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Immunotoxicity of Municipal Effluents to Freshwater Mussels

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Abstract

The release of treated municipal wastewaters to the aquatic environment raises concern about the health impacts on local resident invertebrates such as mussels. The purpose of this study was to examine the immunotoxicity of two types of treated municipal effluents—physico-chemical treated and physico-chemical with additional ozonation—to freshwater mussels. Immunocompetence was followed by tracking changes in hemocyte viability, adherence, phagocytosis, vitellogenin (Vtg)-like proteins and the pro-inflammatory precursors nitric oxide (NOx) production and arachidonate cyclo-oxygenase (COX) activity. The study results revealed that following a two-week continuous-flow exposure to the effluents, a reduction in hemocyte viability, adherence, NOx and COX activities was observed. Vtg-like proteins were also increased, highlighting the estrogenic nature of the effluents. A significant correlation was found between Vtg-like proteins and the phagocytic efficiency index ($r=0.34$; $p<0.001$), which suggests that estrogenic compounds may have been involved in the immunocompetence of mussels exposed to municipal effluents. In conclusion, short-term exposures to treated municipal effluents have the potential to impede the immunocompetence of mussels occurring in the vicinity of an effluent dispersion plume.

Keywords: Freshwater mussels; Phagocytosis; Nitric oxide; Cyclo-oxygenase; Vitellogenin-like proteins; Municipal effluents

Introduction

Municipal effluents are well known to be major sources of contamination in aquatic ecosystems. In addition to the heavy metals and polyaromatic hydrocarbons they contain, some of the urban contaminants in municipal effluents exhibit neuroendocrine-disrupting activity such as 17α-,17β-ethynylestradiol (active ingredient in birth control pills), nonylphenol (a breakdown product of alkylphenol polyethoxylate surfactants), bisphenol A and the natural estrogen 17β-estradiol [1,2,3]. These effluent discharges also contain pharmaceutical and personal care products [4], which can not only act as potential endocrine disruptors but affect other physiological targets such as serotonin and DNA integrity in biota [5,6]. For example, final gamete maturation and spawning depends on both serotonin and prostaglandin signalling (the latter is mediated by arachidonate cyclo-oxygenase or COX activity), two processes that could be influenced by anti-depressive and non-steroidal anti-inflammatory drugs. It was reported that tricylic antidepressants were shown to suppress spawning and fertilization in zebra mussels [7].

The freshwater mussel is a key member of the benthic community in many aquatic habitats worldwide. Because these mussels are sedentary filter-feeders, they are particularly at risk to continuous sources of pollution such as municipal effluents. They could act as surrogate organisms for humans to determine the potential effects of exposure to municipal effluent contamination such as microbes, viruses and chemical pollutants. In addition, mussels and fish could find there way in the food chain and contaminate game fish for human consumption (although some reports exists that freshwater mussels could be eaten by local populations. Mussels possess an open circulatory system wherein the hemolymph contains cells responsible for immunity, hemostatis and nutrition. The immune system in mussels is essentially based on cell-mediated processes wherein phagocytosis and cell-mediated toxicity occurs [8]. The cellular component consists of granulocytes and hyalinocytes, the former accounting for most of the phagocytic activity. Bivalves also release a number of cell mediators involved in inflammation, which contributes to oxidative stress. For example, mussels exhibit NO synthase activity, which is involved in oxidative bursts following phagocytosis [9]. The activity of arachidonate cyclo-oxygenase activity, which is blocked by non-steroidal anti-inflammatory drugs, was shown to be induced by exposure to urban effluents, suggesting the involvement of this enzyme during oxidative stress events [10]. Recent evidence has also revealed that vitellogenin (Vtg), the egg-yolk protein precursor, plays a role in immunity [11]. Indeed, Vtg possesses bactericidal and opsonising activity and helps immunocytes to inactivate bacteria in fish [12].

The purpose of this study was therefore to examine the immunotoxicity of municipal effluents on freshwater mussels. A parallel aim was to determine whether the addition of an ozone treatment could mitigate the responses in mussels exposed to a major municipal effluent from a large and populous city.

Materials and Methods

Mussel handling and exposure to municipal effluents

Freshwater *Elliptio complanata* mussels were collected during the first week of June 2006 in the St. Lawrence River. They were left to stand in 300-L tanks containing UV-treated and charcoal-filtered tap water at 15°C for one month before the exposure experiments. The mussels were fed three times a week with *Pseudokirchneriella subcapitata* algal suspensions (100 million cells per feeding). For the

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exposure experiment, mussels (n=30 individuals per treatment vessel) were placed in 60-L tanks that received a continuous flow (0.2 L/hour) of a physical- and chemical-treated effluent before and after ozonation [6]. The physical- and chemical-treatment consisted of reducing the amount of suspended matter to the low mg/L range (< 5 mg/L). The exposure concentrations were 3, 10, and 20% effluent v/v with dechlorinated (charcoal filter) and UV-treated tap water from the City of Montreal at the same exposure temperature. The ozone treatment consisted of passing effluent through ozone (generated by electric arcs) by a fine bubble diffuser. The ozone concentration in the effluent ranged from 10 to 20 mg/L. Both the physico-chemical-treated and the ozone-treated effluents were maintained for 2 hr in the dark before exposure to the mussels to allow removal of gaseous ozone and equilibration with the atmosphere. The exposure period was terminated after two weeks because of the appearance of mortality (8–10%) at the highest exposure concentration with both the initial effluent and the ozone-treated effluent.

