Chloramination of wastewater effluent: Toxicity and formation of disinfection byproducts

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2   byproducts

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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

2

byproducts (N-DBPs) due to their enrichment in nitrogen-containing compounds (e.g., ammonia
or organic nitrogen such as amino acids and peptides) (Bond et al., 2011; Westerhoff and Mash,
2002). N-DBPs generally form in lower concentrations than non-nitrogenous regulated DBPs
(i.e., trihalomethanes, THMs, and haloacetic acids, HAAs), but may present a higher health risk
(Muellner et al., 2007; Plewa et al., 2004). *In vitro* mammalian cell assays have demonstrated
that N-DBPs such as haloacetonitriles (HANs) (Muellner et al., 2007), halonitromethanes
(HNMs) (Plewa et al., 2004) and haloacetamides (HAcAms) (Plewa et al., 2007) exhibit orders
of magnitude higher levels of cytotoxicity and genotoxicity than THMs and HAAs (Plewa et al.,
2008). Reclaiming wastewater for various agricultural, industrial or municipal applications is of
growing interest but requires the practice of disinfection to prevent outbreaks of waterborne
diseases. Dissolved organic matter isolated from wastewater effluent (EfOM) was found to be
significantly more enriched in nitrogen (i.e., to be a potential source of N-DBPs) than organics
recovered from surface waters (e.g., N/C mass ratios up to 0.17 compared to 0.01-0.06 for river
waters) (Drewes and Croue, 2002; Le Roux et al., 2016; Zheng et al., 2014).

  Moreover, the presence of bromide and iodide ion in wastewater (especially at locations where
potable water is produced through the desalination of seawater or brackish water) favors the
formation of brominated and iodinated byproducts that are often more toxic than their
chlorinated analogues (Plewa et al., 2008; Richardson et al., 2008, 2007). Brominated and
iodinated N-DBPs are among the most cytotoxic/genotoxic disinfection by-products known
today (Muellner et al., 2007; Plewa et al., 2008). Water utilities, especially in the USA, have
been increasingly switching chlorine disinfection to monochloramine to reduce the concentration
of regulated THMs and HAAs (U.S. Environmental Protection Agency, 2006), however,
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 (Liviac 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 - $CHCl_2Br$, 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.

111

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,

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: NH_2Cl (mg/L as Cl_2) = 3 × DOC (dissolved

147 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 ± 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_2 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_2 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_2O 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 µL of DMSO

204 ($10^5\times$ concentration) and stored in Teflon-sealed sample vials at −20°C. For each experiment a

205 50× dilution of each sample stock solution was prepared in F12 cell culture medium+5% fetal

206 bovine serum (FBS).

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 µg/mL streptomycin

211 sulfate, 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37 °C in a humidified

212 atmosphere of 5% CO_2.

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 (~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 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 (LC_{50}^{-1})(10^3) and allowed for easy

224 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 $(50\% \text{ TDNA}^{-1})(10^3)$ 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

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 ≥ 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_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

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_2O (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_2/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_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

