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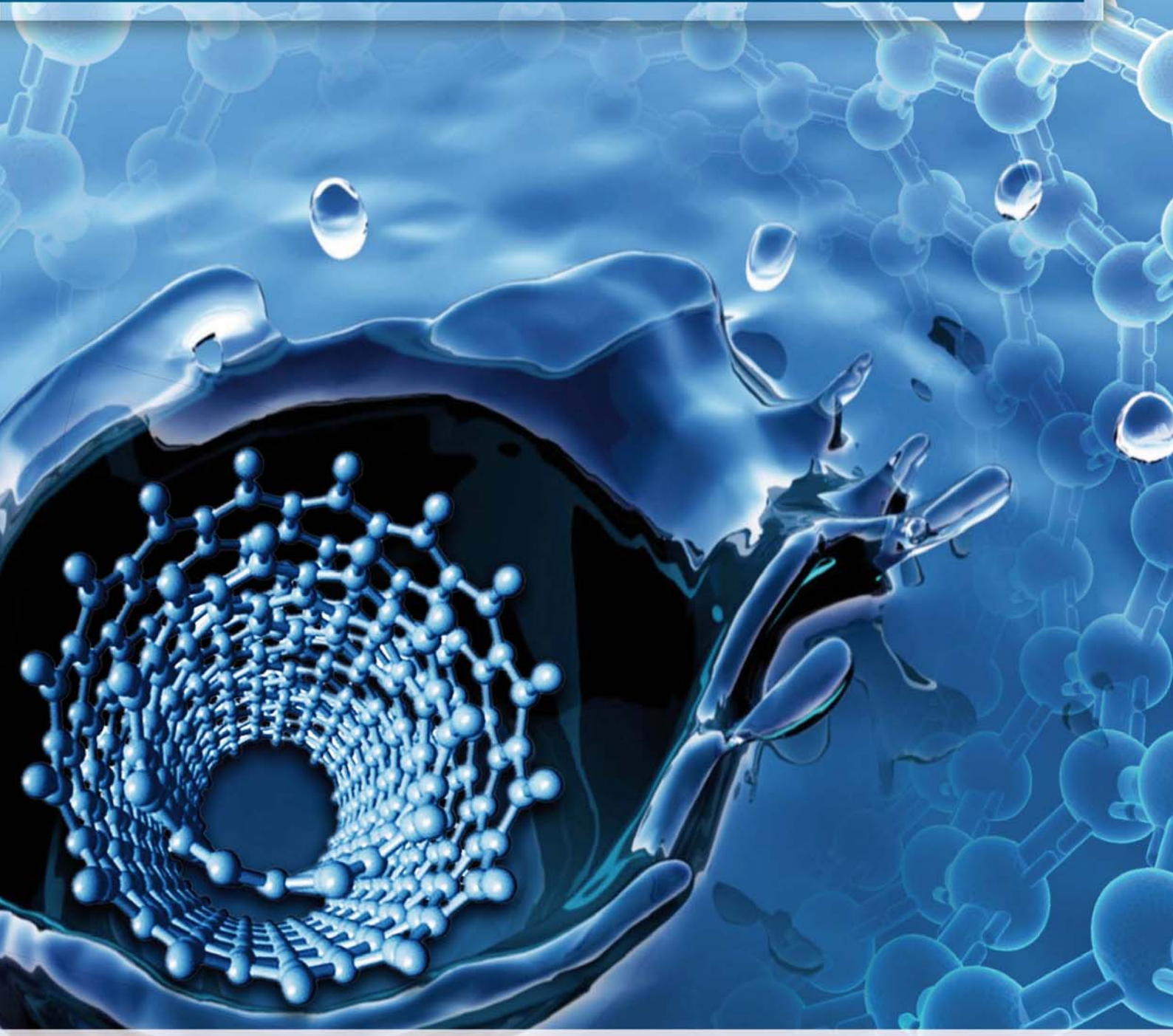

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In vitro immunotoxicity of untreated and treated urban wastewaters using various treatment processes to rainbow trout leucocytes

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Abstract

Municipal effluents are known to impede the immune system of aquatic organisms. The purpose of this study was to examine the immunotoxicity of urban wastewaters before and after 6 treatment processes from 12 cities toward trout leucocytes. Freshly prepared trout leucocytes were exposed to increasing concentrations of solid phase (C18) extracts of wastewaters for 24 hr at 15°C. Immunocompetence was determined by following changes in leucocyte viability and the proportion of cells able to ingest at least one (immunoactivity) and at least three (immunoefficiency) fluorescent beads. The influents were treated by six different treatment strategies consisting of facultative aerated lagoons, activated sludge, biological aerated filter, biological nutrient removal, chemically-assisted physical treatment and trickling filter/solid contact. Water quality parameters of the wastewaters revealed that the plants effectively removed total suspended solids and reduced the chemical oxygen demand. The results revealed that the effluents' immunotoxic properties were generally more influenced by the properties of the untreated wastewaters than by the treatment processes. About half of the incoming influents decreased leucocyte viability while 4 treatment plants were able to reduce toxicity. The influents readily increased phagocytosis activity for 8/12 influents while it was decreased in 4/12 influents. This increase was abolished for 4/12 of the effluents using treatments involving biological and oxidative processes. In conclusion, municipal effluents have the potential to alter the immune system in fish and more research will be needed to improve the treatments of wastewaters to better protect the quality of the aquatic environment.