At the end of the exposure period, the mussels were depurated in clean water overnight in 60-L aquaria at 15°C. Morphological measurements (mussel weight, shell length, soft tissue weight) were taken and the visceral mass containing the gonad and ganglia were dissected out on ice and homogenized using a Teflon pestle tissue grinder. The homogenization buffer consisted of 125 mM NaCl containing 25 mM Hepes-NaOH, pH 7.4, 1 mM dithiothreitol and 10 µg/mL apoprotinin protease inhibitor. The homogenates were stored at -85°C until analysis. Total proteins were determined using the principle of protein-dye binding with serum bovine albumin for calibration [13].

Immunocompetence assessments

Immunocompetence was determined by the microplate method using fluorescently-labelled bacteria [14]. Briefly, hemolymph samples were collected using a syringe and transferred to two black Microtiter plates for phagocytic activity and hemocyte viability determinations. The hemocytes were then settled and allowed to adhere to the bottom of the wells for 1 hr at 20°C. The hemolymph was then removed by aspiration and the hemocytes washed once in phosphate-buffered saline solution diluted 1/3 in water. For phagocytosis, 50 µL of fluorescein-labelled bacteria (at 5 x 10^7 bacteria per well) was added to each well and allowed to incubate for 2 hr at room temperature. At the end of the incubation step, the wells were washed twice in diluted PBS and the remaining traces of external *Escherichia. coli* were quenched by adding Trypan blue as directed and the amount of ingested bacteria was immediately determined by fluorescence at 485 nm and 520 nm for excitation and emission, respectively (Biotek Fluorescence Microplate Reader, USA). Total proteins were determined using the fluorescamine methodology, as described elsewhere [15], using standard solutions of serum bovine albumin. Standard solutions of fluorescein were used for calibration and phagocytic activity was expressed as µmole of fluorescein/mg cell proteins. Cell viability was determined in cells using the carboxyfluorescein diacetate methology. Phagocytic efficiency was obtained by the ratio of phagocytic activity/cell viability responses. The number of adhered cells was estimated by the total protein assay using the fluorescamine method described above. Briefly, at the end of the fluorescein diacetate uptake assay, a 50-µL volume of fluorescamine (10 µg/mL) in acetonitrile was added to 200 µL of the cell suspension, mixed for 5 min, and readings were taken at 400 nm excitation and 450 nm emission. Cell viability was expressed as µmole of fluorescein in cells/mg proteins. Cell adherence was expressed as mg proteins per well.

Inflammation biomarkers

Inflammation was tracked by measuring the total levels of nitrite and nitrate (nitric oxide) and the activity of arachidonic acid cyclo-oxygenase (COX) activity. Since nitric oxide readily oxidizes to form nitrite (NO_2^-) and nitrate (NO_3^-), the nitrate ions were selectively reduced by nitrate reductase. The total levels of nitrites in the cell-free hemolymph were determined using the Griess reagent methodology [16]. After reducing the nitrates to nitrites, 50 µL of the Griess reagent (Sigma Chemical Company, Mississauga, Ontario, Canada) was added to the wells and absorbance was measured at 450 nm. Standards of $NaNO_2$ were used for calibration and the data were expressed as µmole NO_2/mg proteins in the hemolymph sample. COX activity was determined in gonad homogenate supernatant. The gonadal tissues were homogenized using a Teflon pestle tissue grinder in ice-cold 100 mM NaCl, 25 mM Hepes-NaOH, pH 7.4, 1 mM dithiothreitol and 10 µg/mL apoprotinin. The homogenate was immediately centrifuged at 12 000 g for 20 min at 2°C and the supernatant carefully collected from the upper lipid layer. COX activity was determined by following the oxidation of 2,7-dichlorofluosrescein in the presence of arachidonate [17]. The incubation buffer consisted of 50 mM Tris-HCl, pH 8, containing 0.05% Tween-20 and 50 µM arachidonate, 2 µM dichlorofluorescein and 0.1 µg/mL horseradish peroxidase. The formation of fluorescein was measured fluorometrically, as described above, and the data were expressed as the increase in fluorescein units/min/mg proteins.

Data analysis

Biomarkers were determined in N=8 mussels per treatment group. The homogeneity of variance was tested using Levene's test. Where the distribution of data was heterogeneous, the data were log transformed. The data were subject to an analysis of variance and differences from controls or between the physico-chemical-treated and ozone-treated effluents were determined with the Least Square Difference test. Correlation and factorial analyses were determined by the Pearson-moment and principal component procedures, respectively. Significance was set at $p<0.05$.

Results

The physico-chemical treatment of the raw wastewaters consist in several steps to essentially remove the solids and suspended matter. The wastewaters are first passed through grids, sieves and the sludge are allowed to settle at the bottom in clarifiers to remove the bulk materials. The remaining solids are precipitated/floculated using coagulating agents such as the addition of ferric chloride and surfactants. The resulting effluent is generally of low turbidity with a

PARA METER	PHYSICO- CHEMICAL-TREATED EFFLUENT	AFTER OZONATION
Conductivity ($uScm^{-1}$)	850–950	Same
pH	7–7.5	Same
Suspended particulate matter (mg/L)	22	9
BOD_5 (mg/L)	41	39
DOC (mg/L)	95	79
Turbidity (NTU)	13	8
Coliforms (thermotolerant) x 106 fc/100 mL	3.8 x 10^6	4.0x10^2

BOD_5: biochemical oxygen demand (5%); DOC: dissolved organic carbon

Table 1: Physical and chemical characteristics of City of Montreal wastewater effluents.

yellowish color apparence with the presence of suspended matter < 10 mg/L but containing up to 3-5 millions thermotolerant coliforms/100 mL. The addition of ozone comes after this to disinfect the effluent and to breakdown the organic matter [18]. The basic chemical properties of the physical- and chemical-treated municipal effluent before and after ozonation were evaluated (Table 1). The pH and conductivity values were not affected by ozone treatment. Total coliform levels were significantly reduced, going from 3.8 x 10^6 to 4 x 10^2 fecal coliform units/100 mL. The suspended matter content decreased from 22 to 9 mg/L. Turbidity also dropped, starting at 13 and ending at 8 normalized transmission units (NTU). The dissolved organic carbon (DOC) content and the biochemical oxygen demand (BOD) were not strongly affected, with 17% and 5% reductions, respectively. Although the increase in wastewater quality was achieved with ozonation, the ozone-treated effluent was still toxic to freshwater mussels because mortality events were still observed (8-10% mortality after 2 weeks).