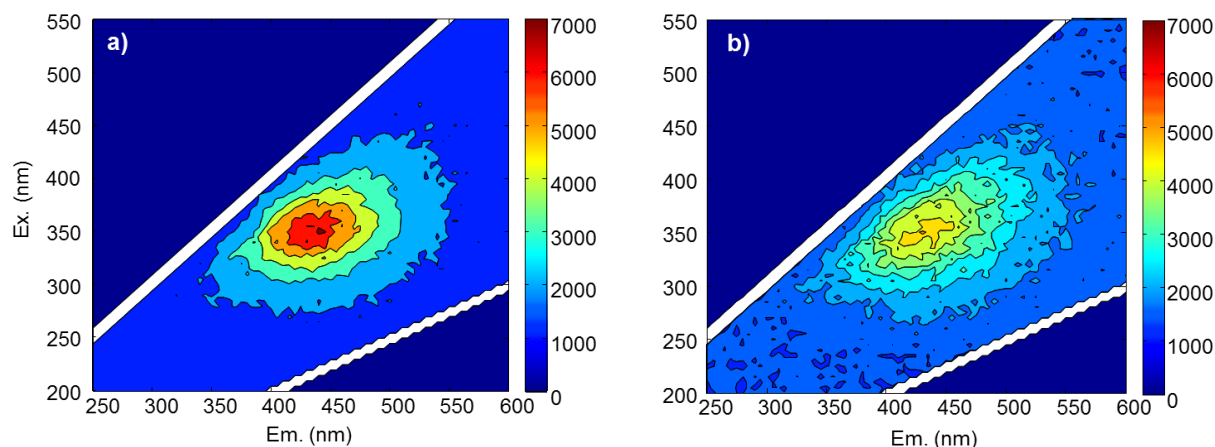|				
WW1 (Non-chloraminated)	4.65	45.8	23.7	30.5
WW2 (Chloraminated)	4.55	34.3	23.1	42.6
WW3 (Chloraminated)	4.60	42.4	22.2	35.4

286

287 Fig. 1. Fluorescence EEMs of a) wastewater effluent WW2 and b) wastewater effluent WW2

288 after 72h chloramination (14.2 mg Cl$_2$/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 ($\mu g/\mu L$)	Chronic Cytotoxicity (LC_{50}) ($\mu g/\mu L$) [a]	r^{2} [b]	Lowest Significant Toxic Concentration ($\mu g/\mu L$) [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$_2$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$_2$Cl)

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.
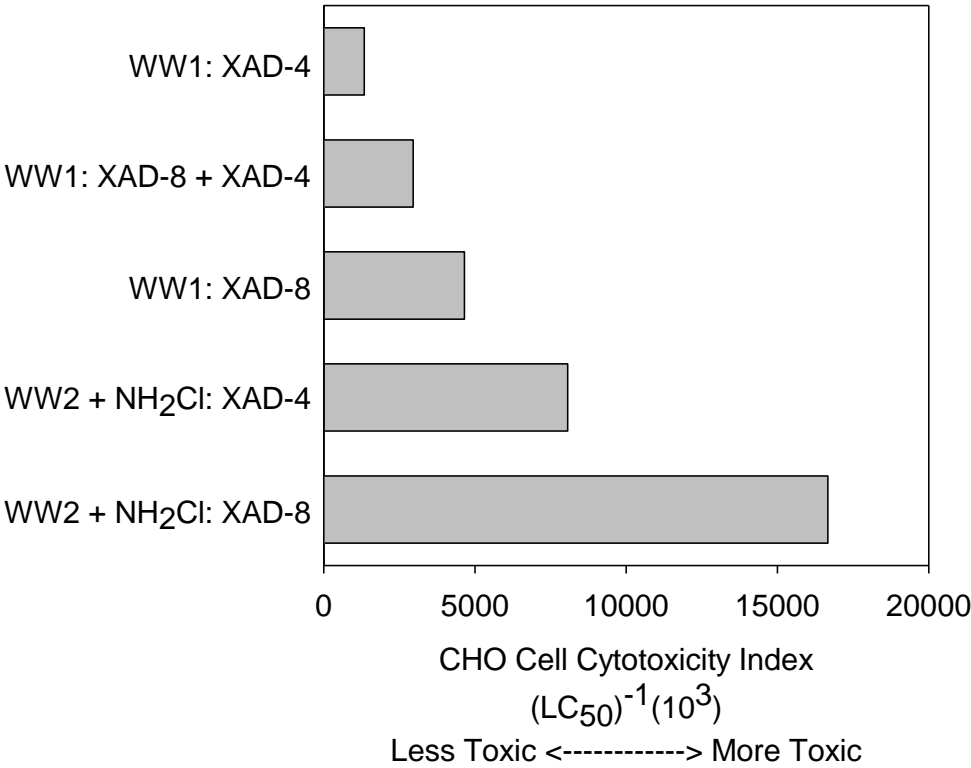

