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1 Chloramination of wastewater effluent: Toxicity and formation of disinfection  
2 byproducts

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18

19 **Abstract**

20 The reclamation and disinfection of waters impacted by human activities (e.g., wastewater  
21 effluent discharges) are of growing interest for various applications but has been associated with  
22 the formation of toxic nitrogenous disinfection byproducts (N-DBPs). Monochloramine used as  
23 an alternative disinfectant to chlorine can be an additional source of nitrogen in the formation of  
24 N-DBPs. Individual toxicity assays have been performed on many DBPs, but few studies have  
25 been conducted with complex mixtures such as wastewater effluents. In this work, we compared  
26 the cytotoxicity and genotoxicity of wastewater effluent organic matter (EfOM) before and after  
27 chloramination. The toxicity of chloraminated EfOM was significantly higher than the toxicity of  
28 raw EfOM, and the more hydrophobic fraction (HPO) isolated on XAD-8 resin was more toxic  
29 than the fraction isolated on XAD-4 resin. More DBPs were also isolated on the XAD-8 resin. N-  
30 DBPs (i.e., haloacetonitriles or haloacetamides) were responsible for the majority of the  
31 cytotoxicity estimated from DBP concentrations measured in the XAD-8 and XAD-4 fractions  
32 (99.4% and 78.5%, respectively). Measured DBPs accounted for minor proportions of total  
33 brominated and chlorinated products, which means that many unknown halogenated compounds  
34 were formed and can be responsible for a significant part of the toxicity. Other non-halogenated  
35 byproducts (e.g., nitrosamines) may contribute to the toxicity of chloraminated effluents as well.

36 **Keywords**

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

39 **Introduction**

40 The disinfection of waters impacted by human activities (e.g., agriculture or wastewater  
41 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

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

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

85 As a result, the aim of this work was (i) to compare the cytotoxicity and genotoxicity of EfOM  
86 resin isolates recovered before and after chloramination, (ii) to analyze the toxicity of resin  
87 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<sub>2</sub>Br, chlorodibromomethane - CHClBr<sub>2</sub>, 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 DCaAcAm and TCAcAm, respectively) were obtained from Sigma-Aldrich. Other HAcs (i.e.,  
104 dibromoacetamide - DBAcAm, tribromoacetamide - TBAcAm, bromochloroacetamide -  
105 BCaAcAm, chloroiodoacetamide - CIAcAm, bromoiodoacetamide - BIAcAm and  
106 diiodoacetamide - DIAcAm) were purchased from Cansyn Chem. Corp. Haloacetaldehydes  
107 (HAcs) 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.

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## **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. AOX concentrations were determined from the respective Cl<sup>-</sup> and Br<sup>-</sup> concentrations. Free chlorine and total chlorine concentrations in the sodium hypochlorite stock solutions were determined by spectrophotometric measurement at 292 nm. NH<sub>2</sub>Cl and NHCl<sub>2</sub> 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,

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

140

### 141 **1.3. Chloramination conditions**

142 Monochloramine stock solutions were prepared by adding sodium hypochlorite (NaOCl) to a  
143 continuously-stirred ammonium chloride solution adjusted to pH 8.5 with sodium hydroxide, at a  
144 Cl:N molar ratio of 1:1.4. The concentration of monochloramine stock solutions was adjusted to  
145 a desired concentration. Chloramination reactions were performed at pH 7.7 without addition of  
146 buffering agents, and doses were calculated as:  $\text{NH}_2\text{Cl (mg/L as Cl}_2\text{)} = 3 \times \text{DOC (dissolved}$   
147  $\text{organic carbon, mg C/L)}$  (Dotson et al., 2009; Krasner et al., 2007). Preformed monochloramine  
148 was used to avoid breakpoint reactions that could occur with residual ammonium concentration  
149 of the effluent (0.2-1 mgN/L after prolonged aeration activated sludge). The pH remained stable  
150 ( $7.7 \pm 0.5$ ) during all the reaction time (72 hr). At the end of the contact time, samples were  
151 acidified at pH 2 with concentrated HCl and immediately subjected to the XAD resins extraction  
152 protocol. No additional quenching was performed in order to preserve the disinfection  
153 byproducts formed (no residual oxidant was measured after acidification), and samples were  
154 directly passed through XAD resins for fractionation and extraction.

155

### 156 **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

180 of acetonitrile) and lyophilization. With this approach the cytotoxicity and genotoxicity values of  
181 samples WW1 and WW2 could be expressed as per mg of EfOM. For effluent WW3, both resins  
182 were eluted with ethyl acetate and the chloraminated EfOM extracts were concentrated under a  
183 stream of N<sub>2</sub> to a final volume of 100 mL to allow the direct quantification of DBPs and the  
184 analysis of toxicity from the same extract, as previously described in the literature (Jeong et al.,  
185 2012; Richardson, 2011). It should be noted that these concentration approaches (i.e.,  
186 lyophilization or N<sub>2</sub> evaporation) are usually suitable for semi- or non-volatile chemicals but not  
187 for volatile compounds (Weinberg, 2009). Nevertheless, volatile DBPs (e.g., THMs) analyzed in  
188 the ethyl acetate extracts after concentration were in accordance with concentrations analyzed in  
189 the wastewater effluent samples (see Section 2.3.4). Since the two chloraminated wastewater  
190 effluents (WW2 and WW3) were not sampled at the same time (and exhibited different DOC  
191 values) and fractions were obtained through different processes (as described above), the toxicity  
192 results of these two types of samples were not comparable.

193

## 194 **1.5. Analytical biology**

### 195 **1.5.1. Sample preparation**

196 The lyophilized or liquid organic fractions were received and stored at -20 °C. Mg amounts of  
197 each lyophilized fraction were placed into separate sterile Supelco sample vials. To sterilize the  
198 sample, DMSO was added to each sample such that for each mg of sample 1 µL of DMSO was  
199 added. After the sample was mixed (vortexed) in DMSO, 1 µL of sterile ddH<sub>2</sub>O was added for  
200 each mg of the lyophilized fraction. The stock solution for each sample was completely  
201 dissolved in this DMSO-water (1:1 v/v) solvent and this was stored at -20°C. Ethyl acetate  
202 organic extracts (liquid form) obtained from WW3 were solvent exchanged into DMSO so that

203 the organic material from 1 L of wastewater effluent was concentrated into 10  $\mu$ L of DMSO  
204 ( $10^5\times$  concentration) and stored in Teflon-sealed sample vials at  $-20^\circ\text{C}$ . For each experiment a  
205  $50\times$  dilution of each sample stock solution was prepared in F12 cell culture medium+5% fetal  
206 bovine serum (FBS).

### 207 **1.5.2. Chinese hamster ovary cells**

208 Chinese hamster ovary (CHO) cell line AS52, clone 11–4–8 was used for the biological assays  
209 (Wagner et al., 1998). CHO cells were maintained on glass culture plates in Ham’s F12 medium  
210 containing 5% FBS, 1% antibiotics (100 U/mL sodium penicillin G, 100  $\mu\text{g}/\text{mL}$  streptomycin  
211 sulfate, 0.25  $\mu\text{g}/\text{mL}$  amphotericin B in 0.85% saline), and 1% glutamine at  $37^\circ\text{C}$  in a humidified  
212 atmosphere of 5%  $\text{CO}_2$ .

