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Short-term physiological effects of a xenobiotic mixture on the freshwater mussel *Elliptio complanata* exposed to municipal effluents

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ABSTRACT

The aim of this study was to investigate the short-term effects of tertiary-treated municipal effluents on the freshwater mussel *Elliptio complanata*. Caged mussels were immersed during 2 weeks in a river located North of Montreal Island, upstream/downstream the outfall and in one reference site located at the beginning of the Rivière des Prairies. A selection of biomarkers was analyzed to depict changes on various physiological systems: general physiology (mussel viability, condition index and gonado-somatic index), immune status (hemocyte viability, cellularity, phagocytosis efficiency, NK-like cytotoxic activity and lysozyme activity), inflammation (cyclo-oxygenase activity), detoxification (glutathione-S-transferases activity) and vitellogenesis (alkali-labile phosphate level). The analysis of total and fecal coliform counts in water and of heterotrophic bacteria levels in mussel tissues showed that the bacteriological quality of the water strongly decreased from the reference site to the downstream site. This was correlated with a significant loss of weight and an increase of mussel mortality. Cellularity and phagocytosis efficiency were significantly increased in the downstream site compared to the reference site. Though not statistically significant, lysozyme activity was also increased. NK-like cytotoxicity, activity of the pro-inflammatory enzyme COX and the levels of ALP and MT were not significantly changed. Conversely, the municipal effluents induced a significant increase of GST activity in downstream site, indicating a stimulation of detoxification metabolism. Altogether, these results confirm that a short-term exposure to a mixture of bacterial and chemical compounds released by the wastewater treatment plant La Pinière induces adverse physiological effects in *E. complanata*, as observed with the modulation of immune response and induction of detoxification metabolism.

1. Introduction

The quality of surface water has been a source of growing concern for the last decade. A major source of water pollution is municipal sewage, which poses a threat to both human health and aquatic ecosystems. Wastewater treatment plants must treat a wide variety of natural and industrial products since they receive wastewater from both domestic and industrial sources (Chambers et al., 1997). The harmful effects of these contaminants have been tested in the field and in the laboratory, mainly for their endocrine disruption effects (Jobling et al., 2003; Quinn et al., 2004; Gust et al., 2010). Recently, their immunotoxicological effects, also investigated (Akaishi et al., 2007; Bouchard et al., 2009), showed that these effluents lead to immunosuppression in mussels. In addition to known chemicals classically found in municipal effluents, an emerging panel of molecules such as pharmaceuticals, personal care products and products from the nanotechnology industry makes the chemical composition of wastewater increasingly more complicated to analyze and to process.

Laval Island (Quebec, Canada) is the largest suburb of Montreal and is separated from the Montreal Island to the south by the Rivière des Prairies. The La Pinière (LP) treatment plant is the largest and the newest (built in 1998) of the three wastewater plants in Laval Island. With a mean flow rate of 240,000 m^3/d, the LP plant processes the sewage of about 75% (i.e. 280,000 people) of the Laval population. Additionally, the LP plant receives the effluents of the southern half of the Laval Island where hospitals and other medical services are located. The LP plant executes primary, secondary and tertiary levels of sewage treatment. It uses a physico-chemical process to remove large particles and

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phosphates and a final disinfection step by UV treatment. The toxicological impacts of tertiary-treated effluents are expected to be low compared to those of effluents undergoing only a primary and secondary treatment, essentially aimed at removing suspended matter and particles.

In the field, contaminants are found in mixtures so that synergistic effects can occur (Cleuvers, 2003). *In situ* approaches, such as mussel caging, allow the determination in the natural environment of the effects of mixtures. Mussel caging has become a recognized approach in ecotoxicological studies (Hyotylainen et al., 2002; Gagné et al., 2001; Gagné and Blaise, 2003; Blaise et al., 2003; Andral et al., 2004; Gagnon et al., 2006; Pytharopoulou et al., 2008; Bouchard et al., 2009). As a filter-feeding sedentary organism, mussels can bioaccumulate contaminants; it is thus a convenient model to conduct caging experiment in the field (Rittschof and McClellan-Green, 2005). Additionally, the use of a local species, such as *Elliptio complanata*, allows the *in situ* analysis of wild animals chronically exposed to the discharges (Gagné et al., 2011).

In bivalve molluscs, protection of the organism is solely dependent on the innate immunity (Mydlarz et al., 2006). Innate immunity is composed of both cellular and humoral components consisting of molecules circulating in the hemolymph. The involvement of circulating hemocytes as the first line of defense against invading microorganisms is well established (Cheng and Auld, 1977). Hemocytes can phagocytose the foreign particles (Lopez et al., 1997; Beaven and Paynter, 1999; Canesi et al., 2002a). They can produce oxygen or nitrogen reactive substances for the killing and destruction of microorganisms (Anderson et al., 1992; Pipe, 1992). NK-killer like cytotoxic activity, which consists in the recognition and destruction of exogenous cells, has been reported in molluscs (Franceschi et al., 1991). The humoral components of bivalve immunity comprise lysozyme activity, lectins, anti-microbial peptides and the phenoloxidase system (Stefano et al., 1990; Munoz et al., 2006). The presence of chemical and bacterial contaminants in the environment could compromise immune functions and facilitate the progression of infectious diseases (Morley, 2010). The effects of primary-treated municipal effluents have been investigated in the field on bivalves, after various exposure periods ranging from 1 month to a year (Gagné and Blaise, 2003; Gagné et al., 2004; Quinn et al., 2004, 2005; Gagnon et al., 2006; Akaishi et al., 2007; Bouchard et al., 2009) but the toxic impacts of tertiary-treated urban effluents on indigenous freshwater mussels are poorly documented. Though a number of studies have been published on municipal effluent ecotoxicology, the chemical characterization of the released effluents and its ecotoxicological effect were rarely analyzed together in the field.

