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Abstract

The reclamation and disinfection of waters impacted by human activities (e.g., wastewater effluent discharges) are of growing interest for various applications but has been associated with the formation of toxic nitrogenous disinfection byproducts (N-DBPs). Monochloramine used as an alternative disinfectant to chlorine can be an additional source of nitrogen in the formation of N-DBPs. Individual toxicity assays have been performed on many DBPs, but few studies have been conducted with complex mixtures such as wastewater effluents. In this work, we compared the cytotoxicity and genotoxicity of wastewater effluent organic matter (EfOM) before and after chloramination. The toxicity of chloraminated EfOM was significantly higher than the toxicity of raw EfOM, and the more hydrophobic fraction (HPO) isolated on XAD-8 resin was more toxic than the fraction isolated on XAD-4 resin. More DBPs were also isolated on the XAD-8 resin. N-DBPs (i.e., haloacetonitriles or haloacetamides) were responsible for the majority of the cytotoxicity estimated from DBP concentrations measured in the XAD-8 and XAD-4 fractions (99.4% and 78.5%, respectively). Measured DBPs accounted for minor proportions of total brominated and chlorinated products, which means that many unknown halogenated compounds were formed and can be responsible for a significant part of the toxicity. Other non-halogenated byproducts (e.g., nitrosamines) may contribute to the toxicity of chloraminated effluents as well.

Keywords

Disinfection byproducts, Chloramination, Toxicity, Wastewater, XAD resins, Haloacetonitriles, Haloacetamides

Introduction

The disinfection of waters impacted by human activities (e.g., agriculture or wastewater effluent discharges) has been associated with the formation of nitrogenous disinfection

42 byproducts (N-DBPs) due to their enrichment in nitrogen-containing compounds (e.g., ammonia
43 or organic nitrogen such as amino acids and peptides) (Bond et al., 2011; Westerhoff and Mash,
44 2002). N-DBPs generally form in lower concentrations than non-nitrogenous regulated DBPs
45 (i.e., trihalomethanes, THMs, and haloacetic acids, HAAs), but may present a higher health risk
46 (Muellner et al., 2007; Plewa et al., 2004). *In vitro* mammalian cell assays have demonstrated
47 that N-DBPs such as haloacetonitriles (HANs) (Muellner et al., 2007), halonitromethanes
48 (HNMs) (Plewa et al., 2004) and haloacetamides (HAcAms) (Plewa et al., 2007) exhibit orders
49 of magnitude higher levels of cytotoxicity and genotoxicity than THMs and HAAs (Plewa et al.,
50 2008). Reclaiming wastewater for various agricultural, industrial or municipal applications is of
51 growing interest but requires the practice of disinfection to prevent outbreaks of waterborne
52 diseases. Dissolved organic matter isolated from wastewater effluent (EfOM) was found to be
53 significantly more enriched in nitrogen (i.e., to be a potential source of N-DBPs) than organics
54 recovered from surface waters (e.g., N/C mass ratios up to 0.17 compared to 0.01-0.06 for river
55 waters) (Drewes and Croue, 2002; Le Roux et al., 2016; Zheng et al., 2014).
56 Moreover, the presence of bromide and iodide ion in wastewater (especially at locations where
57 potable water is produced through the desalination of seawater or brackish water) favors the
58 formation of brominated and iodinated byproducts that are often more toxic than their
59 chlorinated analogues (Plewa et al., 2008; Richardson et al., 2008, 2007). Brominated and
60 iodinated N-DBPs are among the most cytotoxic/genotoxic disinfection by-products known
61 today (Muellner et al., 2007; Plewa et al., 2008). Water utilities, especially in the USA, have
62 been increasingly switching chlorine disinfection to monochloramine to reduce the concentration
63 of regulated THMs and HAAs (U.S. Environmental Protection Agency, 2006), however,
64 monochloramine can be an additional source of nitrogen in the formation of N-DBPs (Kimura et

al., 2013; Le Roux et al., 2016). Chloramines can also be formed unintentionally from the reaction between free chlorine and ammonia during chlorination, which may increase the risk of N-DBP formation when high ammonia concentrations are present.

While toxicity assays have been conducted for many individual DBPs, few studies have been performed with complex mixtures such as natural waters, drinking waters or wastewater effluents. Many of the >500 DBPs reported in the literature were not analyzed for toxicological effects (Richardson and Postigo, 2015). Similarly, many studies characterized DBP occurrence from various sources and their formation conditions, but the evaluation of DBP formation in conjunction with toxicity assays has not been extensively explored. Richardson (2011) published a protocol for DBP extraction, analysis and toxicity assessment, consisting in the extraction of disinfected waters by XAD resins (XAD-8 and XAD-2 in series), followed by an elution with ethyl acetate. The extract is then either directly analyzed by gas chromatography coupled with mass spectrometry (GC-MS) or evaporated and exchanged to dimethylsulfoxide (DMSO) for further genotoxicity/cytotoxicity analyses. This method has been used for swimming pool waters (Liviac et al., 2010; Plewa et al., 2011; Richardson et al., 2010) and drinking waters disinfected with chlorine, ozone or chlorine dioxide (Jeong et al., 2012) and was recently applied to disinfected (i.e., chlorinated and ozonated) wastewater effluents (Dong et al., 2016). N-DBPs are compounds of interest because of their potential toxicity, and the chloramination of EfOM is expected to favor the production of this class of DBPs because nitrogen can be incorporated both from monochloramine and from the nitrogenous moieties present in EfOM.

As a result, the aim of this work was (i) to compare the cytotoxicity and genotoxicity of EfOM resin isolates recovered before and after chloramination, (ii) to analyze the toxicity of resin extracts obtained from chloraminated wastewater effluent in relation with the formed DBPs, and

88 (iii) to estimate the contribution of N-DBPs to the toxicity of chloraminated wastewater
89 effluents.
90
91 **1. Materials and methods**
92 **1.1. Materials**
93 Analytical or laboratory grade reagents and were used without further purification. MilliQ
94 water was produced with a Millipore system (18.2 MΩ.cm). Sodium hypochlorite (NaOCl, 5.65-
95 6%, Fisher Scientific) and ammonium chloride (Acros Organics, 99.6%) were used to prepare
96 chloramine solutions. Methyl tert-butyl ether (MTBE) and ethyl acetate (> 99%, Fisher
97 Scientific) were used for DBP extractions without further purification. A THM calibration mix
98 (chloroform - TCM, dichlorobromomethane - $CHCl_2Br$, chlorodibromomethane - $CHClBr_2$, and
99 bromoform - TBM), a mixed standard (EPA 551B Halogenated Volatiles Mix) containing
100 HANs, trichloronitromethane (TCNM, or chloropicrin) and haloketones (HKs), and a mixed
101 standard containing 9 HAAs (EPA 552.2 Methyl Ester Calibration Mix) were supplied from
102 Supelco (Sigma-Aldrich). Chloro-, bromo-, dichloro-, and trichloroacetamide (CAcAm, BAcAm,
103 DCAcAm and TCAcAm, respectively) were obtained from Sigma-Aldrich. Other HAcAms (i.e.,
104 dibromoacetamide - DBAcAm, tribromoacetamide - TBAcAm, bromochloroacetamide -
105 BCAcAm, chloroiodoacetamide - CIAcAm, bromoiodoacetamide - BIAcAm and
106 diiodoacetamide - DIAcAm) were purchased from Cansyn Chem. Corp. Haloacetaldehydes
107 (HAcAls) were obtained from TCI America, Cansyn Chem. Corp. and Sigma-Aldrich.
108 Decafluorobiphenyl (99%, Sigma-Aldrich, Supelco) was used as a surrogate standard. 2
109 bromopropionic acid (Fluka Analytical) was used as a surrogate for HAA extractions and
110 analyses.