### 213 **1.5.3. CHO cell chronic cytotoxicity assay**

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

#### 225 **1.5.4. CHO cell single cell gel electrophoresis assay**

226 Single cell gel electrophoresis (SCGE, or Comet) assay quantitatively measures genomic  
227 deoxyribonucleic acid (DNA) damage in individual nuclei induced by a test agent (Tice et al.,  
228 2000). We employed a microplate methodology (Wagner and Plewa, 2009). The SCGE metric  
229 for genomic DNA damage was the %Tail DNA value which is the amount of DNA that migrated  
230 from the nucleus into the microgel (Kumaravel and Jha, 2006). Within each concentration factor  
231 range with >70% cell viability, a concentration-response curve was generated and regression  
232 analysis was used to fit the curve. The concentration factor inducing a 50% Tail DNA (TDNA)  
233 value was calculated using a regression analysis for each concentration-response curve. The  
234 genotoxic index (GTI) was a metric that expressed increasing values with increased genotoxic  
235 damage ( $50\% \text{ TDNA}^{-1})(10^3)$  and allowed for easy comparisons among the evaluated wastewater  
236 samples.

#### 237 **1.5.5. Statistical analyses**

238 For the cytotoxicity assay, a one-way analysis of variance (ANOVA) test was conducted to  
239 determine if the sample induced a statistically significant level of cell death as compared to the  
240 concurrent negative controls at a specific concentration factor. If a significant  $F$  value ( $P \leq 0.05$ )  
241 was obtained, a Holm-Sidak multiple comparison versus the control group analysis was  
242 performed to identify the lowest cytotoxic concentration factor. The power of the test statistic  
243 ( $1-\beta$ ) was maintained as  $\geq 0.8$  at  $\alpha = 0.05$ . For the SCGE assay, the %Tail DNA values were not  
244 normally distributed which limits the use of parametric statistics (Box et al., 1978). The mean  
245 %Tail DNA value for each microgel was calculated and these values were averaged among all  
246 the microgels for each wastewater sample concentration factor. Averaged mean values express a  
247 normal distribution according to the central limit theorem. An ANOVA test was conducted on

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

251

## 252 2. Results and discussion

### 253 2.1. EfOM extraction recovery

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

256 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 <sub>2</sub> O	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

257

258 The first experiment consisted in the extraction of 150 L non-chloraminated wastewater  
 259 effluent (WW1, 4.65 mg C/L) followed by an elution with acetonitrile/H<sub>2</sub>O (75:25, v/v). 69.5%  
 260 DOC was retained on both XAD resins (2.13 mg C/L on XAD-8, 1.10 mg C/L on XAD-4)  
 261 (Table 2). 1.42 mg C/L was measured at the output of the XAD-4 resin (consisting in the  
 262 hydrophilic fraction of EfOM). The second extraction was performed with 69 L wastewater  
 263 effluent (WW2, 4.68 mg C/L) after 72 hr of chloramination (14.2 mg Cl<sub>2</sub>/L as preformed  
 264 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<sub>2</sub>/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.

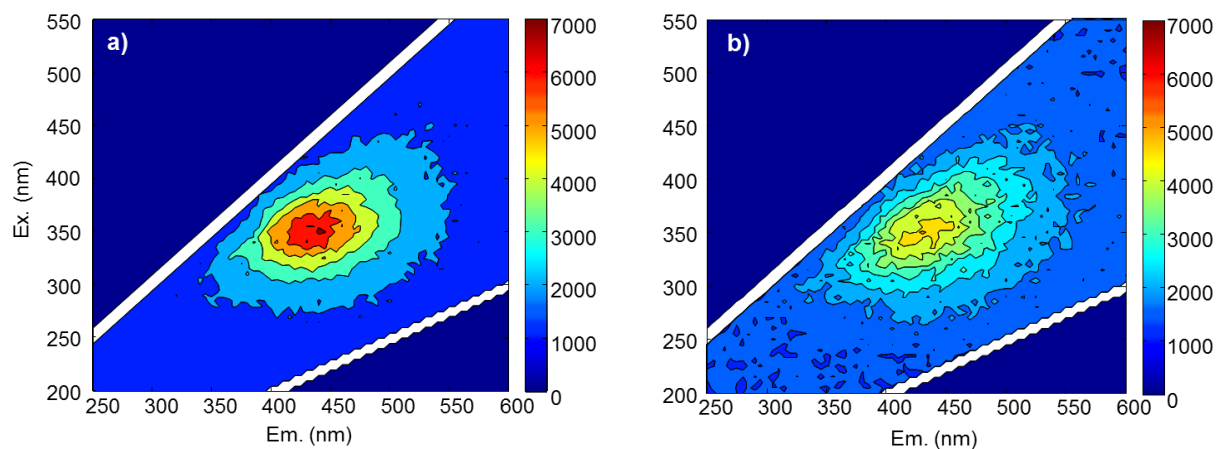
282

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<sub>2</sub>/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
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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

286



287 Fig. 1. Fluorescence EEMs of a) wastewater effluent WW2 and b) wastewater effluent WW2  
 288 after 72h chloramination (14.2 mg Cl<sub>2</sub>/L) at pH 7.7

289

## 290 2.2. Analytical biology results

### 291 2.2.1. CHO cell cytotoxicity results

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

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

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

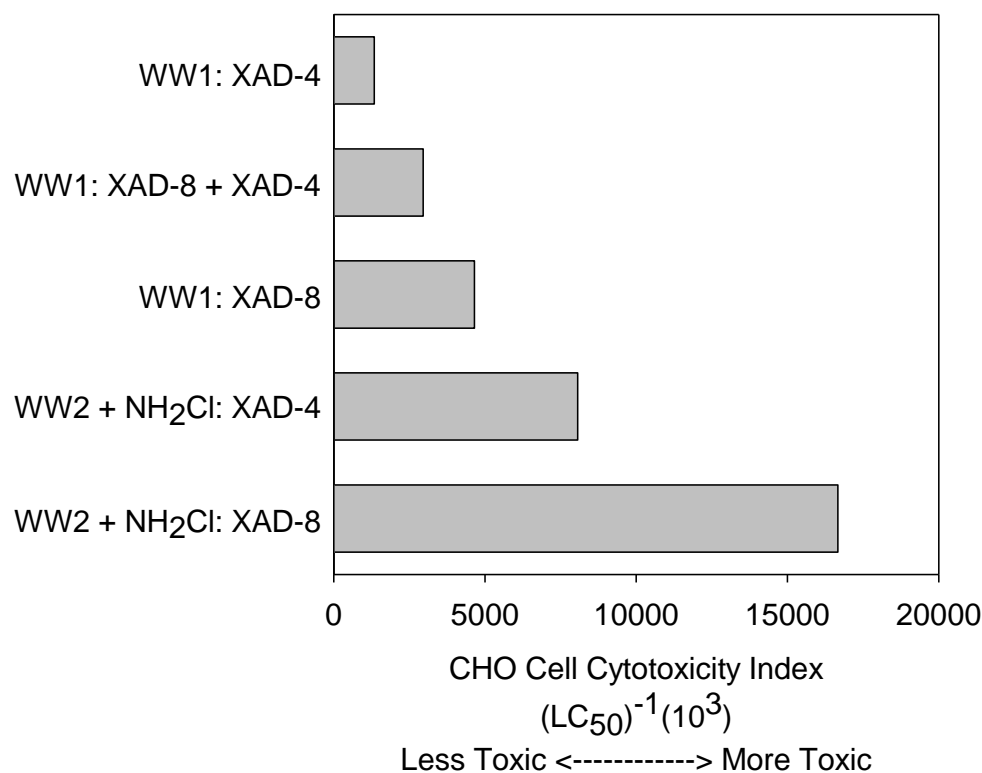
Sample	Fraction	Conc. Range (μg/μL)	Chronic Cytotoxicity (LC <sub>50</sub> ) (μg/μL) <sup>a</sup>	<i>r</i> <sup>2b</sup>	Lowest Significant Toxic Concentration (μg/μL) <sup>c</sup>	ANOVA Test Statistic <sup>d</sup>
WW1	XAD-8	0.05-0.75	0.215	0.99	0.15	$F_{10, 50} = 144.2; P \leq 0.001$
WW1	XAD-4	0.05-1.15	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 <sub>2</sub> Cl)	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<sub>2</sub>Cl)

