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Investigating the relationship between embryotoxic and genotoxic effects of benzo[a]pyrene, 17*-ethinylestradiol and endosulfan on Crassostrea gigas embryos

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Abstract:

Genotoxicity biomarkers are widely measured in ecotoxicology as molecular toxic endpoints of major environmental pollutants. However, the long-term consequences of such damage still have to be elucidated. Some authors have suggested that the accumulation of unrepaired DNA lesions could explain the embryotoxicity of certain chemical pollutants. As embryotoxicity exerts a direct impact on the recruitment rate, genotoxicity could be closely related to disturbances of ecological concern and produce a possible impact upon population dynamics.

The aim of the present work was to study the genotoxicity and the embryotoxicity of three relevant pollutants for oyster embryos: the polycyclic aromatic hydrocarbon, benzo[a]pyrene (BaP), the synthetic estrogenic hormone, 17greek small letter alpha-ethinylestradiol (EE2), and the organochlorine pesticide, endosulfan (ES). For each substance, gamete fertilization was performed and embryo development followed in contaminated reference seawater.

Following exposure, embryotoxicity was evaluated by calculating the percentage of abnormal D-larvae obtained at 20 h development. Genotoxicity was measured in parallel by conducting a comet assay on enzymatically dissociated cells of pre-shelled larvae (16 h development). The oxidized DNA base, 8-oxodGuo, was also measured by HPLC coupled to electrochemical detection. For each contaminant, the relationship between genotoxicity and embryotoxicity was then studied to check for the possible significance of genotoxicity in the population dynamics of marine bivalves from polluted areas.

For BaP, embryotoxicity and DNA strand breakage were both observed from the lowest tested concentration of 0.2 nM. Induction of 8-oxodGuo was significant from 20 nM. Endosulfan exposure resulted in similar effects for oyster embryos but from higher concentrations and followed a concentration-dependent manner. Embryotoxicity and genotoxicity in terms of DNA strand breaks were observed for endosulfan from 300 and 150 nM, respectively. No change in 8-oxodGuo level was observed following endosulfan exposure. EE2 displayed no toxic effect for oyster embryos within the range of tested concentrations (from 0.02 to 1.7 nM).

Taking into account all the data collected during this study, a positive and significant correlation was demonstrated in oyster embryos between genotoxicity as measured by the comet assay and embryotoxicity.

Keywords: Pacific oyster; Embryotoxicity; DNA damage; Endosulfan; BaP; 17*-Ethinylestradiol