1.2. Analytical methods

Total Organic Carbon (TOC) and Total Nitrogen (TN) concentrations were measured using a TOC analyzer equipped with a TN detection unit (TOC-VCSH, Shimadzu). Three-dimensional fluorescence excitation - emission matrices (EEMs) were obtained by a Fluoromax fluorometer (Horiba Scientific, Japan). Samples for Adsorbable Organic Halide (AOX) analyses were processed through adsorption on activated carbon columns using a TOX sample preparatory unit (TXA-03, Mitsubishi Chemical Analytech Co., Ltd., Japan). AOX were then transformed into hydrogen halides by combustion (950 °C) of the activated carbon for at least 30 min via an AOX-200 adsorbable halogen analyzer and then collected in Milli-Q water as chloride and bromide ions. Offline quantification of chloride and bromide ions was performed by a Dionex 1600 reagent free ion chromatograph (IC) equipped with a conductivity detector and a Dionex IonPac AS-15 column (2 × 250 mm) and using an online KOH eluent (30 mM) generator at a flow rate of 0.4 mL/min. AOCl and AOBr concentrations were determined from the respective Cl^- and Br^- concentrations. Free chlorine and total chlorine concentrations in the sodium hypochlorite stock solutions were determined by spectrophotometric measurement at 292 nm. NH_2Cl and $NHCl_2$ concentrations in stock solutions were determined by spectrophotometric measurement using their respective molar extinction coefficients at 245 nm and 295 nm. Residual oxidant during chloramination reactions was analyzed by DPD colorimetric method. Four THMs, four HANs, two HKs, chloropicrin and seven HAcAls were extracted and analyzed following EPA method 551, which consists of a liquid-liquid extraction using MTBE followed by gas chromatography coupled with electron capture detector (GC-ECD) or GC-MS (Munch and Hautman, 1995). Nine HAAs were extracted and analyzed following EPA method 552.2,

which is based on a liquid-liquid extraction with MTBE in acidic conditions followed by derivatization to methyl esters using acidic methanol, and analysis by GC-MS (Munch and Munch, 1995). HAcAms were analyzed following the same EPA method 551 protocol, replacing MTBE by ethyl acetate for the liquid-liquid extraction. Samples were also analyzed in GC-MS full scan mode to search for other DBPs and unknown mass spectra were subjected to library database searching (National Institute of Standards and Technology - NIST).

1.3. Chloramination conditions

Monochloramine stock solutions were prepared by adding sodium hypochlorite (NaOCl) to a continuously-stirred ammonium chloride solution adjusted to pH 8.5 with sodium hydroxide, at a Cl:N molar ratio of 1:1.4. The concentration of monochloramine stock solutions was adjusted to a desired concentration. Chloramination reactions were performed at pH 7.7 without addition of buffering agents, and doses were calculated as: NH_2Cl (mg/L as Cl_2) = 3 × DOC (dissolved organic carbon, mg C/L) (Dotson et al., 2009; Krasner et al., 2007). Preformed monochloramine was used to avoid breakpoint reactions that could occur with residual ammonium concentration of the effluent (0.2-1 mgN/L after prolonged aeration activated sludge). The pH remained stable (7.7 ± 0.5) during all the reaction time (72 hr). At the end of the contact time, samples were acidified at pH 2 with concentrated HCl and immediately subjected to the XAD resins extraction protocol. No additional quenching was performed in order to preserve the disinfection byproducts formed (no residual oxidant was measured after acidification), and samples were directly passed through XAD resins for fractionation and extraction.

1.4. Effluent organic matter (EfOM) extraction procedure

157 Wastewater secondary effluent samples were collected at three different periods (WW1, 2 and
158 3, Table 1) from a wastewater treatment plant (Jeddah, Saudi Arabia) before chlorine
159 disinfection. Samples were transferred to the laboratory, filtered on cartridge filters (Polygard®
160 10 µm pore size, Millipore) prior to the chloramination and fractionation experiments to remove
161 any particulate matter and stored at 4 °C for maximum 7 days. The hydrophobic (HPO) and
162 transphilic (TPI) fractions of the non-chloraminated and chloraminated (laboratory conditions)
163 wastewater EfOM were isolated using a comprehensive isolation protocol described elsewhere
164 (Croué, 2004; Leenheer et al., 2000). Briefly, wastewater effluent samples (before or after 72 hr
165 chloramination) were acidified at pH 2 with hydrochloric acid and passed through XAD-8 and
166 XAD-4 macroporous resins in series at a flow rate of 5.2 L/hr. Resins were then rinsed with
167 formic acid at pH 2 in order to remove the inorganic species present in the void volume without
168 eluting the organics adsorbed onto the resins, which were recovered afterward with either
169 acetonitrile/water (75:25 v/v), acetonitrile or ethyl acetate. During the adsorption phase, 100 mL
170 samples were collected from the output of the resins for DOC and DBP measurements. For
171 effluent WW1, organics adsorbed onto the two XAD resins and eluted with the same volume of
172 acetonitrile/water were concentrated using a rotary evaporator to remove acetonitrile. Equal
173 volumes (50:50 v/v) of the fractions obtained from the two resins were also combined before the
174 rotary evaporation step to obtain a mixed XAD-8+XAD-4 extract. The water concentrates were
175 lyophilized to obtain the XAD-8, XAD-4 and mixed XAD-8+XAD-4 solid organic fractions for
176 use in toxicity analyses. For effluent WW2, the elution of both resins was performed with pure
177 acetonitrile to perform direct DBP identification by full-scan GC-MS on a small volume (i.e., 1.5
178 mL) of the extracts. Milli-Q® water was then added to the acetonitrile extracts to reach 75:25 v/v
179 acetonitrile/water condition (same as used for effluent WW1) before rotary evaporation (removal

of acetonitrile) and lyophilization. With this approach the cytotoxicity and genotoxicity values of samples WW1 and WW2 could be expressed as per mg of EfOM. For effluent WW3, both resins were eluted with ethyl acetate and the chloraminated EfOM extracts were concentrated under a stream of N_2 to a final volume of 100 mL to allow the direct quantification of DBPs and the analysis of toxicity from the same extract, as previously described in the literature (Jeong et al., 2012; Richardson, 2011). It should be noted that these concentration approaches (i.e., lyophilization or N_2 evaporation) are usually suitable for semi- or non-volatile chemicals but not for volatile compounds (Weinberg, 2009). Nevertheless, volatile DBPs (e.g., THMs) analyzed in the ethyl acetate extracts after concentration were in accordance with concentrations analyzed in the wastewater effluent samples (see Section 2.3.4). Since the two chloraminated wastewater effluents (WW2 and WW3) were not sampled at the same time (and exhibited different DOC values) and fractions were obtained through different processes (as described above), the toxicity results of these two types of samples were not comparable.

1.5. Analytical biology

1.5.1. Sample preparation

The lyophilized or liquid organic fractions were received and stored at −20 °C. Mg amounts of each lyophilized fraction were placed into separate sterile Supelco sample vials. To sterilize the sample, DMSO was added to each sample such that for each mg of sample 1 µL of DMSO was added. After the sample was mixed (vortexed) in DMSO, 1 µL of sterile ddH_2O was added for each mg of the lyophilized fraction. The stock solution for each sample was completely dissolved in this DMSO-water (1:1 v/v) solvent and this was stored at −20°C. Ethyl acetate organic extracts (liquid form) obtained from WW3 were solvent exchanged into DMSO so that

the organic material from 1 L of wastewater effluent was concentrated into 10 µL of DMSO ($10^5\times$ concentration) and stored in Teflon-sealed sample vials at −20°C. For each experiment a 50× dilution of each sample stock solution was prepared in F12 cell culture medium+5% fetal bovine serum (FBS).

1.5.2. Chinese hamster ovary cells

Chinese hamster ovary (CHO) cell line AS52, clone 11−4−8 was used for the biological assays (Wagner et al., 1998). CHO cells were maintained on glass culture plates in Ham's F12 medium containing 5% FBS, 1% antibiotics (100 U/mL sodium penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37 °C in a humidified atmosphere of 5% CO_2.

