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Chloramination of wastewater effluent: Toxicity and formation of disinfection byproducts

Julien Le Roux 1, Michael J. Plewa 2, Elizabeth D. Wagner 2, Maolida Nihemaiti 3, Azra Dad 4, and Jean-Philippe Croué 3*

1 LEESU (UMR MA 102), Université Paris-Est – AgroParisTech, 94010 Créteil cedex, France
2 Department of Crop Sciences, and the Global Safe Water Institute, University of Illinois at Urbana–Champaign, Urbana, IL 61801, United States
3 Curtin Water Quality Research Centre, Department of Chemistry, Curtin University, GPO Box U1987, Perth, WA 6845, Australia
4 US Food and Drug Administration National Center for Toxicological Research, Jefferson, AR, 72079, United States

E-mail addresses: julien.le-roux@u-pec.fr (Julien Le Roux), mplewa@illinois.edu (Michael J. Plewa), edwagner@illinois.edu (Elizabeth D. Wagner), maolida.nihemaiti@curtin.edu.au (Maolida Nihemaiti), nfn.azra@fda.hhs.gov (Azra Dad), jean-philippe.croue@curtin.edu.au (Jean-Philippe Croué)

*Corresponding author: Phone: +61 (0)8 9266 9793; email: jean-philippe.croue@curtin.edu.au
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
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
Chloramines can also be formed unintentionally from the reaction between free chlorine and ammonia during chlorination, which may increase the risk of N-DBP formation when high ammonia concentrations are present.

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

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

1. Materials and methods

1.1. Materials

Analytical or laboratory grade reagents and were used without further purification. MilliQ water was produced with a Millipore system (18.2 MΩ.cm). Sodium hypochlorite (NaOCl, 5.65-6%, Fisher Scientific) and ammonium chloride (Acros Organics, 99.6%) were used to prepare chloramine solutions. Methyl tert-butyl ether (MTBE) and ethyl acetate (> 99%, Fisher Scientific) were used for DBP extractions without further purification. A THM calibration mix (chloroform - TCM, dichlorobromomethane - CHCl₂Br, chlorodibromomethane - CHClBr₂, and bromoform - TBM), a mixed standard (EPA 551B Halogenated Volatiles Mix) containing HANs, trichloronitromethane (TCNM, or chloropicrin) and haloketones (HKs), and a mixed standard containing 9 HAAs (EPA 552.2 Methyl Ester Calibration Mix) were supplied from Supelco (Sigma-Aldrich). Chloro-, bromo-, dichloro-, and trichloroacetamide (CAcAm, BAcAm, DCAcAm and TCAcAm, respectively) were obtained from Sigma-Aldrich. Other HAcAmS (i.e., dibromoacetamide - DBAcAm, tribromoacetamide - TBAcAm, bromochloroacetamide - BCAcAm, chloroiodoacetamide - CIAcAm, bromoiodoacetamide - BIAcAm and diiodoacetamide - DIAcAm) were purchased from Cansyn Chem. Corp. Haloacetaldehydes (HAcAlS) were obtained from TCI America, Cansyn Chem. Corp. and Sigma-Aldrich. Decafluorobiphenyl (99%, Sigma-Aldrich, Supelco) was used as a surrogate standard. 2 bromopropionic acid (Fluka Analytical) was used as a surrogate for HAA extractions and analyses.
1.2. **Analytical methods**

