ELSEVIER

Aquatic Toxicology 81 (2007) 36–44

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: In vitro and in vivo studies

Laura Canesi [a,*], Lucia Cecilia Lorusso [b], Caterina Ciacci [b], Michele Betti [b], Marco Rocchi [c], Giulio Pojana [d], Antonio Marcomini [d]

[a] *Dipartimento di Biologia, Università di Genova, Corso Europa 26, Genova 16132, Italy*
[b] *Istituto di Scienze Fisiologiche, Università 'Carlo Bo' di Urbino, Italy*
[c] *Istituto di Biomatematica, Loc. Crocicchia, Università 'Carlo Bo' di Urbino, Italy*
[d] *Dipartimento di Scienze Ambientali, Università 'Cá Foscari' di Venezia, Italy*

Received 7 September 2006; received in revised form 26 October 2006; accepted 28 October 2006

Abstract

Endocrine disrupting compounds (EDCs) are almost ubiquitous in the aquatic environment. In the marine bivalve *Mytilus* the natural estrogen 17β-estradiol (E_2) and different EDCs have been recently demonstrated to affect the function of the immune cells, the hemocytes. The effects were Tamoxifen-sensitive and were mediated by rapid modulation of kinase-mediated transduction pathways. In this work we compared the in vitro effects of individual estrogenic chemicals (E_2, EE: 17α-ethynyl estradiol; MES: mestranol; NP: nonylphenol; NP1EC: nonylphenol monoethoxylate carboxylate; BPA: bisphenol A; BP: benzophenone) on hemocyte parameters: lysosomal membrane stability (LMS), phagocytosis, lysozyme release. LMS was the most sensitive effect parameter, showing a decreasing trend at increasing concentrations of estrogens. EC_{50} values obtained from LMS data were utilized to calculate the estradiol equivalency factor (EEF) for each compound; these EEFs allowed for an estimation of the estrogenic potential of a synthetic mixture with a composition very similar to that previously found in waters of the Venice lagoon. Concentrated mixtures significantly affected hemocyte parameters in vitro and the effects were prevented by Tamoxifen. Significant effects of the mixture were also observed in vivo, at longer exposure times and at concentrations comparable with environmental exposure levels. The results indicate that *Mytilus* immune parameters can be suitably utilized to evaluate the estrogenic potential of environmental samples.

Keywords: Estrogenic chemicals; Estrogen mixtures; Bivalves; Immunity; Hemocytes

1. Introduction

Endocrine disrupting compounds (EDCs) include both natural and synthetic steroid estrogens, as well as a variety of estrogen-mimicking chemicals, such as alkylphenols and alkylphenol ethoxylates, PCBs, dioxins, various pesticides and herbicides. EDCs have been found in freshwater, estuarine and marine environments (Atkinson et al., 2003; Peck et al., 2004; Braga et al., 2005), thus representing a potential hazard for aquatic species (Rotchell and Ostrander, 2003; Sumpter and Johnson, 2005). Most studies on the effects and mechanisms of action of estrogenic chemicals have been focused on vertebrates: many EDCs have been shown to bind both mammalian and fish estrogen receptors (ERs) (McLachlan, 2001; Rotchell and Ostrander, 2003). The estrogenic potency of EDCs is commonly related to that of 17β-estradiol (E_2), the most potent natural estrogen, by using different assays; these are based on the measurement of responses mediated by the common 'genomic' mechanism of action of estrogens in vertebrate cells, that is binding to intracellular ERs that act as ligand-inducible transcription factors thus modulating the expression of estrogen responsive genes (Andersen et al., 1999; Gutendorf and Westendorf, 2001; Brian et al., 2005).

Although invertebrates represent approximately 95% of animal species, and a significant component of aquatic ecosystems, information on the effects and mechanisms of action of EDCs in these organisms is scarce compared to that available for vertebrates (Jobling et al., 2003; Verslycke et al., 2004; Roepke

* Corresponding author. Tel.: +39 010 3538241; fax: +39 010 353267.
E-mail address: Laura.Canesi@unige.it (L. Canesi).

0166-445X/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2006.10.010

et al., 2005; Ohelmann et al., 2006). The evaluation of the effects of EDCs on invertebrates represents a complex task, because of the limited knowledge on the endocrine systems, their differences in a large number of species, and on the mechanisms of action of natural hormones (Ohelmann and Schulte-Ohelmann, 2003; Porte et al., 2006). Among aquatic invertebrates, in molluscs endogenous estrogens have been identified, and evidence for ER-like receptors has been obtained in different species (Reis-Henriques et al., 1990; Gagnè et al., 2001; Di Cosmo et al., 2002; Canesi et al., 2004a; Osada et al., 2003, 2004). ER-like receptors have been recently characterized in gastropods (Thornton et al., 2003; Kajiwara et al., 2006) and cephalopods (Keay et al., 2006). These studies showed that molluscan ERs, although phylogenetically clustered with other steroid receptors, appear to be functionally different: their transcriptional activity is constitutive, and not activated by estrogens.

Bivalves are sessile, filter-feeding molluscs that can represent a primary target for estrogenic chemicals, due to their high bioaccumulation and low biotransformation potential for contaminants (Ortiz-Zarragoitia and Cajaraville, 2006). Recent progress has been made on the role of estrogens on bivalve reproduction (Osada et al., 2003, 2004; Gauthier-Clerc et al., 2005), steroid biosynthesis and metabolism (Janer et al., 2005a, 2005b) and, in analogy with vertebrate studies, on the induction of Vitellogenin (Vtg)-like proteins in males by EDCs, although the relationship between estrogen exposure and Vtg induction has not been fully clarified (Blaise et al., 1999; Gagnè et al., 2001; Riffeser and Hock, 2002; Ortiz-Zarragoitia and Cajaraville, 2006). In the marine mussel *Mytilus*, exposure to E_2 does not affect the expression of ER- and Vtg-like sequences (Puinean et al., 2006). Effects of municipal effluents containing EDC mixtures have been demonstrated in non-reproductive tissues (Gagnè et al., 2001, 2004; Quinn et al., 2004); a role for EDCs in neuroendocrine disruption of reproduction has been suggested (Gagnè and Blaise, 2003).

In *Mytilus* hemocytes, the cells responsible for innate immunity (Canesi et al., 2002a), E_2 was shown to modulate a number of functional parameters, including lysosomal membrane stability (LMS), extracellular lysozyme release, phagocytosis, oxyradical production (Canesi et al., 2004a, 2006). Low nM concentrations stimulated the immune function, whereas higher concentrations were inhibitory. The effects of E_2 were prevented by the antiestrogen Tamoxifen and were mediated by rapid, 'non-genomic' mechanisms of action similar to those identified in mammalian cells (Lösel et al., 2003), involving activation of cytosolic kinases such as MAPKs (mitogen activated protein kinases) and PKC (protein kinase C), and phosphorylation of transcription factors such as STATs (signal transducers and activators of transcription) and CREB (cAMP responsive element binding protein) (Canesi et al., 2004a, 2006), that play a key role in the hemocyte immune response (Canesi et al., 2005a). Different EDCs were also shown to rapidly affect hemocyte lysosomal function and signalling through Tamoxifen-sensitive modulation of kinase pathways, although at higher concentrations, and with distinct effects depending on the compound (Canesi et al., 2003, 2004b). The effects were confirmed in vivo, in mussels injected with bisphenol A, at concentrations similar to those utilised for E_2 (Canesi et al., 2005b).