1.5.3. CHO cell chronic cytotoxicity assay

This assay measures the reduction in cell density on flat-bottom 96-well microplates as a function of the concentration of the test sample over a period of approximately 72 hr (~3 cell cycles). Microliters of the sample were diluted with F12 +FBS medium to analyze a range of concentration factors. This assay was calibrated; the detailed procedure was published (Plewa and Wagner, 2009). For each sample concentration factor, 4-8 replicate wells were analyzed. The experiments were repeated 2-3 times. A concentration-response curve was generated for each sample. The LC_{50} values were calculated from the regression analysis and represent the sample concentration factor that induced a 50% reduction in cell density as compared to the concurrent negative controls. The cytotoxicity index (CTI) value was a metric that expressed increasing values with increased cytotoxic damage (LC_{50}^{-1})(10^3) and allowed for easy comparisons among the wastewater samples isolated in the same conditions.

1.5.4. CHO cell single cell gel electrophoresis assay

Single cell gel electrophoresis (SCGE, or Comet) assay quantitatively measures genomic deoxyribonucleic acid (DNA) damage in individual nuclei induced by a test agent (Tice et al., 2000). We employed a microplate methodology (Wagner and Plewa, 2009). The SCGE metric for genomic DNA damage was the %Tail DNA value which is the amount of DNA that migrated from the nucleus into the microgel (Kumaravel and Jha, 2006). Within each concentration factor range with >70% cell viability, a concentration-response curve was generated and regression analysis was used to fit the curve. The concentration factor inducing a 50% Tail DNA (TDNA) value was calculated using a regression analysis for each concentration-response curve. The genotoxic index (GTI) was a metric that expressed increasing values with increased genotoxic damage ($50\%\ TDNA^{-1}$)(10^3) and allowed for easy comparisons among the evaluated wastewater samples.

1.5.5. Statistical analyses

For the cytotoxicity assay, a one-way analysis of variance (ANOVA) test was conducted to determine if the sample induced a statistically significant level of cell death as compared to the concurrent negative controls at a specific concentration factor. If a significant F value ($P \leq 0.05$) was obtained, a Holm-Sidak multiple comparison versus the control group analysis was performed to identify the lowest cytotoxic concentration factor. The power of the test statistic ($1-\beta$) was maintained as ≥ 0.8 at $\alpha = 0.05$. For the SCGE assay, the %Tail DNA values were not normally distributed which limits the use of parametric statistics (Box et al., 1978). The mean %Tail DNA value for each microgel was calculated and these values were averaged among all the microgels for each wastewater sample concentration factor. Averaged mean values express a normal distribution according to the central limit theorem. An ANOVA test was conducted on

these averaged %Tail DNA values (Lovell and Omori, 2008). If a significant F value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted (power ≥ 0.8; $\alpha = 0.05$).

2. Results and discussion

2.1. EfOM extraction recovery

The EfOM extraction conditions and recovery for the three different batches of wastewater effluents (WW1, 2 and 3) are presented in Table 1 and 2.

Table 1. Summary of experiments and extraction conditions

Wastewater effluent sample	Chloramination	Extracted volumes (L)	DOC before / after chloramination (mg C/L)	Elution solvent	Fractions phase
WW1	No	150	4.65 / N.A.	Acetonitrile/H_2O	Solid
WW2	Yes	69	4.68 / 4.55	Acetonitrile	Solid
WW3	Yes	76	4.97 / 4.60	Ethyl acetate	Liquid

N.A. = Not Applicable; DOC: dissolved organic carbon

The first experiment consisted in the extraction of 150 L non-chloraminated wastewater effluent (WW1, 4.65 mg C/L) followed by an elution with acetonitrile/H_2O (75:25, v/v). 69.5% DOC was retained on both XAD resins (2.13 mg C/L on XAD-8, 1.10 mg C/L on XAD-4) (Table 2). 1.42 mg C/L was measured at the output of the XAD-4 resin (consisting in the hydrophilic fraction of EfOM). The second extraction was performed with 69 L wastewater effluent (WW2, 4.68 mg C/L) after 72 hr of chloramination (14.2 mg Cl_2/L as preformed monochloramine, pH 7.7), followed by an elution with acetonitrile (no water was added to be

265 able to analyze the recovered fractions by GC-MS, see Section 2.3.1). Less DOC (i.e., humic-
266 like substances) was adsorbed onto the XAD-8 resin (1.56 mg C/L) than from the non-
267 chloraminated effluent (2.13 mg C/L), while WW1 and WW2 showed similar DOC content (4.65
268 and 4.55 mg C/L, respectively). Similar amount of DOC was adsorbed onto the XAD-4 resin,
269 i.e., 1.10 and 1.05 mg C/L for WW1 and WW2, respectively. The observed lower retention of
270 chloraminated EfOM onto the XAD-8 resin could be attributed to the breakdown of large organic
271 macromolecules (i.e., humic-like substances) into smaller hydrophilic molecules during
272 oxidation by chloramines. Accordingly, fluorescence EEMs obtained before and after
273 chloramination of wastewater effluent WW2 (Fig. 1) exhibited a decrease of 26.5% in signal
274 intensity after chloramination at the wavelengths showing the most intense response (i.e.,
275 excitation: 350 nm, emission: 420 nm), indicating that EfOM degradation occurred through the
276 elimination of aromatic structures that usually exhibit fluorescence signal at those wavelengths
277 (Chen et al., 2003). The third extraction was performed with 76 L of wastewater effluent (WW3,
278 4.97 mg C/L) after 72 hr of chloramination (14.9 mg Cl_2/L, pH 7.7) followed by an elution with
279 ethyl acetate. As observed for WW2, the DOC content of WW3 slightly decreased after
280 chloramination (i.e., 4.97 vs 4.60 mg C/L). The total amount of DOC adsorbed onto the two
281 XAD resins (64.6%) was comparable to WW1 and WW2.
282
283 Table 2. Percentages of dissolved organic carbon (DOC) isolated on XAD-8 and XAD-4 resins
284 from wastewater effluent and chloraminated wastewater effluent (72 hr chloramination at 14.2
285 mg Cl_2/L and at pH 7.7)

Wastewater effluent sample	DOC before extraction (mg C/L)	% DOC isolated on XAD-8 resin	% DOC isolated on XAD-4 resin	% DOC not isolated

WW1 (Non-chloraminated)	4.65	45.8	23.7	30.5
WW2 (Chloraminated)	4.55	34.3	23.1	42.6
WW3 (Chloraminated)	4.60	42.4	22.2	35.4

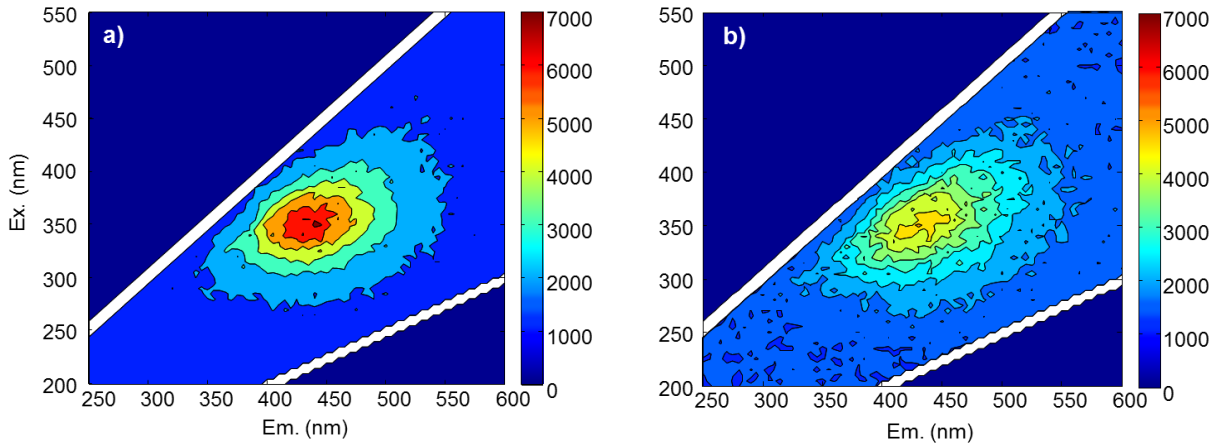


Fig. 1. Fluorescence EEMs of a) wastewater effluent WW2 and b) wastewater effluent WW2 after 72h chloramination (14.2 mg Cl_2/L) at pH 7.7