1. Introduction

The marine environment is under high anthropic pressure, particularly within costal and estuarine areas. The inputs of diverse chemicals, bioavailable for organisms, threaten the ecosystem's health and marine biodiversity. Further to biotransformation, chemical pollutants can exert different kinds of toxicity from molecular to physiological levels, triggering potential consequences on an individual or community scale. Such processes have been the subject of recent research (Matthiessen and Law, 2002, Shahidul Islam and Tanaka, 2004). In ecotoxicological studies, efforts are commonly focused on few toxic endpoints and on a restricted number of individuals. The remaining difficulty is to predict the long-term consequences of such toxic effects on the population, the community and the ecosystem. In the case of xenoestrogens, the situation is different as the effects are related to the reproduction process and have a direct impact on the population dynamics (Miller and Ankley, 2004, Gutjahr Gobell *et al*., 2006). For other toxic endpoints, it is more difficult to stipulate the long-term consequences caused by pollutant exposure. This is the case for genotoxicity, despite its reported involvement in mutagenesis, carcinogenesis, ageing and other pathologies.
Within this context, this study aims to bring response elements to the relationship that may exist between the genotoxicity and the embryotoxicity of certain chemicals. Such a relationship was previously suggested by some authors who propose the accumulation of unrepaired DNA damage as an explicative variable of chemical embryotoxicity (Anderson and Wild, 1994, Wells *et al*., 1997). By its direct effect on the recruitment rate, embryotoxicity is one of the parameters governing population dynamics. Such a relationship will thus establish a link between early markers of genotoxicity and indirect effects on population dynamics.
Three chemicals were studied in the present paper. The first one is benzo[a]pyrene (BaP) which is a polycyclic aromatic hydrocarbon (PAH) often used as a model compound in genotoxicity and cancerogenicity studies. BaP features on the list of the 16 priority PAHs of the US Environmental Protection Agency (EPA) and on the dangerous substances list of the European Water Framework Directive (EU WFD). PAHs such as BaP are known as embryotoxic and teratogenic compounds (Wassenberg and Di Giulio, 2004). The second chemical tested is ethinylestradiol (Ethinyl-17α-dihydroxy-3,17 estratriene-1,3,5(10), EE2), a synthetic hormone and part of the composition of contraceptive pills. EE2 is a water pollutant producing endocrine disruptor effects in exposed organisms, leading to growth and reproduction disturbances (Jobling *et al*., 2004). The last chemical studied is endosulfan, which is an organochlorine pesticide that was banned from production from December 2005 but whose use was permitted until May 2007 in Europe. In fact, its worldwide use is responsible for the high concentrations measured in aquatic systems. Because it is known as one of the most toxic pesticides for aquatic life, endosulfan is considered as a hazardous pollutant by the EPA and as a priority dangerous substance by the EU WFD. Endosulfan is also known as a xenoestrogen (Bulayeva and Watson, 2004).
The Pacific oyster, *Crassostrea gigas* (Lamark, 1819), is the biological model selected for this study. Due to its bio-ecological particularities (e.g. filter-feeding activity, sessile nature, ubiquity), the oyster is an organism of choice for biomonitoring the marine environment. The knowledge of its life cycle and the control of its breeding allow for the development of an oyster bioassay based on the obtention of abnormal D larvae as a measure of embryotoxicity (Chapman and Morgan, 1983, His *et al*., 1997). Further to exposure to these selected chemicals, embryotoxicity was evaluated by calculating the percentage of D-shell larvae presenting shell and/or mantle abnormalities. Genotoxicity was measured in parallel in terms of DNA strand breaks and 8-oxo-7,8-dihydro-2'-desoxyguanosine (8-oxodGuo). Genotoxicity and embryotoxicity data were then analyzed for correlations.

2. Material and methods

2.1. Chemicals and material

The reference sea water used for the experiments was pumped from Argenton (Brittany, France) and was filtered at 0.22 µm before use. RPMI 1640 medium, dimethyl sulphoxide (DMSO), 0.4% trypan-blue solution, benzo[a]pyrene (CAS No 50-32-8), endosulfan (CAS No 115-29-7), 17α-ethinylestradiol (CAS No 57-63-6), ethidium bromide (BET), foetal bovine serum (SVF), sodium dodecyl sulfate (SDS), RNases A and T1, normal and low melting point agarose, collagenase, deferoxamine mesylate, nuclease P1, Triton X-100, pure 8-hydroxy-2'-deoxyguanosine (8-oxodGuo) and pure 2'-deoxyguanosine (dGuo) were purchased from Sigma Aldrich Chemicals. The protease came from Qiagen, and the alkaline phosphatase from Roche Diagnostics.

2.2. Animals

Oysters came from a hatchery specialized in the production of organisms used for bioassay monitoring (Guernsey sea farms, UK). Oysters were dispatched in batches of 5 males and 5 females. They were used within two days. The individuals not used the first day were stored at 4°C.

2.3. Gamete recovery and fertilization

In the laboratory, spawning was induced by scarification as described by Quiniou *et al.* (2005). This method allows for a quick recovery of the gametes in reference sea water. Following rehydratation, the oocytes and the sperm of different oysters were sieved at 100 nm for oocytes and 32 nm for spermatozoa, and observed under an inverted microscope. The oocytes were fertilized by the sperm-dense suspension to raise a ratio of 10 spermatozoa per 1 oocyte. Fifteen minutes after fertilization, embryos were counted and dispatched in each assay beaker at a final concentration of 50,000 fertilized eggs per litre.

