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Plastic Contamination and Water Quality Assessment of Urban Wastewaters

Abstract

The peroxidase toxicity assay (Perotox) represents a quick and inexpensive methodology to screen for the water quality of municipal and industrial wastewaters. The purpose of this study was to assess wastewater from 7 cities using the following 5 treatment processes: aerated lagoons, secondary aerated sludge, biofiltration, trickling filter and primary treatment. In parallel, the presence of plastic nanoparticles and plastic materials in the organic matrix were determined. The untreated (influents) and treated effluents were fractionated on C18 solid-phase cartridges and eluted in ethanol (50 X concentration) before testing with the Peroxtox assay. A DNA protection index was also derived from the recovery of peroxidase (Per) activity in the presence of DNA as a proxy of genotoxicity. The data revealed that the wastewaters contained organic matter in the mg/L range, polyaromatic hydrocarbons (Pahs), humic and fluvic acids (HA/FA), polystyrene nanoplastics (PsNPs) and plastic-related materials. Inhibition of the Per reaction was observed in most wastewaters (90%) leading to reduced elimination of toxic reactive species with a low but significant correlation with plastic levels (r=-0.37). The addition of DNA during the Per reaction and the effluents prevented inhibitions in nearly 60 % of the effluents including the plastic material fraction suggesting the presence of genotoxic compounds in these effluents. The study also revealed that effluents using biofiltration and aeration processes were generally less harmful based on the Perotox and DNA protection assays. The Perotox assay represents a cost-effective alternative for the rapid and cheap screening of municipal wastewater quality.

Keywords

Peroxidase • Wastewaters • DNA protection • Plastics • Alternative methods

Research Article

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Introduction

Municipal wastewaters are produced from domestic and industrial waste products of townships. They also receive hospital wastewaters and leachates from solid waste disposal sites, which are usually connected to the wastewater treatment plant works. They also treat road runoffs containing plastics rubber from tire and street wear/erosion straining the treatment plants capacity to receive excess water from rainfall events [1,2]. Indeed, the intensity of rainfall events are expected to increase by global warming exceeding the capacity of wastewater treatment plants to handle excess volumes [3,4]. In addition to the already known contaminants in municipal wastewaters such as Pahs, heavy metals and critical elements of technology (stemming from solid waste disposal sites), pesticides, pharmaceutical and personal care products and endocrine disrupters, micro and nano-sized plastic materials are found in municipal wastewater treatment plants as well [5,6]. Most wastewater samples

contained microparticles (5 mm – 1 µm size) ranging from 2000 to 3000 particles/L in untreated (influent) wastewaters down to 744 to 1244 particles/L following treatments. The average removal of MP in activate sludge was 57% indicating a significant proportion remaining in the suspended and dissolved organic fraction released in the receiving waters. The most common type of plastics was polystyrene (PS), polypropylene (PP) and polyethylene terephthalate (PET). Although large plastic particles were most abundant in biosolids, there were no significant influences from wastewater treatment types and sludge treatment processes. However, the mobilisation of smaller plastic nanoparticles (1000-1 nm range) in the dissolved organic matter matrix of effluents are less understood at the present time, hence the need to analyse plastic materials in the dissolved organic matter phase in wastewaters. A recent study revealed that nanoplastics are found in the 0.01-10 µg/L concentration range [7]. The effluent released between 3-10% of nanoplastics of the untreated influent and the above plastics were among the most abundant. Some evidence exists that nanoplastics could be genotoxic and producing epigenetic effects in offsprings in fish [8]. Recent studies have also shown that municipal effluents release genotoxic compounds [9, 10]. The long-term release of genotoxic compounds could lead to genetic instability, deleterious mutations, epigenetic changes and cancers in organisms. Thus, the need to have effective methods to screen for genotoxic compounds in environmental studies. Given the number of municipal effluents in a given country, rapid screening methods for wastewater quality are needed for our sustainable development. Moreover, cost effective assays to determine the toxic and genotoxic potential as new alternative methods (NAMs) for the reduction of fish use during screening of various pollutants are becoming a pressing ethical issue.

Peroxidases (Per) are enzymes involved in the elimination of the potent reactive oxygen species (H_2O_2) by oxidizing various types of electron donor molecules such as phenols, herbicides, antioxidant compounds (electron donors), pharmaceuticals, Pahs and unsaturated lipids. Per enzymes are hemoproteins found in all oxygen-living organisms involved in oxygen metabolisms such as oxygen transport, respiration (mitochondria electron transport systems), photosynthesis and oxidative metabolisms of xenobiotics [11]. The production of reactive species is often a critical step in initiating most toxic responses, it is therefore conceivable that Per represents a fundamental unit for oxygen mobilisation during the initiation of toxicity. The manifestation of toxicity involved most of the times the production of oxygen reactive species. Per are involved in 2 main reactions: peroxidative and catalytic oxidation of H_2O_2. Peroxidative oxidation involves the oxidation of an electron donor: $AH_2 + H_2O_2 \rightarrow AOH + H_2O$. Catalytic oxidation involves the transformation into O_2 and H_2O: $2\ H_2O_2 \rightarrow O_2 + H_2O$. Per activity can also oscillate in the presence of O_2 and NADH leading to the formation H_2O and oxidized NAD+, so-called the Peroxidase-oxidase reaction [12]. Moreover, Per have the ability to oxidize a broad spectrum of environmental pollutants [13]. Plant Per have shown the ability to degrade toxic pollutants as well making it a sensitive enzyme for environmental pollution and remediation strategies. The Per reaction using a luminescent substrate luminol with aromatic enhancers such as aminophenols is highly selective, sensitive and simple to determine the presence of environmental pollutants [14]. The first reports of Per inhibitions/activations for water quality assessment used unfiltered water samples containing bacteria, which are sources of Per [15]. In filtered industrial effluents (removing microorganisms), the Per reaction was inhibited leading the accumulation of H202 [16]. Moreover, the effluent concentration inhibiting the Per reaction was concordant with the acute lethality for rainbow trout providing toxicological meaning and its potential as NAMs for reducing fish testing. An interesting add-on to this test was the DNA protection assay where Per inhibition was partly or completely restored to control values when DNA was added in the reaction mixture. The restauration of Per activity in the presence of DNA was associated to genotoxicity 70% of times with no false negatives, with the bacterial SOS Chromotest, making it a rapid genotoxicity pre-screening tool for pollutants and their mixtures [16]. The binding of aromatic hydrocarbons to DNA was catalyzed by horseradish Per and H_2O_2 [17] indicating that oxidation of aromatic hydrocarbon by Per could lead to DNA adducts and strand breaks. Aromatic hydrocarbons with an ionizing potential < 7.35 eV produced DNA strand breaks and involved one electron oxidation with aromatic hydrocarbons. The ionizing potential threshold corresponds to medium and heavy Pahs composed with ≥ 3 aromatic rings, which abound in municipal wastewaters.

