Genotoxicity assessment and detoxification induction in *Dreissena polymorpha* exposed to benzo[a]pyrene

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The use of DNA adduct analysis has previously focused on the use of marine organisms for biomonitoring, whereas similar investigations in freshwater organisms are sparse. In that context, we have investigated the relevance of DNA adducts as biomarkers of genotoxicity in the freshwater mussels *Dreissena polymorpha*. The objective of the present study is to determine the stability of DNA adducts induced by benzo[a]pyrene (B[a]P) in zebra mussels. Mussels were exposed to dissolved B[a]P (10–100 µg/l) for 4 days. Afterwards, mussels were kept in clean water for 28 days and DNA adduct levels were subsequently measured in two different organs, the digestive glands and the gills, using the ^{32}P-postlabelling technique. In parallel, the expression of genes involved in the detoxification system was assessed by qPCR (catalase, superoxide dismutase, glutathione S transferase, HSP70, aryl hydrocarbon receptor, P glycoprotein). We observed a higher level of DNA adducts in the digestive glands compared to the gills. Moreover, in gills, the level of DNA adduct was dependent on the B[a]P concentration. The levels of adducts tended to decrease in both organs after 28 days in clean water. In addition, an early induction of *HSP70*, P*g*P, *AHR* and *SOD* mRNA levels was noticed in the gills compared to the digestive glands. *CAT* and *GST* gene expression increased from 12h exposure in both organs. A higher gene expression level of those genes was observed in the gills, except for *AHR* and *CAT* genes. Data converge towards the fact that DNA adducts hence represent a very promising biomarker of B[a]P contamination and potentially of exposure to polycyclic aromatic hydrocarbons. In addition, for the first time in this study, B[a]P detoxification system was characterised in *D. polymorpha*.

Introduction

Genotoxic endpoints have been widely used as biomarkers of pollution in marine biomonitoring programs (1), while they have been investigated only recently in freshwater studies. Indeed, a few studies in fluvial or lake areas have demonstrated the value of using exposure biomarkers such as DNA adducts (2–4) and DNA strand breaks (5,6) for the biological effects evaluation of chemical contamination. By using the comet assay, we have recently demonstrated the value of measuring DNA strand breaks in field translocated zebra mussels (7,8).

Zebra mussels have been used for decades in pollution monitoring programs in Europe (9) and in North America (10). Currently, *Dreissena polymorpha* (Pallas) has been introduced in some monitoring programs for the evaluation of the biological effects of pollutants (11). These animals present some advantages as bioindicator species: (i) They are sessile filter feeders capable of accumulating large amounts of pollutants and especially metallic as well as hydrophobic contaminants such as polycyclic aromatic hydrocarbons (PAHs) (7,12); (ii) they are widely distributed around the world (13) and also along the Seine River (14); (iii) the fact that they live attached to rocky surfaces makes the sampling easy.

PAHs constitute a major class of pollutants of the aquatic environment. Some of them are known to be procarcinogenic chemicals that require metabolic activation to exert their genotoxicity on organisms (15). Kurelec and co-workers (16) were the first to describe the *in vitro* formation of DNA adducts in the mussel *Mytilus galloprovincialis* homogenate exposed to aminofluorene. Later, DNA adduct formation was observed both in marine mussels (3,17,18) and in freshwater mussels (2,3), exposed to model PAHs, which demonstrates that these species are able to metabolise benzo[a]pyrene (B[a]P) into reactive metabolites that can bind to DNA to form DNA adducts. Laboratory experiments have demonstrated dose- and time-dependent formation of DNA adducts in marine mussels exposed to B[a]P (17,19,20), with a tissue difference in DNA adduct formation (17,18) and a persistence of 2 weeks after the end of the experiment in gills (17), hence constituting a promising biomarker of genotoxicity. In all these studies, the ^{32}P-postlabelling assay (21) was used for the measurement of DNA adducts since it represents the reference technique for DNA adduct measurement *in vitro* and *in vivo*. In marine environmental biomonitoring, this method has been widely applied in mussels (22–25) since it enables the evaluation of environmental genotoxic impact without knowing the nature of pollutants.

In mammals, it is known that B[a]P enters cells through its binding to the aryl hydrocarbon receptor (AHR) and is afterwards metabolised by phase I and II enzymes. These enzymes implicated in the biotransformation of PAHs in mammals have also been characterised in mussels: cytochrome P450 (CYP1a) (phase I), glutathione S transferase (GST) (phase II), superoxide dismutase (SOD) and catalase (CAT) (26,27). Very recently, researches have made a considerable effort to sequence genes encoding for phase I and II enzymes in the mussel *D. polymorpha* and their mRNA expression has been shown to be modulated after mussel exposure to cadmium, copper and mercury chloride (27). SOD represents the first defensive system against reactive oxygen species (ROS) production since it catalyses the dismutation of O^{2-} to

H$_2$O$_2$. CAT catalyses the reduction of H$_2$O$_2$ into H$_2$O, whereas the GST represents quantitatively the most important phase II enzymes of the detoxification system (26). It catalyses the conjugation reaction of glutathione with xenobiotics and also plays a role in preventing oxidative damage by conjugating breakdown products of lipid peroxides to GSH (28). Finally, the HSP70 and the transmembrane protein transporter P-gP1 act to eflux xenobiotics out of cells (29,30).

The present study aimed to get a better understanding of the formation and persistence of DNA adducts in gills and digestive glands of the freshwater mussel *D. polymorpha* exposed to B[a]P, a model PAH, using the ^{32}P-postlabelling technique. Moreover, mRNA levels of phase I and II enzymes were measured by quantitative real-time PCR, in the different conditions of exposure, which enable to propose a B[a]P detoxification signalling in *D. polymorpha*. This mechanistic understanding and adaptation at molecular and genetic levels could increase the knowledge of the survival strategies of the organisms under stressed conditions, so as to, finally, develop efficient approaches to protect ecosystem health (31).

