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Immunotoxic potential of aeration lagoon effluents for the treatment of domestic and hospital wastewaters in the freshwater mussel *Elliptio complanata*

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Abstract

Municipal wastewaters are major sources of pollution for the aquatic biota. The purpose of this study was to determine the levels of some pharmaceutical products and the immunotoxic potential of a municipal wastewater aeration lagoon for the treatment of the domestic wastewaters of a small town with wastewater inputs from a 400-bed hospital complex. Endemic mussels were collected, caged and placed in the final aeration lagoon and at sites 1 km upstream and 1 km downstream of the effluent outfall in the receiving river for a period of 14 days. The results showed that the final aeration lagoon contained high levels of total coliforms, conductivity and low dissolved oxygen (2.9 mg/L) as well as detectable amounts of trimethoprim, carbamazepine, gemfibrozil, and norfloxacin at concentrations exceeding 50 ng/L. The lagoon effluent was indeed toxic to the mussel specimens, as evidenced by the appearance of mortality after 14 days (10% mortality), decreased mussel weight-to-shell-length ratio and loss of hemocyte viability. The number of adhering hemocytes, phagocytic activity, total nitrite levels and arachidonic cyclooxygenase activity were significantly higher in mussels placed in the final aeration lagoon. A multivariate analysis also revealed that water pH, conductivity, total coliforms and dissolved oxygen were the endpoints most closely linked with phagocytic activity, the amount of adhering hemocytes and loss of hemocyte viability. In conclusion, exposure of mussels to treated aerated lagoon wastewater is deleterious to freshwater mussels where the immune system is compromised.

Key words: mussels; municipal wastewaters; immunocompetence; inflammation; cyclooxygenase activity
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Introduction

Effluents emanating from municipal wastewater treatment plants are recognized as major sources of pollution for aquatic ecosystems. In addition to heavy metals, polycyclic aromatic hydrocarbons and other industrial contaminants, containing a cocktail of contaminants such as endocrine-disrupting substances (Sumpter and Jobling, 1995; Vethaak et al., 2005; Chambers et al., 1997). Pharmaceutical and personal care products (PPCPs) have also been identified in municipal effluents; they represent an important source of contamination of the aquatic environment (Kümmerer, 2001; Andreozzi et al., 2003). PPCPs have been found to contain many types of over-the-counter and prescription drugs such as non-steroidal anti-inflammatory drugs (acetaminophen, naproxen and ibuprofen), antibiotics (sulfonamides and macrolides), cholesterol-regulating drugs (clofibrates and statins), anti-hypertensives (β-blockers and inhibitors of angiotensin converting enzyme) and neuro- and/or psychoactive drugs (morphine, selective serotonin-reuptake inhibitors, benzodiazepines and illicit drugs). Most of the PPCPs in municipal effluents are thought to be the by-product of human consumption but they can be released in significant quantities in hospital wastewaters discharged to municipal sewage systems. It is difficult to determine the contribution of hospital wastewaters to the load of PPCPs in the aquatic environment because of the diversity of wastewater treatment methods, the respective sediment-water partition of each PPCP, the size of the towns with respect to hospital effluent flow rates, types of hospital treatment facilities (oncology versus surgical wards) and the dilution properties (hydrology) of the receiving waters. It is expected that the more common PPCPs are likely to be found in municipal effluents. For example, trimethoprim (a bacteriostatic agent used in hand-washing liquids) and roxithromycin (a macrolide antibiotic used to treat respiratory tract infections) were among the products that contributed the most to municipal effluent loadings (Ort et al., 2010).

Benthic invertebrates such as bivalves, annelids and microcrustaceans are particularly at risk from point sources of pollution because of their sessile nature. Bivalves are long-lived (ranging from years to decades) and filter high

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volumes of water and suspended solids during feeding and respiration. The immune system in mussels is found in the hemolymph, which is an open circulatory system of defence against invading micro-organisms. Mussels lack a complementary system (acquired immune capacity or antibody production) but are well equipped with innate immunity that involves cellular- and humoral-based defence capacities (Gliński and Jarosz, 1997). These include phagocytosis, lysozyme excretion, peroxinitrite release during oxidative burst, opiate-dependent immunocyte regulation, and many lectins that can act as biocidal and agglutination (bacteriostatic) agents (Nieto-Fernandez et al., 1999; Arumugam et al., 2000). The immune function of mussels can be determined using a convenient microplate method that measures the capacity of hemocytes to ingest fluorescently labelled bacteria, the relative amount of adhering and viable hemocytes (Blaise et al., 2002). Mussels possess arachidonate cyclooxygenase (CO*x*) activity, which is the rate-limiting enzyme involved in the production of pro-inflammation mediators (eicanosoid) and prostaglandins for the contraction of smooth muscles during spawning (Canesi et al., 2002; Gagné et al., 2004). They also possess nitric oxide (NO) synthase activity, which produces NO, an important cellular messenger involved in many pathophysiological processes like inflammation, vasodilation, and opiate-mediated immunosuppression (Liu et al., 1996; Magazine et al., 1996).

