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DNA oxidation and DNA repair in gills of zebra mussels exposed to cadmium and benzo(a)pyrene

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Abstract Freshwater bivalve molluscs are considered as effective indicators of environmental pollution. The comet assay allows the detection of DNA damage such as DNA strand breaks and alkali-labile sites. The main oxidative lesion, 8-*oxo*-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is a pre-mutagenic lesion, can be detected by the comet assay coupled with the hOGG1 DNA repair enzyme. With this modified assay we recently observed that BaP induced 8-oxodG lesions and with the modified comet-Fpg assay we observed that Cd induced oxidative DNA damage. The aim of this study was to determine the stability of DNA lesions in Cd and BaP exposed zebra mussels using the comet-hOGG1 assay. Mussels were exposed for 24 h to these two chemicals and then placed in clean water for 6 days. We observed that BaP (7, 12 and 18 µg/L) induced an increase of DNA strand break levels as soon as 6 h of exposure and that the two highest concentrations of BaP induced a low level of hOGG1-sensitive sites. After 2 days of depuration, BaP induced DNA lesions returned to the basal level, indicating an effective DNA repair. Cd (3, 32 and 81 µg/L) induced an increase of the DNA strand break levels and a low level of hOGG1-sensitive sites. This study revealed that BaP-induced DNA lesions are repaired more efficiently than Cd-induced DNA lesions. As the level of hOGG1 sensitive sites was increased in Cd and BaP exposed mussels, it seems that these chemicals induce 8-*oxo*-dG.

Keywords Zebra mussel · Comet-hOGG1 assay · Oxidative stress · 8-oxodG · DNA repair

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Introduction

Freshwater bivalve molluscs have been considered as effective indicators of environmental pollution. *Dreissena polymorpha*, also called the zebra mussel is a freshwater bivalve, largely used for biomonitoring in lakes and rivers (Binelli et al. 2001; Guerlet et al. 2007; Bacchetta and Mantecca 2009; Bourgeault et al. 2010). Among all studied biomarkers in zebra mussel, Binelli et al. (2007) have concluded that the prevalence of DNA strand breaks (SB), measured with the comet assay, was the strongest discrimination factor between polluted sites. Due to their filtration activity, bivalves are exposed to numerous pollutants and thus are subject to a considerable amount of oxidative damage (de Almeida et al. 2003; Emmanouil et al. 2007). Some studies have reported a high level of the oxidative DNA lesion 8-*oxo*-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in bivalves exposed in situ to urban pollutants such as PAHs. For example with *Unio tumidus* in the Moselle Basin (Charissou et al. 2004) and with *Ruditapes decussates* inhabiting the Gulf of Gabés (Jebali et al. 2007), high level of 8-*oxo*-dG was detected. We recently detected 8-oxodG in zebra mussels transplanted to an urban area of the Seine River basin, suggesting that DNA oxidation is a valuable indicator of environmental stress (Michel et al. 2013).

Among DNA lesions, the main oxidative lesion is 8-*oxo*-7,8-dihydro-2′-deoxyguanosine (8-oxodG), which is