In respect to plastic nanoparticles, Per was shown to form of protein corona on the surface polystyrene nanoplastics (PsNPs) and inhibited the enzyme activity [18] contributing to elevated levels of reactive oxygen species H2O2 that could reach toxic concentrations.

The purpose of this study was therefore to test the Perotox assay in various municipal wastewaters following 5 different treatment processes in Canadian cities. The influence of DNA on Per activity was also examined to determine potential DNA binding or genotoxic agents in municipal effluents. In parallel, the organic carbon fraction of the municipal wastewater extracts was examined for the presence of Pahs, polystryrene nanoplastics (PsNPs) and plastic leachates. An attempt was made to examine the influence of population size, wastewater treatment types and the occurrence of plastic materials on the Perotox assay.

Materials and Methods

Sample preparation

Horseradish peroxidase (Per) and serum bovine albumin were purchased from Sigma Chemicals (On, Canada) and prepared at 1 mg/mL in phosphate buffered saline (PBS: 140 mM NaCl, 5 mM KH2PO4 and 1 mM NaHCO3, pH 7.5) and kept for non-longer than one week at 4oC in the dark. DNA from salmon sperm were purchased from Sigma Chemicals (On, Canada) and dissolved at 1 mg DNA/mL in 50 mM NaCl containing 0.5 mM KH2PO4, pH 7.4. The sample was heated at 70oC for 15 min to ensure complete dissolutions and stored at 4oC. Hydrogen peroxide (H2O2) solutions were prepared at 1% concentration and stored at 4oC for non-longer than one week. The Per substrate 2,4-dihydrofluorescein diacetate (DHFDA; Sigma Chemical company, ON, Canada) stock solution was dissolved in 10 % DMSO in PBS at 1 mM concentration.

The municipal wastewaters from 7 townships differing in population size were collected for 3 days as 24h-composite samples (Table 1). The wastewaters (1 L) were transported back to the laboratory and stored at 4oC in the dark. The untreated wastewaters were also collected at the wastewater station plants and were termed influent. The samples were filtered on 0.8 µm pore cellulose filter and 250 mL was passed through a reverse phase C18 cartridge (500 mg; Supelco, USA). After washing with MilliQ water (10 ml), the material was eluted with 1 mL ethanol and concentrated to 0.5 mL under nitrogen stream (500 X concentrate). The ethanol samples were stored at -20oC until analysis. Preliminary experiments revealed that fluorescently-labeled 50 nm polystyrene nanoplastics (Polyscience, USA) were retained by the C18 SPE at 99% efficiency.

Wastewater extract characteristics

The dissolved organic matter (DOC) in the ethanol extract was determined by the spectrometric methodology at 254 nm [19] and this metric was used to normalized the following measurements. Levels of plastic-like substances in the organic matter matrix (i.e. the C18 extract) were determined using the copper fluorescence quenching methodology [20]. The decreased in fluorescence for humic/fulvic acids (HA/FA) was determined at 265 nm excitation/463 nm emission and normalized to the organic carbon contents. The polypropylene (PP)-derived materials (250 nm excitation/324 nm emission) and polyvinyl chloride /polystyrene-derived materials (295 nm excitation/411 nm emission) were determined before and after the addition of 0.1 volume of 1 mM of CuCl2. The difference between fluorescence without Cu – fluorescence with Cu was calculated and standard solutions of polystyrene (20 nm diameter) and polypropylene (100 nm diameter) were used for calibration. The data were expressed as ug PP or PS/PVC-equivalents/ mg DOC.

The levels of light, medium and heavy polyaromatic hydrocarbons (Pahs) were determined in the ethanol extracts using fixed wavelength fluorometry [21]. Briefly, 100 µL of ethanol extracts were placed in dark 96-wells microplate and fluorescence was measured at 290 nm excitation/340 nm emission (for light PAhs: naphthalene), 325 nm excitation/370 nm emission (for medium Pahs: pyrene) and 385 nm excitation /440 nm emission (for heavy Pahs: benzo(a)pyrene) using a fluorescence microplate reader (Neo-2 Synergy, Biotek Instruments, USA). Standard solutions of the Pahs were used for calibration. Recovery of each Pahs size class were between 80-95% in the C18 extraction columns. The levels of polystyrene nanoplastics (PsNPs) were determined using a molecular rotor probe 9(dicyanovinyl)-julolidine as previously described [22]. Briefly, 10 µL of

the effluent extract were mixed with 190 uL of 10 uM of the probe (diluted in MilliQ water) and fluorescence were taken at 450 nm excitation and 620 nm emission (Neo-2, Synergy-4, Biotech Instruments, USA). Standard solutions of polystyrene nanoparticles (20 nm Polyscience, USA) were used for calibration. Solvatochromatic analysis of the ethanol extracts were also analyzed to detect plastic-like substances using the Nile red methodology [23]. Briefly, 25 µL of the ethanol extracts were mixed with 225 µL of 50 uM Nile red (in PBS buffer) and the emission spectra were recorded between 520-700 nm at 485 nm excitation. The first derivative of the spectra revealed that PsNPs emitted at 600-620 nm and this signal was taken as a measured of plastic-like materials. The data were expressed as relative fluorescence units (600 nm) corrected for the organic matter contents.

