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Toxicological Effects of Municipal Wastewaters to Rainbow Trout Hepatocytes

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Municipal effluents are known to contain many chemicals that can be released in the aquatic ecosystem. These effluents arise from many different sources such as industrial, domestic and street runoffs during rain/snow precipitations. Although municipal effluents are treated by different processes such as aeration, sedimentation with(out) the addition of non-ionic surfactants (Field and Reed, 1996), phosphate removal and biofiltration, an hazard still remains since they can still contaminate the aquatic biota albeit with much less hazard than with untreated effluents (Ohe et al, 1996 ; Ersnt et al, 1994).

Ecotoxicological evaluations of municipal wastewaters can be assayed by a variety of cost-effective bioassays representative of different trophic levels (Munawar et al, 1992). At the fish trophic level, fish bioassays like the rainbow trout acute lethality test or the fathead minnow test for example, proved to be useful to evaluate the potential hazard of point source pollution. Numerous fish cell systems have been proposed as rapid and cost-effective alternatives to *in vivo* bioassays (Babich and Borenfreund, 1991; Ryan and Hightower, 1994). However, rainbow' trout hepatocytes have been successfully used to screen (sub)lethal effects of different sources of pollution including industrial effluents (Gagné and Blaise, 1997). Moreover, this cell system was recently proposed as an alternative to the acute rainbow trout lethality since it proved successful in the classification of (non)toxic effluents to trouts (Gagné and Blaise, 1997). Trout hepatocytes play a central role in the metabolism of xenobiotics (Baksi and Frazier, 1990) in that they contain most of biotransformation enzymes and proteins which constitute the first line of defense against the toxicity of chemicals. Indeed, not only mortality effects can be measured with hepatocytes. but numerous sublethal effects such as metallothioneins, cytochrome-mediated activity of organic compounds, oxidative stress, estrogenicity and DNA damage. The purpose of the study was therefore to evaluate the cytotoxic effects of municipal effluents from different cities with primary cultures of rainbow trout hepatocytes. Attempts were made to find relationships between municipal characteristics (i.e., population

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size, number of industries etc.) and the toxicological properties of municipal effluents.

MATERIAL AND METHODS

Rainbow trout *(Oncorhynchus mykiss),* 20 cm long, were obtained from a commercial fish hatchery and fed trout chow once daily. Hepatocytes were collected by a double perfusion method with some modifications (Klauning et al, 1985). Cells were distributed in 48-well microplates at a density of 1 x 10^6/mL in L-15 medium supplemented with 1% fetal bovine serum, 100 units of penicillin, 100 µg/L of streptomycin and 0.25 µg/L amphotericin B. The cells were exposed to 0.1, 1, 10, 25 and 50 % v/v of the municipal effluents, to 200 ng/mL of benzo(a)pyrene (BaP), and to 250 ng/mL of Zn. The municipal effluents were 24 h composites, brought back to the laboratory at 4°C in 60 L containers lined with polyethylene bags and tested within 5 days after collection. BaP served as the positive control for both genotoxicity and cytochrome P4501Al induction, while Zn served as the positive control for MT determinations. The cells were incubated for 48 h at 15°C in a humidified incubator.

Cell viability was determined using the propidium iodide (PI) exclusion test, as described previously (Gagné and Blaise, 1996). Cells were resuspended in phosphate buffered saline (PBS) containing 10 mM sodium citrate, 5 mM glucose, 5 mM Hepes-NaOH, pH 7.5, and 0.5 mM pyruvate. Approximately 5 x 10^4 cells were mixed with 25 µg/mL of PI in PBS for 30 min. Fluorescence was then evaluated where dead cells became fluorescent at 600 nm upon excitation at 485 nm. The proportion of viable cells relative to exposure to the effluents was determined with 10 % DMSO, a positive control that permeabilizes cells. Hence, the same amount of cells was exposed to 10 % DMSO in PBS for 30 min, incubated with PI for 10 min and analyzed by microfluorimetry.

Cytochrome P4501Al activity was measured by following deethylation of 7-ethoxyresorufin (Hahn et al, 1996) with some moditications. Briefly, 1 x 10^5 cells were added to 10 µM of 7-ethoxyresorutin in PBS containing 0.015% triton X-100 for 30 min at 22°C. Afterwards, fluorescence was measured at 590 nm upon excitation at 540 nm. Fluorescence intensity was corrected for blank values (t = 0 min) and cell density as measured at 600 nm. Standards of 7-hydroxyresorufin were used to calculate the amount of product formed over time.

MT levels were quantified in hepatocytes using the silver-saturation assay (Scheuhammer and Cherian, 1986) with some modifications (Gagné and Blaise, 1993). Briefly, 2 x 10^5 cells were mixed to 50 µL Ag^{1+} at a concentration of 20 µg/mL, in 500 mM glycine buffer, pH 8.5 for 5 min. After volume adjustment to 500 µL with glycine buffer, 50 µL of 2% Hb was added and allowed to incubate for another 5 min at room temperature. The mixture was heated at 100°C in a

boiling water bath for 2.5 min, centrifuged at 10 000 x g for 2 min, and treated with Hb once more. The remaining Ag in the supernatant was determined by atomic absorption spectrometry using a graphite-tube atomizer with a Zeeman background corrector. Blanks contained no cells, and standard rabbit MT (0.5 and 1 µg/mL) was used. A ratio of 17 moles of Ag/mole of MT was assumed in order to calculate the MT concentration. Finally, MT levels were corrected for cell density by absorbance of the cell suspension, before the lysis step, at 600 nm.