considered as a premutagenic lesion. Indeed 8-oxodG causes misreading of DNA and contributes to G- to T-transversion. 8-oxodG lesion is of particular concern as it has been proven to be associated with carcinogenesis (Kasai 1997) and aging (Zahn et al. 1987). 8-*oxo*-dG is eliminated mainly through the base excision repair pathway, which in eukaryotes is initiated by the OGG1 protein, a DNA glycosylase that catalyzes the excision of 8-oxoG from DNA (Boiteux and Radicella 2000). Very little is known on the repair and on the consequence of this particular DNA lesion in bivalves except for *Mytilus edulis* where the repair activity of 8-oxodG has been demonstrated (Emmanouil et al. 2007). Field experiments confirmed this finding since the high level of 8-oxodG in marine mussels from urban/industrialized sites contaminated PAHs was decreased after a 1 month depuration experiment in clean water (Emmanouil et al. 2008). We have previously detected 8-oxodG in gills of freshwater mussel exposed to BaP (Michel and Vincent-Hubert 2012) as observed for marine mussel (Akcha et al. 2000). Other model contaminants commonly found in freshwater and sea water such as Cr(VI) and Cd can also induced oxidative DNA lesions in bivalves. For zebra mussels, we recently observed an increase of Fpg-sensitive sites measured with the formamidopyrimidine DNA-glycosylase comet assay (Fpg-comet assay) in gills of zebra mussels exposed to Cd (Vincent-Hubert et al. 2011) suggesting that Cd induces 8-*oxo*-dG. However, no evidence of DNA oxidation was noticed with the same assay for marine mussel *M. edulis* exposed to Cd (Emmanouil et al. 2007). Similar mechanisms of genotoxicity are found between mammals and *M. edulis*: low concentrations of Cd enhance the genotoxicity of H_2O_2 and Cd inhibits the DNA repair of 8-oxodG (Boiteux and Radicella 2000, Pruski and Dixon 2002). However, the repair and/or stability of this oxidative DNA lesion was not assessed yet in zebra mussel. It seems important to have firstly a robust test to measure the pre-mutagenic effects before considering the impact on the next generation which could give an ecotoxicological significance as suggested by many authors (Jha 2008).

The alkaline comet assay enables the detection of single or double DNA strand breaks and alkali-labile sites in individual cells. First introduced by Ostling and Johanson (1984) and then modified by Singh et al. (1988), the comet assay is widely used to measure DNA strand breaks in plants, worms, molluscs, fish, amphibians and mammalians in biomonitoring studies (Cotelle and Férard 1999; Frenzilli et al. 2009). DNA strand breaks measured with the comet assay are direct damages and indirect strand breaks resulting from DNA repair. 8-oxodG can also be quantified indirectly by a modified comet assay, a comet assay coupled with lesion-specific DNA glycosylase such as Fpg or hOGG1 (Collins et al. 1997; Smith et al. 2006). As Fpg is not exclusively specific of 8-oxodG (Smith et al. 2006), we recently used the comet-hOGG1 assay to demonstrate that BaP induced 8-oxodG in zebra mussel (Michel and Vincent-Hubert 2012). As this DNA lesion is a pre-mutagenic lesion, it might be important to determine its repair efficiency in zebra mussels in order to evaluate the consequences of the impact of micro-pollutants during field exposure. The aim of this study was (i) to determine whether Cd can induced 8-oxodG in gill cells of *D. polymorpha*, and (ii) to determine the stability of DNA strand breaks induced in Cd and BaP exposed zebra mussels. For this purpose, we have exposed zebra mussels to Cd and BaP during 24 h and then, DNA oxidations were measured with the comet-hOGG1 assay during a depuration step of 6 days in clean water.

Materials and methods

Chemicals and enzymes

The hOGG1 enzyme was purchased from Ozyme (St-Quentin-en-Yvelines, France), Dispase II from Roche Diagnostic (Meylan, France), Benzo(a)pyrene BaP (CAS number 50328) from Acros organics (Geel, Belgium), $CdCl_2$ (CAS number 7790-78-5) and all other reagents were purchased from Sigma–Aldrich (St-Quentin-Fallavier, France).

Mussel sampling and maintenance conditions

Zebra mussels were collected in January 2009, in the Meuse River (France), a reference site used in many studies dealing with zebra mussels. Mussels were manually removed from rocks using a scalpel, selected by length (25–30 mm) and rapidly brought back to the laboratory in water from the sampling site. Mussels were randomly placed in 20-L aerated tanks in Valvert mineral water and acclimatized during 2 weeks to constant temperature (15 °C); a day/night lighting system was implemented. The Valvert mineral water was chosen for its composition, in particular its low calcium concentration. Calcium competes with water contaminants and thus could influence the genotoxic response of mussels' cells during further exposure. Mussels were fed daily with an algal suspension of *Chlorella vulgaris* (Müller Naturhaus, Germany) and water was changed every 2 days.

