

## Effects of selected pharmaceutical products on phagocytic activity in *Elliptio complanata* mussels

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### Abstract

Municipal wastewaters are recognized as a major source of pharmaceutical and personal care products to the aquatic environment, thereby exposing biota to unknown chronic effects. This study sought to examine the immunotoxic effects of pharmaceutical and urban waste products on the freshwater mussel *Elliptio complanata*. Hemolymph samples were collected and treated in vitro with increasing concentrations of various drugs (bezafibrate, carbamazepine, fluoxetine, gemfibrozil, morphine, naproxen, novobiocin, oxytetracycline, sulfamethazole, sulfapyridine and trimethoprim) and urban waste related chemicals (coprostanol, caffeine, cotinine) for 24 h at 15 °C. In a parallel experiment, mussels were caged and placed in two final aeration lagoons for the treatment of domestic wastewaters. At the end of the exposure period, hemolymphs were tested for phagocytic activity, intracellular esterase activity, cell adherence and lipid peroxidation (LPO). The products that most increased phagocytosis were bezafibrate, gemfibrozil and trimethoprim, while novobiocin and morphine reduced its activity. Intracellular esterase activity was reduced most strongly with sulfamethazole, novobiocin, gemfibrozil, bezafibrate and carbamazepine. Cell adherence was decreased by oxytetracycline, novobiocin and naproxen, and increased by gemfibrozil, bezafibrate and sulfapyridine. Exposure to these products also modulated LPO in hemocytes. Coprostanol and naproxen were more potent to reduce LPO while novobiocin and sulfapyridine were the most potent to induce LPO. The potential to induce LPO was positively correlated with the number of functional groups on the molecule (i.e., its nucleophilicity). Mussels exposed to domestic wastewater treatment plant aeration lagoons had decreased intracellular esterase and phagocytic activity as well, suggesting immunosuppression. PPCPs (pharmaceuticals and personal care products) that are recognized to disrupt cytokine signalling network by the nitric oxide pathway and cell permeability were generally the most potent ones. The data suggest that PPCPs have the potential to cause adverse effects on the immune system of bivalves.

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**Keywords:** Immunocompetence; Bivalve; Phagocytosis; Pharmaceutical products; Municipal effluents

### 1. Introduction

Bivalves are important members of aquatic ecosystems and markedly interact with water and sediment. These sessile and long-lived organisms filter large quantities of surface water for feeding and respiration. They are therefore particularly susceptible to environmental stressors, including point-source and diffuse contamination, water-level variations and climatic changes (e.g., temperature fluctuations in shallow water). In bivalves, hemocytes circulate in an open vascular system (i.e.

the hemolymph) that pervade most organs, favouring direct exposure to the external environment and hence to contaminants. Long-term exposure to contaminants emanating from various sources (urban and industrial wastewaters) could compromise immune function and progressively lead to infectious diseases and cancerous disorders such as neoplasia (Krishnakumar et al., 1999; Weinberg et al., 1997).

In mussels, the immune system is comprised of cellular and humoral components, but lacks the lymphoid system (i.e. lymphocytes and immunoglobulins). Hemocytes are tentatively classified as stem, phagocytic, hemostatic and nutritive cells (Glinski and Jarosz, 1997). The cellular immune system participates in various functions such as phagocytosis (hyalinocytes),

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nodule formation, encapsulation, pearl formation and liquefaction/necrosis of tissues. The humoral components of bivalve immunity comprise lysozyme activity, lectin/cytokine production and the phenyloxidase system (Munoz et al., 2006; Stefano et al., 1990). Bivalves also possess the cytokine network that closely resembles vertebrate systems (Hughes et al., 1992) and is influenced by the opiate receptor (Stefano et al., 1990). The activation of opiate receptors in mussel hemocytes by morphine reduces phagocytic activity (Stefano et al., 1993), perhaps through the release of nitric oxide by neural ganglia (Stefano et al., 2004). The release of interleukins or tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) during inflammation could also reduce phagocytic activity and cell motility (Ottaviani et al., 1995). Small pathogens may be agglutinated or opsonized by lectins (agglutinins), facilitating clearance by circulating hemocytes or lysed directly without their involvement. The cellular and humoral defences in bivalves are remarkably efficient in controlling infections, but are susceptible to disruption from various environmental stressors.