315

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 $(50\%\text{Tail DNA}^{-1})(10^3)$ 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 (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$_2$Cl	XAD-4	2.0-3.4	2.45	0.98	2.0	$F_{11, 28} = 153.0$: $P \leq 0.001$
WW2+NH$_2$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. [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.
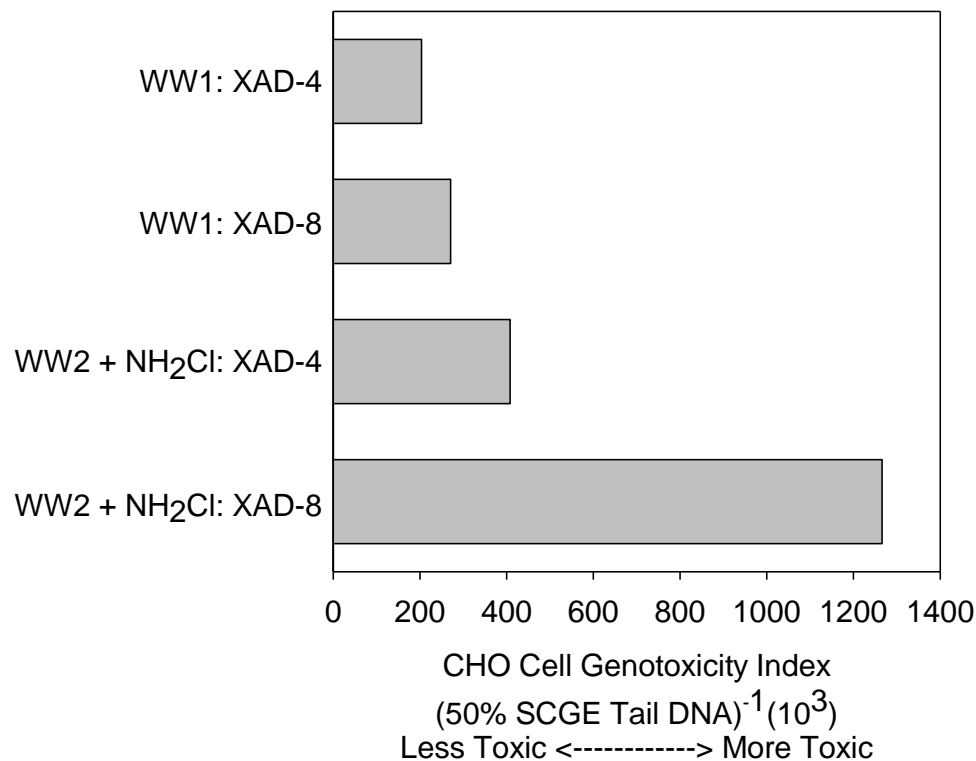
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Fig. 3. Comparison of the CHO cell genotoxicity index values for each fraction of non-

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

CHO: Chinese hamster ovary.

The genotoxicity data indicate that the wastewater EfOM isolated from the XAD-8 resin was

more genotoxic than the XAD-4 extracts of the same wastewaters. This suggests that

hydrophobic EfOM comprised more potent inducers of genomic DNA damage than transphilic

EfOM. Chloramination had a major effect on the resulting genotoxicity of the XAD-8 extracted

organic material, significantly more that for the XAD-4 isolate. These data indicate that the

chloraminated EfOM extracted over XAD-8 or XAD-4 resins were more genotoxic than non-

chloraminated EfOM.