In vivo exposure of zebra mussels

Following overnight equilibrium of plastic tanks with chemical compounds, after renewing the water, 15 mussels were added, in tanks containing 12 L of water each and were exposed for 24 h to three measured concentrations of

BaP (7, 12 and 18 µg/L in 0.001 % DMSO) or $CdCl_2$ (3, 32 and 81 µg/L) as described in Michel *et al*. (Michel et al. 2013). Cadmium in water was measured by means of atomic absorption spectrometry according to (Pellet et al. 2009).These concentrations were chosen as they are equal to the lowest genotoxic concentration (Emmanouil et al. 2007; Binelli et al. 2008). Negative control (clean water) and solvent control (0.001 % of DMSO) were carried out. At the end of the exposure (24 h), the contaminated water was removed and replaced by clean water for a depuration period of 6 days, water was not changed during the depuration period. Three mussels were analysed per treatment. DNA strand breaks and DNA oxidation levels were measured on three mussels per condition after 6 and 24 h of exposure to BaP and Cd and after 48, 72 and 168 h of depuration. During this depuration step, mussels were fed every two days with an algal suspension of *C. vulgaris* (Müller Naturhaus, Germany).

Isolation of gill cells

Gills were removed carefully, rinsed in cold PBS kept at 4 °C under reduced light to prevent UV-induced DNA damage. Gill cells were isolated using enzymatic digestion with dispase (0.8 U/ml, Roche) as previously reported (Vincent-Hubert et al. 2011). The gills were incubated at 37 °C for 30 min. The cellular suspension was removed, centrifuged at 600 g for 5 min; the enzyme was eliminated after a second centrifugation. The cellular suspension was kept on ice to minimize endogenous damage occurring during slide preparation. Cell viability assessment was performed using the trypan blue dye exclusion. The Comet assay was performed only with the cell population that showed a viability of about 90 %.

Comet-hOGG1 assay

Two slides per mussels were used for each condition (hOGG1 and buffer). DNA damage levels were quantified in gill cells using the comet assay as described by Singh et al. (1988) and modified as follows (Vincent-Hubert et al. 2011): 90 µL of 0.5 % low-melting point agarose (LMA) in PBS mixed with 10 µL of cell suspension were spread on pre-coated slides (0.5 % normal melting agarose) and covered with a cover slip. After agarose polymerization at 8 °C for 15 min, the cover slips were removed and the slides were placed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1 % Triton X-100 and 10 % DMSO, pH 10) at 8 °C for 1 h. After this, slides were rinsed three times in PBS in Coplin jar with hOGG1 buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, pH 8). Then, 50 µL of hOGG1 enzyme (0.16 U) in hOGG1 buffer were added to each slide and a cover slip was then placed on each. The slides were incubated at 37 °C in a dark humidified chamber for 45 min. Slides used for the comet assay alone (without hOGG1 enzyme) were incubated with 50 µL of enzyme buffer only. At the end of incubation, cover slips were removed from each slide which were placed in a horizontal electrophoresis tank filled with cold electrophoresis buffer (0.3 M NaOH, 0.001 M EDTA, pH 13, 8 °C) for DNA unwinding during 15 min. Electrophoresis then took place in cold electrophoresis buffer at 0.78 V/cm, 300 mA for 10 min at room temperature.

Finally, all the slides were drained and immersed in 70 % ethanol for 5 min to dehydrate the agarose, and then left to dry. The slides were stored at room temperature with desiccant until staining.

Quantitation of comet assay data

Slides were stained with 30 µl of ethidium bromide (0.8 µg/ml) and examined the next day with a fluorescence microscope (Olympus BX51, 60X lens). For the quantitation of the comet assay data, we used an image analysis system (Komet 5.5, Andor Technology) linked to a CCD camera. One hundred randomly chosen nuclei were examined per mussel. The level of DNA damage was expressed as Olive Tail Moment (OTM = Tail DNA % × (tail mean–head mean)) (Olive et al. 1990) in arbitrary unit (A.U.). All slides were coded and analysed blind. Three slides were analysed per treatment condition and 100 nuclei were scored per mussel.

