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Neuroendocrine disruption and health effects in *Elliptio complanata* mussels exposed to aeration lagoons for wastewater treatment

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Abstract

The purpose of this study was to examine neuroendocrine-disrupting effects of two domestic wastewater aeration lagoons on freshwater mussels. Mussels were caged and placed in two final aeration lagoons for treating domestic wastewaters for 60 days, at a site 1 km downstream of the dispersion plume on the eastern shores of the Richelieu River; the western shore served as the reference site. The mussels were analysed for gonad activity, oxidative metabolism of xenobiotics, stress biomarkers and neuroendocrine status (monoamine and arachidonic acid metabolism). The domestic wastewaters produced many different effects at all levels examined. The gonado-somatic index and vitellogenin-like proteins were significantly induced in both aeration lagoons and gonad pyrimidine synthesis (aspartate transcarbamylase activity) was significantly reduced, indicating that vitellogenin-like proteins were produced while DNA synthesis in gametes remained constant. Biomarkers of oxidative metabolism revealed that global heme oxidase (HO), glutathione S-transferase and xanthine (caffeine) oxydoreductase (XOR) activities were significantly induced in at least one of the aeration lagoons, but not downstream of the dispersion plume. The activities of 7-ethoxyresorufin (cytochrome P4501A1), dibenzoylfluorescein (cytochrome P450 3A4 and 3A5) and benzoyloxyresorurufin (cytochrome P450 3A4 and 2B6) dealkylases were readily induced by substances sharing structural similarities with coplanar polyaromatic hydrocarbons and hydroxylated or aminated aromatic or cyclic hydrocarbon compounds such as pharmaceuticals or steroids in the domestic wastewaters. Biomarkers of toxic stress revealed that exposure to aeration lagoons led to increased production of metallothioneins, lipid peroxidation and DNA strand breaks, with decreased heme oxygenase activity. LPO was significantly correlated with XOR, HO and cytochrome P4501A1 activities. Neuroendocrine effects included significant increases in dopamine and serotonin levels and in monoamine oxidase (MAO). Dopamine transport in synaptosome was significantly increased while serotonin transport activity was significantly decreased, suggesting the mussels were in a state of serotonergicity. Moreover, arachidonic acid cyclooxygenase (COX) activity was also readily increased in one aeration lagoon. Aeration lagoons for the treatment of domestic wastewaters are toxic, estrogenic and disrupt the metabolism of monoamines and COX in freshwater mussels.

Keywords: Domestic effluents; Mussels; Neuro-endocrine disruption; Biomarkers

1. Introduction

Municipal effluents are recognized as important sources of pollution for the aquatic ecosystem. They are reported to have a variety of endocrine-disrupting properties, including estrogenicity and serotonergicity (Sumpter and Jobling, 1995; Gagné et al., 2001; Gagné and Blaise, 2003; Vethaak et al., 2005). More recently, pharmaceutical and personal care products (PPCPs) were found in municipal effluents, where they represent a major source of contamination to the aquatic environment (Kummerer, 2001;

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Andreozzi et al., 2003). Municipal effluents contain a number of PPCPs, including non-prescription drugs (ibuprofen and aspirin), antibiotics (sulphonamides and macrolides), cholesterol-regulating drugs (clofibrates and statins), β-blockers (propanolol), neuroactive drugs (caffeine, carbamazepine, fluoxetine and morphine), perfumes (musks) and miscellaneous medical contrasting agents. Mussels are at high risk of exposure to such contamination because these benthic bivalves are sedentary, they live at the sediment/water interface and they filter high volumes of water, including suspended material and colloids. For example, mussels placed in the immediate area of a municipal effluent outfall were shown to be contaminated by coprostanol, produced by coliform bacteria, and alkylphenol polyethoxylates (Sabik et al., 2003; Hellou et al., 2006). No biomarkers have yet been developed to measure the early biological effects of PPCPs in the field. However, two types of biomarkers were recently proposed to measure PPCP effects in field studies (Gagné and Blaise, 2004). The first are biomarkers encompassing the effects of drugs with no assumption of their mechanism of action and specificity. For example, lipid peroxidation is often induced by the oxidative metabolism of various drugs, like many other compounds (e.g. metals and pesticides). The second class of biomarkers are those that are related to the mode of action of the drug, with possible profound effects on an organism's survival, immune function and reproduction. For example, serotonin reuptake inhibitors (for the treatment of depression in humans) could exacerbate the spawning process, which depends on serotonin signalling (Fong, 1998).

Mussels have the capacity to biotransform foreign organic chemicals such as phase I and II biotransformation enzymes, but with a much lower potential for phase I reactions than in vertebrates. Nevertheless, high activity in glutathione S-transferase (GST, a phase II conjugation enzyme) was detected in bivalves whose activity/expression is inducible by many organic contaminants, including 5–6 ring PAHs and chlorinated aromatic hydrocarbons (Gowland et al., 2002). *Mytilus galloprovincialis* mussels were shown to contain immunoreactive cytochromes P450 of the CYP1A, CYP2B, CYP2E, CYP3A and CYP4A families in microsomes of the digestive gland (Peters et al., 1998). In mammals, pharmaceuticals are processed by five major cytochrome P450 families: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Crespi et al., 1997). CYP3A and CYP2C are considered the most important drug-metabolizing enzymes. However, aquatic species such as bivalves and fish appear to lack the CYP2C family. The lack of cytochrome P4502C in bivalves or fish would limit the elimination of drugs such as barbiturates (phenobarbital), trimipramine (antidepressant), sulfaphenazole and sildenafil or Viagra™ (Richardson and Bowron, 1985; Kirchheiner et al., 2003; Marques-Soares et al., 2003). Biomarkers of toxic stress and tissue damage were assessed by metallothioneins (MT), heme oxygenase (HO), lipid peroxidation (LPO), and DNA strand breaks. The above biomarkers are considered as early warning systems of exposure to metals and organic compounds.

The freshwater mussel *Elliptio complanata* is a sexually dimorphic ovoviviparous invertebrate that broods eggs containing yolk proteins rich in vitellogenins (Vtg). The synthesis of Vtg was shown to be controlled, at least partially, by β-estradiol in mussels (Gagné et al., 2001) and in oysters (Li et al., 1998). In a more recent study, the metabolism of dopamine and serotonin in nerve tissues was disrupted in mussels exposed to a municipal wastewater dispersion plume (Gagné and Blaise, 2003). Monoamines (serotonin, dopamine) are important mediators of gamete maturation and spawning, the latter is activated by serotonin and prostaglandin released by the induction of arachidonic acid cyclooxygenase or COX (Matsutani and Nomura, 1987). The measurement of gonadal dopamine and serotonin was proposed to assess the stages of gametogenesis from early vitellogenesis to spawning (Pani and Croll, 1998; Gagné and Blaise, 2003). Indeed, spawning activity reduces dopamine levels but increases serotonin in oyster gonad, while estradiol-17β (during gametogenesis) follows the level of dopamine in the gonad of the same species (Osada and Nomura, 1989).

The objective of this study was to examine the toxicological effects of two final aeration lagoons for treatment of wastewater on freshwater mussels undergoing gametogenesis. *E. complanata* mussels, endemic to the study area, were caged and placed in the aeration lagoons and also at a site downstream of the dispersion plume to examine the impact of domestic wastewater on general health status, oxidative metabolism, biomarkers of stress and tissue damage. In addition, the endocrine status of gametogenesis was studied by tracking variations in Vtg, monoamines (dopamine and serotonin) and arachidonic acid cyclooxygenase. An attempt was made to relate the neuroendocrine effects to those of tissue damage induced by exposure to domestic wastewaters.