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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

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
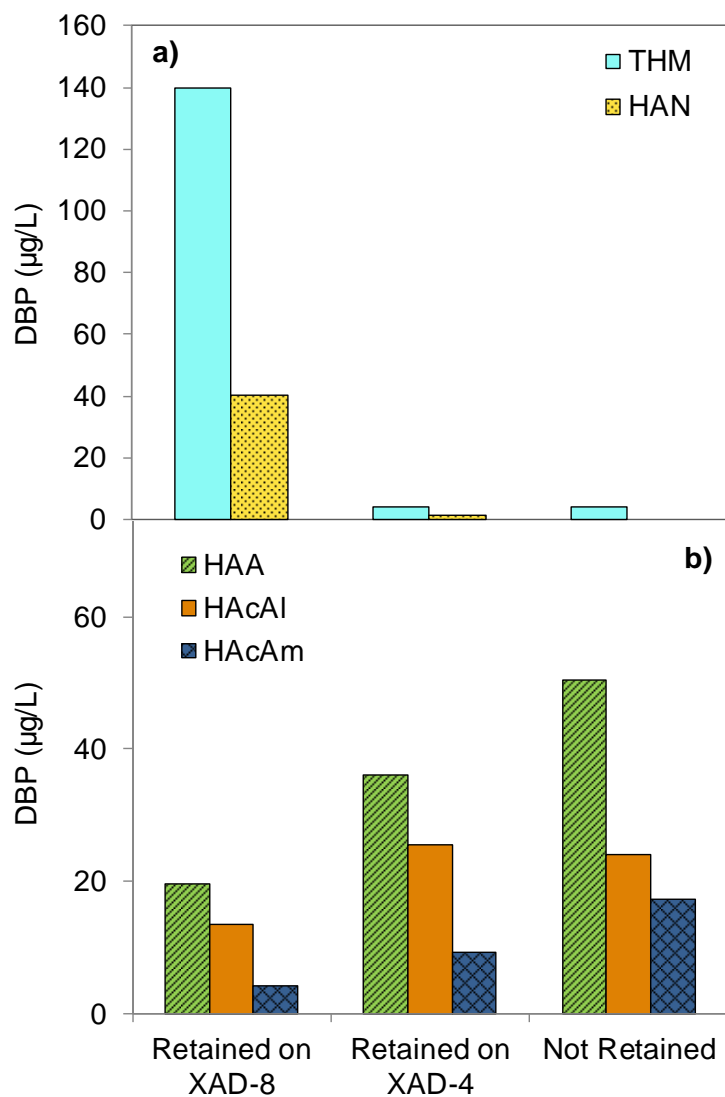
377   fraction).

378

Fig. 4. Retention of a) THMs and HANs and b) HAAs, HAcAl and HAcAms onto XAD-8 and

XAD-4 resins used in series. DBP concentrations are expressed as the total sum of each

individual DBP in a given class of DBP. THMs: trihalomethanes; HANs: haloacetonitriles;

HAAs: haloacetic acids; HAcAls: haloacetaldehydes; HAcAms: haloacetamides; DBP:

disinfection byproduct.

2.3.3. DBP contributions to AOX

387   AOBr and AOCl 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 (AOCl and AOBr) retained

390   on each resin were calculated in the same manner than individual DBPs (i.e., by difference

391   between inlet and outlet concentrations). AOBr and AOCl 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 AOCl and 79.2% of AOBr 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 AOCl and AOBr concentrations (Fig. 5). Unknown AOCl (UAOCl) and

398   unknown AOBr (UAOBr) were determined from the differences between measured AOCl or

399   AOBr and the sum of chlorine or bromine-equivalent concentrations of measured specific DBPs.

400   Br-DBPs contributed to 23.1% of AOBr retained on the XAD-8 resin and to 9.2% of AOBr

401   retained on the XAD-4 resin. Bromoform, DBAN and DBCM were the three major Br-DBPs

402   contributing to AOBr 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 AOCl retained on XAD-8

406   and XAD-4 resins, respectively, which indicates that a majority of chlorine-containing