Key words: municipal effluents; fish leucocytes; viability; phagocytosis
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Introduction

Municipal wastewaters represent a major source of pollution and threaten the integrity of aquatic ecosystems (Weirich et al., 2011; Holeton et al., 2011). Wastewaters are generally treated at wastewater treatment plants (WWTPs) where different technologies are applied to clean up the wastewaters. In most cases, WWTPs operating at optimal flow rates (i.e., during normal conditions) and capacity with respect to the population size and water quantity are well suited in reducing total suspended solids (TSS), ammonia, fecal coliforms and chemical/biochemical oxygen demand. Besides these characteristics, treated municipal effluents still contain a complex array of pollutants such as metals, polycyclic aromatic hydrocarbons, and pharmaceutical and personal care products etc. (Dickenson et al., 2011). They are also recognized to contain compounds capable of disrupting the endocrine system in aquatic organisms, such as estrogenic and serotonergic compounds (Voutsa et al., 2006; Klečka et al., 2009; Gagné et al., 2004). The occurrence of these compounds in effluents will depend on the treatment processes being applied and the nature of the incoming untreated wastewaters (Gagné et al., 2012b). For example, acidic drugs such as naproxen, ibuprofen and salicylates were more effectively removed by activated sludge than with chemically assisted physical treatment in WWTPs (Gagné et al., 2012a). The presence of these compounds in addition to the presence of microorganisms could pose a threat to organisms living near effluent dispersion plumes (Holeton et al., 2011). Although WWTP were not initially designed to remove pollutants, some of the treatment

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processes may be able to remove these pollutants, which is perceived as an additional value for the environment. Indeed, the Neptune European program promotes research studies on areas involved in the removal of micropollutants and ecotoxicity reduction of wastewaters, sludge reuse and nutrient elimination.

Despite the application of various treatment processes, municipal effluents are still considered toxic to aquatic organisms (Holeton et al., 2011). For example, mussel mortality (10% after 14 days) was observed when mussels were placed in the final aeration pond from a moderate size township treating domestic and hospital wastewaters and in the surviving mussels, the immune system was compromised with signs of inflammation (Gagné et al., 2012a). We also considered six commonly used treatment processes from 12 cities across Canada: facultative aerated lagoons (FAL), activated sludge (AS), biological aerated filter (BAF), biological nutrient removal (BNR), chemically-assisted primary treated effluent (CPT) and trickling filter/solids contact (TFSC). It is thought that WWTPs involving biologically-assisted oxidation of the wastewaters are generally more effective in reducing the concentrations of various micro-pollutants in wastewaters. Indeed, using a suite of in vitro bioassays, WWTPs using strong oxidation steps or biological treatments were found to yield less toxic effluents (Kienle et al., 2011) albeit the immune component was not evaluated.

In the context of municipal effluents, the immune system represents a critical physiological system which is involved in the deactivation and removal of foreign bodies. Fish possess both innate and specific immunocompetence where the innate immunity involves phagocytosis by macrophages/granulocytes and the production of natural cytotoxic cells (Secombes, 1996; Watts et al., 2001). Phagocytosis consists of the internalization and destruction of foreign microorganisms or particles by the production of strong oxidizing agents, such as peroxynitrite formation in the phagosomes (Hoeger et al., 2004). The effects of municipal effluents on fish leucocytes are not well understood at the present time because of the complexity of these mixtures. For example, the presence of bacteria in water could increase phagocytosis, while the presence of contaminants could either exacerbate or suppress phagocytosis in wild fish populations over a large urban area (Ménard et al., 2010).

The purpose of this study was therefore to examine the chemically-induced immunotoxicity of 12 WWTP from various city sizes and treatment processes identified above in rainbow trout leucocytes. Because municipal effluents contain a complex array of microorganisms and chemicals capable of affecting the immune response, we focused on the dissolved chemical component of the effluents by preparing solid phase C18 ethanol extracts of the filtered effluents to remove the suspended particles and microbiological components of the wastewaters. Immunocompetence was determined by following changes of cell viability and phagocytosis activities (ingestion of one bead or more and more than 3 beads) in freshly prepared trout leucocyte culture. The immunocompetence of untreated wastewaters or influents was also compared to evaluate the capacity of the various treatment processes to mitigate changes at the immunocompetence level.

1 Methods

1.1 Wastewaters sample preparation

A series of 12 influents and effluents were sampled from various WWTP that use 6 types of wastewater treatments (**Table 1**): FAL, AS, BAF, BNR, CPT and TFSC. The FAL treatment involves a series of inter-connected ponds (3 to 6) where suspended matter is allowed to settle and the organic matter degraded with the combination of aeration, sunlight and microbial activity. The AS treatment involves the addition of active microbial culture to force the biological degradation of the organic matter, and initiate, in some cases, nitrification. The BAF treatment consists of another means of biological treatment where the wastewater is mixed with an aerated mixture of nitrifying microorganisms. The BNR treatment is similar to BAF but includes additional denitrification and biological phosphorus removal in aerobic and anaerobic zones during the activated sludge process. The CPT essentially removes suspended solids using physical means (grids, settling steps) with the addition of flocculating agents ($FeCl_3$, Al and surfactants). The TFSC process involves trickling of the wastewaters through a bed of sand followed with an activated sludge process with short retention time.

For the influents and effluents, 24-hr composite samples (0.4 L at each 30 min interval) of wastewaters were prepared on three consecutive days at each WTP using an HACH Sigma 900 refrigerated apparatus (HACH Company, Loveland Co., USA). On reception of the composite

Table 1 General characteristics of wastewater treatment plants

Treatment type	WWTP code	Population served	Average flow (m^3/day)	Residential (%)
AS	5W	374000	226000	90
AS	7C	32000	20000	90
AS	9F	45000	19000	90
AS	12P	170000	409000	Not available
BAF	2L	105000	60000	70
BNS	6B	650000	400000	Not available
CPT	8H	65000	70000	Not available
CPT	11M	300000	289000	67
FAL	1R	190000	72000	90
FAL	4X	25000	11000	100
FAL	10K	Not available	500	100
TFSC	3A	900000	450000	60

AS: activated sludge; BNR: biological nutrient removal; BAF: biological aerated filter; CPT: chemically assisted primary treatment; FAL: facultative or aerated lagoon: TFSC: trickling filter/solids contact.

wastewaters (influents and effluents), the samples were filtered with a 0.4 μm-pore polycarbonate membrane to remove suspended solids and microorganisms. A volume of 200 mL was then fractionated on a reverse-phase C18 mini-column (360 mg) under vacuum, washed with 6 mL of bi-distilled water and air-dried. The material was eluted with analytical-grade ethanol (Sigma-Aldrich Chemical Co., Canada). The extracts were then stored at –20°C. The 3-day ethanol extracts were pooled for each set of influent and effluent samples. The ethanol extracts were also concentrated further under nitrogen stream to give a concentration factor of 1000× concentrate. The composite wastewaters were analyzed for pH, chemical oxygen demand, total suspended solids and ammonia following standard methods (APHA, 2005).