CHO: Chinese Hamster Ovary; EfOM: effluent organic matter; ANOVA: analysis of variance.

<sup>a</sup> The LC<sub>50</sub> 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. <sup>b</sup>  $r^2$  is the coefficient of determination for the regression analysis upon which the LC<sub>50</sub> value was calculated. <sup>c</sup> 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. <sup>d</sup> 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  $(50\% \text{Tail DNA}^{-1})(10^3)$  and the  
 324 larger the GTI value the greater the genotoxic damage.

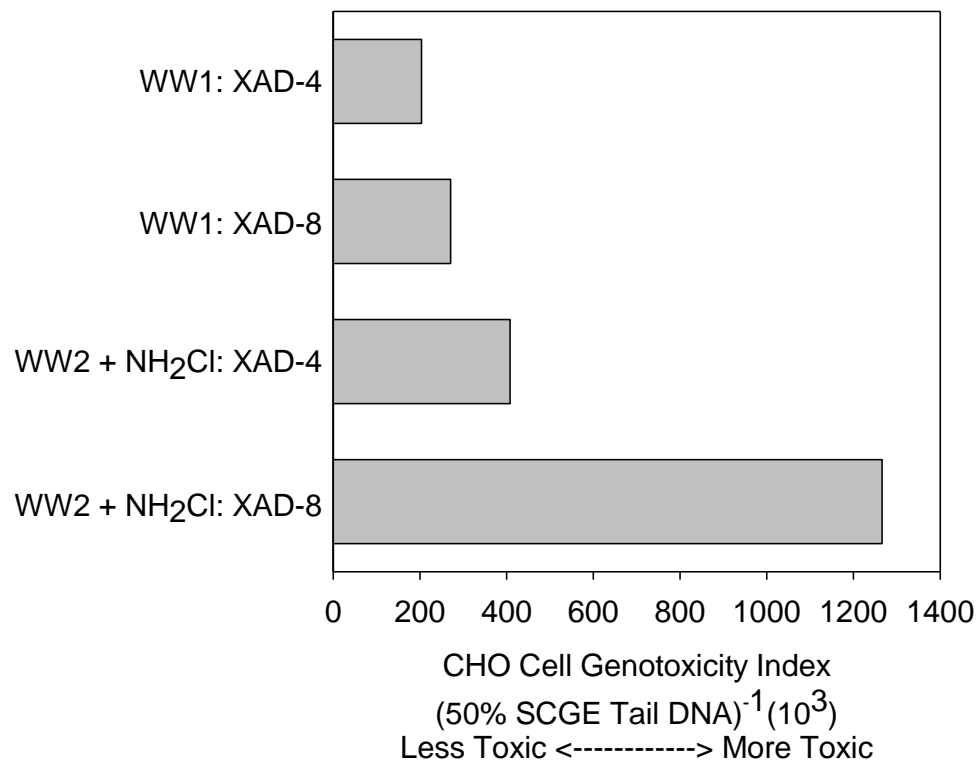
325

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 ( $\mu\text{g}/\mu\text{L}$ )	Acute Genotoxicity (50% Tail DNA) $\mu\text{g}/\mu\text{L}^{\text{a}}$	$r^2$ <sup>b</sup>	Lowest Significant Toxic Concentration ( $\mu\text{g}/\mu\text{L}$ ) <sup>c</sup>	ANOVA Test Statistic <sup>d</sup>
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 <sub>2</sub> Cl	XAD-4	2.0-3.4	2.45	0.98	2.0	$F_{11,28} = 153.0: P \leq 0.001$
WW2+NH <sub>2</sub> Cl	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.  
<sup>a</sup> The SCGE 50% Tail DNA value is the EfOM sample concentration (as  $\mu\text{g}/\mu\text{L}$ ), determined from a regression analysis of the data, that induced the migration of 50% SCGE Tail DNA value.  
<sup>b</sup>  $r^2$  is the coefficient of determination for the regression analysis upon which the SCGE 50% Tail DNA value was calculated. <sup>c</sup> 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. <sup>d</sup> 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

343

## 344 **2.3. DBP formation**

### 345 **2.3.1. DBP identification**

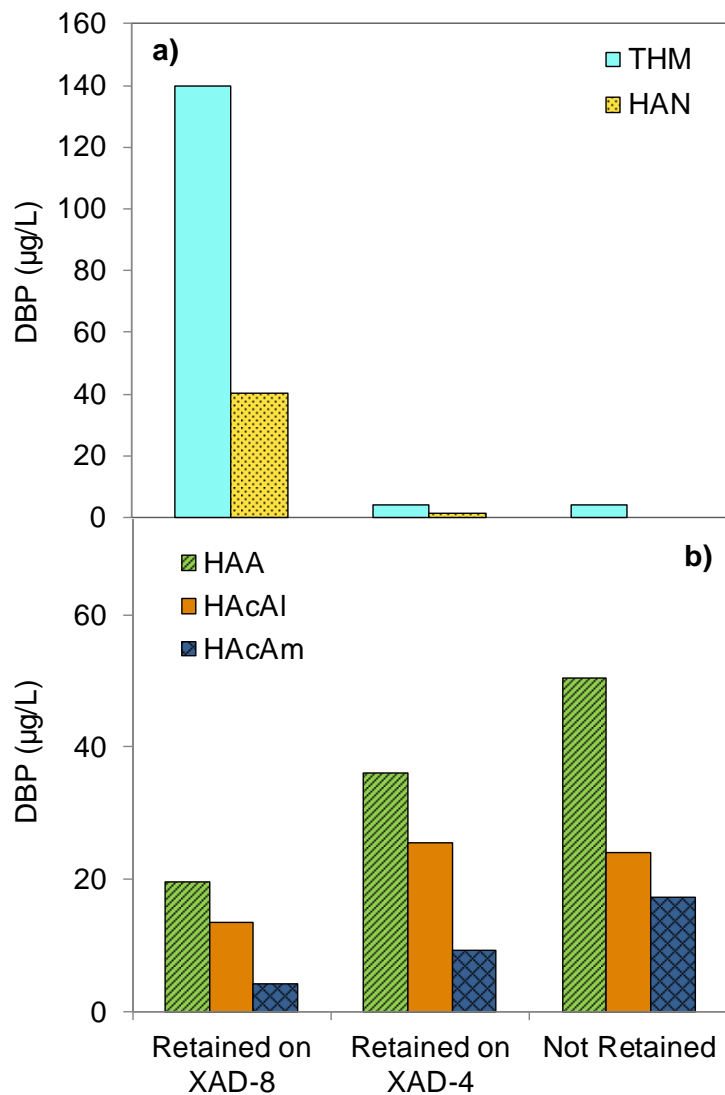
346 A small volume of the acetonitrile extracts obtained from the two resins that received the  
347 chloraminated wastewater effluent WW2 was evaporated under a stream of N<sub>2</sub> and transferred to  
348 ethyl acetate for direct full-scan GC-MS analyses in order to characterize the main products  
349 formed by chloramination and retained onto XAD resins. Tribromoacetaldehyde (TBACAl) was  
350 the most predominant identified peak in the chromatograms (Appendix A, Fig. S2). TBM,  
351 DBAcAm, dibromoacetaldehyde (DBAcAl), chlorodibromoacetaldehyde (CDBAcAl), BIAcAm,  
352 and BCACAm were also present in the chromatograms. Many other brominated and chlorinated  
353 peaks were detected but could not be identified by direct comparison of mass spectra with NIST  
354 database. Brominated species were predominant because of the presence of bromide ion in the  
355 wastewater effluent (2.52 mg/L as Br<sup>-</sup>), and some iodinated products (e.g., BIAcAm) were  
356 detected in the XAD-8 fraction (though iodide ion could not be detected by IC analysis because  
357 of matrix effects). Some of these brominated and iodinated DBPs (i.e., TBACAl, DBAcAl,  
358 BIAcAm) are among the most cytotoxic and genotoxic DBPs identified today (Plewa et al.,  
359 2008). DBPs and AOX were not quantified during this experiment, and lyophilized fractions  
360 were only analyzed for cytotoxicity and genotoxicity.