The purpose of the present study was to examine the impacts of aeration lagoon effluent for the treatment of domestic and hospital wastewaters on the immune system of the endemic mussel *Elliptio complanata*. Changes in hemocyte adherence, viability and phagocytosis were determined in the mussels. The total levels of NO*x*, CO*x* activities and lipid peroxidation were concurrently determined in the hemolymph to verify whether the humoral components of the immune system could be affected as well. The contribution of PPCPs found in the effluent on the invertebrate immune system was discussed in relation to the observed effects on caged mussels.

1 Materials and methods

1.1 Mussel handling and exposure

E. complanata mussels were collected by hand in the Richelieu River (Quebec, Canada) in late May, during the period of late gametogenesis. They were placed in aerated tanks for two weeks at 15°C and fed with commercial algae preparations. The mussels were caged following a standard operating procedure (Salazar and Salazar, 2001), in which 30 mussels (7 ± 0.7 cm shell length) are placed in each cage. The first cage was submerged at a depth of 2 m in the final aeration lagoon treating the domestic wastewaters of a town of some 35,000 inhabitants, with wastewater inputs from a 400-bed hospital complex. Two other cages were set up and submerged at sites located 1 km upstream (reference site) and 1 km downstream (polluted site) of the effluent outfall in the Mille-Iles River (Quebec, Canada) in June 2006. The exposure period lasted 14 days, until the appearance of mortality in more than 10% of mussels in the final aeration lagoon. The wastewater treatment method consisted of aeration, photo- and biological degradation under constant aeration of four interconnected ponds. On occasion, the first lagoon (incoming raw wastewater) is supplemented with ferric chloride to assist the flocculation of particles. A 24-hr composite of the effluent was collected (40 L) for the analysis of a suite of pharmaceutical products using high-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS; ALS Laboratory Group, Environmental Division, Nepean, Ontario, Canada) and gas chromatography (GC-MS/MS after extract derivatization (Lajeunesse and Gagnon, 2007). A total of 42 drugs were screened: six neutral drugs (trimethoprim, pentoxufylline, cyclophosphamide, carbamazepine, caffeine and cotinine), ten acidic pharmaceuticals (bezafibrate, clofibrate, diclofenac, fenoprofen, gemfibrozil, ibuprofen, indomethacin, ketoprofen, naproxen and salicylic acid), 17 sulfonamide antibiotics (sulfacetamide, sulfapyridine, sulfadiazine, sulfamethoxazole, sulfathiazole, sulfamerazine, sulfamoxole, sulfamethizole, sulfabenzamide, sulfamethazine, sulfamethoxypyridazine, sulfameter, sulfachloropyridazine, sulfaquinoxaline, sulfamethoxine, sulfaphenazole and sulfaguanidine), two macrolide antibiotics (roxithromycin and novobiocin), four quinolone antibiotics (norfloxacin, ofloxacin, oxolinate and pipemidate), and three tetracycline antibiotics (oxytetracycline, tetracycline and chlortetracycline). The data were expressed as ng/L in surface water.

After the exposure period, the cages were harvested and the mussels depurated in dechlorinated tap water for 24 hr at 15°C. Condition factor (CF) was determined based on mussel wet weight (g) by longitudinal shell length (cm). A 0.5–0.7 mL sample of the hemolymph was taken by syringe at the anterior adductor muscle and kept on ice in microcentrifuge tubes (Sarstedt, USA). The activity of dihydrofolate reductase (DHFR), a rate-limiting enzyme in the synthesis of purines, was measured in the visceral mass as a biomarker of exposure to municipal effluents known to contain (drug-based) inhibitors of DHFR (Gagné et al., 2010).

1.2 Immunocompetence evaluation

Phagocytic activity was assessed with a fluorescence-based microplate assay that uses fluorescently labelled *Escherichia coli* (Blaise et al., 2002). Briefly, a 100-μL hemolymph sample was added to three wells of a 96-well black polystyrene microplate for determination of phagocytosis, number of adhered hemocytes and viability. The hemocytes were allowed to adhere to the bottom of the wells for 1 hr at 20°C and the wells were washed once with phosphate-buffered saline (PBS) previously diluted one-quarter with distilled water. Fluorescein-labelled bacteria were then added to the wells (25 μL at a density of 5×10^7 *E. coli*/well) and allowed to stand for 2 hr. After the incubation period, the wells were washed twice and the cells resuspended in 50 μL of diluted PBS. The amount of engulfed bacteria was determined after quenching the externally bound bacteria with a solution of Trypan blue

in citrate buffer at pH 5.5 (Hed, 1994). Fluorescence was measured at 485 nm excitation and 520 nm emission (Bioscan, USA) using standard solutions of fluorescein for calibration. Phagocytic activity was expressed as umol fluorescein/mg cell protein. In separate wells, the density of adhered hemocytes and cell viability was determined by the fluorescamine and carboxyfluorescein retention tests, respectively (Lorenzen and Kennedy, 1993; Altman et al., 1993). Standard solutions of fluorescein (for cell viability and phagocytosis) and serum bovine albumin were used for calibration (total proteins).