In vitro peroxidase assay

The in vitro Perotox assay was based on a previous methodology for industrial effluents toxicity screening [16]. This simple, rapid and cheap assay was also associated to toxicity (mortality) in rainbow trout. The Per assay principle consisted of a mixture of Per, albumin and substrates (H2O2 and DHFDA) exposed to municipal effluent samples. DNA could be added to seek out interactions with municipal wastewater extracts by increased Per activity. The reagent DHFDA was used instead of luminol for fluorometric detection at 485 nm excitation and 530 nm emission for fluorescein formation. The reaction media contained Per and albumin at 0.1 µg/mL in 0.1 X PBS in a total volume of 160 µL followed by the addition of 20 µL of the ethanolic extract for 5 min. After this period, 20 µL of 1 µM DCFDA and 0.01 % H2O2 were added. The reaction was allowed to proceed at 25oC for 30 min with readings taken at each 2-3 min interval for fluorescein (excitation 485 nm/emission 530 nm). The same procedure was repeated with the ethanol extract pre-incubated for 5 min with 1 µg/mL DNA to determine the influence of DNA on Per reaction rates. The DNA protection index is defined as: Per activity with DNA/Per activity. Blanks consisted of ethanol only and CdNO3 was used as a positive control (1 µg/L CdNO3 decreases Per activity by 30-40%). This Cd concentration (µg/L) is in the same range to trout toxicity (LC50 between 0.7-3 µg/L) for rainbow trout embryos and larvae.

Data analysis

The in vitro exposure experiments to the various influents/ effluents from 7 cities were repeated three times in the absence and presence of added DNA to determine DNA protection of the Per reaction. The population size of each (anonymous) city and the various types of wastewater treatment were as follows (Table 1): aerated lagoon (Lag), lagoon with facultative aeration (LagF), advanced biofiltration (Adv), biological filtration (Bio), secondary activated sludge (SecA), secondary membrane bioreactor (SecM) and primary physico-chemical treatment (Prim). The untreated effluents were denoted by None. In this study, the influence of treatment processes was obtained from cities differing in population sizes. To account for the influence of population size and the effluents toxic properties from different treatment scenarios, the influents properties were first examined by population size. The effluents properties were then examined by analysis of covariance (ANCOVA) on log-transformed data with the treatment processes as the main variable and population size as the covariate. Critical differences between the absence (None) of wastewater treatments and the following 7 different treatment processes were determined by the LSD test. Significance was set at $\alpha \leq 0.05$. The relationships between the endpoints were examined using the Pearson moment correlation test. Discriminant function analysis was also used to seek the difference of wastewater properties before and after the treatment processes. All statistical analyses were performed using the Statsoft software package (USA).

Results

In this study, the wastewater samples were collected before treatment (influent denoted as none) and after various treatments (effluents) from cities differing in population size (Table 1). Ideally, comparisons between treatment processes should be made with similar population and similar ratio between domestic and industrial inputs. To compensate for population effects (and directly related to effluent flow rates and industrial inputs since industrialisation generally follow the city size), the results obtained between different parameters were controlled statistically using population size as the covariable as explained in the data analysis section. Indeed, we found a strong correlation between population size and effluent

Townships	Population	Treatment Type	Comments
1	4 864	Aerated lagons (Lag)	Only the influent (Inf) and final effluent (Eff) were sampled
2	38000	Secondary activated sludge (SecA)	Influent (Inf), primary (Prim) and the final Eff) effluents
3	63120	Secondary activated sludge (SecA)	Influent (Inf), primary (Prim) and the final Eff) effluents
4	99 860	Primary (Prim)	Influent (Inf), primary (Prim) and the final Eff) effluents
5	108 840	Biological filtration (BF)	Influent (Inf), primary (Prim) and the final Eff) effluents
6	662 250	Trickling filter (TF)	Influent (Inf), primary (Prim) and the final Eff) effluents
7	749 610	Secondary activated sludge (SecA)	Influent (Inf), primary (Prim) and the final Eff) effluents

Table 1. City size and wastewater treatment plants

flow rates from the wastewater treatment plant (r=0.94; p<0.001). It is noteworthy, that the aeration lagoons with secondary UV-treatment were collected at 3 cities with populations of 38 000, 61 120 and 749 610 residents, making easier comparisons for this treatment at least. Basic physicochemical characteristics were also provided in (Table 1S) (supplementary information). In general, the wastewater originated from cities with relatively low industries input (5-10%) with the exception of city #5 with a reported population of 108 840 inhabitants.

The DOC was determined in the ethanol extracts following C18 extraction (Table 2). The population size did not influence the DC in the influents. ANCOVA revealed that DC was significantly lower in BF-Prim, BF-Eff and higher for TF-Prim and TF-Eff compared to the influents (none). The levels of light, medium and heavy Pahs were also determined (Table 2). The population size did not influence the levels of the light Pahs in the untreated influents. The light Pahs were significantly higher in the SecA Eff1, SecAPrim2, SecAPrim3, Prim-Eff and SecAEff2 samples. For medium Pahs, covariance analysis revealed no significant changes between treatments when population size was taken in account. For heavy Pahs, the population size was not significant but the levels were significantly decreased for SecA-Eff and SecA-Prim2 and higher for SecA-Prim3 and Prim-Eff samples. Correlation analysis revealed that light, medium and high Pahs were significantly correlated with DC and with each other (Table 3). HA/FA levels were significantly decreased in the Lag-Eff, SecA-Prim 1 and 2, SecA-Eff 1 and 2 in respect to the influent. They were significantly higher in the Prim-Eff in respect to the influent. Correlation analysis revealed that HA/FA levels were not correlated with population but with DOC (r=0.37), light- (r=0.52), med- (r=0.96), and high-Pahs (r=0.71) levels (Table 3), which provides information on the origin of surface water input in the station plant.

