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JOURNAL OF ENVIRONMENTAL SCIENCES
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Effects of a municipal effluent on the freshwater mussel *Elliptio complanata* following challenge with *Vibrio anguillarum*

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ARTICLE INFO

Article history:
Received 26 January 2015
Revised 11 March 2015
Accepted 31 March 2015
Available online 3 July 2015

Keywords:
Elliptio complanata
Immunocompetence
Oxidative stress
Inflammation
Vibrio anguillarum

ABSTRACT

The purpose of this study was to examine the cumulative effects of exposure to a pathogenic bacteria and municipal effluent in the freshwater mussel *Elliptio complanata*. Mussels were exposed to increasing concentrations of an ozone-treated effluent at 15°C for 7 days. A sub-group of mussels was inoculated with *Vibrio anguillarum* and exposed to the same conditions as above. After the exposure period, mussels were collected to assess hemocyte count and viability, immunocompetence (phagocytosis and nitrite production), oxidative stress/inflammation (cyclooxygenase and lipid peroxidation) and oxygen radical/xenobiotic scavenging activity (metallothioniens, glutathione S-transferase). The results showed that mussels exposed to municipal effluent had increased hemocyte counts, phagocytosis, nitrites, lipid peroxidation and metallothioneins. In the inoculated mussels, the same responses were observed, in addition to cyclooxygenase and glutathione S-transferase activities. Multivariate analyses revealed that (1) the response pattern changed with effluent concentration, where increased responses observed at low effluent concentrations (>10%, V/V) were attenuated at higher effluent concentrations, (2) the effluent produced more pronounced changes in lipid peroxidation, metallothionein and hemocyte viability, and (3) the simultaneous presence of *V. anguillarum* led to more important changes in hemocyte count and viability and nitrite levels. In conclusion, the presence of *V. anguillarum* could alter the response of mussels to municipal effluent, which could lead to increased inflammation in mussels.

Introduction

The aquatic environment in the vicinity of urban areas is contaminated by municipal wastewaters or effluents, which are from both domestic and industrial sources. They contain polyaromatic hydrocarbons, polybrominated ethers, heavy metals, and pharmaceutical and personal care products, which can contain endocrine disrupters (Chambers et al., 1997; Wang et al., 2013). Municipal wastewaters are usually treated to remove suspended solids, ammonia and high loadings of microorganisms from the water column. The removal and destruction of microorganisms are usually achieved using biological treatment (aeration ponds/sludges), ozone treatment and UV radiation. Ozonation of municipal effluent (ME) represents an effective and sound method for removing not only bacteria, but also viruses and other parasites (Gehr et al., 2003). The ozone concentration usually applied is in the range of 5–20 mg/L, depending on the complexity of the organic matrix of the effluent and the target fecal bacteria (Świetlik and Sikorska, 2004; von Gunten, 2007).

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http://dx.doi.org/10.1016/j.jes.2015.03.029
1001-0742/

ME have various toxic effects on freshwater mussels, such as oxidative stress, altered immunocompetence, and neuroendocrine disruption of reproduction (Gagné et al., 2008a, 2011; Gillis et al., 2014a, 2014b). However, the interaction between exposure to ME and to potentially pathogenic bacteria is not well understood. In caged mussels exposed to high concentrations of untreated sewage water, between 50%–100% of mussels died following challenge with *Vibrio anguillarum* (Akaishi et al., 2007). The bacterial clearance rate appeared to be independent of mussel mortality, indicating that pathogenicity was not solely related to the removal of bacteria by the organisms, but also to the pathogenicity of the bacteria strain. Infiltration of hemocytes in the infected tissues was also observed. *V. anguillarum* was found to be a more potent pathogen than *Vibrio splendidus*, as it led to higher levels of oxidative and immune stress in clams (Liu et al., 2013). *V. anguillarum* is a Gram-negative (resistance to lysozyme) rod bacterium with a flagellum that thrives in saline and brackish waters. This bacterial species was selected as a potential pathogen for freshwater mussels exposed to brackish waters.