2. Methods

2.1. Mussel collection and exposure to aeration lagoons

E. complanata mussels were collected by hand in the Richelieu River (Quebec, Canada) in late May, during their period of late gametogenesis. They were placed in aerated tanks for two weeks at 15 °C and fed with *Selenastrum capricornutum* algal preparations (5–10 million cells). The mussels were then caged at $N = 40$ mussels per cage according to Applied Biomonitoring's procedure (Salazar and Salazar, 2001). The cages were then submerged at a depth of two metres inside two final aeration lagoons (AL1, AL2) for the treatment of domestic wastewaters; the effluent dispersion plume of AL1 was located on the eastern shore of the Richelieu River at a site 1 km downstream the point of discharge of the last aeration lagoon, while a site on the western shore of the river was considered as the reference (Fig. 1). The aeration lagoon AL2 is a fur-

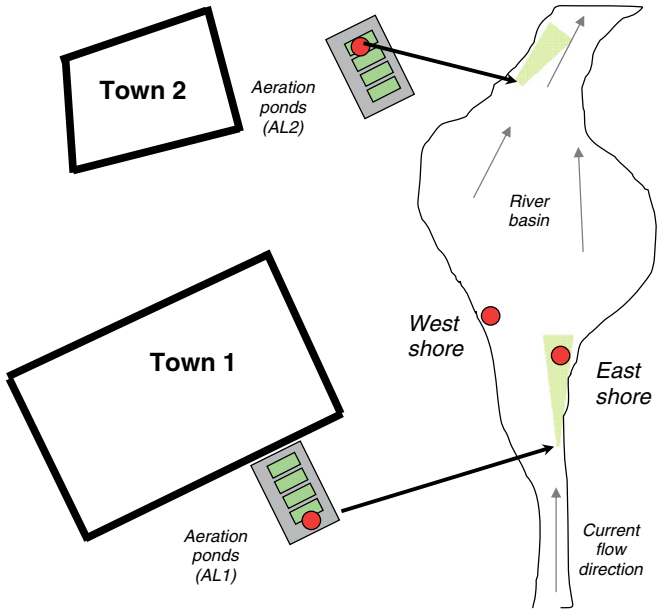

Fig. 1. Map of the study sites. The aeration ponds are identified for townships 1 and 2. The red dots represent the sites where mussels were caged. Town 1 or AL1 was located about 10 km upstream AL2 in the River. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ther 10 km downstream. The mussels were exposed for 60 days (July to the end of August) in the summer of 2004. The lagoons treat (essentially) domestic wastewater from two towns (population 20 000 and 12 000 each) with no large industrial-source inputs. The type of wastewater treatment is considered biological consisting in four interconnected series of lagoons gradually degrading wastewater by a combination of aeration (with hydraulic pumps), photodegradation by natural sunlight and by endogenous bacterial and algal communities. In some cases, particles were flocculated by the addition of ferric solutions to the first aeration lagoon. After the exposure period, the cages were removed and the mussels were allowed to depurate in clean dechlorinated water for 24 h at 15 °C. Mussels were then dissected for the gills, digestive gland and gonads (including nerve ganglia) and stored at −85 °C. For biomarker measurements, tissues were thawed on ice and then homogenized in ice-cold 10 mM Hepes-NaOH buffer, pH 7.4, containing 100 mM NaCl, 0.1 mM EDTA and 0.1 mM dithiothreitol. The tissues were homogenized using a Teflon pestle tissue grinder and centrifuged first at 3000*g* for a sub-sample of the supernatant for synaptosome preparation (dopamine and serotonin transport activity) in the gonad and then at 15 000*g* for 20 min at 2 °C to obtain the supernatant (S15 fraction). Mussel soft tissues were also collected ($N = 8$ animals) for total silver (Ag), aluminium (Al), arsernic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb) and zinc (Zn) analyses by ion-coupled plasma atomic emission spectroscopy as described elsewhere (Gagnon et al., 2006; National Laboratory for Environmental Testing, 1994).

2.2. Gonad activity assessment

Gonad activity was studied in the following endpoints: the gonado-somatic index (GSI; weight of gonad/soft tissue weight), the relative levels of vitellogenin-like proteins (Vtg) and the rate-limiting enzyme aspartate transcarbamylase (ATC) activity for pyrimidine synthesis. Sex was determined by microscopic examination of gonad smears at 400 × enlargement. The presence of Vtg in gonad was determined by the organic alkali-labile phosphate (ALP) technique (Gagné et al., 2001) using the acetone fractionation procedure. Briefly, the gonad homogenate 15 000*g* supernatant was mixed with ice-cold acetone to yield a 30% final concentration and centrifuged at 10 000*g* for 5 min at 4 °C. The pellet was dissolved in NaOH 1 M at 60 °C for 30 min to release inorganic (alkali-labile) phosphates. These were determined according to the phosphomolybdenum method developed by Stanton (1968). Vtg-like protein levels were expressed as μg of alkali-labile phosphate (ALP)/mg of gonad protein. Gamete activity was determined by measuring the activity of ATC in the S15 fraction of gonad homogenate (Mathieu, 1987) using the spectrophotometric detection method for phosphate measurements in the presence of labile carbamoylphosphate (Herries, 1967). The data were expressed as ug phosphate/min/mg proteins. Total proteins were determined by the method of Bradford (1976).

2.3. Xenobiotic metabolism

Phase I and II biotransformation activity in the digestive gland was determined by examining the following activities: hemoprotein oxidase (HO), 7-ethoxyresorufin (EROD), benzyl resorufin ether (BzRes), dibenzylfluorescein dealkalases (DBF dealkylase), GST and xanthine oxidoreductase (XOR) activities in the S15 fraction of digestive gland homogeanates. HO, ERDOD, BzRes and DBF dealkylases activities were determined as described elsewhere (Quinn et al., 2004). Xanthine oxidoreductase (XOR) activity, implicated in the oxidative catabolism of xanthines, was determined by the method described by Zhu et al. (1994). Glutathione S-transferase (GST) was determined by the spectrophotometric method of Boryslawskyj et al. (1988) using 2,4-dichloronitrobenzene as the substrate. Data are expressed as increase in absorbance at 412 nm/min/mg proteins.

2.4. Biomarkers of toxic stress

Lipid peroxidation (LPO) was also measured according to the thiobarbituric acid method of Wills (1987). Thiobarbituric acid reactants (TBARS) were determined by fluorescence at 520 nm for excitation and 590 nm for emission using a fluorescence microplate reader (Dynatech, Fluorolite-1000). Because the reagent could react with other aldehydes, results were expressed as μg of TBARS/mg of homogenate protein. Metallothionein (MT) levels

were determined in the digestive gland of collected clams by the thiol spectrophotometric assay (Viarengo et al., 1997). The MT fractionation and assay procedure was performed on the 15 000*g* supernatant. Standards of reduced glutathione (GSH) were used for calibration. The data are expressed as μmol of GSH equivalents per mg protein. DNA damage (double and single DNA strand breaks) was assessed in gills, digestive glands and gonad by means of an alkaline precipitation assay (Olive, 1988) with fluorescence quantification of DNA strands in the presence of trace amounts of detergents (Bester et al., 1994). The assay principle is based on K-assisted SDS precipitation of genomic DNA (associated with proteins), which leaves protein-free DNA breaks (double and single strands) in the supernatant. Salmon sperm DNA standards were added for DNA calibration. The results were expressed as μg of supernatant DNA/mg of proteins. Heme oxygenase activity was determined according to the formation of bilirubin at 460 nm using hematin as the substrate (Sunderman, 1987). An aliquot sample of the S15 fraction of the digestive gland was mixed with 100 μM NAPDH, 10 μM hemin in the 50 mM potassium phosphate buffer at pH 7.4. Absorbance readings at 460 nm were taken at 10 min intervals for 60 min at 30 °C. Data were expressed as change in absorbance at 460 nm/(min × mg proteins).

