome P450 content and biotransformation enzymes such as NAD(P)H-dependent flavoprotein reductases that can respectively generate $O_2^{\cdot-}$ and/or H_2O_2 and $O_2^{\cdot-}$ through their own catalytic cycle (Livingstone *et al.* 1990). As B[*a*]P appears to stimulate ROS production in mussel, it is quite surprising that 8-oxodGuo level was not affected in B[*a*]P-exposed mussels. In fact, as a filter-feeding organism, the mussel is a high bioaccumulator of organic pollutants such as PAHs (Akcha *et al.* 1999). B[*a*]P metabolism has been demonstrated *in vivo* in *Mytilus* sp., leading to the formation of predominant B[*a*]P dione metabolites (Michel *et al.* 1995). The potential of benzo[*a*]pyrene diones and known cycling compounds to stimulate *in vitro* ROS production has been well-demonstrated in mussel digestive gland microsomes (Sjölin and Livingstone 1997). *In vivo*, increased ROS production by PAH exposure was indirectly demonstrated by the study of both induced damage to cellular macromolecules (Herbert and Zahn 1990) and variations in antioxidant defences (Solé *et al.* 1994).

In this study, B[*a*]P-stimulated ROS production presumably is likely to have occurred, given the high B[*a*]P bioconcentration reached in whole mussel tissues. The observation of an induction of benzo[*a*]pyrene hydroxylase activity after B[*a*]P exposure is indicative of higher phase I metabolism (Akcha *et al.* in press). Although the measurement of NAD(P)H-dependent cytochrome P450 reductase (EC 1.6.2.4) activity in digestive gland microsomes revealed no increase in quinone reduction (data not shown), a rise in catalase (CAT, EC 1.11.1.6) activity was highly significant in B[*a*]P-exposed mussel, indicating an increased $O_2^{\cdot-}$ production (Akcha *et al.* in press). The absence of oxidative DNA damage in gills was thus not consistent with our findings. Few data in the literature are helpful in clarifying these results. Canova *et al.* (1998) showed increased 8-oxodGuo levels in gill DNA after B[*a*]P exposure for 48 h to 5, 50 and 100 ppb and for 72 h to 5, 50, 100 and 1000 ppb, with values ranging from 2.24 ± 0.27 (72 h, control) to 7.66 ± 0.69 8-oxodGuo per 10^5 dGuo (72 h, 100 ppb). Because the B[*a*]P concentration recorded in whole mussel tissues in our study (about 100 mg kg^{-1} ww) was similar to those estimated from the study of Canova *et al.* (1998) with the 1000 ppb B[*a*]P concentration (111.9 mg kg^{-1} ww at 72 h exposure), increased 8-oxodGuo levels could have been observed in gills for the B[*a*]P exposure level reached during the present experiment. Because mussels were respectively contaminated via the feed

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supply and the water column in our study and that of Canova *et al.* (1998), the difference in the effect recorded on gill 8-oxodGuo level may be linked to the way of exposure. As a matter of fact, by contaminating mussels via the feed supply, gills were not the target tissue of exposure, presumably explaining why no increase in 8-oxodGuo was recorded in this tissue.

Other explanations may account for the lack of a B[*a*]P effect on gill 8-oxodGuo level. As in the case of CAT activity, an increase in antioxidant defences could have overcome B[*a*]P stimulated ROS production and limited its toxic effects in mussel. The absence of oxidative DNA damage could also be related to the HPLC/ECD assay, particularly to the DNA extraction procedure used for the analysis. In fact, methodological problems have been observed in 8-oxodGuo measurement, mainly concerning artifactual oxidation of DNA during workup process, leading to an overestimation of measured 8-oxodGuo levels (Cadet *et al.* 1997b). Because in the present paper, gill 8-oxodGuo levels were up to 10 times higher than the values reported in the same tissue by Canova *et al.* (1998), the absence of any effect of B[*a*]P exposure on the occurrence of 8-oxodGuo could be due to artifactual sample oxidation that could have masked the possible B[*a*]P oxidative effects. The results of the methodological work presented in this paper support this assumption. In fact, the addition of a stronger ionic chelator in the homogenization buffer and the reduction of the number of DNA precipitation steps led to a decrease in the 8-oxodGuo level recorded by the SEVAG method in gill tissue (6.6 against 25.8 8-oxodGuo per 10^5 dGuo). Although no difference in level was observed between phenol- and SEVAG-extracted gill and digestive gland DNA, the chaotropic method allowed detection of significant lower values in both tissues.

The induction and time-dependent increase of B[*a*]P–DNA adducts in mussel gills confirm that DNA adduct measurement is a promising biomarker of genotoxicity in aquatic organisms. The higher DNA adduct levels observed in gills as compared with digestive gland indicate the greater sensitivity of the former tissue to macromolecular adduct formation. Thus, measurement of bulky aromatic adduct formation appears to be more pertinent for mussel gills than digestive gland since it allows earlier detection of marine pollutant genotoxic effects. The absence of B[*a*]P effects on 8-oxodGuo level in mussel gill DNA could be due to the chosen way of B[*a*]P exposure or to artifactual oxidation during sample processing. Although 8-oxodGuo measurement represents a potent biomarker for oxidative stress in aquatic biomonitoring, further studies are needed to fully assess the trace level of oxidative damage to DNA in aerobic organisms.

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