The relative levels of plastic related materials were determined for PsNPs and plastic-leachates of PP and PVC- PS (Figure 1). The PS-like materials were determined by the fluorescence Cu quenching (Figure 1) and NR solvatochromic methodologies (Figures 1) respectively. The levels of PsNPs were significantly related with population size (r=0.34; p=0.01) indicating that plastic nanoparticles somewhat increase with population (Figure 1). In respect to different treatment processes, prim-Effl and SecA-prim1 treatments increased the levels of PSNPs compared to untreated wastewaters (influents) even after correcting against population size (ANCOVA p<0.001 for population and treatment processes). PsNPs levels were significantly correlated with med Pahs (r=0.61) and HA/FA levels (r=0.59). The relative levels of plastic leachates were determined by the Cu fluorescence quenching

Townships	Population	Treatment Type	Industrial Input (%)	pH	Alkalinity (mg/L CaCO3)	TSS (mg/L)	BOD (mg/L)	COD (mg/L)	NH4-N (mg/L)	TKN (mg/L)	NO3/NO2 (mg/L N)
1	4 864	Aerated lagons (Lag)	<5%	7.64	100	8	4	38	9	11	2.4
2	38000	Secondary activated sludge (SecA)	10%	7.29	110	< 5.18	< 1.12	50	0.33	1.72	21.4
3	63120	Secondary activated sludge (SecA)	10%	7.64	187	8,3	5,8	60	21	23.5	0.07
4	99 860	Primary (Prim)	10%	7.06	133	48	117	251	24.7	34.9	0.031
5	108 840	Biological filtration (BF)	30%	6.71	62,7	12	18	60	2.67	6.9	16
6	662 250	Trickling filter (TF)	5%	7.45	179	6	8,1	81	30.3	32,3	0.065
7	749 610	Secondary activated sludge (SecA)	10%	6.75	248	6	9	100	26.8	34	0.58

Note: Supplementary materials

Table 1S. General characteristics of the municipal wastewaters.

Treatments	OC mg/L	Light Pahs mg/L	Med Pahs mg/L	High Light Pahs mg/L	Humic and fluvic acid (RFU/mg OC)
Influent (untreated WW)	1.13± 0.01	2.2± 0.4	0.26± 0.08	0.03± 0.006	0.18± 0.05
SecA-Prim1	1.17± 0.01	2.7± 0.8	0.10± 0.03*	0.02± 0.009	0.094± 0.02*
SecA-Eff1	1.18± 0,013	4.7± 1.4*	0.07± 0.01*	0.005± 0.004	0.04± 0.01*
SecA-Prim2	1.17± 0.03	5.6± 1*	0.12± 0.03*	0.014± 0.002*	0.05± 0.03*
SecA-Eff2	1.18± 0.003	5.0± 0.8*	0.17± 0.01	0.03± 0.009	0.048± 0.001*
SecA-Prim3	1.11± 0.02	4.9± 1.8*	0.18± 0.06	0.06± 0.03*	0.2± 0.02
SecA-Eff3	1.07± 0.006	1.6± 0.4	0.13± 0.03*	0.03± 0.008	0.09± 0.04
TF-Eff	1.17 ±0.01*	3.3± 1.7	0.07± 0.02*	0.03± 0.01	0.07± 0.02*
Prim-Eff	1.1± 0.005*	6.4± 2*	0.7± 0.1*	0.08± 0.005*	0.58± 0.06*
BF-Prim	1.1± 0.008*	1± 0.6	0.13± 0.003	0.02± 0.006	0.12± 0.01
BF-Eff	1.075 ±0.007	2.2± 0.9	0.17± 0.02	0.032± 0.004	0.16± 0.01
Lag-Eff	1.1± 0.3	0.4± 0.04	0.15± 0.06	0.02± 0.01	0.06± 0.02*

Note: The star symbol * indicates significance from the untreated wastewaters (WW).

Table 2. Organic matter content and PAHs profiles.