Material and methods

Chemical reagents

B[a]P and DMSO were purchased from Sigma (France), acetone and dichloromethane were supplied by Merck and heptane by Promochem. B[a]P standard was obtained from Dr. Ehrenstorfer's laboratory. Stock solution of B[a]P was prepared in DMSO.

Mussel sampling and maintenance conditions

Adult zebra mussels, *D. polymorpha*, 18–22 mm long, were collected in October 2010 at a reference site at Commercy (France) (48°45′33″N, 5°36′05″W). Animals were transferred to the laboratory, cleaned of all fouling organisms and kept in a tank containing raw water originating from Commercy site (16°C) for 2 weeks acclimation before the experiments.

In vivo exposure of zebra mussels to benzo[a]pyrene

Mussels were exposed to B[a]P (nominal concentration: 10, 100 μg/l) for 4 days in glass tanks containing 12 l of Aquarel water (Nestlé), under artificial light and without feeding. Control mussels were incubated in water containing 0.01% DMSO as a solvent carrier. In the first study, water, DMSO and B[a]P were renewed every 2 days; then, in the second study, water and B[a]P were completely renewed twice a day. After the exposure time, mussels were maintained in clean water for a recovery period of 28 days and fed every 2 days. Digestive glands and gills (20 mussels per condition) were dissected at 5 days after the beginning of exposure and at days 14 and 28 of recovery. Animals that had not become attached to the tank were removed regularly. Tissues were then stored at −80°C for further analysis.

Benzo[a]pyrene concentration in soft tissue

B[a]P was extracted as described by Bourgeault *et al.* (7). B[a]P analysis was performed on 10 freeze-dried mussels that had been pooled and reduced to a powder. B[a]P was microwave-extracted (Mars Xpress, CEM) with 20 ml acetone/heptane (v/v) (50/50) spiked with deuterated B[a]P as internal standard. Following the microwave extraction, the sample was filtered through a 0.7 μm glass fibre filter (GF/F Whatmann). The filtrate was then evaporated down to a final volume of 500 μl. The extract was purified using a silica gel column and eluted with 10 ml heptane/dichloromethane (v/v) (80/20). The volume was finally reduced to 100 μl by a gentle nitrogen gas stream. Extract was analysed by gas chromatography-mass spectrometry (Thermo Trace GC Ultra-DSQII). Results were expressed in micrograms of B[a]P per gram of dry weight tissue (μg/g dw). This extraction and analysis protocol was validated using a certified reference material Mussel Homogenate (IAEA-432). Results were expressed in micrograms of B[a]P per gram of dry weight tissue (μg/g dw).

^{32}P-postlabelling analysis of DNA adducts

DNA isolation. Briefly, digestive glands or gills were homogenised in 0.8 ml of a solution containing NaCl (0.1 M), EDTA (20 mM) and Tris-HCl, pH 8 (50 mM) (SET). To the homogenate, 100 μl of SDS (20%) was added, and

following incubation for 10 min at 65°C, 800 μl of potassium acetate (6 M, pH 5) was added and the reaction mixture was kept at 0°C for 30 min. After centrifugation for 25 min at 0°C (10 000*g*), the supernatant, which contained nucleic acids, was collected and nucleic acids were precipitated overnight at −20 °C by adding two volumes of cold ethanol. DNA pellets were collected and washed once with 1 ml of 90% ethanol and dissolved in 500 μl of SET (15 min at 37°C). The total extract was mixed with 10 μl of a mixture of RNase A (20 mg/ml) and RNase T1 (10 000 U/ml) (note: before use, the RNases were boiled at 100°C for 15 min to destroy DNases) and incubated for 1 h at 37°C; this treatment was repeated twice. Samples were then treated with 25 μl of proteinase K solution (20 mg/ml SET) for 1 h at 37 °C. After digestion, 500 μl of Rotiphenol was added and the mixture was moderately shaken for 20 min at room temperature and centrifuged for 15 min at 15°C (10 000*g*). The aqueous phase was collected after two extractions. After a final extraction with one volume of chloroform/isoamyl alcohol (24:1), the aqueous phase was collected and 50 μl of sodium acetate (3 M, pH 6) was added. The DNA was precipitated by the addition of two volumes of cold ethanol overnight at −20°C, and the precipitate was collected by centrifugation at 10 000*g* for 30 min. The DNA pellet was washed four times with 90% ethanol. The purity of the DNA was checked by recording UV spectra between 220 and 320 nm (32).