### 361 **2.3.2. DBP quantification and estimated retention on XAD resins**

362 During the XAD resins extraction conducted on WW3 (initial DOC 4.97 mg C/L, 14.9 mg  
363 Cl<sub>2</sub>/L), aqueous samples were collected before and after each XAD resin (Appendix A, Table S1)  
364 in order to quantify THMs, HAAs, HANs, HAcAms and HAcAls and evaluate the retention of  
365 each class of DBPs onto both resins (calculated as the difference between DBP concentrations at  
366 the inlet and at the outlet of a resin column). Results indicated a high retention of total THMs

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

378



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.

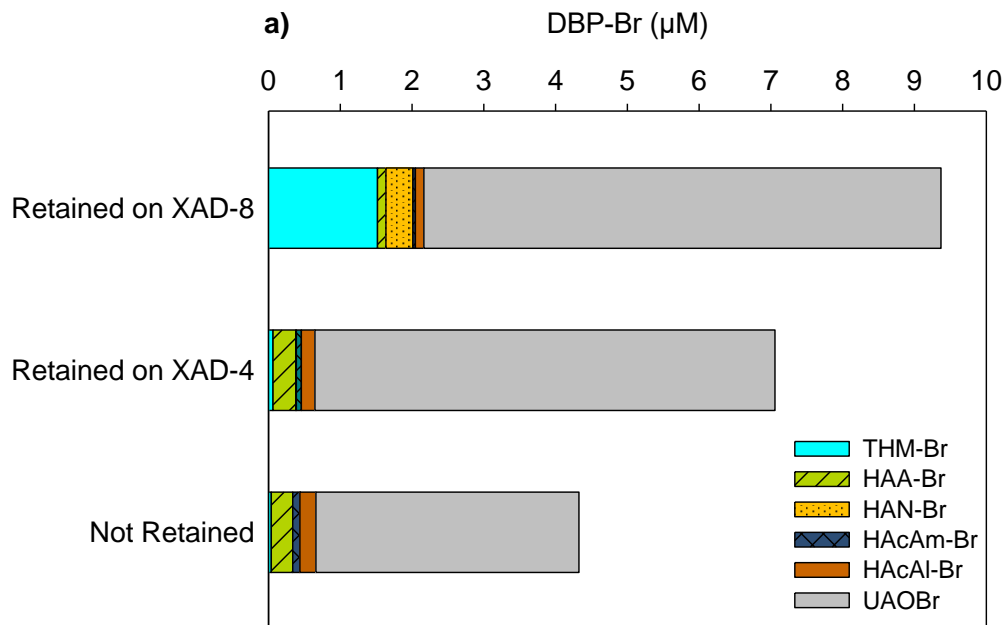
385

386 **2.3.3. DBP contributions to AOX**

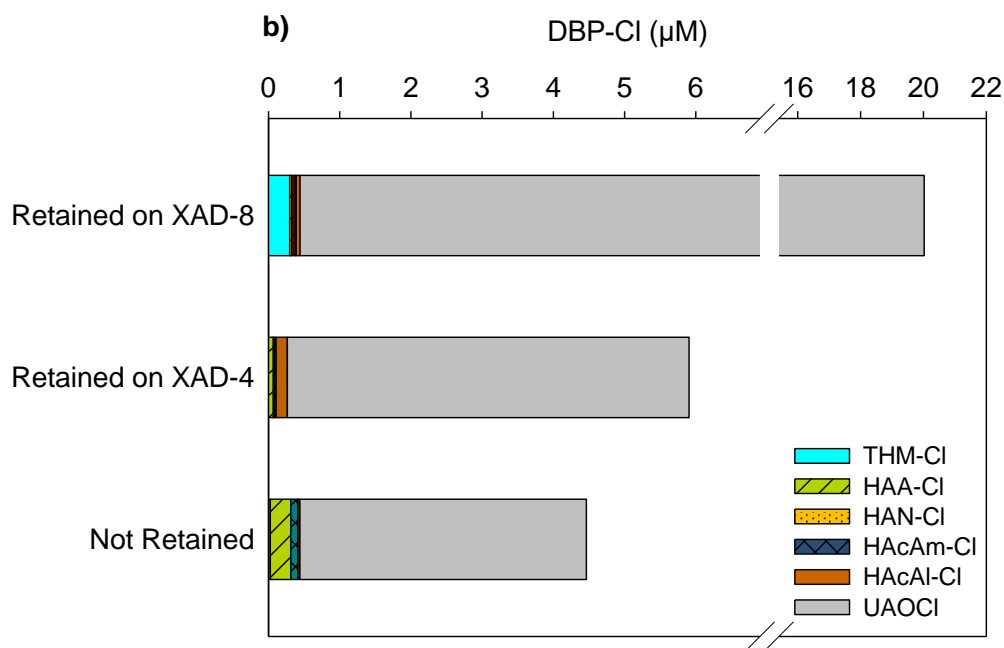
387 AOB<sub>r</sub> and AOC<sub>l</sub> concentrations in chloraminated wastewater effluent were 1658.2 µg/L as Br  
388 and 1078.9 µg/L as Cl, which is in accordance with the presence of a majority of brominated  
389 DBPs detected in full-scan chromatograms. The proportions of AOX (AOC<sub>l</sub> and AOB<sub>r</sub>) retained  
390 on each resin were calculated in the same manner than individual DBPs (i.e., by difference  
391 between inlet and outlet concentrations). AOB<sub>r</sub> and AOC<sub>l</sub> were mostly retained on the XAD-8  
392 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  
393 and 209.6 µg/L as Cl), which may explain the higher toxicity observed in the isolated  
394 XAD-8/hydrophobic (HPO) fraction than that of the XAD-4/transphilic (TPI) fraction (Fig. 5).  
395 Overall, 85.3% of AOC<sub>l</sub> and 79.2% of AOB<sub>r</sub> were retained on both resins. All analyzed DBP  
396 concentrations were expressed in µg/L as Cl (Cl-DBP) or µg/L as Br (Br-DBP) to obtain their  
397 relative contribution to AOC<sub>l</sub> and AOB<sub>r</sub> concentrations (Fig. 5). Unknown AOC<sub>l</sub> (UAOC<sub>l</sub>) and  
398 unknown AOB<sub>r</sub> (UAOB<sub>r</sub>) were determined from the differences between measured AOC<sub>l</sub> or  
399 AOB<sub>r</sub> and the sum of chlorine or bromine-equivalent concentrations of measured specific DBPs.  
400 Br-DBPs contributed to 23.1% of AOB<sub>r</sub> retained on the XAD-8 resin and to 9.2% of AOB<sub>r</sub>  
401 retained on the XAD-4 resin. Bromoform, DBAN and DBCM were the three major Br-DBPs  
402 contributing to AOB<sub>r</sub> retained on the XAD-8 resin, while DBAA, CDBAcAl and DBAcAm were  
403 the major Br-DBPs retained on the XAD-4 resin. Br-DBPs present in the fraction not retained  
404 onto the two resins exhibited concentrations of the same order of magnitude of Br-DBPs retained  
405 onto the XAD-4 resin. Cl-DBPs only represented 2.2% and 4.5% of AOC<sub>l</sub> retained on XAD-8  
406 and XAD-4 resins, respectively, which indicates that a majority of chlorine-containing  
407 compounds were not quantified.

