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In vitro immunotoxicity of environmentally representative antibiotics to the freshwater mussel *Elliptio complanata*

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ABSTRACT

The separate and combined *in vitro* toxic effects of antibiotics (ciprofloxacin, erythromycin, novobiocin, oxytetracycline, sulfamethazole and trimethoprim) commonly found in urban wastewater effluents were assessed on the immune parameters of *Elliptio complanata* at environmentally relevant concentrations. The observed responses were then compared to those produced by the physicochemical-treated wastewater effluent of a major city before and after the removal of microorganisms. Most of the selected antibiotics, separately and as mixture, induced changes in immune responses. The removal of microorganisms and fine particles from the effluent increased or decreased the resulting immunotoxic effects, depending of the observed parameter. The immunotoxic effects of erythromycin, sulfamethoxazole and trimethoprim were closely associated to the antibiotic mixture and the filtered effluent. In conclusion, the data revealed that the removal of fine particles and microorganisms from municipal effluents can alter the toxic nature of the effluent that is closely associated with the cumulative effects of antibiotics.

1. Introduction

Current risk-assessment methods focus on the intrinsic toxic properties of single chemicals. This is not especially environmentally realistic because organisms are often exposed to a variety of compounds at low concentrations in the environment (Fent et al., 2006). In this respect, municipal wastewater effluents represent the largest source of pharmaceutical products in the environment (Kolpin et al., 2002). Antibiotics are among the most frequently detected group of potentially toxic pharmaceuticals; this underscores the following ecotoxicological concerns: 1) the cumulative toxic effects of antibiotics on aquatic animals are not well understood, 2) their continuous presence leads to the development of antibiotic-resistant bacteria, and 3) antibiotics can act, at very low concentrations, as signaling agents and change the natural microbial diversity in aquatic ecosystems (Fatta-Kassinos et al., 2011).

The interaction between antibiotics and the immune parameters is better understood in mammals than in aquatic invertebrates, having been extensively reviewed (Labro, 2000), particularly for macrolide (Kanoh and Rubin, 2010) and quinolone (Dalhoff, 2005) antibiotics. Macrolide antibiotics have heterogeneous effects, but appear to reduce the production of proinflammatory cytokines and impair cell signaling pathways (Kanoh and Rubin, 2010). Quinolone antibiotics attenuate cytokine responses (Interleukin-1, Il-1 and Tumor Necrosis Factor, TNF). Sulfamethoxazole exerts an inhibitory effect on phagocyte function. Most reports suggest an inhibitory action of tetracyclines on various phagocyte functions, but they paradoxically increase Il-1b secretion by liposaccharide (LPS)-stimulated human monocytes (Labro, 2000). Novobicin is reported to inhibit cytokine signaling by preventing LPS-induced TNF-α and Il-1, 6 and 10 secretion (Lhurmann et al., 1998). However, little information exists about the individual or combined effects of antibiotics on the immune parameters of bivalves and other invertebrates.

In bivalve molluscs, protection of the organism against pathogens is solely dependent on innate immunity (Mydlarz et al., 2006). Innate immunity is composed of both cellular and humoral components consisting of cells and molecules circulating in the hemolymph. The involvement of circulating hemocytes as the first line of defence against invading microorganisms is well established (Farcy et al., 2011). Hemocytes can ingest foreign particles by phagocytosis (Canesi et al., 2002a). This is followed by the production of reactive oxygen species or nitrogen-reactive substances for

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the destruction of ingested microorganisms (Pipe, 1992). Lysozymes are another non-specific immune response against invading bacteria in bivalves (Mydlarz et al., 2006). They are produced by the hemocytes and secreted in the hemolymph after pathogen recognition by the host or during physiological stress (Carballal et al., 1997; Pipe, 1990). Nitric oxide (NO) plays an important role in destroying bacteria and microparasites (Tafalla et al., 2003; Villamil et al., 2007). NO is produced by NO-synthase in hemocytes during phagocytosis and reacts with hydrogen peroxide to form peroxynitrite, a highly potent bactericide (Gourdon et al., 2001). Moreover, as it does in vertebrates, NO may act as an immunomodulator and mediate the effects of estrogens and opioids on immunity and inflammation (Galloway and Depledge, 2001; Stefano et al., 2003). Cyclooxygenase (COX) is involved in the first step of arachidonic acid oxidation leading to the production of prostaglandins, which are readily induced during inflammatory reactions in many tissues of the mussel. COX is also involved in the signaling pathways leading to hemocyte bactericidal activity (Canesi et al., 2002b). Gluthatione (GSH) is an important scavenger of free radicals and is involved in the maintenance of the redox status in cells, especially after oxidative bursts. In vertebrates, GSH controls the ability of lymphocytes to respond to proliferation cues in their environment and its depletion may repress immunity (Brousseau et al., 1998). Thus GSH status may be important in the function of bivalve hemocyte.

This study sought to examine the immunotoxicity of different antibiotics alone with different modes of action found in municipal wastewater effluents. These effects were then compared to those of their mixture based on the reported concentrations and relative proportions found in the municipal effluent of a large city. The mixture was composed of ciprofloxacin (CIP), erythromycin (ERY), novobiocin (NOV), oxytetracycline (OTC), sulfamethazole (SMZ), and trimethoprim (TMT). These antibiotics, with the exception of NOV, appear on the priority list of Besse et al. (2008). The effects of the antibiotics alone and as a mixture were then compared to the overall effects of filtered (bacteria-free) and unfiltered municipal effluents. The purpose was to determine if the responses patterns of the effluent could be explained by its antibiotic load, and if so, which the antibiotics with the highest environmental relevance were.