In molluscs, defence against invading microorganisms is ensured by non-specific immunity, which involves ingestion of foreign bodies by hemocytes and release of cytokines, enzymes such as lysozymes, and other biocidal compounds (Brousseau et al., 2012). Circulating hemocytes, namely granulocytes and hyalinocytes, are cells involved in immunity and in the transport of nutrients to various tissues in mussels respectively. Granulocytes are involved in phagocytosis, which can be easily determined by flow cytometry or fluorescence microplate methods (Goedken and De Guise, 2004; Auffret et al., 2006; Blaise et al., 2002a). Following ingestion of bacteria, an oxidative burst occurs in the resulting phagosome, producing nitric oxide and hydrogen peroxide (Buggé et al., 2007). During that process, radical oxygen species (ROS) are formed which could, in turn, be harmful to the mussels if this response is sustained or antioxidant pathways are saturated or altered by the presence of contaminants. Inflammation could also be monitored by measuring arachidonic acid cyclooxygenase (COX) activity (Gagné et al., 2008a). COX is involved in the production of prostaglandins and other mediators of inflammation and pain. Interestingly, ME contains high amounts of COX inhibitors, such as acetaminophen, ibuprofen and other non-steroidal anti-inflammatory drugs (Lajeunesse and Gagnon, 2007). Nevertheless, exposure to a chemically processed ME produced an inflammation response in freshwater mussels (Gagné et al., 2005). Indeed, COX activity was significantly induced in gonad tissues of *Elliptio complanata* mussels, even though ibuprofen was able to inhibit COX when injected in mussels. COX activity in the gonad could also be related to gamete maturation and spawning, as with serotonin. Exposure of mussels to pathogenic bacteria such as *Vibrio* sp. could also stimulate the mobilization of peripheral hemocytes, phagocytosis and oxidative stress through oxidative burst (Perrigault and Allam, 2012). The interaction of exposure to *Vibrio* bacteria and ME is poorly understood at the present time. It is expected that the bacterial loading of ME and direct exposure to bacteria should contribute to stimulate the immune response in freshwater mussels, but the interaction with chemicals found in ME could either stimulate or decrease immunity in mussels when challenged with bacteria. As reported by Parry and Pipe (2004), the interactive effects of temperature and copper could influence immune response and disease susceptibility. Cumulative effects are often encountered when the agents act on the same pathways *e.g.*, both copper and *Vibrio* challenge will lead to the production of ROS, which could alter immunocompetence.

The purpose of this study was therefore to examine the effects of the interaction of ME and *V. anguillarum* exposure on immunocompetence, oxidative stress/inflammation and ROS mobilization in tissues in freshwater mussels. Both non-inoculated and inoculated mussels were exposed to increasing concentrations of a treated ME for 7 days, after which immune, oxidative stress and inflammation responses were examined. An attempt was made to understand the cumulative toxicity of ME and a potential pathogen to freshwater mussels.

1. Materials and methods

1.1. Mussel collection and exposure to municipal effluents

Freshwater mussels, *Elliptio complanata*, were collected by hand in June at the Richelieu River (Quebec, Canada). The species is abundant in a sector of the river that is not heavily impacted by domestic or industrial activities. The mussels were taken to the laboratory of Environment Canada and allowed to stand in 300-L tanks under constant aeration at 15°C using City of Montreal tap water that was treated with UV-radiation and charcoal filter. The mussels were held under these conditions for 4 weeks before initiating exposure to ME and challenge with *V. anguillarum*. Municipal effluents were collected for 3 consecutive days in the morning (9:00 to 10:30 am), placed in plastic containers lined with polyethylene bags and stored at 4°C in darkness for no longer than 2 weeks. The ME was ozone-treated ME (10 mg/L injected ozone) from a physically and chemically treated effluent (removal of suspended solids by sieving and addition of flocculants) from a largely populated area (>100,000 inhabitants). About 20 mussels were transferred to 40-L tanks and exposed to increasing concentrations (0, 1%, 3%, 10% and 20%, V/V) of the ozone-treated ME for 7 days at 15°C under constant aeration. Exposure for long periods (7–14 days) is usually required to observed toxicity in treated municipal effluents. The ME concentrations were changed every 24 hr to ensure continuous exposure to the ME.

Concurrently, a sub-group of mussels was placed in another 60-L tank and injected intra-muscularly with 50 μL of a *V. anguillarum* suspension, as described by Akaishi et al. (2007). Briefly, a pure strain of *V. anguillarum* was obtained from microbiology laboratory of Fisheries and Oceans Canada (Moncton, New Brunswick). The bacteria were kept in Trypticase soy agar with 2% NaCl. Bacterial density was estimated by the plate dilution method. The concentration of bacteria during the exponential phase was on the order of $10^{15–17}$ counts/mL. The bacterial challenge was performed in $N = 10$ mussels exposed to increasing concentrations of the ME as described above. A volume of 50 μL of bacteria suspension was injected into the adductor muscles and the mussels were allowed to stand in air for 30 min before being returned to water. Control mussels were injected with Trypticase soy agar

