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Contribution of pharmaceuticals, fecal bacteria and endotoxin to the inflammatory responses to inland waters

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HIGHLIGHTS

- The present study was performed to determine the distribution of pro-inflammatory effects to inland waters.
- Three different wastewater treatment plants and their recipient lakes were studied.
- The levels of pharmaceuticals in the WWTPs and lakes were determined and compared to the immune response elicited by urinary bladder and immune cells.
- The results of the present study indicate that the cytokine profiles of exposed cells correlate to the endotoxin load of the waters rather than to the levels of pharmaceuticals or live bacteria load.

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ABSTRACT

The increasing contamination of freshwater with pharmaceuticals, surfactants, pesticides and other organic compounds are of major concern. As these contaminants are detected at trace levels in the environment it is important to determine if they elicit biological responses at the observed levels. In addition to chemical pollutants, there is also a concern for increasing levels of bacteria and other microorganisms in freshwater systems. In an earlier study, we observed the activation of inflammatory systems downstream of a wastewater treatment plant (WWTP) in southern Sweden. We also observed that the water contained unidentified components that were pro-inflammatory and potentiated the immune response in human urinary bladder epithelial cells. In order to determine if these effects were unique for the studied site or represent a common response in Swedish water, we have now performed a study on three WWTPs and their recipient waters in central Sweden. Analysis of immune responses in urinary bladder epithelial cells, monocyte-like cells and blood mononuclear cells confirm that these waters activate the immune system as well as induce pro-inflammatory responses. The results indicate that the cytokine profiles correlate to the endotoxin load of the waters rather than to the levels of pharmaceuticals or culturable bacteria load, suggesting that measurements of endotoxin levels and immune responses would be a valuable addition to the analysis of inland waters.

1. Introduction

Industrialization and population growth has led to increased levels of pollution in the environment, resulting in an elevated risk to human health. The quality of wastewater is of major concern. Chemical and pharmaceutical contaminants have been detected in wastewater treatment plant (WWTP) effluents and inland waters over the years, indicating that the sewage treatment process does not eliminate all substances

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(Palmer et al., 2008; Pal et al., 2010). Chemicals that pass through the WWTPs end up in recipient rivers and water systems. It has been shown that the compounds that are released in WWTP effluents have the potential to create health problems even at trace quantities (Ferrari et al., 2004). While WWTPs are efficient in reducing the release of certain contaminants to the environment, there remains room for improvement of the processes. Commonly WWTPs use a three-step process to treat wastewater. Generally, the first step comprises mechanical separation, followed by chemical treatment and then biological treatment. However, while the WWTP effluents retain many compounds (Marklund et al., 2005), it is likely that new and emerging chemicals will pass through the system.

The release of organic contaminants in WWTP effluents, especially pharmaceuticals and endocrine disrupting compounds, into inland waters and ground water is of major concern (Fent et al., 2006; Pal et al., 2010). Among pharmaceuticals, the prevalence of non-steroidal anti-inflammatory drugs (NSAIDs) (Antonić and Heath, 2007) and antibiotics (Kümmerer, 2001; Bendz et al., 2005; Palmer et al., 2008; Zhou et al., 2009) has increased over the years. Since these pharmaceuticals often occur at trace levels, the health risk for organisms is not always considered a direct threat (Jones et al., 2001). However, our recent findings show that even at low environmental levels these compounds can trigger inflammatory responses in human *in vitro* systems (Khalaf et al., 2009). Studies using rainbow trout have also shown that chronic exposure to WWTP effluents alters the immune system functions in aquatic animals (Hoeger et al., 2004; Müller et al., 2009).

Different approaches have been used to assess effects of unknown chemicals on biological systems. In this study, we analyzed WWTPs and their recipient lake waters using *in vitro* methods to determine if the waters induce inflammatory responses in human cell lines and primary cultures. Inflammation is a biological response to pathogens or harmful stimuli that elicit epithelial cell signaling to the immune system through the production of pro-inflammatory cytokines, immuno-regulatory growth factors and chemokines initiating an immune response (Svanborg et al., 1999). The release of chemokines, such as interleukin-8 (CXCL8), facilitates the migration of immune cells including monocytes and macrophages to the site of infection (Svanborg et al., 1999). Macrophages play a central role in inflammation due to their ability to regulate and activate other immune cells such as T-cells (Bowdish et al., 2007). Nuclear factor-κB (NF-κB) is an important regulator of the initial events of the inflammatory response and can be activated by many factors including lipopolysaccharide (LPS) from Gram-negative bacteria, tumor necrosis factor (TNF) and interleukin-1β (IL-1β) (Ghosh and Hayden, 2008). The interaction of LPS with Toll-like receptor-4 (TLR4) on macrophages and epithelial cells results in the activation of NF-κB complex (Classen et al., 2009) that subsequently regulates the induction of inflammatory cytokines including the TNF, IL-1β, CXCL8 and interleukin-6 (IL-6) (Ghosh and Hayden, 2008; Classen et al., 2009).