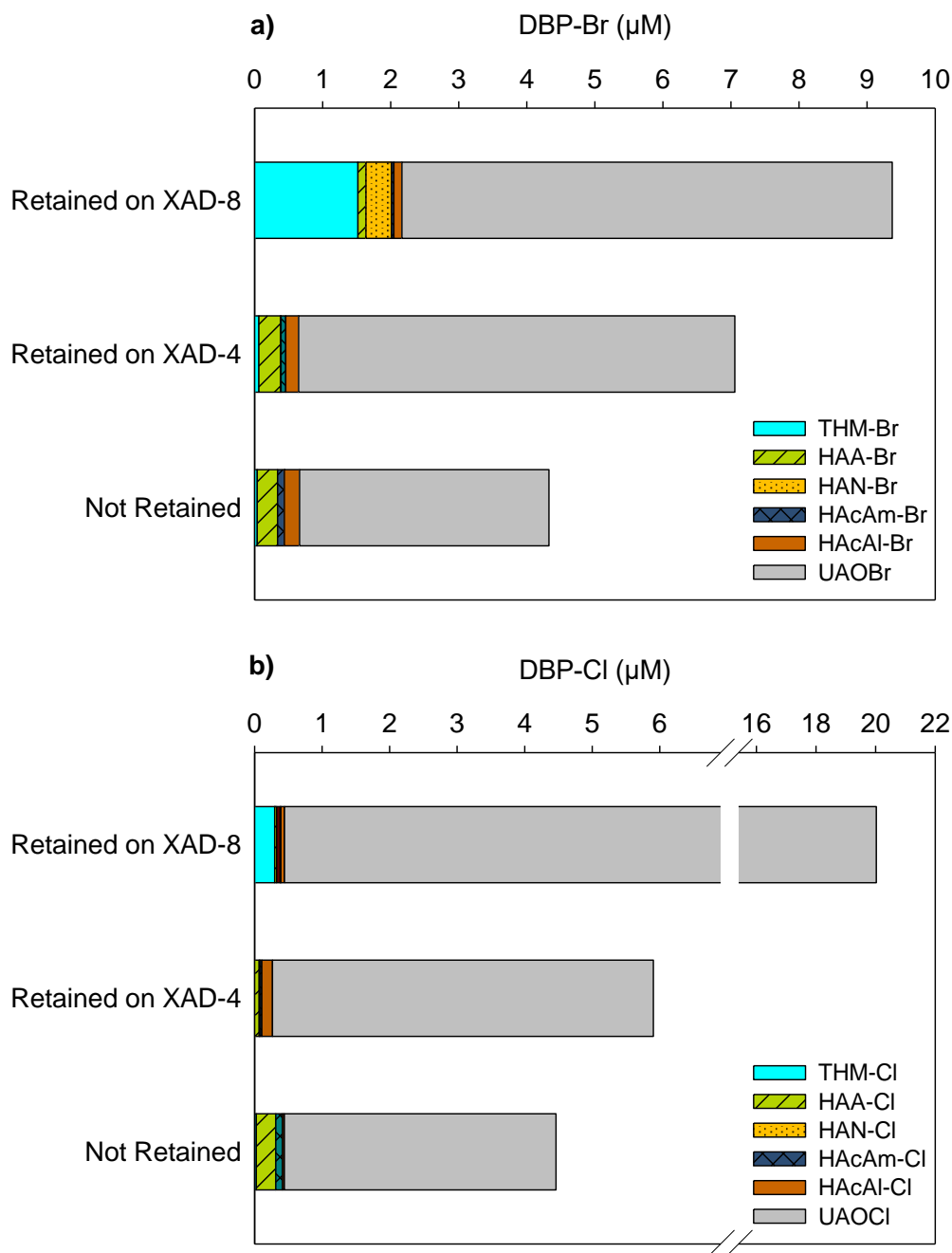407   compounds were not quantified.

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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

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_{50}

420 concentration factor as discussed previously (Appendix A, Table S2). Cytotoxicity index (CTI)

421 value derived from LC_{50} 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_{50} value available in the literature (Eq. 1) (Allard et al., 2015; Smith et al., 2010).