2.4. Chemical exposure

BaP, EE2 and endosulfan are not water soluble and were previously dissolved in dimethyl sulfoxide (DMSO) after having determined their solubility limits. Chemical solutions were prepared so that the DMSO concentration in the sea water was 0.1%. For each chemical, three concentrations of exposure were selected considering the environmental concentrations described in the bibliography. The lowest concentration was within the range of the highest environmental concentrations reported by the authors, the other two were multiplied by a factor of 10 to 10,000 (Table 1).
For the embryotoxicity assays, embryos were exposed for 20hrs at 24°C in the dark in beakers containing 30 mL of filtered sea water (four replicates per condition). This incubation time enables the embryo to develop until the D-shell stage.
For the genotoxicity assays, embryos were incubated in 2 L beakers for 16h at 24°C in the dark. This incubation period allows for the recovery of unshelled larvae whose cells can later be enzymatically digested for the comet assay. Three replicates were performed per condition and each replicate consisted of four beakers containing a total of 400,000 larvae.

2.5. Embryotoxicity assay

Following exposure, the larvae of each beaker were fixed using formol (0.5 mL/beaker). For each assay beaker, one hundred larvae were observed under an inverse microscope (g x 40) to determine the rate of D-shell larvae presenting mantle and/or shell abnormalities (Figure 1).

2.6. Genotoxicity assays

2.6.1. Measurement of DNA strand breaks (Comet assay)

2.6.1.1 Dissociation of embryo cells

The comet assay was performed on isolated cells as described in Akcha *et al.* (2003). As a consequence, it is necessary after pollutant exposure to dissociate larvae cells prior to comet measurement. Different protocols of cell dissociation were tested in our laboratory: mechanic dissociation by using a potter and enzymatic dissociation comparing the action of three different enzymes (trypsine, collagenase and accutase). Larvae digestion by collagenase was selected for our study due to a high dissociation efficiency, good cell viability (trypan blue exclusion test > 80%), and low comet parameter values (unpublished data). Digestion occurred as follows. Following 16hrs of exposure, the larvae were recovered by sieving at 32 mm and incubated in 15 mL of a collagenase solution at 1.11 $g.L^{-1}$. The reaction was stopped by centrifugation for 10 min at 1,300 rpm and 4°C. The cell pellet was then resuspended in 2.8 mL of freezing medium made up of 40% CMFS, 12% DMSO, 15% SVF, and 33% RPMI. Before and after freezing in liquid nitrogen, cell viability was determined for each sample by a trypan-blue exclusion test. In both cases, cell viability was at least equal to 75%, allowing the conduction of the comet assay.

2.6.1.2 Slide preparation and comet measurement

Cell samples were slowly defrosted at room temperature. Slides were prepared as follows: *First layer:* The slides were immersed in a 0.5% NMP agarose solution in PBS and allowed to dry at room temperature. *Second layer:* For each sample, 30 µl of the cell suspension were added in a 225 µl aliquot of a 0.5% LMP agarose solution in PBS. Eighty-five microliters of this mixture were then deposited on a slide and spread using a micro-cover glass. The slides were immediately placed on ice in the dark for 1 min to allow for the agarose to polymerise. For each sample, three slides were prepared from the same aliquot. *Third layer:* Once the cover glass had been withdrawn, 90 µl of the LMP agarose solution were deposited on the slide and spread using a micro-cover glass. The slides were then stored on ice.