1.2 Leucocyte preparation and immunotoxicity assessments

The anterior kidney of rainbow trout was collected in anesthetized fish (0.1% MSS-222; Boreal Laboratories, Canada). The kidneys were crushed with a glass grinder (Wheaton Scientific, USA) containing 1 mL of RPMI 1640 cell culture media with 10 U/mL heparin, 10 mmol/L Hepes-NaOH, pH 7.4, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% fetal bovine serum (BioMedia, Canada). The cell suspension was adjusted to 5 mL with the RPMI media and the cell suspension was overlaid on 5 mL of lymphocyte poly gradient media (Cedarlane Laboratories, ON, Canada) and centrifuged at 275 ×*g* for 30 min. Leucocytes remained at the RPMI-gradient media interface and were collected by a Pasteur pipette, washed twice in RPMI media without heparin (centrifugation at 500 ×*g* for 10 min) and the final concentration adjusted to 1×10^6 cells/mL (hematocytometer counting under microscope at 200× enlargement (Bright-line, USA).

Cells were exposed to increasing concentrations of influent and effluent extracts (0.12%, 0.25%, 0.5% and 1%, *V*/*V*) at a cell density of 1×10^6 live cells/mL for 6 hr at 15°C in a saturated humidity incubator supplied with 5% CO_2 and 95% air atmosphere. At the end of the exposure period, the cells were centrifuged at 500 ×*g* for 10 min and resuspended in RPMI media at a cell density of 1×10^6 cells/mL.

Cell viability was determined using trypan blue stain (0.4%; Sigma-Aldrich Chemical Co., Canada). Viable and dead (stained) cells were then counted on a hematocytometer as described above. For phagocytosis, a duplicate sample of 500 μL of the cell suspension was mixed with fluorescent latex beads (diameter of circa 1.8 μm; Polysciences, USA) at 100:1 bead to cell number ratio. The suspension was allowed to incubate at room temperature for another 18 hr in the dark. After this second incubation period, the cell suspension was layered over 3 mL of RPMI containing 3% bovine serum albumin (Sigma Chemical Company, MO, USA) and centrifuged at 150 ×*g* at 4°C for 8 min. The cell pellet was resuspended in a fixative media composed of phosphate-buffered saline (Hematall, Becton Dickinson, USA) and 0.5% formaldehyde. The cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson, USA) and 5000 cells were measured. Analyses were performed using two endpoints corresponding to the percentage of leucocytes containing one bead or more (M1; immunoactivity) and the percentage of cells containing three beads or more (M2; immunoefficiency).

1.3 Data analysis

The cells were exposed in quadruplicate. The normal distribution of the data and homogeneity of variances were then checked using the Shapiro-Wilk and Barlett tests respectively. The data were then subjected to an analysis of variance with the exposure concentration as the main treatment group. Critical differences from the controls were appraised using the Dunnett *t* test. The threshold concentrations were calculated as the geometric mean of the lowest observable effect and the no observable effect concentrations (threshold = $(\text{LOEC} \times \text{NOEC})^{1/2}$. The LOEC was determined by ANOVA followed by comparison with controls using the Least Square Difference test. To test the efficacy of the various cities in removing the observed changes in the receiving effluent, analysis of covariance was performed with the exposure concentration as the main factor and the influent effects as the covariable. Multivariate analysis of covariance (MANCOVA) was performed using the exposure concentration (0, 0.12, 0.25, 0.5 and 1%, *V*/*V*) and treatment type (not treated or influent, BAF, FAL, AS, CPT, TFSC) as the main variables with the population size, WWTP flow rate, TSS, ammonia and DOC as covariables. Discriminant function analysis was performed to highlight the global changes in the capacity of the various treatment processes to change the toxic responses of the incoming influents and to compare the global responses of the 12 untreated wastewaters with each other. Significance was set at $p < 0.05$ and all statistical tests were performed using the Statistica software package (USA).

2 Results

The wastewaters were collected at the various WWTPs serving different population sizes and employing six different treatment processes (**Table 2**). The flow rates were significantly correlated with population size ($r = 0.83$; $p < 0.001$). No clear trend between the flow rates and the treatment processes was observed. The highest flow rates involved FAL and TFSC processes, however. The mean effluent temperature during the sampling events varied from 13.5°C to 21.2°C (data not shown). Based on influent and effluent comparisons, the WWTPs were able to reduce TSS and COD. TSS was reduced with an efficiency ranging from 66% to 98%, with the AS and BAF processes