The activity of arachidonate cyclooxygenase (CO*x*) and NO levels were determined in the hemolymph. The CO*x* activity was determined in hemocytes according to the oxidation rate of 2,4-dichlorofluorescin in the presence of arachidonate. The reaction mixture (200 μL) consisted of 50 μmol/L arachidonate, 50 μL hemolymph, 5 μmol/L dichlorofluorescin, 0.1 μg/mL horseradish peroxidase in 50 mmol/L Tris-HCl, at pH 8 and containing 0.05% Tween-80. The mixture was allowed to stand at 20°C for 0, 10, 20 and 30 min. Fluorescence readings at 485 nm excitation and 520 nm emission were taken. The production of NO was estimated by measuring the levels of nitrite concentration in the plasma. Because NO readily reacts with oxygen to yield nitrite (NO_2^-) and nitrates (NO_3^-), nitrate reductase was added to measure the total nitrite concentration in the plasma (Verdon et al., 1995). Briefly, 50 μL of hemolymph was added to 25 μL of nitrate reductase 80 U/L (Sigma) and 50 μL NADPH at 1 μmol/L (Sigma) prepared in 25 mmol/L potassium phosphate pH 7.4. After incubation at room temperature for 30 min, the concentration of NO_2 was measured by the addition of 100 μL of Griess reagent (Sigma) to the wells. Absorbance was read after 30 min at 450 nm and sodium nitrite standards (Sigma) were used to express the results in μmol NO_2/mg proteins. The levels of lipid peroxidation (LPO) were also determined in the hemolymph using the thiobarbituric acid methodology that reacts with malonaldehyde following the oxidative breakdown of unsaturated phospholipids (Wills, 1987). Standard solutions of tetramethoxypropane (a stabilized form of malonaldehyde) were used for calibration. Fluorescence was measured at 540 and 620 nm for excitation and emission, respectively.

1.3 Data analysis

Biomarkers were determined in 12 mussels per site. The homogeneity of variance was examined by the Bartlett test. In all cases, the distribution of the data was considered homogeneous and normal. The data were then subjected to an analysis of variance and the difference among the final aeration lagoon and the downstream sites from the upstream site was determined using the least square difference test. Correlation, factorial and site classification analyses were determined by the Pearson-moment, principal-component and discriminant-function procedures, respectively, using the Statistica software package (version 8.0). Significance was set at $p < 0.05$.

2 Results

The mussels were exposed to the effluent in the final aeration lagoon and the municipal effluent dispersion plume at sites 1 km upstream and 1 km downstream of the outfall for 14 days each. The retention time of the aeration lagoon wastewater was estimated between 15 to 25 days. The flow rate of incoming wastewaters is subject to change depending on the rainfall. The general physico-chemical characteristics of the final aeration lagoon and the upstream and downstream sites are shown in Table 1. The water temperature did not change significantly but changes were observed in the other parameters. The pH of the lagoon water was 0.6, unit below the receiving water sites. Lagoon water conductivity was 1018 μS/cm, while values for the upstream and downstream sites were 235 and 265 μS/cm, respectively. Based on these values, the estimated dilution of the effluent at the downstream site was 4% (for dissolved components). The dissolved oxygen content of the aeration lagoon and the downstream and upstream sites were, respectively, 2.9 ± 0.6, 5.8 ± 1.0 and 7.7±1.0 mg/L, indicating an hypoxic environment in the final aeration lagoon. The total coliform levels in the final aeration lagoon were 2200 ± 400 counts/100 mL, dropping to 1000 ± 200 counts/100 mL at the downstream site, compared to 500 ± 125 counts/100 mL at the upstream site. This suggests that the upstream site is also influenced by other sources of thermotolerant coliforms in this river system.

Concentrations of pharmaceutical products were determined in the final aeration lagoon since this treatment system also treats the wastewater generated by a hospital with a top capacity of 400 beds (Table 2). Of the 43 drugs and metabolites analyzed, 17 (or 40%) were detected at levels exceeding the limit of detection of the instrument. Recovery experiments revealed that all compounds were well extracted, with a mean recovery of 80% ± 15%. The following compounds were the most abundant (i.e. found at concentrations > 0.1 μg/L): the neurorelaxant carbamazepine and the bacteriostatic drug trimethoprim, which is a DHFR inhibitor. The other compounds were found at concentrations < 0.1 μg/L, including caffeine, bezafibrate, diclofenac, gemfibrozyl, clofibrate and many quinoline antibiotics plus one sulfonamide antibiotic (sul-

Table 1 Physico-chemical characteristics of lagoon effluent and sites in receiving waters*

Site	Temperature (°C)	pH	Conductivity (μS/cm)	Dissolved oxygen (mg/L)	Total coliforms (counts/100 mL)	Fecal coliforms (counts/100 mL)
Aeration lagoon	18.0–21.0	7.40 ± 0.25	1018 ± 150	2.9 ± 0.6	2200 ± 400	110 ± 20
Upstream	18.7–20.5	8.06 ± 0.16	235 ± 16	7.7 ± 1.0	500 ± 125	< 100
Downstream	18.9–21.5	8.40 ± 0.20	265 ± 3	5.8 ± 1.0	1000 ± 200	< 100

* Gagné et al., 2010.