Total Organic Carbon (TOC) and Total Nitrogen (TN) concentrations were measured using a TOC analyzer equipped with a TN detection unit (TOC-VCSH, Shimadzu). Three-dimensional fluorescence excitation - emission matrices (EEMs) were obtained by a Fluoromax fluorometer (Horiba Scientific, Japan). Samples for Adsorbable Organic Halide (AOX) analyses were processed through adsorption on activated carbon columns using a TOX sample preparatory unit (TXA-03, Mitsubishi Chemical Analytech Co., Ltd., Japan). AOX were then transformed into hydrogen halides by combustion (950 °C) of the activated carbon for at least 30 min via an AOX-200 adsorbable halogen analyzer and then collected in Milli-Q water as chloride and bromide ions. Offline quantification of chloride and bromide ions was performed by a Dionex 1600 reagent free ion chromatograph (IC) equipped with a conductivity detector and a Dionex IonPac AS-15 column (2 × 250 mm) and using an online KOH eluent (30 mM) generator at a flow rate of 0.4 mL/min. AOCl and AOb concentration 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. NH₂Cl and NHCl₂ concentrations in stock solutions were determined by spectrophotometric measurement using their respective molar extinction coefficients at 245 nm and 295 nm. Residual oxidant during chloramination reactions was analyzed by DPD colorimetric method. Four THMs, four HANs, two HKs, chloropicrin and seven HAcAls were extracted and analyzed following EPA method 551, which consists of a liquid-liquid extraction using MTBE followed by gas chromatography coupled with electron capture detector (GC-ECD) or GC-MS (Munch and Hautman, 1995). Nine HAAs were extracted and analyzed following EPA method 552.2,
which is based on a liquid-liquid extraction with MTBE in acidic conditions followed by derivatization to methyl esters using acidic methanol, and analysis by GC-MS (Munch and Munch, 1995). HACs were analyzed following the same EPA method 551 protocol, replacing MTBE by ethyl acetate for the liquid-liquid extraction. Samples were also analyzed in GC-MS full scan mode to search for other DBPs and unknown mass spectra were subjected to library database searching (National Institute of Standards and Technology - NIST).

1.3. Chloramination conditions

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

1.4. Effluent organic matter (EfOM) extraction procedure
Wastewater secondary effluent samples were collected at three different periods (WW1, 2 and 3, Table 1) from a wastewater treatment plant (Jeddah, Saudi Arabia) before chlorine disinfection. Samples were transferred to the laboratory, filtered on cartridge filters (Polygard® 10 µm pore size, Millipore) prior to the chlorination and fractionation experiments to remove any particulate matter and stored at 4 °C for maximum 7 days. The hydrophobic (HPO) and transphilic (TPI) fractions of the non-chloraminated and chloraminated (laboratory conditions) wastewater EfOM were isolated using a comprehensive isolation protocol described elsewhere (Croué, 2004; Leenheer et al., 2000). Briefly, wastewater effluent samples (before or after 72 hr chloramination) were acidified at pH 2 with hydrochloric acid and passed through XAD-8 and XAD-4 macroporous resins in series at a flow rate of 5.2 L/hr. Resins were then rinsed with formic acid at pH 2 in order to remove the inorganic species present in the void volume without eluting the organics adsorbed onto the resins, which were recovered afterward with either acetonitrile/water (75:25 v/v), acetonitrile or ethyl acetate. During the adsorption phase, 100 mL samples were collected from the output of the resins for DOC and DBP measurements. For effluent WW1, organics adsorbed onto the two XAD resins and eluted with the same volume of acetonitrile/water were concentrated using a rotary evaporator to remove acetonitrile. Equal volumes (50:50 v/v) of the fractions obtained from the two resins were also combined before the rotary evaporation step to obtain a mixed XAD-8+XAD-4 extract. The water concentrates were lyophilized to obtain the XAD-8, XAD-4 and mixed XAD-8+XAD-4 solid organic fractions for use in toxicity analyses. For effluent WW2, the elution of both resins was performed with pure acetonitrile to perform direct DBP identification by full-scan GC-MS on a small volume (i.e., 1.5 mL) of the extracts. Milli-Q® water was then added to the acetonitrile extracts to reach 75:25 v/v acetonitrile/water condition (same as used for effluent WW1) before rotary evaporation (removal
of acetonitrile) and lyophilization. With this approach the cytotoxicity and genotoxicity values of samples WW1 and WW2 could be expressed as per mg of EfOM. For effluent WW3, both resins were eluted with ethyl acetate and the chloraminated EfOM extracts were concentrated under a stream of N₂ to a final volume of 100 mL to allow the direct quantification of DBPs and the analysis of toxicity from the same extract, as previously described in the literature (Jeong et al., 2012; Richardson, 2011). It should be noted that these concentration approaches (i.e., lyophilization or N₂ evaporation) are usually suitable for semi- or non-volatile chemicals but not for volatile compounds (Weinberg, 2009). Nevertheless, volatile DBPs (e.g., THMs) analyzed in the ethyl acetate extracts after concentration were in accordance with concentrations analyzed in the wastewater effluent samples (see Section 2.3.4). Since the two chloraminated wastewater effluents (WW2 and WW3) were not sampled at the same time (and exhibited different DOC values) and fractions were obtained through different processes (as described above), the toxicity results of these two types of samples were not comparable.