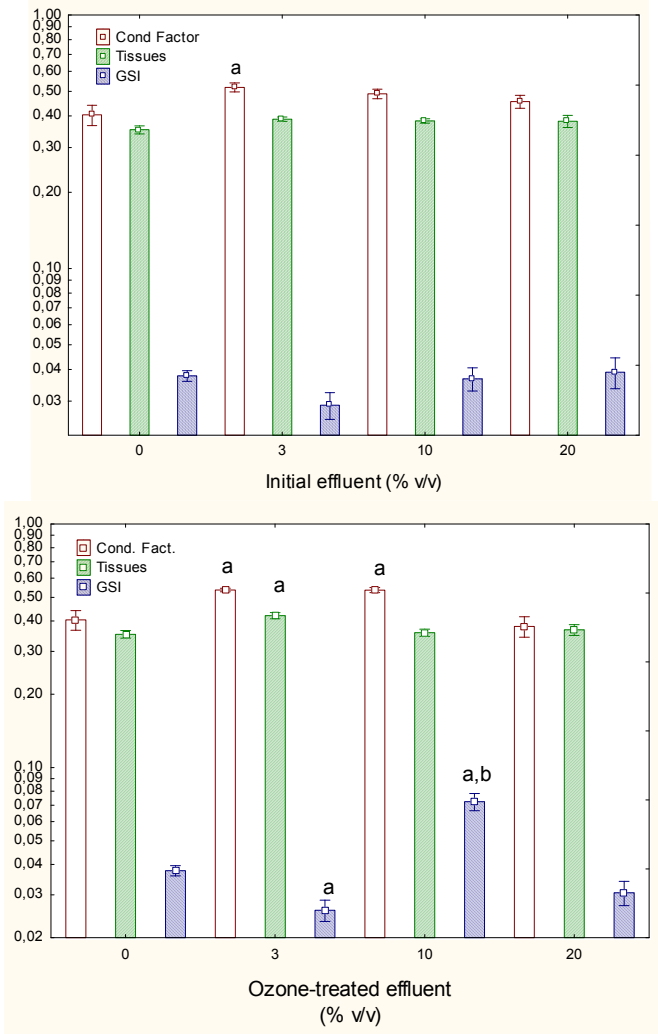

Figure 1: Morphological characteristics of freshwater mussels exposed to urban effluents. Morphological characteristics consisted of condition factor (mussel weight/shell length), soft tissue content, and gonado-somatic index (GSI). The letter 'a' denotes a significant difference from the unexposed (control) group; the letter 'b' denotes a difference between the ozone-treated and the initial effluent.

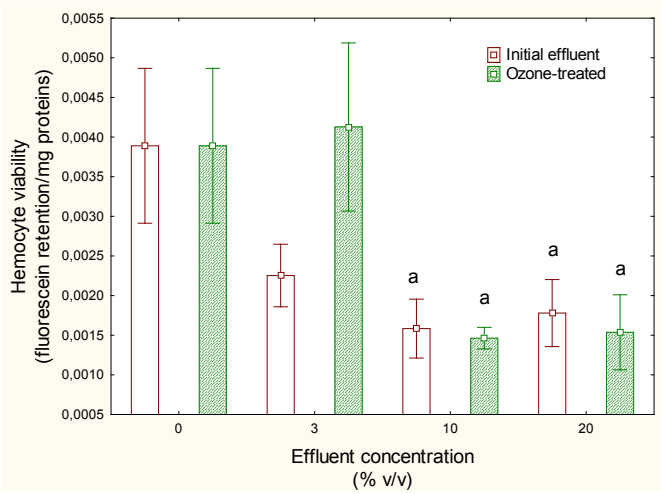

Figure 2: Change in hemocyte viability in mussels exposed to a physical- and chemical- treated effluent before and after ozonation. Hemocyte viability was determined by the fluorescein retention test. The letter 'a' denotes a significant difference from the unexposed (control) group; the letter 'b' denotes a difference between the ozone-treated and the initial effluent.

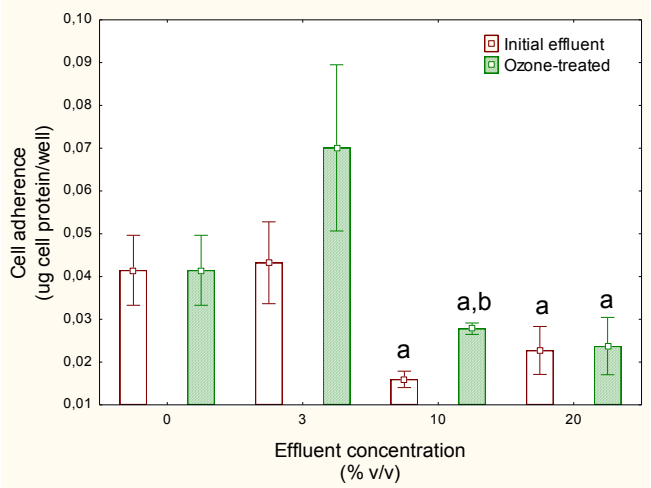

Figure 3: Change in hemocyte adherence in microplate wells from mussels exposed to the municipal effluents.
Hemocyte adherence was determined by the amount of cellular proteins adhered to the bottom of the microplate wells. The letter 'a' denotes a significant difference from the unexposed (control) group; the letter 'b' denotes a difference between the ozone-treated and the initial effluent.