Table 2 Load of pharmaceutical compounds in the final aeration lagoon for the treatment of domestic and hospital wastewaters

Chemical class	Mode of action	Drug	Concentration (ng/L)
Neutral	Antibiotic; inhibitor of dihydrofolate reductase	Trimethoprim	200 ± 60
	Mood stabilizing and anticonvulsant	Carbamazepine	170 ± 35
	Central nervous system stimulant	Caffeine	12 ± 4
	Metabolite of nicotine	Cotinine	22 ± 6
Acidic	Agonist of peroxisome proliferation activated receptors (hypolipidemic)	Bezafibrate	13 ± 3
		Clofibrate	13 ± 4
		Gemfibrozil	63 ± 18
	Anti-inflammatory; cyclooxygenase activity inhibitors	Naproxen	46 ± 15
Sulfonamide antibiotic	Sulfonamide antibiotic; dehydropteroate synthetase inhibitor	Sulfamethizole	36 ± 12
Quinoline antibiotic	Antibiotic: DNA gyrase inhibitor	Norfloxacin	59 ± 20
		Ofloxacin	58 ± 30
		Oxolinic acid	20 ± 5
		Pipemidic acid	40 ± 12

famethizole).

Mussels exposed to the final aeration lagoon showed signs of acute stress, as revealed by the appearance of mortality (10% mortality) after 14 days. The condition factor (mussel weight/shell length) and mussel size (weight) were significantly affected in mussels exposed to the final aeration lagoon (Fig. 1a and b). Indeed, both the mussel weight-to-length ratio and mussel weights dropped 1.5-fold in mussels exposed to the final aeration lagoon but this effect was not observed at the downstream site. The activity of DHFR was used as a marker enzyme of DHFR inhibitor drugs such as methotrexate and/or timethoprim (Fig. 1c). The DHFR activity was significantly reduced 1.4-fold in exposed mussels at the site downstream of the municipal effluent plume, but not significantly the final aeration lagoon. No significant correlation between DHFR activity and mussel condition factor was observed.

The immune system in mussels was studied at the cellular level by monitoring changes in hemocyte viability, adhered hemocyte counts, and phagocytosis, and at the inflammation level, as determined by NO*x*, CO*x* and LPO in the hemolymph (Figs. 2 and 3). As with mortality events observed in the final aeration lagoon, hemocyte viability in mussels was significantly reduced 1.5-fold relative to the upstream site (Fig. 2a). No significant correlations were observed with either DHFR activity or condition factor. Relative levels of adhered hemocyte were also determined in mussels (Fig. 2b). The proportion of adhering hemocytes was significantly increased in the caged mussels in the aeration lagoon and at the downstream site relative to the upstream site. Levels of hemocyte were negatively correlated with viability ($r = –0.50$; $p = 0.05$; Table 3). Phagocytic activity was also affected by the aeration lagoon effluent (Fig. 2c). Phagocytic activity was significantly induced in mussels at both the downstream site and in the aeration lagoon, reaching 6.6-fold induction in the latter. Phagocytic activity was significantly correlated with the relative amount of adhered hemocyte ($r = 0.61$; $p = 0.04$; Table 3).

At the humoral level, total nitrite levels and CO*x* activity were determined (Fig. 3a and b). Total NO*x* levels were readily elevated in mussels placed in the final aeration lagoon (Fig. 3a). However, no changes were observed between the upstream and downstream sites. NO*x* levels were significantly correlated with the condition factor ($r = –0.78$; $p < 0.01$) and cell viability ($r = –0.54$; $p < 0.01$), highlighting the negative effects on mussel health (Table 3). Hemolymph CO*x* activity was readily increased in mussels placed in the final aeration lagoon (Fig. 3b); no difference was observed between the upstream and downstream sites. The CO*x* activity was marginally correlated with hemocyte viability ($r = –0.46$; $p = 0.06$; Table 3). The extent of LPO in the hemolymph was also determined (Fig. 3c). LPO activity was generally lower in mussels at both the downstream site and the aeration lagoon. However, LPO was higher in the latter mussels compared to those from the downstream site. A correlation analysis revealed that LPO was negatively correlated with phagocytic activity ($r = –0.67$; $p < 0.01$) and hemocyte adherence ($r = –0.48$; $p < 0.01$; Table 3).

In an attempt to describe the general effects of the aeration lagoon on the immune system of freshwater mussels, factorial and discriminant-function analyses were performed (Fig. 4a and b). The factorial analysis revealed

Table 3 Correlation analysis of biomarker data

	CF	Phag	Via	Adh	NO*x*	CO*x*	LPO	DHFR
Cond. factor	1	–0.11 ($p < 0.1$)	0.43 ($p < 0.1$)	–0.01 ($p > 0.1$)	**–0.78*** ($p < 0.05$)	0.06 ($p > 0.1$)	0.02 ($p > 0.1$)	–0.14 ($p > 0.1$)
Phag		1	–0.18 ($p > 0.1$)	**0.61** ($p < 0.05$)	0.38 ($0.05 < p < 0.1$)	0.21 ($p > 0.1$)	**–0.67** ($p < 0.01$)	–0.15 ($p > 0.1$)
Via			1	**–0.50** ($p = 0.05$)	**–0.54** ($p < 0.01$)	–0.46 ($0.05 < p < 0.1$)	–0.03 ($p > 0.1$)	–0.33 ($p > 0.1$)
Adh				1	0.08 ($p > 0.1$)	0.06 ($p > 0.1$)	**–0.48** ($p < 0.01$)	–0.01 ($p > 0.1$)
NO*x*					1	0.16 ($p > 0.1$)	0.20 ($p > 0.1$)	0.02 ($p > 0.1$)
CO*x*						1	–0.14 ($p > 0.1$)	0.03 ($p > 0.1$)
LPO							1	0.36 ($p > 0.1$)

CF: condition factor; Phag: phagocytosis; Via: viability; Adh: adhered hemocyte; NO*x*: nitric oxide; CO*x*: cyclooxygenase; LPO: lipid peroxidation; DHFR: dehydrofolate reductase.