Statistical analysis

Statistical analysis has been performed to determine whether the mean OTM of buffer treated slides and the mean OTM of hOGG1 treated is different. For the statistically different values of OTM, the level of hOGG1-sensitive sites was calculated as followed: hOGG1-sensitive sites = (OTM of hOGG1 treated slide)- (OTM of no hOGG1 treated slide). The Shapiro–Wilk test was used to verify the normality of the variance of the comet data. As the normality was not respected, the Kruskall–Wallis test was performed to evaluate potential significant differences between control and treated slides. All statistical analysis was performed with R software 3.1.3.

Results

DNA strand breaks and hOGG1-sensitive sites in BaP exposed mussels

As soon as 6 h after the beginning of exposure, BaP induced a rapid and significant increase of the DNA strand

breaks levels in mussel gill cells as we observed an increase of the OTM ($p < 0.001$) (Fig. 1). BaP (12 µg/L) induced the highest level of DNA SB, corresponding to a three-fold increase than control mussels ($p < 0.001$). 24 h after the beginning of exposure, DNA SB levels decreased but stayed significantly higher than in the control mussels ($p < 0.001$). After 2 and 6 days of depuration, these levels reached the basal level corresponding to the DNA strand break level measured in control mussels or in mussels exposed to DMSO (data not shown), indicating that zebra mussels had repaired DNA lesions induced during BaP exposure.

The comet-hOGG1 assay allowed the detection of a significant higher level of DNA damage in BaP (12 and 18 µg/L) exposed mussels, compared with the standard comet assay ($p < 0.001$) (Fig. 1). These data allowed us to calculate hOGG1 sensitive sites (see Materials & methods) (Table 1). The highest level of hOGG1 sensitive site was 0.52 A.U. for BaP (12 µg/L) exposed mussels. However, for some BaP exposed mussels, the comet hOGG1 assay did not detect a higher level of DNA damage compared with the standard comet assay which does not reveal the presence of hOGG1 site.

DNA strand breaks and hOGG1-sensitive sites in Cd exposed mussels

Cadmium induced a dose-dependent increase of DNA SB as soon as 6 h after the beginning of the exposure ($p < 0.001$) (Fig. 2). The highest level of DNA strand breaks, about 3 times more than in control mussels, were measured in Cd (32 and 81 µg/L) exposed mussels. Only the OTM of mussels exposed to Cd 32 µg/L decreased significantly from 6 h of exposure until the end of depuration, to reach the basal level on day 7. On the contrary, the OTM of two other groups of mussels (3 and 81 µg/L) never returned to the OTM of the control mussels.

Compared with the standard comet assay, the comet-hOGG1 assay allowed the detection of significant higher levels of DNA strand breaks in Cd exposed mussels (Fig. 2). The highest level of hOGG1-sensitive sites was obtained for the highest Cd concentration (81 µg/L). After 72 h, no hOGG1-sensitive sites were detected in Cd exposed mussels (Table 2).

Discussion

Generation of DNA lesions by BaP and repair

The development of methods to assess the level of DNA damage in marine organisms is of great interest for ecotoxicological investigations on the effects and the causal mechanisms of damage induced by environmental pollutants. Among DNA alterations commonly measured, such as strand breaks, micronuclei and adducts (Canova et al. 1998; Akcha et al. 2003) detection of 8-*oxo*-dG is recognized as a useful marker of oxidative-mediated genotoxicity (Lopez-Barea and Pueyo 1998).

We confirmed that the comet-hOGG1 assay detected a higher level of DNA SB than the standard comet assay in mussels exposed to BaP, suggesting the presence of 8-*oxo*-dG (Michel and Vincent-Hubert 2012a). Enhanced levels of 8-*oxo*-dG have been documented within aquatic invertebrates, after natural or artificial exposure to environmental pollutants (Canova et al. 1998; Akcha et al. 2000). Various methods have been developed to detect oxidative DNA damage in situ without cell disruption, DNA isolation