Morphological characteristics were determined by measuring the condition factor (CF, mussel weight/shell length), soft tissue ratio and gonado-somatic index (GSI) (Figure 1). In the initial effluent, CF was only significantly elevated at a 3% effluent concentration and returned to control values for effluent concentrations of 10 and 20%. No significant changes were observed in the proportion of soft tissue and GSI in the exposed mussels. For the ozonated effluent, the CF was significantly elevated at 3 and 10% effluent concentration and returned to control values at 20% v/v. The proportion of soft tissues rose at the lowest effluent concentration and returned to control values as the effluent concentration increased. The GSI showed a biphasic response (i.e. was significantly lower) at 3% v/v, followed by an increase at the

10% effluent concentration. At the highest concentration tested, the GSI was not significantly different from the controls. The increase in GSI was also significantly higher than the GSI for the initial effluent. No significant correlations were observed between CF, soft tissue index and GSI.

Hemocyte viability was determined in mussels exposed to both municipal effluents (Figure 2). In the physico-chemical-treated effluent, hemocyte viability dropped 2.5-fold, reaching 40% cell viability at the 10% v/v effluent concentration. The same was also observed for the ozonated effluent, which reached a residual viability of 40%, the same as the initial effluent. A correlation analysis revealed that hemocyte viability was significantly related with CF (r=-0.53; p=0.01). Cell adherence was also determined in mussels exposed to both the initial and the ozonated effluent (Figure 3). For the initial effluent, a significant reduction in adhering cellular proteins was observed at the 10% and 20% effluent concentrations. For the ozonated effluent, a similar decrease was observed for the hemocyte but adherence was higher at the 10% effluent concentration compared to the same concentration of the unozonated effluent. A correlation analysis revealed the number of adhering cellular proteins was significantly correlated with hemocyte viability (r=0.62; p<0.01; see Table 2 for correlation results). Phagocytic activity was also determined in mussels (Figure 4). In the initial effluent, no significant changes were observed. However, a significant increase in phagocytic activity was observed at the highest concentration tested in the ozonated effluent. The phagocytic efficiency index was determined by calculating the ratio between phagocytic activity and cell viability (results not shown). The results revealed that the phagocytic efficiency index was significantly higher at the 10% and 20% concentrations of the initial effluent. Ozonation of the effluent also produced this response. A correlation analysis revealed that phagocytic efficiency was significantly correlated with hemocyte adherence (r=-0.43; p<0.001) and with GSI (r=0.30; p<0.05). Levels of Vtg-like proteins were also determined (Figure 5) due to their potential role in estrogenic urban effluents and in the immune system. The data revealed that Vtg-like proteins were significantly induced at the lowest effluent concentration of the initial effluent. The same was also observed for the ozone-treated effluent. A correlation analysis revealed that Vtg-like proteins were significantly correlated with phagocytic efficiency (r=0.34; p<0.05) and hemocyte viability (r=-0.47; p<0.05).

Inflammation status was determined by measuring changes in NO production and arachidonate cyclo-oxygenase (COX) activity (Figures 6A and B). A significant decrease in the production of NO in hemocytes was observed at the 3% effluent concentration for both types of urban effluents. A correlation analysis revealed that NO production was significantly correlated with the phagocytic efficiency index (r=-0.24; p=0.05). COX activity was also significantly reduced at the 10% and 20% concentrations of the initial effluent. This decrease was less severe in the ozone-treated effluent, where a significant decrease was now observed at the 10% effluent concentration relative to the control but the activity was significantly higher at 3% concentration of the unozonated effluent. A correlation analysis revealed that COX activity was significantly correlated with phagocytic efficiency (r=-0.27; p<0.05), CF (r=-0.33; p=0.01), GSI (r=-0.27; p<0.05) and NOx (r=0.60; p<0.001) levels.

A factorial analysis of the biomarker data revealed that cell adherence, viability, NOx and COX activities were the principal components with factorial weights > 0.7 (Figure 7). These biomarkers explained 50% of the total variance. Cell viability and adherence were grouped together, which suggests that viable cells tend to adhere to the bottom of the wells. Interestingly, Vtg-like proteins were closely related with phagocytosis and phagocytic efficiency. The inflammation biomarkers NOx and COX activity were also closely related to each other.

Discussion

Municipal effluents are well recognized to contain numerous potentially toxic contaminants where local organisms might be at risk. The effluent was shown to contain nonylphenols and nonylphenol$_{1-17}$ethoxylates at 1 and 144 µg/L respectively [19]. Moreover, mussels exposed to the same municipal effluent accumulated significant quantities of alkylphenol polethoxylates reaching 10 µg/g concentrations in sediments and mussels [2]. Mussels exposed to these wastewaters also contained similar amounts of coprostanol (16 µg/g),