Municipal effluents represent a major source of pollution, releasing contaminants such as metals, polyaromatic hydrocarbons, pesticides, nonylphenol and surfactants into aquatic ecosystems. These effluents were recently shown to release a number of pharmaceutical and personal care products (PPCPs) into the environment (Boyd et al., 2003; Buxton and Kolpin, 2002). For example, carbamazepine, caffeine and various antibiotics are usually found at  $\mu\text{g/L}$  concentrations in effluents discharging to surface waters (Gagné et al., 2005, in press; Clara et al., 2004; Standley et al., 2000). The effects of PPCPs on sessile invertebrates such as mussels remain largely unknown at the present time. Recent studies have shown that municipal effluents mimic a physiological state close to inflammation in mussels, in addition to their endocrine-disrupting activity (Gagné et al., 2005, in press, 2001). It thus appeared justified to examine the influence that PPCPs commonly found in municipal wastewaters might have on the immune function of endemic mussels exposed to municipal effluent outfalls. Our study therefore sought to examine the immunotoxic effects of various PPCPs and urban related compounds found in significant quantities in specific municipal effluents of interest on *Elliptio complanata* hemolymph exposed in vitro. Immunocompetence was assessed by phagocytosis of fluorescent bacteria, intracellular esterase activity, adherence to microplate wells and the formation of lipid peroxidation. In a parallel study, immunocompetence was assessed in mussels exposed to two domestic wastewater treatment plant aeration lagoons as a source of PPCPs for 60 days. We also attempted to relate the pharmacological properties of PPCPs to observed immunotoxic responses in the mussel specimens.

## 2. Materials and methods

### 2.1. Mussel maintenance and handling

Mussels were collected by hand in a Laurentian lake known to abound in *E. complanata* mussels (Downing and Downing, 1992). They were placed in coolers ( $4^{\circ}\text{C}$ ) and brought back to

the laboratory where they were acclimated in 300-L tanks filled with a continuous flow of charcoal-filtered and UV-treated tap water at  $15^{\circ}\text{C}$ . Mussels were allowed to acclimatize for one month and fed three times weekly with commercial coral reef feed solution enriched with *Selenastrum capricornutum* algae ( $1\text{--}10 \times 10^6$  algae/mL).

### 2.2. Hemolymph extraction and drug treatment

Hemolymph (about 1 mL) was collected through the posterior adductor muscle by means of a syringe needle from each of 10 mussels. After pooling and mixing by inversion,  $200\ \mu\text{L}$  aliquots of hemolymph were dispensed into individual wells of opaque polystyrene microplates. Drugs (bezafibrate, carbamazepine, fluoxetine, gemfibrozil, morphine, naproxen, novobiocin, oxytetracycline, sulfamethazole, sulfapyridine and trimethoprim) and urban waste products (caffeine, coprostanol and cotinine) were added separately ( $100\ \mu\text{L}$  in  $50\ \text{mM}$  NaCl,  $1\ \text{mM}$  Hepes–NaOH, pH 7.4) to obtain a final concentration of 0, 2.5, 25, 50 and  $100\ \mu\text{M}$ . The microplates were incubated in darkness for 24 h at  $15^{\circ}\text{C}$  with saturated humidity. Afterwards, supernatants were carefully removed by aspiration and wells washed with  $100\ \mu\text{L}$  PBS (phosphate buffered saline diluted to  $50\ \text{mM}$  NaCl for freshwater bivalves). Phagocytic activity, intracellular esterase activity, cell adherence and lipid peroxidation were evaluated as described elsewhere (Blaise et al., 2002). For phagocytosis,  $25\ \mu\text{L}$  of fluorescein-labeled bacteria (corresponding to  $5 \times 10^7$  bacteria/well) were added to the wells and left to incubate for 2 h at room temperature. The wells were then washed once with PBS as described above and resuspended in  $100\ \mu\text{L}$  of  $0.125\ \text{mg/L}$  of Trypan Blue, pH 4.4 ( $50\ \text{mM}$  sodium-citrate), to quench fluorescence from any residual bacteria adhering to the cell wall (Hed, 1995). Fluorescence was measured at 485 nm excitation and 520 nm emission (Fluorolite 1000, Dynatech Microplate Reader). Phagocytic activity was expressed as  $\mu\text{mole}$  of fluorescein/relative fluorescence units for proteins, as determined, for the latter, by the fluorescamine method (Lorenzen and Kennedy, 1993). Lipid peroxidation was also determined in the hemolymph suspension according to the thioabarbitoric acid reactants methodology using tetramethoxypropane as the standard (Wills, 1987). The data were expressed as  $\mu\text{mole}$  of thiobarbituric acid reactants or TBARS/relative protein fluorescence units. Cell viability (intracellular esterase activity) and cell adherence (total proteins in adhered cells) were determined in separate wells following the carboxyfluorescein diacetate (Altman et al., 1993) and fluorescamine methods (Lorenzen and Kennedy, 1993), respectively. Briefly, after the 24-h exposure time, the hemolymph was removed and wells were washed once with PBS. Then,  $100\ \mu\text{L}$  of  $10\ \mu\text{M}$  carboxyfluorescein diacetate were added to the cells and left to incubate for 15 min at room temperature. The medium was removed, and wells were washed once in PBS and resuspended in  $100\ \mu\text{L}$  of PBS prior to fluorescence readings. For protein determinations,  $130\ \mu\text{L}$  of  $30\ \text{mM}$  NaOH were added to the plates for 30 min to lyse the cells. Then,  $70\ \mu\text{L}$  of  $500\ \mu\text{g/mL}$  fluorescamine (in 100% acetonitrile) were added and the plate was left to stand for 10 min. Fluorescence was measured at 400 nm excitation and

