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Immunotoxicological effects of an activated-sludge-treated effluent on rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Municipal effluents are often treated by biological processes designed to enhance natural biodegradation that introduce important changes to the local bacterial community and contaminant status. The purpose of this study was to examine the immunotoxicological effects of an activated sludge treated urban effluent on rainbow trout (*Oncorhynchus mykiss*). Trout specimens were exposed to increasing concentrations of the effluent at 0.1%, 1% and 10% v/v dilutions for 30 days at 15 °C. After the exposure period, the leukocytes were isolated from the anterior kidney and the following parameters were determined: phagocytic activity, lymphoblastic transformation and natural cytotoxic activity. The results revealed that phagocytic activity was not significantly affected by the biologically treated effluent. Natural cytotoxic activity was significantly increased at an effluent threshold concentration of 3% v/v at an effector/target cell ratio of 20:1 and 40:1. Stimulated lymphocyte T and B proliferation was significantly decreased at a threshold concentration of 0.3%. However, neither the unstimulated lymphocyte T nor lymphocyte B proliferation was significantly affected by effluent exposure. The results suggest that the activated-sludge-treated effluent increased cell-mediated cytotoxic activity with a concomitant decrease in cells responsible for antibody and cytokine production, indicating that the immune system of rainbow trout was directed towards the elimination of transformed cells, virus-infected cells and protozoan parasites at the expense of cytokine and antibody production.

1. Introduction

Municipal effluents contain a number of contaminants from anthropogenic and industrial activities. The continuous release of these chemicals could pose a threat to organisms living in receiving waters near effluent outfalls. For example, these effluents contain microorganisms, viruses and chemicals such as polycyclic aromatic hydrocarbons, highly polar organic compounds such as pharmaceutical products, bisphenol A, phthalates, pesticides, and perhaps even compounds derived from nanotechnology. Incoming wastewaters are usually first treated to remove solids and suspended matter by adding various precipitating agents such as ferric chloride or alum. In Canada, biological treatment of the clarified primary effluent represents a common strategy for removing human pathogens and pollutants. Biological treatment consists of steps designed to enhance the "natural" degradation processes of pollutants and to selectively remove pathogenic bacteria and viruses by continuous aeration and mixing of the wastewaters by an activated-sludge process. These optimized conditions favor microbial growth and degradation of the various contaminants. In the case of activated sludge, the primary effluent is bubbled/mixed with air for a given period and the suspended material/flocs are allowed to settle (decantation), after which the supernatant (effluent) is removed and released to the receiving water and the settled sludge is re-injected with the incoming primary-treated effluent during the air bubbling/mixing process. Although enhanced microbiological degradation of municipal wastewaters represents a more natural means of removing toxic chemicals and human pathogens, the "local" bacterial community that is produced might have some deleterious effects on aquatic biota, in addition to the effects of the remaining, more persistent chemicals. The discharge of these effluents from municipal wastewater treatment plants raises concerns about the possible impact on fish health. It was estimated that 50% of fish in Swiss lakes and rivers were affected by municipal effluent discharges, with higher rates of parasitic and bacterial infections being found in fish from the Alte Aare River (Escher et al., 1999). Fish mortality, reduced growth and infectious diseases were observed in fish collected adjacent to a municipal effluent dispersion plume (Grizzle et al., 1988). Signs of disease were apparent in the incidence of skin and fin erosion, hyperplasia, hypertrophy, necrosis, inflammation and leukocyte infiltration in skin, fins, jaws, gills, livers and kidneys (Burkhard-Holm et al., 1997; Bucher and Hofer, 1993). Hepatic cytochrome *P4501A* activity and the induction of vitellogenin in fish collected near an urban effluent outfall suggested the presence of

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polycyclic aromatic hydrocarbons and (xeno)estrogenic compounds (Gagné and Blaise, 1999; Björkblom et al., 2009).

The immune systems of fish exposed to sewage effluents could also be compromised which increases the incidence of disease in wildlife (Vos et al., 2000; Fournier et al., 2000). To the best of our knowledge, studies on the impacts of biologically-treated municipal effluents (which carry a high load of endogenous microorganisms) on fish immune systems are lacking at present. Macrophages are responsible for phagocytosis, a key process of the innate and non-specific aspects of fish immunity. Phagocytosis is responsible for the removal and destruction of foreign bacteria and particles (Anderson and Zeeman, 1995). Another function of innate immunity consists of natural cytotoxic cell (NCC) activity, which is involved in the destruction of tumors and virus-infected cells (Faisal et al., 1991; Nakanishi et al., 1999). In fish, cells possess NCC activity, which is demonstrated in spontaneous cytotoxicity against fish or mammalian cancerous cell lines, virus-infected cells and protozoan parasites (Evans and Jaso-Friedmann, 1994). Adapted and specific immune responses can be determined by the ability of B and T lymphocytes to proliferate, which confers protection against both biotic and abiotic agents. The production of cytokines or other humoral factors are generally coupled with the profileration of lymphocytes in vertebrates (Blohm et al., 2003; Yun et al., 2006). This represents a measure of adaptive immunity, which implicates antigen–antibody interactions and antibody production for lymphocyte B and cytokine production and destruction of foreign or transformed cells by the lymphocyte T family (Utke et al., 2008).

The purpose of this study was to examine the potential immunotoxicity of an activated-sludge-treated effluent of a fairly large Canadian city, on rainbow trout *Oncorhynchus mykiss*. The immune systems of specimens were evaluated by tracking changes in phagocytic activity, NCC activity and lymphocyte B and T proliferation.