(2% NaCl) only. After the exposure period, a 200-μL hemolymph sample was collected for immunocompetence assessment and a digestive gland sample was collected for metallothionein (MT) levels and glutathione S-transferase (GST) activity. The digestive gland was homogenized in ice-cold 140 mmol/L NaCl containing 25 mmol/L Tris-acetate, pH 7.8, 1 mmol/L EDTA and dithiothreitol using a Teflon pestle tissue grinder (5–6 passes). The homogenate was centrifuged at 15 000 ×*g* for 20 min at 2°C and the supernatant stored at −85°C until biochemical analysis. Total proteins were determined using the Coomassie Brilliant Blue dye-binding method (Bradford, 1976) using standard solutions of serum bovine albumin for calibration. The presence of bacteria in the hemolymph was also determined by qPCR methodology using the cpn60 marker gene as described by Douville et al. (2010). Briefly, DNA was extracted from the hemolymph using the commercial DNeasy® Blood & Tissue Kit (Qiagen, Mississauga, Ontario, Canada). The isolated DNA was measured at 260 nm and purity determined by the 260/280 nm ratio using the NanoDrop™ 1000 (Thermo Scientific Wilmington, Delaware, USA). The primers used for *V. anguillarum* were forward primer (5′-CCAAGCACTGCAAGATGAAC-3′) and reverse primer (5′-CCAATGCTTCACCTTCAACA-3′), giving a band at 239 base pairs (2% agarose gel electrophoresis with SYBR staining of DNA) with a melting point of 84.5°C.

1.2. Immunocompetence evaluation

Hemolymph (approximately 1 mL sample) was collected from the posterior adductor muscle with a 23-gauge needle syringe and placed on ice. Hemocyte count and viability were determined by flow cytometry (Guava EasyCyte Plus), using the Viacount cell kit from Guava Technologies (Hayward, CA, USA). A 20-μL sample of the hemolymph was mixed with 80 μL of Viacount reaction solution for 10 min before analysis, following the supplier's instructions. Phagocytosis activity was also determined by flow cytometry as described elsewhere (Brousseau et al., 1998). Briefly, cells were first gated by size and granulosity from the other particles/debris. For each organism, 200 μL of hemolymph was exposed to yellow latex FluoSpheres (2 μm diameter, Molecular Probes Inc., Eugene, OR, USA) at a cell-to-bead ratio of 1:30. The samples were allowed to incubate for 18 hr at 20°C in saturated humidity atmosphere. After this incubation period, cells were collected, washed in PBS (pre-diluted 1/3 in distilled water for freshwater mussels) containing 0.1% serum bovine albumin and resuspended in 200 μL of PBS for flow cytometric analysis. A total of 5000 cells were counted to determine the proportion of cells that engulfed at least three particles or more (yellow fluorescence window). The data were expressed as % of cells that engulfed at least 3 beads or more. For nitrite levels, the hemolymph was centrifuged at 200 ×*g* for 5 min at 4°C, and 100 μL of the supernatant was mixed with 100 μL of Griess reagent (Sigma Chemical Company, Mississauga, ON, Canada). The hemocyte pellet was stored at −85°C for oxidative stress biomarkers. Absorbance readings were taken at 450 nm after 30 min using standard solutions of sodium nitrite ($NaNO_2$) for calibration. Total hemolymph proteins were determined using the protein-dye binding principle (Bradford, 1976). Serum bovine albumin was used for calibration. The results were expressed as μmole NO_2/mg protein.

1.3. Oxidative stress assessment

Cyclooxygenase (COX) activity in hemocytes was measured by the oxidation of 2,7-dichlorofluorescin in the presence of arachidonic acid (Fujimoto et al., 2002). The pellet was resuspended in 250 μL of pre-diluted PBS (as described above). A volume of 50 μL of this cell suspension was mixed with 200 μL of reaction media composed of 50 mmol/L Tris–HCl, pH 8.0, containing 0.05% tween-20, 50 μmol/L arachidonic acid, 2 μmol/L dichlorofluorescein and 0.1 μg/mL horseradish peroxidase. The reaction was allowed to proceed for 0, 10, 20 and 30 min at 30°C. Fluorescence readings were taken at each time using 485 nm excitation and 520 nm for emission (Bioscan, Chameleon II, USA). The data were expressed as relative fluorescence units/min/mg proteins. Lipid peroxidation (LPO) in the digestive gland was also evaluated using the thiobarbituric acid method (Wills, 1987). The formation of thiobarturic acid reactants (TBARS) in the homogenate was measured by fluorescence at 520 and 590 nm for excitation and emission wavelengths respectively (Bioscan, USA). The data were expressed as μg TBARS/mg digestive gland. MT levels were assessed as a marker for oxygen radical/metal scavenging activity. MT levels were determined by the thiol spectrophotometric assay (Viarengo et al., 1997). The digestive gland homogenate was centrifuged at 15,000 ×*g* for 20 min at 2–4°C and the supernatant was kept for MT fractionation and quantitation. The data were expressed as μmol of GSH equivalent/mg protein in the supernatant fraction. GST activity was determined for reactive oxygen radical scavenging and conjugation of xenobiotics. GST was analyzed by a spectrophotometric method using GSH and 2,4-chlorodinitrobenzene as the chromophore substrate (Boryslawskyj et al., 1988). The assay was performed in the 15 000 ×*g* supernatant as described above, and the data were expressed as the increase in absorbance (412 nm)/(min·mg protein) in the supernatant.