Table 2 Efficacy of municipal wastewater treatment processes

Treatment type	WWTP code	Untreated wastewaters				Treated wastewaters (effluent)			
		pH	Total ammonia (mg/L N)	COD (mg/L)	TSS (mg/L)	pH	Total ammonia (mg/L N)	COD (mg/L)	TSS (mg/L)
AS	5W	7.6 ± 0.03	20 ± 5	351 ± 50	190 ± 30	7.4 ± 0.1	**4 ± 2**	**46 ± 4**	**10 ± 1**
AS	7C	7.6 ± 0.7	26 ± 5	347 ± 70	140 ± 65	7.4 ± 0.2	**0.7 ± 0.7**	**44 ± 8**	**< 3**
AS	9F	7.2 ± 0.2	21 ± 1	413 ± 40	203 ± 30	7.6 ± 0.1	21 ± 1	**60 ± 8**	**8 ± 2**
AS	12P	7 ± 0.03	19 ± 8	420 ± 200	130 ± 30	7 ± 0.1	11 ± 5	Na	**29 ± 40**
BAF	2L	7.5 ± 0.2	22 ± 5	278 ± 80	140 ± 10	7.70 ± 0.01	**0.4 ± 0.2**	**34 ± 2**	**8 ± 1**
BNR	6B	7.6 ± 0.1	34 ± 6	384 ± 80	190 ± 60	7.4 ± 0.1	**0.8 ± 0.1**	**30 ± 2**	**8 ± 2**
CPT	8H	7.1 ± 0.03	7 ± 0.9	165 ± 9	76 ± 7	7 ± 0.2	7 ± 1	**102 ± 4**	**29 ± 7**
CPT	11M	7.3 ± 0.2	10 ± 1	248 ± 80	160 ± 70	7.3 ± 0.3	12 ± 5	**75 ± 40**	**23 ± 1**
FAL	1R	7.3 ± 0.1	27 ± 0.8	349 ± 90	130 ± 70	7.4 ± 0.2	**0.2 ± 0.053**	**41 ± 4**	**6 ± 1**
FAL	10K	7.6 ± 0.02	27 ± 1	290 ± 30	130 ± 8	**8.0 ± 0.1**	**3 ± 0.5**	**100 ± 10**	**43 ± 3**
FAL	4X	7.4 ± 0.1	14 ± 1	237 ± 50	90 ± 20	**8 ± 0.04**	**0.7 ± 0.1**	**46 ± 2**	**26 ± 2**
TFSC	3A	7 ± 0.1	23 ± 2	489 ± 40	180 ± 9	7.50 ± 0.1	**30 ± 0.3**	**78 ± 3**	**7 ± 2**

COD: chemical oxygen demand; TSS: total suspended solids.
Significant differences between the untreated wastewaters and treated effluents are highlighted in boldface.

showing the best performance. The WWTPs were also effective in reducing the COD by a mean of 78%. COD was significantly correlated with TSS (r = 0.94), as expected, since treatment processes are designed to reduce these parameters. Plants 1R, 2L, 4X, 6B, 7C and 10K were able to reduce ammonia by at least 90%. Correlation analysis revealed that total ammonia was significantly correlated with TSS (r = 0.77), and COD (r = 0.80).

2.1 Immunotoxicity of the influents

The toxicity of the incoming untreated influents from the various WWTPs were examined in rainbow trout leucocytes (**Fig. 1**). For cell viability, the following influents were cytotoxic to the hemocytes: 1R (threshold

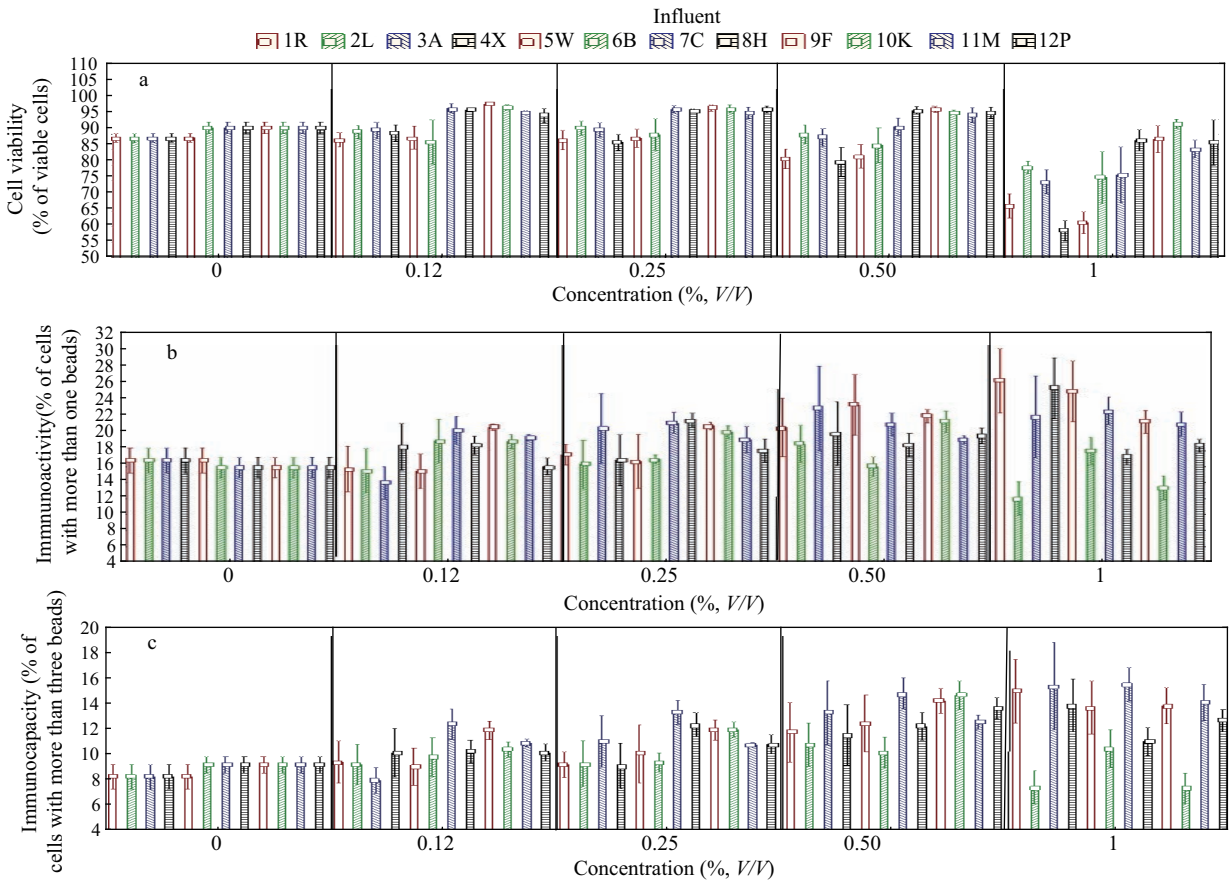

Fig. 1 Immunotoxicity of untreated municipal wastewaters from WWTPs (plants are named as 1R to 12P) to rainbow trout leucocytes. Trout leucocytes were exposed to increasing concentrations of the wastewater extracts before (influent) treatment and cell viability (a), immunoactivity (b) and immunoefficiency (c) were determined.