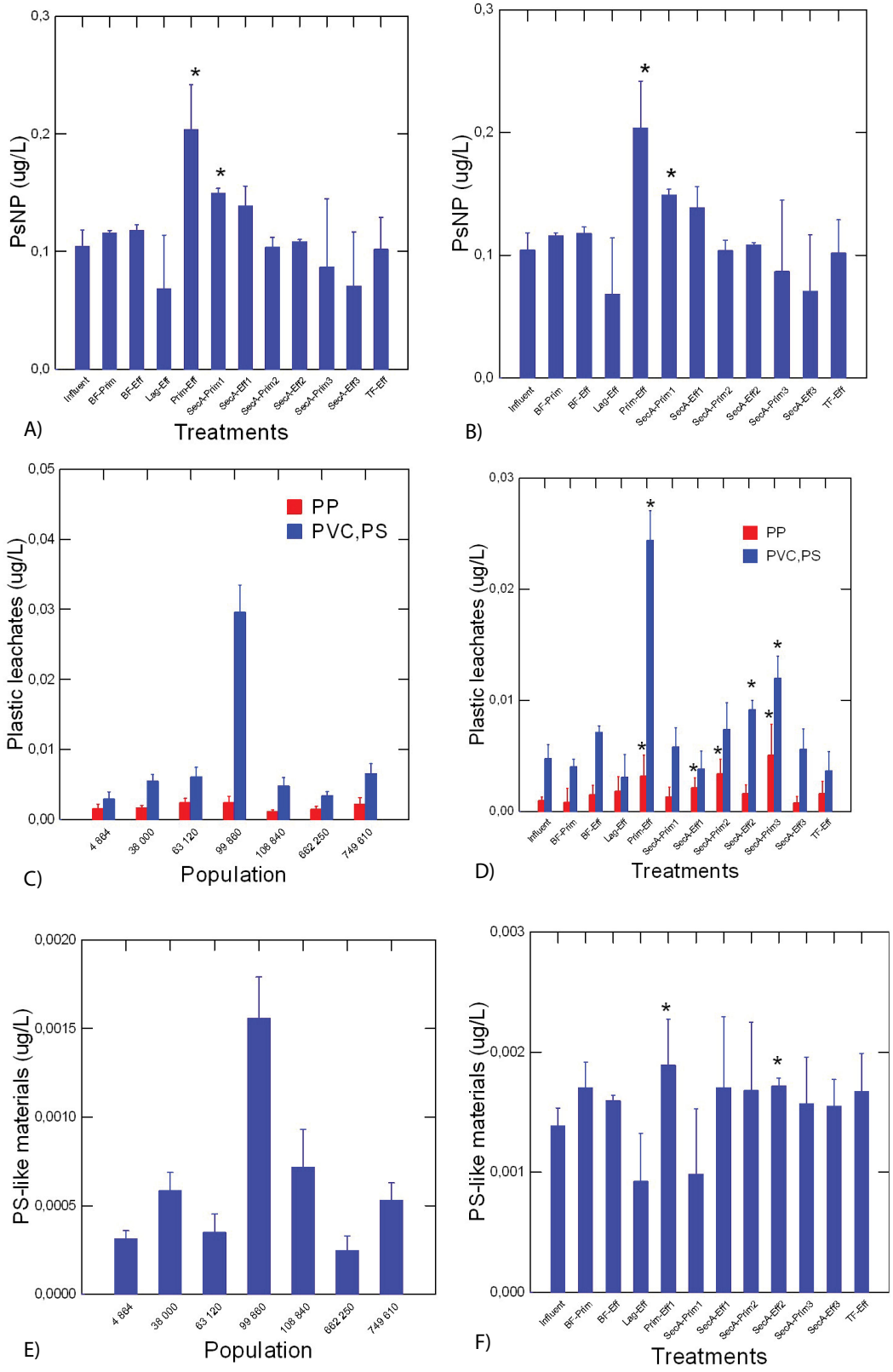
	Population	OC Pahs	light Pahs	Med	High	HA/FA	PP	PVC, PS	Perox	Perox -NA	PSNPs	PS (NR)
Population	1											
OC	0.02	1										
light Pahs	0.03	0.86	1									
Med Pahs	0.06	**0.47**	0.59	1								
High Pahs	-0.11	**0.83**	**0.83**	**0.73**	1							
HA/FA	-0.09	**0.37**	**0.52**	**0.96**	**0.71**	1						
PP	0.07	**0.91**	**0.98**	**0.56**	**0.86**	**0.46**	1					
PVC,PS	-0.06	**0.62**	**0.78**	**0.93**	**0.84**	**0.91**	**0.72**	1				
Perox	**-0.46**	-0.15	-0.23	**-0.37**	-0.23	-0.28	-0.27	-0.25	1			
Perox(DNA)	**-0.44**	0.14	0.08	-0.2	0.01	-0.13	0.1	-0.09	**0.40**	1		
DNA Prot	0.06	0.22	0.18	0.18	0.20	0.15	0.21	0.14	**-0.65**	**0.35**	1	
PSNPs	0.22	0.20	0.27	**0.61**	0.23	**0.59**	0.25	**0.55**	**-0.37**	0.10	**0.41**	**1**
PS(NR)	-0.05	-0.26	-0.16	0.28	-0.07	**0.50**	0.04	**0.46**	-0.05	0.09	0.1	**0.48**

Note: Significant correlations are highlighted in **bold.**

Table 3. Correlation analysis.

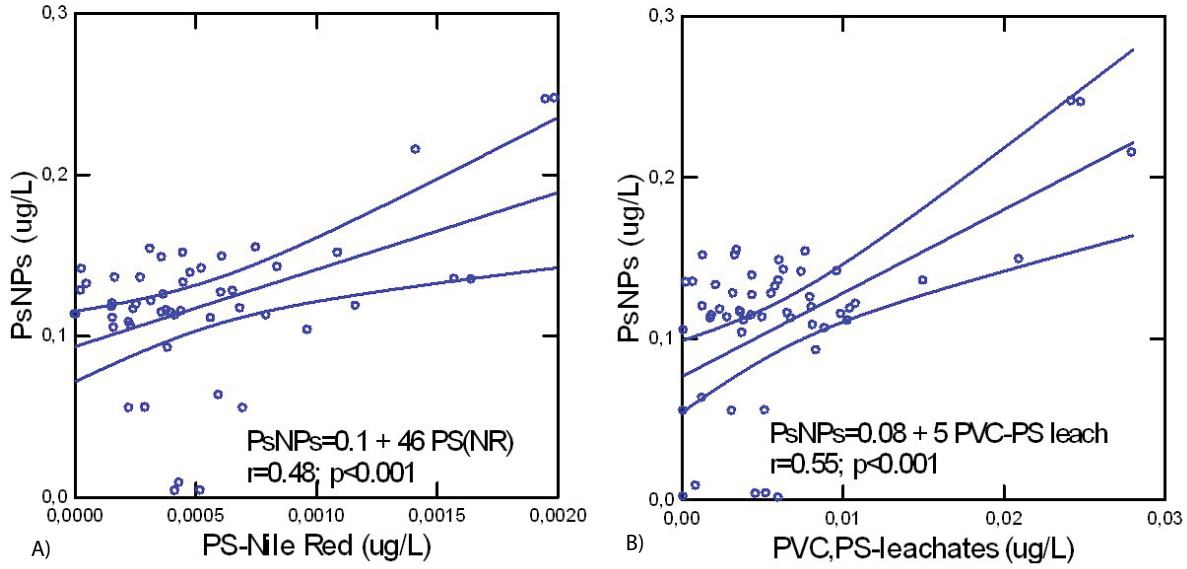
methodology and revealed that population was not related with PP and PVC/PS levels in the wastewater extracts. In respect to the different treatment processes, PP levels were increased in the Prim-Efff, SecA-Effl1, SEcA-Prim1 and SecA-Prim3 compared to the influent. Correlation analysis revealed that PP was significantly correlated with DC, light-, med-, high-Pahs and HA/FA (Table 3). The PVC-PS levels were significantly higher in the Prim-Eff, SecA-Prim2 and 3 effluents compared to the untreated influents. PVC-PS levels were significantly correlated with DC (r=0.62), light-(r=0.78), med-(r=0.93), high-(r=0.84), HA/FA (r=0.91) and PP (r=0.72). The levels of PS-like materials determined by the solvatochromism of NR generally followed the same trend with PVC-PS leachates (r=0.5) with no influence of population size but only the Prim-Eff treatment was significantly higher than the influent samples. The PS-like leachates were related to HA/FA (r=0.5) and PsNPs (r=0.48). We examined the relationships between the PSNPs and PS-materials as determined by the Cu fluorescence quenching and solvatochromic NR assays in the attempt understand the degradation profile of nanoplastics and plastics materials in municipal wastewaters (Figure 2). The analysis revealed the PsNPs were significantly related to PS materials based on NR (r=0.48) and Cu fluorescence quenching (r=0.55). This suggests that the PS materials did not entirely originate from the nanoparticles in the effluents (i.e. contamination arise from a continuum of plastic micro/nanoparticles and smaller polymers) since we would have obtained a negative relationship. This was further confirmed with an analysis of covariance with the PsNPs as the covariate showing significance between the wastewater treatment types with PS and PVC/PS-materials.