1.4. Data analysis

Non-inoculated and inoculated mussels were exposed to increasing concentrations of ME in 10 replicates ($N = 10$). Normality of the data was checked by Shapiro–Wilks test, followed by an analysis of variance. Critical differences between controls or treatment group were determined by the Least Square Difference Test at $\alpha < 0.05$. Correlation and canonical analysis of the biomarker data and physiological groups of biomarkers were performed (hemocyte abundance, immune function, oxidative stress/inflammation and xenobiotic metabolisms). The data were also subjected to factorial and discriminant function analyses to seek out differences between the ME exposure concentrations and the presence of *V. anguillarum*. All statistical tests were performed using Statistica 8.0 (France).

2. Results

The municipal effluents were first treated to remove suspended solids (sieving), clarified using flocculants and then disinfected using ozone (10 mg/L injected O_3). The composite effluent had a

pH of 7.1 with a conductivity of 800 ± 20 μS/cm. The dissolved organic carbon (DOC) concentration was 18 ± 3 mg/L with relatively low ammonia (3.3 ± 0.5 mg/L). The effluent contained $1.8 \times 10^4 \pm 0.2 \times 10^4$ fecal coliforms/100 mL. Mussels were inoculated with *V. anguillarum* through intramuscular injection into the adductor muscles. The inoculum contained 514 ng of target DNA per ng of isolated DNA. The primers used for bacterial cpn60 were not uniquely specific for *V. anguillarum* since the control mussels are not expected to contain this strain. Mussels injected with the inoculum contained 0.4 ± 0.1 and 1 ± 0.1 pg of target DNA after exposure for 96 hr in aquarium water and municipal effluents (all exposure concentrations combined). Mussels exposed to ME removed three times less target DNA (cpn 60) than those maintained in aquarium water.

Mortality (15%) was observed in both non-inoculated and inoculated mussels at the highest exposure concentration. Peripheral hemocytes were determined in both the non-inoculated and inoculated mussels (challenged with *V. anguillarum*) exposed to increasing concentrations of ME (Fig. 1A). In the non-inoculated mussels exposed to ME, hemocyte counts were significantly increased at 1% and 3% effluent concentrations, reaching a 1.8-fold increase compared with the controls. In the inoculated mussels, hemocyte concentration was unchanged as compared to the control mussels, but readily increased at 1, 3 and 10% effluent concentrations, reaching a maximum of a 3.5-fold increase compared with the controls. No correlation was observed between hemocyte concentrations in the non-inoculated and inoculated mussels exposed to ME, which suggests that mussels exposed to both *V. anguillarum* and ME responded differently than mussels exposed to ME alone. Hemocyte viability was not significantly affected by exposure to ME either alone or in the presence of *V. anguillarum* (Fig. 1B). Only a transient change (increase) in cellular metabolic activity was found at 10% effluent concentration for inoculated mussels.

Immunocompetence was examined by measuring phagocytosis capacity and nitrite levels in the hemolymph (Fig. 2). In non-inoculated mussels exposed to ME, significant increases in phagocytosis activity (% of cells that ingested at least 3 beads) were observed at the lowest concentrations (1% and 3%). In the inoculated mussels, phagocytosis activity was not significantly increased compared with the control mussels. Phagocytosis capacity was significantly higher in the inoculated mussels than in the mussels exposed to the 10% ME concentration only. Nitrite levels were increased at 3% ME concentration in both the non-inoculated and inoculated mussels. Nitrite levels were not significantly increased in the inoculated mussels compared with the controls. However, nitrite levels were significantly correlated with hemocyte concentration ($r = 0.33$; $p < 0.001$) and phagocytosis activity ($r = 0.27$; $p = 0.001$).

The occurrence of oxidative stress and inflammation was also determined in the mussels (Fig. 3). Cyclooxygenase (COX) activity was not significantly affected in the non-inoculated mussels exposed to ME. In the inoculated mussels, COX activity was significantly increased at 1% and 3% compared to the control mussels. Correlation analysis revealed that COX activity between non-inoculated and inoculated mussels was not significant, suggesting a difference in the effects of the ME between the two groups. LPO levels were significantly induced at 3% effluent concentration in the mussels exposed to ME. In the inoculated mussels, LPO was significantly increased at 1% and 3% effluent concentrations. Correlation analysis revealed that LPO was significantly correlated with hemocyte concentration ($r = 0.27$; $p < 0.01$) in the mussels exposed to ME. In the inoculated mussels, LPO was more strongly correlated with hemocyte concentration ($r = 0.55$; $p < 0.001$) in the presence of ME. Oxygen radical and xenobiotic scavenging were followed by MT production and GST activity (Fig. 4). MT levels in the non-inoculated mussels were significantly induced at the highest exposure concentration (20%) reaching a 2-fold increase compared with the controls. In the inoculated mussels, MT levels were not significantly affected, although a similar trend was observed. MT levels at 20% effluent concentration were significantly lower compared to mussels not treated with *V. anguillarum*. Correlation analysis revealed that MT levels of inoculated mussels were significantly

