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The effects of a primary-treated municipal effluent on the immune system of rainbow trout (*Oncorhynchus mykiss*): Exposure duration and contribution of suspended particles

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ABSTRACT

Municipal sewage effluents are complex mixtures of contaminants known to disrupt both immune and endocrine functions in aquatic organisms. The present study sought to determine the impacts of municipal effluent on the immune systems of juvenile rainbow trout (*Oncorhynchus mykiss*), by exposing specimens to low concentrations (0.01%, 0.1%, 1% or 10%) of sewage effluent for periods of 28 or 90 days. The soluble and insoluble fractions of the effluent were also studied to assess the contribution of fractions rich in microorganisms and particles on fish immune systems. To this end, the trout were also exposed to soluble and insoluble fractions of the effluent for a period of 28 days. Immunocompetence was assessed by the following three parameters: phagocytosis, natural cytotoxic cells (NCC) and blastogenesis of lymphocytes under mitogen stimulation. Fish exposed to the 1% sewage effluent concentration for 28 days had enhanced phagocytic activity; at 90 days, phagocytic activity was reduced. T and B lymphocyte proliferation in fish from both groups was similarly stimulated. Phagocytosis and NCC activities were influenced more by the insoluble fraction than the soluble fraction of the effluent. Conversely, mitogen-stimulated T lymphocyte proliferation was enhanced in cells of fish exposed to the soluble fraction of the effluents, with a dampening effect on the insoluble (particulate) fraction of the effluent. In conclusion, the effects of the effluent and its fractions were higher at the cellular-mediated immunity level than at the acquired immunity level. Immunotoxicity data on the soluble fraction of the effluent were more closely associated to data on the unfractionated effluent, but the contribution of the particulate fraction could not be completely ignored for phagocytosis and B lymphocyte proliferation.

1. Introduction

Municipal wastewaters are a mixture of chemical and microbiological contaminants from commercial, institutional and industrial sources, sanitary wastewater, underground infiltration and surface runoff events. The municipal effluent from the city of Montréal (Quebec, Canada) is considered a major source of contamination for the St. Lawrence River. Indeed, with a mean flow rate of 2 500 000 m^3/day and potential maximum capacity of 7600 000 m^3/day during rainfalls, it is considered one of the highest-volume emitters of wastewater in North America (Purenne, 2002). The Montreal wastewater treatment plant currently uses a physicochemical process (primary treatment) to remove large particles (gate and grit removal) and settle the finer ones by coagulation with alum and/or ferric chloride and anionic surfactants. No disinfection steps are currently applied (although it is being considered) to the wastewater before discharging it to the St. Lawrence River (Wagner et al., 2002). Although a major proportion of the particles are removed during the treatment process, the final effluent contains detectable amounts of suspended solids (SS), biochemical oxygen demand (BOD), chemical oxygen demand (COD) and microorganisms (Wagner et al., 2002; Payment et al., 2000).

Municipal effluents are made up of a complex organic matrix collectively termed dissolved and suspended organic matter (DOM and SOM, respectively). DOM is comprised of many hydrophilic and hydrophobic binding sites (Leenheer and Croué, 2003). Interactions between DOM and xenobiotics involve various modes of binding such as polyvalent cation interactions, hydrogen bonding, charge transfers, ion exchanges, nonpolar interaction, hydrophobic adsorption, etc. (Leenheer and Croué, 2003). There are many contaminants that bind to the fine SPM contained in primary-treated wastewater, including polycyclic musk fragrances, polycyclic aromatic hydrocarbons (PAH), surfactants,

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polychlorinated biphenyls (PCB), sterol (cholesterol), and metal ions (Alberts et al., 1989; Hassett and Anderson, 1979; Paxéus, 1996; Rimkus, 1999). Municipal effluents were found to produce many effects at the endocrine, immunological and neurological levels in fish and mussels (Matozzo et al., 2008; Salo et al., 2007; Tarrant et al., 2008). Salo et al. (2007) reported that the exposure of rainbow trout to municipal effluent for four weeks suppressed spontaneous proliferation of lymphocytes and elevated plasma lysozyme activity. The increase in lysozyme activity suggests that the presence of bacteria/microorganisms in the primary-treated effluent could have caused this stimulation. In addition to chemical contamination, the release of microorganisms could also saturate the process of phagocytosis and increase the susceptibility of fish to harmful bacteria. Indeed, fish exposed to sewage effluents had alterations specific to infection and disease to both external (e.g. skin, fins, jaw) and internal (liver and pronephros) organs, with symptoms of inflammation resulting from the infection and tissue necrosis (Burkhardt-Holm et al., 1997; Bucher and Hofer, 1993; Escher et al., 1999; Grizzle et al., 1988). In addition to the effects of microorganisms, exposure to various contaminants could also increase the susceptibility of wild fish populations to disease. For example, trout exposed to the effluent in the Alte Aare River experienced elevated rates of parasitic infestations and bacterial infections (Escher et al., 1999). Moreover, the prevalence of neoplastic diseases was observed in teleost at sites contaminated by municipal and industrial wastewaters (Black and Baumann, 1991). To our knowledge no studies have yet examined the effects of particle/microorganism removal from municipal effluents on (non)specific immune function in fish.