2.2. Analytical biology results

2.2.1. CHO cell cytotoxicity results

From the data presented in Table 3 and Fig. 2, the XAD-8 EfOM isolate recovered from non-chloraminated wastewater was more cytotoxic than the XAD-4 EfOM extract of the same wastewater. According to the isolation protocol used, hydrophobic EfOM (i.e., XAD-8 isolate, humic substances-like) was more cytotoxic than transphilic EfOM (i.e., XAD-4 isolate). The mixed XAD-8+XAD-4 fraction (50:50 v/v) exhibited an intermediate cytotoxicity, consistent with its composition (see Section 1.4) and with the toxicity of the XAD-8 and XAD-4 fractions. The structural characterization performed on similar isolates obtained from the effluent collected from the same wastewater treatment plant by Zheng et al. (2014) indicated that the XAD-8

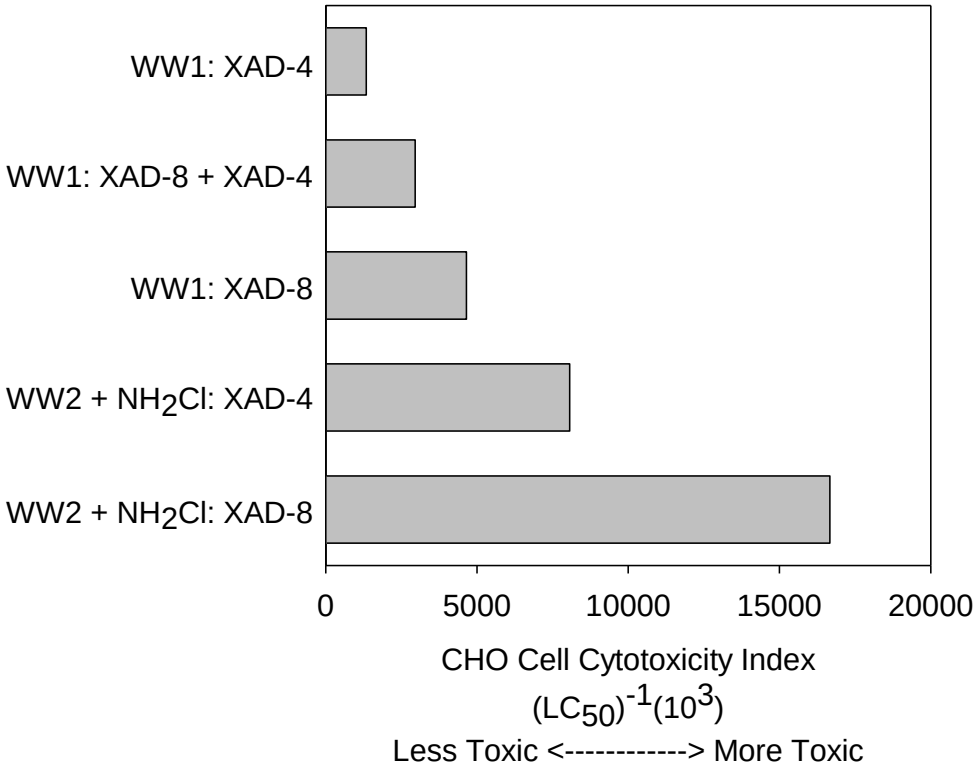
isolate was more enriched in aromatic structures including phenolic moieties than the XAD-4 extract that also incorporated carbohydrates. From the chloraminated wastewater effluent WW2, the XAD-8 extract was also more cytotoxic than the XAD-4 extract, and was significantly more cytotoxic than the non-chloraminated XAD-8 extract (WW1). The XAD-4 extract of WW2 was more cytotoxic than that of WW1 as well. This indicates a significant increase in cytotoxicity of the XAD-8 and XAD-4 extractable EfOM after chloramination. Since the amount of DOC isolated on each resin was lower from the non-chloraminated effluent than from the chloraminated one (i.e., 69.5 and 57.4%, respectively), the cytotoxicity of the chloraminated effluent was even higher when normalized to the total amount of DOC extracted on each resin (Appendix A, Fig. S1). Cytotoxicity results of chloraminated effluent WW3 obtained from ethyl acetate extracts (liquid form) exhibited a greater difference between XAD-8 and XAD-4 EfOM toxicity (i.e., XAD-8 EfOM was 7.6× more cytotoxic). These results are further discussed in paragraph 3.3.4.

Table 3. Comparative CHO cell chronic cytotoxicity of EfOM fractions isolated from wastewater effluents before (WW1) and after (WW2) chloramination.

Sample	Fraction	Conc. Range (µg/µL)	Chronic Cytotoxicity (LC_{50}) (µg/µL) [a]	r^2 [b]	Lowest Significant Toxic Concentration (µg/µL) [c]	ANOVA Test Statistic [d]
WW1	XAD-8	0.05-0.75	0.215	0.99	0.15	$F_{10, 50} = 144.2$: $P \leq 0.001$
WW1	XAD-4	0.05-1.15	0.746	0.99	0.35	$F_{10, 55} = 57.6$: $P \leq 0.001$
WW1	XAD-8 + XAD-4	0.05-0.75	0.338	0.99	0.20	$F_{10, 51} = 174.1$: $P \leq 0.001$
WW2 (+NH_2Cl)	XAD8	.005-0.4	0.060	0.97	0.025	$F_{10, 53} = 81.1$: $P \leq 0.001$
WW2	XAD-4	0.05-0.5	0.124	0.99	0.05	$F_{10, 53} = 105.0$: $P \leq 0.001$

(+NH_2Cl)

CHO: Chinese Hamster Ovary; EfOM: effluent organic matter; ANOVA: analysis of variance.
[a] The LC_{50} value is the concentration of the wastewater EfOM sample (as μg/μL), determined from a regression analysis of the data, that induced a cell density of 50% as compared to the concurrent negative controls. [b] r^2 is the coefficient of determination for the regression analysis upon which the LC_{50} value was calculated. [c] Lowest cytotoxic concentration was the lowest concentration of the wastewater EfOM sample in the concentration-response curve that induced a statistically significant reduction in cell density as compared to the concurrent negative controls. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.



315

316 Fig. 2. Comparison of the CHO cell cytotoxicity index values for each fraction of non-

317 chloraminated wastewater effluent (WW1) and chloraminated wastewater effluent (WW2).

318 XAD-8+XAD-4 fraction from WW1 was obtained by mixing equal volumes of XAD-8 and

319 XAD-4 fractions (50:50 v/v). CHO: Chinese hamster ovary.

320

321 **2.2.2. CHO cell genotoxicity results**

322 The results of the genomic DNA damage analyses are presented in Table 4 and the genotoxicity
323 index (GTI) values are presented in Fig. 3. The GTI is defined as (50%Tail DNA^{-1})(10^3) and the
324 larger the GTI value the greater the genotoxic damage.