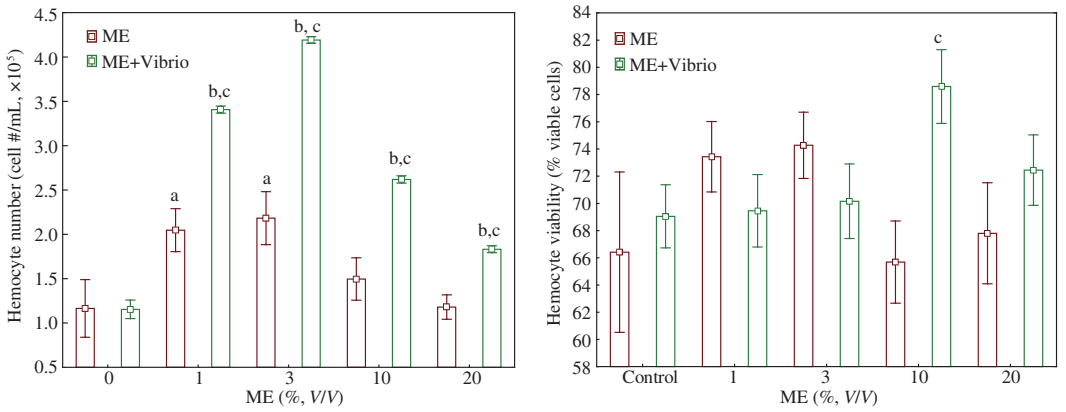

Fig. 1 – Variation in hemocyte number in mussels exposed to a municipal effluent and challenged with *V. anguillarum*. Mussels were exposed to the municipal effluent (ME) and mussels inoculated with *V. anguillarum* were exposed to increasing concentrations of ME. Hemocyte concentration (A) and viability (B) were determined in the hemolymph. The letter "a" indicates significance between control and ME concentration. The letter "b" indicates a difference in ME concentration in mussels inoculated with *V. anguillarum*. The letter "c" indicates significant difference between mussels and those inoculated with *V. anguillarum* at a given exposure concentration.

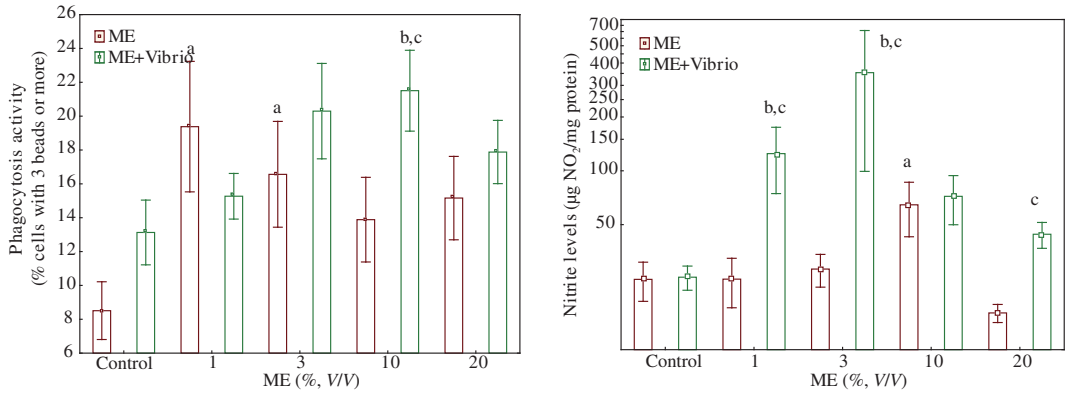

Fig. 2 – Immunoresponse of freshwater mussels exposed to ME and V. *anguillarum*. Mussels and mussels challenged by V. *anguillarum* were exposed to increasing concentrations of ME. Phagocytosis capacity and released nitrites in the hemolymph were determined. The letter "a" indicates significance between control and ME concentration. The letter "b" indicates a difference in ME concentration in mussels inoculated with V. *anguillarum*. The letter "c" indicates significant difference between mussels and those inoculated with V. *anguillarum* at a given exposure concentration.

correlated with hemocyte concentration ($r = -0.29$; $p < 0.05$) and phagocytosis capacity ($r = 0.41$; $p < 0.01$). GST activity was not affected in the non-inoculated mussels (Fig. 4B). In the inoculated mussels, GST activity was significantly elevated at 3% effluent concentration. Correlation analysis revealed that GST activity in the inoculated mussels was significantly correlated with phagocytosis activity ($r = 0.29$; $p < 0.05$) and MT levels ($r = 0.69$; $p < 0.001$). GST activity was also correlated with MT levels ($r = 0.70$; $p < 0.001$) and ($r = 0.69$; $p < 0.001$) in the non-inoculated mussels.