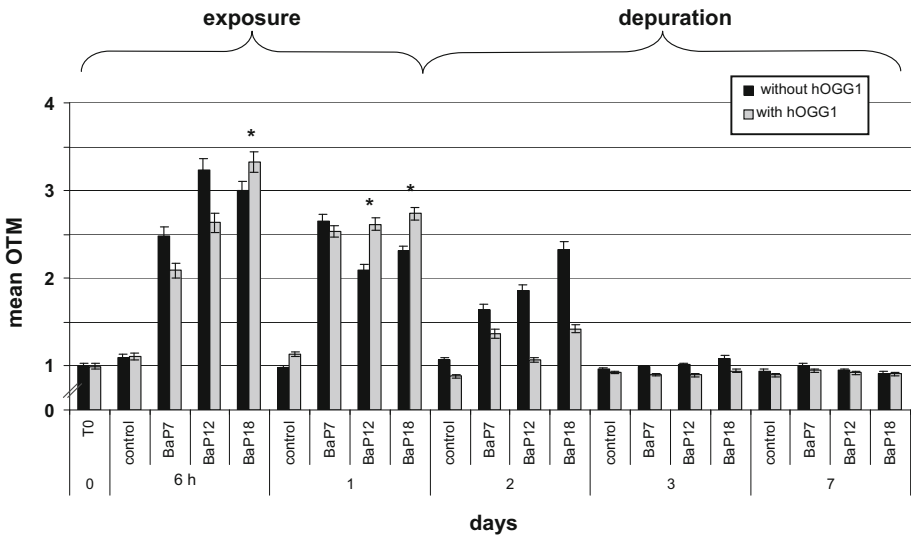

Fig. 1 DNA strand breaks measured with the comet assay and the comet-hOGG1 assay in mussel gill cells after 24 h of exposure to BaP and during 6 days of depuration. DNA strand breaks were measured with the comet assay during a 24 h exposure to BaP (7, 12 and 18 µg/L) and during 6 days of depuration in clean water. DNA strand break levels are expressed as the mean OTM ± SE. Statistical differences obtained between hOGG1 and its respective control (no hOGG1) with the Kruskall-Wallis test, *$p < 0.001$ ($n = 3$) is reported

Table 1 hOGG1-sensitive sites calculated for BaP exposed mussels

	6 h		24 h		
	Control	BaP 18 µg/L	Control	BaP 12 µg/L	BaP 18 µg/L
Untreated	0.6	2.5	0.49	1.60	1.81
hOGG1	0.62	2.82	0.64	2.12	2.24
hOGG1 sensitive sites	0.02	0.33	0.15	0.52	0.43

hOGG1-sensitive sites calculated as: (OTM of hOGG1 treated slides)—(OTM of hOGG1 untreated slides) after 24 h in vivo of exposure to BaP and during 6 days of depuration

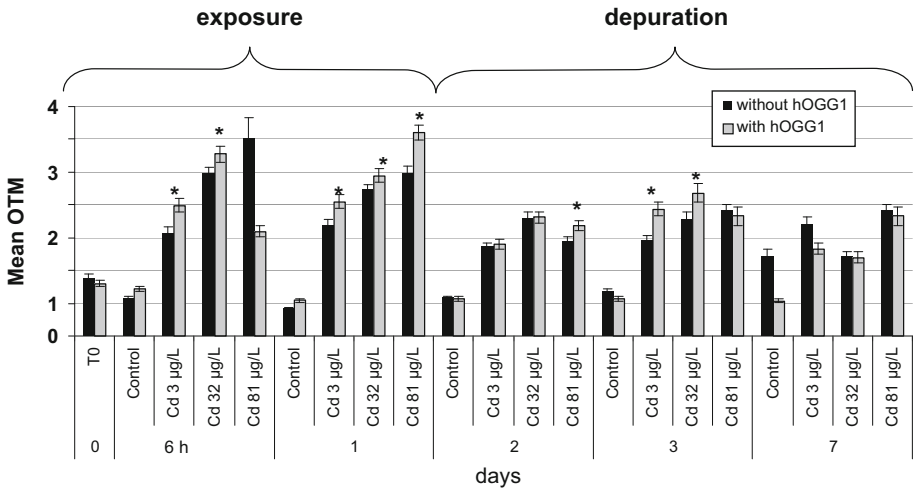

Fig. 2 DNA strand breaks measured with the comet assay and the comet-hOGG1 assay in mussel gill cells after 24 h of exposure to Cd and during 6 days of depuration. DNA strand breaks data were measured with the comet assay during 24 h of Cd exposure (3, 32 and 81 µg/L) and during 6 days of depuration in clean water. DNA strand breaks levels are expressed as the mean OTM ± SE. Statistical differences obtained between hOGG1 and its respective control (no hOGG1) with the Kruskall–Wallis test, *$p < 0.001$ ($n = 3$) is reported