Once the last cover glass was withdrawn, the slides were immersed in a ice-cold lysis buffer (2.5 M NaCl, 0.1 M $EDTANa_2$, 0.01 M Tris base, 1% N-sarcosinate, 10% DMSO, 1% Triton X100, pH 10) for 1hr at room temperature in the dark. DNA unwinding was then performed by pre-incubating the slides in a freshly prepared electrophoresis buffer (0.3 M NaOH, 0.001 M EDTA, pH 13) for 15 min at room temperature in the dark. DNA migration was performed in the same buffer for 20 min at 23 V (390 mA, E = 0.66 $V.cm^{-1}$). At the end of electrophoresis, slides were washed by incubation for 3 x 5 min in Tris base 0.4 M, pH 7.5. In order to obtain permanent preparations, the slides were immersed for 10 min in absolute ethanol for deshydrating, and allowed to dry at room temperature. Just before analysis, 75 µL of BET at 8 $µg.mL^{-1}$ were deposited on the slide and spread over it using a cover glass. Slides were placed for at least 30 min at 4°C for coloration.

Slides were analysed using an optical fluorescence microscope (Olympus BX60, x 40) fitted to a CDD camera (Olympus) and an image analysis system (Komet 4, Kinetic Imaging Ltd). Several parameters were calculated for each observed cell (75 cells/slide), including the percentage of DNA present in the comet tail and the Olive tail moment (OTM).

2.6.2. Measurement of 8-oxodGuo (HPLC/ECD)

2.6.2.1 DNA extraction

DNA was isolated from embryo cells using the chaotropic NaI method which derives from that of Helbock *et al.* (1998), slightly modified by Akcha *et al.* (2000a). Samples were centrifuged at 1,500 g for 10 min at 4°C. The supernatants were discarded and the pellets were suspended in 2 mL of 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). Following centrifugation (1,500 g, 10 min, 4°C), pellets were recovered and resuspended in 600 µL of Buffer B (5 mM EDTA-Na_2, 10 mM Tris-HCl, 0.15 mM deferoxamine mesylate, pH 8). After addition of 35 µL of 10% SDS, RNA digestion was performed by incubation with 120 µg of RNase A and 20 U of RNase T1 for 15 min at 50°C. Protein digestion was carried out by incubation with 600 µg of protease for 1hr at 37°C. Samples were then centrifuged at 5,000 g for 15 min at 4°C, and supernatants recovered in 15 mL sterile tubes. Following addition of 1.2 mL of sodium iodide solution (20 mM EDTA-Na2, 7.6 M NaI, 40 mM Tris-HCl, 0.3 mM deferoxamine mesylate, pH 8) and 2 mL isopropanol, the tubes were centrifuged for 15 min at 5,000 g. The pellets were then recovered and resuspended in 2 mL 40% isopropanol. Following centrifugation (5,000 g, 15 min, 4°C), pellets were washed in 2 mL of 70% glacial ethanol, and centrifuged at 5,000 g for 5 min at 4°C. Ethanol was then discarded using a pipette, and the pellets were left to dry for 1 hr at room temperature. DNA was finally resuspended in 100 µL of deferoxamine mesylate 0.1 mM and left to dissolve overnight at 37°C.

2.6.2.2 DNA quantification

Measurement was performed by spectrophotometry at 280, 260, and 230 nm wavelengths by using a ND1000 NanoDrop (NanoDrop Technologies, Inc). Pure DNA solution presents a 260/280 ratio between 1.8 and 2, and a 260/230 ratio of approximately 2. DNA concentration of 50 $\mu g.mL^{-1}$ is obtained for a theoric absorbance of 1 OD unity at 260 nm. Following quantification, DNA samples were stocked in liquid nitrogen prior to analysis.

2.6.2.3 DNA digestion

For each sample, 15 µg of DNA were digested by incubation with 5 U of nuclease P1 for 2hrs at 37°C. Four units of alkaline phosphatase were then added for an additional 1 hr incubation at 37°C. The sample was centrifuged (5 min, 7,000g, 4°C) and the supernatant was recovered for injection.