2.5. Neuroendocrine assessments

Neuroendocrine characterizations of gonad tissues were assessed by measuring the following parameters: serotonin, dopamine with corresponding ATP-dependent synaptosome transport activity (a measure of synaptic reuptake), monoamine oxidase (MAO) and COX activity measurements. The gonad (containing nerve ganglia) homogenates were centrifuged at 3000*g* for 15 min at 2 °C. The supernatant was overlaid on 0.8 M sucrose containing 10 mM Hepes-NaOH, pH 7.4, and 1 mM EDTA, and centrifuged at 10 000*g* for 20 min at 4 °C to separate the synaptosomes (in the 0.8 M sucrose phase) from mitochondria (pellet). The synaptosomes were immediately mixed with two volumes of distilled water to prevent osmotic shearing of membranes. Monoamine (serotonin and dopamine) levels were measured in gonad homogenates using fluorescence-based methodologies (Orsinger et al., 1980; Szabo et al., 1983). For serotonin and dopamine determinations, a sub-sample of homogenates was mixed with one volume of n-butanol containing 0.1 N HCl and 0.2 mM reduced GSH. The mixture was centrifuged at 2000*g* for 15 min to remove precipitated material, and the supernatant was extracted once with one volume of hexane. For serotonin, the aqueous phase was mixed with one volume of *o*-phthalaldehyde in 2 N HCl and heated to 70 °C for 15 min. Fluorescence was measured at 360 nm excitation and 480 nm emission. For dopamine, the aqueous phase was mixed with 0.1 volume of alkaline iodide solution (I_2/KI, Lugol, Sigma Chemical Co.), heated at 75 °C for 5 min and fluorescence was measured at 325 nm excitation and 385 nm emission. Standards of serotonin and dopamine were used for calibration. Data were expressed as ng of monoamine/mg proteins. Serotonin and dopamine transport in the presence of ATP was determined in synaptosomes using the liberation of inorganic phosphate from ATP methodology (Kakko et al., 2003). The levels of inorganic phosphates liberated from ATP hydrolysis were determined using the phosphomolybdate assay (Stanton, 1968). MAO activity was determined using the serotonin analogue tryptamine as the substrate. Mitochrondria (resuspended in 125 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4 to give 0.1–0.5 mg/ml proteins) were incubated in the presence of 2 μM dichlorofluorescin, 100 μM tryptamine in 10 mM Hepes-NaOH, pH 7.4, containing 125 mM NaCl, 0.1 M aminotriazole (a catalase inhibitor) and 1 μg/ml of horseradish peroxidase (Sigma-Aldrich Co., USA). The reaction was allowed to proceed at 30 °C for 0, 15, 30 and 60 min. Fluorescence was measured at 485 nm excitation and 520 nm emission. Enzyme activity was expressed as nmole of fluorescein formed/(min × mg proteins). Standard solutions of fluorescein were used for calibration. Finally, cyclooxygenase (COX) activity was measured by the oxidation of 2,7-diclhorofluorescin in the presence of arachidonate (Fujimoto et al., 2002). Briefly, 50 μl of gonad 15 000*g* supernatant were mixed in 200 μl of 50 μM arachidonate, 2 μM dichlorofluorescin and 0.1 μg/ml of horseradish peroxidase in 50 mM Tris–HCl buffer, pH 8, containing 0.05% Tween 20. The reaction mixture was incubated for 0, 10, 20 and 30 min at 30 °C, and fluorescence was measured at 485 for excitation and 520 for emission (Dynatech, Fluorolite 1000). The data were expressed as increase in relative fluorescence units/(min × mg proteins).

2.6. Data analysis

For each determination, a replicate number of $n = 10$ mussels was used for every aeration lagoon and river sites (eastern and western shore). Data were analysed using a two-way analysis of variance (two-way ANOVA) with gender and sites as the effect variables after verifying for homogeneity of variance and normality in the data distribution with Bartlett's test. If the data proved not to be parametric, then they were analysed using rank-based ANOVA. Critical differences in controls were appraised using the ranked-based Mann–Whitney *U* test. A correlation analysis was performed according to the Pearson-moment correlation test. Significance was set at $p < 0.05$.

3. Results

Mussels were caged in two aeration lagoons, dubbed AL1 and AL2, downstream of the dispersion plume of AL1 (East shore) and compared to the reference site located on the western shore (Fig. 1). The pH of the last aeration lagoon was roughly one to two pH units below that of the receiving water: pH 6.1–7.5 (Table 1). The temperature of the aeration lagoons, taken every 15 min using temperature

Table 1
Study site characteristics

Site	Population[a]	pH/conductivity (μS cm^{-1})	Temperature changes	
			Minimum–maximum	Mean ± SD
Aeration lagoon 1 (AL1)	20 342	6.1–7.1/400–800	7.6–27.4	23 ± 2
Aeration lagoon 2 (AL2)	12 395	7–7.5/600–900	14–29	23 + 1.5
Western shore (reference)	–	8.1–8.6/180	8.5–25.7	23 ± 1.5
Eastern shore (1 km downstream of the plume)	–	8.1–8.6/180	NA[b]	

[a] According to the last 2001 census (Statistics Canada).
[b] NA: not analysed.

probes, fluctuated widely, from about 8 to 27.4 °C, but only for short periods of time. However, the mean temperature was the same (was not significantly different), 23 °C, for each lagoon and also downstream of the plume. At the end of the exposure period, mortality events were observed at AL1 (30% mortality) and AL2 (45% mortality), but no mortality at the eastern shore (within the plume) and reference (western shore) sites. The condition factor (total weight/shell length) and soft tissue ratio (tissue weight/total weight) were only reduced at AL2 (results not shown). Gonad activity was examined in mussels by measuring the GSI and pyrimidine biosynthesis by ATC activity and Vtg-like proteins (Fig. 2). The GSI was significantly increased in both aeration lagoons, but not in the plume along the eastern shore. The rate-limiting enzyme ATC for pyrimidine synthesis of gamete formation was significantly reduced at site AL2 only. The egg yolk protein Vtg was significantly induced at all sites including the site downstream of the dispersion plume (eastern shore). Vtg induction was more strongly induced in males, suggesting (two-way ANOVA for site and gender $p < 0.05$) the presence of xeno-oestrogens in the aeration lagoons.