^{32}P-postlabelling

DNA (4 μg) was digested at 37°C for 4 h with 10 μl of the mix containing 1 μl of micrococcal nuclease (2 mg/ml corresponding to 500 mU), spleen phosphodiesterase (105 mU/sample or 26 mU/μg DNA), 1 μl of sodium succinate (200 mM) and 1 μl of calcium chloride (100 mM, pH 6). The digested DNA was then treated with 5 μl of the mix containing nuclease P1 (4 mg/ml), ZnCl$_2$ (1 mM) and sodium acetate (0.5 M, pH 5) at 37°C for 45 min. The reaction was stopped by adding 3 μl of Tris base. The DNA adducts were labelled as follows. To the NP1 digest, 5 μl of the reaction mixture containing 2 μl of bicine buffer [Bicine (800 μM), dithiothreitol (400 mM), MgCl$_2$ (400 mM) and spermidine (400 mM) adjusted to pH 9.8 with NaOH], 9.6 U of polynucleotide kinase T4 and 100 μCi of [^{32}P]ATP (specific activity 6000 Ci/mmol) was added and incubated at 37°C for 45 min. Normal nucleotides, pyrophosphate and excess ATP were removed by chromatography on PEI/cellulose TLC plates in 2.3 M NaH$_2$PO$_4$ buffer, pH 5.7, overnight (D1). The origin areas containing labelled adducted nucleotides were cut out and transferred to another PEI/cellulose TLC plate, which was run in 5.3 M lithium formate and 8.5 M urea (pH 3.5) for 3 h (D2). A further migration was performed after turning the plate 90° anticlockwise in 1 M LiCl, 0.5 M Tris and 8 M urea (pH 8) for 2 h (D3). Finally, the chromatogram was washed in the same direction in 1.7 M NaH$_2$-PO$_4$, pH 6, for 2 h (D4). Autoradiography was carried out at −80°C for 48 h in the presence of an intensifying screen. Radioactive spots were detected by autoradiography on Kodak super X-Ray film (33).

Quantitation of DNA Adducts

Quantitation of DNA adducts was obtained by storage phosphor imaging techniques employing intensifying screens at room temperature for 18 h. The screens were scanned using a Typhoon 9210 (Amersham). Software used to process the data was ImageQuant (version 5.0). After background subtraction, the levels of DNA adducts were expressed as relative adduct labelling (RAL) in total nucleotides. To calculate the levels of screen response (screen pixel) in dpm, samples of [^{32}P]ATP at different concentrations from 10 to 500 dpm were appropriately diluted and spotted on a TLC plate. The plate was then scanned with the samples to obtain a radioactivity scale.

RNA extraction, RT-PCR and qRT-PCR analysis

Total RNA from control and B[a]P-treated mussels was extracted using TRIzol Reagent as described (34). RNA concentration and purity were measured by spectrophotometric absorption at 260 and 280 nm. First strand cDNA synthesis was carried out on 1 μg of total RNA extract with oligo-dT primers according to Improm II Reverse Transcriptase kit (Promega). Preparations of digestive glands and gills cDNA were used to quantify specific transcripts in LightCycler 480 Real Time PCR System (Biorad) using SYBR Green Power Master Mix (Invitrogen) with the primers pairs listed in Table I. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (Cp). Cp values for target genes (TG) were compared to the corresponding values for a reference gene (ribosomal S3 gene) to obtain ΔCp values (ΔCp = Cpref − CpTG). PCR efficiency values for reference and tested genes were calculated as described (27), and assumed to be close to 100% from these calculations.

Condition index

Three condition indices (CI) were used so as to measure the well-being/nutritional status of the bivalves according to Lundebye *et al.* (1997):

Table I. Primer sequences (5′-3′) used in qRT-PCR

Gene	Short name	Forward primer	Reverse primer	Accession number
Ribosomal protein S3	S3	CAGTGTGAGTCCCTGAGATACAAG	AACTTCATGGACTTGGCTCTCTG	AJ517687
P-glycoprotein	P-gp1	CACCTGGACGTTACCAAAGAAGATATA	TCACCAACCAGCGTCTCATATTT	AJ506742
Aryl-hydrocarbon receptor	AH-R	ATCACAGCGATGAGCCTCAG	AGACAGCATTGCGAGGTCAC	DQ159188
Superoxide dismutase	SOD	GACAGCATGGCTTCCATGTG	AGGAGCCCCGTGAGTTTTG	AY377970
Catalase	CAT	ATCAGCCTGCGACCAGAGAC	GTGTGGCTTCCATAGCCGTT	EF681763
Glutathione S-transferase	GST	ATGATCTATGGCAACTATGAGACAGG	GAAGTACAAACAGATTGTAGTCCGC	EF194203
Heat-shock protein 70	HSP70	GCGTATGGACTTGATAAGAACCTCA	GAACCCTCGTCGATGGTCA	EF526096

CI-1: Soft tissue wet weight (g) × 1000/shell weight (g)
CI-2: Soft tissue wet weight (g) × 1000/(length × width/height (mm))
CI-3: Soft tissue wet weight (g) × 1000/total weight (soft tissue + shell) (g)

Statistical analysis

Adduct and RT-qPCR results are given as mean values ± SD of three values (three mussels per condition pooled and three repetitions of each test). The measured values were compared among different groups (model agents' concentration) using an analysis of variance (ANOVA) followed by a Tukey post hoc test. Statistical significance was accepted at $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).
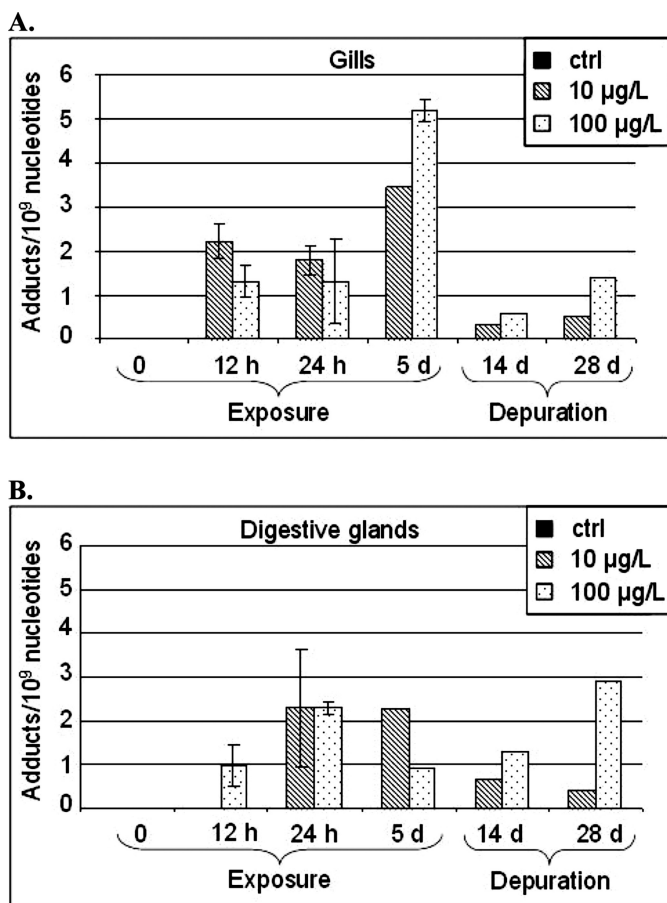