In the present study, we analyzed WWTP waters to determine the contribution of released pharmaceuticals, bacteria and endotoxin on immune responses observed in the recipient waters. We focused on the inflammatory responses following the exposure of human carcinoma bladder cell line (5637), human acute myeloid monocyte leukemic cell line (THP-1), macrophage-differentiated THP-1 cells and human primary macrophage cells to waters collected from lakes in central Sweden. Samples analyzed included those from passive samplers placed in the influent and effluent WWTPs, and water grab samples collected from the lakes into which the effluent was released. The study areas were selected based on the presence of WWTPs and recipient lakes with unique characteristics.

2. Materials and methods

2.1. Water sampling

The samples from the 3 WWTPs were collected using Polar Organic Chemical Integrative Samplers (POCIS, ALS Scandinavia, Sweden). The passive samplers were kept for 3 weeks in influent and effluent water during May 2009. Water samples were collected from five Swedish lakes during May 2009 and stored in aliquots at −20 °C. Lake water was sampled at 0.5 m depth using autoclaved 1 L glass flasks ($n = 4$). The lakes investigated were Ratusenjärvi (R; Lat 67°740′, Long 22°.163′) as a pristine control site; Öresjö in Borås (B; Lat 57°764′, Long 12°943′); Möckeln in Karlskoga (K; Lat 59°295′, Long 14°517′); Hjälmaren in Örebro (Ö; Lat 59°278′, Long13°286′); and Mälaren in Västerås (V; Lat 59°569′, Long 16°600′). The control lake, Lake Ratusenjärvi, is located in a pristine area in the Northern part of Sweden and is unaffected by human activities. Lake Hjälmaren is a large lake, covering an area of 480 km^2. It is divided into three section with a shallow western section (average depth 1.5 m) and middle section with a depth of about 10 m and a large eastern section with a maximum depth of 22 m. The average depth of the lake is 6.1 m, and the estimated volume is 3 km^3. Svartån is the main contributor of water entering the western shallow section of the lake. Örebro WWTP releases its effluents into river Svartån about 1 km upstream of lake Hjälmaren. Örebro WWTP receives water from more than 100000 people, and the industrial contribution is calculated to be comparable to 33000 people. River Svartån runs through an agricultural landscape. Lake Möckeln is a smaller lake covering an area of 18 km^2, with an average depth of 10 m and a volume of 0.214 km^3. The WWTP in Karlskoga releases its effluent water into Lake Möckeln. The WWTP receives water from a population of 30000 people of which industry contributes the equivalent of about 6000 people. The area around and upstream of Lake Möckeln is mostly forest with only limited agricultural activity. Lake Mälaren is the third largest lake in Sweden. It has an average depth of 10 m, an estimated volume of 14.2 km^3 and covers an area of 1140 km^2. The lake is downstream of Lake Hjälmaren and Lake Mälaren is the main water source for 1.3 million people in Sweden. The WWTP in Västerås receives water from about 125000 people, including the contribution from local industry. It delivers its effluent into a bay of Lake Mälaren and was chosen as the fresh water intake for the city of Västerås is located only 8 km away from where the WWTP effluent enters the bay. Lake Öresjö in the municipality of Borås was included in the study as we have earlier detected pro-inflammaroty effects in this lake (Khalaf et al., 2009). Lake Öresjö has an average depth of 14.2 m, covers an area of 6 km^2 and has an estimated volume of 0.091 km^3. It receives water from river Viskan and is surrounded by agricultural land.

2.2. Sample preparation

The substances collected using the POCIS passive samplers were extracted with 50 mL methanol and concentrated in DMSO (Applichem, Germany). Methanol was evaporated at room temperature using nitrogen gas. All the cell types were cultured and maintained in RPMI 1640 growth medium (Hyclone, UK) containing 10% FBS (Hyclone, UK) at 37 °C and 5% CO_2. Dry media (RPMI 1540; Invitrogen, UK) were added to the lake waters and supplemented with 10% FBS and 2 mM L-glutamine (Hyclone, USA) followed by filter-sterilizing through 0.2 μm filters prior to bioassay.

2.3. Chemical analysis

The POCIS samples from the WWTP, and the lake waters were sent to ALS Scandinavian AB Sweden for chemical analysis of selected pharmaceuticals. The substances collected using the POCIS passive samplers were extracted with 50 mL methanol. The methanol extracts were thereafter concentrated by SPE (Oasis HLB) and analyzed by LC-MS/MS against calibration standards. The addition of ^{13}C-labeled internal standards to the extracts was used to compensate for matrix effects. Extraction recovery of the different pharmaceuticals was determined, and the presented concentrations were compensated for extraction recoveries. The concentrations obtained by POCIS samplers have