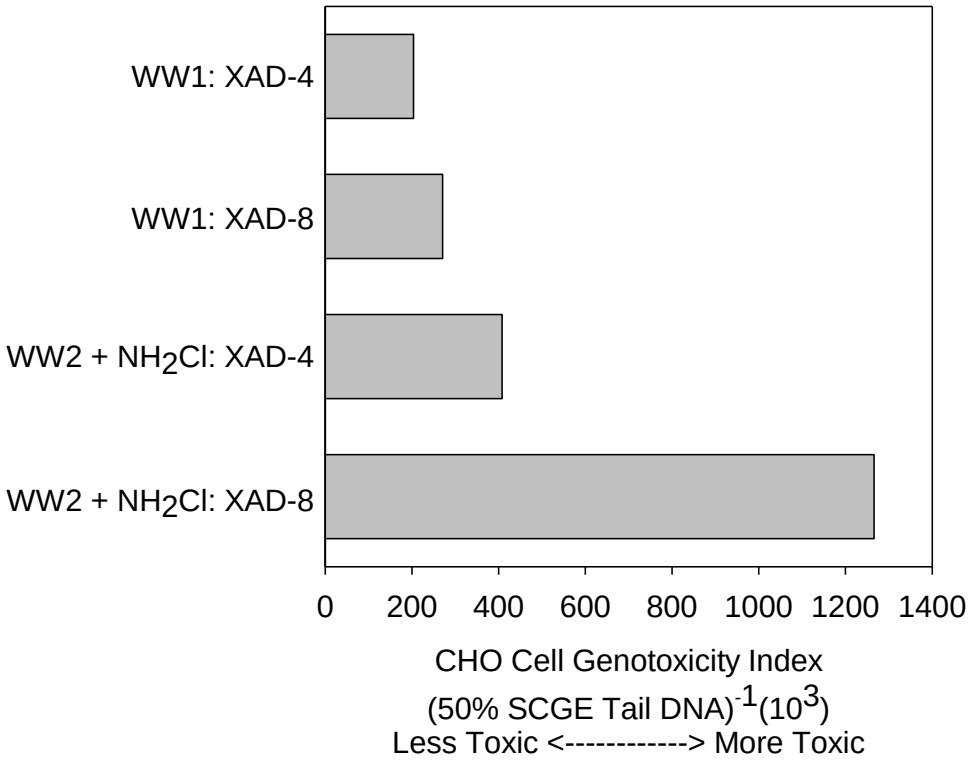
325

326
327 Table 4. Comparative CHO cell genotoxicity of EfOM fractions isolated from wastewater
328 effluents before (WW1) and after (WW2) chloramination.

Sample	Fraction	Conc. Range (μg/μL)	Acute Genotoxicity (50% Tail DNA) μg/μL[a]	r^2 [b]	Lowest Significant Toxic Concentration (μg/μL) [c]	ANOVA Test Statistic [d]
WW1	XAD-4	0.3-5.0	4.91	0.98	4.25	$F_{8, 23} = 23.0$: $P \leq 0.001$
WW1	XAD-8	0.2-4.0	3.69	0.99	2.5	$F_{9, 28} = 49.0$: $P \leq 0.001$
WW2+NH_2Cl	XAD-4	2.0-3.4	2.45	0.98	2.0	$F_{11, 28} = 153.0$: $P \leq 0.001$
WW2+NH_2Cl	XAD-8	0.075-0.9	0.79	0.96	0.5	$F_{10, 28} = 20.8$: $P \leq 0.001$

CHO: Chinese hamster ovary; EfOM: effluent organic matter; ANOVA: analysis of variance. [a] The SCGE 50% Tail DNA value is the EfOM sample concentration (as μg/μL), determined from a regression analysis of the data, that induced the migration of 50% SCGE Tail DNA value. [b] r^2 is the coefficient of determination for the regression analysis upon which the SCGE 50%Tail DNA value was calculated. [c] The lowest genotoxic concentration was the lowest concentration of the wastewater sample in the concentration-response curve that induced a statistically significant amount of genomic DNA damage as compared to the negative control. [d] The degrees of freedom for the between-groups and residual associated with the calculated *F*-test result and the resulting probability value.

329



330

331 Fig. 3. Comparison of the CHO cell genotoxicity index values for each fraction of non-
332 chloraminated wastewater effluent (WW1) and chloraminated wastewater effluent (WW2).
333 CHO: Chinese hamster ovary.

334

335 The genotoxicity data indicate that the wastewater EfOM isolated from the XAD-8 resin was
336 more genotoxic than the XAD-4 extracts of the same wastewaters. This suggests that
337 hydrophobic EfOM comprised more potent inducers of genomic DNA damage than transphilic
338 EfOM. Chloramination had a major effect on the resulting genotoxicity of the XAD-8 extracted
339 organic material, significantly more that for the XAD-4 isolate. These data indicate that the
340 chloraminated EfOM extracted over XAD-8 or XAD-4 resins were more genotoxic than non-
341 chloraminated EfOM.

342

2.3. DBP formation

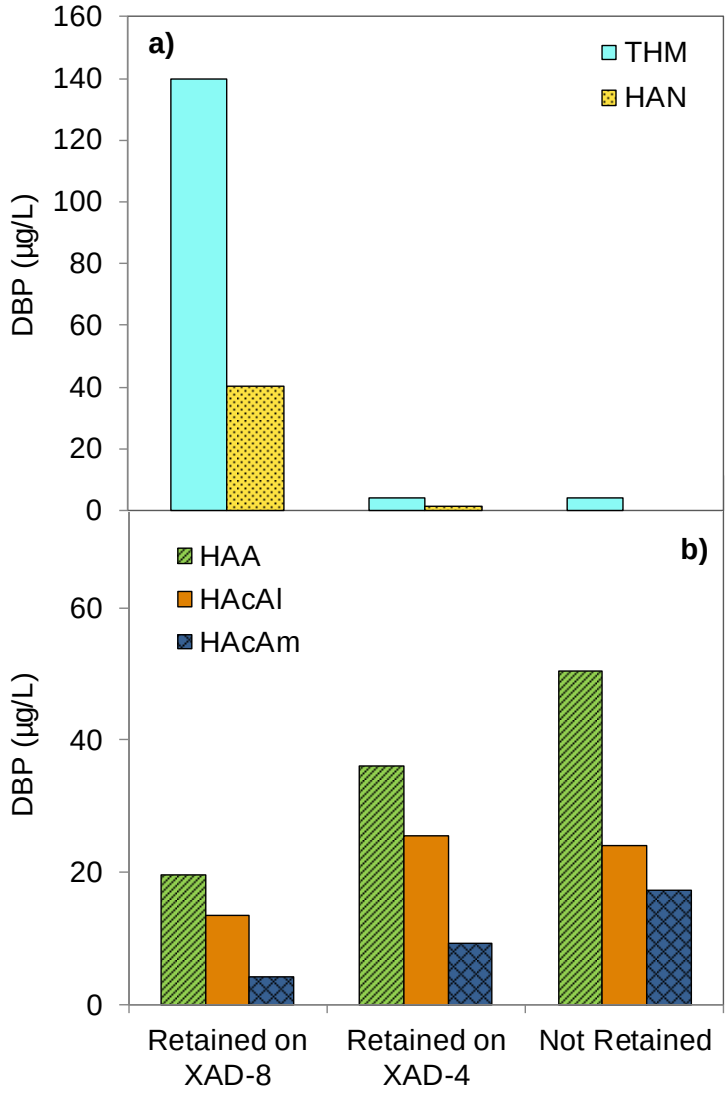
2.3.1. DBP identification

A small volume of the acetonitrile extracts obtained from the two resins that received the chloraminated wastewater effluent WW2 was evaporated under a stream of N_2 and transferred to ethyl acetate for direct full-scan GC-MS analyses in order to characterize the main products formed by chloramination and retained onto XAD resins. Tribromoacetaldehyde (TBAcAl) was the most predominant identified peak in the chromatograms (Appendix A, Fig. S2). TBM, DBAcAm, dibromoacetaldehyde (DBAcAl), chlorodibromoacetaldehyde (CDBAcAl), BIAcAm, and BCAcAm were also present in the chromatograms. Many other brominated and chlorinated peaks were detected but could not be identified by direct comparison of mass spectra with NIST database. Brominated species were predominant because of the presence of bromide ion in the wastewater effluent (2.52 mg/L as Br^-), and some iodinated products (e.g., BIAcAm) were detected in the XAD-8 fraction (though iodide ion could not be detected by IC analysis because of matrix effects). Some of these brominated and iodinated DBPs (i.e., TBAcAl, DBAcAl, BIAcAm) are among the most cytotoxic and genotoxic DBPs identified today (Plewa et al., 2008). DBPs and AOX were not quantified during this experiment, and lyophilized fractions were only analyzed for cytoxicity and genotoxicity.