Fig. 1. Formation of DNA adducts in gills (**A**) and digestive glands (**B**) of *D. polymorpha*. Mussels were exposed to 10 and 100 μg/l of B[a]P for 4 days (B[a]P was added two times a day in aquaria) and the depuration period was of 28 days.

Results

Benzo[a]pyrene bioaccumulation and condition index

When the mussels were exposed to B[a]P (10 μg/l) by renewing the water every 2 days, B[a]P concentration in soft tissues of mussels was 21 μg/g dried weight while B[a]P content in mussels exposed to 100 μg/l was 20 times higher than the 10 μg/l exposed mussels (449 μg B[a]P/g dry weight) (Table II).

When mussels were exposed to B[a]P (10 μg/l) by renewing the water two times a day, the concentration of B[a]P in soft tissues was of 175 μg B[a]P/dry weight whereas 25 539 μg of B[a]P/g were detected in animals exposed to 100 μg/l of B[a]P (Table II).

As shown in Table III, the condition indices of mussels exposed to B[a]P for 5 days did not vary significantly whatever the condition of exposure or B[a]P concentrations tested.

DNA adduct formation

We did not observe any formation of DNA adduct in mussels present in aquariums when B[a]P was renewed every 2 days, for both tissues tested (data not shown). On the other hand, when B[a]P was added twice a day in the water, DNA adducts were

Table II. B[a]P tissue concentration in whole tissue of *D. polymorpha* exposed to 10 or 100 μg/l B[a]P for 4 days

B[a]P concentration added in aquaria (μg/l)	B[a]P accumulation (μg/g dried tissue)	
	B[a]P renewed every two days	B[a]P renewed twice a day
0	0	0
10	21	175
100	449	25539

Table III. Condition indices of mussels *D. polymorpha* exposed to benzo(a)pyrene

(A) B[a]P exposure	CI 1	CI 2	CI 3
Control	544 ± 133	24 ± 9	35 ± 6
10 μg/l	515 ± 100	23 ± 6	34 ± 5
100 μg/l	539 ± 61	27 ± 6	35 ± 3
(B) B[a]P exposure	CI 1	CI 2	CI 3
Control	632 ± 168	28 ± 10	38 ± 6
10 μg/l	561 ± 148	30 ± 8	35 ± 6
100 μg/l	590 ± 121	28 ± 8	37 ± 5

CI were calculated as described in the section "Materials and Methods": B[a]P was renewed every 2 days (A) or two times a day (B) in aquaria.
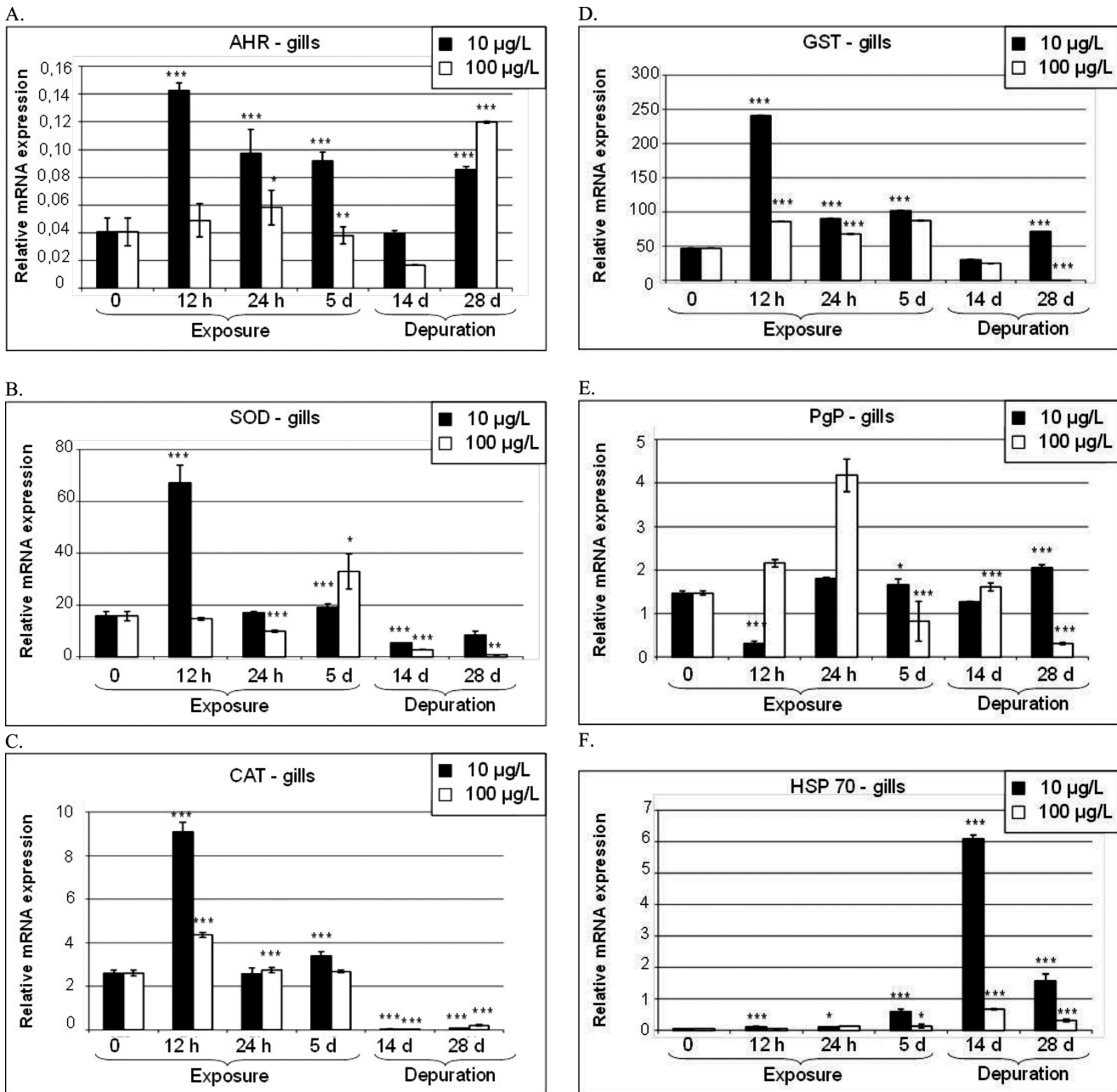
Fig. 2. Relative mRNA abundance values of *AHR* (**A**), *SOD* (**B**), *CAT* (**C**), *GST* (**D**), *P-gP* (**E**) and *HSP70* (**F**) in the gills of *D. polymorpha*. Mussels were exposed to B[a]P (added two times a day), mRNA levels were analysed by q-RTPCR. Results are normalised with the reference gene S3. (*): data significantly different compared to control ($P < 0.05$).