* Significant correlations are shown in bold.

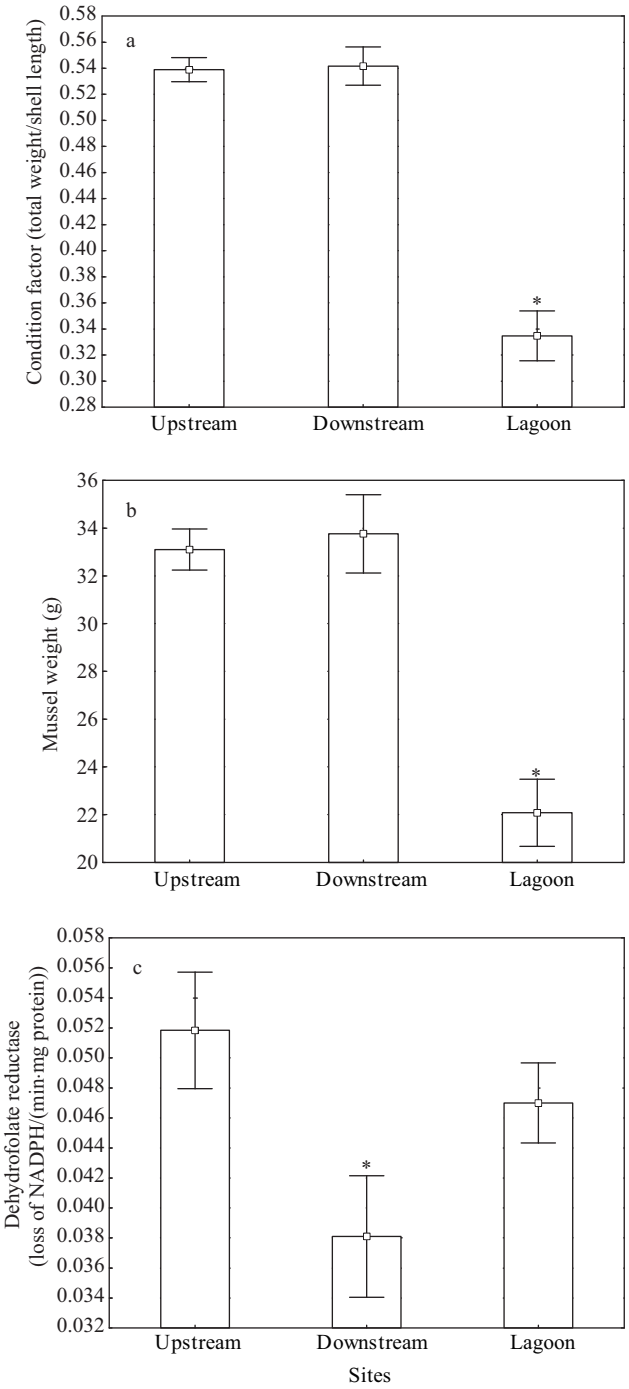

Fig. 1 Condition factor of mussels (a), the change in weight (b) and dehydrofolate reductase (DHFR) activity (c) in mussels exposed to an aeration lagoon for the treatment of domestic and hospital wastewaters. Freshwater mussels were placed in experimental cages for 14 days in the final aeration lagoon and at sites 1 km downstream and 1 km upstream of the effluent dispersion plume. The data represent the mean with standard error. * Significance at $p < 0.05$ compared to upstream reference site.