The influence of the aeration lagoons on xenobiotic (oxidative) metabolism was investigated in mussels by following HO, phase I biotransformation mono-oxygenases, XOR and glutathione S-transferase activities (Fig. 3). Mono-oxygenases were examined by EROD (CYP1A1), DBF (CYP 3A4 and 3A5), BzRes (CYP3A4 and 2B6) dealkylase activities. An ANOVA revealed that total HO activity was significantly induced at site AL1 only (1.4-fold induction). However, the mono-oxygenases tested were all significantly induced at all sites, including the downstream site on the eastern shore for BzRes dealkyase. This enzyme activity exhibited the highest responses with respect to the western shore, with 12.5-, 23- and 28-fold induction for the downstream site along the eastern shore, AL1 and AL2, respectively. DBF dealkylase was induced 1.33-and 2-fold for AL1 and AL2, respectively. The activity of EROD, a well known marker enzyme for polyaromatic hydrocarbons and dioxins, was also induced 2.8- and 1.6-fold for lagoons AL1 and AL2, respectively. EROD activity at the downstream site (eastern shore) was somewhat reduced but at the $p = 0.08$ level (Mann–Whitney U test). DBF and BzRes dealkylases were significantly correlated ($R = 0.7$; $p < 0.05$). The phase-2 conjugating enzyme GST displayed a more complex pattern of response. Its activity was induced 4-fold at AL2, while its activity at AL1 was significantly reduced by 0.3-fold relative to the reference site (western shore). GST activity at the downstream site was marginally elevated 1.7-fold but at $p = 0.08$ (Mann–Whitney U test). The enzyme XOR, implicated in the oxidative catabolism of xanthines, was significantly induced 1.4-fold at AL1, but significantly reduced 0.7-fold at AL2. Various stress-related biomarkers were examined in mussels exposed to the aeration lagoons (Fig. 4). MT levels in digestive gland were induced 3.7- and 2.8-fold for AL1 and AL2, respectively. Total metal tissue contents of Ag, Al, As, Cd, Cr, Cu, Pb and Zn was also significantly changed through the sites (Table 3). Total Ag, As, Cr, Cu and Zn contents were significantly lower at AL1 in respect to the reference site located at the Western shore. Total Al and Cd contents were significantly increased at AL2 while Cu and Zn in tissues were significantly increased at the downstream site located at the Eastern shore. HO activity was reduced at 0.6- and 0.7-fold in lagoons AL1 and AL2, respectively. LPO in digestive gland was increased 1.4-fold at AL1 while only DNA damage was increased (5.2-fold) at site AL2 alone.

The neuroendocrine biomarkers also revealed many changes in the aeration lagoons (Fig. 5). Increased gonad dopamine (1.2- and 2-fold for AL1 and AL2, respectively) and serotonin (5.5-fold in AL2) levels were observed in aeration lagoons treating domestic wastewaters. Dopamine levels were also significantly elevated 1.6-fold at the site downstream of the dispersion plume on the eastern shore. On the one hand, serotonin transport activity was significantly reduced 3.3-fold in both aeration lagoons and 2.6-fold at the downstream site. On the other hand, dopamine transport activity was significantly elevated at both lagoons (2.25- and 1.6-fold for AL1 and AL2, respectively) and 1.75-fold at the downstream site. MAO activity tended to be higher in the aeration lagoons but was only significant at AL2. At the downstream site, MAO activity was significantly reduced. COX activity was significantly increased at AL2 only.

A correlation analysis was performed on the biomarker data to identify possible relationships between biomarkers of early biological effects or tissue damage and, neuroendocrine perturbations (Table 2). The GSI was significantly correlated with ATC ($R = -0.55$), HO ($R = 0.39$) and serotonin transport activity ($R = -0.39$), suggesting that as the

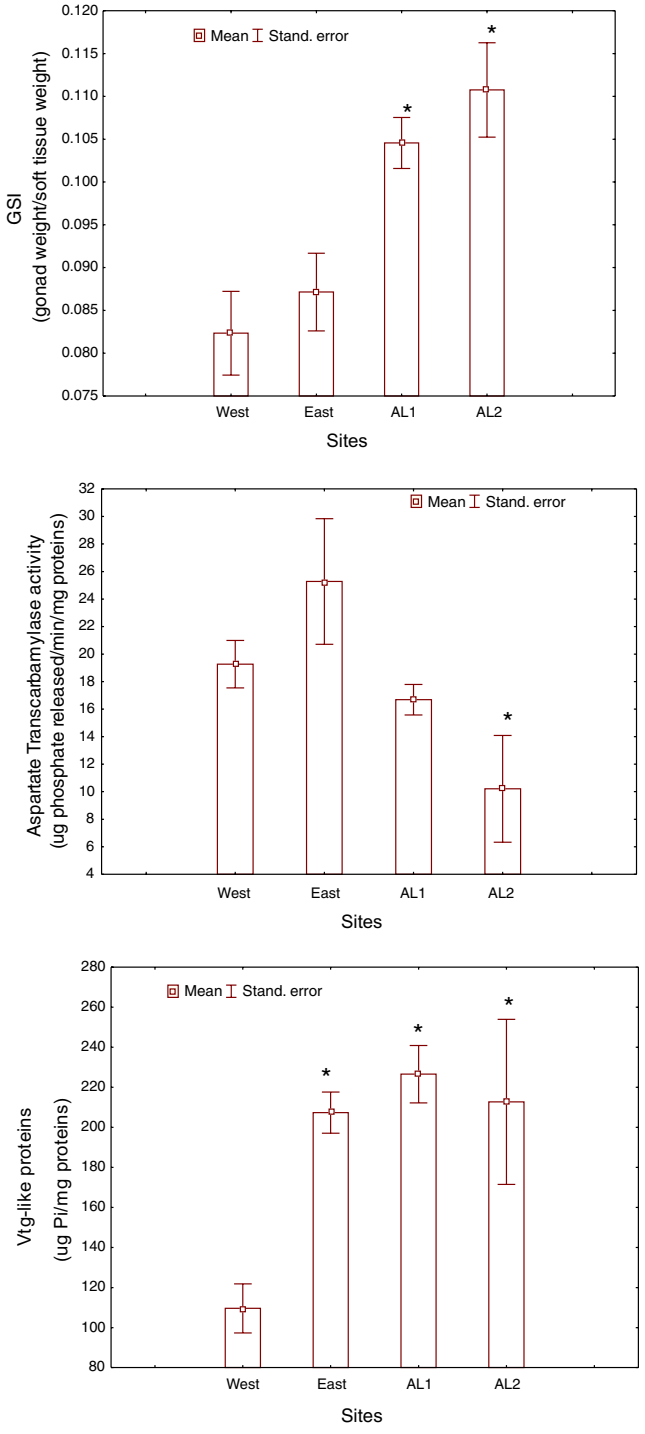

Fig. 2. Gonadal activity in mussels exposed to aeration lagoons. Gonad activity was determined by the GSI, pyrimidine biosynthesis activity (ATC) and Vtg-like proteins of $n = 8$ individuals at each site. Asterisks (*) indicate significance at $p < 0.05$ relative to the western shore.

GSI increases, serotonergic effect, HO also increases and pyrimidine synthesis for the eggs/spermatozoa decreases. The levels of Vtg-like proteins were correlated with DBF and BzRes dealkylases ($R = 0.66$ and 0.6, respectively), DNA strand breaks ($R = 0.49$), serotonin ($R = 0.36$) and dopamine ($R = 0.55$). LPO in digestive gland was correlated with HO ($R = 0.58$), XOR ($R = 0.45$), EROD ($R = 0.37$) and COX activity ($R = -0.53$). DNA damage was correlated with serotonin ($R = 0.52$) and dopamine ($R = 0.46$). A factorial analysis was performed to identify which biomarker explains most of the observed responses (Table 4). The analysis revealed that 61% of the variance was explained by three factors. The biomarkers associated with these factors were: Vtg-like proteins, GSI, DBF dealkylase, EROD and DNA damage for the first factor, HO, GST and LPO for the second factor, and ATC for the last factor. The biomarkers EROD (−0.68), MT (−0.69) and HO (0.68) were close to the 0.7 threshold in factor 1 biomarker group.