450 nm emission. Standards of fluorescein (phagocytosis and cell viability) and bovine serum albumin (proteins) were used for calibration.

### 2.3. Field exposure to aerated lagoons for treating domestic wastewater

Freshwater mussels were placed in experimental cages according to an established method (Salazar and Salazar, 2001). Briefly, mussels were individually placed in cylindrical nets ( $n=8$  mussels per net) and four nets were attached to a 1-m<sup>2</sup> PVC frame. The frames were attached to cinder blocks (25 kg) marked with buoys for easy location. Three frames were placed in the final aeration lagoons for the treatment of domestic wastewaters of two small cities (population approximately 15 000 each) for 60 days (July and August) in 2004.

### 2.4. Data analysis

The experiments were repeated  $n=4$  times. The threshold concentration was calculated as the square root of the product between the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC): threshold concentration ( $\mu\text{M}$ ) =  $(\text{NOEC} \times \text{LOEC})^{1/2}$ . The LOEC was determined using the Mann–Whitney  $U$  test. Threshold effect concentrations were analysed to identify different groups of responses by analysis of variance (ANOVA). Correlations between immune parameters were examined using the Pearson-product moment procedure (Statistica, version 7). Significance was set at  $p < 0.05$ . The environmental hazard ratio was derived by assuming the presence of a theoretical concentration of 1  $\mu\text{g/L}$  in the municipal

effluent and the most sensitive immune parameter response. A safety factor of 100 for acute to chronic exposure and a factor of 10 for interspecies difference were applied. For example, the lowest threshold value for carbamazepine was 3  $\mu\text{M}$  or 708  $\mu\text{g/L}$  and the calculated hazard ratio was:  $(1/708) \times 1000 = 1.4$ . The relative risk potential based on the threshold effect concentration was derived for each compound in the following manner. For phagocytosis or cell adherence or intracellular esterase activity, ANOVA on the threshold effect concentration revealed that three groups were significantly different: a value of 1 was given for products in the low risk (high threshold concentration) group, a value of 2 for products in the moderate risk (intermediate threshold concentration) group and a value of 3 for products in the high (low threshold concentration) group.

## 3. Results

Hemocytes were exposed to a wide variety of PPCPs and urban-related pollutants (coprostanol, caffeine and cotinine) usually found in municipal effluents containing both domestic and industrial wastewaters (Table 1). The values reported for these compounds ranged from 0.02 to 10  $\mu\text{g/L}$ , with caffeine, carbamazepine, gemfibrozil, bezafibrate, novobiocin, oxytetracycline, cotinine, coprostanol and morphine being the most abundant (i.e.  $> 0.07 \mu\text{g/L}$ ). Hemocytes were exposed to increasing concentrations of carbamazepine, a pharmaceutical product commonly found in municipal wastewaters, for 24 h at 15 °C (Fig. 1). Phagocytosis (the amount of ingested fluorescent bacteria) was increased at a threshold concentration of 61  $\mu\text{M}$  (14 mg/L), while lipid peroxidation was not affected. Hemocyte adherence was also reduced at a threshold concentration of 61  $\mu\text{M}$ . However,

Table 1  
Pharmacological properties of selected drugs and occurrence in municipal wastewaters