2.3.2. DBP quantification and estimated retention on XAD resins

During the XAD resins extraction conducted on WW3 (initial DOC 4.97 mg C/L, 14.9 mg Cl_2/L), aqueous samples were collected before and after each XAD resin (Appendix A, Table S1) in order to quantify THMs, HAAs, HANs, HAcAms and HAcAls and evaluate the retention of each class of DBPs onto both resins (calculated as the difference between DBP concentrations at the inlet and at the outlet of a resin column). Results indicated a high retention of total THMs

and HANs on XAD-8 resin (94% and 97%, respectively), while HAAs, HAcAls and HAcAms exhibited a poor retention on both resins (Fig. 4). The hydrophobic character of DBP classes are in the order HAcAm < HAA < HAcAl < HAN < THM when estimating logD values at pH 2 for both chlorinated and brominated species using ChemAxon Marvin Suite. The XAD-8 resin is known to adsorb less hydrophilic molecules with higher molecular weights than molecules retained on the XAD-4 resin. This is in agreement with the retention of more hydrophobic DBPs (THMs and HANs) on the XAD-8 resin, possibly because they may exhibit stronger affinity with the hydrophobic macromolecules retained on this resin, rather than because of their direct adsorption on the resin. More hydrophilic compounds were better isolated on the XAD-4 resin (as part of the transphilic/TPI fraction) or not retained by both resins (i.e., hydrophilic/HPI fraction).



379

380 Fig. 4. Retention of a) THMs and HANs and b) HAAs, HAcAl and HAcAms onto XAD-8 and
381 XAD-4 resins used in series. DBP concentrations are expressed as the total sum of each
382 individual DBP in a given class of DBP. THMs: trihalomethanes; HANs: haloacetonitriles;
383 HAAs: haloacetic acids; HAcAls: haloacetaldehydes; HAcAms: haloacetamides; DBP:
384 disinfection byproduct.

385

386 **2.3.3. DBP contributions to AOX**

AOBr and AOCl concentrations in chloraminated wastewater effluent were 1658.2 µg/L as Br and 1078.9 µg/L as Cl, which is in accordance with the presence of a majority of brominated DBPs detected in full-scan chromatograms. The proportions of AOX (AOCl and AOBr) retained on each resin were calculated in the same manner than individual DBPs (i.e., by difference between inlet and outlet concentrations). AOBr and AOCl were mostly retained on the XAD-8 resin (748.7 µg/L as Br and 710.8 µg/L as Cl), compared to the XAD-4 resin (563.8 µg/L as Br and 209.6 µg/L as Cl), which may explain the higher toxicity observed in the isolated XAD-8/hydrophobic (HPO) fraction than that of the XAD-4/transphilic (TPI) fraction (Fig. 5). Overall, 85.3% of AOCl and 79.2% of AOBr were retained on both resins. All analyzed DBP concentrations were expressed in µg/L as Cl (Cl-DBP) or µg/L as Br (Br-DBP) to obtain their relative contribution to AOCl and AOBr concentrations (Fig. 5). Unknown AOCl (UAOCl) and unknown AOBr (UAOBr) were determined from the differences between measured AOCl or AOBr and the sum of chlorine or bromine-equivalent concentrations of measured specific DBPs. Br-DBPs contributed to 23.1% of AOBr retained on the XAD-8 resin and to 9.2% of AOBr retained on the XAD-4 resin. Bromoform, DBAN and DBCM were the three major Br-DBPs contributing to AOBr retained on the XAD-8 resin, while DBAA, CDBAcAl and DBAcAm were the major Br-DBPs retained on the XAD-4 resin. Br-DBPs present in the fraction not retained onto the two resins exhibited concentrations of the same order of magnitude of Br-DBPs retained onto the XAD-4 resin. Cl-DBPs only represented 2.2% and 4.5% of AOCl retained on XAD-8 and XAD-4 resins, respectively, which indicates that a majority of chlorine-containing compounds were not quantified.