In this work, we extended our studies on the in vitro effects of EDCs on the hemocyte function in terms of number and type of individual compounds, concentration range, and effect parameters. The effects of different concentrations of a set of widely distributed estrogenic compounds on LMS were evaluated and the results compared to those obtained with the natural estrogen E_2. Specific immune parameters, such as phagocytosis and release of hydrolytic enzymes, were also evaluated. The same effect endpoints were measured in hemocytes exposed to a synthetic mixture containing E_2 and the examined estrogens in proportions similar to those previously found in environmental samples from the Venice Lagoon (Pojana et al., 2004). Finally, the effects of the synthetic mixture on immune parameters were evaluated by an in vivo experiment, conducted at longer exposure times, in the hemocytes of mussels injected with the mixtures at environmental concentrations.

2. Materials and methods

2.1. Chemicals

All reagents were of analytical grade. 17β-Estradiol (E_2) and 17α-ethinylestradiol (EE) were from Sigma (St. Louis, MO); bisphenol A (BPA) and 4-*n*-nonylphenol (NP) were from Riedel-de Haen (Germany); mestranol (MES) and benzophenone (BP) were obtained from Fluka (Büchs, Switzerland); nonylphenol monoethoxylate carboxylate (NP1EC) (purity ~ 90%) was purchased by Ciba Speciality Chemicals (Basel, Switzerland) and further purified by semipreparative HPLC up to a final >99% purity. Stock solutions of E_2, EE, BP and NP in ethanol and of MES, BPA and NP1EC in methanol were prepared and kept at −20 °C.

2.2. Animals, hemolymph collection, preparation of hemocyte monolayers and hemocyte treatment

Mussels (*Mytilus galloprovincialis* Lam.) 4–5 cm long, sampled from an unpolluted area at Cesenatico (RN) were obtained from SEA (Gabicce Mare, PU) and kept for 1–3 days in static tanks containing artificial sea water (ASW) (1 l/mussel) at 16 °C. Average dry weight of whole animal soft tissues was about 1 g. Sea water was changed daily. Hemolymph was extracted from the posterior adductor muscle of 8–20 mussels, depending on the experiment, and hemocyte monolayers were prepared as previously described (Canesi et al., 2002b). Hemocytes were incubated at 16 °C for 30 min with different EDCs (from stock solutions in ethanol or methanol) suitably diluted in ASW. Untreated and control vehicle hemocyte samples were run in parallel.

2.3. Lysosomal membrane stability

Lysosomal membrane stability in control hemocytes and hemocytes incubated with different concentrations of EDCs for 30 min was evaluated by the Neutral Red Retention time assay

as previously described (Canesi et al., 2003) according to Lowe et al. (1995).

2.4. Phagocytosis assay

Phagocytosis of Neutral Red-stained zymosan by hemocyte monolayers was used to assess the phagocytic ability of hemocytes as previously described (Canesi et al., 2005c) according to Pipe et al. (1995).

2.5. Lysosomal enzyme release

Lysosomal enzyme release by mussel hemocytes was evaluated by measuring lysozyme activity in the extracellular medium as previously described (Canesi et al., 2003). Lysozyme activity in aliquots of serum of control and treated hemocytes incubated for different periods of time (from 0 to 60 min), was determined as described by Chu and La Peyre (1989).

2.6. Mixtures of estrogenic chemicals

A binary mixture was obtained from stock solutions of E_2 and EE suitably diluted in ASW at final concentrations of 5 and 10 nM, respectively (1:2). A synthetic mixture of E_2, EE, BPA, NP, NP1EC, BP and MES, was obtained from stock solutions of individual compounds suitably diluted in ASW in order to obtain the same peak concentration of each compound recorded in a coastal lagoon such as the Venice Lagoon, Italy (Pojana et al., 2004). This synthetic mixture (MIX1×) contained: E_2: 52 ng/l = 0.19 nM; EE: 125 ng/l = 0.42 nM; MES: 75 ng/l = 0.24 nM; BP: 1040 ng/l = 5 nM; NP: 201 ng/l = 0.91 nM; NP1EC: 256 ng/l = 0.92 nM; BPA: 88 ng/l = 0.38 nM, corresponding to a total amount of 7.96 nM/l (1767 ng/l). Concentrated mixtures were also utilised (from MIX10× to MIX10^3×, corresponding to total concentrations from 79.6 to 7960 nM/l). Untreated and control vehicle hemocyte samples were run in parallel.

2.7. In vivo exposure to the synthetic EDC mixture

For in vivo experiments, mussels were collected in September–November 2005. Average concentration of free E_2 in the hemolymph of control mussels was 800 pg/ml (evaluated by a commercial competitive chemiluminescent enzyme immunoassay kit-Immulite 2000 Estradiol). The EDC mixture, whose relative composition has been described above, was injected into the posterior adductor muscle of groups of 10–12 mussels using a sterile 1 ml syringe with a 18G1/2" needle as previously described (Canesi et al., 2005b, 2006). Taking into account that the average volume of hemolymph that can be withdrawn from mussels of this size (4–5 cm) was 0.8–1 ml, each mussel was injected with 50 μl of a concentrated mixture (20×) suitably diluted in ASW. This gave a nominal concentration of total EDCs of 7.96 pmol/ml hemolymph (1767 pg/g dry weight), corresponding to that of MIX1× (7.96 nM/l). Lower concentrations of the mixture were also utilised in order to obtain a nominal amount of total EDCs of 0.0796, 0.796 pmol/ml hemolymph/mussel (or 17.67, 176.7 pg/g dry weight), corresponding to Mix0.1×, Mix0.01×. Experiments were repeated three times. For each experiment, a parallel set of control mussel were injected with 50 μl of a solution of ASW containing an equal amount of vehicle (final concentration methanol/ethanol ≤0.005%). Mussels were then placed in plastic tanks containing ASW at 16 °C (0.5 l/mussel). After 24 h hemolymph was withdrawn from groups of vehicle- and EDC-injected mussels pooled in 50 ml Falcon tubes at 16 °C and samples analysed for LMS, phagocytosis and lysozyme release as described above. Animals were not fed during the experiment.

2.8. Data analysis

The results are the mean ± S.D. of at least four experiments (three for in vivo experiments) and assays were performed in triplicate. Statistical analysis was performed by using the Mann–Whitney *U*-test with significance at $P \leq 0.05$. LMS data obtained from in vitro experiments with individual compounds were log transformed and EC_{50} were calculated from a regression model of the original data and analysed by one-way ANOVA. NOEC and LOEC were calculated by one-way ANOVA followed by Dunnett's test. All statistical analyses were performed at a 95% confidence level.