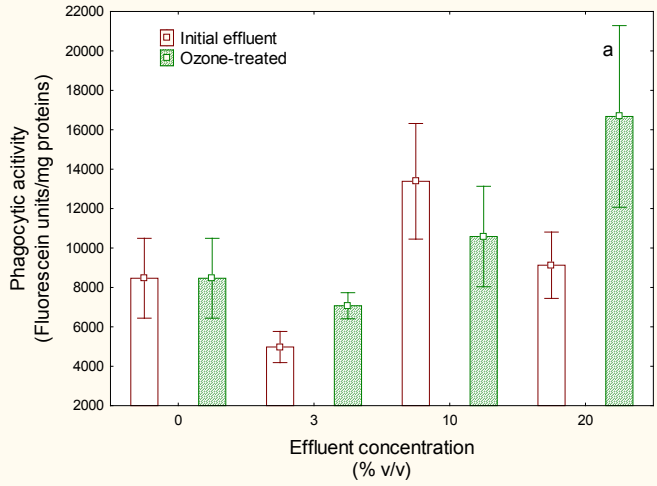

Figure 4: Phagocytic activity in freshwater mussels exposed to the municipal effluents. Phagocytic activity was determined by the fluorescent microplate method using fluorescently-labelled bacteria. The letter 'a' denotes a significant difference from the unexposed (control) group; the letter 'b' denotes a difference between the ozone-treated and the initial effluent.

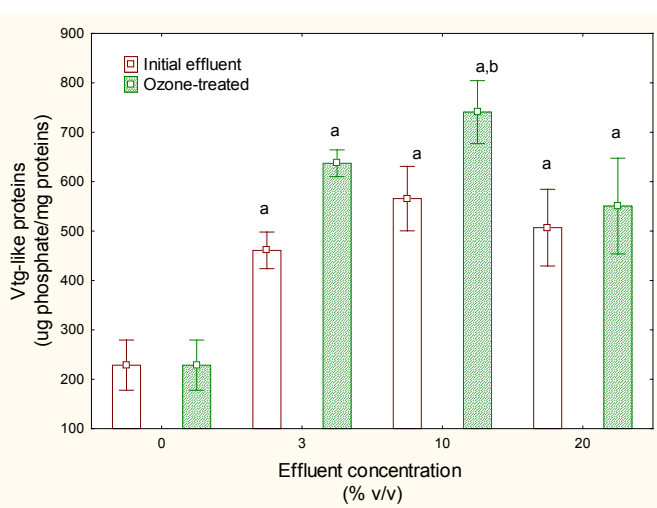

Figure 5: Changes in vitellogenin-like proteins in mussels exposed to the municipal effluents. The levels of Vtg-like proteins were determined in the gonadal tissues. The letter 'a' denotes a significant difference from the unexposed (control) group; the letter 'b' denotes a difference between the ozone-treated and the initial effluent. Significance was set at p<0.05.

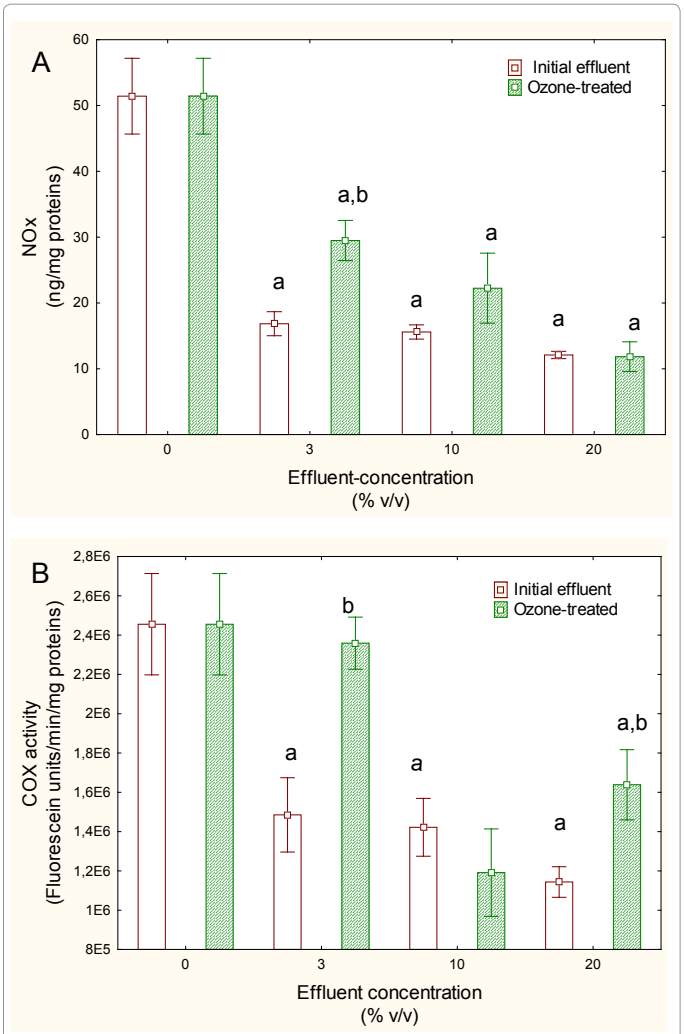

Figure 6: Inflammation biomarkers in mussels exposed to the municipal effluents. The production of NOx (A) and COX (B) activity was determined in the visceral mass. The letter 'a' denotes a significant difference from the unexposed (control) group; the letter 'b' denotes a difference between the ozone-treated and the initial effluent.

a reduced form of bile salts [20]. In another study, the presence of anti-infectives and selective reuptake inhibitors were also found in this effluent indicating the release of pharmaceutical products from human therapeutic use [21,22] Municipal effluents are well known to influence the immune system in both marine and freshwater mussels [14, 23] . Both the microbiological and chemical components in the effluents can influence the immune system. Indeed, the presence of microorganisms in municipal effluents was associated with reduced hemocyte density and increased phagocytic activity in freshwater mussels [24]. Moreover, the presence of bacteria with strong activity in leucine aminopeptidase and acid phosphatase was associated with decreased hemocyte viability and natural cellular cytotoxic activity. The latter decrease can render the mussel immunocytes more susceptible to virus-mediated or other (neoplastic?) transformations. In another study, fecal coliform levels in the water column were also associated with increased phagocytosis in feral fish at sites downstream from municipal discharge points [25]. However, bacterial clearance rates were seemingly not affected in mussels placed downstream of municipal effluent outfalls [23], but serious lesions were observed in gills, as revealed by hemocyte infiltration, epithelium proliferation, lamellar fusion, and dilated hemolymphatic sinus. The reaction of mussel hemocytes to bacteria is species-specific relative to immune/inflammatory responses [26]. Indeed, a *Vibrio splendidus* challenge in *Mytilus galloprovincialis* stimulated the immune response and the expression of antioxidant genes, but had no effect on the down-regulation of some antioxidant genes when *Vibrio anguillarum* was injected instead.