2.6.2.4 HPLC analysis

The 8-oxodGuo level was determined by HPLC (Agilent 1200 series) coupled to electrochemical (Coulochem III, ESA) and UV (Agilent 1200 series) detection. Separation of 8-oxodGuo and 2'-deoxyribosides was carried out by using an Ultrasphere pre-column (5C18, Interchim) and an Uptisphere column (5ODB, Interchim). Elution was performed in isocratic mode using a mobile phase composed of 10% methanol and 100 mM sodium acetate, at pH 5.2. The elution flow rate was set at 1 $mL.min^{-1}$. The guard and the measure cells were respectively set at an oxidation potential of 460, 150 and 380 mV. The quantification of 8-oxodGuo was performed in accordance with a calibration curve previously obtained with known pmole amounts of authentic 8-oxodGuo. For a standard expression in the number of 8-oxodGuo residues per 10^6 dGuo, deoxyguanosine was also quantified by fitting a UV detector to the output of the HPLC column. The UV detector was set at a wavelength of 254 nm. A calibration curve was also obtained for this compound within the nmole range. For the conditions described, the retention times of both 8-oxodGuo and dGuo were respectively 11.5 and 8.5 min at 35°C.

2.7. Statistical analyses

Embryotoxicity and genotoxicity data were exploited by performing an analysis of variances, ANOVA, using Statistica 6.0 (Statsoft. Inc). Normality of the data was checked (normal p-plot) and when necessary normalization was performed by mathematical processing. Each time a significant difference was demonstrated, an *a posteriori* test (Tuckey's test) was performed. Correlations were also checked using the same software.

3. Results

3.1. Benzo[a]pyrene exposure

The percentage of abnormal D-shell larvae significatively increased following exposure to BaP ($p<0.001$). Embryotoxicity was observed from the lowest tested concentration of 0.2 nM and did not appear to be concentration-dependent (Figure 2).
Following 16hrs exposure to BaP, a significant increase in DNA strand breaks was observed by the comet assay ($p<0.001$). The increase was significant from the lowest tested concentration of 0.2 nM and was independent of the concentration (Figure 3). An increase in the level of 8-oxodGuo was also observed from the exposure concentration of 20 nM ($0.01<p<0.05$) (Figure 4).

3.2. 7α-ethinylestradiol exposure

Ethinylestradiol had no effect on larvae development in the oyster for the range of tested concentrations (0.02-1.7 nM) ($p>0.05$) (Figure 2).
The results of the comet assay revealed no difference between the control and the exposed groups in the extent of DNA strand breaks ($p>0.05$) (Figure 3). For this treatment, 8-oxodGuo measurement could not be carried out.

3.3. Endosulfan exposure

Following endosulfan exposure, an increase in abnormal D larvae was observed ($p<0.001$) (Figure 2). The increase became statistically significant from the highest tested concentration of 300 nM.
The level of DNA strand breaks increased with the concentration of endosulfan. However, the increase became statistically significant only at the highest tested concentration of 150 nM ($0.001<p<0.005$) (Figure 3). No difference in the level of 8-oxodGuo was observed for the tested range of endosulfan concentration ($p>0.05$) (Figure 4).

3.4. Relationship between embryotoxicity and genotoxicity

The data collected in the present study were analyzed together in order to check for correlations between the embryotoxic parameter (% of abnormal D-larvae) and the genotoxicity measurement ($\sqrt{Tail\ DNA}$ and 8-oxodGuo level). The results obtained are described in Table 2. A significant positive correlation was demonstrated between the comet parameter, $\sqrt{Tail\ DNA}$ and the percentage of abnormal D-shell larvae ($R^2 = 0.651$, $p < 0.001$). No correlation was observed between 8-oxodGuo level and embryotoxicity ($p = 0.91$) nor between the two genotoxic parameters measured ($p = 0.93$).