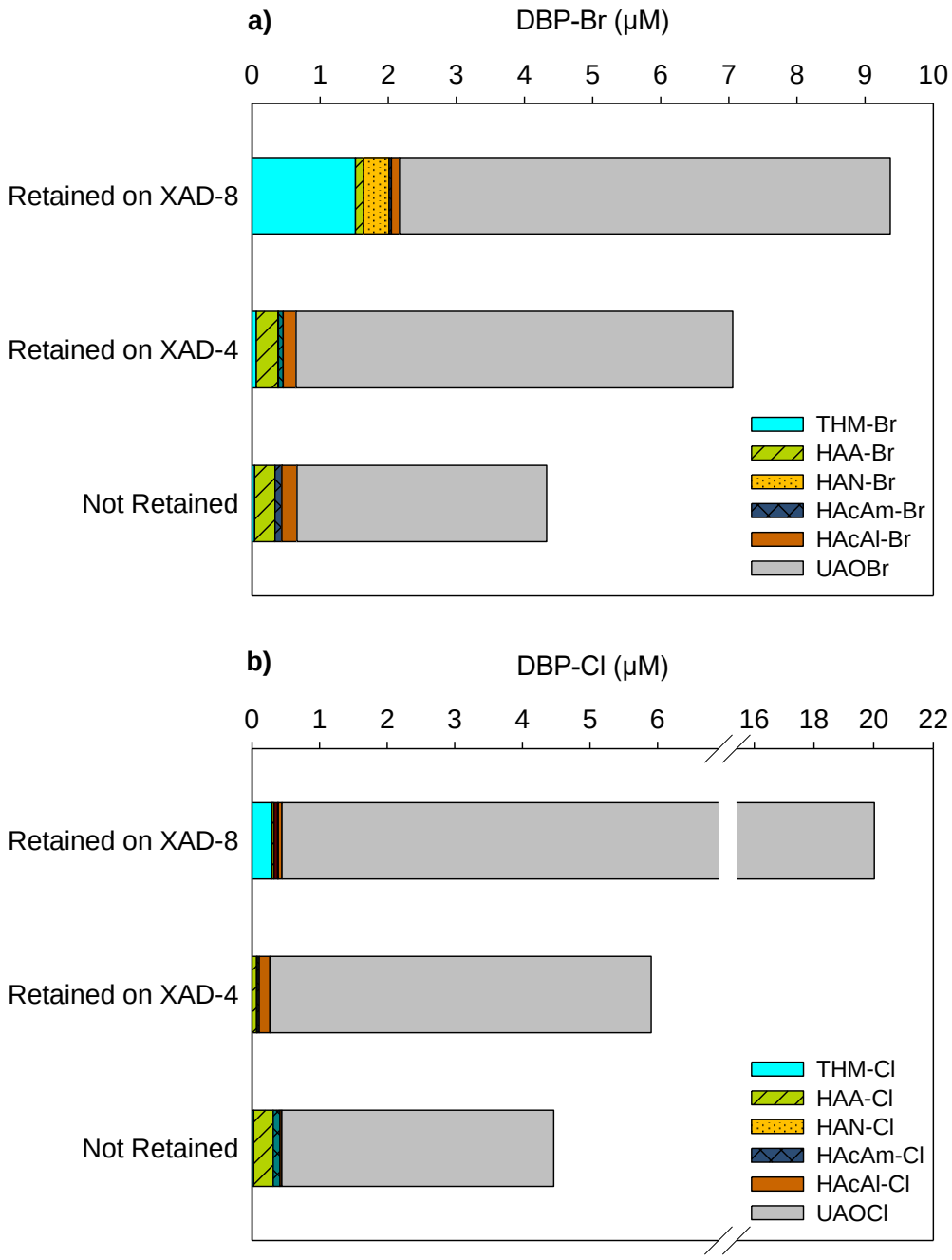


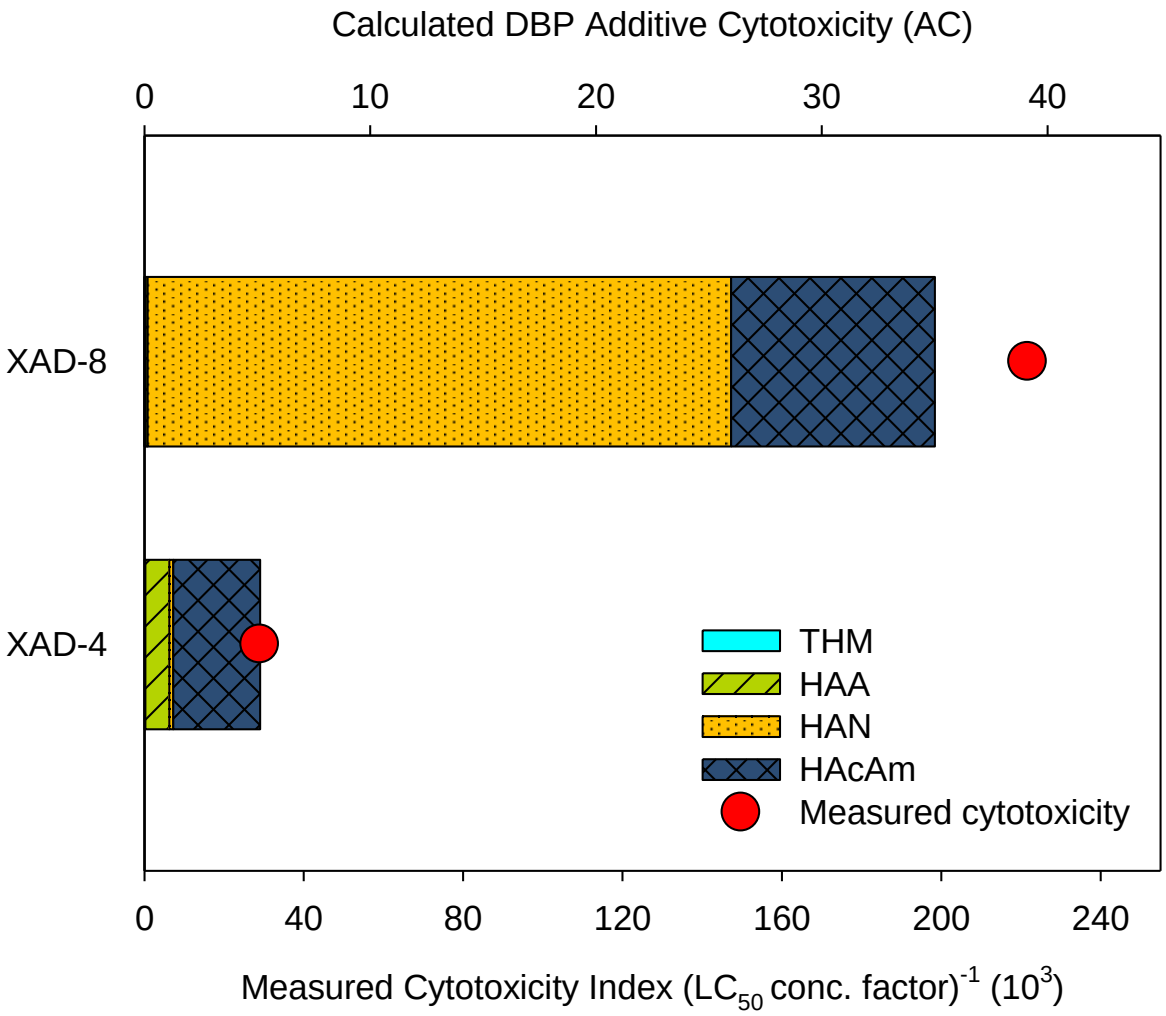
Fig. 5. DBP contributions to a) AOBr and b) AOCl retained on XAD-8 and XAD-4 resins, based on concentrations analyzed in water samples at the inlet and outlet of each resin. UAOCl and UAOBr stand for unknown AOCl and unknown AOBr.

2.3.4. DBP quantification in XAD resin-isolated fractions and associated toxicity

Ethyl acetate was used as the eluting solvent of the organics adsorbed onto the two resins from the chloraminated wastewater effluent WW3, in order to directly quantify DBPs present in each recovered fraction. The cytotoxicity of the two fractions was also assessed and expressed as LC_{50} concentration factor as discussed previously (Appendix A, Table S2). Cytotoxicity index (CTI) value derived from LC_{50} concentration factor of the XAD-8 fraction was 222, i.e, ~20-fold more toxic than values reported from surface waters and secondary effluents subjected to various oxidation processes (i.e., ozone, chlorine, UV or chlorine dioxide) and CTI value of XAD-4 fraction (CTI = 29) was in the same range than chlorinated secondary effluents (Dong et al., 2016; Jeong et al., 2012; Plewa et al., 2012). Small volumes of ethyl acetate extracts were diluted in ethyl acetate (for HAcAms analyses) or in MTBE (for THMs and HAAs analyses) and spiked with internal standards before injection in GC-MS. After correction with concentration factor of extraction and evaporation steps (concentrations × 760), concentrations obtained by analysis of the ethyl acetate extracts (Appendix A, Table S3) were in the same range as concentrations calculated by the difference between inlet and outlet of the columns (i.e., ~72% and 99% recovery for XAD-8 and XAD-4 resins, respectively, losses possibly occurring during elution and subsequent preparation of samples for analysis). More DBPs and AOX were quantified in the chloraminated effluent isolated from the XAD-8 resin as compared to the XAD-4 resin. Some DBPs (e.g., THMs) that were detected at high concentrations exert much lower toxicity than others such as HANs. In order to compare DBP results with the measured cytotoxicity and genotoxicity of each recovered fraction, the total DBP additive cytotoxicity (AC) was calculated from the measured concentration of each individual DBP divided by its respective cytotoxicity LC_{50} value available in the literature (Eq. 1) (Allard et al., 2015; Smith et al., 2010).

$$AC = \sum_{i}^{n} \frac{C_i}{LC_{50,i}} \qquad (1)$$

Where C_i is the measured concentration of a given DBP *i* in a mixture of *n* DBP, $LC_{50,i}$ is its LC_{50} cytotoxicity value and the calculated AC is a dimensionless value. Following this approach, the ratio between XAD-8 and XAD-4 resin calculated additive cytotoxicity was similar to the ratio of measured cytotoxicity index values (Fig. 6). While THMs were the major DBPs present in the XAD-8 fraction, most of the calculated additive cytotoxicity was attributed to HANs and HAcAms. DBAN, BAcAm, BCAN and DBAcAm were the dominant cytotoxic DBPs, accounting for 68.7%, 14.0%, 8.2% and 6.7% of the calculated AC, respectively. In the XAD-4 fraction, HAcAms and HAAs accounted for the majority of the calculated additive cytotoxicity, with DBAcAm, BCAcAm and MBAA accounting for 64.8%, 16.5% and 13.4%, respectively. Overall, N-DBPs (i.e., HANs and HAcAms) were responsible for the majority of the calculated cytotoxicity in both fractions (99.4% and 78.5% for XAD-8 and XAD-4 fractions, respectively).



452

453 Fig. 6. DBP additive cytotoxicity (AC) calculated from measured concentrations of DBPs in the
454 XAD-8 and XAD-4 resin fractions and individual DBP cytotoxicity LC_{50} values, compared to
455 the measured cytotoxicity index values of the two fractions.

456

457 Another approach is to assess a calculated cytotoxicity index (CCTI) value ($(LC_{50})^{-1}$) to the
458 isolated fraction according to a concentration addition model based on the individual cytotoxicity
459 LC_{50} value of each analyzed DBPs and their respective measured molar fraction (calculated from
460 the total measured DBP concentration) (Eq. 2) (Tang et al., 2013; Zeng et al., 2016).

$$CCTI = \sum_{i=1}^{n} \frac{p_i}{LC_{50,\,i}} \qquad (2)$$

Where p_i is the molar fraction of DBP *i* in a mixture of *n* DBP and $LC_{50,i}$ is the individual LC_{50} value of DBP *i.* The obtained CCTI values are expressed as $(LC_{50})^{-1}$, which facilitates the comparison with measured cytotoxicity index values (expressed as (LC_{50} concentration factor)$^{-1}$).