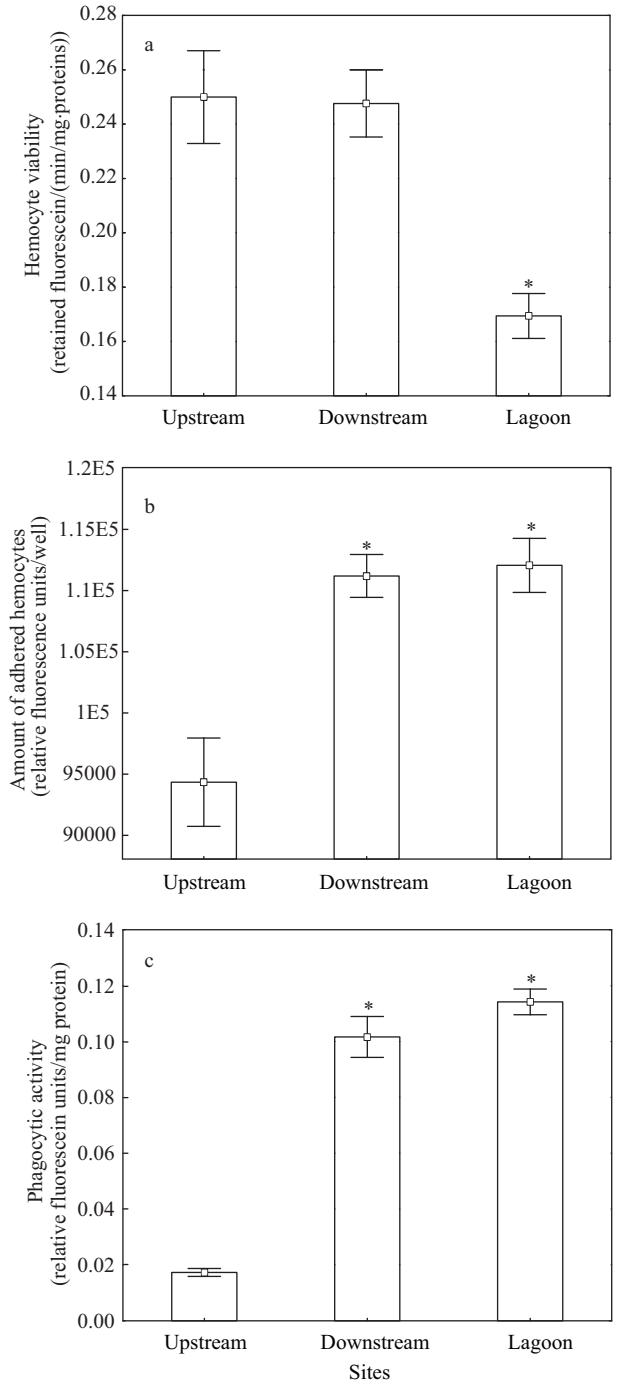

Fig. 2 Changes in viability of hemocytes (a), amount of adherent hemocytes (b) and phagocytic activity (c) in mussels exposed to aeration lagoon effluent. Mussels were exposed to the lagoon effluent and the upstream/downstream sites for 14 days. Phagocytic activity was determined in the hemolymph. The data represent the mean with standard error. * Significance $p < 0.05$ compared to upstream reference site.

that 60% of the total variance was explained by the parameters and that the following measurements exhibited high factorial weights (> 0.7): water conductivity, pH, total coliforms, dissolved O_2, NOx, and phagocytosis (Fig. 4a). Phagocytic activity and hemocyte adherence were clustered together, suggesting that these two properties are closely associated. Water conductivity and total coliforms were closely associated with NOx in the hemolymph, suggesting an interaction between ambient water salinity and bacterial loadings with the level of inflammatory mediator NOx in the hemolymph. Mussel condition factor and hemocyte viability were also closely associated with pH and negatively with water conductivity and coliform levels. A discriminant function analysis of the biological responses revealed that the three sites were correctly identified with upstream, downstream and aeration lagoon sites (95%, 88% and 79% correctness, respectively). The aeration lagoon was sometimes classified similarly to the downstream site, but it differed readily from the upstream

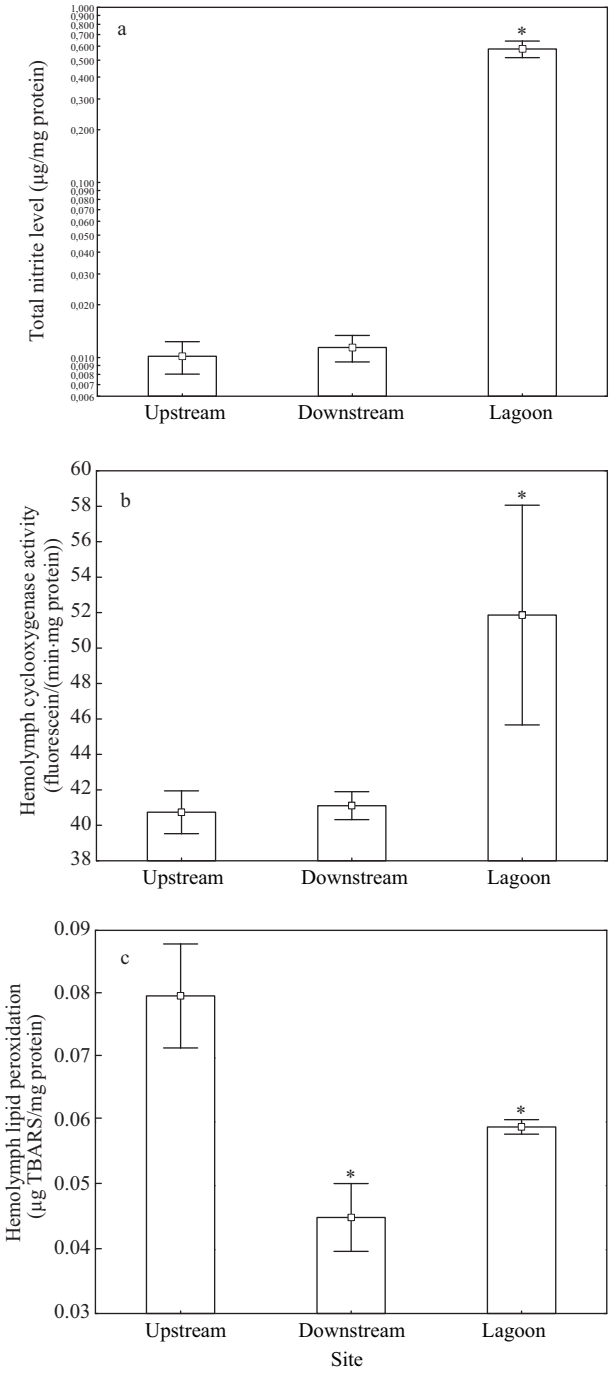

Fig. 3 Change in mediators of inflammation in freshwater mussels exposed to municipal lagoon wastewaters. The levels of total nitric oxide (a), cyclooxygenase activity (b) and LPO (c) were determined in the hemolymph. * Indicate significance from the upstream reference site at $p < 0.05$ level.