detected in B[a]P exposed mussels compared to control animals (Figure 1A and 1B). Three different adduct spots were detected in all conditions of B[a]P exposure for the gills and digestive glands. No DNA adducts were observed in DMSO-treated mussels. In the digestive glands of mussels exposed to 10 µg/l of B[a]P, DNA adduct was not detected after 12 h, while they were observed for 24 h and 5 days of exposure (Figure 1B). In mussels exposed to 100 µg/l of B[a]P, the DNA adduct level was progressively increased from 12 to 24 h, before decreasing after 5 days of exposure (Figure 2B). In contrast to the digestive glands, DNA adduct levels detected in the gills increased in a time-dependent manner for both B[a]P concentrations (Figure 1A). Lower levels were detected in 100 µg/l exposed mussels for 12 h and 24 h of exposure, compared to bivalves treated with 10 µg/l. After 5 days of exposure, the levels of

DNA adducts was higher in mussels exposed to B[a]P 100 µg/l as compared to mussels exposed to 10 µg/l. After 5 days of exposure, the DNA adduct level was higher in gills than in digestive glands.

After 14 days and 28 days of depuration, mussels presented a decrease in the DNA adduct level in gills whatever the concentration of B[a]P used for the exposure, whereas after 28 days of depuration, DNA adduct level re-increased in the digestive glands of mussels exposed to 100 µg/l of B[a]P, reaching a higher value than for the exposure period (Figure 2A).