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

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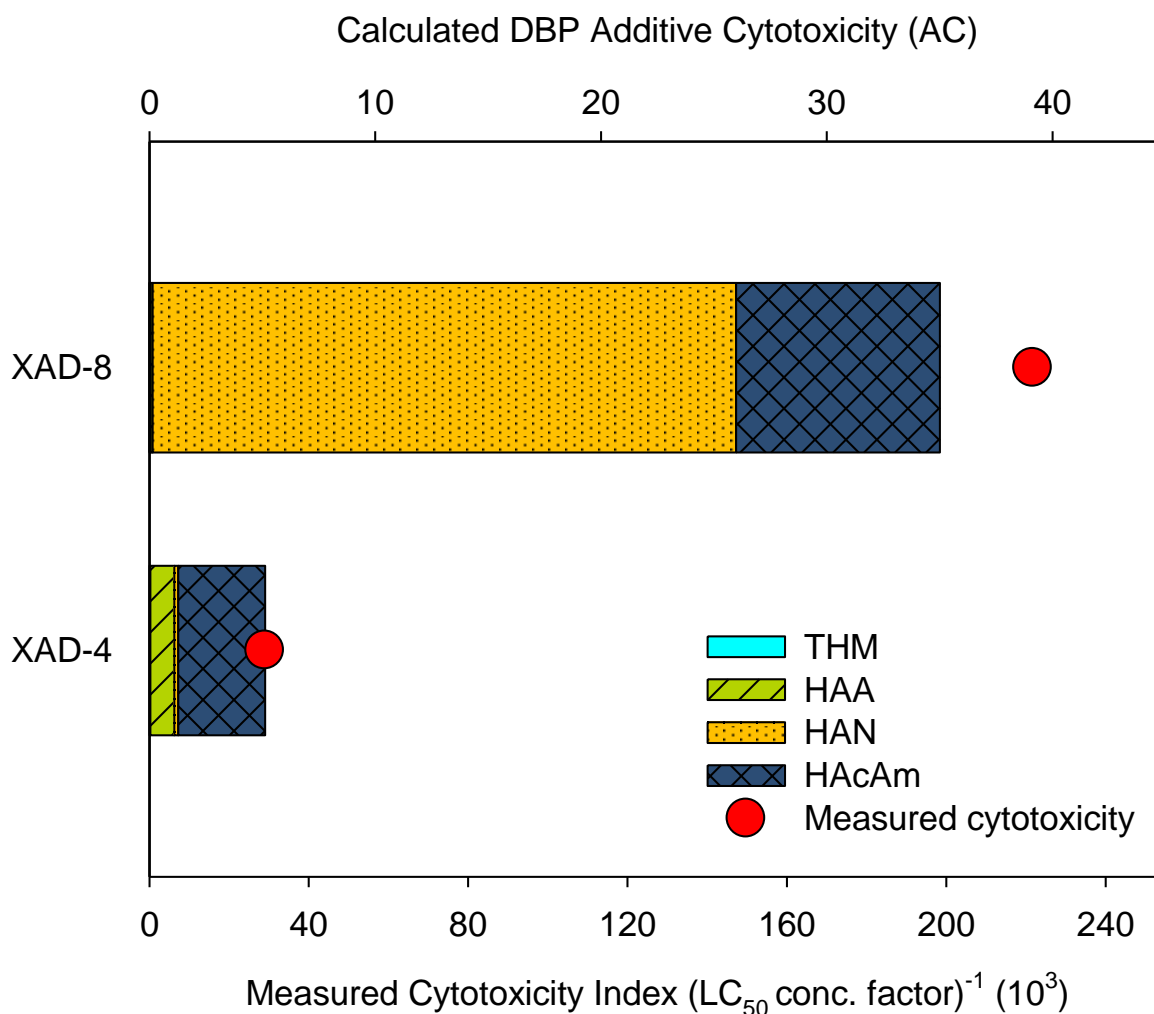
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Fig. 6. DBP additive cytotoxicity (AC) calculated from measured concentrations of DBPs in the XAD-8 and XAD-4 resin fractions and individual DBP cytotoxicity LC_{50} values, compared to the measured cytotoxicity index values of the two fractions.

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

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

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

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).