4. Discussion

4.1. BaP

In this paper, embryotoxicity was observed for BaP at a concentration of 0.2 nM, which is environmentally relevant (Kolpin *et al.*, 2002), suggesting a risk for the oyster recruitment rate in severely polluted areas.
The embryotoxicity observed for BaP is in accordance with the results of previous studies conducted on the prenatal toxicity of PAH exposure for aquatic organisms. The applications of the oyster embryo-larval bioassay already contributed to a sounder knowledge of the embryotoxic and teratogenic effects of PAHs (Geffard *et al.*, 2002 and 2003). In echinoderm, PAH exposure results in a dose-dependent disruption of axal development on the sea urchin embryo (Pillai *et al.*, 2003). In the early life stages of the zebrafish (48-96 hpf), certain PAHs have been associated with the observation of what is called the “blue sac syndrome”, including various developmental effects such as reduced growth, crania-facial malformations, yolk sac and pericardial edema, and subcutaneous hemorrhaging, generally associated with planar halogenated aromatic hydrocarbons. The pathways by which PAHs disturb individual development in fish are complex and seem to be dependent on the structure of the compound itself (Incardona *et al.*, 2006, Billiard *et al.*, 2006). For tricyclic aromatic hydrocarbons such as phenanthrene, embryonic cardiac dysfunction is mainly observed and seems to be mediated in an aryl hydrocarbon receptor (AHR)- and CYP1A-independent manner, by direct action on cardiac ion channels (Incardonna *et al.*, 2005). On the other hand, tetracyclic aromatic hydrocarbons such as benz[a]anthracene appear to display their embryotoxicity through a classical AHR pathway (Incardona *et al.*, 2006). In this latter case, ligation to the AHR could result in an increase in the transcription level of a pro-apoptotic gene as demonstrated recently for 7,12-dimethylbenz(a)anthracene (Detmar *et al.*, 2006).
In parallel to embryotoxicity, genotoxic effects, as measured by DNA strand breaks and the oxidized DNA base 8-oxodGuo, were observed following oyster embryo to BaP exposure, confirming the ability of early life stages to bioactivate PAH (Fong *et al.*, 1993). The genotoxicity and the cancerogenicity of BaP have been well described in humans and rodents (Akcha *et al.*, 2004, Xue and Warshawsky, 2005) and similar pathways of bioactivation have been proposed in marine organisms (van der Oost *et al.*, 2003). Bioactivation of BaP to electrophilic metabolites resulted in DNA adduct formation as already demonstrated in mussels (Canova *et al.*, 1998, Akcha *et al.*, 2000b). BaP can also be oxidized to prooxidant metabolites such as quinones and o-quinones responsible for a redox-cycling production of reactive oxygen species (Sjölin and Livingstone, 1997). The resulting oxidative stress damages the macromolecules such as the DNA. The formation of the oxidized DNA base, 8-oxodGuo has been reported in bivalves following BaP contamination via the water column or the trophic supply (Canova *et al.*, 1998, Akcha *et al.*, 2000a, Machella *et al.*, 2004). DNA strand breaks can also be produced in response to oxidative stress as already demonstrated *in vivo* following exposure of flounders, oysters and mussels to BaP via the sediment, the water column or by trophic transfer (Nasci *et al.*, 1999, Taban *et al.*, 2004). An increase in the level of DNA strand breaks in shrimp embryos was also reported by Hook and Lee (2004) following a 12h exposure to 0.2 µM BaP .
In the present paper, DNA strand breaks appear more sensitive than 8-oxodGuo to BaP exposure, with both lesions significantly increasing from a concentration of respectively 0.2 and 20 nM. This could be explained by the measurement of DNA strand breakage by alkaline comet assay that also takes into account the presence of abasic and other alkali-labile sites that can result from the incomplete repair of DNA damage such as adducts by excision repair systems.