In an attempt to gain a global view of the observed responses, a discriminant function and factorial analyses of the data was performed (Fig. 5). The analysis revealed that mussels exposed to ME were clustered together in the 2-D plot and differed from the inoculated mussel group. The analysis also showed that the more concentrated the effluent, the more similar the resulting effects are to those of the mussels exposed to ME alone (*i.e.*, the effects are dominated by the ME). For example, the inoculated mussels exposed to 20% ME (20% + *Vibrio*) were within the effluent-dominated effects (see red square in Fig. 5). This suggests that as the effluent concentration increases, the observed effects are more influenced by the ME than by inoculation with the pathogen. Based on the effluent concentration, the effects related to the effluent were also concentration dependent and could be separated into two groups: the low exposure concentration group (1%, 3% and 10%) and the high exposure concentration group (20%). The biomarkers that best described these changes (*i.e.*, between the low and high ME concentrations) were LPO, MT and hemocyte viability. A similar pattern was also found in inoculated mussels exposed to low (1% and 3%) and high (10% and 20%) ME concentrations. The biomarker responses caused by the presence of *Vibrio* alone were similar to those of the control mussels. The interaction between the ME and *Vibrio* treatments was stronger in the low-exposure ME concentration group (1% and 3%). The major effects explaining this interaction were hemocyte concentration, hemocyte viability and nitrites. Canonical analysis between

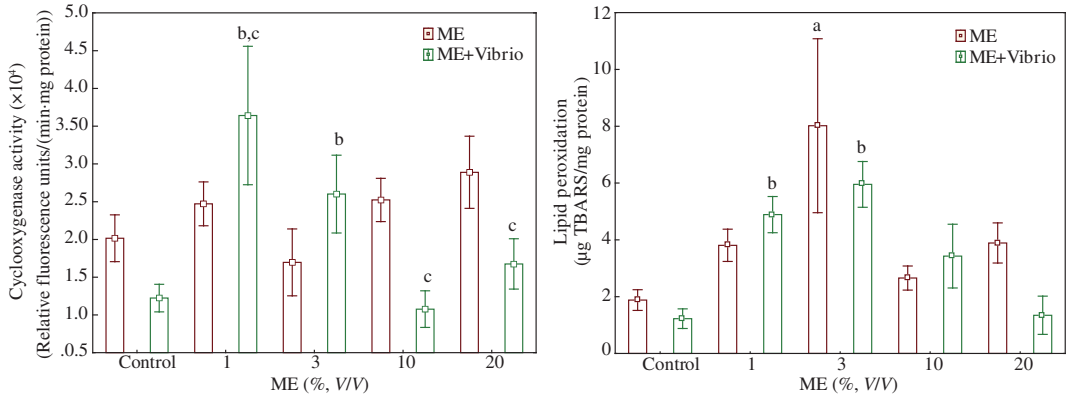

Fig. 3 – Oxidative stress and inflammation in non-inoculated mussels and mussels inoculated with V. *aguillarum* exposed to ME. Non-inoculated mussels and mussels inoculated with V. *anguillarum* were exposed to increasing concentrations of ME. COX and LPO were determined in the digestive gland. The letter "a" indicates significance between control and ME concentration. The letter "b" indicates a difference in ME concentration in mussels inoculated with V. *anguillarum*. The letter "c" indicates significant difference between mussels and those inoculated with V. *anguillarum* at a given exposure concentration.

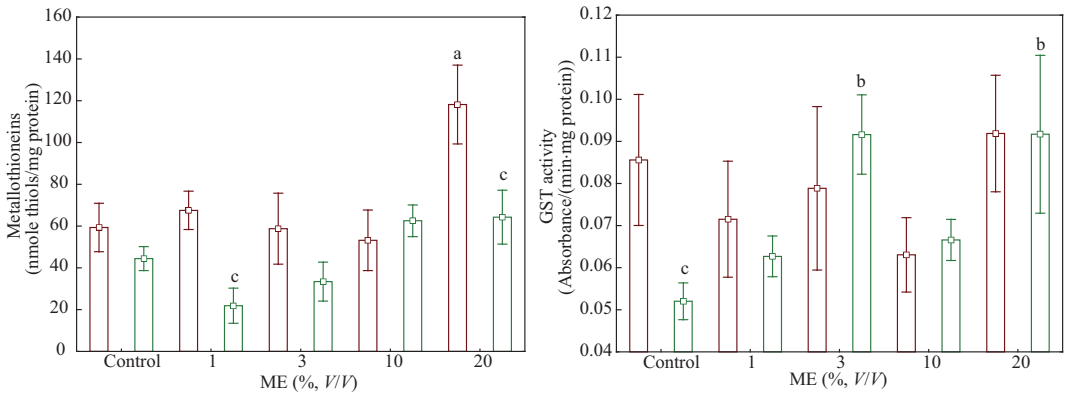

Fig. 4 – Xenobiotic metabolism of mussels treated with ME and bacteria. Non-inoculated mussels and mussels inoculated with *V. anguillarum* were exposed to increasing concentrations of ME. Total MT and GST activity were determined in the digestive gland. The letter "a" indicates significance between control and ME concentration. The letter "b" indicates a difference with ME concentration in mussels inoculated with *V. anguillarum*. The letter "c" indicates significant difference between mussels and those inoculated with *V. anguillarum* at a given exposure concentration.