408

409



410



411

412 Fig. 5. DBP contributions to a) AOBBr and b) AOCl retained on XAD-8 and XAD-4 resins, based  
 413 on concentrations analyzed in water samples at the inlet and outlet of each resin. UAOCi and  
 414 UAObBr stand for unknown AOCl and unknown AOBBr.

415

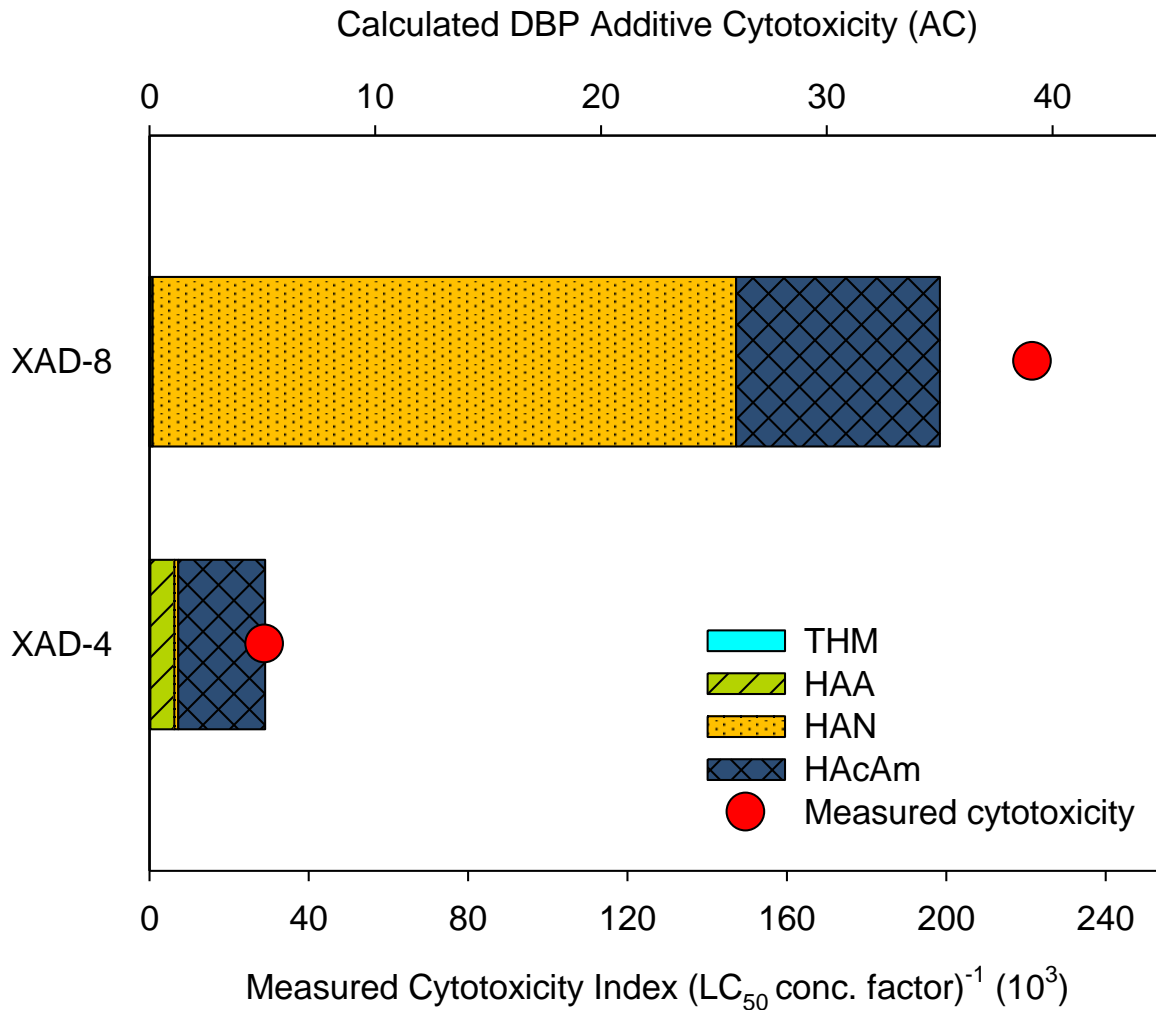
416 **2.3.4. DBP quantification in XAD resin-isolated fractions and associated toxicity**