3. Results

3.1. Effects of individual compounds on hemocyte parameters

Lysosomal membrane stability (*LMS*): the effects of different concentrations of individual estrogenic chemicals on lysosomal membrane stability of *Mytilus* hemocytes were first evaluated and compared to those of E_2. The mean annual values of NRR time in control hemocytes, evaluated over a 18-month period, were 122 ± 12 min (mean ± S.D.) and no significant seasonal-related differences were observed. However, in order to minimize the chance of seasonal changes in LMS sensitivity to EDC treatment, experiments were repeated utilising hemocytes from mussel sampled at different times of the year and data were pooled. The complete concentration–response curve of each compound was determined and the results, expressed as percent lysosomal destabilisation with respect to controls, are shown in Fig. 1. E_2 induced lysosomal destabilisation in the nM range (1–100 nM). A similar effect was observed with all the estrogenic compounds tested (MES, NP1EC, BP, NP, EE and BPA), although at higher concentrations (nM–μM).

Phagocytosis: All EDCs induced a small but significant stimulation of phagocytosis at lower concentrations, from 0.1 to 5 μM (about + 20/30% with respect to controls; $P \leq 0.05$); higher concentrations (25–100 μM) were inhibitory (Fig. 2A–F). A similar biphasic effect was previously observed with E_2: concentrations from 5 to 25 nM increased phagocytosis, whereas higher concentrations decreased the phagocytic activity (Canesi et al., 2006).

Lysosomal enzyme release: Most of the compounds tested induced a significant stimulation of extracellular enzyme release

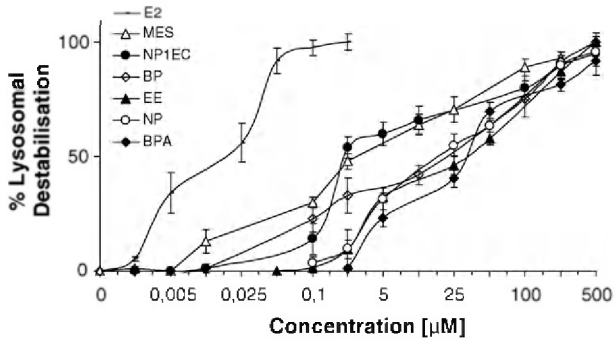

Fig. 1. In vitro effects of different concentrations of E_2 and of individual estrogenic chemicals on lysosomal membrane stability (LMS) of mussel hemocytes. LMS was measured as NR retention time in control cells and in cells pretreated with different EDCs for 30 min, as described in Section 2, and the results were expressed as % lysosomal destabilisation with respect to controls. Results are mean ± S.D. of at least five experiments in triplicate.

from 15 min of incubation, that was maximal at 30–60 min; no significant differences in the time course were observed for different compounds (data not shown). A comparison of the effects of different estrogenic compounds is shown in Table 1, where results are reported as % increase in lysozyme activity with respect to controls at 30 min after exposure; at this time point maximal lysozyme release was induced by both bacterial challenge (Pruzzo et al., 2005), and E_2 exposure (Canesi et al., 2004a). MES, NP1EC, BP (1 μM) induced a significant increase in lysozyme release (between +30 and 60% with respect to controls; $P \leq 0.05$) that was comparable to that induced by nM E_2. At this concentration all EDCs, except for NP, stimulated phagocytosis. EE and BPA did not significantly affect lysozyme release at concentrations up to 5 μM.

Table 1
Effects of individual estrogenic chemicals on hemocyte lysosomal enzyme release

	Lysozyme activity (mU/mg) protein, % increase vs. controls at 30 min
E_2 25 nM	45 ± 5*
MES 1 μM	35 ± 4*
NP1EC 1 μM	60 ± 5*
BP 1 μM	43 ± 4*
NP 1 μM	32 ± 3*
EE 5 μM	13 ± 7 n.s.
BPA 5 μM	14 ± 6 n.s.

Hemocytes were incubated with the estrogenic compounds for 30 min and lysozyme activity was evaluated in the extracellular medium as described in methods. Reported values are the mean ± S.D. of at least four experiments in triplicate. *$P \leq 0.05$, Mann–Whitney U test. n.s.: not significant.

3.2. Comparison of the effects of individual compounds

Since LMS was the most sensitive exposure parameter to individual compounds and showed a clear dose-dependent response in a full concentration range, the potency of different EDCs was compared to that of E_2 utilising lysosomal stability data. For each compound the EC_{50} was calculated from a regression model after logit transformation of the original data and analysed by ANOVA ($P < 0.05$); the NOEC (no-observed-effect concentration) and LOEC (lowest-observed-effect concentration) were evaluated using ANOVA and Dunnett's test ($P < 0.05$) and the results are reported in Table 2. The NOEC and LOEC for E_2 were, respectively, 1 and 5 nM, and the EC_{50} was 13.34 nM. The LOEC of the different estrogenic compounds ranged from 0.01 for MES to 5 μM for BPA. The EC_{50} were all in the μM range, with EC_{50}

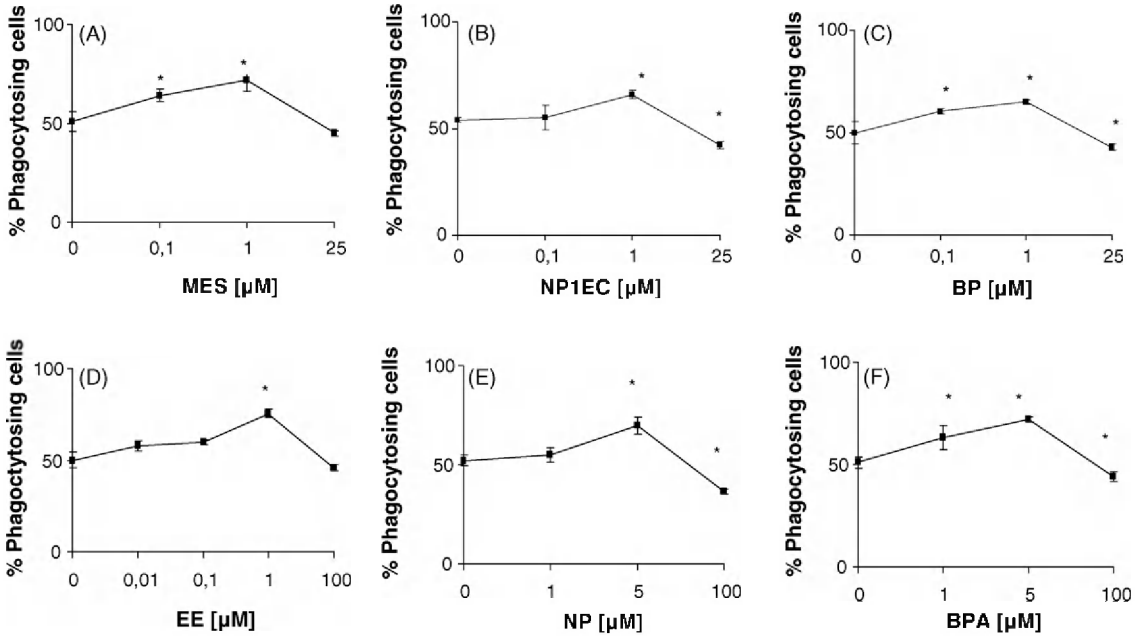

Fig. 2. In vitro effects of different concentrations of individual estrogenic chemicals (A–F) on phagocytosis of NR-conjugated zymosan particles by mussel hemocytes. Results, expressed as % of phagocytic cells, are mean ± S.D. of at least four experiments in triplicate. *$P \leq 0.05$, Mann–Whitney U test.