Table 2 hOGG1-sensitive sites calculated for Cd exposed mussels

	6 h			24 h				48 h		72 h		
	Control	Cd 3 µg/L	Cd 32 µg/L	Control	Cd 3 µg/L	Cd 32 µg/L	Cd 81 µg/L	Control	Cd 81 µg/L	Control	Cd 3 µg/L	Cd 32 µg/L
Untreated	0.6	1.58	2.48	0.49	1.69	2.23	2.47	0.58	1.43	0.68	1.46	1.78
hOGG1	0.62	1.99	2.77	0.64	2.05	2.45	3.09	0.57	1.69	0.57	1.94	2.18
hOGG1 sensitive sites	0.02	0.42	0.29	0.15	0.36	0.22	0.62	nd	0.25	nd	0.48	0.4

hOGG1-sensitive sites calculated as: (OTM of hOGG1 treated slides)—(OTM of hOGG1 untreated slides) after 24 h in vivo of exposure to Cd and during 6 days of depuration

or hydrolysis; these approaches include the comet assay joined with the use of bacterial endonuclease III and formamidopyrimidine glycosylase (which respectively nicks oxidized pyrimidines and recognizes ring opened purines, (reviewed in Collins et al. 2002), or the use of specific antibodies developed to detect 8-*oxo*-dG (Santella 1999). However, while the comet assay is largely used ecotoxicology, the application of modified comet assay in non-human models is very limited. To our knowledge this is the first application of hOGG1 comet assay to mussels.

After 24 h of depuration, the hOGG1-sensitive sites were no longer detected in BaP exposed mussels suggesting a DNA repair. The same observation was made in marine mussels, since the levels of 8-oxodG measured in

mussels reached the level measured in control mussels even though the concentration of BaP was much higher in that study (Akcha et al. 2000). In human cells, the main 8-oxodG repair is the base excision repair (BER) which induces the elimination of the oxidized guanine by DNA glycosylase (Boiteux and Radicella 2000; Smart et al. 2006) and then the DNA polymerase β (pol β) inserts the correct base according to the bases complementarity (Hoeijmakers 2001). Even though the BER pathway is extremely well conserved among organisms very little information is available for bivalves. Repair systems do exist in bivalves as has been shown by time-course differences in strand break levels and by the excision of 8oxodG in mussel gill (Emmanouil et al. 2007).

Our data indicated that zebra mussels are able to rapidly repair DNA lesions induced by BaP. Indeed, after 48 h of depuration, the level of DNA SB detected with the standard comet assay return to the basal level. Bihari et al. (1990), reported similar evolution of DNA lesions in mussel *Mytilus galloprovincialis* treated with a single dose of BaP, an increase after 1.5 h followed by a decrease after 48 h. Moreover, the authors highlighted that the DNA repair was dependent of the chemical: for 4-nitroquinoline-N-oxide (4 NQO), a strong mutagen, treated mussels, the level of DNA damage returned to the basal level only after 5 days, instead of 48 h for BaP.

BaP concentrations used in that study are low compared with other studies (Le Goff et al. 2006) which highlights mussel capacities of metabolisation. Even though bivalve BaP metabolisation remain lower than in vertebrates, zebra mussel gill cells are able to transform the BaP into active metabolite which can induce DNA lesions such as bulky DNA adducts as we recently observed in gills and digestive gland (Chatel et al. 2012). BaP induced DNA SB has been largely observed in bivalve species such as for example *Mytilus sp.* (Bihari et al. 1990) and *Crassostrea gigas* (Nacci et al. 1996) zebra mussel (Binelli et al. 2008). Mitchelmore et al. (1998) suggested the role of free radicals in BaP induced DNA strand breaks which could also explain the formation of 8-oxodG detected in marine mussels (Akcha et al. 2000; Machella et al. 2004).