2. Materials and methods

2.1. Mussel maintenance and handling

Wild freshwater *Elliptio complanata* mussels were collected in June 2011 in the Achigan Lake (Quebec, Canada), which is not subjected to any direct sources of pollution. The animals were maintained for three months in aquariums at 15 °C, with a 16-h-light/8-h dark cycle. *At this time, mussels were at the post-spawning-resting phase.* They were fed daily with both concentrates of phytoplankton (Phytoplex®) and laboratory-cultured *Pseudokirchneriella subcapitata* algae.

2.2. Hemolymph collection and exposure scenario

Hemolymph was collected individually from the posterior adductor muscle of twelve mussels per treatment using a syringe with a 23G-gauge needle. The twelve repeated four times (12 mussels per antibiotic separated and combined, filtered and unfiltered effluent). All experiments were performed individually, the hemolymph of each mussel being its own control. The hemocyte concentration in hemolymph was counted using flow cytometry (see corresponding paragraph). A nominal quantity of 200 000 hemocytes was dispensed into individual wells of 300 µL polystyrene microplates, and the volume was completed to 200 µL with PBS (140 mM NaCl, 5 mM KH_2PO_4 and 5 mM $NaHCO_3$, pH 7.4) diluted one-quarter with distilled water. Thus all wells contained the same volume (200 µL), and the same amount of cells (200 000).

The selected antibiotics (CIP, ERY, NOV, OTC, SMZ, TMT; Sigma–Aldrich Canada Ltd., Oakville, ON, Canada) were tested, both individually and as a mixture, at environmentally relevant concentrations and proportions. The concentrations corresponding to 1× were in accordance with the reported concentrations in the effluents (Tables 1 and 3): 50 ng/L for ERY (CAS 114-07-8), SMZ (CAS 723-46-6) and TMT (CAS 738-68-5), 100 ng/L for CIP (CAS 85721-33-1) and NOV (CAS 1476-53-5) and 200 ng/L for OTC (CAS 6153-64-4). Antibiotics were added separately (50 µL in deionized water or DMSO) to obtain a final concentration of 0, 0.2, 1, 5 and 25×. These concentration corresponded to 40, 200, 1000 and 5000 ng/L for OTC; 20, 100, 500 and 2500 ng/L for CIP and NOV; and 10, 50, 250 and 1250 ng/L for ERY, SMZ and TMT. An exposure to the mixture of these antibiotics was also performed. The composition of the mixture consisted in all the antibiotics (CIP, ERY, NOV, OTC, SMZ, TMT) at the same concentrations (0.2, 1, 5 and 25×) that tested individually, and according to the literature (Table 1). The exposures lasted 24 h at 17 °C in saturated humidity in the dark. A solvent control (DMSO 0.01%) was added for ERY, SMZ, and TMT. The hemocytes were also exposed to a 24-h composite effluent from the wastewater treatment plant of the City of Montreal (Quebec, Canada). The primary treated effluents were collected as a 24 h composited samples (at 1 L per hour rate during three days). A subsample was filtered with a Millipore 0.22-µm Durapore filter. The exposure concentrations were 1, 10, 25 and 50%, plus control, for 24 h at 17 °C with saturated humidity in the dark.

For phagocytosis, hemolymph was mixed with green-yellow latex FluoSpheres, mean diameter of 1.6 µm (Molecular Probes Inc., Eugene, OR, USA), at a hemocyte-

Table 1
Characteristics of the antibiotics and exposure concentrations.

	Family	Mode of action	Therapeutic dose (mg/L/d)	Effluent concentration	1× exposure concentration
Ciprofloxacin	Quinolone	Gyrase B inhibitor	80	10–110 ng/L (Spongberg and Witter, 2008) 5–7000 ng/L (Verlicchi et al., 2010 and cited references) ND-2610 ng/L med 410 (Terzic et al., 2008) 250 ng/L (Zuccato et al., 2005)	100 ng/L
Erythromycin	Macrolide	50S ribosomal subunit synthesis inhibition	200	30–2000 ng/L (Verlicchi et al., 2010 and cited references) 20–420 ng/L med 130 (Terzic et al., 2008) 50 ng/L (Zuccato et al., 2005)	50 ng/L
Novobiocin	Coumeromycin	Gyrase B inhibitor	50	170 ng/L[a] (Gagne et al., 2006a)	100 ng/L
Oxytetracycline	Tetracycline	30S ribosomal subunit synthesis inhibition	100	220 ng/L[a] (Gagne et al., 2006a)	200 ng/L
Sulfamethoxazole	Sulfamide	Tetrahyfolate synthesis inhibitor	30	50 ng/L[a] (Gagne et al., 2006a) 80–470 ng/L (Spongberg and Witter, 2008) 2–5000 ng/L (Verlicchi et al., 2010 and cited references) 20–11 600 ng/L med 1180 (Terzic et al., 2008) 130 ng/L (Zuccato et al., 2005)	50 ng/L
Trimethoprim	Benzylpyrimidines	Dihydrofolate reductase inhibitor	80	70 ng/L[a] (Gagne et al., 2006a) 20–8000 ng/L (Verlicchi et al., 2010 and cited references) 40–2550 ng/L med 780 (Terzic et al., 2008) 10–190 ng/L (Metcalfe et al., 2003)	50 ng/L

[a] In the effluent of the Montreal wastewater treatment plant.