site. Phagocytosis, cell adherence and condition factor were the biomarkers that discriminated the sites the most (component 1 on the *X*-axis).

3 Discussion

The municipal wastewater treatment lagoon in this study treats the wastewaters generated by a moderate-sized population (approx. 35,000 residents) and receives the wastewater of a 400-beds hospital. The contribution of the hospital effluent to the municipal effluent in respect

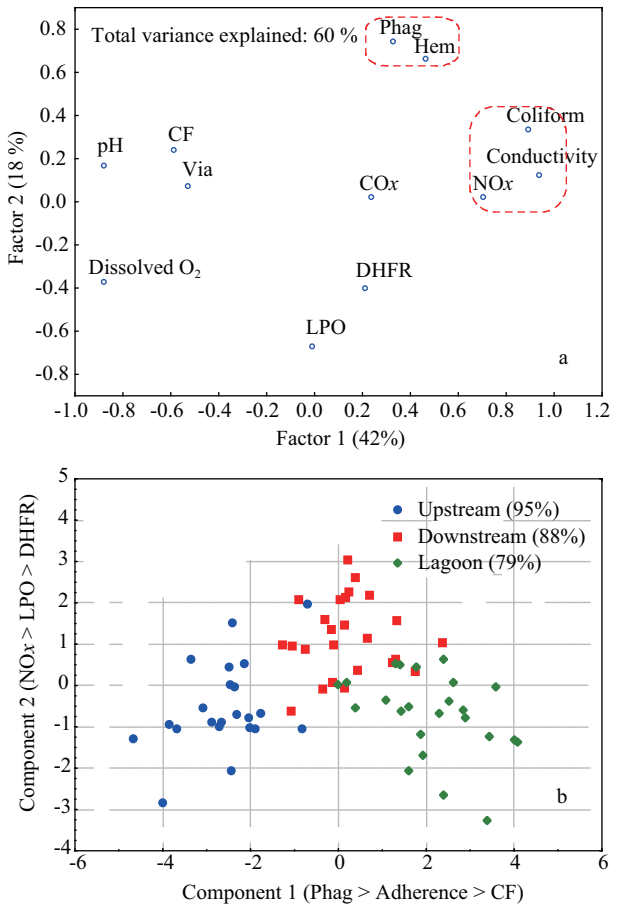

Fig. 4 Results of factorial and discriminant function analyses. Factorial (a) and discriminant function (b) analyses were performed based on the physiological responses for the latter. Total nitrates and nitrites (NO_x), amount of adhered hemocyte (Hem), phagocytosis (Phag), lipid peroxidation in hemolymph (LPO), condition factor (CF), viability (Via) and dehydrofolate reductase activity (DHFR).

to pharmaceuticals is difficult to determine because of the paucity of data on the relative levels of pharmaceutical products stemming from hospital wastewaters. The size of the hospital and type of wards (i.e., number of beds and proportion of specialized treatment units in psychiatry or oncology, for example), in addition to the population size and wastewater treatment methods, are other factors that could influence the occurrence of therapeutic agents in wastewaters. A recent study on cytostatic drugs in wastewaters from 21 hospitals of different sizes in China found methotrexate and cyclophosphamide at concentrations between 20 and 100 ng/L, respectively (Yin et al., 2010). Methotrexate was detected in the influent (raw, untreated wastewaters) of a sewage treatment plant at a concentration of 59 ng/L; the levels were below the detection limit after undergoing physico-chemical treatment (Garcia-Ac et al., 2009). In another study, the contribution of an average-sized hospital (200 beds) was examined to determine the proportion of pharmaceuticals entering the municipal treatment facilities (Ort et al., 2010). Of the 85 compounds analyzed, only 15% of the drugs contributed significantly to the load in the municipal effluent, with trimethoprim and roxithromycin being the major drugs. The proportion of pharmaceuticals from the hospital wastewaters to the treated municipal effluent represented 1% to 11% of the

total loads for some compounds (Langford and Thomas, 2009). For example, 11% of the propanol (a β-blocker) and 2% of the atenolol, carbamazepine, metaprolol and atorvastatin originated from the hospital wastewater. This suggests that most of the drugs detected in the municipal effluents are rather the result of human drug consumption than the release of (untreated) hospital wastewaters to the aeration ponds.