4. Discussion

The aeration lagoons treat what is essentially domestic wastewater from towns with no significant industrial inputs. In the near future, additional lagoons will be introduced at the site corresponding to AL1 townships for the treatment of industrial wastewaters. In keeping with the estrogenicity of municipal effluents, gonad levels of Vtg-like proteins were elevated in both lagoons as well as at the downstream site on the eastern shore. This suggests that the ponds release estrogenic compounds in the receiving water, perhaps by estradiol/estrone naturally excreted in mammals and by anticontraceptive drugs (i.e., ethynylestradiol-17β). The potential of municipal wastewaters to induce synthesis of the egg-yolk precursor, Vtg, is well established in fish (Sumpter and Jobling, 1995; Diniz et al., 2005) and, more recently, in freshwater mussel *E. complanata* (Gagné et al., 2001). Interestingly, the synthesis of Vtg-like proteins was correlated with GSI but not with pyrimidine synthesis (ATC activity). The close relationship between GSI and Vtg-like protein was also shown in oysters such that they were dependent on estradiol-17β (Li et al., 1998). The induction of vitellogenesis, however, was not accompanied by the synthesis of pyrimidines (ATC activity), which was actually decreased in AL2, suggesting that the amount of DNA in gametes was not increasing but accumulating Vtg. This could be associated with the observation that the lagoons led to mortality perhaps associated to the harsh exposure conditions (high temperature and conductivity). Another interpretation for this could be associated with the novel biological function of Vtg that was recently proposed for Vtg in fish: as a defence against pathogenic bacteria (Shi et al., 2006). Indeed, *Puntius conchonius* males challenged with *Escherichia coli* synthesize Vtg where it exhibited both antibacterial and hemagglutinating activities *in vitro*. However, it is not known whether bivalve Vtg shares similarities with fish Vtg with respect to its role as a defence mechanism against pathogens. This should be examined further because of the implication that the Vtg biomarker is considered specific to estrogenic signalling by the estrogen receptor.

Oxidative metabolism was readily increased in both aeration lagoons and sometimes in the dispersion plume

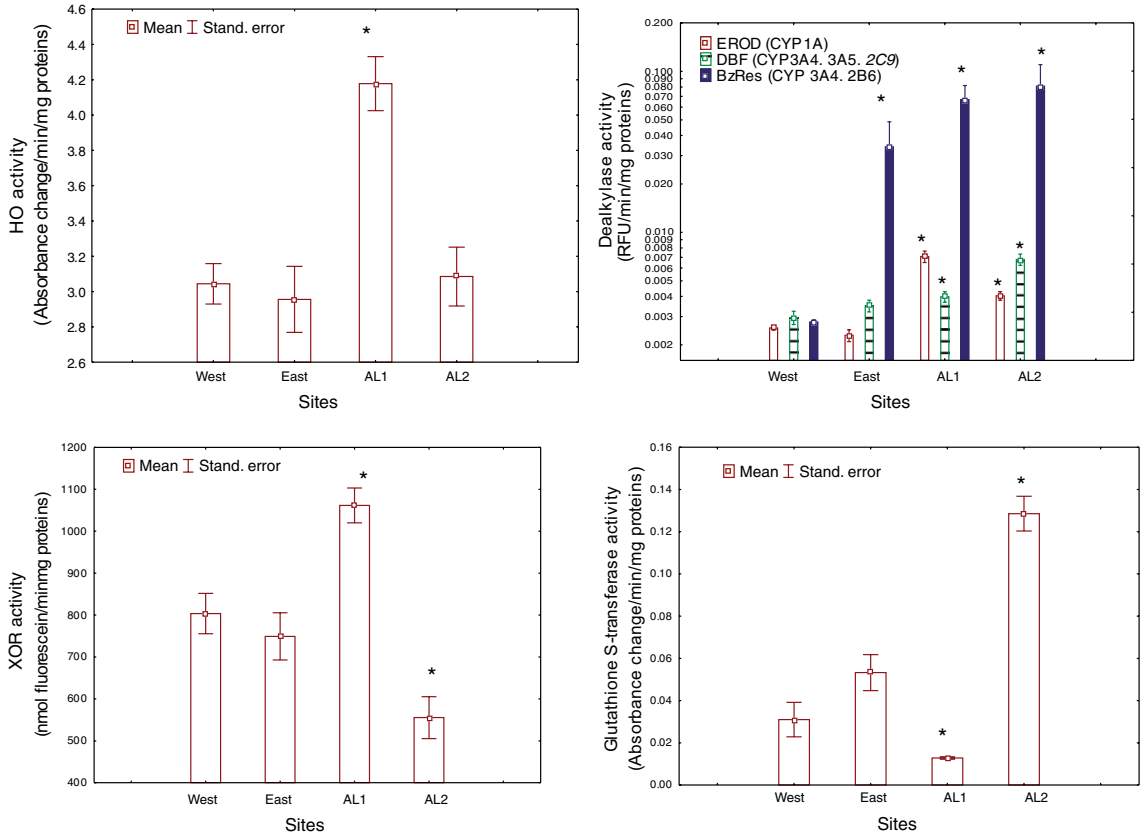

Fig. 3. Oxidative and xenobiotic metabolism in mussels exposed to aeration lagoons. The activities of EROD, DBF, BZRes dealkylases, GST and xanthine oxidase were determined in the digestive glands of $n = 8$ individuals at each site. RFU: relative fluorescence units. Asterisks (*) indicate significance at $p < 0.05$ relative to the western shore.

downstream along the eastern shore indicating impact in field, hence implying more ecological relevance than observed effects directly in the lagoons. Levels of CYP1A activity as determined by EROD activity were readily induced albeit somewhat with GST activity, suggesting the presence of polyaromatic hydrocarbons in treated wastewaters. This was shown in another study where benzo(*a*)pyrene hydroxylase (another marker enzyme for CYP1A1 like EROD) and GST activities were induced in mussels collected at polluted sites (Gowland et al., 2002). Municipal wastewaters are recognized to contain pharmaceutical products and most of them are biotransformed by five cytochrome P450 families in mammals (humans): CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Crespi et al., 1997). The substrates DBF and BzRes used in the present study were supposedly responsive to CYP3A4, 3A5, 2B6 and 2C9 activities. In mussels, immuno-reactivity with at least five cytochrome P450s, CYP1A, CYP2B, CYP2E, CYP3A and CYP4A, in digestive gland microsomes was detected (Peters et al., 1998), where CYP1A was significantly induced in mussels transplanted to Venice lagoons (Peters et al., 1998). In another study, blue mussels collected at sites contaminated by PAHs had increased immunopositive CYP1A protein, as expected, but with a concomitant decrease in CYP3A immunopositive protein (Shaw et al., 2004). An analysis of covariance on mussels exposed to aeration lagoons revealed that EROD activity (CYP1A related) was neither significantly correlated with DBF nor BzRes dealkylases and EROD activity was significantly induced in the aeration lagoons. In a previous study of carp collected from AL2, DBF dealkylase activity in the liver was readily induced, with EROD activity being decreased and not correlating with the former (Gagné and Blaise, 2004), thus corroborating the findings of the present study for DBF activity. Hydra exposed to carbamazepine, a drug consistently found in urban wastewaters above the µg/l range, had induced BzRes dealkylase activity, but not EROD activity after 96 h (Quinn et al., 2004). In addition to its role as a major drug metabolizing enzyme (Crespi et al., 1997), CYP3A activity is responsible for the 6β hydroxylation of testosterone, and thus it participates in the turnover of androgen steroids. CYP3A activity was found to be dependent on the pregnate X receptor (PXR) in Atlantic salmon (Meucci and Arukwe, 2006), where juvenile salmon exposed to environmentally realistic concentrations of the xenoestrogen nonylphenol had increased CYP3A and CYP1A1 mRNA levels that correlated with their corresponding receptor mRNA levels: PXR and AhR, respectively. In another fish study, treatment of zebrafish larvae to dexamethasone, the macrocyclid antibiotic rifampicn and 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin elicited induction