1.5. Analytical biology

1.5.1. Sample preparation

The lyophilized or liquid organic fractions were received and stored at −20 °C. Mg amounts of each lyophilized fraction were placed into separate sterile Supelco sample vials. To sterilize the sample, DMSO was added to each sample such that for each mg of sample 1 µL of DMSO was added. After the sample was mixed (vortexed) in DMSO, 1 µL of sterile ddH₂O was added for each mg of the lyophilized fraction. The stock solution for each sample was completely dissolved in this DMSO-water (1:1 v/v) solvent and this was stored at −20°C. Ethyl acetate organic extracts (liquid form) obtained from WW3 were solvent exchanged into DMSO so that
the organic material from 1 L of wastewater effluent was concentrated into 10 µL of DMSO (10^5×
concentration) and stored in Teflon-sealed sample vials at −20°C. For each experiment a
50× dilution of each sample stock solution was prepared in F12 cell culture medium + 5% fetal
bovine serum (FBS).

1.5.2. Chinese hamster ovary cells

Chinese hamster ovary (CHO) cell line AS52, clone 11−4−8 was used for the biological assays
(Wagner et al., 1998). CHO cells were maintained on glass culture plates in Ham’s F12 medium
containing 5% FBS, 1% antibiotics (100 U/mL sodium penicillin G, 100 µg/mL streptomycin
sulfate, 0.25 µg/mL amphotericin B in 0.85% saline), and 1% glutamine at 37 °C in a humidified
atmosphere of 5% CO₂.

1.5.3. CHO cell chronic cytotoxicity assay

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

Single cell gel electrophoresis (SCGE, or Comet) assay quantitatively measures genomic deoxyribonucleic acid (DNA) damage in individual nuclei induced by a test agent (Tice et al., 2000). We employed a microplate methodology (Wagner and Plewa, 2009). The SCGE metric for genomic DNA damage was the %Tail DNA value which is the amount of DNA that migrated from the nucleus into the microgel (Kumaravel and Jha, 2006). Within each concentration factor range with >70% cell viability, a concentration-response curve was generated and regression analysis was used to fit the curve. The concentration factor inducing a 50% Tail DNA (TDNA) value was calculated using a regression analysis for each concentration-response curve. The genotoxic index (GTI) was a metric that expressed increasing values with increased genotoxic damage (50% TDNA⁻¹)(10³) 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 $\geq 0.8$ at $\alpha = 0.05$. For the SCGE assay, the %Tail DNA values were not normally distributed which limits the use of parametric statistics (Box et al., 1978). The mean %Tail DNA value for each microgel was calculated and these values were averaged among all the microgels for each wastewater sample concentration factor. Averaged mean values express a normal distribution according to the central limit theorem. An ANOVA test was conducted on
these averaged %Tail DNA values (Lovell and Omori, 2008). If a significant $F$ value of $P \leq 0.05$ was obtained, a Holm-Sidak multiple comparison versus the control group analysis was conducted (power $\geq 0.8; \alpha = 0.05$).