concentration = 0.71%), 2L (0.71%), 3A (0.71%), 4X (0.71%), 5W (0.71%), and 7C (0.71%) (**Fig. 1a**). The other influents did not significantly influence cell viability. Multivariate analysis of the influents revealed that cell viability was influenced by the following WWTP characteristics in decreasing order of intensity: TSS > flow rate > population > city > exposure concentration. Hemocyte viability was significantly correlated with population size (r = –0.21; p < 0.01), TSS (r = –0.53, p < 0.001), total ammonia (r = –0.50, p < 0.001), and COD (r = –0.45; p < 0.001). For immunoactivity (i.e., the proportion of cells that engulfed of at least one bead), the following influents led to increased phagocytosis activity: 1R (threshold concentration = 0.71%), 5W (0.35%), 7C (< 0.12%), 8H (0.17%), 9F (< 0.12%), 10K (0.17%), and 11M (< 0.12%) (**Fig. 1b**). Multivariate analysis of the influents revealed that the following WWTP properties significantly influenced phagocytosis activity in decreasing order of intensity: flow rate > city ≫ total ammonia > TSS. Immunoactivity was significantly correlated with cell viability (r = –0.59; p < 0.01) and TSS (r = 0.15; p < 0.05). For immunoefficiency, i.e. the proportion of hemocytes that engulfed at least three beads, the following WWTPs significantly increased phagocytosis: 1R (threshold concentration = 0.71%), 3A (0.71%), 7C (< 0.12%), 8H (0.17%), 9F (< 0.12%), 10K (0.17%), 11M (0.35%) and 12P (0.35%) (**Fig. 1c**). Multivariate analysis of covariance revealed that the following parameters significantly influenced immunoefficiency in decreasing order of intensity: TSS > ammonia ~ DOC > city flow rate > exposure concentration. Correlation analysis revealed that immunoefficiency was significantly related with TSS (r = 0.21; p < 0.01). DOC (r = 0.20; p < 0.01), hemocyte viability (r = –0.60; p < 0.001) and immunoactivity (r = 0.96; p < 0.001).

2.2 Immunotoxicity of the effluents

Cell viability was significantly decreased by the following WWTP: 1R (0.06%), 2L (< 0.002%), and 4X (0.22%) (**Fig. 2a**). Multivariate analysis of covariance revealed