Drug	Chemical properties <sup>a</sup>	Class	Mode of action	Reported level in municipal wastewaters <sup>b</sup>	
				$\mu\text{g/L}$	nM
Caffeine	C <sub>8</sub> H <sub>10</sub> N <sub>4</sub> O <sub>2</sub> /194/0.3	Excitant/stimulatory	$\beta$ -adrenergic xanthine	10	50
Carbamazepine	C <sub>15</sub> H <sub>12</sub> N <sub>2</sub> O/236/0.1	Anticonvulsant	Narcosis (membrane depolarisation)	0.1	0.4
Trimethoprim	C <sub>14</sub> H <sub>18</sub> N <sub>4</sub> O <sub>3</sub> /290/0.2	Bacteriostatic agent	Prevents the reduction of dihydrofolate	0.07	0.2
Fluoxetine	C <sub>17</sub> H <sub>18</sub> NOF <sub>3</sub> ·HCl/346/0.14	Anti-depressive	Inhibitor of serotonin reuptake	0.05	0.14
Naproxen	C <sub>14</sub> H <sub>14</sub> O <sub>3</sub> /230/0.1	Anti-inflammatory/analgesic	COX inhibitor	0.3	1.3
Gemfibrozil	C <sub>15</sub> H <sub>22</sub> O <sub>3</sub> /250/0.08	Anti-cholesteremic agent	Peroxisome proliferator-activated receptor (PPAR) agonist.	0.07	0.28
Bezafibrate	C <sub>19</sub> H <sub>20</sub> ClNO <sub>4</sub> /362/0.15	Anti-cholesteremic agent	Lipoprotein lipase (LPL) activator Peroxisome proliferator-activated receptor (PPAR) agonist.	0.07	0.19
Novobiocin	C <sub>31</sub> H <sub>35</sub> N <sub>2</sub> NaO <sub>11</sub> /635/0.2	Antibiotic	Inhibition of DNA synthesis (topoisomerase II inhibitor)	0.2	0.3
Sulfapyridine	C <sub>11</sub> H <sub>11</sub> N <sub>3</sub> O <sub>2</sub> S/249/0.27	Treatment of inflammatory bowel disease	A metabolite of sulfasalazine	0.02	0.08
Sulfamethoxazole	C <sub>10</sub> H <sub>11</sub> N <sub>3</sub> O <sub>3</sub> S/253/0.3	Antibiotic	Tetrahydrofolate synthesis inhibitor	0.05	0.2
Oxytetracycline	C <sub>22</sub> H <sub>24</sub> N <sub>2</sub> O <sub>9</sub> /460/0.2	Antibiotic	Inhibition of bacterial protein synthesis (30S ribosome binding)	0.2	0.4
Coprostanol	C <sub>27</sub> H <sub>48</sub> O/389/0.01	Reduced cholesterol	Fecal bacteria reductive metabolism	0.1	0.26
Cotinine	C <sub>10</sub> H <sub>12</sub> N <sub>2</sub> O/176/0.14	Metabolite of nicotine	Oxidation metabolite of nicotine	0.2	1.1
Morphine	C <sub>17</sub> H <sub>19</sub> NO <sub>3</sub> /285/0.1	Opiate receptor agonist	Block pain perception	0.1 <sup>c</sup>	0.35

<sup>a</sup> Chemical properties are molecular formula/molecular weight/nucleophilicity. Nucleophilicity was calculated as the number of functional groups on the carbon backbone: (number of O+N+S+F)/(number of C+H).

<sup>b</sup> According to Clara et al., 2004 and Gagné et al., 2005, in press.

<sup>c</sup> According to Gagné et al., 2004.

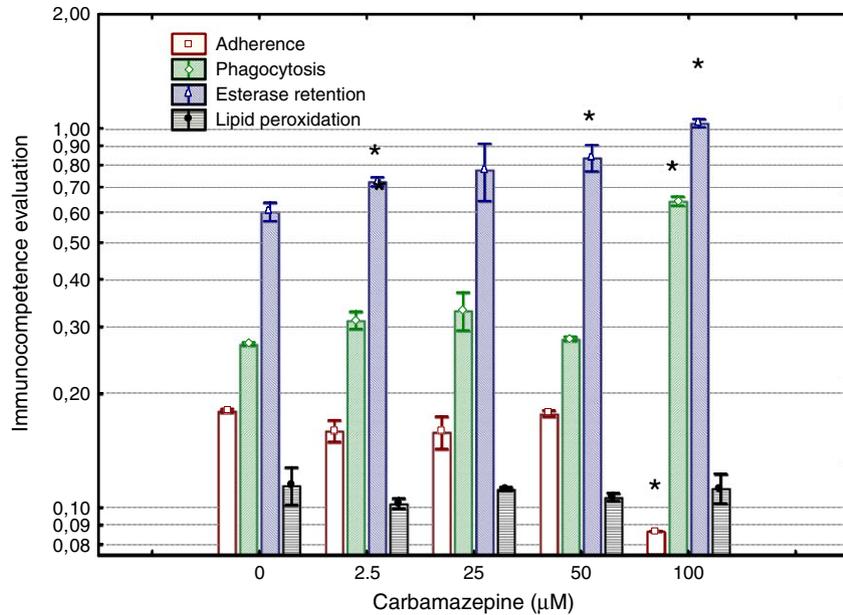


Fig. 1. Effects of carbamazepine on immune activity of hemocytes. Hemocytes were incubated for 24 h at 15 °C with increasing concentrations of carbamazepine prior to immunocompetence evaluation. The data represent the mean with standard error from  $n=4$  replicates. The asterisk \* indicates significance at  $p < 0.05$  with respect to untreated cells.