Following this approach, the CCTI value of the XAD-8 fraction was still higher than that of the XAD-4 fraction, in accordance with measured cytotoxicity index values, but the ratio between the calculated cytotoxicity of XAD-8 and XAD-4 isolates was much lower than from the first approach (Appendix A, Fig. S3), because this calculation is based on a number *n* of measured DBPs, while many other unknown Cl-DBPs and Br-DBPs were measured in the XAD-8 fraction as UAOCl and UAOBr. This result shows that analyzed DBPs did not significantly represent the global cytotoxicity of the samples, i.e., that other toxic products (e.g., chlorinated, brominated or even iodinated byproducts) accounted for the difference observed between the XAD-8 and the XAD-4 fraction. In order to correct the CCTI values to take into account the unknown halogenated products, molar fractions of DBPs were calculated based on total concentrations of measured AOX (expressed as mol/L as X) (Eq. 3).

$$CCTI = \sum_{i=1}^{n} \frac{\frac{C_i}{AOX}}{LC_{50,\,i}} \qquad (3)$$

Where Ci is the measured concentration (expressed as mol/L as X) of a given DBP *i* and $LC_{50,i}$ is its LC_{50} cytotoxicity value.

With this approach, the proportion of N-DBPs (HANs and HAcAms) in AOX was a good proxy of the global toxicity measured in the samples (Fig. 7).

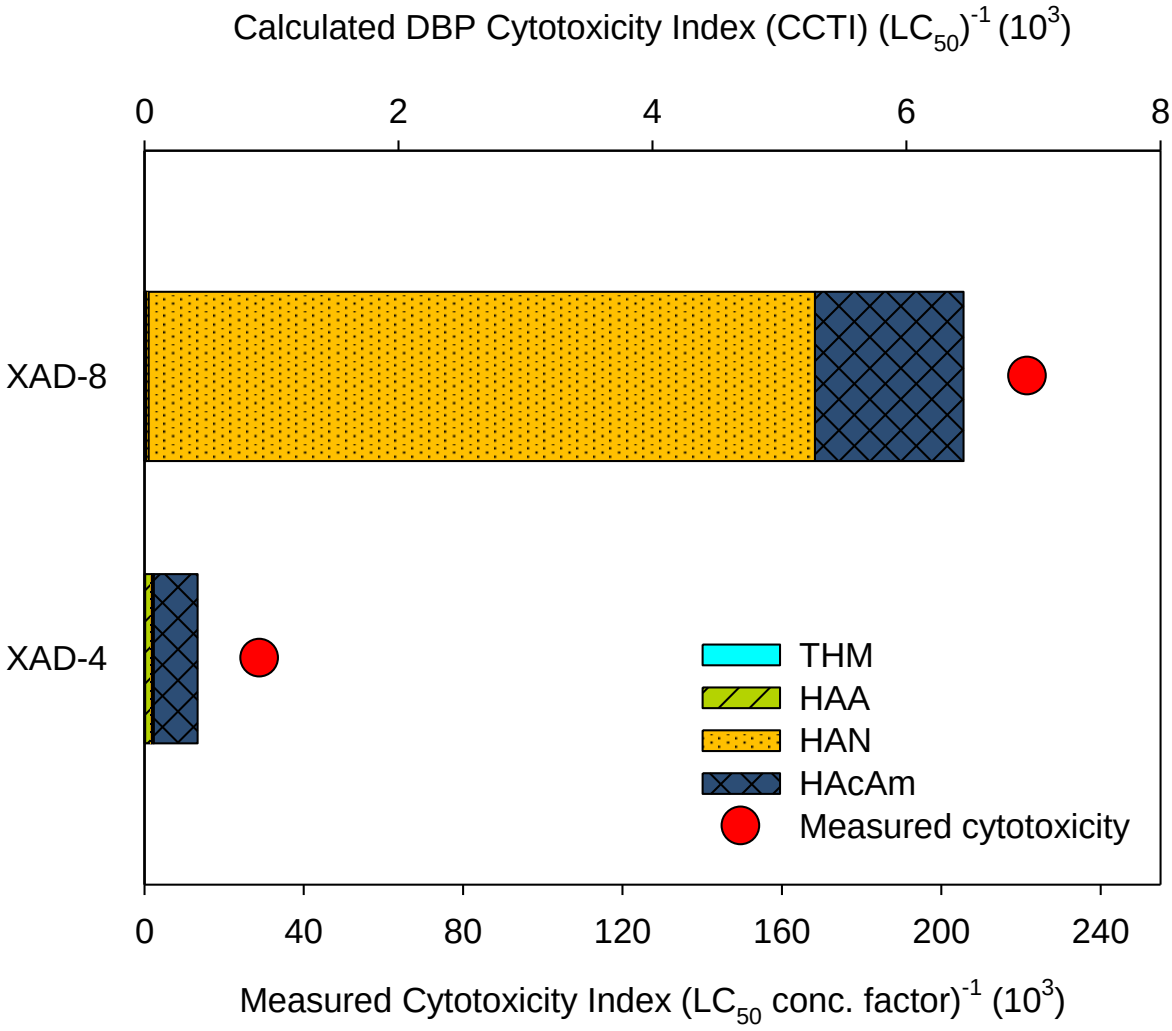


Fig. 7. DBP additive cytotoxicity index (CCTI) values calculated from molar fractions of each DBP (as fractions of measured AOX), divided by their respective individual cytotoxicity LC_{50} values, compared to the measured cytotoxicity index values of the XAD-8 and XAD-4 fractions. AOX: adsorbable organic halide.

3. Conclusions

- The toxicity of chloraminated wastewater effluents was significantly higher than the toxicity of raw wastewater effluents

- More DBPs were isolated from the XAD-8 resin, i.e., hydrophobic (HPO) EfOM fraction, which is in accordance with the higher toxicity observed for this fraction, when compared to the results obtained with the XAD-4 resin.

- Isolation recovery of DBPs onto XAD resins depends on their hydrophilicity: most hydrophobic THMs and HANs were mostly retained on the XAD-8 resin (also showing a larger proportion of DOC adsorbed), while more hydrophilic HAAs, HAcAms and HAcAls were less retained on both resins, leading to higher concentrations in the organic fraction that did not adsorb onto the two superposed resins. The toxicity of the non-retained organic fraction needs to be evaluated.

- Measured DBPs accounted for minor proportions of the brominated and chlorinated products analyzed as AOBr and AOCl, which means that most AOX consisted in unknown brominated and chlorinated compounds.

- Estimated cytotoxicity of XAD-8 resin/hydrophobic (HPO) and XAD-4 resin/transphilic (TPI) isolates calculated based on additive cytotoxicity of measured DBPs did not account for the difference observed between the measured cytotoxicity of the two fractions. Other products were probably responsible for the higher toxicity of the HPO fraction. These products may include chlorinated, brominated and iodinated byproducts, or non-halogenated byproducts (e.g., nitrosamines) and unknown high-molecular weight compounds.

- Among the measured DBPs, N-DBPs (i.e., HANs and HAcAms) contributed to the majority of the estimated additive DBP cytotoxicity of each fraction (78.5% and 99.4% for

514 TPI and HPO fractions, respectively), and were a good proxy of the global cytotoxicity
515 when expressed as molar fractions of AOX.

516 **Acknowledgments**.

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519 Disinfection By-Products in Reclaimed Wastewater Effluents: Chemistry, Toxicity and Control
520 Strategies"

521 **Appendix A. Supplementary Material**

522 Supplementary data to this article can be found online at
523 http://dx.doi.org/10.1016/j.jes.2017.04.022.

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