Table 2
Relative potency of E_2 and the examined estrogenic compounds on lysosomal membrane destabilization (LMS) in *Mytilus* hemocytes

	NOEC (μM)	LOEC (μM)	EC_{50} (μM)	EEF ($EC_{50\,E2}$/$EC_{50\,test\,compound}$)
E_2	0.001	0.005	0.013	1
MES	0.001	0.01	2.080	6.41×10^{-3}
NP1EC	0.01	0.1	6.686	1.99×10^{-3}
BP	0.01	0.1	8.535	1.56×10^{-3}
NP	0.1	1	19.324	0.69×10^{-3}
EE	0.1	1	24.764	0.53×10^{-3}
BPA	1	5	34.486	0.38×10^{-3}

Values are expressed as % lysosomal destabilisation with respect to controls. EC_{50} were calculated from a regression model after logit transformation of the original data and analysed by one-way ANOVA. NOEC and LOEC were calculated by one-way ANOVA followed by Dunnett's test. All statistical analyses were performed at a 95% confidence level.

MES < NP1EC ≅ BP < NP ≅ EE ≅ BPA, and estradiol equivalency factors (EEFs) (i.e., the ratio $EC_{50}E_2/EC_{50\,test\,compound}$, conventionally set at 1 for E_2), from 6.41×10^{-3} for MES to 0.38×10^{-3} for BPA.

3.3. Effects of a binary mixture E_2/EE

Hemocyte parameters were evaluated following cell treatment with a binary mixture containing E_2 and the synthetic estrogen EE at the ratio 1:2, this proportion reflecting the relative amounts of the two compounds previously measured in samples from different sites of the Venice Lagoon (Pojana et al., 2004). The E_2/EE mixture was arbitrarily tested at the concentration of 5 nM E_2 (LOEC E_2) plus 10 nM EE (<NOEC EE), and the results are reported in Fig. 3. The effect of the binary mixture on LMS was not statistically different from that observed with E_2 alone (Fig. 3A); however, EE induced a significant increase in E_2-stimulated phagocytosis (Fig. 3B). Interestingly, 10 nM EE significantly reduced basal extracellular enzyme release by control cells and prevented that induced by E_2 (Fig. 3C).

3.4. Effects of a synthetic EDC mixture

A synthetic mixture was prepared containing seven compounds (E_2, MES, NP1EC, BP, EE, NP, BPA) according to their distribution found in samples from the Venice Lagoon (Pojana et al., 2004). In MIX1×, each compound was present at concentrations corresponding to maximal values found in environmental samples (Table 3); all compounds were present at sub-nM concentrations (ng/l), except for BP, that exhibited the highest concentration (up to 1.040 μg/l) (Pojana et al., 2004), resulting in a total concentration of the mixture of 7.96 nmol/l (1767 ng/l). On the basis of the EEFs obtained from LMS data and on the concentration of each compound in the mixture, the EEQ (estradiol equivalent concentration) of the mixture was calculated. EEQ of MIX1× was 53 ng/l, corresponding to about 0.2 nmol/l, with E_2 representing about 97.5% and 94% of the mixture on a ng/l and nmol/1 basis, respectively.

Hemocytes were treated with MIX1× and concentrated mixtures following the same protocol of exposure to individual compounds; functional parameters were evaluated and the results are reported in Fig. 4. As shown in Fig. 4A, MIX1× and 10× did not significantly affect hemocyte LMS; however,

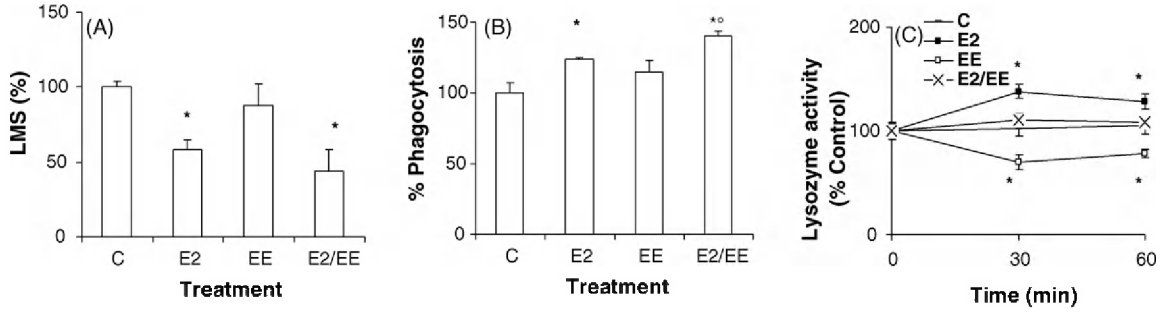

Fig. 3. In vitro effects of a binary mixture of E_2/EE (1:2) on hemocyte parameters. Hemocytes were exposed to 5 nM E_2, 10 nM EE or E_2/EE (5/10 nM) as described in methods and results are expressed as % of control values: (A) LMS; (B) phagocytosis; (C) lysozyme release. Data are the mean ± S.D. of at least four experiments in triplicate. $^*P \leq 0.05$, Mann–Whitney *U* test.

Table 3
Composition of the synthetic mixture MIX1× and resulting EEQs

Compound (MW)	Relative potency (EEF)	Concentration in MIX1× (nmol/l)	EEQ (pmol/l)	EEQ (ng/l)
E_2 (272.4)	1	0.19	190	51.75
MES (310.43)	6.41×10^{-3}	0.24	1.53	0.477
NP1EC (278)	1.99×10^{-3}	0.92	1.83	0.510
BP (182.22)	1.56×10^{-3}	5	7.8	0.142
NP (220.36)	0.69×10^{-3}	0.81	0.55	0.121
EE (296.4)	0.53×10^{-3}	0.42	0.22	0.065
BPA (228.29)	0.38×10^{-3}	0.38	0.14	0.031
Total		7.96	202.07	53.09

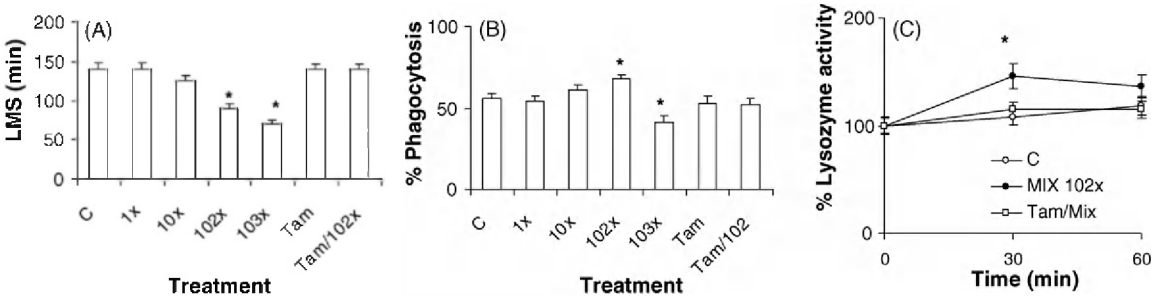

Fig. 4. In vitro effects of a synthetic EDC mixture on mussel hemocytes. Hemocytes were incubated with the mixture at different concentrations (1×, 10×, 10^2×, 10^3×, corresponding to 7.96–79.6–796–7960 nmol/l, respectively) as described for individual compounds (see Section 2). Hemocytes were also pre-incubated with the antiestrogen Tamoxifen (10 min, 100 nM) before exposure to the mixture 10^2× (Tam/Mix): (A) LMS, expressed as NR retention time (min); (B) phagocytosis; (C) lysosomal enzyme release. C = control. Results are the mean ± S.D. of at least four experiments in triplicate. $^*P \leq 0.05$, Mann–Whitney *U* test.