intracellular esterase activity was significantly increased at a threshold concentration of 2.5 µM (0.7 mg/L). Hemocytes were also exposed to various PPCPs in vitro (Table 2). Phagocytic activity was generally stimulated (78% of time) by PPCPs, while morphine and the antibiotics novobiocin, sulfapyridine and sulfamethazole decreased this activity. Phagocytic activity was negatively correlated with the number of polar functional groups of the compound ( $R = -0.61$ ;  $p = 0.02$ ), suggesting that a drug's potential to decrease phagocytosis is related to its polarity. An analysis of variance (ANOVA) of threshold concentrations for phagocytosis revealed three distinct groups of PPCP products: the low effect (risk) group (sulfamethazole, coprostanol, carbamazepine, fluoxetine), the moderate effect group (sulfapyridine,

morphine, oxytetracycline, naproxen, caffeine, cotinine) and the high effect group (novobiocin, bezafibrate, gemfibrozil and trimethoprim). The relative cumulative risk potential (RCRP) based on threshold values are summarized in Fig. 2 where a value of 1 was assigned for the low risk group, 2 for the moderate risk group and 3 for the high risk group. Intracellular esterase activity was always increased with the PPCPs tested, but caffeine and morphine had no effect at the highest concentration tested. A negative and marginal correlation was found between esterase activity and cell adherence ( $R = -0.52$ ;  $p = 0.06$ ), suggesting that low adherence is somewhat associated with increased esterase activity. An ANOVA of the threshold concentration for esterase activity identified three groups: the low effect (risk) group

Table 2  
Effects of selected drugs on the immune function of *Elliptio complanata*

Drug	Phagocytic activity (threshold effect concentration in µM)	Intracellular esterases (µM)	Lipid peroxidation (µM)	Adherence (µM)	Hazard ratio <sup>b</sup>
Caffeine	41 (+) <sup>a</sup>	ND	370 µM (+)	370 µM (-)	0.12
Cotinine	45 (+)	400 (+)	200 (+)	400 (-)	0.13
Carbamazepine	61 (+)	3(+)	ND	61 (-)	1.4
Trimethoprim	3 (+)	3(+)	ND	120 (+)	1.15
Fluoxetine	100 (+)	100(+)	ND	100 (+)	0.03
Coprostanol	182 (+)	21(+)	21 (-)	182 (+)	0.12
Naproxen	35 (+)	152(+)	35 (-)	4 (+)	1.09
Gemfibrozil	3 (+)	3(+)	140 (-)	3 (-)	1.33
Bezafibrate	2 (+)	2(+)	ND	2 (-)	2.1
Oxytetracycline	17 (+)	76(+)	ND	2 (+)	1.1
Novobiocin	1 (-)	12 (+)	112 (+)	1 (+)	1.6
Sulfapyridine	32 (-)	140(+)	140 (+)	3 (-)	1.33
Sulfamethazole	280 (-)	3(+)	ND	280 (+)	1.31
Morphine	31 (-)	ND	NA	31 (-)	1.47

<sup>a</sup>+: Induction of response; -: Inhibition of response.

<sup>b</sup>Risk ratio as described in Materials and methods section (data analysis).

NA: not analysed; ND: no effect found at the highest concentration tested.