417 Ethyl acetate was used as the eluting solvent of the organics adsorbed onto the two resins from  
418 the chloraminated wastewater effluent WW3, in order to directly quantify DBPs present in each  
419 recovered fraction. The cytotoxicity of the two fractions was also assessed and expressed as LC<sub>50</sub>  
420 concentration factor as discussed previously (Appendix A, Table S2). Cytotoxicity index (CTI)  
421 value derived from LC<sub>50</sub> concentration factor of the XAD-8 fraction was 222, i.e., ~20-fold more  
422 toxic than values reported from surface waters and secondary effluents subjected to various  
423 oxidation processes (i.e., ozone, chlorine, UV or chlorine dioxide) and CTI value of XAD-4  
424 fraction (CTI = 29) was in the same range than chlorinated secondary effluents (Dong et al.,  
425 2016; Jeong et al., 2012; Plewa et al., 2012). Small volumes of ethyl acetate extracts were diluted  
426 in ethyl acetate (for HAcAms analyses) or in MTBE (for THMs and HAAs analyses) and spiked  
427 with internal standards before injection in GC-MS. After correction with concentration factor of  
428 extraction and evaporation steps (concentrations × 760), concentrations obtained by analysis of  
429 the ethyl acetate extracts (Appendix A, Table S3) were in the same range as concentrations  
430 calculated by the difference between inlet and outlet of the columns (i.e., ~72% and 99%  
431 recovery for XAD-8 and XAD-4 resins, respectively, losses possibly occurring during elution  
432 and subsequent preparation of samples for analysis). More DBPs and AOX were quantified in  
433 the chloraminated effluent isolated from the XAD-8 resin as compared to the XAD-4 resin.  
434 Some DBPs (e.g., THMs) that were detected at high concentrations exert much lower toxicity  
435 than others such as HANs. In order to compare DBP results with the measured cytotoxicity and  
436 genotoxicity of each recovered fraction, the total DBP additive cytotoxicity (AC) was calculated  
437 from the measured concentration of each individual DBP divided by its respective cytotoxicity  
438 LC<sub>50</sub> value available in the literature (Eq. 1) (Allard et al., 2015; Smith et al., 2010).

$$439 \quad AC = \sum_i^n \frac{C_i}{LC_{50,i}} \quad (1)$$

440 Where  $C_i$  is the measured concentration of a given DBP  $i$  in a mixture of  $n$  DBP,  $LC_{50,i}$  is its  
441  $LC_{50}$  cytotoxicity value and the calculated AC is a dimensionless value. Following this approach,  
442 the ratio between XAD-8 and XAD-4 resin calculated additive cytotoxicity was similar to the  
443 ratio of measured cytotoxicity index values (Fig. 6). While THMs were the major DBPs present  
444 in the XAD-8 fraction, most of the calculated additive cytotoxicity was attributed to HANs and  
445 HAcAms. DBAN, BAcAm, BCAN and DBAcAm were the dominant cytotoxic DBPs,  
446 accounting for 68.7%, 14.0%, 8.2% and 6.7% of the calculated AC, respectively. In the XAD-4  
447 fraction, HAcAms and HAAs accounted for the majority of the calculated additive cytotoxicity,  
448 with DBAcAm, BCACAm and MBAA accounting for 64.8%, 16.5% and 13.4%, respectively.  
449 Overall, N-DBPs (i.e., HANs and HAcAms) were responsible for the majority of the calculated  
450 cytotoxicity in both fractions (99.4% and 78.5% for XAD-8 and XAD-4 fractions, respectively).  
451



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).

461 
$$CCTI = \sum_{i=1}^n \frac{p_i}{LC_{50,i}} \quad (2)$$

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

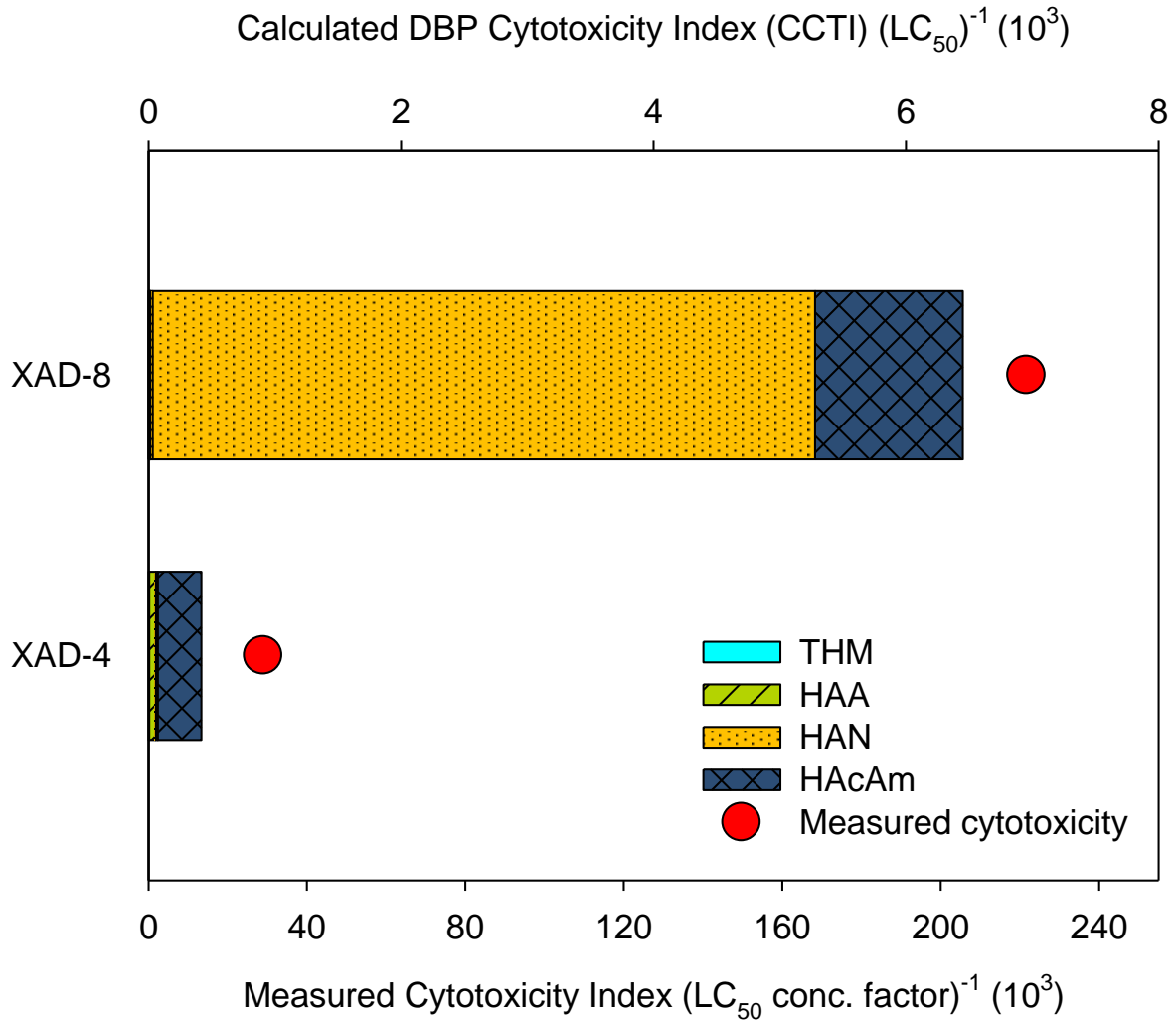
466 Following this approach, the CCTI value of the XAD-8 fraction was still higher than that of the  
 467 XAD-4 fraction, in accordance with measured cytotoxicity index values, but the ratio between  
 468 the calculated cytotoxicity of XAD-8 and XAD-4 isolates was much lower than from the first  
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

