Immunotoxicity of Municipal Effluents to Freshwater Mussels

Gagné F1, André C1, Cejka P2, Hausler R3 and Fournier M4

1Fluvial Ecosystem Research, Environment Canada, Montréal, Que, Canada
2Montreal Wastewater Treatment Plant, Montréal, Que., Canada
3STEPPE, École de Technologie Supérieure, Montréal, Que, Canada
4INRS-Institut Armand-Frappier, Université du Québec, Pointe-Claire, Que, Canada

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 cyclooxygenase (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; Cyclooxygenase; 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 arachidonic cyclooxygenase or COX activity), two processes that could be influenced by anti-depressive and non-steroidal anti-inflammatory drugs. It was reported that tricyclic 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 their 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, hemostasis 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 hyalineocytes, 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 cyclooxygenase 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...
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 10^6 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 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 cyclooxygenase (COX) activity. Since nitric oxide readily oxidizes to form nitrite (NO_3^-) and nitrate (NO_2^-), 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 nitrites to nitrates, 50 μL of the Griess reagent (Sigma Chemical Company, Mississauga, Ontario, Canada) was added to the wells and absorbance was measured at 540 nm. Standards of NaNO_2 were used for calibration and the data were expressed as μmole NO_3^-/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-dichlorofluorescein 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 homogeneous, 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 remaining solids are precipitated/floculated as suspended particulate matter. The wastewaters are first passed through grids, sieves 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 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.

**Table 1:** Physical and chemical characteristics of City of Montreal wastewater effluents.
yellowish color appearance 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).

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 phagocytosis 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 arachidonic 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, ethoxylates at 1 and 144 μg/L respectively [19]. Moreover, mussels exposed to the same municipal effluent accumulated significant quantities of alkylphenol polyethoxylates reaching 10 μg/g concentrations in sediments and mussels [2]. Mussels exposed to these wastewaters also contained similar amounts of coprostanol (16 μg/g),...
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 *Ellipcho 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 like compounds in effluents which are known to stimulate NO release [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 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.
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]. Estrogen 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 led 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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