Genotoxicity was tested using the alkaline precipitation assay (APA), with some modifications (Olive, 1988; Gagné and Blaise, 1997). Briefly, 1 x 10^5 cells were mixed with 250 µL of 2% SDS containing 50 mM NaOH, 10 mM EDTA and 10 mM Tris-HCl, pH 12.3. An equal volume of KCI, at a concentration of 120 mM, was added and allowed to incubate at 60°C for 10 min. The mixture was cooled on ice for at least 15 min and centrifuged at 9 000 x g for 5 min. DNA remaining in the supernatant was analyzed by microfluorimetry using Hoechst dye. DNA levels were normalized by cell density (A_{600}).

Cells were exposed to effluents in quadruplicate (n=4). The data underwent an analysis of variance and critical differences between unexposed and exposed cells were determined with Dunnett's t test. Significance was invariably set at $p<0.05$. For environmental samples, the Lowest Observable Effect Concentration (LOEC) and the No Observable Effect Concentration (NOEC), expressed in % v/v, were determined. A toxicity threshold (TT) was then calculated from the following equation: $TT = (NOEC \times LOEC)^{1/2}$. Environmental sample data were further transformed into toxicity units (TU) with the help of the following equation: TU = 100% v/v / TT. This transformation renders data directly proportional to toxic strength. Pearson-Moment correlation and principal component analysis were also performed to observe any trends between the toxicological effects and municipal characteristics of the wastewaters.

RESULTS AND DISCUSSION

Municipal effluents were obtained from different cities having different characteristics (Table 1). These wastewaters are released by cities having different population size and number of industries. Cities # 4, 13 5 and 3 are the most populated and industrialized while cities #14, 15, 9, and 10 where the smallest ones with consistently less industries. Indeed, we found a significant correlation between population size and the number of industries ($R=0.98$, $p<0.001$), number of employees ($R= 0.97$, $p<0.001$), flow rate ($R=0.99$, $p<0.001$) and surface area of the towns ($R=0.641$, $p=0.007$). However, the predominant industrial sector in the most populated towns were from food processing while the predominant sector of the less populated townships was from wood processing. The treatment procedure of the wastewaters varied: (non-)aerated lagoons, activated sludge, physical/chemical treatments and biofiltration. The last two treatments are generally considered more

performant water treatment procedures in respect to chemical contamination. Most of the municipal effluents were treated for the removal of phosphates by alum or iron chloride treatment. Five cities did not use any phosphate removal treatments at all (effluents # 1, 8, 11, 14 and 15).

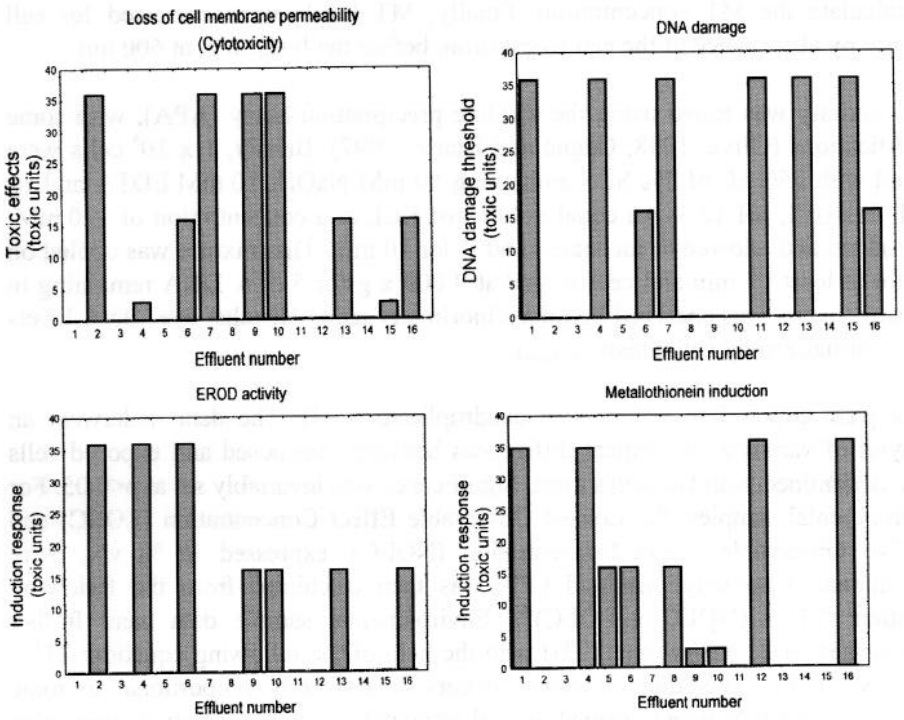

Figure 1. Cytotoxic effects of municipal effluents to rainbow trout hepatocytes.