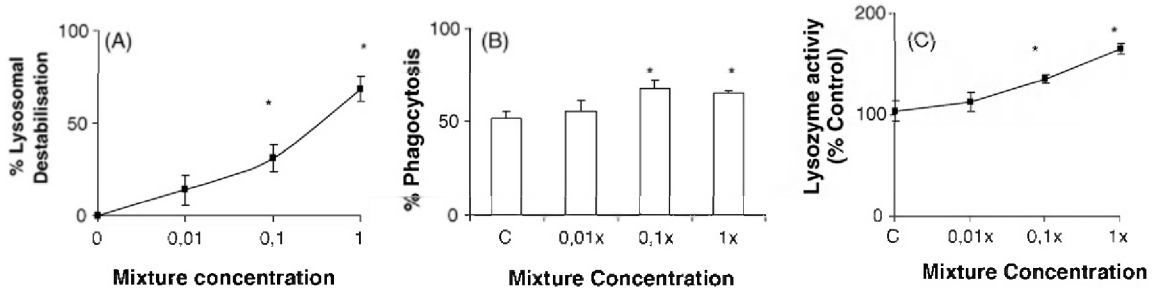

Fig. 5. In vivo effects of a synthetic EDC mixture on mussel hemocytes. Mussels were injected with different amounts of the mixture as described in Section 2 and hemocytes were collected 24 h post-injection. Nominal mixture concentrations 0.01, 0.1, and 1, corresponded to 0.0796, 0.796, and 7.96 pmol/ml hemolymph/mussel or 17.67, 176.7 and 1767 pg/g dry weight, respectively: (A) LMS; (B) phagocytosis; (C) lysosomal enzyme release. Results are the mean ± S.D. of three experiments in triplicate. $^*P \leq 0.05$, Mann–Whitney *U* test.

higher concentrations induced lysosomal membrane destabilisation (+39 and +50%, respectively for MIX10^2×, MIX10^3×; $P \leq 0.05$). The effect of MIX10^2× (EEQ = 5.3 μg/l–20.2 nM) was prevented by cell pre-treatment with the antiestrogen Tamoxifen (100 nM). At this concentration, the antiestrogen was previously shown to prevent the effects of different estrogens without affecting hemocyte parameters or kinase-mediated cell signalling (Canesi et al., 2004a).

Phagocytosis was also unaffected by lower concentrations of the mixture (Fig. 4B). However, MIX10^2× stimulated the phagocytic activity (+22%; $P \leq 0.05$) and the effect was prevented by Tamoxifen. Higher concentrations were inhibitory (−27% with MIX10^3×; $P \leq 0.05$). MIX10^2× also induced a significant stimulation of lysosomal enzyme release (+30% at 30 min with respect to controls; $P \leq 0.05$); the effect was prevented by Tamoxifen (Fig. 4C).

In in vitro experiments of short-term exposure to different estrogens, alone or in combination, hemocyte viability was evaluated by propidium iodide staining and flow cytometry as previously described (Betti et al., 2006). Only in samples treated with the highest concentrations of individual estrogens, and that showed largest lysosomal destabilisation (>80%) cell viability decreased from 97 to 85% (data not shown).

3.5. *In vivo effects of the EDC mixture*

The effects of the EDC mixture on hemocyte parameters were also evaluated in vivo, at longer exposure times, and the results are summarised in Fig. 5. Mussels were injected into the adductor muscle sinus with 50 μl of solutions containing different amounts of the mixture (nominal concentrations of 0.0796, 0.796 and 7.96 pmol/ml hemolymph/mussel, respectively, or 17.67, 176.7 and 1767 pg/g dry weight, corresponding to those of Mix0.01×, 0.1× and 1×, respectively, in nmol/l). Hemocytes were sampled at 24 h post-injection and analysed for LMS, phagocytosis and lysozyme release. No mortality or significant changes in hemocyte counts were recorded (not shown) in EDC-injected mussels with respect to controls; however, significant changes in hemocyte parameters were observed. As shown by Fig. 5A, exposure to the mixture induced a clear dose-dependent lysosomal destabilization (up to 71% with 7.96 pmol; $P \leq 0.05$). The two higher concentrations also induced a significant stimulation of the phagocytic activity (+20 and 28%, respectively, with 0.796 and 7.96 pmol; $P \leq 0.05$) (Fig. 5B) and lysozyme release (+32 and +61%, respectively) (Fig. 5C).

4. Discussion

The results demonstrate that in mussel hemocytes short-term exposure to individual estrogenic compounds induced rapid changes in hemocyte parameters in vitro. A complete dose–response curve was obtained for LMS, clearly showing that all the EDC tested were effective at concentrations higher (nM–μM) than those of E_2 (low nM). The results confirm that in the hemocytes the lysosomal vacuolar system, that plays a key role in different aspects of the immune function, rep-

resents a sensitive target for the rapid action of estrogenic compounds (Burlando et al., 2002; Canesi et al., 2004b, 2006). Lower concentrations (0.1–5 μM) induced a significant stimulation of phagocytosis and extracellular lysozyme release; in these conditions, EDC-induced lysosomal membrane destabilisation was generally smaller than 50%, indicating increased intracellular membrane fusion events leading to activation of the hemocyte function (Burlando et al., 2002). Higher concentrations were generally inhibitory of the phagocytic process, this probably reflecting disruption of the intracellular vacuolar system, as indicated by concomitant large decreases in LMS. A similar concentration-dependent pattern of responses was previously observed with E_2 both in vitro and in vivo (Canesi et al., 2004a, 2006). Inhibition of phagocytosis has been subsequently demonstrated also in clam hemocytes in vivo after injection with 10–20 nmol E_2/animal (Gauthier-Clerc et al., in press).

The effects of both E_2 and estrogenic chemicals (DES, EE, BPA, NP) on hemocyte functional parameters, including LMS, have been previously shown to be mediated by common mechanisms of action, involving rapid modulation of the kinase-mediated transduction pathways responsible for immune activation (Canesi et al., 2004a, 2004b, 2006). Individual EDCs were shown to exert a distinct effect (either increases or decreases) in the phosphorylation (activation) state of different kinases, MAPKs in particular (Canesi et al., 2004a, 2004b). The effects of EDCs on hemocyte function and signaling were prevented by the antiestrogen Tamoxifen, indicating involvement of ER-like receptors. Because the transcriptional activity of molluscan ERs does not appear to be activated by estrogenic compounds, conserved non-genomic mechanisms via 'alternative' modes of action, involving distinct membrane ER-like receptors of receptor-independent mechanisms that lead to modulation of kinase cascades, are likely candidates for this role (Keay et al., 2006).