4.2. EE2

As an endocrine disruptor, EE2 interferes with the normal physiological functions related to reproduction, justifying studies of its potential developmental toxicity. The morphological disorders of the reproductive tract associated with EE2 exposure and other environmental xenoestrogens have been closely studied in recent years (Porte *et al.*, 2006, Goksoyr, 2006). Nevertheless, there remains a paucity of literature ascertaining the embryotoxic effect of EE2. A

negative effect on embryo development of *Oryzias latipesa* was reported by Papoulias *et al.* (2000) from concentrations ranging from 0.5 to 2.5 ng per egg. A marked embryotoxic effect from 0.1 mM was also reported *in vitro* for rodent embryos by Beyer and Juchau (1988). Embryotoxic effects were also reported for its natural hormonal counterpart, 17β-estradiol, to which fish at early life stages were exposed over a 12 week period and subsequently shown to suffer malformations and incomplete ossification from a concentration of 10 nM (Iguchi *et al.*, 2001). In the present paper, no embryotoxic effect of EE2 was observed for oyster embryos within the range of tested concentrations (0.02 to 1.7 nM).
EE2 is known to be a strong promotor of hepatocarcinogenesis due to its effects on cell proliferation. The significance of direct and indirect genotoxicity of EE2 metabolites has also been proposed in carcinogenesis, thus justifying the interest in studying EE2 genotoxicity. Following exposure of human lymphocytes to EE2 (3.4-300 µM) with or without S9 metabolic activation, an increase in sister chromatide exchange and chromosomal aberrations was reported (Hundal *et al.*, 1997, Siddique *et al.*, 2005). In rat testicular cells, EE2 exposure resulted in an increase in the level of the oxidized base, 8-oxodGuo, reaching a maximum at a concentration of 10 nM, the formation of this oxidative DNA lesion following a bell-shaped concentration-response relationship (Wellejus and Loft, 2002, Wellejus *et al.*, 2004). Base oxidation was also demonstrated *in vivo* in female rats orally exposed to EE2 (0.075 or 0.75 mg/day) (Ogawa *et al.*, 1995). In the latter study, the decrease in 8-oxodGuo observed after the combined administration of antioxidant vitamins underlined the significance of oxidative stress in the induced genotoxicity. In the rat, the formation of hepatic DNA adducts was also detected by the application of the ^{32}P post-labeling technique in parallel to the observation of hyperplastic nodules in the liver (Shimomura *et al.*, 1992).
Similarities were found between the metabolism of PAH hydroquinones and catechol estrogens produced by the hydroxylation of the aromatic A ring of estradiol and estrone at positions C2 and C4 (Penning, 1993). The 2,3 and 3,4 catechol estrogens can produce highly reactive quinone metabolites involved in the *in vitro* formation of stable and depurinating DNA adducts (Stack *et al.*, 1996, 1998, Cavalieri and Rogan, 1998, Debrauwer *et al.,* 2002, Jouanin *et al.,* 2002, Belous *et al.*, 2007). Following reduction, these quinones can also lead to the redox-cycling production of highly reactive radical semi-quinones that can covalently bind to the DNA (Akanni and Abul-Hajj, 1997, Cavalieri *et al.*, 2006). Redox-cycling reactions also generate reactive oxygen species (ROS) involved in oxidative damage to cellular macromolecules such as the DNA (Yager, 2000, Hiraku *et al.*, 2001).
In the present study, the absence of embryotoxicity for EE2 could be explained by the low concentrations tested (max 1.7 nM), that remain well below the effective concentration measured by Beyer and Juchau in rodents (0.1 mM). In fish, malformations were induced for an EE2 concentration within the same range (10 nM), although fish were exposed for 12 weeks compared to only 16hrs for oyster embryos used in this experiment.
It is possible that the range of tested concentrations was too low for the expression of potential EE2 genotoxicity.