### 2. Results and discussion

#### 2.1. EfOM extraction recovery

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

<table>
<thead>
<tr>
<th>Wastewater effluent sample</th>
<th>Chloramination</th>
<th>Extracted volumes (L)</th>
<th>DOC before / after chloramination (mg C/L)</th>
<th>Elution solvent</th>
<th>Fractions phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>WW1</td>
<td>No</td>
<td>150</td>
<td>4.65 / N.A.</td>
<td>Acetonitrile/H$_2$O</td>
<td>Solid</td>
</tr>
<tr>
<td>WW2</td>
<td>Yes</td>
<td>69</td>
<td>4.68 / 4.55</td>
<td>Acetonitrile</td>
<td>Solid</td>
</tr>
<tr>
<td>WW3</td>
<td>Yes</td>
<td>76</td>
<td>4.97 / 4.60</td>
<td>Ethyl acetate</td>
<td>Liquid</td>
</tr>
</tbody>
</table>

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

The first experiment consisted in the extraction of 150 L non-chloraminated wastewater effluent (WW1, 4.65 mg C/L) followed by an elution with acetonitrile/H$_2$O (75:25, v/v). 69.5% DOC was retained on both XAD resins (2.13 mg C/L on XAD-8, 1.10 mg C/L on XAD-4) (Table 2). 1.42 mg C/L was measured at the output of the XAD-4 resin (consisting in the hydrophilic fraction of EfOM). The second extraction was performed with 69 L wastewater effluent (WW2, 4.68 mg C/L) after 72 hr of chloramination (14.2 mg Cl$_2$/L as preformed monochloramine, pH 7.7), followed by an elution with acetonitrile (no water was added to be
able to analyze the recovered fractions by GC-MS, see Section 2.3.1). Less DOC (i.e., humic-like substances) was adsorbed onto the XAD-8 resin (1.56 mg C/L) than from the non-chloraminated effluent (2.13 mg C/L), while WW1 and WW2 showed similar DOC content (4.65 and 4.55 mg C/L, respectively). Similar amount of DOC was adsorbed onto the XAD-4 resin, i.e., 1.10 and 1.05 mg C/L for WW1 and WW2, respectively. The observed lower retention of chloraminated EfOM onto the XAD-8 resin could be attributed to the breakdown of large organic macromolecules (i.e., humic-like substances) into smaller hydrophilic molecules during oxidation by chloramines. Accordingly, fluorescence EEMs obtained before and after chloramination of wastewater effluent WW2 (Fig. 1) exhibited a decrease of 26.5% in signal intensity after chloramination at the wavelengths showing the most intense response (i.e., excitation: 350 nm, emission: 420 nm), indicating that EfOM degradation occurred through the elimination of aromatic structures that usually exhibit fluorescence signal at those wavelengths (Chen et al., 2003). The third extraction was performed with 76 L of wastewater effluent (WW3, 4.97 mg C/L) after 72 hr of chloramination (14.9 mg Cl2/L, pH 7.7) followed by an elution with ethyl acetate. As observed for WW2, the DOC content of WW3 slightly decreased after chloramination (i.e., 4.97 vs 4.60 mg C/L). The total amount of DOC adsorbed onto the two XAD resins (64.6%) was comparable to WW1 and WW2.

Table 2. Percentages of dissolved organic carbon (DOC) isolated on XAD-8 and XAD-4 resins from wastewater effluent and chloraminated wastewater effluent (72 hr chloramination at 14.2 mg Cl2/L and at pH 7.7)

<table>
<thead>
<tr>
<th>Wastewater effluent sample</th>
<th>DOC before extraction (mg C/L)</th>
<th>% DOC isolated on XAD-8 resin</th>
<th>% DOC isolated on XAD-4 resin</th>
<th>% DOC not isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ex (nm)</td>
<td>Em (nm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
<td>---------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WW1 (Non-chloraminated)</td>
<td>4.65</td>
<td>45.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WW2 (Chloraminated)</td>
<td>4.55</td>
<td>34.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WW3 (Chloraminated)</td>
<td>4.60</td>
<td>42.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Fluorescence EEMs of a) wastewater effluent WW2 and b) wastewater effluent WW2 after 72h chloramination (14.2 mg Cl₂/L) at pH 7.7