Gene expression

In order to investigate some of the mechanisms of detoxification, we performed gene expression analysis in the freshwater
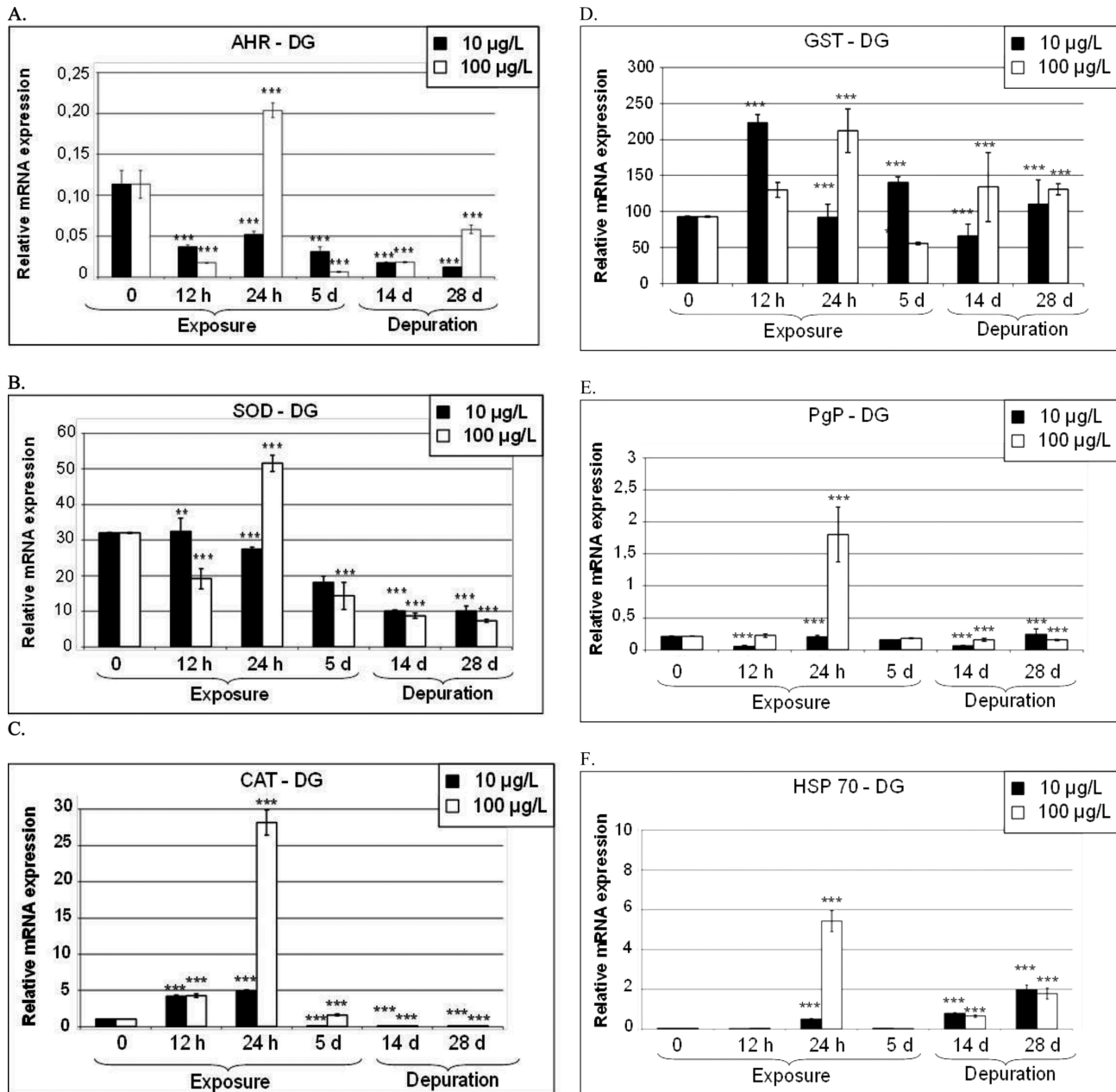
Fig. 3. Relative mRNA abundance values of *AHR* (**A**), *SOD* (**B**), *CAT* (**C**), *GST* (**D**), *P-gP* (**E**) and *HSP70* (**F**) in the digestive glands of *D. polymorpha*. Mussels were exposed to B[a]P (added two times a day), mRNA levels were analysed by q-RTPCR. Results are normalised with the reference gene S3. (*): data significantly different compared to control (*P* < 0.05).

mussel using quantitative RT-PCR, according to the primer sequences recently published (27,35). As referred to this latter work, S3 ribosomal gene was chosen as the reference gene for gene expression normalisation and t0 was used as control of the experiment.

When B[a]P medium was changed twice a day, we observed significant variations in the mRNA levels of all detoxification genes in *D. polymorpha*. Indeed, *AHR* mRNA expression in gills was early induced after 12 h of exposure, then remained high until 5 days, decreased after 14 days in depuration period before being re-enhanced after 28 days of recovery in freshwater, compared to control levels. In addition, *AHR* mRNA levels were higher after 10 µg/l B[a]P exposure than 100 µg/l (Figure 2A).

Concerning the digestive glands, *AHR* mRNA expression only increased after 24 h exposure with 100 µg/l of

B[a]P, whereas for the other time points tested, a significant down-regulation was observed (Figure 3A).

SOD as well as *CAT* gene expressions were enhanced earlier in the gills compared to the digestive glands. For both organs, it was noticed that they decreased when mussels were placed in recovery period, being significantly lower to the basal level (Figures 2B, 2C, 3B and 3C).

GST mRNA level increased in both tissues after 12 h exposure. In addition, as for *CAT* and *SOD* genes, *GST* levels tended to decrease in depuration period following an exposure to 10 µg/l. It could be nevertheless noticed, for those time points, that levels remained higher in digestive glands compared to the gills (Figures 2D and 3D).

PgP gene expression was induced after 24 h of exposure to 100 µg/l in the digestive glands while in the gills, its expression was detected after 12 h. In addition, in this tissue, levels

707

remained elevated after 28 days in depuration period (Figures 2E and 3E).

HSP70 gene expression increased for both tissues during B[a]P exposure, reaching a maximum for the gills after 5 days of exposure with 10 µg/l and after 24 h exposure to 100 µg/l for the digestive glands. On the contrary to the other genes, *HSP70* mRNA levels was more elevated in the depuration period than in the exposure and moreover its expression was highly elevated in the depuration period of 14 and 28 days for both tissues analysed (Figures 2F and 3F).

Discussion

Formation of DNA adducts

In the present study, analysis of DNA adducts revealed that the renewal of aquaria with B[a]P two times per day was necessary for DNA adduct formation in the freshwater mussel. The ^{32}P-postlabelling technique is sensitive enough to detect 1 adduct per 10^{10} nucleotides (36,37) suggesting that when mussels were exposed to B[a]P every 2 days, B[a]P concentration was too low in the aquaria to generate DNA adduct formation or that DNA adduct level is under the limit of quantitation or cells were able to remove formed adducts. In cell culture, it was demonstrated that the B[a]P DNA adducts are quickly removed (38).

We showed that DNA adducts were formed early after 12 h of B[a]P exposure in both organs tested, indicating that B[a]P metabolism occurred rapidly. Indeed, B[a]P diol epoxide (BPDE), the major reactive metabolite of B[a]P, has a high reactivity to DNA and especially to the N^2 position of guanine, hence forming anti-BPDE adducts (39,40).

Number of adducts ranged from 1.3 to 5.19 adducts/10^9 nucleotides in gills and from 0.9 to 2.29 adducts/10^9 nucleotides in the digestive glands, which is comparable to those obtained for the same species (2) and for marine mussels (17,19,20) also exposed in the laboratory to B[a]P.