In addition, there are many chemicals present in effluents that could also compromise the molluscan immune system. For example, pharmaceutical products such as bezafibrate, gemfibrozil and trimethoprim increased phagocytic activity in *Elliptio complanata* hemocytes exposed *in vitro* [27]. Antibiotics, cholesterol synthesis inhibitor drugs (fibrates) and neuroleptics (carbamazepine) were also cytotoxic in hemocytes. Although the contribution of pharmaceutical products in wastewaters is difficult to predict in mussel immune systems, current evidence reveals that this class of contaminant contributes at least in part to the observed effects in effluent-exposed mussels. The breakdown product of nonylphenol polyethoxylates, nonylphenol, was shown to inhibit COX activity and the production of prostaglandins in rabbit kidney medulla microsomes [17]. This is in keeping with the reduced activity of COX in mussels exposed continuously to a municipal effluent before and even after ozone treatment. The proinflammation mediator NOx was also reduced in mussels exposed to both effluents. This finding suggests that mussels were actively involved in clearing bacterial loadings given the observed changes in phagocytic activity and inflammation. The addition of an ozone treatment step mitigated the reduction in COX activity and NOx production. The decrease in NOx could also represent a compensation mechanism from opiate-like compounds in effluents which are known to stimulate NO release in mussels [28]. COX and NOx were significantly correlated ($r=0.60$; $p<0.001$), suggesting that these inflammation mediators might be acting together to maintain phagocytic activity. The release of NOx by hemocytes is also involved in the process of destruction of foreign

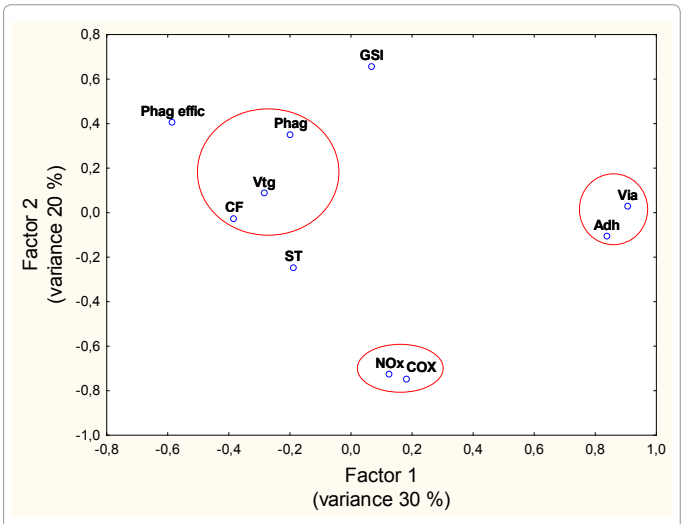

Figure 7: Factorial analysis of the immune-related responses in mussels exposed to a physical-and chemical-treated effluent before and after ozone treatment. A factorial analysis was performed using the principal component procedure for the extraction of the factors. Abbreviations: CF: condition factor; ST: soft tissue weight ratio; GSI: gonado-somatic index; phag: phagocytic activity; phag effic: phagocytic efficiency or phagocytic activity/cell viability; Vtg: vitellogenin-like proteins; Adh: hemocyte adherence; Via: hemocyte viability, NOx: total nitrites and nitrates; COX: cyclooxygenase. The biomarkers that are clustered or close together indicate a significant and proportional response.

	Phag	Via	Adherence	Phag/via	CF	Vtg (ALP)	GSI	NOx	COX Gon
Phag	1	-0.06 p>0.1	-0.07 p>0.1	**0.65** **p<0.001**	0.06 p>0.1	0.02 p>0.1	0.14 p>0.1	-0.08 p>0.1	-0.17 p>0.1
Via		1	**0.81** **p<0.001**	**-0.42** **p<0.001**	**-0.29** **p<0.05**	-0.23 p=0.1	-0.03 p>0.1	0.07 p>0.1	0.12 p>0.1
Adherence			1	**-0.43** **p<0.001**	-0.16 p>0.1	-0.05 p>0.1	-0.07 p>0.1	0.16 p>0.1	*0.21* *p<0.1*
Phag/via				1	0.09 p>0.1	**0.34** **p<0.05**	**0.30** **p<0.05**	**-0.24** **p=0.05**	**-0.27** **p<0.05**
CF					1	0.27 p=0.1	-0.09 p>0.1	-0.11 p>0.1	**-0.32** **p<0.05**
Vtg (ALP)						1	0.12 p>0.1	0.09 p>0.1	-0.15 p>0.1
GSI							1	*-0.18* *p=0.1*	**-0.27** **p<0.05**
NOx								1	**0.60** **p<0.001**

Table 2: Correlation analysis of immunocompetence data.

particles in phagosomes. NOx combines with hydrogen peroxide to form highly biocidal peroxynitrite, which can lead to oxidative damage [29]. The increase in NOx could have a dampening effect on phagocytic activity and reduce immune alertness [30]. This agrees well with the maintenance and elevation of phagocytic activity with reduced COX activity and NOx concentrations in mussels exposed to municipal effluents.