The results obtained in vitro with individual compounds indicate that LMS, a general indicator of bivalve cellular stress associated to both anthropogenic and natural perturbations (Lowe et al., 1995), including endogenous steroids (Moore et al., 1978) and bacterial challenge (Canesi et al., 2005a), represents the most sensitive effect parameter of EDC exposure in hemocytes. Therefore, EC_{50} obtained for LMS data were utilised to compare the effects of individual estrogenic compounds in terms of EEFs (estradiol equivalency factors, i.e., the ratio $EC_{50}E_2/EC_{50\,\text{test compound}}$, conventionally set at 1 for E_2). The obtained EC_{50} for E_2 was 13.34 nM, whereas the EC_{50} of the different EDCs were all in the μM range (EC_{50} of MES ≅ NP1EC ≅ BP < NP = EEBPA). These data support previous results indicating that, in mussel hemocytes, the effects of the steroidal estrogens MES and EE are comparable to those of non-steroidal EDCs such as BPA and NP (Canesi et al., 2004b). On the other hand, EE is a potent synthetic estrogen in vertebrates; in a fish Vtg-assay, EE was shown to be more potent than E_2 (Thorpe et al., 2003). EE is often found in relevant concentrations in environmental samples (Atkinson et al., 2003; Braga et al., 2005). In particular, in samples from the Venice Lagoon, E_2 and EE were found in a characteristic ratio of 1:2 (Pojana et al., 2004). Therefore, we evaluated the in vitro effects of a binary mixture of E_2/EE (1:2, arbitrarily chosen as 5 and 10 nM, respectively) on hemocyte parameters. In the presence of EE (at a concentration < NOEC), no significant changes were observed in E_2-induced lysosomal destabilisation; however, a significant increase in phagocytosis was observed with respect to E_2 alone. Moreover, EE prevented the E_2-induced release of hydrolytic enzymes. The results suggest that, although the potency of all tested estrogens is much lower than that of E_2, some compounds at low concentrations may interfere with the mechanisms of action of the natural estrogen, at least in vitro.

This hypothesis was tested by utilising a synthetic mixture containing 7 estrogenic compounds, including E_2, at relative concentrations (MIX1×) corresponding to maximal values found in samples from the Venice Lagoon (Pojana et al., 2004). On the basis of EEFs evaluated utilising LMS data, and on the concentration of each individual compound in the mixture, the calculated EEQ (estradiol equivalent concentration) of MIX1× was about 53 ng/l (0.2 nM), with E_2 representing more than 97% of the mixture on a weight basis (Table 3). This value is lower, although in the same concentration range, than that previously estimated (EEQ = 172 ng/l; Pojana et al., 2004) by averaging literature EEF values obtained by different bioassays based on mammalian and fish ER transcriptional activation. However, the mechanisms underlying the observed responses to EDCs in molluscs can only be speculated (Jobling et al., 2003), since this mode of action of estrogens has been not demonstrated yet in these organisms (Keay et al., 2006). Our results indicate that the estrogenic potential of environmental mixtures can be evaluated by a simple and rapid assay that measures the response of molluscan immunocytes to individual estrogenic compounds induced by modulation of kinase pathways.

The effects of the synthetic mixture were first evaluated in vitro. Since in MIX1× the concentration of each compound, including E_2, was lower than the calculated NOECs, concentrated mixtures were also tested. Concentrated mixtures induced a pattern of responses similar to those observed with the individual compounds in the same experimental conditions: a dose-dependent effect on LMS, a biphasic effect on phagocytosis, and stimulation of lysosomal enzyme release; moreover, the effects were prevented by cell pre-treatment with the antiestrogen Tamoxifen. The effects of the mixture were similar to, and even slightly lower than, those expected on the basis of the concentrations of E_2 in the mixture. As indicated by the results obtained with the binary mixture E_2:EE, the presence of other estrogenic compounds, although at low concentrations, may affect the hemocyte response to the natural estrogen in vitro. Mutual interferences among individual compounds may be due not only to their relative concentrations in a mixture, but also to their distinct mechanisms of action on the hemocyte; certain estrogens, like DES, were shown to induce phosphorylation (activation) state of signalling components involved in the immune response, indicating mechanisms of action similar to those of E_2 (Canesi et al., 2004b); some others, such as BPA and NP, showed opposite effects (Canesi et al., 2004b, 2005b).

The effects of the synthetic mixture were also evaluated in vivo, at lower concentrations and longer incubations times (24 h), in hemocytes collected from mussels injected with different amounts of the mixture (nominal concentrations of 0.0796, 0.796 and 7.96 pmol/ml hemolymph/animal of total estrogens respectively, or 17.67, 176.7 and 1767 pg/g dry weight). In vivo treatment with 0.796 and 7.96 pmol EDCs induced significant changes in all parameters tested. These concentrations corresponded to those of MIX1× and MIX0.1×, in nmol/l, that is to the maximal and mean concentrations, respectively, previously found in environmental samples of the Venice Lagoon (Pojana et al., 2004). Although in bivalves injected into the adductor muscle a large part of the administered estrogen is lost from the open circulatory system (Le Curieux-Belfond et al., 2005), in the experimental same conditions utilised in the present work, comparable effects were observed with higher amounts of E_2 (5 pmol; Canesi et al., 2006) and BPA (25 pmol; Canesi et al., 2005a).

These results indicate that in vivo, at longer exposure times, mixtures of natural and synthetic estrogens may exert additive effects on hemocyte parameters that cannot be observed in vitro after short term exposure; at the same time, interferences by certain compounds like those observed for EE may not be as significant as those observed in vitro. In vivo, EE has been shown to induce significant disruption of mollusc reproductive output; however, molluscs appear to be less sensitive than fish (Jobling et al., 2003). The differences observed between in vitro and in vivo effects of EDC mixtures on mussel immunocytes may result from indirect effects of estrogenic compounds occurring at the organism level, in particular on steroid metabolism and on circulating levels of endogenous immunomodulators. Exogenous E_2 can be rapidly esterified in mussel tissues, this representing a homeostatic mechanisms for steroid hormones in bivalves (Janer et al., 2005a, 2005b). Moreover, long term exposure of freshwater bivalves to municipal effluents reduced phagocytic activity (Blaise et al., 2002) and altered levels and metabolism of the endogenous immunomodulators dopamine and serotonin (Gagnè and Blaise, 2003; Gagnè et al., 2004).

Potential responses induced by additive combinations of E_2 and xenoestrogens are receiving increasing attention (Rajapakse et al., 2002; Thorpe et al., 2003; Brian et al., 2005). Our results, although preliminary, indicate that also in mussel hemocytes the effects of EDC mixtures could be stronger than those expected on the basis of the individual concentrations, and demonstrate that significant effects on hemocyte function can be observed following in vivo exposure to environmental concentrations. Overall, these data indicate that the immune system, in marine bivalves like in mammals (Ahmed, 2000; Okai et al., 2004), represents a significant target for the action of environmental estrogens, thus supporting the importance of investigating full range responses to estrogenic chemicals in ecologically relevant invertebrate species.

Acknowledgment

The Authors thank Prof. John Sumpter, of the Brunel University (London, U.K.) for his critical reading of the manuscript.