The Perotox assay was used to screen in the municipal effluent extracts at 50 X concentration in the attempt to identify potentially toxic effluents (Figure 3). This assay is based on the reduction of Per activity, which was related to fish toxicity in a previous study with industrial effluents [16]. In respect to population size, smaller cities inhibited less Per activity (r=-0.46) suggesting a lower toxic potential as the population decreases (Figure 3). The Per activity was significantly decreased by nearly all treatment plants with the Prim-Eff, TF-Eff and SecA-Eff2 samples showing the strongest inhibitions. Since the exposure concentration of the extracts corresponded to 50 X concentration, fish toxicity should be confirmed for effluents for concentrations at lower than 100%. Correlation analysis revealed that the



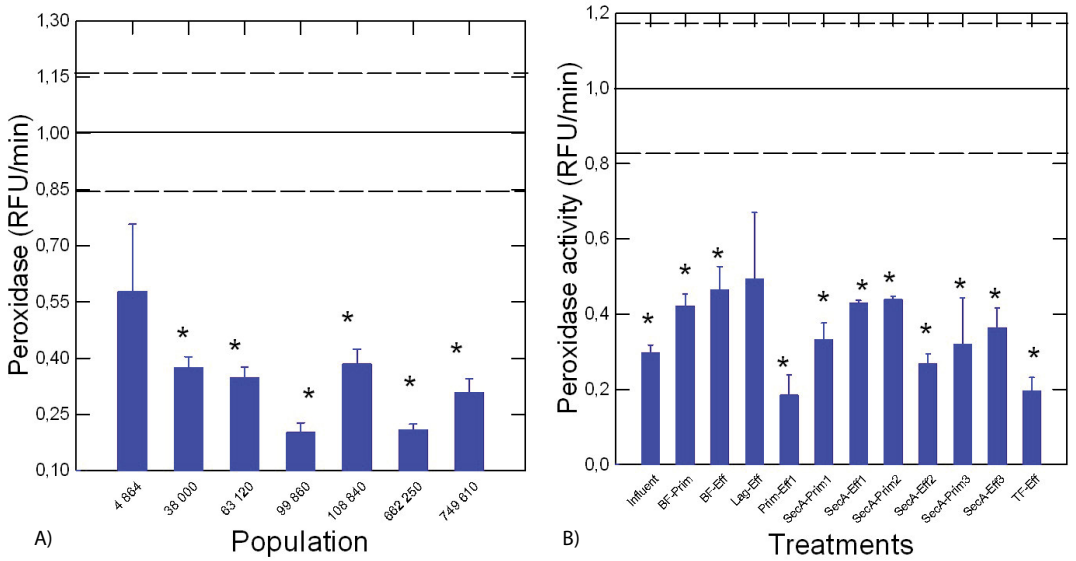
Note: The municipal wastewaters before and after various treatments were concentrated using C18 SPE and eluted with ethanol. The levels of nanoparticles (A, B), plastic materials (C, D and E, F by the Cu-quenching and NR methodologies respectively) were determined based on population density (untreated influents A, C, E) and various treatment processes (B, D, F). The data represent the mean with standard error. The star * symbol indicates significance in respect to untreated wastewaters (Influent).

Figure 1. Occurrence of plastic-like substances and nano plastics in municipal wastewaters.



Note: Regression analysis between the concentration of PSNPs and the levels of plastic like substances from NR solvatochromic (A) and the Cu quenching (B) methodologies.

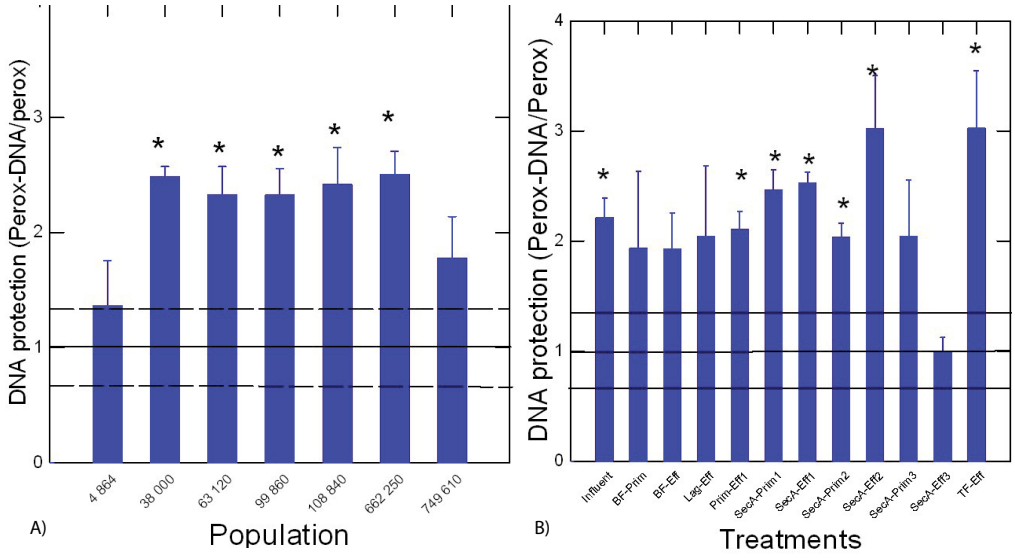
Figure 2. Relationship between PsNPs and plastic substances.