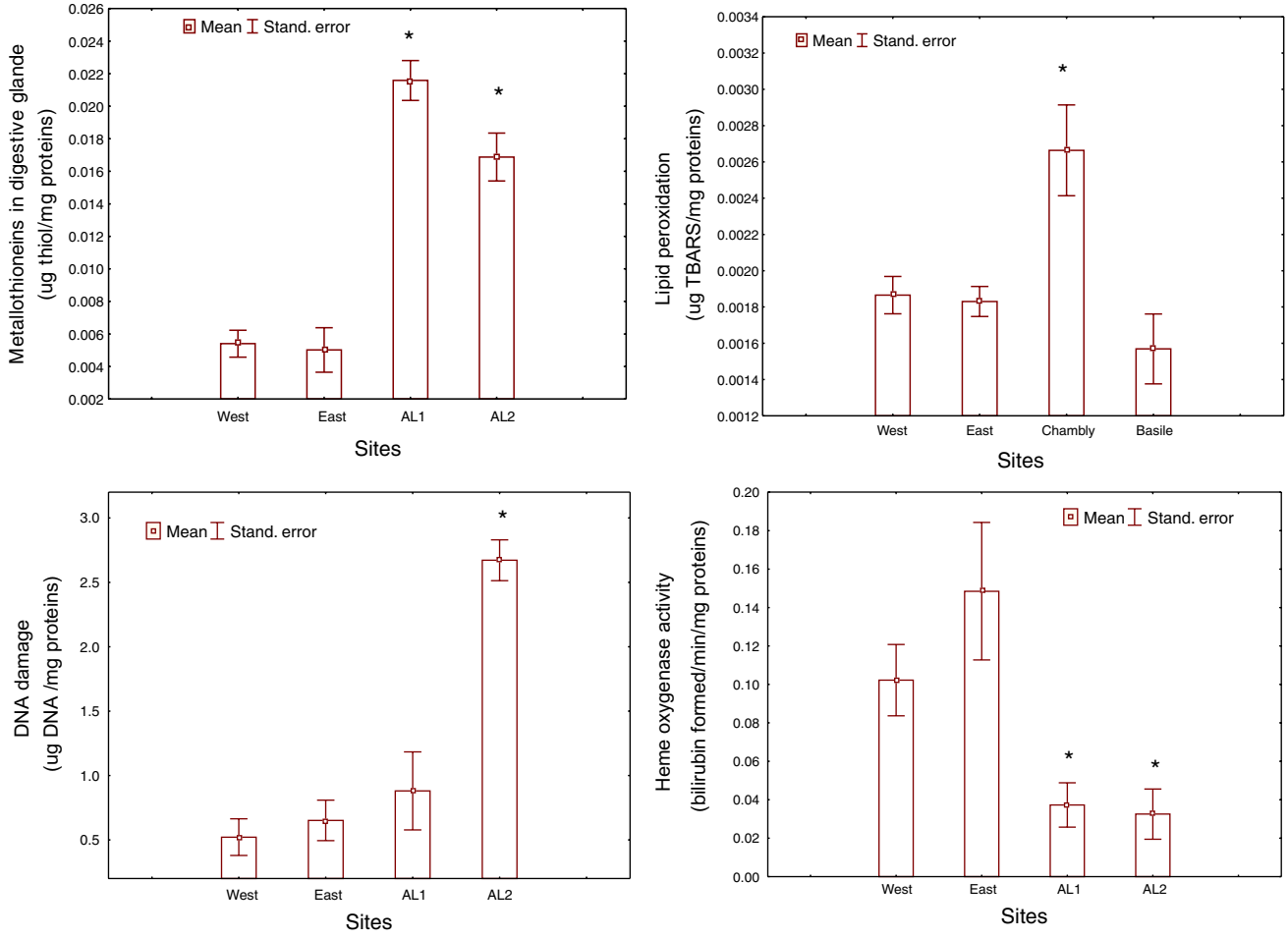

Fig. 4. Stress and tissue damage biomarkers in mussels exposed to aeration lagoons. Biomarkers of toxic effects were determined in the digestive glands of $n = 8$ individuals at each site. Asterisks (*) indicate significance at $p < 0.05$ relative to the western or eastern shores.

of CYP3A65 transcription in the foregut (Tseng et al., 2005). Hence, CYP3A activity is not only regulated by the occurrence of xenobiotics (i.e., considered one of the major drug metabolizing cytochromes in vertebrates), but also with androgen metabolism (i.e., 6β-testosterone hydroxylase). The levels of Vtg-like proteins (an indicator of estrogenic stimulation) were highly correlated with both DBF and BzRes dealkylases activity, which agrees with the finding of CYP3A activity was influenced by estrogenic compounds (i.e., nonylphenol). This hypothesis is further supported by an analysis of covariance of DBF dealkylase, which showed that its activity was significantly induced in both aeration lagoons and influenced by Vtg rather than by EROD or total HO activities. Furthermore, the analysis showed that BzRes activity was more strongly influenced by Vtg levels than with DBF activity: BzRes was not significantly induced at sites when Vtg was included as a covariate. Aquatic organisms (fish and mussels) appear to lack the CYP2C family that is required for phenobarbital metabolism, for example. This study shows for the first time that urban waste pollution stemming from biological treatments induced drug-metabolizing enzymes of the CYP3A and 2B families. The increase in MT in mussels exposed to the aeration lagoons suggested heavy metals but cannot exclude other factors that were recognized to increase MT synthesis such as inflammation and oxidative stress. The correlations between MT levels with oxidative metabolism enzymes (i.e., HO, EROD, DBF dealkylase) suggest a common source heavy metals and polar polycylic (aromatic) hydrocarbons. Analysis of covariance revealed that HO and Vtg-like proteins and sites were all significant factors ($F_{\text{sites}} > F_{\text{HO}} > F_{\text{Vtg}}$; $p < 0.01$ for each factor) in respect to MT levels suggesting that heavy metals contribute, in part at least, to the MT signal.