2. Materials and methods

2.1. Exposure to the biologically treated effluent

Juvenile rainbow trout (*O. mykiss*) were collected from a local hatchery (Aquipro, Quebec, Canada) and kept in 300-L tanks at 15 °C under constant aeration one month prior to the initiation of the exposure experiments. Fish were placed in 90-L glass aquaria (three per treatment concentration) and acclimated for five days at 14 °C, with a photoperiod of 12 h light/12 h dark, in dechlorinated city water. They were fed daily at a rate of 1% body weight with commercial G1 food (Aquipro, Quebec, Canada). Half of the water was renewed every two days to remove food and waste products from the aquaria.

A total volume of 180 L of the biologically treated effluent was collected daily (60 L) for a three-day period during the morning hours (10–11 am) in June. The effluent was from a town of approximately 200,000 inhabitants. The effluents were mixed together and kept at a temperature of 4 °C in the dark in containers lined with polyethylene bags. The exposure experiments were initiated immediately after the last day of effluent collection (T0 corresponds to the last 60 L input). Four groups of 15 juvenile rainbow trout were exposed to 0.1%, 1% and 10% dilutions of the municipal effluent for 30 days at 15 °C. The control group was exposed to dechlorinated and UV-treated city drinking water. Half of the water was renewed every two days (semi-static conditions) and the fish were fed at the same frequency. During the experiment, parameters such as water temperature, dissolved oxygen content, pH and conductivity were routinely measured with a Multi 340i set (Hoskin Scientific, Montreal, Quebec). Data on nitrite, nitrate and ammonia concentrations were analyzed using commercial kits as described above. After the exposure experiment, fish were anaesthetized with MS222 (0.1% v/v; Boreal Laboratories, Ontario, Canada) and the head-kidney was removed under sterile conditions and mashed with a 2-mL glass grinder (Wheaton Scientific, New Jersey, USA) containing 1 mL of sterile RPMI 1640 (Bio Media, Quebec, Canada) supplemented with heparin (10 U/mL) (Organon Teknika, Ontario, Canada), 10% (v/v) Fetal Bovine Serum (FBS) (Bio Media), penicillin (100 U/mL)/streptomycin (100 mg/mL) (Bio Media) and HEPES (10 mM) (Bio Media). The cell suspension of head-kidney cells was layered over 5 mL of Lympholyte Poly (Cedarlane Laboratories, Ontario, Canada) and centrifuged at 275×*g* for 30 min at 20 °C. After centrifugation, leukocytes were partitioned at the culture media/Lympholyte Poly and collected with a sterile Pasteur pipette, washed twice in RPMIc (by centrifugation–resuspension steps), counted and adjusted to 10^6 cells/mL in RPMIc. Cell viability was determined by the Trypan Blue exclusion test at 0.004% concentration (Sigma-Aldrich Chemical Co, MO, USA). The proportions of viable and dead (blue) cells were determined microscopically with a hemacytometer (Brightline, PA, USA).

2.2. Phagocytosis assay

Phagocytic activity was determined using a flow cytometer-based methodology (Brousseau et al., 1998). Briefly, a volume of 500 µL of each cell leukocyte suspension (1×10^6 cells/mL) was incubated for 18 h at 20 °C with fluorescein-labelled latex beads ($d=1.86$ µm; Polysciences, PA, USA) at a 100:1 bead-to-cell ratio. After the incubation period, the leukocyte suspension was centrifuged (150×*g* at 4 °C for 8 min) over a layer of 3% of bovine serum albumin (in RPMI media) to remove the latex spheres adsorbed at the surface of cell membranes. The cell pellet was resuspended and fixed in 0.5% formaldehyde (Sigma) and diluted in phosphate-buffered saline (Hematall, Becton Dickinson, CA, USA). Cells were analyzed by flow cytometry using a FACScan (Becton Dickinson). For each sample, 5000 individual cells were recorded. Events showing a fluorescence intensity corresponding to the cumulative fluorescence of one bead or more (basal activity or M1) and three beads or more (active phagocytosis or M2) were considered for phagocytic activity. These endpoints were expressed as the percentage of macrophages having engulfed beads over total number of macrophages.

2.3. Mitogenic assay

A sample volume (1×10^5 cells) was incubated with 20 µg/mL of phytohemagglutinin (PHA; Sigma) for lymphocyte T proliferation or with 200 µg/mL of lipopolysaccharide (LPS; Sigma.) for lymphocyte B proliferation. The cells (unstimulated, PHA- and LPS-treated cells) were incubated for 72 h at 20 °C in sterile L-15 media supplemented with 10% (v/v) fetal bovine serum, HEPES (10 mM; pH 7.4), penicillin (100 U/mL) and streptomycin (100 mg/mL). Unstimulated cells were incubated with supplemented L-15 medium only and all samples were tested in triplicate. After the incubation period, a volume of 0.5 µCi of (^{3}H)-methylthymidine (ICN Biomedicals, CA, USA) was added to each well and incubated for another 18 h. The cells were collected on a filter using a cell harvester (Skatron Instruments As, Lier, Norway). Radioactivity was determined with a ß-scintillation counter (LKB Wallac 1217 Rackbeta) (ChemGen Corp., MD, USA). The results were expressed as disintegration per min (DPM) and a stimulation index (SI) was calculated as follows: [LPS or PHA treatment group/unstimulated cells]×100.

2.4. Natural cytotoxic cell activity

First, target YAC-1 tumor cells were pre-stained with a non-toxic dye. An aliquot of 1 mL containing 1×10^7 YAC-1 tumor cells was mixed with 10 µL of 3 mM perchlorate 3,3-dioctadecyloxacarbocyanine dye (DIO) (Sigma) and incubated at 37 °C with 5% of CO_2 for 20 min. After incubation, the cell suspension was washed twice in RPMIc media and adjusted to a cell concentration of 1×10^6 cells/mL. Effector (fish leukocytes) and target (YAC-1 tumor cell line) cells were mixed at