Recent studies have revealed the involvement of Vtg in the immune system, in addition to its responsiveness to various environmental estrogens. Indeed, Vtg possesses biocidal activity and is inducible by bacterial exposure in fish [11,31]. Moreover, in caged mussels exposed to municipal effluents, the levels of Vtg-like proteins were significantly correlated with phagocytic activity, which suggests that this interaction exists in freshwater mussels as well [32]. However, the presence of estrogenic compounds in municipal effluents could also alter the immune response in mussels. Compounds such as nonylphenol, estradiol-17β, ethynylestradiol and bisphenol A were all capable of stimulating phagocytosis at low environmentally relevant doses and inhibited this response at higher concentrations in *Mytilus galloprovincialis* [33]. In another study of the clam *Mya arenaria*, E2 significantly inhibited phagocytic activity in hemocytes, with a concomitant mobilization of glycogen in support of Vtg production [34]. Estrogenic signalling also involves nitric oxide release in the *Mytilus edulis* nervous system [35]. These authors found that the addition of estradiol-17β (E2) to pedal ganglia immediately released NO in a dose-dependent manner. E2 conjugated to albumin also stimulated NO release, suggesting mediation through a membrane binding site (receptor). Tamoxifen, an estrogen receptor antagonist, inhibited the action of both free and protein-bound E2. The release of NO, in turn, could influence the activity of immunocytes in mussels [36]. It was found that non-stimulated immunocytes do not stimulate glanglionic NO release when co-incubated with pedal ganglia. However, following activation with interleukin 1β, the significant release of NO from pedal ganglia was observed. Moreover, longer NO exposure times in ganglia significantly reduced immunocyte activity, highlighting the down-regulating role of NO on immunocyte activity. Given the observation that estrogens could increase NO production, the significant decrease of NO in mussels exposed to the municipal effluent could reflect a compensatory mechanism to maintain the immune alertness of mussel hemocytes exposed to municipal effluents. In a previous study on blue mussels exposed to untreated municipal effluents, phagocytic activity and NOx production were significantly increased, indicating that this raw effluent stimulated the immune response after 14 days, but phagocytosis returned to control values at 21 days [23]. If this holds true, then the measurement of NOx and phagocytic activity could provide insight into the balance between active phagocytosis and inflammatory status in organisms challenged by municipal effluents.

In conclusion, the exposure of mussels to a physico-chemical treated effluent before and after ozone treatment leds to mortality events after 2 weeks of continuous exposure (flow through). Exposure to these effluents led to significant changes at the immune system level in mussels. Indeed a decrease in cell viability, hemocyte adherence, NOx levels and COX activities was observed in these two types of effluents, with no important changes in their capacity to ingest foreign bacteria. The addition of an ozone step led to increased water quality parameters (coliforms, DOC, suspended solids) and improved some of the immune parameters such as phagocytosis, NOx and COX activity. The presence of estrogenic compounds and Vtg-like protein induction was partly associated with the observed responses, thus highlighting the complex interplay between the presence of microorganisms and pollutants in these wastewaters.

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References

1. Sumpter JP, Jobling S (1995) Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ Health Perspect 103: 173-178.
2. Sabik H, Gagné F, Blaise C, Marcogliese DJ, Jeannot R (2003) Occurrence of alkylphenol polyethoxylates in the St. Lawrence River and their bioconcentration by mussels (Elliptio complanata). Chemosphere 51: 349-356.
3. Vethaak AD, Lahr J, Schrap M, Belfroid AC, Rijs GBJ, et al. (2005) An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. Chemosphere 59: 511-524.
4. Kummerer K (2001) Drugs in the environment: Emission of drugs, diagnostic aids and desinfectants into wastewater by hospitals in relation to other sources – a review. Chemosphere 45: 957-969.
5. Lajeunesse A, Gagnon C, Gagné F, Louis S, Cejka P, et al. (2011) Distribution of antidepressants and their metabolites in brook trout exposed to municipal wastewaters before and after ozone treatment: Evidence of biological effects. Chemosphere 83: 564-571.
6. Gagné F, André C, Cejka P, Hausler R, Fournier M (2011) Alterations in DNA metabolism in Elliptio complanata mussels after exposure to municipal effluents. Comp Biochem Physiol 154: 100-107.