2.2. Analytical biology results

2.2.1. CHO cell cytotoxicity results

From the data presented in Table 3 and Fig. 2, the XAD-8 EfOM isolate recovered from non-chloraminated wastewater was more cytotoxic than the XAD-4 EfOM extract of the same wastewater. According to the isolation protocol used, hydrophobic EfOM (i.e., XAD-8 isolate, humic substances-like) was more cytotoxic than transphilic EfOM (i.e., XAD-4 isolate). The mixed XAD-8+XAD-4 fraction (50:50 v/v) exhibited an intermediate cytotoxicity, consistent with its composition (see Section 1.4) and with the toxicity of the XAD-8 and XAD-4 fractions. The structural characterization performed on similar isolates obtained from the effluent collected from the same wastewater treatment plant by Zheng et al. (2014) indicated that the XAD-8
isolate was more enriched in aromatic structures including phenolic moieties than the XAD-4 extract that also incorporated carbohydrates. From the chloraminated wastewater effluent WW2, the XAD-8 extract was also more cytotoxic than the XAD-4 extract, and was significantly more cytotoxic than the non-chloraminated XAD-8 extract (WW1). The XAD-4 extract of WW2 was more cytotoxic than that of WW1 as well. This indicates a significant increase in cytotoxicity of the XAD-8 and XAD-4 extractable EfOM after chloramination. Since the amount of DOC isolated on each resin was lower from the non-chloraminated effluent than from the chloraminated one (i.e., 69.5 and 57.4%, respectively), the cytotoxicity of the chloraminated effluent was even higher when normalized to the total amount of DOC extracted on each resin (Appendix A, Fig. S1). Cytotoxicity results of chloraminated effluent WW3 obtained from ethyl acetate extracts (liquid form) exhibited a greater difference between XAD-8 and XAD-4 EfOM toxicity (i.e., XAD-8 EfOM was 7.6× more cytotoxic). These results are further discussed in paragraph 3.3.4.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction</th>
<th>Conc. Range (µg/µL)</th>
<th>Chronic Cytotoxicity (LC50) (µg/µL)</th>
<th>ANOVA Test Statistic d</th>
<th>( F_{10, 50} = 144.2: P \leq 0.001 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WW1</td>
<td>XAD-8</td>
<td>0.05-0.75</td>
<td>0.215</td>
<td>0.15</td>
<td>( F_{10, 55} = 57.6: P \leq 0.001 )</td>
</tr>
<tr>
<td>WW1</td>
<td>XAD-4</td>
<td>0.05-1.15</td>
<td>0.746</td>
<td>0.35</td>
<td>( F_{10, 51} = 174.1: P \leq 0.001 )</td>
</tr>
<tr>
<td>WW1</td>
<td>XAD-8 + XAD-4</td>
<td>0.05-0.75</td>
<td>0.338</td>
<td>0.20</td>
<td>( F_{10, 53} = 81.1: P \leq 0.001 )</td>
</tr>
<tr>
<td>WW2 (+NH₂Cl)</td>
<td>XAD8</td>
<td>0.05-0.4</td>
<td>0.060</td>
<td>0.025</td>
<td>( F_{10, 53} = 105.0: P \leq 0.001 )</td>
</tr>
<tr>
<td>WW2</td>
<td>XAD-4</td>
<td>0.05-0.5</td>
<td>0.124</td>
<td>0.05</td>
<td>( F_{10, 53} = 105.0: P \leq 0.001 )</td>
</tr>
</tbody>
</table>
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.

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**Fig. 2.** Comparison of the CHO cell cytotoxicity index values for each fraction of non-chloraminated wastewater effluent (WW1) and chloraminated wastewater effluent (WW2).

XAD-8+XAD-4 fraction from WW1 was obtained by mixing equal volumes of XAD-8 and XAD-4 fractions (50:50 v/v). CHO: Chinese hamster ovary.