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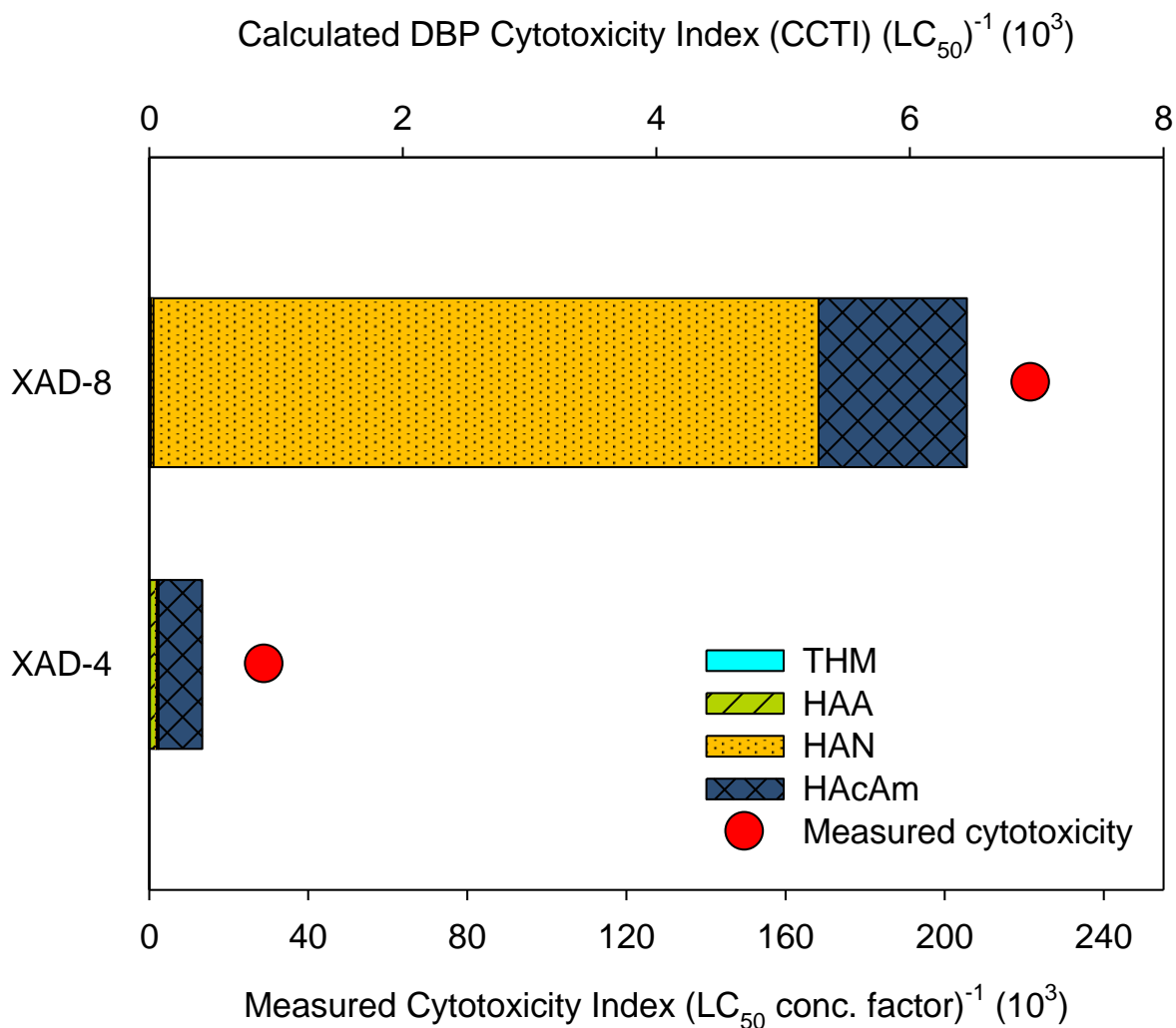
Fig. 7. DBP additive cytotoxicity index (CCTI) values calculated from molar fractions of each

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

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 AOBr 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.

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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