References

Andersen, H.R., Andersson, A.M., Arnold, S.F., Autrup, H., Barfoed, M., Beresford, N., Bjerregaard, A., Christiansen, P., Gissel, L.B., Hummel, B., Jorgensen, R., Korsgaard, E.B., Le, B., Guevel, R., Leffers, H., McLachlan, J., Moller, A., Nielsen, J.B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K.L., Perez, P., Skakkeboek, N.E., Sonnenschein, C., Soto, A.M., et al., 1999. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Environ. Health Perspect. 107 (Suppl. 1), 89–108.

Ahmed, S.A., 2000. The immune system as a potential target for environmental estrogens (endocrine disrupters): a new emerging field. Toxicology 156, 191–206.

Atkinson, S., Atkinson, M.J., Tarrant, A.M., 2003. Estrogens from sewage in coastal marine environments. Environ. Health Perspect. 111, 531–535.

Betti, M., Ciacci, C., Lorusso, L.C., Canonico, B., Falcioni, T., Gallo, G., Canesi, L., 2006. Effects of tumor necrosis factor alpha (TNFalpha) on *Mytilus* hemocytes: role of stress-activated MAP kinases. Biol. Cell. 98, 233–244.

Blaise, C., Gagne, F., Pellerin, J., Hansen, P.D., 1999. Determination of vitellogenin-like properties in *Mya arenaria* hemolymph (Saguenay Fijord, Canada): a potential biomarker for endocrine disruption. Environ. Toxicol. 14, 455–465.

Blaise, C., Trottier, S., Gagne, F., Lallement, C., Hansen, P.-D., 2002. Immunocompetence of bivalve hemocytes as evaluated by a miniaturised phagocytosis assay. Environ. Toxicol. 17, 160–169.

Braga, O., Smythe, G.A., Schafer, A.I., Feitz, A.J., 2005. Steroid estrogens in ocean sediments. Chemosphere 61, 827–833.

Brian, J.V., Harris, C.A., Scholze, M., Backhaus, T., Booy, P., Lamorea, M., Pojana, G., Jonkers, N., Runnalls, T., Bonfà, A., Marcomini, A., Sumpter, J.P., 2005. Accurate prediction of the response of freshwater fish to a mixture of estrogenic chemicals. Environ. Health Perspect. 113, 721–728.

Burlando, B., Marchi, B., Panfili, I., Viarengo, A., 2002. Essential role of Ca^{2+}-dependent phospholipase A2 in estradiol-induced lysosome activation. Am. J. Physiol. Cell Physiol. 283, C1461–C1468.

Canesi, L., Gavioli, M., Pruzzo, C., Gallo, G., 2002a. Bacteria-hemocyte interactions and phagocytosis in marine bivalves. Microsc. Res. Technol. 57, 469–476.

Canesi, L., Betti, M., Ciacci, C., Scarpato, A., Citterio, B., Pruzzo, C., Gallo, G., 2002b. Signaling pathways involved in the physiological response of mussel hemocytes to bacterial challenge: the role of stress-activated p38 MAPK. Dev. Comp. Immunol. 26, 325–334.

Canesi, L., Ciacci, C., Betti, M., Scarpato, A., Citterio, B., Pruzzo, C., Gallo, G., 2003. Effects of PCB congeners on the immune function *of Mytilus* hemocytes: alterations of tyrosine kinase-mediated cell signaling. Aquat. Toxicol. 63, 293–306.

Canesi, L., Ciacci, C., Betti, M., Lorusso, L.C., Marchi, B., Burattini, S., Falcieri, E., Gallo, G., 2004a. Rapid Effect of 17beta-estradiol on cell signaling and function of *Mytilus* hemocytes. Gen. Comp. Endocrinol. 136, 58–71.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Gallo, G., 2004b. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. Gen. Comp. Endocrinol. 138, 58–69.

Canesi, L., Betti, M., Ciacci, C., Lorusso, L.C., Gallo, G., Pruzzo, C., 2005a. Interactions between *Mytilus* hemocytes and different strains of *Escherichia coli* and *Vibrio cholerae* O1 E1 Tor: role of kinase-mediated signalling. Cell. Microbiol. 7, 667–674.

Canesi, L., Betti, M., Lorusso, L.C., Ciacci, C., Gallo, G., 2005b. 'In vivo' effects of Bisphenol A in *Mytilus* hemocytes: modulation of kinase-mediated signalling pathways. Aquat. Toxicol. 71, 73–84.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Gallo, G., 2005c. Effects of the brominated flame retardant Tetrabromobisphenol-A (TBBPA) on cell signaling and function of *Mytilus* hemocytes: involvement of MAP kinases and protein kinase C. Aquat. Toxicol. 75, 277–287.

Canesi, L., Ciacci, C., Lorusso, L.C., Betti, M., Guarnieri, T., Tavolari, S., Gallo, G., 2006. Immunomodulation by 17β-Estradiol in bivalve hemocytes. Am. J. Physiol. Regul. Integr Comp. Physiol. 291, R664–R673.

Chu, F.L.E., La Peyre, J.F., 1989. Effect of environmental factors and parasitism on hemolymph lysozyme and protein of American oysters (*Crassostrea virginica*). J. Invert. Pathol. 54, 224–232.

Di Cosmo, A., Di Cristo, C., Paolucci, M., 2002. An estradiol-17β receptor in the reproductive system of the female of *Octopus vulgaris*: characterization and immunolocalization. Mol. Reprod. Dev. 61, 367–375.

Gagnè, F., Blaise, C., 2003. Effects of municipal effluents on serotonin and dopamine levels in the freshwater mussel *Elliptio complanata*. Comp. Biochem. Physiol. 136C, 117–125.

Gagnè, F., Blaise, C., Hellou, J., 2004. Endocrine disruption and health effects of caged mussels, *Elliptio complanata*, placed downstream from a primary-treated municipal effluent plume for 1 year. Comp. Biochem. Physiol. 138C, 33–44.

Gagnè, F., Blaise, C., Salazar, M., Salazar, S., Hansen, P.D., 2001. Evaluation of estrogenic effects of municipal effluents to the freshwater mussel *Elliptio complanata*. Comp. Biochem. Physiol. 128C, 213–225.

Gauthier-Clerc, S., Pellerin, J., Amiard, J.-C, 2005. Estradiol-17beta and testosterone concentrations in male and female *Mya arenaria* (Mollusca bivalvia) during the reproductive cycle. Gen. Comp. Endocrinol. 145, 133–139.

Gauthier-Clerc, S., Pellerin, J.,Fournier, M., Amiard, J.-C, in press. Immunological and biochemical responses in *Mya arenaria* (Mollusca, Bivalvia) exposed in vivo to estradiol-17β. Comp. Biochem. Physiol., partC. doi:10.1016/cbpc.2006.08.008.

Gutendorf, B., Westendorf, J., 2001. Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens, and xenoestrogens. Toxicology 166, 79–89.

Janer, G., Lavado, R., Thibaut, R., Porte, C., 2005a. Effects of 17β-estradiol exposure in the mussel *Mytilus galloprovincialis*: a possible regulating role for steroid acyltransferases. Aquat. Toxicol. 75, 32–42.

Janer, G., Msia-Vela, S., Le Blanc, G.A., Porte, C., Kauffman, F.C., 2005b. Esterification of vertebrate-type steroids in the Eastern oyster (*Crassostrea virginica*). Steroids 69, 129–136.