The increase in XOR activity could be explained by the presence of xanthines such as caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine) or increased catabolism of purines (which degrade into urate). Increased catabolism of purines occurs with extensive DNA repair that releases DNA strand breaks. However, an analysis of covariance for XOR with DNA strand breaks as the covariate revealed that DNA strand breaks (salvaged in turn by purine catabolic pathways) had only a marginal effect between site differences in XOR activity. Thus, the possibility that XOR activity was induced by exogenous xanthines remains. Caffeine is often found in municipal

Table 2
Correlation analysis of biomarker data[a]

Biomarkers	Correlation coefficient (R)	p-Value
Reproductive function		
GSI		
HO	0.64	<0.01
DNA damage	0.63	<0.01
EROD	0.62	<0.01
COX	0.57	0.03
Serotonin transport	−0.52	0.03
ATC	−0.55	<0.01
Serotonin	0.49	0.03
MT	0.47	0.05
Vtg-like proteins	0.38	0.09
Vtg-like proteins		
DBF	0.66	<0.01
BzRes	0.6	0.014
Dopamine	0.55	<0.01
Serotonin	0.55	<0.01
EROD	0.42	0.03
ATC		
Heme oxygenase	0.68	0.01
XOR	*0.41*	*0.06*
Oxidative metabolism		
EROD		
MT	0.78	<0.01
HO	0.77	<0.01
Xanthine oxidase	0.55	<0.01
Heme oxygenase	−0.5	0.03
Dopamine transport	0.49	0.05
Serotonin transport	−0.44	0.05
LPO	0.37	0.03
Serotonin	0.38	0.08
DBF		
GST	0.78	<0.01
BzRes	0.7	0.02
DNA damage	0.7	<0.01
MT	0.68	<0.01
Xanthine oxidase	−0.61	<0.01
COX	0.6	<0.01
Dopamine	0.55	<0.01
MAO	0.5	0.03
Serotonin	0.41	0.05
BzREs		
Serotonin	0.55	0.04
GST	0.48	0.04
XOR		
LPO	0.56	<0.01
GST	−0.61	<0.01
HO		
LPO	0.59	<0.01
Xanthine oxidase	0.52	<0.01
MT	0.48	0.02
Dopamine transport	0.44	0.05
GST	−0.34	0.04
Dopamine	−0.34	0.07
Neuroendocrine effects		
COX		
LPO	−0.53	<0.01
GST	0.53	0.01
DNA damage	0.54	0.04
Serotonin		
DNA damage	0.52	0.02
GST	0.3	0.09
Dopamine		
Serotonin transport	−0.49	0.02
GST	0.31	0.07
DNA damage	0.46	0.03
Serotonin transport		
DNA damage	−0.46	0.06
MT	−0.57	0.065
Dopamine transport		
LPO	0.36	0.08
MAO		
Dopamine transport	0.49	0.05
Stress and damage		
Heme oxygenase		
MT	−0.53	0.05
GST		
LPO	−0.57	<0.01
DNA damage	0.6	<0.01
DNA strand breaks		
HO	−0.57	0.06
MT	See above	
LPO	See above	
HO	See above	

Marginal correlations ($0.05 < p < 0.1$) are in *italic*.
[a] Only significant correlations were shown ($p < 0.05$).

wastewaters, reaching well above the 10 μg/l range (Standley et al., 2000; Gagné et al., 2006). The metabolism of xanthine by XOR leds to the production reactive oxygen species, which in turn, produce oxidative stress in tissues. Indeed, LPO was significantly correlated with XOR in mussels exposed to domestic wastewaters, suggesting that increased xanthine metabolism and cytochrome P450 activity could lead to oxidative stress in mussels. To the best of our knowledge this is the first report of increased XOR activity, independently of DNA damage, in aquatic animals exposed to municipal effluents. The levels of MT were significantly induced in each aeration ponds suggesting perturbation in metal mobilization in tissues. Indeed, total Al and Cd levels in soft tissues were increased in the second aeration lagoon. However, many metals were depressed in AL1 indicating perhaps that the susceptibility of the organisms toward metal exposure changes in environments polluted by urban wastewaters. It is also possible that the MT response is not entirely specific to heavy metal in such chemically-complex environments.

Although dopamine levels were increased about 2-fold in AL2, its synaptosome transport activity was also elevated to the same level of intensity, suggesting reduced dopamine residence time in synapse, and hence its corresponding effect. Moreover, MAO activity was increased

Table 3
Heavy metal bioaccumulation in mussels from the study sites

Study sites	Ag (μg/g)	Al (×10) (μg/g)	As (μg/g)	Cd (μg/g)	Cr (μg/g)	Cu (μg/g)	Pb (μg/g)	Zn (×10) (μg/g)
AL1	0.15 ± 0.02*	5.3 ± 0.6	3.5 ± 0.09*	1.03 ± 0.04*	3.6 ±0.3*	8.6 ± 0.3*	0.63 ± 0.04	16.4 ± 0.6*
AL2	0.23 ± 0.05	13 ± 2*	5.40 ± 0.3	1.8 ± 0.2*	7.7 ± 0.6	15 ± 2	2.1 ± 0.9	19 ± 1
East shore	0.25 ± 0.03	4.2 ± 0.8	5.5 ± 0.3	1.30 ± 0.09	8 ± 1	37 ± 8*	1.5 ± 0.2	24 ± 2*
West shore (reference site)	0.34 ± 0.08	4.4 ± 0.3	5.4 ± 0.3	1.21 ± 0.04	6.5 ± 0.8	19 ± 4	1 ± 0.1	19.4 ± 0.5

Total metal levels were determined from soft tissues from $N = 8$ animals. The star (*) indicates a statistically difference at $p < 0.05$ level in respect to the reference site.