Given the fact that mussels are filter-feeders and exposed to both the dissolved and suspended particulate components of wastewaters, these organisms are relevant test species to bio-monitor the toxicity of complex mixtures such as municipal effluents. Total coliforms, conductivity and dissolved oxygen levels were significantly affected by the aeration lagoon discharges and these were considered major components under a factorial analysis. Moreover, total NO*x* levels were also highly elevated in the hemolymph of mussels exposed to the final aeration lagoon, where a 32% drop in hemocyte viability was observed. The effects of dissolved oxygen on the immune responses were studied in the scallop (Chen et al., 2007). Scallops were cultivated under various dissolved oxygen conditions (8.5 to 2.5 mg/L) for 21 days at a mean water temperature of 16°C. Lower levels of dissolved oxygen (2.5 and 4.5 mg/L) resulted in lower survival rates (82%). Total hemocyte counts were also decreased (2.5 mg/L), which is similar to the reported value of 2.9 mg/L in the final aeration lagoon in this study. This finding suggests that the hypoxic conditions of the aeration lagoon have contributed, at least in part, to the loss of hemocyte viability in mussels.

A study on *Elliptio complanata* mussels exposed to a physical- and chemical-treated effluent in real time for 7 weeks showed significant reductions in phagocytosis and cell viability (Gagné et al., 2008), although both NO*x* levels and CO*x* activities were readily increased. Furthermore, the response of the immune system to urban effluents is dependent on the duration of exposure. For example, increased phagocytic activity, NO*x* levels and H_2O_2 production were observed in *Mytilus edulis* mussels exposed to effluents for 14 days followed by decreased activity and production at 21 days, with the exception of the sustained elevation of H_2O_2 (Akaishi et al., 2007). Hence, the increase in phagocytic activity and inflammatory agents represents the first line of response but if the duration of exposure is maintained, then only the inflammatory mediators remain elevated. Interplay takes place between inflammation and phagocytosis wherein sustained inflammation can reduce phagocytosis in mussels exposed to municipal effluents (Gagné et al., 2008).

The presence of pharmaceuticals in the treated effluents can also affect the immune system in mussels. Estrogenic compounds such as 17β-estradiol were shown to inhibit phagocytic activity and oxygen radical production in *Mytilus galloprovincialis* hemocytes at concentrations above 25 nmol/L, while activating them at lower concentrations (Canesi et al., 2006). The production of oxygen radicals was mediated by NO*x* production since the presence of NO synthetase inhibitors prevented the accumulation of nitrite in tissues. The effects of estradiol-17β were also prevented by tamoxifen, an estrogen receptor blocker. In a follow-up study, loss of lysosomal membrane stability in hemocyte (another marker of cell viability) was significantly induced with many estrogenic compounds such as nonyphenol-polyethoxylate and mestranol (Canesi et al., 2007a). These last two compounds were the most potent after estradiol-17β (EC50 13 nmol/L; LOEC 5 nmol/L). Carbamazepine is commonly found in municipal effluents and mussel exposure to 0.1 to 10 μg/L of carbamazepine for 7 days resulted in a significant decrease in hemocyte lysosomal membrane stability (Martin-Diaz et al., 2009). It also reduced cell signalling in hemocytes by reducing cAMP levels and protein kinase activity. The blood lipid-lowering drugs bezafibrate and gemfibrozil were also able to induce a rapid lysosomal membrane destabilization, NO*x* production and increased phagocytic activity in mussels injected with these compounds (Canesi et al., 2007b). In a previous study using freshwater mussel hemocytes exposed *in vitro* to a selection of pharmaceuticals, those compounds capable of disrupting the cytokine signalling network by the nitric oxide pathway and cell membrane permeability were generally the most potent ones (Gagné et al., 2006). The β-blocker propanolol was shown to affect cAMP-dependent signalling pathways, which is consistent with its therapeutic mode of action (Franzellitti et al., 2011). This drug also compromised lysosomal membrane stability in hemocytes. Dopamine β-hydroxylase was induced by liposaccharide stimulation in scallop hemocytes, suggesting a role for dopamine in the activation of immunocytes (Zhou et al., 2011). Given that municipal effluents are known to alter dopamine metabolism in mussels, this could represent another physiological pathway by which immunocompetence is altered. Indeed, mussels caged in the same effluents had elevated levels of dopamine and decreased monoamine oxidase activity in their gonadal tissues (Gagné et al., 2010). This is consistent with the observed changes in hemocyte function in this study, where high increases in phagocytosis and cell adherence were found. The levels of non-steroidal anti-inflammatory drugs can reach concentrations as high as 10 μg/L in municipal effluents. Ibuprofen was shown to destabilize hemocyte lysosomal membranes at a threshold concentration of 0.2 μg/L and DNA damage (micronuclei frequency) at 2 μg/L after 96 hr exposure in zebra mussels (Paolini et al., 2011), suggesting that the presence of this drug class could be harmful to the mussel immune system.

In conclusion, municipal effluents have the potential to activate some immune responses in caged freshwater mussels exposed for two weeks. On the one hand, exposure to the final aeration lagoon leads to decreased condition factor (mussel weight-to-shell-length ratio), hemocyte viability and LPO. On the other hand, exposure to the final aeration lagoon led to increased hemocyte adherence, phagocytic activity, NO*x* levels and CO*x* activity, suggesting a sustained state of inflammation in mussels. Some of these effects were still observed at the downstream site (phagocytosis, hemocyte adherence, DHFR activity and LPO), suggesting negative impacts on the receiving waters of river.

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