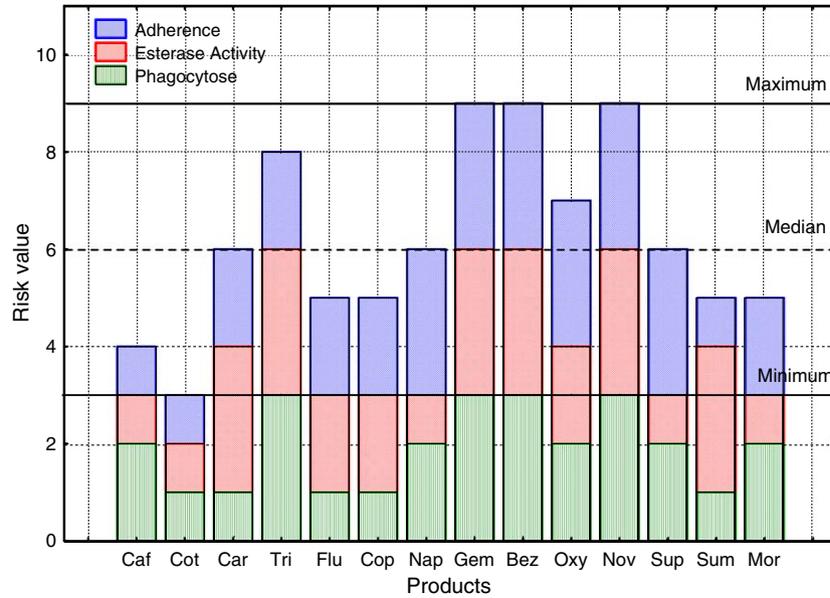


Fig. 2. Relative cumulative risk potential based on threshold values for phagocytosis, esterase activity and cell adherence. The cumulative risk potential for each drug was calculated using ANOVA as described in Materials and methods section (data analysis). The minimum, median and maximum values are shown. The maximum value of 9 is calculated as follows: 3 (phagocytosis)+3 (esterase activity)+3 (cell adherence). The abbreviations in the abscissa are: caffeine (Caf), cotinine (Cot), carbamazepine (Car), trimethoprim (Tri), fluoxetine (Flu), coprostanol (Cop), naproxen (Nap), gemfibrozil (Gem), bezafibrate (Bez), oxytetracycline (Oxy), novobiocin (Nov), sulfapyridine (Sup), sulfamethazole (Sum) and morphine (Mor).

(sulfapyridine, naproxen, cotinine, morphine, fluoxetine and caffeine), the moderate effect group (oxytetracycline, coprostanol, fluoxetine) and the high effect group (sulfamethazole, novobiocin, gemfibrozil, bezafibrate, trimethoprim and carbamazepine). In examining the adherence of hemocytes to microplate wells in the presence of various PPCPs, half had an effect on hemocyte adherence. Compounds that decreased hemocyte adherence were caffeine, cotinine, carbamazepine, gemfibrozil,

bezafibrate and sulfapyridine. An ANOVA of the effect threshold concentrations for cell adherence revealed three distinct groups: the low effect group (caffeine, cotinine, sulfamethazole), the moderate effect group (carbamazepine, trimethoprim, fluoxetine, coprostanol and morphine) and the high effect group (gemfibrozil, bezafibrate, oxytetracycline, novobiocin, sulfapyridine and naproxen). The data show that the following products were the most potent to affect immune responses (phagocytosis,

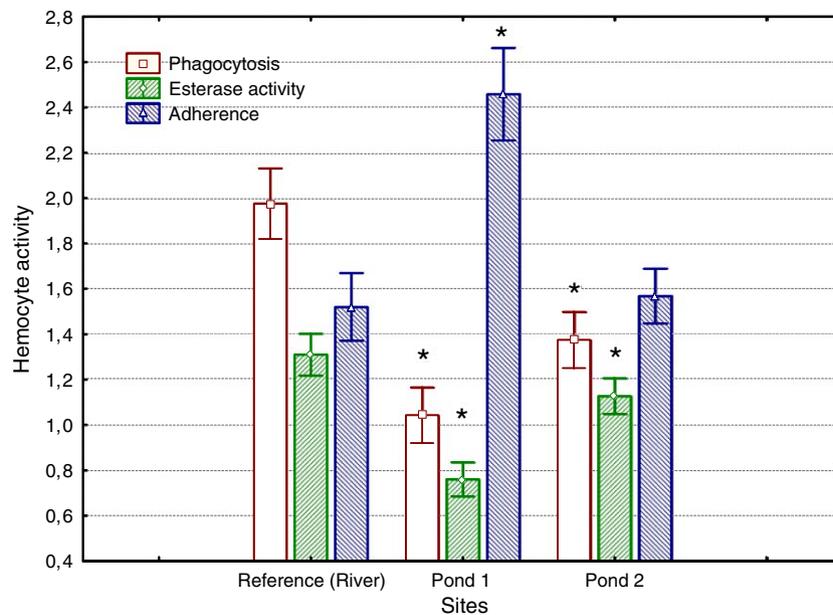


Fig. 3. Immunotoxic effects of domestic wastewater aeration lagoons on *Elliptio complanata* mussels. Mussels ( $n=10$ ) were caged and exposed to two domestic wastewater aeration lagoons and to the Richelieu River as the reference for 60 days in July and August 2004. The data represent the mean with standard error. The asterisk \* indicates significance at  $p < 0.05$  in respect to reference site.

esterase activity and cell adherence): carbamazepine, trimethoprim, naproxen, gemfibrozil, bezafibrate, oxytetracycline, novobiocin and sulfapyridine.