We have demonstrated, in the freshwater mussel, a tissue specific formation of DNA adducts after 5 days of exposure, as also previously noticed in the marine mussel *M gallopro-vincialis* exposed to the same compound (17). In addition, we have also reported a significantly higher level of DNA adducts in the gills compared to the digestive glands, hence confirming the interest of studying the gills for DNA adduct measurement. Gills are constantly exposed to dissolved contaminants and are capable of metabolising mutagens and carcinogens into reactive products as the digestive glands (41,42).

In contrast to these observations, studies have demonstrated a higher binding of B[a]P to DNA in digestive glands compared to gills in *M. galloprovincialis* also exposed to B[a]P (43). Some authors have suggested the presence of tissue-specific CYP leading to the formation of different metabolites and, hence, inducing different kinds of DNA adducts in the gills and in the digestive glands, explaining the difference in DNA adduct levels observed in gills and digestive glands (17). In addition, some studies have demonstrated a seasonal impact on DNA adduct levels as well as on PAH tissue content in marine (44) as well as in freshwater mussels (31; Michel *et al*., unpublished data), probably caused mainly by a difference in lipid content.

In the present study, a dose-dependent formation of DNA adducts following B[a]P exposure was not observed. When it enters in the cells, B[a]P, as a lipophilic substance, is entrapped in the lipid surface of plasmic membranes and hence does not interact with DNA. However, when storage sites are saturated, a small increase of exposure is characterised by a formation of a DNA adduct level that is not dose dependent.

Moreover, there is a constant balance between free B[a]P and lipid-bound B[a]P, and a decrease in the free concentration levels leads to a release of B[a]P from lipids, hence explaining the difference between DNA adduct levels and B[a]P content in mussels (44).

Persistence of DNA adducts

Our data have shown, for the first time in the freshwater mussel, that DNA adducts formed in both gills and digestive glands after exposure to B[a]P remained persistent 28 days after the end of the exposure. Studies have noticed a 2-week persistence of DNA adducts in gills of *M. galloprovincialis* exposed to 17 µg/l B[a]P, whereas B[a]P tissue concentration was not detectable after 2 weeks (17), indicating that (i) B[a]P is efficiently detoxified by marine mussels and that (ii) DNA adduct measurement represented a more integrated evaluation of a B[a]P exposure than B[a]P bioaccumulation. The removal of the 4-NQO (4-nitroquinoline N-oxide) DNA adduct in *Mytilus* spp. has been shown to be biphasic, with an initial rapid decline (22), also observed with B[a]P in the present study, followed by a slower one, hence probably reflecting involvement of different cell mechanisms such as DNA repair. Among the DNA repair pathways existing in mammals, nucleotide excision repair (NER) is mainly involved in bulky DNA adduct removal (45). Proliferating cell nuclear antigen (PCNA) and Gadd45 proteins are known to be induced by p53 and bind to DNA for DNA repair (45,46). Little is known about the DNA repair system in mussels but a clear increase in PCNA expression has been observed following mussel exposure to B[a]P, indicating that this protein may also play a role in mussel DNA repair (47).

In the present study, it was also noticed in the digestive glands, a re-increase in DNA adduct levels after 28 days of depuration. These results suggested that in freshwater mussels, metabolic activation of B[a]P was biphasic.

Metabolism gene expression

In mammalian systems, it is known that B[a]P detoxification occurs through the recruitment of AHR. Inactive AHR, present in the cytoplasm, is bound to two molecules of HSP90 stress protein. Ligand binding to AHR results in the dissociation of HSP90 and the association with ARNt (AH receptor nuclear translocator) and the complex thus formed acts as a transcription factor of many genes such as the CYP450 family and other detoxification enzymes (48,49). In the present study, we have shown an up-regulation of the gene encoding for AHR in gills and in digestive glands after mussel exposure to B[a]P. Concerning the gills, expression of AHR and formation of DNA adducts were simultaneously detected after 12 h of B[a]P exposure. However, for the digestive glands, presence of DNA adducts occurred after 12 h exposure and *AHR* mRNA levels were only increased after 24 h, hence suggesting that B[a]P enters in the mussel cells via two ways: the binding to AHR receptor and the direct diffusion through cell membrane (50). Only few studies have investigated the effect on contaminants on *AHR* gene expression in *D. polymorpha*. Those works have shown that AHR gene expression was regulated by pharmaceutical active compounds (PhACs) (37,51), suggesting that those compounds, as well as B[a]P, are metabolised via CYP450

enzymes, as reported in mammalian systems (52). B[a]P metabolic activation by CYP450 leads to the formation of highly toxic metabolites and especially BPDEs that covalently bind to DNA-forming DNA adducts (17). Moreover, AHR is known to play an important role in cell functions such as cell growth, death and migration (50), suggesting a toxic effect on mussel global health.

So as to characterise the induction of ROS by B[a]P exposure, gene expression of two markers of oxidative stress was analysed. SOD and CAT are the two major antioxidant enzymes that catalyse the transformation of ROS into H_2O_2 and H_2O, respectively. According to the results concerning *AHR* mRNA levels, those of *CAT* and *SOD* were also enhanced earlier in the gills than in the digestive glands. *CAT* mRNA expression was strongly increased by B[a]P (about 4-fold in gills compared to control).

Previous studies have demonstrated, in the marine mussel *M. galloprovincialis*, that CAT and *SOD* enzyme activities were dependent on PAH content in tissues both in laboratory (53) and in field studies (54). In addition, a correlation between DNA adduct formation, B[a]P concentration in whole mussel and CAT activity has been observed in the digestive glands of *M. galloprovincialis* exposed to B[a]P (20). The same correlation has also been observed in our study but only for the highest concentration of B[a]P tested. At the enzymatic levels, data of the literature appeared to be contradictory; hence it is suggested that CAT activity was dependent on the animal tested, the nature of chemicals and the intensity of exposure (55).