7. Hardege JD, Duncan J, Ram JL (1997) Tricyclic antidepressants suppress spawning and fertilization in the zebra mussel, Dreissena polymorpha. Comp Biochem Physiol 118: 59-64.
8. Glinski Z, Jarosz J (1997) Molluscan immune defenses. Arch Immunol Ther Experim 45: 149-155.
9. Banakou E, Dailianis S (2010) Involvement of Na+/H+ exchanger and respiratory burst enzymes NADPH oxidase and NO synthase, in Cd-induced lipid peroxidation and DNA damage in haemocytes of mussels. Comp Biochem Physiol 152: 346-352.
10. Gagné F, Bérubé E, Fournier M, Blaise C (2005) Inflammatory properties of municipal effluents to Elliptio complanata mussels: Lack of effects from anti-inflammatory drugs. Comp Biochem Physiol 141: 332-337.
11. Shi X, Zhang S, Pang Q (2006) Vitellogenin is a novel player in defence reactions. Fish Shellf Imm 20: 769-772.
12. Zhang S, Sun Y, Pang Q, Shi X (2005) Hemagglutinating and antibacterial activities of vitellogenin. Fish and Shellfish Immun 19: 93-95.
13. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal.Biochem 72: 248-254.
14. Blaise C, Trottier S, Gagné F, Lallement C, Hansen P-D (2002) Immunocompetence of bivalve hemocytes as evaluated by a miniaturized phagocytosis assay. Environ Toxicol 17: 160–169.
15. Lorenzen A, Kennedy S (1993) A fluorescence-based protein assay for use with a microplate reader. Anal Biochem 214: 346–348.
16. Verdon CP, Burton BA, Prior RL (1995) Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by NADP+ when the Griess reaction is used to assay for nitrite. Anal Biochem 224: 502–508.
17. Fujimoto Y, Usa K, Sakuma S (2005) Effects of endocrine disruptors on the formation of prostaglandin and arachidonoyl-CoA formed from arachidonic acid in rabbit kidney medulla microsomes. Prost Leuko and Essent Fatty Acids 73: 447-452.
18. Gehr R, Wagner M, Veerasubramanian P, Payment P (2003) Disinfection efficiency of peracetic acid, UV and ozone after enhanced primary treatment of municipal wastewater. Water Res 37: 4573-4586.
19. Sabik, H, Gagnon C, Houde F and Deblois C (2004) Distribution, Fate, and Behavior of Nonylphenol Ethoxylates and Degradation Products in the Dispersion Plume of a Major Municipal Wastewater Effluent. Environ Forensics 5: 61-70.
20. Gagné F, Blaise C, Lachacne B, Sunahara GI and Sabik H (2001) Evidence of coprostanol estrogenicity to the freshwater mussel Elliptio complanata. Environ Poll 115 : 97-106.
21. Segura PA, Gagnon C, Sauvé S (2007) A fully automated on-line preconcentration and liquid chromatography-tandem mass spectrometry method for the analysis of anti-infectives in wastewaters. Anal Chim Acta 604: 147-57.
22. Lajeunesse A, Gagnon C, Sauvé S (2008) Determination of basic antidepressants and their N-desmethyl metabolites in raw sewage and wastewater using solid-phase extraction and liquid chromatography-tandem mass spectrometry. Anal Chem 80: 5325-5333.
23. Akaishi FM, St-Jean SD, Bishay F, Clarke J , Rabitto IdS, et al. (2007) Immunological responses, histopathological finding and disease resistance of blue mussel (Mytilus edulis) exposed to treated and untreated municipal wastewater. Aquat Toxicol 82: 1-14.
24. Douville M, Gagné F, Zhu B, Fortier M, Fournier M (2010) Characterization of commercial microbial products by polymorphic DNA markers and enzymatic activity diversity: Occurrence and potential effects on freshwater mussels exposed to municipal effluents. Res J Biotech 5: 31-48.
25. Ménard L, Escarné R, Marcogliese DJ, Cyr D, Fournier M, et al. (2010) The impacts of urban pollution on the immune system of spottail shiners (Notropis hudsonius) in the St. Lawrence River. Fres Environ Bull 19: 1369-1374.
26. Canesi L, Barmo C, Fabbri R, Ciacci C, Vergani L, et al. (2010). Effects of vibrio challenge on digestive gland biomarkers and antioxidant gene expression in Mytilus galloprovincialis. Comp Biochem Physiol 152C: 399-406.
27. Gagné F, Blaise C, Fournier M, Hansen P-D (2006) Effects of selected pharmaceutical products on phagocytic activity in Elliptio complanata mussels. Comp Biochem Physiol C Toxicol Pharmacol. 143: 179-186.
28. Mantione KJ, Kim C, Stefano GB (2006) Morphine regulates gill ciliary activity via coupling to nitric oxide release in a bivalve mollusk: Opiate receptor expression in gill tissues. Med Sci Monitor 12: 195-200.
29. Ducrocq C, Blanchard B, Pignatelli B, Ohshima H (1999) Peroxinitrite: An endogenous oxidizing and nitrating agent. Cell Mol Life Sci 55: 1068-1077.
30. Stefano GB, Digenis A, Spector S, Leung MK, Bilfinger TV, et al. (1993) Opiate-like substances in an invertebrate, an opiate receptor on invertebrate and human immunocytes, and a role in immunosuppression. Proc Nation Acad Sci USA 90: 11099-11103.
31. Li Z, Zhang S, Zhang J, Liu M, Liu Z (2009) Vitellogenin is a cidal factor capable of killing bacteria via interaction with lipopolysaccharide and lipoteichoic acid. Mol Immunol 46: 3232-3239.
32. Bouchard B, Gagné F, Fortier M, Fournier M (2009) An in-situ study of the impacts of urban wastewater on the immune and reproductive systems of the freshwater mussel Elliptio complanata. Comp Biochem Physiol C Toxicol Pharmacol 150: 132-140.
33. Canesi L, Lorusso LC, Ciacci C, Betti M, Rocchi M et al. (2006) Immunomodulation of Mytilus hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: In vitro and in vivo studies. Aquat Toxicol 81: 36-44.
34. Gauthier-Clerc S, Pellerin J, Fournier M, Amiard JC (2006) Immunological and biochemical responses in Mya arenaria (Mollusca: Bivalvia) exposed in vivo to estradiol-17β. Comp Biochem Physiol C Toxicol Pharmacol 144: 228-234.
35. Stefano GB, Cadet P, Mantione K, Cho JJ, Jones D, et al. (2003) Estrogen signaling at the cell surface coupled to nitric oxide release in Mytilus edulis nervous system. Endocrinology 144: 1234-1240.
36. Stefano GB, Mantione K, Jones D, Zhu W, Casares F, et al. (2004) Immunocytes modulate ganglionic nitric oxide release which later affects their activity level. Neuro Endocrinol Lett 25: 57-61.