4.3. ES

ES is known as a xenoestrogen and is potentially toxic during the development of exposed organisms. As for EE2, there is a paucity of literature ascertaining its embryotoxicity and teratogenicity, and this is exclusively reported in rodents (Gupta *et al.*, 1978, Singh *et al.*, 2007) .
Oxidative DNA damage has been associated with ES exposure. In yeast, rodent, and human cells, ES was shown to cause oxidative stress, resulting in a dose-dependent increase in DNA strand breaks (Sohn *et al.*, 2004, Bajpayee *et al.*, 2006). Following 96hr exposure to 1.23 nM, ES was shown to increase the frequency of micronuclei and nuclear abnormalities in the erythrocytes of juveniles of *Sparus aurata* in a dose-dependent manner (Neuparth *et al.*, 2006). The induction of micronuclei has already been reported in amphipods following 96hr exposure to concentrations ranging from 6 to 25 nM (Lajmanovich *et al.*, 2005).
In mammals, ES is metabolized into endosulfan sulfate and to endosufan ether, the latter being further transformed to endosulfan ether, hydroxyether and lactone (Bajpayee *et al.*, 2006). The genotoxicity of ES and its metabolites appears to be mediated via the production of oxyradicals. However, ES was also shown to form DNA adducts in primary rat hepatocytes and human liver

hepatoblastoma cells, indicating the biotransformation of the parent compound into electrophilic metabolites (Dubois *et al.*, 1996).
In the present paper, the embryotoxicity and the genotoxicity of ES was confirmed for oyster embryos at concentrations from 300 and 150 nM, respectively. These concentrations may be considered rather high in the light of available bibliographic data but should be interpreted upon consideration of the short duration of the exposure period.

4.4. The statistical relationship between embryotoxicity and genotoxicity

A positive correlation was demonstrated between embryotoxicity and genotoxicity when DNA strand break levels were considered as DNA damage. Despite the fact that a cause/effect relationship cannot be established, a statistical link is demonstrated between these two mechanisms of toxicity.
A large number of research papers highlight the significance of genotoxicity in the pathophysiology of reproduction in human and other vertebrates such as fish (Agarwal *et al.*, 2003, 2006, Zhou *et al.*, 2006). There is much less data concerning the potential link between genotoxicity and embryotoxicity. As suggested by Wells *et al.* (1997), embryotoxicity is thought to result from the bioactivation of pro-teratogens to reactive metabolites for the cellular macromolecules. The formation of DNA and protein adducts is proposed as a crucial event for teratogenesis initiation. Oxidative damage to these macromolecules is also considered a key event in the molecular mechanisms of embryotoxicity and teratogenicity. The specific target genes or proteins, however, remain unknown.
It is important to note that in the present paper we did not observe a correlation between comet and 8-oxodGuo data and between embryotoxicity and 8-oxodGuo data. As already mentioned, it is possible that DNA adduct formation represents an important pathway of BaP and ES genotoxicity. In this case, the comet assay may be more appropriate for a global measurement of DNA damage.

Conclusion

Following exposure of oyster embryos to EE2, no embryotoxicity and genotoxicity was observed for the range of concentrations tested (0.02-1.7 nM). Exposure of oyster embryos to BaP and ES resulted in embryotoxicity as measured by the percentage of abnormal D-shell larvae. These environmental pollutants also displayed genotoxic effects as measured by oxidative DNA damage, in terms of DNA strand breaks, alkali labile sites and 8-oxodGuo. These results confirm the ability of the oyster to biotransform these xenobiotics from early life stages. Bioactivation is thought to result in the induction of an oxidative stress involved in the measured oxidative DNA damage. DNA adduct formation is expected to occur for the selected exposure conditions.
The positive correlation observed between oyster embryotoxicity and the comet data tends to confirm the significance of genotoxicity in the molecular mechanisms of BaP and ES embryotoxicity. The genotoxicity of chemical pollutants would therefore have an indirect effect on population size and as a consequence on population dynamics.
For BaP, toxic effects are observed from a concentration of 0.2 nM that is situated within the same range as the highest concentrations recorded in the aquatic environment, suggesting a risk for oyster recruitment in highly polluted coastal areas.

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