Note: The influents and effluents were extracted on a C18-SPE columns and eluted with ethanol (corresponding to 500X). The extracts (50 X) were then tested with the peroxidase assay for the untreated effluents (A) and different wastewater treatment (WWT) type (B). The solid lines correspond to the control activity of peroxidase with the dotted lines corresponding to normal variation of 15%. The star symbol * indicates significant difference from the control activity.

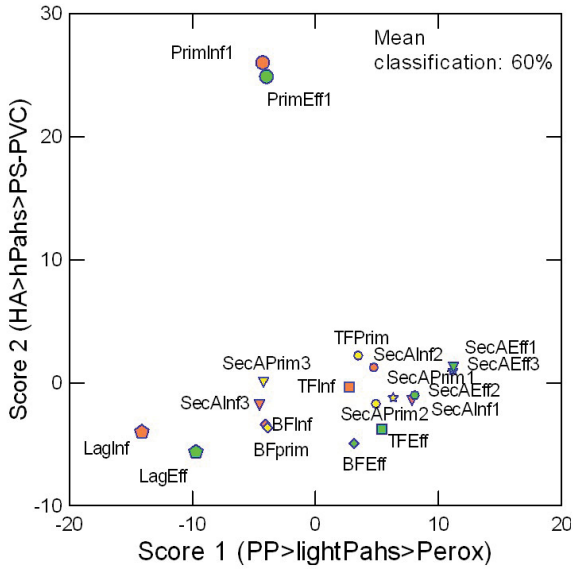
Figure 3. Peroxidase activity in municipal influents and effluents.

Per activity was negatively correlated with medium Pahs (r=-0.37) and PsNPs (r=-0.37). In the attempt to identify potential genotoxic effluents, the DNA protection assay was used during the Per activity assays (Figure 4). The principle of the assay resides in the measurement of Per activity in the presence of added DNA and the effluent extracts relative to the assay without added DNA where interactions with DNA prevent protect Per against inhibition. In respect to population size, DNA protection was found in 5/7 wastewaters but with a significant contribution with population size (Figure 4). For the treatment types,



Note: The influents and effluents were extracted on a C18-SPE column and eluted with ethanol (corresponding to 500X). The extracts (50 X) were then tested with the peroxidase assay in the presence of DNA for the untreated effluents (A) and different wastewater treatment (WWT) type (B). The DNA protection was defined as Peroxidase with DNA (0.1 µg/mL) /peroxidase without DNA. The solid lines correspond to the absence of effects of DNA and the dotted lines corresponding to normal variation of 15%. In B, the none treatment group consists of the mean value of untreated effluents in A. The star symbol * indicates significant difference from the control (absence of DNA effects).

Figure 4. DNA protection assay of the peroxidase activity



Note: In the attempt to determine the global effects of the various treatments on the observed effects, a discriminant function analysis was performed. The influents, primary step and the final effluents were denoted by dark red, yellow and green colors respectively.

Figure 5. Discriminant function analysis of municipal wastewaters quality.

significant protective effects were found in 7/12 (58%) suggesting that municipal effluents might have genotoxic activity relatively often (Figure 4). Correlation analysis revealed the DNA protection potential was correlated with Per (r=-0.65) and PsNPs (r=0.41) suggesting that the stronger inhibition of Per is associated to increased DNA protection and the presence of plastic nanoparticles.

A discriminant function analysis was performed to identify the most important descriptors allowing the best discrimination between the treatment types (Figure 5). The analysis permitted the correct classification in 60 % of the cases with the following endpoints: PP, light Pahs, Per, HA-FA, high Pahs and PVC-PS materials. The treatment process that separated the influents from the corresponding effluents were those using aeration and biological degradation: aerated lagoons, biological filtration and aeration lagoons with secondary UV. The first primary treatments of the wastewaters were generally closer to the corresponding influents, suggesting that removal of coarse suspended matter had minimal effects on water quality relative to untreated influents.