LPO in hemocytes was either increased or decreased with the PPCPs tested. LPO was increased only with 31% of the drugs tested: caffeine, cotinine, novobiocin and sulfapyridine. LPO thresholds were positively correlated with the number of polar compounds ( $R=0.7$ ;  $p=0.01$ ), indicating that a compound's polarity produces more oxidative damage. Gemfibrozil, naproxen and coprostanol reduced LPO in hemocytes. A threshold effect concentration analysis revealed only two distinct groups: the high effect group (coprostanol, naproxen, novobiocin) and the low effect group (caffeine, cotinine, novobiocin and sulfapyridine). A significant negative correlation ( $R=-0.72$ ;  $p<0.01$ ) was obtained between LPO and cell adherence to microplate wells. The hazard ratio calculation (Table 2) revealed that the relative risk varied from 0.03 to 2.1 (70 fold). An ANOVA on the hazard ratio revealed three distinct groups: the low risk group (caffeine, cotinine, fluoxetine, coprostanol), the moderate risk group (trimethoprim, naproxen, oxytetracycline), and the high risk group (sulfamethazole, sulfapyridine, novobiocin, gemfibrozil, carbamazepine, morphine and bezafibrate).

To determine the global effects of municipal wastewater on freshwater mussels, they were placed in aeration lagoons and exposed to the domestic wastewater of two different cities for 60 days (Fig. 3). For logistical reasons, the reference site chosen was markedly downstream in the Richelieu River well outside the influence of the effluent discharge. Phagocytic activity and intracellular esterase activity were significantly reduced in both lagoons, indicating hemocyte immunosuppression. Cell adherence was significantly elevated ( $p<0.05$ ) in pond 1 only.

#### 4. Discussion

Municipal effluents are known to contain a variety of PPCPs, which are released into aquatic ecosystems. Carbamazepine and caffeine are often found at concentrations in the  $\mu\text{g/L}$  range in municipal effluents (Gagné et al., 2005, in press; Clara et al., 2004). Moreover, carbamazepine (log octanol water partition coefficient or  $\log k_{ow}=2.45$ ) is persistent in these effluents (up to 100 days), hence it, along with caffeine ( $\log k_{ow}=0.85$ ), were proposed as chemical tracers for urban pollution (Clara et al., 2004; Termes et al., 2001; Standley et al., 2000). In the present study, carbamazepine was shown to induce phagocytic and intracellular esterase activity, as well as to reduce cell adherence to microplate wells. Because of its lipophilic properties, carbamazepine acts on membranes (narcosis-induced membrane depolarization) and this might augment their permeability and contribute to loss of cell adherence. This is corroborated by the negative ( $R=-0.52$ ) correlation observed between esterase activity and cell adherence. It appears that drugs found in municipal wastewaters affect the immune system of bivalves, but with varying intensity. Novobiocin, sulfapyridine, sulfamethazole and morphine inhibited phagocytic activity. Morphine was identified as a potentially high risk drug to hemocytes because it reduces phagocytosis at relatively low concentrations. It is known to modulate peritoneal inflammation reactions

in fish and mice by reducing leukocyte mobility (Chadzinska et al., 1999). Morphine also reduces cell adherence in mussel hemolymph, suggesting that hemocyte motility might also be affected. Morphine receptors have been reported in hemocytes where their activation reduces phagocytic activity and motility in hemocytes of the blue mussel *M. edulis* (Ottaviani et al., 1995) through nitric oxide production. Cell adherence was negatively correlated ( $R=-0.57$ ) with intracellular esterase activity and LPO in both cases. LPO in hemocytes was positively associated with the number of nucleophilic groups of the parent drug. This was also observed in rainbow trout hepatocytes, where the induction of LPO was positively related with the number of polar functional groups on the drug's carbon backbone (Gagné et al., 2005, in press). Thus, the number of functional groups on drugs is related to their potential to elicit oxidative stress in mussel hemocytes which in turn decreases cell adherence but not necessarily phagocytosis.