477 
$$CCTI = \sum_{i=1}^n \frac{C_i}{LC_{50,i}} \quad (3)$$

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

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

482



484

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

488 AOX: adsorbable organic halide.

489

490 **3. Conclusions**

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

- 493 - More DBPs were isolated from the XAD-8 resin, i.e., hydrophobic (HPO) EfOM fraction,  
494 which is in accordance with the higher toxicity observed for this fraction, when compared  
495 to the results obtained with the XAD-4 resin.
- 496 - Isolation recovery of DBPs onto XAD resins depends on their hydrophilicity: most  
497 hydrophobic THMs and HANs were mostly retained on the XAD-8 resin (also showing a  
498 larger proportion of DOC adsorbed), while more hydrophilic HAAs, HAcAms and HAcAls  
499 were less retained on both resins, leading to higher concentrations in the organic fraction  
500 that did not adsorb onto the two superposed resins. The toxicity of the non-retained organic  
501 fraction needs to be evaluated.
- 502 - Measured DBPs accounted for minor proportions of the brominated and chlorinated  
503 products analyzed as AOB<sub>r</sub> and AOCl, which means that most AOX consisted in unknown  
504 brominated and chlorinated compounds.
- 505 - Estimated cytotoxicity of XAD-8 resin/hydrophobic (HPO) and XAD-4 resin/transphilic  
506 (TPI) isolates calculated based on additive cytotoxicity of measured DBPs did not account  
507 for the difference observed between the measured cytotoxicity of the two fractions. Other  
508 products were probably responsible for the higher toxicity of the HPO fraction. These  
509 products may include chlorinated, brominated and iodinated byproducts, or non-  
510 halogenated byproducts (e.g., nitrosamines) and unknown high-molecular weight  
511 compounds.
- 512 - Among the measured DBPs, N-DBPs (i.e., HANs and HAcAms) contributed to the  
513 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.

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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>.

## 524 **References**

- 525 Allard, S., Tan, J., Joll, C.A., von Gunten, U., 2015. Mechanistic Study on the Formation of Cl-  
526 /Br-/I-Trihalomethanes during Chlorination/Chloramination Combined with a Theoretical  
527 Cytotoxicity Evaluation. *Environ. Sci. Technol.* doi:10.1021/acs.est.5b02624
- 528 Bond, T., Huang, J., Templeton, M.R., Graham, N., 2011. Occurrence and control of nitrogenous  
529 disinfection by-products in drinking water – A review. *Water Res.* 45, 4341–4354.
- 530 Box, G.E.P., Hunter, W.G., Hunter, J.S., 1978. *Statistics for Experimenters: An Introduction to*  
531 *Design, Data Analysis, and Model Building.* Wiley & Sons Inc.: New York, NY.
- 532 Chen, W., Westerhoff, P., Leenheer, J.A., Booksh, K., 2003. Fluorescence Excitation-Emission  
533 Matrix Regional Integration to Quantify Spectra for Dissolved Organic Matter. *Environ. Sci.*  
534 *Technol.* 37, 5701–5710.
- 535 Croué, J.-P., 2004. Isolation of humic and non-humic NOM fractions: structural characterization.  
536 *Environ. Monit. Assess.* 92, 193–207.
- 537 Dong, S., Lu, J., Plewa, M.J., Nguyen, T.H., 2016. Comparative Mammalian Cell Cytotoxicity of  
538 Wastewaters for Agricultural Reuse after Ozonation. *Environ. Sci. Technol.* 50, 11752–11759.  
539 doi:10.1021/acs.est.6b04796

540 Dotson, A., Westerhoff, P., Krasner, S.W., 2009. Nitrogen enriched dissolved organic matter  
541 (DOM) isolates and their affinity to form emerging disinfection by-products. *Water Sci. Technol.*  
542 60, 135. doi:10.2166/wst.2009.333

543 Drewes, J.E., Croue, J.-P., 2002. New approaches for structural characterization of organic  
544 matter in drinking water and wastewater effluents, *Water Science and Technology: Water*  
545 *Supply*.

546 Jeong, C.H., Wagner, E.D., Siebert, V.R., Anduri, S., Richardson, S.D., Daiber, E.J., McKague,  
547 A.B., Kogevinas, M., Villanueva, C.M., Goslan, E.H., Luo, W., Isabelle, L.M., Pankow, J.F.,  
548 Grazuleviciene, R., Cordier, S., Edwards, S.C., Righi, E., Nieuwenhuijsen, M.J., Plewa, M.J.,  
549 2012. Occurrence and toxicity of disinfection byproducts in European drinking waters in relation  
550 with the HIWATE epidemiology study. *Environ. Sci. Technol.* 46, 12120–12128.

551 Kimura, S.Y., Komaki, Y., Plewa, M.J., Mariñas, B.J., 2013. Chloroacetonitrile and N,2-  
552 dichloroacetamide formation from the reaction of chloroacetaldehyde and monochloramine in  
553 water. *Environ. Sci. Technol.* 47, 12382–12390. doi:10.1021/es4029638

554 Krasner, S.W., Scilimenti, M.J., Mitch, W., Westerhoff, P., Dotson, A., 2007. Using formation  
555 potential tests to elucidate the reactivity of DBP precursors with chlorine versus with  
556 chloramines, in: *Proc. of the 2007 AWWA Water Quality Technology Conference*, Denver.  
557 AWWA.

558 Kumaravel, T.S., Jha, A.N., 2006. Reliable Comet assay measurements for detecting DNA  
559 damage induced by ionising radiation and chemicals. *Mutat. Res. Toxicol. Environ. Mutagen.*  
560 605, 7–16. doi:10.1016/j.mrgentox.2006.03.002

561 Le Roux, J., Nihemaiti, M., Croué, J.-P., 2016. The role of aromatic precursors in the formation  
562 of haloacetamides by chloramination of dissolved organic matter. *Water Res.* 88, 371–379.  
563 doi:10.1016/j.watres.2015.10.036

564 Leenheer, J.A., Jean-Philippe Croué, Mark Benjamin, Gregory V. Korshin, Cordelia J. Hwang,  
565 Auguste Bruchet, George R. Aiken, 2000. Comprehensive Isolation of Natural Organic Matter  
566 from Water for Spectral Characterizations and Reactivity Testing, in: *Natural Organic Matter*  
567 *and Disinfection By-Products*, ACS Symposium Series. American Chemical Society, pp. 68–83.

568 Liviac, D., Wagner, E.D., Mitch, W.A., Altonji, M.J., Plewa, M.J., 2010. Genotoxicity of Water  
569 Concentrates from Recreational Pools after Various Disinfection Methods. *Environ. Sci.*  
570 *Technol.* 44, 3527–3532. doi:10.1021/es903593w

571 Lovell, D.P., Omori, T., 2008. Statistical issues in the use of the comet assay. *Mutagenesis* 23,  
572 171–182. doi:10.1093/mutage/gen015

573 Muellner, M.G., Wagner, E.D., McCalla, K., Richardson, S.D., Woo, Y.-T., Plewa, M.J., 2007.  
574 Haloacetonitriles vs. Regulated Haloacetic Acids: Are Nitrogen-Containing DBPs More Toxic?  
575 *Environ. Sci. Technol.* 41, 645–651. doi:10.1021/es0617441

576 Munch, D.J., Hautman, D.P., 1995. EPA Method 551.1 Determination of Chlorination  
577 Disinfection Byproducts, Chlorinated Solvents, and Halogenated Pesticides/Herbicides in