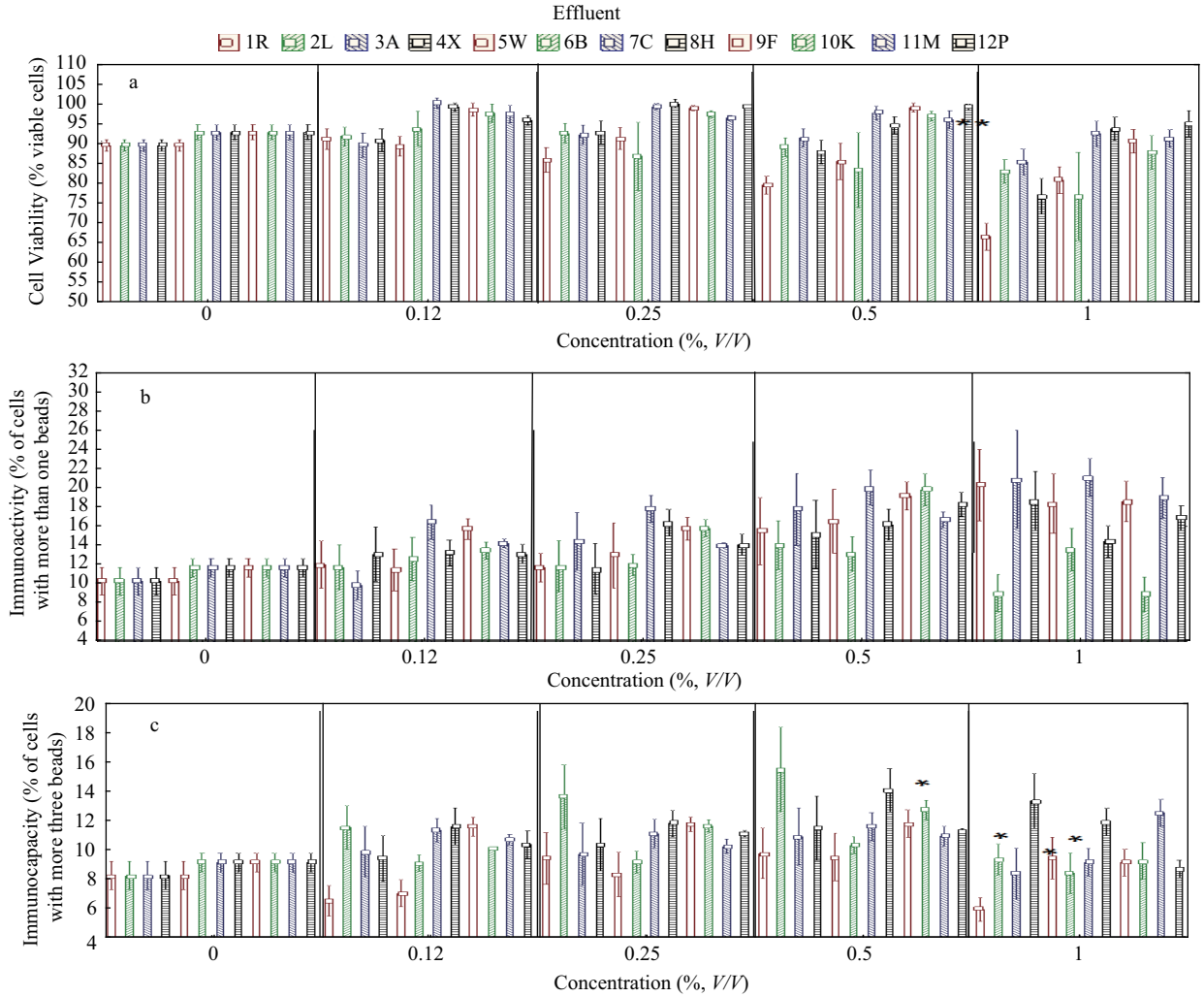

Fig. 2 Change in immunotoxicity of treated municipal effluents in trout leucocytes. Leucocytes were exposed to increasing concentrations of the effluent extracts and cell viability (a), immunoactivity (b) and immunoefficiency (c) were determined. The star symbol * indicates significant difference between the influent and the effluent at a given wastewater concentration.

that hemocyte viability was significantly influenced by these parameters in decreasing order: TSS > flow rate > population > exposure concentration > total ammonia > city. The toxic effects of the effluents were significantly influenced by the incoming influent for some WWTPs. The following WWTPs were affected as follows: 1R (influent only), 3A (influent > effluent), 4X (influent > effluent), 5W (influent > effluent), 6B (influent only), 7C (influent > effluent), 8H (influent only), 9F (influent only), 10K (influent only), 11M (influent only) and 12P (influent > effluent). Hence, WWTPs from 1R, 6B, 8H, 9F, 10K and 11M were principally affected by the incoming effluents, where the applied treatments had no influence on toxicity. The effects of the effluents on immunoactivity were examined (**Fig. 2b**). The following WWTP significantly affected this endpoint: 2L (< 0.12%), 3A (0.71%), 4X (0.71%), 8H (0.35%), 9F (< 0.12%), 10K (0.35%), 11M (0.71%) and 12P (0.35%). Multivariate analysis of covariance revealed that the following parameters influenced immunoactivity or the capacity of immunocytes to engulf at least one bead, in decreasing order of intensity: total ammonia, exposure concentration and flow rate. Correlation analysis revealed that immunoactivity was negatively correlated with cell viability (r = –0.51). The observed effects on immunoactivity in leucocytes exposed to the effluents were influenced by the influents most of the time, with the exception of effluent 12P. The effluents that were entirely explained by the influents (ANCOVA) were 2L, 6B, 9F, 10K, and 11M which involved the following processes: BAF, BNS, AS, FAL, and CPT.

The effects on immunoefficiency was also analysed to determine the impacts of municipal wastewaters on the capacity of immunocytes to ingest at least three fluorescent beads (**Fig. 2c**). The following WWTPs were able to increase the immunoefficiency potential in immunocytes: 2L (0.17%), 4X (0.71%), 8H (0.35%), 9F (< 0.12%), 10K (0.17%), 11M (0.71%) and 12P (0.17%). Multivariate analysis of variance revealed that the following characteristics significantly influenced the observed responses in decreasing order of intensity: total ammonia > TSS > exposure concentration. Correlation analysis revealed that immunoefficiency was significantly correlated with TSS (r = 0.21), ammonia (r = 0.13; marginal), DOC (r = 0.20), hemocyte viability (r = –0.51) and immunoactivity (r = 0.96). Analysis of covariance of the observed changes in immunoefficiency with respect to the incoming influents revealed that all influents displayed significant effects on the corresponding effluents (ANCOVA, $p < 0.05$). Among these, the following WWTPs were entirely explained (significant effects for the influent with no effects of the effluent) by the incoming influents: 5W (AS), 6B (BNS), 10K (FAL) and 11M (CPT).

In an attempt to gain a global understanding on the effects of the untreated and treated municipal effluents, discriminate function analysis was performed to discriminate the influents and effluents with respect to the observed changes to the immune system and the capacity of the various treatment processes to influence the toxicity of the untreated wastewaters (**Fig. 3**). Discriminate function analysis revealed that untreated wastewaters (influents) were relatively dispersed throughout the 2 components, suggesting that these influents differed with each other. However, some clustering (an indication of similarity) was observed with the following influents: 7C, 8H, 9F, 10K, 11M and 12P. The introduction of the various treatment processes induced important changes in the response pattern in the effluent for the following stations: 1R, 2L, 3A, 4X, and 5W. The effluents 6B, 7C, 8H, 9F, 10K, 11M and 12P were relatively closer to the corresponding influents, suggesting that the processes did not produce important changes in the effluents' toxic properties.

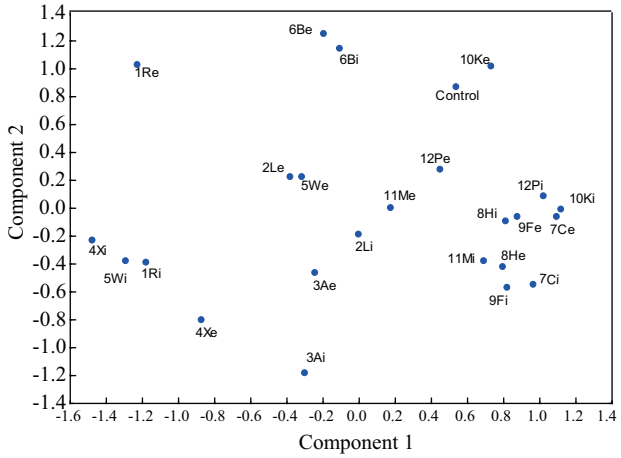

Fig. 3 Discriminant function analysis of immunocompetence data. Immunocompetence data were analyzed by discriminant function analysis to seek out differences between the influents and the effluents. e: effluent, i: influent.