Rainbow hepatocytes were exposed to effluent concentrations for 48 h at 15°C. Afterwards, the hepatocytes were collected for cell viability, DNA damage, EROD activity and metallothionein determinations. Data bar represent toxic strength as expressed in toxicity units.

The effects of municipal effluents on rainbow trout hepatocytes were investigated (Figure 1). We found that 50 % of the municipal effluents were cytotoxic to trout hepatocytes. No linear relationships between cytotoxicity and the municipal characteristics, such as population and industrial numbers, were observed. About 44 % of the municipal effluent were able to induce cytochrome P4501Al activity suggesting the presence of polyaromatic hydrocarbons. Exposure of BaP was able to induce EROD activity at a threshold of 100 ng/ml. The presence of these organic compounds were found in sediments and in the water column from municipal and industrial areas (Huntley et al, 1995; Wenning et al, 1994). Again no linear relationships were obtained with the municipal characteristics. About 56 % of those

Table 1. Demographic characteristics of the municipalities[1].

Towns	Population	Area (Km^2)	Number of industries[2]	Number of employees	Predominant industrial sector[3]	Treatment procedure
1	0.101	0.44	0.35	0.58	Foods, clothings, furnitures	activated sludge, aeration
2	0.41	1.7	0.15	0.08	Printing, machines, foods	biofiltration, alum
3	1.3	2.1	0.55	1	Foods, plastics, clothings	physical/chemical
4	10	8.5	10	10	Foods, plastics, leathers	physical/chemical
5	1.7	4.3	1	1	Foods, Clothing, wood	biofiltration
6	0.14	0.7	0.1	0.4	Foods, rubber, textiles [5]	activated sludge, aeration, filtration with added chlorine
7	0.06	1.2	0.1	0.18	Printing, foods, textiles	activated sludge, aeration
8	0.58	10	0.2	0.7	Metals, minerals, foods	activated sludge, aeration
9	0.03	0.2	0.07	0.04	Woods, foods, plastics	aerated lagoons
10	0.03	0.16	0.05	0.07	Woods, plastics, textiles [5]	aerated lagoons
11	0.015	0.5	0.02	0.05	Woods, plastics, textiles [5]	aerated lagoons
12	0.009	0.56	0.008	0.0035	Woods	aerated lagoons
13	2.7	3.6*	0.37	0.54	Foods, woods, printing	aerated lagoons
14	0.02	0.14	nd[4]	nd	nd [6]	aerated lagoons
15	0.005	2.3	nd	nd	nd [6]	lagoons

(1) All municipal data were normalized between 0 and 10 to ensure anonymity.
(2) Factory-based industries excluding agriculture.
(3) the first three principal industries are given in decreasing order of importance (based on the number of industries and employees).
(4) no data.
(5) mainly first transformation products i.e. chemical fibbers and tissues productions only.
(6) principally domestical municipalities.

effluents were able to induce MT suggesting the presence of bioavailable heavy metals in these effluents apparently obvious to treatment procedures, dephosphatation steps, and municipal characteristics. Zn was able to induce significantly MT at a threshold of 140 ng/ml after a 48 h exposure period. In another study, urban effluents were shown to contain enough metals to produce toxicity to *Chlorella* (Luek-Wong, et al, 1995). Although MT induction are not entirely specific to heavy metals (Kagi, 1991 ; Bauman et al, 1991), the presence of metals in these effluents are likely the be principal class of pollutants at play in rainbow trout hepatocytes exposed to municipal effluents. However, chemicals producing oxidative stress, alkylating agents and those interacting with the glucocorticoid receptor are also potential inducers of MT (Bauman et al, 1991; Hyllner et al, 1989; Penketh et al, 1996).We found that 50 % of the municipal effluents produced DNA damage in rainbow trout hepatocytes suggesting the presence of chemicals that can lead (in)directly to DNA damage. Municipal effluents were also found to contain genotoxins in respect to E coli (White and Rasmussen, 1998) and to polychaetes worms (Hutchinson et al, 1998). This suggests also that municipal effluents are also genotoxic to fish cells as well.

Large cities contain also industrial inputs in addition to domestical wastes and street rain runoffs. This industrial input is likely to increase the contamination loading of the municipal effluents. Indeed, if we compared the overall toxicity (i.e., sum of the toxic units) of the larger cities (having a population higher than 0.1) with the one of smaller cities (having a population number lower 0.1) then a significant difference is observed between the 2 groups *(t* test : p=0.002). This suggest that large cities tend to be more toxic and possess a larger spectrum of effects than with smaller cities. Moreover, principal component analysis of the toxic responses show that hepatocyte viability and EROD induction explain most the observed toxicological responses. This suggest that the principal effects arise from the presence of coplanar polyaromatic compounds and toxic chemicals as defined by loss of cell membrane permeability endpoint.

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