biomarker physiological groups (hemocyte presence, concentration and metabolic activity), immunocompetence (phagocytosis and nitrites), oxidative stress/inflammation (LPO, COX) and xenobiotic/oxygen radical metabolism (MT, GST) revealed that hemocyte presence had the strongest correlation with oxidative stress/inflammation (Rc = 0.23; $p = 0.01$) and xenobiotic/oxygen radical metabolism (Rc = 0.25; $p < 0.001$).

3. Discussion

The ME examined in this study consists of a typical effluent resulting from a physical–chemical treatment process followed by ozonation (10 mg/L) to reduce bacteria, viruses and other microorganisms. The ME still contained on the order of 10^4 fecal coliforms/100 mL with relatively low levels of DOC, significant conductivity (about 2.5 times the levels of the receiving water) and relatively high quantities of ammonia. The chronic toxicity of ammonia (28 days) was reported to be in the same range *i.e.*, between 0.37 and 1.2 mg/L at pH 8.2 (Wang et al., 2007). However, the pH of the exposure water was 6.8 ± 0.2, which was well below the threshold for ammonia toxicity (pH > 7.8).

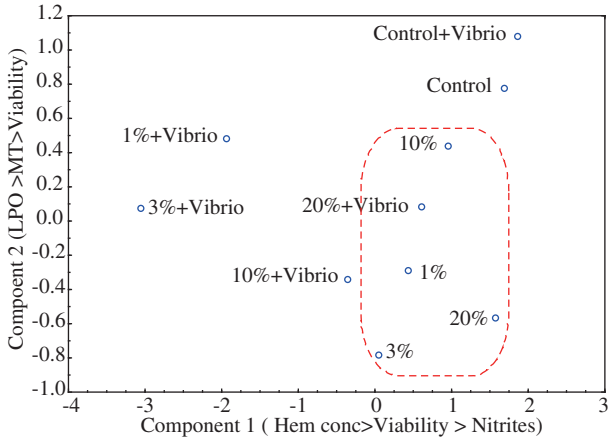

Fig. 5 – Discriminate function analysis of biomarker data. The biomarker data were modeled using linear discriminate function analysis to seek out differences between the effluent exposure concentrations in the inoculated and non-inoculated mussels. The treatments located in the red circle represent effects of the ME alone.

Exposure to ME leads to increased concentrations of hemocytes in the hemolymph, reaching a 2.5- and 3.7-fold increase in the non-inoculated and inoculated mussels respectively. The presence of residual microorganisms could account for this increase, as the addition of *V. anguillarum* enhanced this response. The increase in hemocyte concentration was also found in mussels caged in aeration lagoon effluents for the treatment of domestic and hospital effluents (Gagné et al., 2012). These effluents contain two orders of magnitude fewer fecal coliforms than the ozonated effluent in this study, indicating that microbial loading of this ME was still significant for mussels. Not all *Vibrio* sp. strains could increase the concentration of circulating hemocytes. Interestingly, *Vibrio* species that induce mortality were shown to increase hemocytes, while non-pathogenic species did not have any effect in the soft-shell clam (Mateo et al., 2009). In *Elliptio complanata*, treatment with ME had significant effects on phagocytosis at low effluent concentrations. Moreover, *Vibrio* exposure also increased phagocytosis, but at a higher ME concentration (10%). The reason for this is unclear since enteric bacteria apparently have no effects on phagocytosis in injected blue mussels (Gauthier-Clerc et al., 2013). Mussels were injected to low amounts of *V. anguillarum* compared to the ME (1.8×10^4 fecal coliforms/100 mL, and mussels were exposed to 60 L of ME). In the inoculated mussels, the presence of *Vibrio* could render the mussels less sensitive to ME bacteria, because mussels would direct their activity towards the removal of a potentially pathogenic bacteria species. This is in keeping with the observed increase in hemocyte number and metabolic activity (viability) when mussels were exposed to a pathogenic strain of *Vibrio* compared to a non-pathogenic strain (Mateo et