3 Discussion

Municipal effluents are recognized to influence the immune system in various aquatic organisms (Müller et al., 2009; Blaise et al., 2002). In rainbow trout exposed to municipal wastewaters from a CPT process, increased phagocytosis was observed after 28 days of exposure to the entire effluent, which was followed by a decrease at day 90 (Hébert et al., 2008). In another study, a 45-day exposure to a CPT effluent also increased phagocytosis at 3% (V/V) effluent concentration in rainbow trout (Escarné et al., 2008). This is in keeping with the observed increased phagocytosis activities in leucocytes exposed *in vitro* to effluent extracts applying CPT (8H and 11M). In rainbow trout exposed to an AS-treated effluent, no changes in phagocytosis activity were observed after 30-day exposure at 15°C, although cell-mediated cytotoxicity or killer cells were enhanced (Müller et al., 2009). This also agrees with our findings in the present study where no changes in either immuno-activity or immuno-efficiency were observed in

leucocytes exposed to the 4 AS-treated effluents. These results also suggest that *in vitro* models using primary cultures of rainbow trout leucocytes seems to respond similarly to fish exposed short-term to municipal effluents with respect to phagocytosis activity. If this holds true, then the impacts of municipal effluents on the immune system seem to be principally explained by the dissolved fraction of the effluent. In a recent study, snails exposed to environmentally representative antibiotics and a CPT-treated effluent before and after microfiltration revealed that the response pattern of an antibiotic mixture was closely related to the immune responses of snail hemocytes *in vitro* exposed to the filtered CPT-treated effluent (Gust et al., 2012). This study also showed that erythromycin, sulfamethoxazole and trimethoprim were closely associated to the global response of the antibiotic mixture and the filtered CPT-treated effluent. Although the immunotoxicity properties of a CPT municipal effluent in rainbow trout were more closely explained by the dissolved components as opposed to the whole effluent, the influence of particles/microorganisms could not be ignored (Hébert et al., 2008). When comparing with the incoming influents, which differed in their immunotoxicity properties from each other, phagocytosis was most of the time induced by the influents and effluents, albeit with less intensity by the latter. This suggests that the various treatment processes could modulate the distribution of suspended and dissolved materials, which in turn impact on the resulting immunotoxicity. This is consistent with the observed correlations of the levels of TSS with cell viability (r = –0.36), immunoactivity (r = 0.2) and immune efficiency responses (r = 0.23).

The survey also revealed that a low number of effluents (3/12) reduced phagocytosis activity in trout leucocytes, which would suggest immunosuppressive effects. Phagocytosis activities were more strongly suppressed in leucocytes exposed to WWTPs using FAL and BAF processes. The suppressive effects on phagocytosis could indicate compromised immunocompetence in fish. Moreover, the decreased phagocytosis could also be an indication of changes in the inflammation signalling or status. For example, it was shown that decreased *A. salmonicida* specific antibodies and blood lymphocyte numbers were found in mature female fish exposed to a secondary-treated effluent (Hoeger et al., 2005). Sustained phagocytosis could lead to inflammation by the production of reactive oxygen species, which in turn, depress phagocytosis. Exposure to municipal effluents could also lead to inflammation in mussels exposed to CPT-treated effluent (Gagné et al., 2005). Indeed, mussels exposed to municipal effluents and extracts had increased cyclooxygenase activities, a rate-limiting enzyme in the production of prostaglandins. In another study, the removal of bacteria by ozonation decreased the microbial loadings in mussels and loss of hemocyte viability, but was not successful in removing inflammation, as evidenced by cyclooxygenase and the production of nitrite in the hemolymph (Gagné et al., 2008).

Municipal effluents are well recognized to release estrogenic compounds in the environment. Recent evidence suggests that the immune function could be altered in aquatic organisms by estrogenic compounds. The potent estrogen, 17α-ethynylestradiol (EE2), which has been reported in municipal effluents at the low ng/L range (Chimchirian et al., 2007), reduced both phagocytosis activity and oxidative burst in seabream leucocytes *in vitro* (Cabas et al., 2012). However, EE2 was able to increase gene expression involved in inflammation such as interleukin-1β, interleukin-6, tumor necrosis factor α and tumor growth factor β in macrophages. *In vitro* exposure of tilapia leucocytes to estradiol-17β, EE2 and dexamethasone (a sterol-based anti-inflammatory drug acting through the cortisol receptor pathway) suppressed phagocytosis activity (Law et al., 2001). A recent study in three American municipal wastewater treatment plants (one TFSC and 2 AS) revealed that estradiol-17β and estriol were detected in most treatment plants while EE2 and 17α-dihydroequilin (a horse estrogen) were found at 1/3 of the treatment plants (Chimchirian et al., 2007).

4 Conclusions

In conclusion, the effects of influents and effluents from 12 WWTPs were examined in rainbow trout leucocytes. The study revealed that these wastewaters were cytotoxic towards trout leucocytes whereas the application of various types of water treatments had little influence. Indeed, the immunotoxicity of the effluents were most of the time more strongly associated with the untreated influents than with the treatment processes, since only 4/12 WWTPS significantly reduced toxicity. These involved biological and aeration (oxidation) processes. With respect to phagocytosis activity, we found that the various treatment processes tended to reduce the observed increase in phagocytosis by the influents for only 3/12 WWTPs, suggesting that the applied treatments were not successful in removing these effects. More research is needed to better understand the immunotoxicological properties of urban effluents in aquatic organisms and to improve the treatment of urban wastewaters to protect the water quality of aquatic habitats.

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References

APHA (American Public Health Association), AWWA (American Water Works Association), WEF (Water Environment

Federation), 2005. Standard Methods for the Examination of Water and Wastewater (21st ed.). Published by APHA, Washington DC, USA.