Discussion

The Perotox assay was initially developed to determine the quality of urban water and wastewaters [10, 12]. Initially, this assay was practiced directly on unfiltered waters to detect the presence of bacteria [11]. In principle, this assay could detect the chemical oxygen demand where radical scavengers would reduce H2O2 levels and/or directly inhibit the enzyme in vitro. It is noteworthy that inhibitions in Per activity occurred at threshold concentrations of wastewaters where fish mortality occurred making this a potential new alternative methodology (NAM) for rainbow trout [16]. In the present study, nearly all (92%) the tested wastewaters significantly inhibited Per activity suggesting a potential hazard, since the assay was run on concentrated extracts, to aquatic organisms by the accumulation of reactive oxygen species. However, given that the exposure extract concentration corresponding to 50 X of the wastewaters, toxicity in the original effluent (1X) are unlikely but raises a flag to potential toxic effects and should be checked directly in the wastewaters. Per inhibitions occurring at concentrations below 30% (0.3X) of the effluents were always toxic to rainbow trout [16]. Decreased Per activity would lead to sustained H2O2 levels, a powerful oxidant leading to damaged lipid (peroxidation), proteins (carbonylation) and DNA (8-hydroxyguanosine) [24, 25]. The negative correlation between PsNPs and Per activity suggests that plastics could also contribute to oxidative stress often observed in organisms exposed to municipal wastewaters. Decreased glutathione Per activity with increased superoxide dismutase and catalase were also observed in the liver of carp exposed to PsNPs resulting also to DNA damage [26]. The PsNPs were in the 20-30 nm diameter size range and negatively charged (R-COO-) at the surface. A meta-analysis on the oxidative damage of micro/nanoplastics in rodents revealed that these chemicals systematically reduce antioxidant defence mechanisms such as reduced Per, catalase and superoxide dismutase activities [27]. Oxidative stress from nanoplastics was also associated with DNA damage as well [28]. In the marine mussels Mytilus galloprovincialis exposed to plastic nanoparticles for 21 days, the antioxidant enzymes were overwhelmed leading to oxidative damage at the DNA level (DNA strand breaks formation) in both gills and digestive gland. A recent survey with microplastic particles in Canadian biosolids revealed substantial amounts of microplastics at 636 particles/g biosolids [6]. Fibers and fragments were the most common form of plastics in the biosolids. This suggests that biosolids could act as sink of microplastics in municipal wastewaters where degradation to smaller and more soluble nanoparticles might occur. This could also explain why in some cases plastic materials were more abundant in the treated effluents as for the Prim-Eff and SecA-Prim/Eff samples in the present investigation. Moreover, plastic materials were showed to induce biofilms (formation of gel-like polymeric extracellular substances) disturbing flocculation of bacterial in sludges and contribute to lower flocculation rates and persist longer as suspended matter [29]. The process involved the production of extracellular matrix by bacteria allowing them to adhere to plastic particles and reduce the settling of biosolids. In another study with tire related micro/nanoplastics, tire materials were shown to incorporate in the biomass in aerated lagoons and increased the release of soluble tire substances suggesting biodegradation [30]. Bacteria of the genera Rubrivivax, Ferruginibacter, and Xanthomonas where more abundant in the biomass. Hence, the increase in small PSNPs, PVC-PS and PP-substances observed in some effluents could be the result of increase biodegradation processes from the biomass.

The DNA protection assay is a simple means to evaluate the influence of DNA towards Per inhibition observed in environmental samples. DNA protection was coined from the increase in Per activity when DNA was added in the reaction mix and the effluents [16]. Moreover, DNA protection observed in industrial effluents was related to bacterial genotoxicity in most cases (70% of

the wastewaters were genotoxic to bacteria). Based on the present study, it appears that circa 60% of the wastewaters significantly increased DNA protection index suggesting common occurrence of genotoxic compounds in municipal effluents. In a previous study, acetone extracts of industrial effluents but not with raw sewage (influent) were genotoxic in Alliam root using the micronucleus and anaphase aberration assays [30]. This suggests that the industrial component of municipal wastewaters could drive genotoxicity in the extracts. Wastewaters for various industrial plants were able to produce DNA strand breaks in exposed Hep G2 cells further supporting the genotoxic potential of effluents [31]. However, the genotoxic potential of domestic waste contaminated surface waters (City of Montreal) was observed using a bacterial DNA repair test [9]. Municipal effluents from a primary effluent were also genotoxic to bacteria and calculations revealed that 90% of the genotoxicity was of domestic origin (non-industrial) suggesting that domestic and street runoffs accounted for most of the genotoxic potential. This study also suggested a strong relationship between genotoxicity and population. ANCOVA of the DNA protection index with the treatment processes with population size and organic carbon contents (covariables) revealed a significant influence of organic carbon and population size on DNA protection responses treatment processes. The Prim-effl sample, similar to the type used for the City of Montreal wastewater treatment, were among the highest DNA protection responses even after removing the contribution of population size and organic carbon contents by covariance analysis. This suggests that the genotoxic potential is inherently associated to the treatment processes applied to the influents in addition to population density and organic carbon contents. The genotoxicity evaluation of 2 municipal wastewater treatment stations to Tradescantia pallida plants revealed higher incidence of micronuclei [33]. They also observed that DNA damage followed the elevated concentrations of organic carbon/matter in the effluents. The increase in DNA damage in some effluents compared to the incoming influents was also observed previously [34]. The use of chlorination for disinfection and activated sludge treatment were found to increase the mutagenicity (Salmonella) and sister chromatid exchange (CHO cells) for all the 6 wastewater treatment plants. This was also observed in the present study where DNA protection index was higher at the secondary aeration sludge effluents (SecA-Eff) and the primary settling pond (SecA-Prim).

In a previous study with municipal effluent extracts, effluents produced by aerated sludge, aerated lagoons were less influenced by the incoming influents suggesting that aeration processes produce effluents differing the most from the influents [35]. In the present study, aerated lagoons (with/without secondary UV treatments) and biological filtration treatments produced effluents that differed the most from the influents as shown by discriminant function analysis (Figure 5). The data suggests that wastewater treatments involving biological and oxygenation processes were performing the best in changing the properties of the untreated waters. These influents contain relatively less plastic-related compounds, OC as HA/FA and Pahs.

Conclusion

A simple and inexpensive assay is presented to evaluate wastewater quality and potential toxicity. The Perotox assay is based on the Per reaction in the presence of proteins (albumin) and DNA. The inhibition of Per activity was found in most effluent C-18 extracts (92%) suggesting potentially toxicity related to the persistence of H202 leading to oxidative stress and damage. The DNA protection index is a measure the reversal of Per induced inhibition by DNA was found in nearly 60 % of the effluents suggesting that municipal effluents could damage DNA in the aquatic biota. The presence of plastic nanoparticles and related materials were detected in these extracts and were generally associated to Per inhibitions and DNA protection effects suggesting their contribution in part at least towards long-term toxic impacts of wastewaters. The study also revealed that effluents using biological and aeration processes produced the most difference following treatment of the influents and were generally less harmful based on the Perotox assay. The Perotox assay could also represent an interesting and cost-effective alternative to reduce fish use in effluent toxicity monitoring for the protection of aquatic life. Future directions of this research involve further investigations as a NAMs for fish testing of complex mixtures. The relationships between DNA protection assay and DNA damage/repair activity should be more closely examined as well to validate this assay towards genotoxicity in fish.

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