al., 2009). Another explanation for the lack of phagocytosis activity at high ME concentration is the presence of contaminants that could decrease this activity in mussels or that this response reached a saturation kinetically and decreased at the time of analysis. For example, Hg and Zn exposure to marine and freshwater bivalves hemocytes led to hormesis-type of responses where an increase in phagocytosis occurred at low concentrations followed by a inhibition at high exposure concentrations (Sauvé et al., 2002). In another study, clams exposed to representative polycyclic aromatic hydrocarbons mixtures (coke dust, smelters soot) for 14 days had reduced phagocytosis in males with increased LPO in the digestive gland (Frouin et al., 2007). The representative polycyclic aromatic hydrocarbons (PAHs) mix consisted of coke dust and smelter soot at total PAHs concentrations of 17 and 22 μg/g sediment (soot or dust) at diluted at 2% (V/V) concentration in the sediment bed (sand) for clam exposure. Low salt concentrations in water were also associated with increased phagocytosis activity in *Mya arenaria* clams in the Saguenay fjord (Blaise et al., 2002b). This suggests that increased salts could have resulted in reduced phagocytosis at high ME concentrations (10% and 20%, V/V) due to the high conductivity of the ME. This could also contribute to the apparent biphasic nature of many of the biomarkers in this study, where effects were found at low ME concentrations compared to high ME concentrations (see discriminant function analysis data). This biphasic response was also observed in mussels inoculated with a pathogen and ME (Akaishi et al., 2007). Mussels treated with *V. anguillarum* and exposed to high concentrations of ME (100% and 50%) had a mortality rate of >30%, while those exposed to low effluent concentrations (12.5% and 25%) survived and eliminated the pathogen. This was further supported by the lower capacity of mussels to eliminate *Vibrio* sp. DNA when exposed to ME. Increased hemocyte counts and viability were associated with increased resistance to brown ring disease in *Ruditapes* sp. clams (Allam et al., 2000, 2001). In clam species with high hemocyte (granulocyte) counts and phagocytosis activity, increased resistance to *V. tapetis* (the causative agent of brown ring disease) was found compared with other species with lower hemocyte counts or phagocytosis activity. Again, these immune endpoints were activated when the *Vibrio* species led to pathogenicity *via* the activation of non-specific immunity in bivalves.

In the inoculated mussels exposed to ME, COX activity (inflammation) was significantly higher than in the non-inoculated mussels. Moreover, COX activity was not related to LPO damage in either the non-inoculated or inoculated ME-exposed mussels. One possible explanation for this is that *Vibrio* treatment increased the levels of GST, whereas MT levels were induced by the ME in both non-inoculated and inoculated mussels. Although these markers are recognized to conjugate polar organic and metallic compounds respectively, they are also involved in ROS scavenging. Indeed, the production of ROS could be neutralized by MT during infections (Gagné et al., 2008a; Figueira et al., 2012) and GST (Srikanth et al., 2013). It appears that GST could be involved in the conjugation of metals, metalloids and reactive oxygen species. GST is believed to react to organic compounds with oxygen radicals. The production of ROS during inflammation was shown to oxidize MT and liberate potentially toxic metals from which it binds (Gagné et al., 2008a). In *Mytilus galloprovincialis* challenged with *V. anguillarum*, significant oxidative stress and disturbance in energy metabolism were observed, and GST activity was also found to be induced by bacteria alone (Ji et al., 2013). Analysis of covariance of GST activity, MT and LPO levels in the inoculated mussels revealed that GST was equally induced by oxidative stress and the ME, but the major factor was MT levels in the inoculated mussels exposed to ME. Nevertheless, the effects of this ozone-disinfected ME on GST activity remained significant. In mussels exposed to ME alone, GST activity was linked to MT levels only and not LPO. The oxidative effects and perhaps the inflammatory effects of ozone-treated ME could also be enhanced in mussels (Gagné et al., 2008b). In ozonated ME, COX activity and nitrite levels were readily increased after ozone treatment, which could also contribute to the release of ROS and inflammatory properties of ME. Indeed, caged mussels exposed to various ME also showed increased LPO and GST activity (Gillis et al., 2014b). In another study, exposure of the hard clam *Mercenaria mercenaria* to a protist (quahog parasite) led to increased MT levels in addition to increased hemocyte mobility, phagocytosis and reactive oxygen production (Perrigault and Allam, 2012). These studies suggest that the production of ROS results from the occurrence of both contaminants and the presence of pathogenic microbes *i.e.*, those that elicit strong immune response in mussels.

In conclusion, the data suggest that exposure of mussels to a disinfected ME by ozone can stimulate the immune response such as hemocyte density, phagocytosis, nitric oxide, MT and LPO levels. Co-exposure to *V. anguillarum* exacerbated these responses and induced inflammation as suggested by increased COX activity (but not MT). Although more data is needed, the modulation of immunocompetence in mussels exposed to complex mixtures indicates that the immune system can be influenced by the cumulative effects of exposure to ME-related contaminants and microorganisms. The combined exposures could bring a dual response where activation mechanisms could be induced at low effluent concentrations and reduced when the effluent concentration increases.

Acknowledgments

This project was funded by Environment Canada's St. Lawrence Action Plan. The authors thank Sophie Trépanier for technical assistance in mussel exposure and tissue preparations.

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