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

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fraction</th>
<th>Conc. Range (µg/µL)</th>
<th>Acute Genotoxicity (50% Tail DNA) µg/µL</th>
<th>$r^2$</th>
<th>Lowest Significant Toxic Concentration (µg/µL)</th>
<th>ANOVA Test Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>WW1</td>
<td>XAD-4</td>
<td>0.3-5.0</td>
<td>4.91</td>
<td>0.98</td>
<td>4.25</td>
<td>$F_{8, 23} = 23.0$: $P \leq 0.001$</td>
</tr>
<tr>
<td>WW1</td>
<td>XAD-8</td>
<td>0.2-4.0</td>
<td>3.69</td>
<td>0.99</td>
<td>2.5</td>
<td>$F_{9, 28} = 49.0$: $P \leq 0.001$</td>
</tr>
<tr>
<td>WW2+NH$_2$Cl</td>
<td>XAD-4</td>
<td>2.0-3.4</td>
<td>2.45</td>
<td>0.98</td>
<td>2.0</td>
<td>$F_{11, 28} = 153.0$: $P \leq 0.001$</td>
</tr>
<tr>
<td>WW2+NH$_2$Cl</td>
<td>XAD-8</td>
<td>0.075-0.9</td>
<td>0.79</td>
<td>0.96</td>
<td>0.5</td>
<td>$F_{10, 28} = 20.8$: $P \leq 0.001$</td>
</tr>
</tbody>
</table>

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.
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.
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 N₂ 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 cytotoxicity 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 Cl₂/L), aqueous samples were collected before and after each XAD resin (Appendix A, Table S1) in order to quantify THMs, HAAs, HANs, HAcAms and HAcAls and evaluate the retention of each class of DBPs onto both resins (calculated as the difference between DBP concentrations at the inlet and at the outlet of a resin column). Results indicated a high retention of total THMs
and HANs on XAD-8 resin (94% and 97%, respectively), while HAAs, HAcAls and HAcAms exhibited a poor retention on both resins (Fig. 4). The hydrophobic character of DBP classes are in the order HAcAm < HAA < HAcAl < HAN < THM when estimating logD values at pH 2 for both chlorinated and brominated species using ChemAxon Marvin Suite. The XAD-8 resin is known to adsorb less hydrophilic molecules with higher molecular weights than molecules retained on the XAD-4 resin. This is in agreement with the retention of more hydrophobic DBPs (THMs and HANs) on the XAD-8 resin, possibly because they may exhibit stronger affinity with the hydrophobic macromolecules retained on this resin, rather than because of their direct adsorption on the resin. More hydrophilic compounds were better isolated on the XAD-4 resin (as part of the transphilic/TPI fraction) or not retained by both resins (i.e., hydrophilic/HPI fraction).
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; HAcAl: haloacetaldehydes; HAcAms: haloacetamides; DBP: disinfection byproduct.

2.3.3. DBP contributions to AOX
AOBr and AOCl concentrations in chloraminated wastewater effluent were 1658.2 µg/L as Br and 1078.9 µg/L as Cl, which is in accordance with the presence of a majority of brominated DBPs detected in full-scan chromatograms. The proportions of AOX (AOCl and AOBr) retained on each resin were calculated in the same manner than individual DBPs (i.e., by difference between inlet and outlet concentrations). AOBr and AOCl were mostly retained on the XAD-8 resin (748.7 µg/L as Br and 710.8 µg/L as Cl), compared to the XAD-4 resin (563.8 µg/L as Br and 209.6 µg/L as Cl), which may explain the higher toxicity observed in the isolated XAD-8/hydrophobic (HPO) fraction than that of the XAD-4/transphilic (TPI) fraction (Fig. 5).

Overall, 85.3% of AOCl and 79.2% of AOBr were retained on both resins. All analyzed DBP concentrations were expressed in µg/L as Cl (Cl-DBP) or µg/L as Br (Br-DBP) to obtain their relative contribution to AOCl and AOBr concentrations (Fig. 5). Unknown AOCl (UAOCl) and unknown AOBr (UAOBr) were determined from the differences between measured AOCl or AOBr and the sum of chlorine or bromine-equivalent concentrations of measured specific DBPs.