Rainbow trout exhibit both innate and specific immune responses (Watts et al., 2001). Innate immunity is performed principally by macrophages, granulocytes and natural cytotoxic cells (NCC). Phagocytosis by macrophages and granulocytes consists of the internalization and destruction of foreign microorganisms (Secombes, 1996). NCCs represent one of three types of lymphocytes (the other two being T and B cells) and have demonstrated a capacity for spontaneous cytotoxic action against fish and mammalian cancerous cell lines, virus-infected cells and protozoan parasites (Evans and Jaso-Friedmann, 1994). NCC cells can induce both apoptic and necrotic lesions in target cells (Greenlee et al., 1991). The adaptive and specific immune responses are mediated principally by B and T cells, which are implicated in the production of specific antibodies (B cells) and cytotoxic cells (cytokines) to assist the destruction of pathogen-infected cells. The clonal production of lymphocytes exposed to given antigens forms the basis of adaptive immunity in organisms which are active in aquatic vertebrates.

The purpose of this study was therefore to characterize the impact of wastewater on the immune system of juvenile rainbow trout (*Oncorhynchus mykiss*) before and after the removal of microorganisms and other particles in a primary-treated effluent. An attempt was made to determine the contribution of the soluble and insoluble (particulate) fractions of the effluent to the whole effluent to the immunological responses.

2. Materials and methods

2.1. Animal care

Juvenile rainbow trout *O. mykiss* (Salmonidae) (Aquipro, QC, Canada), with an initial body mass of 7–9 g, were maintained at 15 °C with 16 h/8 h light and dark cycles for two weeks in 90-L glass aquaria containing chlorine-free tap water. For the experiments, the fish were transferred into 45-L glass aquaria. The fish were fed Aquipro commercial fish food daily at a rate of between 1 and 2% body weight, depending on their individual weights. The water was renewed by half every two days (semi-static conditions).

2.2. Exposure to primary-treated effluent before and after particle fractionation

Fish were exposed to a 10% v/v concentration of the primary-treated effluent and to the soluble and insoluble (particulate) fractions (5 mg/L) of the effluent for a period of 28 days. The soluble and insoluble fractions of the effluent were prepared by centrifugation. A volume of 100 L of the effluent was collected at the urban wastewater treatment plant during the morning and centrifuged at 15 000 ×*g* for 10 min in 250 mL polypropylene bottles (Nalgene Products, Rochester, NY, USA) using a centrifuge adapted for large volumes (Biotechnology Instrumentation, Albertville, MN, USA). The supernatant (soluble fraction) was carefully removed from the pellet (insoluble fraction) rich in microorganisms and particles. The soluble portion was filtered on a 0.22-μm pore membrane filter (Millipore Corporation, Bedford, MA, USA) to eliminate any residual suspended matter during handling. The pellet was resuspended in aquarium water to yield the initial concentration of total suspended matter (5 mg/L) and stored at 0–4 °C until the exposure experiments the following week. For the effluent exposure experiments, five groups of 30 juvenile rainbow trout were exposed to 0.01%, 0.1%, 1% and 10% effluent concentrations for a period of either 28 (primary and the soluble/insoluble fractions) or 90 days (primary effluent only). Each experiment had its own chlorine-free tap water control group. The effluent concentrations were renewed each week by replacing 2/3 of the volume.

2.3. Post-exposure fish handling and leucocytes preparation

After the exposure period, the fish were killed in a 0.1% solution of MS-222 (Boreal Laboratories, ON, Canada) and measured for weight and length. The anterior kidney of each fish was removed aseptically and mashed with a 2-mL glass grinder (Wheaton Scientific, N.J., USA) containing 1 mL of sterile RPMI 1640 (Bio Media, QC, Canada) supplemented with 10 U/mL heparin (Organon Teknika, ON, Canada), 10 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) Fetal Bovine Serum (FBS; Bio Media). The cellular suspension was then transferred to a sterile 15-mL conical polypropylene tube (Sarstedt, NC, USA) and the final volume adjusted to a total of 5 mL with RPMI. This cell suspension (5 mL) was laid over 5 mL of Lympholyte Poly gradient media (Cedarlane Laboratories, ON, Canada) and centrifuged at 275 ×*g* for 30 min. Leucocytes between the two layers were removed by aspiration and transferred to a sterile conical polypropylene tube. The cells were washed twice in RPMI media without heparin and adjusted to 1×10^6 cells/mL.

2.4. Leucocyte viability and phagocytosis assessment

Viability was determined using Trypan Blue dye exclusion (0.4%) (Sigma-Aldrich Chemical Co., MO, USA). Viable and dead (stained) cells were determined microscopically with a hemacytometer (Bright-line, PA, USA). For phagocytosis, a duplicate sample of 500 μL of the cellular suspension (previously adjusted to a concentration of 1×10^6 cells/mL) was mixed with fluorescent latex beads (diameter of 1.7 to 1.9 μm; Polysciences, PA, USA) at a 100:1 bead-to-cell ratio. The suspension was incubated at room temperature for 18 h in the dark. After the incubation period, the cell suspensions were layered over 3 mL of RPMI supplemented with 3% bovine serum albumin (BSA; Sigma) and 10% FBS. Removal of the free beads was achieved by centrifugation at 150 ×*g* at 4 °C for 8 min. The cell pellets were then resuspended and fixed in 0.5 mL of 0.5% formaldehyde diluted in phosphate-buffered saline (Hematall, Becton Dickinson, CA, USA). The cells were finally analysed by flow cytometry using a FACScan (Becton Dickinson) and 5000 events were recorded. Analyses were done using two endpoints corresponding to the percentage of phagocytes containing one bead or more (M1) and the percentage of phagocytes containing three beads or more (M2). These cell populations were operationally defined as basal