Generation of DNA lesions by Cd and repair

Although Cd is known to induce low level of genotoxic damages, we observed that Cd induced a rapid and significant increase of DNA strand break levels in mussels, following a dose–response relationship. Our data confirmed our previous observation in zebra mussels (Vincent-Hubert et al. 2011), the same rapid DNA strand break induction was observed after 10 h of exposure with 10 µg/L of Cd. In marine mussel, Emmanouil et al. (2007) have also detected an increase of DNA SB levels after Cd exposure. Cd is not a direct genotoxic (Valverde et al. 2001), it affects the genome stability either by induction of reactive oxygen species (ROS) or by inhibition of DNA repair systems. Indeed, Cd induces the depletion of glutathione and protein-bound sulfhydryl groups, which results in enhanced production of ROS like hydrogen peroxide, superoxide ion and hydroxyl radical (Bertin and Averbeck 2006). In bivalve, like zebra mussels, DNA is subject to considerable amounts of oxidative damage, which are higher than those commonly found in mammals (de Almeida et al. 2003).

We recently showed that Cd induced an increase of Fpg-sensitive sites in zebra mussels suggesting DNA oxidation (Vincent-Hubert et al. 2011). With the present study using the comet-hOGG1 assay, we noticed an increase of hOGG1-sensitive site, suggesting DNA lesion as being the 8-oxodG. (Emmanouil et al. 2008) observed an increase in lipid peroxidation end products in mussels exposed to Cd (200 µg/L). Cd is known to be implicated in the production of ROS such as hydrogen peroxide, superoxide ion and hydroxyl radical which may induce DNA oxidation (Bertin and Averbeck 2006). Cd is also known to inhibit the DNA repair pathways interfering with the last step of ligation of the BER. Thus, inhibition of this final step induced an increase of SB accumulation formed by incomplete oxidative DNA repair (Emmanouil et al. 2007).

Our data show that the DNA strand break level has not returned to the basal level in two groups of mussels, those exposed to low and to high cadmium concentrations. These observations suggest that DNA repair or Cd detoxification could be dependent on Cd concentration. Indeed, low concentrations of Cd could be too low to induce rapid detoxification by metallothionein (MT) as we recently observed that MT mRNA expression was increased only after 3 days in zebra mussels (Vincent-Hubert et al. 2014). On the opposite, high concentration of Cd could induce MT saturation and thus, limit the decrease of DNA strand break levels. This hypothesis suggests a concomitant increase in MT mRNA and MT protein level, which however is not always observed. Moreover other proteins such as HSP and GST could be implicated in cadmium detoxication.

Our data show that oxidative lesions induced by the Cd were repaired less efficiently than those induced by the BaP. Emmanouil et al. (2007) showed that metals (Cd and Cr(VI)) showed differential inhibitory potential towards DNA repair enzyme activity with Cd exhibiting inhibition of DNA cutting activity towards an oligonucleotide containing 8-*oxo*-7,8-dihydro-2′-deoxyguanosine. A similar mechanism may exist in zebra mussel.

In conclusion, BaP and Cd induce DNA lesions and probably 8-*oxo*-dG in gill cells of zebra mussels. The repair

of DNA damage vary according to the chemical: for BaP, DNA strand breaks were repaired efficiently during the depuration step, while for Cd, only the lesions induced by the medium concentration were repaired. Future works could be to determine the role of Cd in DNA repair of chemicals induced DNA lesions in zebra mussel. The application of the comet assay with modified enzymes is a promising tool to go further in the biomonitoring of DNA damage. However, it should be compared with other method of detection such as the standard HPLC-EC. The presence of a premutagenic lesion, the 8-*oxo*-dG, reinforce the comet assay data and therefore its application in ecotoxicology.

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Compliance with ethical standards

Conflict of interest None.

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