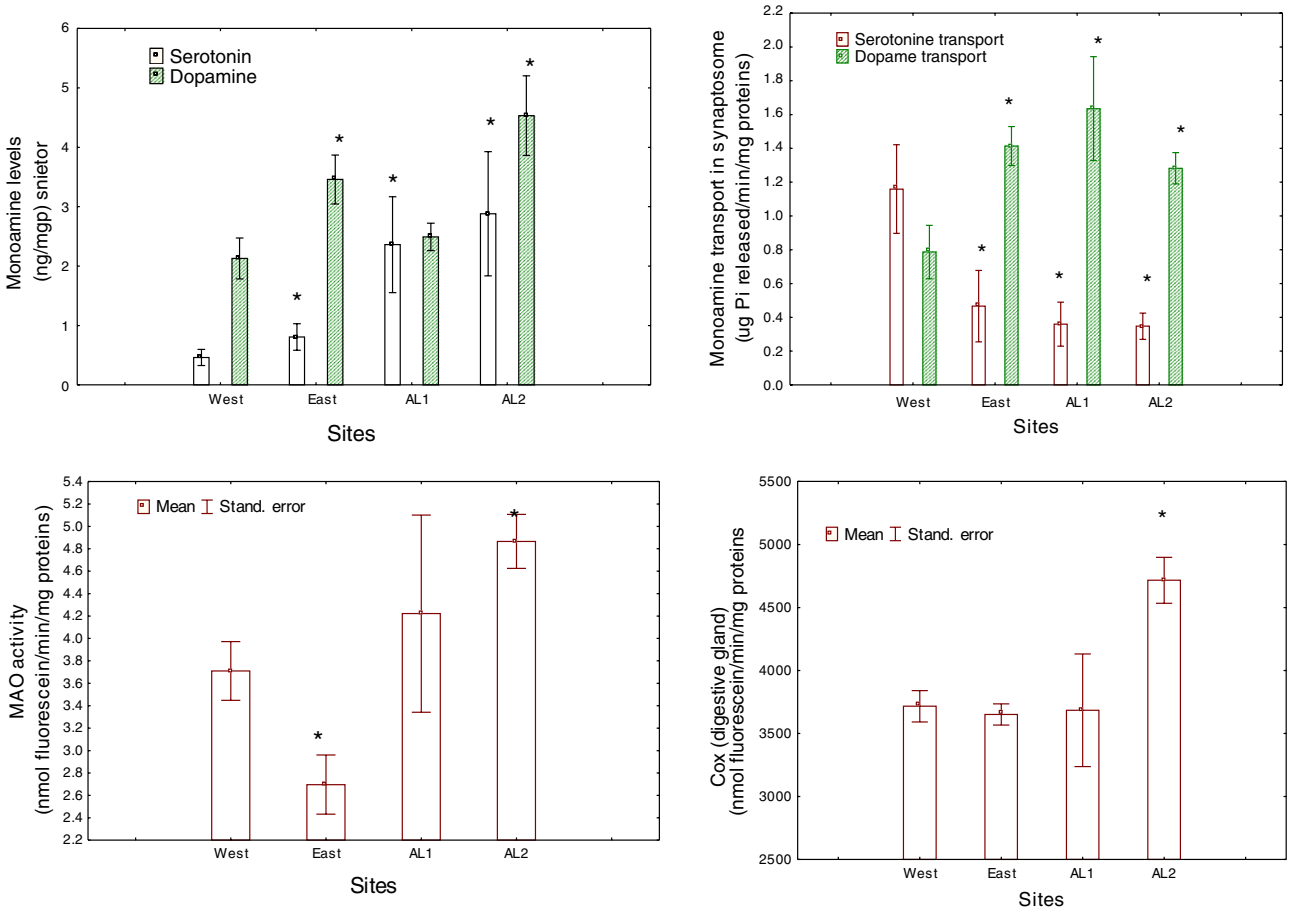

Fig. 5. Neuroendocrine effects in mussels exposed to aeration lagoons. Biomarkers to assess neuroendocrine integrity were determined in mussels exposed to aeration lagoons contaminantion. Asterisks (*) indicate significance at $p < 0.05$ relative to the western shore.

1.3-fold in AL2, again contributing to the increased turnover of dopamine. The opiate morphine was shown to block dopamine stimulation of adenylate cyclase activity in a naloxone-dependent manner in ganglia homogenates (Stefano and Leung, 1982). In another study, the antagonizing effects of opiates on dopamine-dependent cilioinhibitory activity were overcome by increasing dopamine concentration (Aiello et al., 1986). In addition, opiates such as morphine are metabolized by CYP3A4 in fish by *N*-demethylation of morphine (Projean et al., 2003), which was corroborated in the present study by the induction of DBF and BzRes dealkyases. As well, dopamine and morphine synthesis were coupled in *Mytilus edulis* ganglia, where their production depended on tyramine and tyrosine levels (Zhu et al., 2005). The increased dopamine production and synaptosome transport represents perhaps an adaptive mechanism against the presence of opiates in domestic wastewaters. Morphine was recently found in a primary-treated effluent at the 0.1 μg/l range (Gagné et al., 2004). While the effect of dopamine was dampened by increased dopamine transport and MAO activity (increased dopamine turnover), serotonin effects were enhanced by its increased tissue levels and reduced reuptake in synaptosomes at a much higher intensity than with

Table 4
Factorial analysis of biomarker data

Biomarker	Factor 1	Factor 2	Factor 3
GSI	**−0.77**[a]	−0.08	0.40
Vtg-like proteins	**−0.7**	−0.08	−0.57
ATC	0.46	−0.13	**−0.84**
LPO DG	−0.006	**−0.80**	−0.10
XOR DG	−0.12	−0.58	−0.42
GST	−0.46	**0.78**	−0.02
Hemoprotein oxidase	−0.44	**−0.79**	0.23
EROD	−0.68	−0.61	0.01
DBF	**−0.85**	0.30	−0.26
BzREs	−0.58	0.12	−0.31
MT	−0.69	−0.29	0.08
Heme oxygenase	0.68	0.07	−0.31
COX DG	−0.46	0.53	0.10
DNA damage	**−0.74**	0.39	0.14
MAO	−0.36	−0.16	0.11
Serotonin	−0.59	0.03	−0.14
Dopamine	−0.38	0.30	−0.61
Serotonin transport	0.53	0.03	0.19
Dopamine transport	−0.28	−0.51	−0.008
Cumulative variance	31%	50%	61%

[a] Factorial weights >0.7 are highlighted in bold (i.e., considered important).

the increase in MAO activity. The serotonergic properties of urban effluents have been reported in freshwater mussels (Gagné and Blaise, 2003). Hence, aeration lagoons appear to produce a series of changes in monoamine levels, synaptosome transport and turnover that favour a serotonergic status in mussels even though Vtg production is still at play. During vitellogenesis in the scallop *Argopecten purpuratus*, dopamine levels follow the increase in estradiol-17β while serotonin (and COX) gradually increases for final gamete maturation, fertilization and spawning steps (Martinez and Rivera, 1994). However, COX activity was not significantly correlated with either serotonin or its synaptosomal transport activity, indicating that perhaps COX was activated by a process other than spawning. Contaminant inducers of COX are not well understood in aquatic species, but their activity is involved in the inflammation processes that underlie many pathophysiological conditions (Esch and Stefano, 2002). For example, COX-2 mRNA levels in colon cancer cells were increased significantly by compounds that activate transcription through the xenobiotic responsive element and/or the antioxidant responsive element (Sherratt et al., 2003). COX activity in mussels was significantly correlated with DBF dealkylase, which supports perhaps its activation by the xenobiotic responsive element. The reduction in serotonin reuptake activity is consistent with the presence of serotonin reuptake inhibitors such as fluoxetine (Prozac) in municipal effluents. The drug fluoxetine was shown to increase the serotonergic response of spawning in zebra mussels (Fong, 1998). The bioavailability of fluoxetine was confirmed in fish collected in streams dominated by urban effluents (Brooks et al., 2005). However, this needs to be confirmed by measuring the compounds in mussel tissues. The lack of correlation between COX activity and serotonin and dopamine levels, and the positive correlations with oxidative metabolism enzymes raise the possibility that municipal wastewater contaminants produce an inflammation reaction which underlies many (patho)physiological conditions. This hypothesis is supported by the following arguments: (1) exposure to the aeration lagoons increases proportionally COX activity and LPO in mussels; (2) opiates, which are reported to occur in municipal wastewaters, are known to have phagocytosis-dampening effects (Ottaviani et al., 1995), perhaps through the production of nitric oxide which, in turn, leads to oxidative stress (e.g. LPO); and (3) COX is significantly associated with CYP3A activity, which involves xenobiotic metabolism. Although not confirmed by chemical analyses in tissue, these wastewaters produce effects in mussels that are consistent with the presence of estrogenic compounds (ethynylestradiol-17β), caffeine or other xanthines, serotonin reuptake inhibitors, and opiate-like substances, but not with the occurrence of COX inhibitors such as non-steroidal anti-inflammatory drugs because COX activity was significantly induced in one aeration lagoon at least. MAO activity was induced in the aeration lagoons, but significantly reduced in the dispersion plume, complicating the possible actions of MAO inhibitor drugs currently used for the treatment of depression. The combined effects of aspirin, paracetamol and caffeine (used for the treatment of migraines) was COX inhibition and dopamine depletion in rat striatal slices with a dramatic increase in norepinephrine release (Fiebich et al., 2006), effects that were not observed in mussels exposed to domestic wastewaters (increased COX and dopamine).

In conclusion, the present study found that many neuroendocrine-related effects were significantly associated with oxidative metabolism and stress endpoints, which are associated with tissue damage. It also confirmed that urban wastewater treated by aeration lagoons disrupts the monoamine metabolism that favours a serotonergic response over a dopaminergic one, in addition to its estrogenicity, and leads to oxidative stress, possibly through inflammation (COX activity). The observed effects are consistent with the occurrence of estrogens, polycyclic- hydroxylamine-containing compounds like pharmaceuticals, caffeine and other xanthines, serotonin reuptake inhibitors, and opiate-like substances, but not for COX and MAO inhibitors. Thus, the continuous release of wastewaters from aeration lagoons (biological treatment) in aquatic ecosystems poses a risk to the survival of local populations of freshwater mussels.

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