Based on the analysis of threshold concentrations (i.e. the potency of various compounds to produce a biological effect), gemfibrozil, bezafibrate, novobiocin, trimethoprim, sulfapyridine, morphine and carbamazepine were the drugs most often found in the high risk group. It appears that the activation of peroxisome proliferator receptors, inhibition of DNA synthesis, activation of opiate receptors and decrease in membrane permeability are all sensitive targets in freshwater mussel hemocytes. The mussel immune system depends on a cytokine network that resembles that of vertebrates (Betti et al., in press). Indeed, the exposure of circulating hemocytes to TNF- $\alpha$  induced cellular stress led to decreased phagocytosis in the absence of hemolymph, but increased phagocytic activity when present. Blue mussel hemocytes were shown to react to various pro-inflammatory cytokines such as interleukin-1, 6 and TNF- $\alpha$ , again suggesting a common and ancestral signalling system with vertebrates (Hughes et al., 1992). Although the coumeromycin antibiotic novobiocin is a potent inhibitor of ADP-ribosylation that could prevent liposaccharide (LPS)-induced TNF- $\alpha$  and interleukin-1, 6 and 10 secretions, elevated levels of TNF- $\alpha$  by LPS were not reduced by novobiocin in human peripheral blood mononuclear cells (Lhurmann et al., 1998). Moreover, morphine was shown to increase nitric oxide production, which in turn down-regulates immunocyte activity and perhaps phagocytosis (Mantione et al., 2002; Magazine et al., 1996). The peroxisome proliferator receptor agonists (PPRAs) gemfibrozil and bezafibrate have been shown to enhance phagocytic activity and decrease cell adherence in mussels. It has been shown that PPARs inhibit interleukin-1 production by nitric oxide production in lacrimal gland acinar cells and perhaps maintain phagocytic activity in hemocytes by a similar mechanism (Beauregard and Brandt, 2003). Thus, PPARs have some anti-inflammatory properties (Cernuda-Morollon et al., 2002) and appear to stimulate phagocytic activity in mussel hemocytes. This suggests interplay between interleukin-1 and TNF- $\alpha$  signalling by NO production and the maintenance of phagocytic activity in hemocytes.

Both phagocytosis and intracellular esterase activity were significantly reduced in caged mussels exposed to aeration lagoons treating municipal wastewaters. Immunosuppression

was also observed in hemocytes of *E. complanata* mussels exposed for 60 days to the plume of a major municipal effluent discharging to the Saint-Lawrence River (Blaise et al., 2002). Municipal effluents following either primary treatment (Blaise et al., 2002) or aeration lagoon treatment (as shown in this study) clearly inhibit phagocytosis activity in mussels. This suggests that exposing mussels to municipal treatment plant wastewaters in turn leads to a state closely related to inflammation that causes decreased phagocytosis, perhaps through TNF- $\alpha$  or cytokine interactions. Phagocytosis activity could be theoretically decreased by high levels of micro-organisms in municipal effluents but their removal by filtration had no significant effects on phagocytosis activity in mussels (Blaise et al., 2002). Moreover, bacterial densities in the two aerated lagoon treatment ponds investigated (Fig. 3) were relatively low as suggested by total and fecal coliform data reported (<100 to 500 total coliforms and <100 fecal coliforms per 100 mL). However, the contribution of PPCPs contained in these aeration lagoons to the observed immunotoxic effect remains to be confirmed. Municipal effluents were recently identified as causing pro-inflammatory conditions in exposed mussels, as determined by the elevation of both lipid peroxidation and cyclooxygenase activity (Gagné et al., 2005, in press). In addition, municipal effluents are known to release estrogens such as estradiol-17 $\beta$  (E2), estrone and the anti-contraceptive drug ethinyl-estradiol-17 $\beta$ . A study revealed that *Mytilus edulis* hemocytes exposed to E2 (25 nM) increased intracellular free calcium (loss of cell viability and initiation of apoptosis) and affected the phosphorylation state of proteins through a tyrosine kinase-mediated signal (Canesi et al., 2004). Estrogens were also shown to stimulate the release of NO in the mussel nervous system (Stefano et al., 2003) and possibly contribute to the reduction of phagocytosis as well. In another study, carp injected with E2 displayed reduced phagocytic activity as well as superoxide anion and nitric oxide production in macrophages (Watanuki et al., 2002).

In conclusion, this study has shown that immunocytes from freshwater mussels are sensitive to various pharmaceutical compounds usually found in municipal effluents. Novobiocin, the opiate morphine sulfate, gemfibrozil, bezafibrate and carbamazepine were among the most potent PPCPs tested in altering immune function. The respective threshold concentrations following a 24-h exposure in vitro to the PPCPs were about 10–100 times higher than those observed in effluents, but it is not known if longer periods of exposure to smaller amounts of PPCPs would result in similar immunotoxic effects. Moreover, the bioaccumulation potential of these drugs in aquatic organisms is not well understood at the present time, which makes risk assessment difficult. Nevertheless, the immunotoxic properties of these drugs seem to implicate a common pro-inflammatory mechanism through cytokine signalling, oxidative stress and loss of membrane permeability. The observed responses of the immune system of freshwater mussels exposed to aeration lagoons for the treatment of domestic wastewater are consistent with the manifestation of inflammatory-associated effects. However, the exact nature of the contaminants

contained in these treatment lagoons that act on the immune systems of mussels is presently unknown.

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