- 578 Drinking Water by Liquid-Liquid Extraction and Gas Chromatography with Electron-Capture  
579 Detection, Revision 1.0. United States Environmental Protection Agency, Cincinnati, OH.
- 580 Munch, D.J., Munch, J.W., 1995. EPA Method 552.2 Determination of Haloacetic Acids and  
581 Dalapon in Drinking Water by Liquid-liquid Extraction, Derivatization and Gas Chromatography  
582 with Electron Capture Detection, Revision 1.0. United States Environmental Protection Agency,  
583 Cincinnati, OH.
- 584 Plewa, M.J., Muellner, M.G., Richardson, S.D., Fasano, F., Buettner, K.M., Woo, Y.-T.,  
585 McKague, A.B., Wagner, E.D., 2007. Occurrence, synthesis, and mammalian cell cytotoxicity  
586 and genotoxicity of haloacetamides: an emerging class of nitrogenous drinking water  
587 disinfection byproducts. *Environ. Sci. Technol.* 42, 955–961.
- 588 Plewa, M.J., Wagner, E.D., 2009. Mammalian Cell Cytotoxicity and Genotoxicity of  
589 Disinfection By-Products. Water Research Foundation: Denver, CO.
- 590 Plewa, M.J., Wagner, E.D., Jazwierska, P., Richardson, S.D., Chen, P.H., McKague, A.B., 2004.  
591 Halonitromethane Drinking Water Disinfection Byproducts: Chemical Characterization and  
592 Mammalian Cell Cytotoxicity and Genotoxicity. *Environ. Sci. Technol.* 38, 62–68.  
593 doi:10.1021/es0304771
- 594 Plewa, M.J., Wagner, E.D., Metz, D.H., Kashinkunti, R., Jamriska, K.J., Meyer, M., 2012.  
595 Differential Toxicity of Drinking Water Disinfected with Combinations of Ultraviolet Radiation  
596 and Chlorine. *Environ. Sci. Technol.* 46, 7811–7817. doi:10.1021/es300859t
- 597 Plewa, M.J., Wagner, E.D., Mitch, W.A., 2011. Comparative Mammalian Cell Cytotoxicity of  
598 Water Concentrates from Disinfected Recreational Pools. *Environ. Sci. Technol.* 45, 4159–4165.  
599 doi:10.1021/es104284h
- 600 Plewa, M.J., Wagner, E.D., Muellner, M.G., Hsu, K.-M., Richardson, S.D., 2008. Comparative  
601 Mammalian Cell Toxicity of N-DBPs and C-DBPs, in: *Disinfection By-Products in Drinking  
602 Water*, ACS Symposium Series. American Chemical Society, pp. 36–50.
- 603 Richardson, S.D., 2011. XAD resin extraction of disinfectant by-products from drinking water:  
604 SOP - RSB-003.1- Revision No. 1. Environmental Protection Agency, Athens, GA.
- 605 Richardson, S.D., DeMarini, D.M., Kogevinas, M., Fernandez, P., Marco, E., Lourencetti, C.,  
606 Ballesté, C., Heederik, D., Meliefste, K., McKague, A.B., Marcos, R., Font-Ribera, L., Grimalt,  
607 J.O., Villanueva, C.M., 2010. What's in the Pool? A Comprehensive Identification of  
608 Disinfection By-products and Assessment of Mutagenicity of Chlorinated and Brominated  
609 Swimming Pool Water. *Environ. Health Perspect.* 118, 1523–1530. doi:10.1289/ehp.1001965
- 610 Richardson, S.D., Fasano, F., Ellington, J.J., Crumley, F.G., Buettner, K.M., Evans, J.J., Blount,  
611 B.C., Silva, L.K., Waite, T.J., Luther, G.W., others, 2008. Occurrence and mammalian cell  
612 toxicity of iodinated disinfection byproducts in drinking water. *Environ. Sci. Technol.* 42, 8330–  
613 8338.
- 614 Richardson, S.D., Plewa, M.J., Wagner, E.D., Schoeny, R., Demarini, D.M., 2007. Occurrence,  
615 genotoxicity, and carcinogenicity of regulated and emerging disinfection by-products in drinking

616 water: A review and roadmap for research. *Mutat. Res. Mutat. Res.* 636, 178–242.  
617 doi:10.1016/j.mrrev.2007.09.001

618 Richardson, S.D., Postigo, C., 2015. Formation of DBPs: State of the Science, in: Karanfil, T.,  
619 Mitch, B., Westerhoff, P., Xie, Y. (Eds.), *Recent Advances in Disinfection By-Products*.  
620 American Chemical Society, Washington, DC, pp. 189–214.

621 Smith, E.M., Plewa, M.J., Lindell, C.L., Richardson, S.D., Mitch, W.A., 2010. Comparison of  
622 Byproduct Formation in Waters Treated with Chlorine and Iodine: Relevance to Point-of-Use  
623 Treatment. *Environ. Sci. Technol.* 44, 8446–8452. doi:10.1021/es102746u

624 Tang, J.Y.M., McCarty, S., Glenn, E., Neale, P.A., Warne, M.S.J., Escher, B.I., 2013. Mixture  
625 effects of organic micropollutants present in water: Towards the development of effect-based  
626 water quality trigger values for baseline toxicity. *Water Res.* 47, 3300–3314.  
627 doi:10.1016/j.watres.2013.03.011

628 Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y.,  
629 Rojas, E., Ryu, J.-C., Sasaki, Y.F., 2000. Single cell gel/comet assay: Guidelines for in vitro and  
630 in vivo genetic toxicology testing. *Environ. Mol. Mutagen.* 35, 206–221.  
631 doi:10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J

632 U.S. Environmental Protection Agency, 2006. National primary drinking water regulations:  
633 Stage 2 disinfectants and disinfection byproducts rule. *Federal Register* 71:387-493.

634 Wagner, E.D., Plewa, M.J., 2009. Microplate-Based Comet Assay, in: Dhawan A, Anderson D  
635 (Eds.), *The Comet Assay in Toxicology*. Royal Society of Chemistry: London, Pp. 79-97.

636 Wagner, E.D., Rayburn, A.L., Anderson, D., Plewa, M.J., 1998. Analysis of mutagens with  
637 single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone  
638 of Chinese hamster ovary cells. *Environ. Mol. Mutagen.* 32, 360–368. doi:10.1002/(SICI)1098-  
639 2280(1998)32:4<360::AID-EM10>3.0.CO;2-T

640 Weinberg, H.S., 2009. Modern approaches to the analysis of disinfection by-products in drinking  
641 water. *Philos. Trans. R. Soc. Lond. Math. Phys. Eng. Sci.* 367, 4097–4118.  
642 doi:10.1098/rsta.2009.0130

643 Westerhoff, P., Mash, H., 2002. Dissolved organic nitrogen in drinking water supplies: A review.  
644 *J. Water Supply Res. Technol. - AQUA* 51, 415–448.

645 Zeng, T., Plewa, M.J., Mitch, W.A., 2016. N-Nitrosamines and halogenated disinfection  
646 byproducts in U.S. Full Advanced Treatment trains for potable reuse. *Water Res.* 101, 176–186.  
647 doi:10.1016/j.watres.2016.03.062

648 Zheng, X., Khan, M.T., Croué, J.-P., 2014. Contribution of effluent organic matter (EfOM) to  
649 ultrafiltration (UF) membrane fouling: Isolation, characterization, and fouling effect of EfOM  
650 fractions. *Water Res.* 65, 414–424. doi:10.1016/j.watres.2014.07.039

651