Blaise C, Trottier S, Gagné F, Lallement C, Hansen P D, 2002. Immunocompetence of bivalve hemocytes as evaluated by a miniaturized phagocytosis assay. *Environmental Toxicology*, 17(3): 160–169.

Cabas I, Liarte S, García-Alcázar A, Meseguer J, Mulero V, García-Ayala A, 2012. 17α-Ethynylestradiol alters the immune response of the teleost gilthead seabream (*Sparus aurata L.*) both *in vivo* and *in vitro*. *Developmental and Comparative Immunology*, 36(3): 547–556.

Chimchirian R F, Suri R P, Fu H X, 2007. Free synthetic and natural estrogen hormones in influent and effluent of three municipal wastewater treatment plants. *Water Environment Research*, 79(9): 969–974.

Dickenson E R, Snyder S A, Sedlak D L, Drewes J E, 2011. Indicator compounds for assessment of wastewater effluent contributions to flow and water quality. *Water Research*, 45(3): 1199–1212.

Escarné R, Gagné F, Dautremepuits C, Cyr D G, Finnson K, Cejka P, Fournier M, 2008. Effects of municipal sewage effluent on non-specific immune and thyroid functions of rainbow trout (*Oncorhynchus mykiss*). *Trends in Comparative Biochemistry and Physiology*, 13: 27–39.

Gagné F, André C, Cejka P, Hausler R, Fournier M, Blaise C, 2008. Immunotoxic effects on freshwater mussels of a primary-treated wastewater before and after ozonation: A pilot plant study. *Ecotoxicology and Environmental Safety*, 69(3): 366–373.

Gagné F, André C, Fortier M, Fournier M, 2012a. Immunotoxic potential of aeration lagoon effluents for the treatment of domestic and hospital wastewaters in the freshwater mussel *Elliptio complanata*. *Journal of Environmental Sciences*, 24(5): 781–789.

Gagné F, Bérubé E, Fournier M, Blaise C, 2005. Inflammatory properties of municipal effluents to *Elliptio complanata* mussels-lack of effects from anti-inflammatory drugs. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 141(4): 332–337.

Gagné F, Fournier M, Blaise C, 2004. Serotonergic effects of municipal effluents: induced spawning activity in freshwater mussels. *Fresenius Environmental Bulletin*, 13(11): 1099–1103.

Gagné F, Smyth S A, André C, Douville M, Gélinas M, Barclay K, 2012b. Stress-related gene expression changes in rainbow trout hepatocytes exposed to various municipal wastewater treatment influents and effluents. *Environmental Science and Pollution Research*, 20(3): 1706–1718.

Gust M, Gélinas M, Fortier M, Fournier M, Gagné F, 2012. *In vitro* immunotoxicity of environmentally representative antibiotics to the freshwater mussel *Elliptio complanata*. *Environmental Pollution*, 169: 50–58.

Hébert N, Gagné F, Cejka P, Cyr D, Marcogliese D J, Blaise C et al., 2008. The effects of a primary-treated municipal effluent on the immune system of rainbow trout (*Oncorhynchus mykiss*): exposure duration and contribution of suspended particles. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 148(3): 258–264.

Hoeger B, Hitzfeld B, Köllner B, Dietrich D R, van den Heuvel M R, 2005. Sex and low-level sampling stress modify the impacts of sewage effluent on the rainbow trout (*Oncorhynchus mykiss*) immune system. *Aquatic Toxicology*, 73(1): 79–90.

Hoeger B, van den Heuvel M R, Hitzfeld B C, Dietrich D R, 2004. Effects of treated sewage effluent on immune function in rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology*, 70(4): 345–355.

Holeton C, Chambers P A, Grace L, 2011. Wastewater release and its impacts on Canadian waters. *Canadian Journal of Fisheries and Aquatic Sciences*, 68(10): 1836–1859.

Kienle C, Kase R, Werner I, 2011. Evaluation of bioassays and wastewater quality: *In vitro* and *in vivo* bioassays for the performance review in the Project "Strategy MicroPoll". Swiss Centre for Applied Ecotoxicology, Eawag-EPFL, Duebendorf.

Klečka G M, Staples C A, Clark K E, Van der Hoeven N, Thomas D E, Hentges S G, 2009. Exposure analysis of bisphenol A in surface water systems in North America and Europe. *Environmental Science and Technology*, 43(16): 6145–6150.

Law W Y, Chen W H, Song Y L, Dufour S, Chang C F, 2001. Differential *in vitro* suppressive effects of steroids on leukocyte phagocytosis in two teleosts, tilapia and common carp. *General and Comparative Endocrinology*, 121(2): 163–172.

Ménard L, Escarné R, Marcogliese D J, Cyr D, Fournier M, Gagné F, 2010. The impacts of urban pollution on the immune system of spottail shiners (*Notropis hudsonius*) in the St. Lawrence River. *Fresenius Environmental Bulletin*, 19(7): 1369–1374.

Müller C, Ruby S, Brousseau P, Cyr D, Fournier M, Gagné F, 2009. Immunotoxicological effects of an activated-sludge-treated effluent on rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, 150(3): 390–394.

Secombes CJ, Hardie L J, Daniels G, 1996. Cytokines in fish: an update. *Fish and Shellfish Immunology*, 6(4): 291–304.

Siegrist H, 2010. Neptune workshop, Technical solutions for nutrient and micropallutants removal in WWTPs. Laval University, Québec.

Voutsa D, Hartmann P, Schaffner C, Giger W, 2006. Benzotriazoles, alkylphenols and bisphenol A in municipal wastewaters and in the Glatt River, Switzerland. *Environmental Science and Pollution Research-International*, 13(5): 333–341.

Watts M, Munday B L, Burke C M, 2001. Immune responses of teleost fish. *Australian Veterinary Journal*, 79(8): 570–574.

Weirich S R, Silverstein J, Rajagopalan B, 2011. Effect of average flow and capacity utilization on effluent water quality from US municipal wastewater treatment facilities. *Water Research*, 45(14): 4279–4286.

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