In the present work, we analyze the short-term effects of a characterized treated municipal sewage effluent on caged *E. complanata* freshwater mussels in the field. The composition of the tertiary-treated effluents of the La Pinière plant was chemically analyzed for organic contaminants and metals. The physico-chemical and bacteriological qualities of the receiving water were evaluated in each exposure site. Parameters depicting general mussel physiology were evaluated: survival rate, condition index and gonado-somatic index. Hemocyte viability, cellularity, phagocytosis efficiency, NK-like cytotoxic activity and lysozyme activity were analyzed in hemocytes and hemolymphe to investigate the immunotoxicological effects of the effluents. A selection of biomarkers was also analyzed in gonad, digestive gland and hemocytes to investigate the reproductive (vitellogenin-like proteins), inflammatory (cyclo-oxygenase activity) and detoxification functions (glutatione-S-transferase activity, metallothionein). This paper brings new informations about the short-term response of caged *E. complanata* mussels exposed to tertiary treated municipal effluent in the field. A particular emphasis was put on the characterization of the chemical and bacteriological contamination in order to improve our understanding of mixture effects.

2. Material and Methods

2.1. Mussel handling and exposure set-up

Wild freshwater mussels *E. complanata* were collected in June 2009 in the Richelieu River (Quebec, Canada). The animals were maintained for 3 months in filtered water at 15 °C, with a 16 h-light/8 h-dark cycle. The mussels were fed daily with concentrates of phytoplankton (Phytoplex®, Kent Marine, Franklin, WI, USA) and cultured *Pseudokirchneriella subcapitata* microalgae. After this depuration period, the mussels were placed in experimental cages according to a standard method (ASTM, 2001). Briefly, 15 mussels (69 ± 6 mm length, 36 ± 9 g total weight) were distributed in cylindrical nets, which were attached to a plastic frame (1 m^2 of surface area) covered by a rough net cage for protection from predators. Two cages of 15 mussels each were immersed on each site. At the end of the experiment, analyses were done on 10 mussels per site.

The exposure experiment took place in the Rivière des Prairies, which separates the Montreal Island in the South and the Laval Island in the North. This river receives treated wastewater from the La Pinière treatment plant (Fig. 1). The location of the point of release of La Pinière effluents was determined by the increase of conductivity in water. For 2 weeks in September 2009, when water temperature was approximately 16–20 °C, two cages were deployed at each study site: 300 m upstream and 200 m downstream from the point of release and at a reference site located in the Lac des Deux Montagnes, where Rivière des Prairies originates. The cages were maintained in the water column thanks to a 6-kg weights and a buoy for localization.

2.2. Physico-chemical parameters

Water parameters (temperature, pH and conductivity) and the survival of mussels were checked every week. At the end of the experiment, suspended matter and total organic carbon in the water were determined by Environment Canada (Montreal, QC, Canada). Total and fecal coliforms in water and the amount of heterotrophic bacteria in total tissues of a pool of 5 mussels were measured by EXOVA (Quebec, QC, Canada) according to standard procedures.

The contamination spectrum of the La Pinière plant effluents was investigated from a 24-h composite of the effluents. Chemical analyses were done by CEAEQ (Centre d'Expertise en Analyse Environnementale du Quebec, QC, Canada). A large panel of substances was measured: nonylphenol ethoxylates (NPE), polycyclic aromatic hydrocarbons (PAH), pesticides, polychlorinated biphenyls (PCB), polybrominated diphenyl ethers (PBDE), volatile organic compounds (VOC), perfluorates, dioxins, furans, pharmaceuticals and metals.

2.3. Biological parameters

Mussel mortality was checked every week. At the end of the experiment, the mussels were brought back to the laboratory where total mussel weight, soft tissue weight, gonad tissue weight and shell length were determined at 4 °C. Before dissection, hemolymph was collected from the posterior and anterior adductor muscles using a syringe with a 23 G needle, and immediately put on ice. The mussels were then dissected. For biomarkers measurement, the gonad and the digestive gland were stored in −80 °C until analysis.

2.3.1. Immunological parameters

Cellularity and viability were evaluated by flow cytometry using a Guava PCA flow cytometer and a Viacount kit (GuavaTechnologies, Hayward, CA, USA) according to the supplier's instructions.

Phagocytosis was measured according to a flow cytometry method developed for bivalve hemocytes (Brousseau et al., 1998). Briefly, hemocytes were mixed with yellow-green latex Fluoresbrite™ Carboxylate microspheres (Polysciences, Inc., Warrington, PA, USA), which had a diameter of 1.6 µm, at a ratio of 1:100 (hemocytes:beads). The cells were incubated at 16 °C in the dark. After 18 h, an aliquot of 0.5 mL of each cell suspension was layered over a 3% bovine serum albumin (BSA) gradient in RPMI-1640 medium and the cells recovered after centrifugation at 150g for 8 min at 4 °C to remove the free beads. The cell pellets were re-suspended in 0.5 mL of 0.5% formaldehyde fixation solution and acquisitions were performed with the flow cytometer. A FACSCalibur flow cytometer (Becton Dickinson, Mississauga, ON, Canada) equipped with a 488 nm argon laser was used. Hemocyte populations were defined on the basis of their forward and right angle scatter properties (FSC and SSC, respectively). For each sample, the fluorescence of 10,000 events was recorded. The data were initially analyzed for the green fluorescence frequency distribution histogram of the hemocyte