Jobling, S., Casey, D., Rogers-Gray, T., Oehlmann, J., Schulte-Ohelmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2003. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 66, 207–222.

Kajiwara, M., Kuraku, S., Kurokawa, T., Kato, K., Toda, S., Hirose, H., Takahashi, S., Shibata, Y., Iguchi, T., Matsumoto, T., Miyata, T., Miura, T., Takahashi, Y., 2006. Tissue preferential expression of estorgen receptor gene in the marine snail, *Thais clavigera*. Gen. Comp Endocrinol. 148, 315–326.

Keay, J., Bridgham, J.T., Thornton, J.W., 2006. The *Octopus vulgaris* estrogen receptor is a constitutive transcriptional activator: evolutionary and functional implications. Endocrinology 147, 3861–3869.

Le Curieux-Belfond, O., Fievet, B., Seralini, G.F., Mathieu, M., 2005. Short-term bioaccumulation, circulation and metabolism of estradiol-17beta in the oyster *Crassostrea gigas*. J. Exp. Mar. Biol., Ecol. 325, 125–133.

Lösel, R.F., Falkestein, E., Feuring, M., Schultz, A., Tillmann, H.-C, Rossol-Haseroth, K., Wehling, M., 2003. Non genomic steroid action: controversies, questions, and answers. Physiol. Rev. 83, 965–1016.

Lowe, D.M., Fossato, V.U., Depledge, M.H., 1995. Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: an in vitro study. Mar. Ecol. Prog. Ser. 129, 189–196.

McLachlan, J.A., 2001. Environmental signalling: what embryos and evolution teach us about endocrine disrupting chemicals. Endocr. Rev. 22, 319–341.

Moore, M.N., Lowe, D.M., Fieth, P.E., 1978. Responses of lysosomes in the digestive cells of the common mussel, *Mytilus edulis*, to sex steroids and cortisol. Cell Tissue Res. 188, 1–9.

Ohelmann, J., Schulte-Ohelmann, U., 2003. Endocrine disruption in invertebrates. Pure Appl. Chem. 75, 2207–2218.

Ohelmann, J., Schulte-Ohelmann, U., Bachmann, J., Oetken, M., Lutz, I., Kloas, W., Ternes, T.A., 2006. Bisphenol A induces superfeminsation in the ramshorn snail *Marisa cornuarietis* (Gastropoda: prosobrnachia) at environmental relevant concentrations. Environ. Health Perspect. 114, 127–133.

Okai, Y., Sato, E.F., Higashi-Okai, K., Inoue, M., 2004. Enhancing effect of the endocrine disruptor *para*-nonylphenol on the generation of reactive oxygen species in human blood neutrophils. Environ. Health Perspect. 112, 553–556.

Ortiz-Zarragoitia, M., Cajaraville, M.P., 2006. Biomarkers of exposure and reproduction-related effects in mussels exposed to endocrine disruptors. Arch. Environ. Contam. Toxicol. 50, 361–369.

Osada, M., Takamura, T., Sato, H., Mori, K., 2003. Vitellogenin synthesis in the ovary of the scallop, *Patinopecten yessoensis*: control by estradiol-17β and the central nervous system. J. Exp. Zool. 299A, 172–179.

Osada, M., Tawarayama, H., Mori, K., 2004. Estrogen synthesis in relaton to gonadal development of Japanese scallop *Patinopecten yessoensis*: gonadal profile and immunolocalization of P450 aromatase and estrogen. Comp. Biochem. Physiol. 139B, 123–128.

Peck, M., Gibson, R.W., Kortenkamp, A., Hill, E.M., 2004. Sediments are major sinks of steroidal estrogens in two United Kingdom rivers. Environ. Toxicol. Chem. 23, 945–952.

Pipe, R., Coles, J.A., Farley, S.R., 1995. Assays for measuring immune response in the mussel *Mytilus edulis*. In: Stolen, J., Fletcher, T.C., Smith, S.A., Zelikoff, J.T., Kaattari, S.L., Anderson, R.S. (Eds.), Techniques in Fish Immunology: Fish Immunology Technical Communications, Immunological and Pathological Techniques of Aquatic Invertebrates, 4. SOS Publications, Fair Haven, NJ, pp. 93–100.

Pojana, G., Bonfà, A., Busetti, F., Collarin, A., Marcomini, A., 2004. Estrogenic potential of the Venice, Italy, Lagoon waters. Environ. Toxicol. Chem. 23, 1874–1880.

Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: Approaches and perspectives. Comp. Biochem. Physiol. 143C, 303–315.

Puinean, A.M., Labadie, P., Hill, E.M., Osada, M., Kishida, M., Nakao, R., Novillo, A., Callard, I.P., Rotchell, J.M., 2006. Laboratory exposure to 17β-estradiol fails to induce vitellogenin and estrogen receptor gene expression in the marine invertebrate *Mytilus edulis*. Aquat. Toxicol. 79, 376–383.

Pruzzo, C., Gallo, G., Canesi, L., 2005. Persistence of vibrios in marine bivalves: the role of interactions with hemolymph components. Environ. Microbiol. 7, 761–772.

Quinn, B., Gagnè, F., Costello, M., McKenzie, C., Wilson, J., Mothersill, C., 2004. The endocrine disrupting effect of municipal effluent on the zebra mussel (*Dreissena polymorpha*). Aquat. Toxicol. 66, 279–292.

Rajapakse, N., Silva, E., Kortenkamp, A., 2002. Combining xenoestrogens at levels below individual no-observed-effect concentrations dramatically enhances steroid hormone action. Environ. Health Perspect. 110, 917–921.

Reis-Henriques, M.A., Le Guellec, D., Remy-Martin, J.P., Adessi, G.L., 1990. Studies on endogenous steroids from the marine mollusc *Mytilus edulis* L. by gas chromatography and mass spectrometry. Comp. Biochem. Physiol. 95B, 303–309.

Riffeser, M., Hock, B., 2002. Vitellogenin levels in mussel hemolymph—a suitable marker for the exposure for estrogens? Comp. Biochem. Physiol. 132C, 75–84.

Roepke, T.A., Snyder, M.J., Cherr, G.N., 2005. Estradiol and endocrine disrupting compounds adversely affect development of sea urchin embryos at environmentally relevant concentrations. Aquat. Toxicol. 71, 155–173.

Rotchell, J.M., Ostrander, G.K., 2003. Molecular effects of endocrine disrupters in aquatic organisms. J. Toxicol. Environ. Health 6B, 453–495.

Sumpter, J.P., Johnson, A.C., 2005. Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. Environ. Sci. Technol. 39, 4321–4332.

Thornton, J.W., Need, E., Crews, D., 2003. Resurrecting the ancestral steroid receptor: ancient origin of estrogen signalling. Science 301, 1714–1717.

Thorpe, K.L., Cummings, R.I., Hutchinson, T.H., Scholze, M., Bright, G., Sumpter, J.P., Tyler, C.R., 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37, 1142–1149.

Verslycke, T.A., Fockedey, N., Mckenney Jr., C.L., Roast, S.D., Jones, M.B., Mees, J., Janssen, C.R., 2004. Mysid crustaceans as potential test organisms for the evaluation of environmental endocrine disruption: a review. Environ. Toxicol. Chem. 23, 1219–1234.