Br-DBPs contributed to 23.1% of AOBr retained on the XAD-8 resin and to 9.2% of AOBr retained on the XAD-4 resin. Bromoform, DBAN and DBCM were the three major Br-DBPs contributing to AOBr retained on the XAD-8 resin, while DBAA, CDBAcAl and DBAcAm were the major Br-DBPs retained on the XAD-4 resin. Br-DBPs present in the fraction not retained onto the two resins exhibited concentrations of the same order of magnitude of Br-DBPs retained onto the XAD-4 resin. Cl-DBPs only represented 2.2% and 4.5% of AOCl retained on XAD-8 and XAD-4 resins, respectively, which indicates that a majority of chlorine-containing compounds were not quantified.
Fig. 5. DBP contributions to a) AOBr and b) AOCl retained on XAD-8 and XAD-4 resins, based on concentrations analyzed in water samples at the inlet and outlet of each resin. UAOCl and UAOBr stand for unknown AOCl and unknown AOBr.

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

$$AC = \sum_{i}^{n} \frac{C_{i}}{LC_{50, i}}$$

(1)
Where \( C_i \) is the measured concentration of a given DBP \( i \) in a mixture of \( n \) DBP, \( LC_{50,i} \) is its LC\(_{50}\) cytotoxicity value and the calculated AC is a dimensionless value. Following this approach, the ratio between XAD-8 and XAD-4 resin calculated additive cytotoxicity was similar to the ratio of measured cytotoxicity index values (Fig. 6). While THMs were the major DBPs present in the XAD-8 fraction, most of the calculated additive cytotoxicity was attributed to HANs and HAcAms. DBAN, BAcAm, BCAN and DBAcAm were the dominant cytotoxic DBPs, accounting for 68.7%, 14.0%, 8.2% and 6.7% of the calculated AC, respectively. In the XAD-4 fraction, HAcAms and HAAs accounted for the majority of the calculated additive cytotoxicity, with DBAcAm, BCAcAm and MBAA accounting for 64.8%, 16.5% and 13.4%, respectively. Overall, N-DBPs (i.e., HANs and HAcAms) were responsible for the majority of the calculated cytotoxicity in both fractions (99.4% and 78.5% for XAD-8 and XAD-4 fractions, respectively).
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\textsubscript{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\textsubscript{50})\textsuperscript{-1}) to the isolated fraction according to a concentration addition model based on the individual cytotoxicity LC\textsubscript{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).
\[
\text{CCTI} = \sum_{i=1}^{n} \frac{p_i \cdot LC_{50,i}}{LC_{50,i}} \tag{2}
\]

Where \(p_i\) is the molar fraction of DBP \(i\) in a mixture of \(n\) DBP and \(LC_{50,i}\) is the individual LC50 value of DBP \(i\). The obtained CCTI values are expressed as \((LC_{50})^{-1}\), which facilitates the comparison with measured cytotoxicity index values (expressed as \((LC_{50}\text{ concentration factor})^{-1}\)).

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

\[
\text{CCTI} = \sum_{i=1}^{n} \frac{C_i \cdot AOX}{LC_{50,i}} \tag{3}
\]

Where \(C_i\) is the measured concentration (expressed as mol/L as X) of a given DBP \(i\) and \(LC_{50,i}\) is its LC50 cytotoxicity value.

With this approach, the proportion of N-DBPs (HANs and HAcAms) in AOX was a good proxy of the global toxicity measured in the samples (Fig. 7).
Calculated DBP Cytotoxicity Index (CCTI) $(LC_{50})^{-1} \times 10^3$

XAD-8

XAD-4

Measured Cytotoxicity Index (LC$_{50}$ conc. factor)$^{-1} \times 10^3$

0 40 80 120 160 200 240

THM

HAA

HAN

HAcAm

Measured cytotoxicity

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

AOX: adsorbable organic halide.

3. Conclusions

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

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

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

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

Among the measured DBPs, N-DBPs (i.e., HANs and HAcAms) contributed to the majority of the estimated additive DBP cytotoxicity of each fraction (78.5% and 99.4% for
TPI and HPO fractions, respectively), and were a good proxy of the global cytotoxicity when expressed as molar fractions of AOX.

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Appendix A. Supplementary Material

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

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