On the contrary, *GST* mRNA levels increased from 12 h of exposure in both organs but each tissue depicted a specific profile. *GST* was strongly induced by B[a]P and especially in gills of *D. polymorpha* (five times compared to control). GST is a phase II enzyme that plays a role in detoxification by GSH conjugation of electrophilic xenobiotic compounds. It is expressed as multiple isoforms (56). Studies have reported that the different isoforms of GST displayed a tissue-specific pattern in mussels (57). Indeed, GST activity has been shown to be more elevated in gills, maybe due to an intensive antioxidative stress mechanism due to permanent contact with O_2, capable of generating ROS (58,59). According to our results, authors have also demonstrated in *M. galloprovincialis* exposed to high concentrations of B[a]P via food supplies that only the gills presented modification in GST enzyme activity, compared to the digestive glands (20). An inhibition of *GST* gene expression has been observed after marine mussel exposure to B[a]P, whereas mussels collected at a site highly contaminated with PAH exhibited an induction of its expression (60). GSTs are a multigenic family of enzymes named alpha, mu, pi, theta, sigma, zeta and omega (61–63) and in the present study, we analysed the gene expression of pi-GST. In the clam *Ruditapes decussatus*, it has been demonstrated that the expression of the different isoforms seems to be differentially regulated and xenobiotics such as B[a]P preferentially induce one class of GST, the pi-GST (60,64). According to our results, B[a]P also regulated the pi-class in *D. polymorpha* as demonstrated for other bivalves (65).

The transmembrane P-glycoprotein (P-gP) gene expression was also induced after mussel exposure to B[a]P, but in the gills its expression remained high after 28 days of depuration period. P-gP is a part of the multixenobiotic resistance (MXR) mechanism, an established marker of xenobiotic exposure and especially PAH exposure (66). This increase in P-gP levels is not surprising since it is involved in the excretion of xenobiotics.

It is known that the excretion mechanism of *P-gP* mRNA operates non-specifically, exporting not only non-metabolised parent xenobiotics, but also their metabolites as well as waste products derived from potential cell damage (67). Hence, mRNA levels of this gene can be modified with time exposure. An up-regulation of *P-gP* was observed after mussel exposure to the β-blocker metroprolol (35) and to propanolol (68), thus demonstrating elimination process. In general, PAHs have been demonstrated to have an effect on *P-gP* expression (69) and our results give additional support of the relevance of P-gP as biomarker of PAH contamination.

HSP70 gene expression was also analysed as it gives indication concerning the requirement of some processes such as protein repair, transport and protection from oxidative stress (35). Our results showed that B[a]P was a strong inducer of *HSP70* mRNA expression both in gills and in digestive glands of *D. polymorpha* (about 6-fold increase compared to control). Indeed, levels were enhanced following B[a]P treatment and remained elevated after 28 days of depuration in freshwater. The major gene induced after B[a]P exposure was *HSP70* gene (levels were six times more elevated in depuration period than in control levels). Our results showed an earlier induction of *HSP70* mRNA expression in digestive glands compared to gills. In contrast, in freshwater mussels exposed to copper, it has observed an increase in *HSP70* mRNA levels in gills after 1 day while this augmentation was only detected after 7 days in digestive glands (27). *HSP70* gene expression has been shown to be up-regulated in the earthworm *Eisena fetida* exposed to B[a]P (70). HSP70 protein expression was enhanced in marine mussels exposed to PAHs (71), with a correlation to the formation of DNA adducts and oxidative damage (72). This strong increase in *HSP70* mRNA levels could be due to the fact that this gene possesses an anti-oxidant response element to which the transcription factor Nrf2 binds as suggested in *D. polymorpha* for GST and CAT (73).

Conclusion: perspectives

In conclusion of this study, we have demonstrated the value of measuring DNA adducts as biomarkers of genotoxic pollution in freshwater ecosystem since they were formed for all concentrations and time exposure of B[a]P tested. In our experimental conditions, a time-dependent DNA adduct formation was not observed. However, we clearly showed that gills are more sensitive to B[a]P than the digestive glands. Indeed, they presented a higher DNA adduct level that was time dependent and an earlier induction of detoxification genes, which make this organ more suitable for future biomonitoring studies, as previously demonstrated in the laboratory for DNA strand breaks (74). But the most significant finding of the study is that DNA adducts were present in the freshwater mussels for a period of 28 days after the end of the exposure, which is of great interest since it can provide information of an exposure to a genotoxic xenobiotic, weeks after the initial event.

For the first time in the mussel *D. polymorpha*, the expression of some genes implicated in B[a]P detoxification has been characterised. Biomarkers based on mRNA levels reflect the initial steps of events caused by an environmental stress and hence represent promising candidates for ecotoxicological studies. Concentrations of B[a]P tested in this study were relatively high compared to concentrations found in the environment. DNA adduct levels measured in this study were comparable to field studies; Le Goff detected 10 adducts per 10^9

nucleotides while we detected 5 adducts per 10^9 nucleotides in the laboratory (2). Our study gave information concerning the formation and persistence of DNA adducts while this was not published before. Moreover, we performed the analysis of genes implicated in detoxification mechanisms to support our conclusion that B[a]P is at least metabolised by zebra mussels. All these data show the potential to assess DNA adducts in field mussels with this sensitive method, especially as compounds other than B[a]P induce DNA adducts, as we have recently shown (unpublished data).

Acknowledgements

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